APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2002, p. 5769–5772 Vol. 68, No. 11
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.11.5769–5772.2002

Regulation of *freA*, *acoA*, *lysF*, and *cycA* Expression by Iron Availability in *Aspergillus nidulans*

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Received 3 June 2002/Accepted 2 August 2002

In the filamentous fungus *Aspergillus nidulans*, iron homeostasis is regulated at the transcriptional level by the negative-acting GATA factor SREA. In this study the expression of a putative heme-containing metalloreductase-encoding gene, *freA*, was found to be upregulated by iron limitation independently of SREA, demonstrating the existence of an iron-regulatory mechanism which does not involve SREA. In contrast to *freA*, various other genes encoding proteins in need of iron-containing cofactors—*acoA*, *lysF*, and *cycA*—were downregulated in response to iron depletion. Remarkably, SREA deficiency led to increased expression of *acoA*, *lysF*, and *cycA* under iron-replete growth conditions.

Virtually all organisms require iron for their growth. The electron transfer ability of the iron atom makes it essential for redox reactions ranging from respiration to ribonucleotide synthesis. Despite the fact that iron is the fourth most abundant element in the earth's crust, the amount of bioavailable iron is very limited since this metal is most commonly found as insoluble Fe(III)-hydroxide. Thus, microorganisms need specialized iron mobilization systems (14). On the other hand, an excess of iron in the cell can be detrimental, because iron can catalyze the production of cell-damaging hydroxyl radicals in the presence of oxygen. Therefore, the concentration of iron in biological fluids is tightly regulated, and control is accomplished primarily by the rate of uptake.

Under iron starvation, most fungi synthesize and excrete low-molecular-weight, Fe(III)-specific chelators, termed siderophores, in order to solubilize environmental iron. Subsequently, cells recover the iron from the ferrisiderophore complexes via specific uptake mechanisms (17). Furthermore, most fungi possess intracellular siderophores as an iron storage compound. In this respect *Saccharomyces cerevisiae* is an exception since it lacks the ability to synthesize siderophores, although it can utilize siderophores produced by other species. This yeast employs two distinct high-affinity iron uptake systems which are both regulated by the paralogous transcriptional activators Aft1p and Aft2p (2, 32). The first mechanism—termed reductive iron assimilation—requires the action of surface metalloreductases with different substrate specificities (Fre1p to Fre4p) to reduce Fe(III) to Fe(II), which is subsequently transported into the cell by the permease-oxidase complex Ftr1p/Fet3p (1, 5, 27, 34). This system allows the uptake of both siderophore-bound and unbound iron (33). The second iron uptake system—called nonreductive iron assimilation—is specialized for the uptake of siderophore-bound iron and depends on members of the major facilitator superfamily (16, 18, 33).

In ascomycetes and basidiomycetes, siderophore biosynthesis and siderophore-mediated iron uptake are controlled by orthologous, negative-acting GATA transcription factors, e.g., *Aspergillus nidulans* SREA, *Neurospora crassa* SRE, and *Ustilago maydis* URBS1 (15, 29, 35). In *A. nidulans*, deletion of *sreA* results in derepressed intracellular and extracellular siderophore biosynthesis as well as increased accumulation of iron under sufficient iron supply due to derepressed siderophore uptake (21). Recently various members of the SREA regulon which are presumably involved in biosynthesis, transport, and utilization of siderophores have been identified, e.g., *mirA*, which encodes an orthologue of the *S. cerevisiae* siderophore permeases (21, 22). Notably, neither the available *A. nidulans* cDNA and genomic sequences nor the publicly accessible complete genomes of the close relatives *Aspergillus fumigatus* (http://www.sanger.ac.uk/Projects/A_fumigatus/) and *N. crassa* (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) seem to contain orthologues of *S. cerevisiae AFT1* or *AFT2*. Furthermore, *S. cerevisiae* does not possess an orthologue of *A. nidulans* SREA. Thus, the question remains if SREA represents the major iron regulator or if it is specific for control of siderophore metabolism.

