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Aft1p and Aft2p Mediate Iron-responsive Gene Expression in Yeast through Related Promoter Elements*[S]

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The transcription factors Aft1p and Aft2p from *Saccharomyces cerevisiae* regulate the expression of genes that are involved in iron homeostasis. *In vitro* studies have shown that both transcription factors bind to an iron-responsive element (FeRE) that is present in the upstream region of genes in the iron regulon. We have used DNA microarrays to distinguish the genes that are activated by Aft1p and Aft2p and to establish for the first time that each factor gives rise to a unique transcriptional profile due to the differential expression of individual iron-regulated genes. We also show that both Aft1p and Aft2p mediate the *in vivo* expression of *FET3* and *FIT3* through a consensus FeRE. In addition, both proteins regulate *MRS4* via a variant FeRE with Aft2p being the stronger activator from this particular element. Like other paralogous pairs of transcription factors within *S. cerevisiae*, Aft1p and Aft2p are able to interact with the same promoter elements while maintaining specificity of gene activation.

Saccharomyces cerevisiae contains paralogous gene pairs that code for transcription factors that can have both overlapping and distinct functions. These transcription factors bind to the same promoter elements but generate distinct transcriptional profiles. Included in this group are Ace2p/Swi5p, Pdr1p/Pdr3p, and Yap1/Yap2p. Ace2p and Swi5p regulate the expression of cell cycle-specific genes, are 83% identical in their zinc finger DNA-binding domains and recognize the same DNA binding site *in vitro* (1, 2). However, Ace2p and Swi5p can regulate separate genes, where discrimination between promoter elements is achieved through the interaction of these factors with other DNA-binding proteins (2). Alternatively, in cases where both transcription factors can induce the expression of the same gene, one is often the more potent activator (3). There are genes that require both Ace2p and Swi5p for maximal expression and others that are antagonistically regulated by these factors (4). Pdr1p and Pdr3p share 36% identity and regulate the expression of genes that are involved in pleiotropic drug resistance. Both these factors are able to bind to the same DNA element (PDRE) *in vivo* as either hetero- or homodimers (5). Differential expression of Pdr1p/Pdr3p target genes may therefore involve different combinations of Pdr1p/Pdr3p at different PDREs (5). Yap1p and Yap2p are 88% identical in their DNA binding regions and bind to the same consensus site (6). Analysis of global gene expression using microarrays has shown that although Yap1p and Yap2p are both involved in the response to cellular stresses, they regulate different regulons (7). The mechanism(s) of Yap1p/Yap2p gene selectivity are not understood but may include variations in the base pairs flanking the consensus Yap binding site (7).

The iron regulon of *S. cerevisiae* is well characterized and includes genes that are involved in the uptake, compartmentalization and use of iron. These include genes that encode siderophore transporters (*ARN1–4*), iron reductases (*FRE1–6*), iron permeases (*FTR1, FET4*), and multicopper oxidases that are involved in the coordinated oxidation and transport of iron across membranes (*FET3, FET5*) (8–12). In addition, three related genes (*FIT1-FIT3*) encode proteins that localize within the yeast cell wall and whose function is partially related to siderophore uptake (13).

Genes within the iron regulon are induced under low iron conditions and are regulated by the transcription factors, Aft1p and Aft2p (14, 15, 18). Aft1p and Aft2p are 39% identical within their N-terminal regions, which contain the DNA binding domain of each protein (15). The activation domain of Aft1p is within its C-terminal 413–572 amino acid residues which, when fused to the Gal4p DNA binding domain, activates transcription in an iron-independent manner (16). In addition, the N-terminal region of Aft1p contains a nuclear export signal that mediates differential cellular localization of Aft1p in response to iron (16). Aft1p is localized to the cytosol under iron-replete conditions, but is nuclear and active in iron-deficient conditions. Mutant alleles of *AFT1* and *AFT2* (*AFT1-1^{up}* and *AFT2-1^{up}* respectively) have been identified that result in the iron-independent activation of the iron regulon (14, 15). These gain of function mutations result from a single cysteine to phenylalanine substitution in identical regions of Aft1p and Aft2p.

A strain that lacks a functional Aft1p (*aft1*Δ) grows poorly in iron-limiting conditions and exhibits reduced iron uptake and cell surface iron-reductase activity (14, 17). The vector-borne *AFT2-1^{up}* allele is able to partially restore iron uptake in the *aft1*Δ strain (15). The *aft2*Δ strain has no growth phenotype on iron-limiting medium (15). However, the *aft1*Δ *aft2*Δ strain is more sensitive to iron-limiting conditions than the single *aft1*Δ strain (15, 18). The *aft1*Δ strain also fails to grow with glycerol as a respiratory carbon source, and this phenotype is partially restored by complementation with low copy plasmids containing the *AFT2* or *AFT2-1^{up}* alleles (15). Analysis of global gene expression has shown that the *AFT2-1^{up}* allele activates the expression of a subset of the iron regulon (15). However, our previous microarray analysis did not compare transcript pro-

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[S] The on-line version of this article (available at *http://www.jbc.org*) contains EXCEL spreadsheets containing the normalized, filtered, and averaged data.

