The distinct methods by which manganese and iron regulate the Nramp transporters in yeast

Matthew E. PORTNOY^{*1,2}, Laran T. JENSEN^{†2} and Valeria Cizewski CULOTTA^{*†3}

^{*}Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, 615 N. Wolfe St., Baltimore, MD 21205, U.S.A., and [†]Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, 615 N. Wolfe St., Baltimore, MD 21205, U.S.A.

The bakers yeast *Saccharomyces cerevisiae* expresses three Smf metal transport proteins that are differentially regulated by metal ions. Smf1p and Smf2p are regulated at the post-translational level by manganese, whereas Smf3p is regulated by iron through a mechanism that, up until now, was unknown. Through promoter and protein-domain swapping experiments, we now demonstrate that the manganese regulation of Smf1p involves an internal protein-coding region that is separate from the N-terminal domain of this transporter. By comparison, iron regulation of Smf3p involves the upstream non-coding region of the gene. Using *SMF3-lacZ* reporter constructs, we identified two distinct regions of the *SMF3* promoter that contribute to iron regulation: (1) approx. nt –435 to –350 that contain dual

consensus recognition sites for the Aft1 iron transcription factor; and (2) nt –348 to –247 that do not contain obvious Aft1 binding sites. The –348 to –247 region by itself can confer strong iron regulation to the heterologous *CYC1* core promoter, and therefore harbours a putative upstream activating sequence for iron. Iron regulation of *SMF3* was dramatically reduced, but not completely eliminated, in strains lacking both the *AFT1* and *AFT2* iron regulatory factors. Together with the promoter mapping studies, these results suggest that both Aft-dependent and Aft-independent pathways may contribute to iron regulation of *SMF3*.

Key words: Aft1, Smf1, Smf3, metals.

INTRODUCTION

Nramp represents a large family of metal transporters that have been well conserved from bacteria to mammals [1]. For the most part, these proteins have been proposed to play physiological roles in the transport of manganese and iron [2–5]. However, it appears that they also have the capacity for transporting a host of divalent cations, including nickel, zinc, copper, cobalt and cadmium [6,7]. How is it then that Nramp transporters maintain specificity for their cognate metal? One likely possibility involves regulated expression of the transport protein.

An emerging theme is that many Nramp transporters are regulated by their physiological metal ion substrate. For example, mammalian divalent metal transporter [DMT1; also known as divalent cation transporter ('DCT1')/Nramp2] is believed to function primarily in iron uptake and distribution, and, accordingly, DMT1 expression is highly induced by iron starvation conditions [2,3,6]. Likewise, the plant *Arabidopsis thaliana* *Nramp1* as well as *Nramp3* and *Nramp4* genes are induced by iron deprivation, and the encoded proteins are believed to function as iron transporters in plant cells [8,9]. The bakers yeast, *Saccharomyces cerevisiae*, expresses three Nramp transporters (Smf1p, Smf2p and Smf3p) that are differentially regulated by iron and manganese. Smf1p and Smf2p function in manganese uptake and trafficking [4,10,11], and levels of both transport proteins are induced by manganese starvation, and to a lesser extent by iron starvation [12–14]. By comparison, Smf3p shows no regulation by manganese, but is strongly induced under

conditions of iron starvation. We have localized Smf3p to the vacuolar membrane of yeast, and genetic studies indicate that this Nramp transporter helps mobilize vacuolar stores of iron [14].

What accounts for the differential regulation of yeast Nramp transporters by manganese and iron? In the case of Smf1p and Smf2p, regulation by manganese occurs at the level of protein stability and protein trafficking through the secretory pathway. When cells have ample manganese, Smf1p and Smf2p are targeted to the vacuole for degradation and this movement to the vacuole involves the product of the *BSD2* gene [12–14]. Conversely, when cells are starved of manganese, Smf1p and Smf2p fail to arrive at the vacuole and the transporters accumulate to high levels, Smf1p at the cell surface and Smf2p in intracellular vesicles [14]. By comparison, Smf3p is not recognized by Bsd2p for vacuolar degradation, in spite of the high degree of homology among the three Smf proteins (approx. 50% identity at the amino acid level). Notably, Smf1p and Smf2p do contain an approx. 60-amino-acid extension at their N-termini that is absent in Smf3p [14]. Whether or not this accounts for the differential response to manganese is unclear. Likewise, it is not clear how Smf3p responds to iron. Unlike the case with manganese and Smf1p/Smf2p, iron starvation does not affect Smf3p localization or protein turnover [14].