Up to now, it was not known if *A. nidulans* has the ability for reductive iron uptake. Searches for putative components of this system in various *A. nidulans* sequence databases led to the identification of expressed sequence tag clone o5f06a1, whose translation product displayed significant similarity to metalloreductases. The sequence information was used to isolate corresponding genomic clones from a cosmid library provided by the Fungal Genetic Stock Center (4). The five hybridizing clones, L4F02, L28H11, L25F03, L23A09, and L32A010, localized *freA* to chromosome IV, and the entire sequence of *freA* was sequenced directly from cosmid L23A09. Comparison of the genomic and cDNA sequences, obtained by 5′ and 3′ rapid amplification of cDNA ends according to the protocols of Frohman et al. (8), revealed an open reading frame of 1,797 bp

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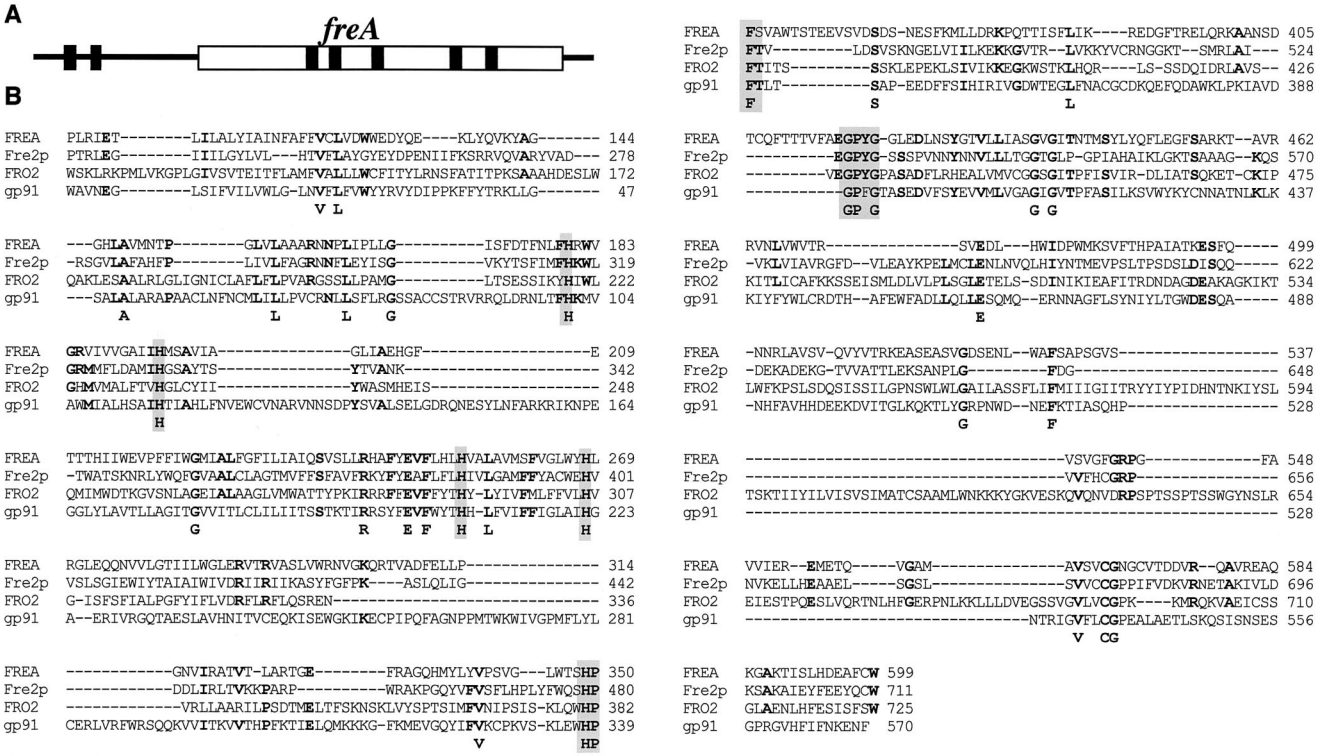

FIG. 1. *A. nidulans freA*. (A) Intron-exon structure of *freA*. (B) Alignment of *A. nidulans* FREA, *S. cerevisiae* Fre2p (P36033), *A. thaliana* FRO2 (CAA70770), and human gp91^{phox} (NP_000388). Amino acid residues identical in three of the four proteins are in boldface, and amino acid residues potentially involved in the bis-heme binding (H), NADPH binding (HPFT), and flavin adenine dinucleotide binding (GPYG) are shaded in gray.