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files for both *AFT2-1^{up}* and *AFT1-1^{up}* cells. Additionally, the *AFT1-1^{up}* and the *AFT2-1^{up}* alleles differentially regulate the expression of two iron-regulated genes (*MRS4* and *FIT2*) (15).

Aft1p mediates transcriptional regulation through an iron-responsive element (FeRE)[1] that has the consensus sequence PyPuCACCCPu (14). The FeRE was identified by the *in vivo* analysis of the *FET3* promoter region using *lacZ* fusion constructs and DNA footprinting (14). Aft1p binds to the *FET3* FeRE *in vivo* in an iron-dependent manner. Sequences similar to the *FET3* FeRE were identified in the promoter regions of five other known iron-regulated genes. *In vitro* assays confirmed the binding of Aft1p to these particular sequences, from which the consensus FeRE sequence was derived (14). *SMF3*, that encodes a metal transporter, is also iron-regulated in an Aft1p/Aft2p-dependent manner through a consensus FeRE (19). N-terminal truncates of Aft1p and Aft2p bind to the *FET3* FeRE *in vitro*, consistent with both factors regulating the iron regulon through the consensus FeRE (15). Expression of *AFT2* from a high copy plasmid, but not the chromosomal copy of *AFT2*, activates the expression of a *lacZ* reporter under the control of the *FET3* FeRE in an iron-dependent manner (18). However, the high expression of one of a pair of transcription factors can result in the aberrant activation of genes that are specifically regulated by the other factor (2). It is therefore not clear to what extent Aft1p and Aft2p interact with the same responsive element *in vivo*.

The evidence is therefore consistent with Aft1p and Aft2p having overlapping but non-redundant roles in the transcriptional regulation of the iron regulon in *S. cerevisiae*. We are interested in understanding the selective advantage of this organism having two iron-responsive transcription factors. In this study, microarray experiments have been used to further define the activities of the gain of function mutants and demonstrate that they give rise to different global transcriptional profiles. We have also analyzed the ability of Aft1p and Aft2p to activate transcription through the same consensus FeRE. *In vivo lacZ* reporter constructs show clearly that Aft1p and Aft2p activate gene expression through the consensus FeREs in the *FET3* and *FIT3* promoter regions. Both Aft1p and Aft2p also induce the activation of *MRS4* through a variant FeRE. An extended FeRE has been identified that is overrepresented in the genes that are most highly induced by the *AFT1-1^{up}* and *AFT2-1^{up}* alleles.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions—Haploid *aft1*Δ*aft2*Δ strains were isolated following the mating of the strains, BY4741*aft2*Δ (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 aft2*::*kanMX4*) and BY4742*aft1*Δ (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 aft1*::*kanMX4*), which were purchased from Research Genetics. A strain containing a copy of the *AFT2-1^{up}* allele integrated at the *HIS3* locus was generated by transforming a haploid *aft1*Δ*aft2*Δ strain (MATα *his3Δ1 leu2Δ0 ura3Δ0 aft1*::*kanMX4 aft2*::*kanMX4*) with plasmid p*AFT2-1^{up}INT* that had been linearized using *Msc*I and selecting for growth on agar plates lacking histidine. The allele status of the *aft1*Δ*aft2*Δ strains and the integration of the *AFT2-1^{up}* allele at the *HIS3* locus were verified using PCR and DNA sequencing. Cells were grown with 2% glucose as a carbon source, in 1% yeast extract, 2% peptone medium (YPD), complete-synthetic medium (CMD) or, when appropriate, complete-synthetic medium lacking uracil and/or histidine (CMD-Ura, CMD-His, CMD-Ura/His).

Vectors and Fusion Genes—The YCp plasmids, p*AFT1-1^{up}* and p*AFT2-1^{up}* are *HIS3* derivatives of p*AFT1-1^{up}* and p*AFT2-1^{up}* (15) from which the *AFT1* (*Xho*I/*Sac*I) and *AFT2* (*Bam*HI/*Xba*I) sequences have been subcloned into the YCp plasmid, pRS413. Consequently, the gain of function mutant alleles of *AFT1* and *AFT2* within p*AFT1-1^{up}* and p*AFT2-1^{up}* are under the control of their own promoters (15). The integrative vector p*AFT2-1^{up}INT* was generated by subcloning the *AFT2* sequences (*Xho*I/*Xba*I) from p*AFT2-1^{up}* into the YIp plasmid, pRS403. The *lacZ* reporter constructs, pFC-W and pFC-LM2 were a gift from Andrew Dancis (14). These contain, respectively, a functional and non-functional copy of the *FET3* FeRE in a minimal promoter. The plasmid p*FET3-lacZ* (0.5 kb of the 5′-region of *FET3*, inclusive of the start codon, fused to the *lacZ* gene in YEp354, Ref. 20) was a gift from Jerry Kaplan (21). To generate a version of p*FET3-lacZ* in which the same FeRE that is present in pFC-W was mutated to a non-functional FeRE (CACCC to CAGGG), p*FET3-lacZ* was used as template for QuikChange mutagenesis (Stratagene) using the primer 5′-GGCAAGGCCCATCTTCAAAAGTGCAGGGATTTGCAGGTGCTCTTATTCTCGCC-3′ and its complement to generate p*FET3-lacZ* (mut). The 0.5-kb fragment containing the *FET3* sequences from p*FET3-lacZ* and p*FET3-lacZ* (mut) were excised and ligated into the *Pst*I/*Sma*I sites of Yep354 to generate p*FET3-lacZ* and p*FET3-lacZ* (mut) respectively. To generate p*FIT3-lacZ* (1 kb of 5′-region of *FIT3*, inclusive of the start codon, fused to the *lacZ* gene in YEp354) yeast-genomic DNA from strain BY4741 was isolated and used as template for PCR using the primers 5′-CGGGATCCTCCATAAACATTTCCTTTGTC-3′ and 5′-CCAAGCTTTCATTTTAGGGATTATTGTTATTAG-3′. The resulting 1-kb fragment was ligated into the *Bam*HI/*Hind*III sites of Yep354 to generate pSJDW36. Plasmid pSJDW36 was used as template for QuikChange mutagenesis using the primer 5′-CATCAAAAAATATGGGATAGCGCCCTGCGCAACAAACACCCTGCAAAAAAAAAATCTAGGACATAGG-3′ and its complement to mutate two potential FeREs to non-functional FeREs (CACCC to CAGGG) and thereby generate pSJDW36 (mut). The *FIT3* sequences from both pSJDW36 and pSJDW36 (mut) were excised and ligated to the *Bam*HI/*Hind*III sites of Yep354 to generate p*FIT3-lacZ* and p*FIT3-lacZ* (mut), respectively. To generate plasmid p*MRS4a-lacZ* (1 kb of 5′-region of *MRS4,* inclusive of the start codon, fused to the *lacZ* gene in YEp354) yeast-genomic DNA from strain BY4741 was used as template for PCR using the primers 5′- CGCACAGGATCCTCGAAGATAGCGTAGCGTTC-3′ and 5′-GGGAGCAAGCTTCATAATATTAACTGATATTTCGGTTG-3′ (primer Mrs4a). The resulting 1-kb fragment was ligated into the *Bam*HI/*Hind*III sites of pHOLLY to generate pJRDW25. Plasmid pJRDW25 was used as template for PCR using primer Mrs4a with, individually, primers 5′-CGCACAGGATCCCATATTTGGAATTCAGC-3′, 5′-CGCACAGGATCCCCCACAGGAATCGCTAC-3′, 5′-CGCACAGGATCCCGTGTCTCTTTTCGGTA-3′, 5′-CGCACAGGATCCGAATGAGAGCATGGCGA-3′, and 5′-CGCACAGGATCCCTTTTGCCTACCATTGG-3′ to generate sequential truncations of the *MRS4* promoter region. The resulting fragments, and the 1-kb *Bam*HI/*Hind*III fragment from pJRDW25, were ligated into the *Bam*HI/*Hind*III sites of Yep354 to generate plasmids p*MRS4lacZ(a-f)*. Plasmid p*MRS4-lacZd* was used as template for QuikChange mutagenesis using the primer 5′-CGAAGACTGAAAGGCAAGAACAGGGTGCTATCTTTTGCCTACCATTG-3′ and its complement and primer 5′-GTCTCTTTTCGGTATTTTGGCAGGGTTTCTTGAATGAGAGCATGGC -3′ and its complement to generate, respectively, plasmids, p*MRS4-lacZd* (mut1) and p*MRS4-lacZd* (mut2) that contain CACCC to CAGGG mutations in individual FeRE-like sequences. To generate a reporter construct that contains the FeRE of *MRS4* within the ΔUAS *CYC1* promoter fused to the *lacZ* gene, 200 pmol of each of the partial overlapping oligos 5′-tcgaAAGAAAGGGTGCCAAAA-3′ and 5′-ctagTTTTGGCACCCTTTCTT-3′ were mixed in SSC (final 0.32×), boiled for 15 min, and then incubated overnight at 55 °C. The annealed products were then ligated into the *Xho*I/*Xba*I sites of pNB404 (22) to generate p*MRS4*-FeRE. All PCR-generated sequences were confirmed by DNA sequencing. All yeast transformations were performed using the lithium acetate procedure. In the case of transformations using *aft1*Δ*aft2*Δ strains, cells were pregrown in YPD under nitrogen and agar plates supplemented with $FeCl_2$ (100 μM).