interrupted by five introns, 53, 50, 43, 47, and 45 nucleotides (nt) in length (Fig. 1A). Additionally, two introns, 66 and 58 nt in length, are present in the 827-bp 5′ untranslated region. The 3′ untranslated region was found to be 84 nt in length. The deduced FREA protein has a calculated molecular mass of 67.2 kDa and shows significant similarity to various metalloreductases, e.g., 24% identity (blastp E-value of $8e^{-32}$) to *S. cerevisiae* Fre2p. An alignment of *A. nidulans* FREA, *S. cerevisiae* Fre2p (10), *Arabidopsis thaliana* FRO2 (24), and the gp91^{phox} subunit of the NADPH oxidase (25), which is critical for production of microbicidal oxidants in human neutrophils, is shown in Fig. 1B. FREA possesses all typical features of metalloreductases (7, 12): a flavin adenine dinucleotide cofactor binding site, an NADPH binding motif, and four typically spaced histidine residues predicted to coordinate a bis-heme structure between transmembrane domains of the protein (Fig. 1B). *S. cerevisiae* possesses nine paralogous, metalloreductase-encoding genes which display different expression profiles: *FRE1* is upregulated by iron and copper depletion, *FRE2* to *FRE6* are upregulated by iron starvation only, *FRE7* is specifically upregulated by copper limitation, and YGL160w and YLR047c are regulated by neither copper nor iron availability (12, 19). Iron regulation of these genes is mediated by Aft1p, and copper regulation is mediated by Mac1p. Fre1p to Fre4p are involved in reduction of siderophore-bound and unbound iron (5, 34), Fre1p and Fre2p additionally function in copper uptake (11), and the function of Fre5p to Fre7p is unknown. To study the expression pattern of *freA*, *A. nidulans* wild-type and *sreA* deletion strains were grown for 24 h at 37°C under standard conditions, iron limitation, and copper starvation as described previously (20). Northern blot analysis revealed that the *A. nidulans freA* expression pattern resembles that of *Saccharomyces FRE2* to *FRE4* by being iron but not copper regulated (Fig. 2). These data indicate that *Aspergillus* FREA is involved in securing iron homeostasis. It might be a component of a possible reductive iron assimilation system or function as an intracellular metalloreductase. In contrast to typical members of the SREA regulon, e.g., *mirA*, SREA deficiency did not lead to derepressed *freA* expression under iron-replete conditions. These data show that in *A. nidulans* an iron-regulatory mechanism exists which does not involve SREA.

Furthermore, SREA-independent expression of *freA* confirms that SREA indeed acts as a direct repressor of extracellular siderophore biosynthesis and uptake. SREA deficiency results in 20-fold-increased accumulation of the intracellular siderophore ferricrocin during iron-replete growth (21). Therefore, it could have been alternatively hypothesized that SREA acts only as a repressor of ferricrocin biosynthesis and that SREA deficiency causes iron deprivation via sequestration of intracellular iron. But in this case, the expression of all iron starvation-induced genes, including *freA*, would be expected to be upregulated under iron-replete conditions in an *sreA* deletion strain.

Iron depletion can lead to upregulation of expression, as in the case of genes involved in high-affinity iron uptake. But the opposite regulatory pattern can also be found: expression of

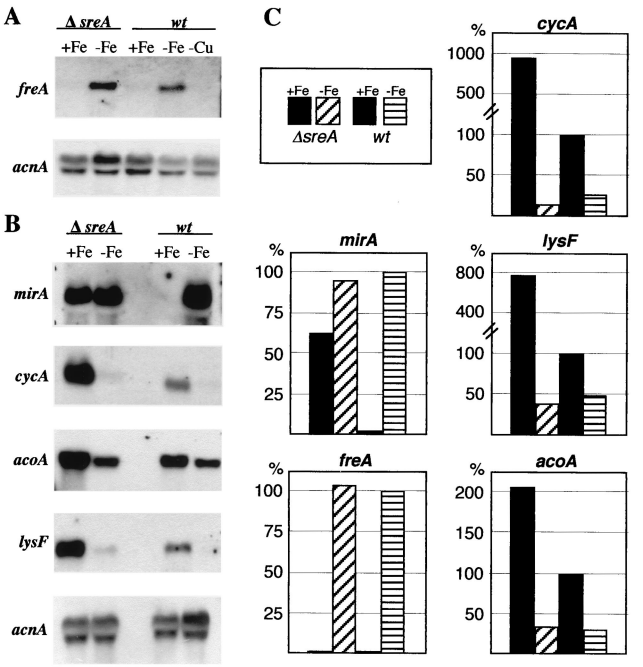

FIG. 2. Expression of *freA*, *mirA*, *cycA*, *acoA*, and *lysF* under standard (+Fe), iron depletion (−Fe), and copper depletion (−Cu) conditions in *A. nidulans* wild-type (*wt*) and SREA-deficient (Δ*sreA*) strains. Fungal strains were grown for 24 h in minimal medium containing 10 μm $FeSO_4$ and 10 μm $CuSO_4$ as described previously (20); for iron- and copper-depleted growth the addition of the respective metal was omitted. As a control for loading and RNA quality, blots were hybridized with the γ-actin-encoding *acnA* gene (6). (A and B) Northern blot analysis was performed with 1 μg of mRNA (A) or 10 μg of total RNA (B), respectively. (C) Quantification of mRNA levels normalized to *acnA* levels with a PhosphorImager. Bars represent mean values of two independent experiments; standard deviations did not exceed 20%.