mRNA Quantification by S1 Nuclease Analysis—Strain *aft1*Δ*aft2*Δ (*MAT α his3Δ1 leu2Δ0 ura3Δ0 aft1*::*kanMX4 aft2*::*kanMX4*) was used for transformation of all *lacZ* reporter constructs with separately p*AFT1-1^{up}*, p*AFT2-1^{up}* or pRS413. Cells were harvested at mid-log phase, and total RNA was extracted using the hot acidic phenol method (31). DNA oligonucleotides, with sequences complementary to *lacZ*, *FIT3* and *CMD1* mRNA (calmodulin as an internal loading control), were end-labeled with ^{32}P using T4 polynucleotide kinase (New England Biolabs). S1 analysis was carried out as previously described (1). Briefly, the ^{32}P-labeled oligonucleotides were hybridized with 12 μg of total RNA in HEPES buffer (38 mM HEPES, pH 7.0, 0.3 M NaCl, 1 mM EDTA, 0.1% Triton X-100) at 55 °C overnight. The reactions were then

[1] The abbreviations used are: FeRE, iron-responsive element; ORF

TABLE I
Genes induced by AFT1–1^{up}

Microarray analysis was carried out comparing transcript levels of control *aft1*Δ*aft2*Δ cells and cells containing p*AFT1–1^{up}*. The 25 most highly induced genes are shown. The mean and S.D. of the Cy5/Cy3 ratios of three independent experiments are listed. Also shown are the equivalent data (mean, S.D.) from the experiments involving p*AFT2–1^{up}*. Listed is the number of consensus FeRE sites (PyPuCACCC) within 1 kb of the start codon of each gene. Notes: (1) *AFT1* is not present in the control strain. (2) YDR271C overlaps with *CCC2* - a known iron regulated gene. (3) A transcript is not detected (ND) when the signal is not greater than the background signal.

Gene name	Mean	S.D.	AFT2–1^{up}	S.D.	# FeRE
FIT3	43.4	8.6	14.6	2.1	3
FIT2	30.9	11.0	2.4	0.2	5
FET3	25.2	12.3	3.7	0.2	2
FIT1	8.7	2.0	14.3	1.8	3
YOR387C	6.7	0.6	2.0	0.7	2
ENB1 (ARN4)	6.7	1.0	2.0	0.7	3
SIT1 (ARN3)	6.3	2.2	1.9	0.3	1
TIS11	5.1	2.0	3.6	1.0	2
FTR1	5.0	0.5	2.6	0.2	2
AFT1 (1)	4.8	2.0	ND[a]		1
FRE2	4.1	0.6	ND		2
FRE1	4.0	0.7	3.5	0.6	1
TAF1 (ARN2)	3.4	0.6	1.1	0.1	1
FRE3	3.0	0.5	2.0	0.4	3
YHL035C	3.0	0.5	2.2	0.2	1
FRE5	2.9	0.4	1.9	0.3	3
BIO5	2.8	0.3	1.7	0.2	0
YDR271C (2)	2.6	0.2	1.9	0.5	0
ARN1	2.5	0.8	1.6	0.2	1
FRE6	2.4	0.2	2.2	0.2	2
ATX1	2.4	0.2	2.0	0.6	1
OYE3	2.3	0.3	2.7	0.4	0
COT1	2.3	0.2	2.3	0.2	1
YMR034C	2.2	0.4	2.2	0.1	1
AKR1	2.2	0.1	1.5	0.1	1

[a] ND, not detected.

heat-denatured in formamide buffer. The samples were then separated using an 8% polyacrylamide/8.3 M urea gel.

Microarray Analysis—Strain *aft1*Δ*aft2*Δ (*MATa his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 aft1*::*kanMX4 aft2*::*kanMX4*) was transformed with separately, p*AFT1-1^{up}*, p*AFT2-1^{up}* and their corresponding parental vectors as controls (pRS316, pRS416). Cells were grown in 300 ml of CMD-Ura medium and harvested at an $OD_{600\ nm}$ of 0.4. Total RNA was isolated by the hot acidic phenol method and mRNA was isolated from total RNA using the PolyATtract mRNA Isolation System IV kit (Promega) following the manufacturer's instructions. mRNA (1.2 μg) was used to generate cDNA probes by reverse transcription (Superscript II, GIBCO) with incorporation of Cy3-dCTP or Cy5-dCTP (Amersham Biosciences). The arrayed slides were produced as follows. The PCR amplification products of all the *S. cerevisiae* open reading frames (ORFs) were purchased from Research Genetics. Each PCR product was reamplified and purified on glass fiber filter plates (Millipore). The reamplified products representing ~6000 ORFs (4% not represented) were diluted to 50% Me_2SO and spotted in duplicate on 3-aminopropylmethyldiethoxy silane-coated slides using a Generation III Microarray Spotter (Amersham Biosciences). The cDNA-labeled probes were then hybridized onto an arrayed slide and fluorescence was captured with a GEN III dual-laser confocal scanner (Molecular Dynamics). Fluorescent intensities were quantified using Arrayvision 6.0 (Imaging Research). The ratios of signal intensities (Cy5/Cy3) were normalized by the median output signal intensity for all genes, and the normalized data for three independent experiments were averaged. The data were filtered to remove those measurements that were not higher than the slides' background fluorescence and assembled into EXCEL spreadsheets. Analysis of the potential regulatory sequences within the most highly induced genes was carried out using the program GeneSpring 3.2 (Silicon Genetics). The EXCEL spreadsheets containing the normalized, filtered, and averaged data are available as Supplemental Material.

RESULTS

Microarray Analysis of Aft1p and Aft2p Regulons—Previous studies have shown that Aft1p and Aft2p differentially activate the expression of a subset of the iron-regulated genes in *S. cerevisiae* (15). We were interested to learn the effect of Aft1p and Aft2p on global gene expression in cells of the same genetic and, separately, the *AFT2-1^{up}* allele. The *aft1*Δ*aft2*Δ strain was used into which p*AFT1-1^{up}*, p*AFT2-1^{up}* and control vectors had been separately transformed. The *AFT1-1^{up}*/*AFT2-1^{up}* alleles were expressed from their own promoters in low copy plasmids, so as to minimize the overexpression of the gain of function alleles. The transcriptional profiles of the p*AFT1-1^{up}*- and the p*AFT2-1^{up}*-containing strains were separately compared with that of control strains. Each experiment was carried out independently on three occasions. The average induction ratio for each gene was calculated, and the genes that were most highly induced in cells containing the *AFT1-1^{up}* and *AFT2-1^{up}* alleles were identified (Tables I and II, Fig. 1). These data will include genes that are the direct targets of Aft1p and/or Aft2p or genes that are activated as the result of the indirect effects of Aft1p/Aft2p. A complete data set can be found in the Supplemental Material.

Many of the genes identified as being induced as the result of the *AFT1-1^{up}*/*AFT2-1^{up}* alleles are known to code for proteins that are involved in iron metabolism. Some of these genes are induced to a similar extent as the result of either Aft1p or Aft2p (e.g. *FRE6, ATX1*, *COT1*). However, the more highly induced genes tend to be activated to a greater extent when Aft1p is present (*e.g. FIT3*, *FIT2*, *FTR1*). There are examples of genes that contain a consensus FeRE but which are only induced as the result of one of the Aft1p/Aft2p factors. These genes include the Aft1p-specific *FRE2* and *ARN2* and the Aft2p-specific *BNA2* and *ECM4*. *BNA2* codes for a protein with similarity to indoleamine 2,3-dioxygenases and Ecm4p is involved in cell wall biosynthesis, although its precise function is not known. Included in the group of genes that are preferentially induced as the result of Aft1p is YOR387C. Interestingly, YOR387C is located within a region of chromosome XV that contains a group of genes (*FIT2*, *FIT3*, *FRE3*) that are highly induced in cells containing either *AFT1-1^{up}* or *AFT2-1^{up}*. YOR387C codes

TABLE II
Genes induced by AFT2–1^{up}

Microarray analysis was carried out comparing transcript levels of control *aft1Δaft2Δ* cells and cells containing p*AFT2–1^{up}*. The 25 most highly induced genes are shown. The mean and the S.D. of the Cy5/Cy3 ratios of three independent experiments are listed. Also shown are the equivalent data (mean, S.D.) from the experiments involving p*AFT1–1^{up}*. Listed is the number of consensus FeRE sites (PyPuCACCC) within 1 kb of the start codon of each gene. Notes: (1) *AFT2* is not present in the control strain (2) YOR225w overlaps with *ISU2*- a known iron regulated gene (3). A transcript is not detected (ND) when the signal is not greater than the background signal.