catB, encoding a heme-containing catalase, is downregulated at the transcript level under iron starvation (21). To investigate if this regulatory pattern is specific for *catB* or holds for other proteins in need of iron-containing cofactors, the expression of the genes encoding the iron-sulfur cluster containing aconitase (*acoA*) and homoaconitase (*lysF*), as well as the heme-containing cytochrome *c* (*cycA*), was studied (23, 30). For partial analysis of the putative *A. nidulans* aconitase gene *acoC*, the expressed sequence tag clone c8d09 was sequenced. It contains the C-terminal 398 amino acids of ACOC displaying 88 and 73% identity to the aconitases of *Aspergillus terreus* and *S. cerevisiae*, respectively. Northern blot analysis proved that expression of genes involved in pathways as distinct as the citric acid cycle (*acoA*) and respiration (*cycA*), as well as lysine and penicillin biosynthesis (*lysF*), is downregulated between two- and eightfold under iron limitation in the wild-type and the SREA-deficient strains (Fig. 2). With an eightfold-decreased transcript level, *cycA* was the gene most dramatically affected by iron depletion. Notably, CYCA-deficient *A. nidulans* mutants are viable, and it was suggested previously that this is due to the ability of *Aspergillus* to ferment and to use alternative respiratory pathways (3). Taken together, these data suggest that, during iron depletion, decreased expression of *cycA* saves energy and iron for other processes essential for survival under iron limitation. Assuming that FREA is involved in iron homeostasis, as has been shown previously for four of the six iron-regulated *S. cerevisiae* paralogues (5, 10, 34), the opposite regulation of *freA* versus *acoA*, *lysF*, and *cycA* by iron availability suggests that under iron depletion the flow of this limiting metal might be directed from various metabolic pathways to systems needed to secure iron homeostasis.

Interestingly, the transcript levels of *acoA*, *lysF*, and *cycA* were elevated between two- (*acoA*) and ninefold (*cycA*) under iron-replete conditions in the *sreA* deletion strain (Fig. 2). Therefore, expression of these genes might be subject to SREA regulation. Alternatively, upregulation of these genes might be caused indirectly since SREA deficiency leads to increased iron accumulation and increased oxidative stress (21): (i) it may reflect the increased bioavailability of iron within SREA-deficient cells, or (ii) it may represent an oxidative stress response. In the latter case, the increased expression of these genes could represent a compensatory response invoked to maintain cellular enzyme activities because, e.g., iron-sulfur cluster-containing enzymes are particularly sensitive to inactivation by oxidative attack (9). In this respect it is noteworthy that, in *Escherichia coli*, expression of aconitase-encoding *acnA* is specifically induced by iron and oxidative stress (13), and it was suggested previously that the aconitase proteins serve as a protective buffer against oxidative stress by acting as a sink for reactive oxygen species (28). The upregulation of *cycA* expression might also be a response to oxidative stress because cytochrome *c* plays an important role in the antioxidant system of mitochondria (26). Remarkably, the promoter region of *lysF* contains several GATA motifs which potentially represent SREA binding sites. But since mutational analysis showed that at least two of these GATA sites mediate a positive effect on *lysF* expression, a direct involvement of the repressor SREA seems to be doubtful (31).

Hybridization probes. The hybridization probes used in this study were generated by PCR with oligonucleotides 5′-AGCC CGGTGTGAAAAGAG and 5′-AACAGGAGGAGGATTG CGCC for *mirA*, 5′-AGATCATGGGAGTTGACCTG and 5′-AGACGGATTGTATGGCGATGAG for *freA*, 5′-ACCCTTT CTCTCTACCTC and 5′-CGCGATTAGACGAGATAA for *cycA*, 5′-TATCCATGTAGTCCGCCC and 5′-GGTCCCACT GTCCAATGC for *acoA*, 5′-GCTGACGAACGAAGAAG and 5′-GCGTTCTTAACCCATTTC for *lysF*, and 5′-CGGTG ATGAGGCACAGT and 5′-CGGACGTCGACATCACA for γ-actin-encoding *acnA*.

Nucleotide sequence accession number. The *freA* and *acnA* sequences were assigned GenBank accession no. AF515629 and AF515630, respectively.

We are grateful to Bruce A. Roe et al. for the information supplied by the *A. nidulans* cDNA sequencing project and to the Whitehead Institute/MIT Center for Genome Research for access to the *N. crassa* genome sequence, as well as to the Sanger Institute and its collaborators David Denning and Andrew Brass at the University of Manchester for access to the *A. fumigatus* genome sequence. We thank Axel Brakhage for a plasmid containing a *lysF* fragment.

This project was supported by Austrian Science Foundation grant FWF-P13202-MOB (to H.H.).

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