Gene name	Mean	S.D.	AFT1–1^{up}	S.D.	# FeRE
FIT3	14.6	2.1	43.4	8.6	3
FIT1	14.3	1.8	8.7	2.0	3
AFT2 (1)	4.4	1.2	ND[a]		0
FET3	3.7	0.2	25.2	12.3	2
TIS11	3.6	1.0	5.1	2.0	2
FRE1	3.5	0.6	4.0	0.7	1
BNA2	3.0	0.6	ND		1
ECM4	2.8	0.5	ND		1
YAP2	2.8	0.2	1.7	0.2	0
YKR104W	2.7	0.2	ND		0
OYE3	2.7	0.4	2.3	0.3	0
SMF3	2.6	0.2	1.9	0.1	2
FTR1	2.6	0.2	5.0	0.5	2
YOL083W	2.5	0.3	1.8	0.2	1
MRS4	2.5	0.3	1.7	0.4	0
FIT2	2.4	0.2	30.9	11.0	5
YMR041C	2.4	0.2	1.5	0.4	0
YBR012C	2.4	0.2	ND		0
LAP4	2.3	0.4	1.6	0.1	1
YOR225W (2)	2.3	0.5	1.7	0.2	0
HSP26	2.3	0.6	1.5	0.4	0
COT1	2.3	0.2	2.3	0.2	1
YGR146C	2.3	0.2	1.5	0.1	1
ISU1	2.2	0.4	1.5	0.3	1
YHL035C	2.2	0.2	3.0	0.5	1

[a] ND, not detected.

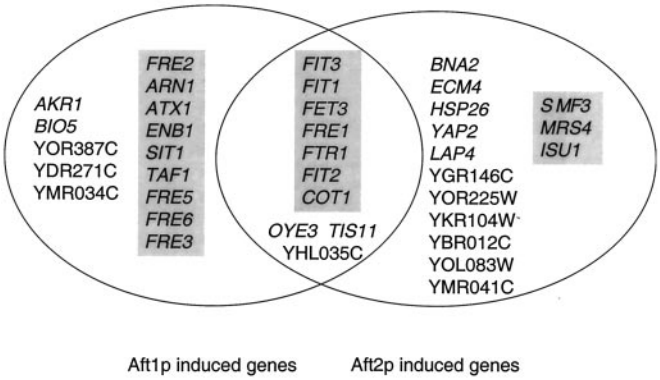

FIG. 1. **Summary of the genes induced by Aft1p and Aft2p.** The overlap represents those genes that are induced by both Aft1p and Aft2p. Those genes that are known to encode proteins that are involved in iron homeostasis are *shaded*. *AFT1* and *AFT2* are excluded.

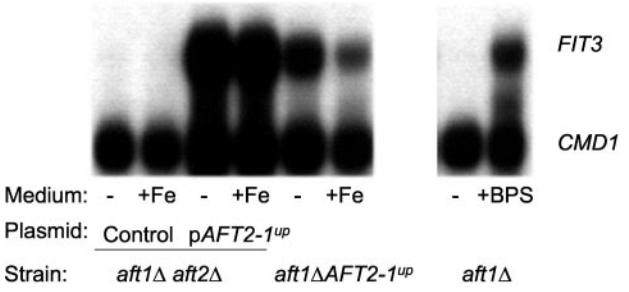

FIG. 2. **Comparison of activation of the expression of FIT3 by the chromosomal and plasmid-borne AFT2-1^{up} allele.** S1 nuclease protection assays were used to quantify mRNA level of *FIT3*. RNA was isolated from the *aft1Δaft2Δ* strain transformed with a control plasmid or p*AFT2-1^{up}* and the *aft1Δaft2Δ* strain into which the *AFT2-1^{up}* allele had been integrated into the *HIS3* locus (*aft1ΔAFT2-1^{up}*). Each strain was grown in CMD (-His) medium with or without added iron (100 μM) to mid-log phase. RNA was also isolated from the *aft1Δ* strain that had been grown in CMD with and without the iron chelator BPS (100 μM). The *upper band* for each sample is the *FIT3* gene, and the *lower band* is the calmodulin-loading control (*CMD1*).

experiments are *OYE3* and YHL035C, which have not previously been identified as targets of the Aft1p/Aft2p factors. *OYE3* codes for a NADPH dehydrogenase and does not contain a consensus FeRE within its 5′-upstream region and consequently may be induced as an indirect result of the Aft1p/Aft2p factors. YHL035C has one consensus FeRE within its 5′-upstream region and codes for a member of the ATP-binding cassette (ABC) family of transporters, however its localization and function are unknown.

Integration of AFT2-1^{up} Allele—To confirm that the use of plasmid-borne constitutive alleles for the microarray experiments was an appropriate strategy, the *AFT2-1^{up}* locus, under the control of its own promoter and terminator, was integrated into the *HIS3* locus of the *aft1Δaft2Δ* strain. S1 nuclease protection assays were used to analyze the expression of *FIT3* in the *aft1Δaft2Δ* strain containing a control plasmid or p*AFT2-*

the plasmid-borne *AFT2-1^{up}* allele was only slightly greater than that of the integrated *AFT2-1^{up}* allele (Fig. 2). Consequently, it is unlikely that the microarray data include genes that have been artificially induced through overexpression of the *AFT1-1^{up}* and *AFT2-1^{up}* alleles. Interestingly, the *AFT2-1^{up}* allele still responds to iron to some extent when it is present in the chromosome, but that response is lost in the strain containing the plasmid-borne *AFT2-1^{up}* allele.

In Vivo Analysis of FET3 FeRE—Truncated Aft1p and Aft2p that contain the N-terminal basic regions of each protein, are able to bind to the *FET3* FeRE *in vitro* (15). As both the *AFT1-1^{up}* and *AFT2-1^{up}* alleles activate the expression of many of the same genes, we initially used two reporter constructs to learn if both factors act through the same FeRE *in vivo*. The

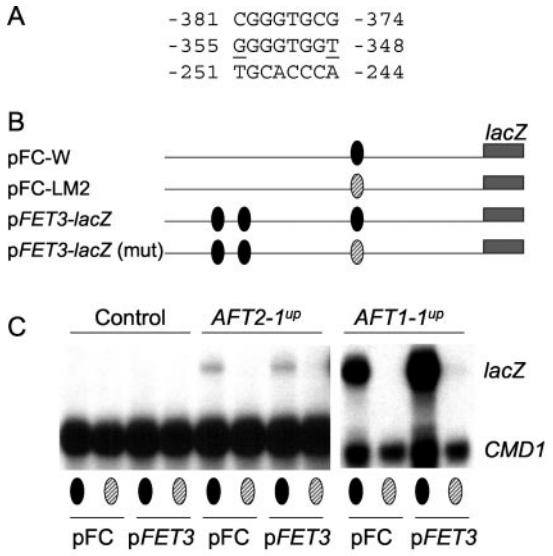

FIG. 3. **Aft1p and Aft2p regulate the expression of FET3 through the same FeRE.** *A*, the nucleotide sequences of 3 FeRE-like elements within 500 bp of the start codon of *FET3*. Nucleotides that deviate from the consensus FeRE sequence identified in Ref. 14 are *underlined*. *B*, plasmid pFC-W contains the −251 FeRE that has been inserted into the *CYC1* promoter and fused to the *lacZ* gene. Plasmid p*FET3-lacZ* contains 500 bp of the upstream region of *FET3* fused to the *lacZ* gene. Ovals represent FeRE-like sequences (*filled in*) or mutated FeREs (*hatched*). *C*, S1 nuclease protection assays were used to quantify mRNA levels of *lacZ*. RNA was isolated from the *aft1*Δ*aft2*Δ strain that had been separately transformed with pRS413 (control), p*AFT1-1*^{up} and p*AFT2-1*^{up} together with the *lacZ* reporter constructs pFC or p*FET3-lacZ* and their respective control plasmids [pFC-W and p*FET3-lacZ* (mut)]. Each strain was grown in CMD (-His/Ura) to mid-log phase. The *upper band* for each sample is the *lacZ* gene. and the *lower band* is the calmodulin-loading control (*CMD1*).

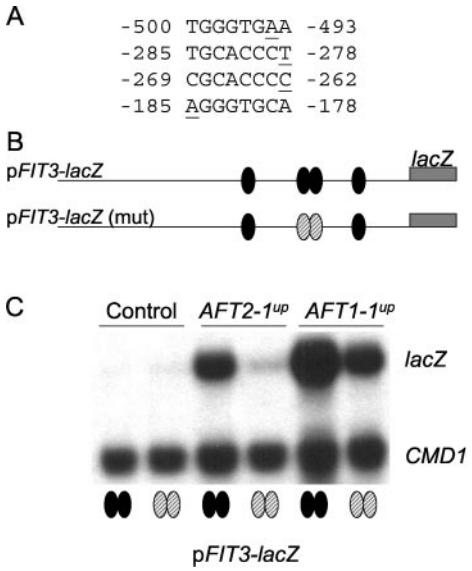

FIG. 4. **Aft1p and Aft2p regulate the expression of FIT3 through the same FeRE.** *A*, the nucleotide sequences of 4 FeRE-like elements within 1 kb of the start codon of *FIT3*. Nucleotides that deviate from the consensus FeRE sequence identified in Ref. 14 are *underlined*. *B*, plasmid p*FET3-lacZ* contains 1 kb of the upstream region of *FIT3* fused to the *lacZ* gene. Ovals represent FeRE-like sequences (*filled in*) or mutated FeREs (*hatched*). *C*, S1 nuclease protection assays were used to quantify mRNA levels of *lacZ*. RNA was isolated from the *aft1*Δ*aft2*Δ strain that had been separately transformed with pRS413 (control), p*AFT1-1*^{up} and p*AFT2-1*^{up} together with the *lacZ* reporter constructs p*FIT3-lacZ* and its control p*FIT3-lacZ* (mut). Each strain was grown in CMD (-His/Ura) to mid-log phase. The *upper band* for each sample is the *lacZ* gene, and the *lower band* is the calmodulin-loading control (*CMD1*).

lacZ reporter gene (p*FET3-lacZ*)(Fig. 3*B*). A mutated fusion gene was generated in which the core CACCC sequence of the FeRE closest to the *lacZ* start codon was changed to the sequence, CAGGG. This mutation is known to create a non-functional FeRE (14). The second reporter construct consists of the same *FET3* single FeRE within a minimal *CYC1* promoter/*lacZ* reporter construct that lacks an upstream activating sequence (pFC-W). The corresponding control vector contains a non-functional CAGGG sequence within the FeRE (pFC-LM2) (14). Plasmids p*FET-lacZ* and pFC-W, and their corresponding control vectors, were transformed separately into the *aft1*Δ*aft2*Δ strain containing either p*AFT1-1*^{up} or p*AFT2-1*^{up}. S1 nuclease protection assays were used to analyze the expression of the *lacZ* reporter genes. Both the *AFT1-1*^{up} and *AFT2-1*^{up} alleles induced the expression of *lacZ* in p*FET-lacZ* and pFC-W, but not the control reporter genes containing a non-functional FeRE (Fig. 3*C*). In both cases, the *AFT2-1*^{up} induced expression of the reporter genes was weaker than the activation by the *AFT1-1*^{up} allele. The *AFT2-1*^{up} allele is a strong activator of the chromosomal *FET3* (15), so the minimal effects of this allele with these particular reporter constructs may be due to the lack of upstream sequences of the *FET3* promoter region that are absent in p*FET3-lacZ* and pFC-W.

In Vivo Analysis of FIT3 FeRE—The most highly induced gene by the *AFT1-1*^{up} and *AFT2-1*^{up} alleles is *FIT3* (Tables I and II). To analyze the extent that both Aft1p and Aft2p activate the expression of *FIT3* through the same FeRE, we generated a reporter construct, containing 1 kb of the *FIT3* 5′-region fused to *lacZ* (p*FIT3-lacZ*). It was anticipated that the larger 5′-upstream region would be representative of the *AFT2-*

that each deviate from the consensus FeRE by one nucleotide (Fig. 4*A*). To see if loss of an FeRE would have the same effect for both Aft1p- and Aft2p-dependent activation, we generated a *FIT3-lacZ* fusion gene that contained mutations in two of these potential FeRE sites. The core CACCC FeRE sequence was changed to the non-functional CAGGG (Fig. 4*B*). Plasmid p*FIT3-lacZ* and its control were transformed separately into the *aft1*Δ*aft2*Δ strain that contained either p*AFT1-1*^{up} or p*AFT2-1*^{up}. Disruption of the two FeRE sites attenuates both the *AFT1-1*^{up} and *AFT2-1*^{up} induced expression of the *FIT3-lacZ* fusion (Fig. 4*C*). Both of these FeRE sites deviate from the consensus FeRE in their most 3′-nucleotide, consistent with this nucleotide being unimportant for recognition by Aft1p and Aft2p. In agreement with the microarray data, the *AFT1-1*^{up} allele is a stronger activator than the *AFT2-1*^{up} allele of the expression of *FIT3*. Together, the analysis of the expression of the *FET3-lacZ* and *FIT3-lacZ* fusion genes is consistent with the *AFT1-1*^{up} and *AFT2-1*^{up} alleles inducing expression through the same core FeRE.

In Vivo Analysis of MRS4 FeRE—We have previously shown that *MRS4* is preferentially induced by the *AFT2-1*^{up} allele (15). Interestingly, the 1-kb 5′-upstream region of *MRS4* contains 5 FeRE-like sequences that each deviate from the consensus FeRE by 2 nucleotides (Fig. 5*A*). To determine whether the *AFT1-1*^{up} and *AFT2-1*^{up} alleles activate the expression of *MRS4* through these non-consensus FeRE sites, a series of *MRS4-lacZ* fusions were generated which contain truncations of the *MRS4* upstream region (Fig. 5*B*). These reporter constructs were transformed separately into the *aft1*Δ*aft2*Δ strain containing either p*AFT1-1*^{up} or p*AFT2-1*^{up}. The presence of at least two putative FeRE-like sites resulted in *AFT2-1*^{up}-dependent *lacZ* expression, whereas the fusion with only one

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