The goal of the present study was to help elucidate the mechanism underlying the disparate regulation of Smf1p/Smf2p and Smf3p. The studies in the present paper show that the N-terminal extension of Smf1p is not responsible for mediating

Abbreviations used: BPS, bathophenanthroline-disulphonic acid; DMT1, divalent metal transporter; HA, haemagglutinin; MDM, metal-depleted minimal defined medium; SD, synthetic dextrose; SD/LF, SD/low iron; UAS, upstream activating sequence.

¹ Present address: Genome Technology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, U.S.A.

² These authors contributed equally to this work.

³ To whom correspondence should be addressed (e-mail vciculotta@jhps.edu).

manganese regulation, but rather protein sequences within the central coding region facilitate control by manganese and Bsd2p. By comparison, iron regulation of Smf3p is transcriptional and involves a complex promoter region of the *SMF3* gene.

MATERIALS AND METHODS

Yeast strains, media and growth conditions

The parental strain BY4741 and the *aft1Δ* derivative 1090 were purchased from Research Genetics (Huntsville, AL, U.S.A.). *AFT1* was disrupted in BY4741 and 1090 using plasmid pLJ176 linearized with *HindIII*, resulting in strains LJ194 (*aft1Δ*) and MP127 (*aft1Δaft2Δ*). The *bsd2Δ* strain XL115 and the *pep4Δ* mutant XL126 were derived from AA255 [12], and the isogenic YPH250 and YPH250Δ*aft1* strains used for Northern-blot analysis were as previously described [15]. Stocks of strains were maintained on standard yeast extract/peptone/dextrose ('YPD') medium [16], and experimental trials involved either synthetic dextrose (SD) medium [16], or two types of metal-limiting medium as follows. A metal-depleted minimal defined medium (MDM) was prepared through use of an ion-exchange resin [13,17] and was supplemented with 2.4 mM MgSO_4 , 13 mM KCl, 2.0 mM CaCl_2 , 0.86 mM NaCl and 0.36 mM ZnCl_2 . When needed, 10 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and/or 10 μM MnSO_4 were added. Alternatively, an SD/low iron (SD/LF) medium was prepared in a manner similar to standard SD medium [16], except that a specialized yeast nitrogen base not supplemented with metals (BIO 101, Vista, CA, U.S.A.) was utilized. The final SD/LF medium was supplemented with 4.2 mM MgSO_4 , 1.4 mM CaCl_2 , 3.2 μM MnCl_2 , 2.9 μM ZnCl_2 and 250 nM CuSO_4 .

Molecular biology

To create the *SMF3-SMF1* plasmid pMP059, a *BamHI* restriction site was introduced by site-directed mutagenesis (Quikchange; Stratagene) at *SMF1* positions -2 to +4 in plasmid pSF10 [13]. The plasmid was then digested with *BamHI* and *XhoI* to remove the upstream non-coding region of *SMF1*, which was replaced by ligation with nt -530 to +3 of *SMF3* (obtained by PCR amplification with *XhoI*- and *BamHI*-modified primers). The resulting plasmid contained *SMF3* nt -530 to the start codon fused in-frame with the full coding sequence of *SMF1* and two copies of a haemagglutinin (HA) epitope at the C-terminus, all contained on a CEN, *URA3* vector. By a similar strategy, two *SMF1-SMF3* fusion constructs were obtained that were expressed either from a 2 μm , *LEU2* (pMP051) or a CEN, *URA3* (pMP058) plasmid. A *BamHI* site was introduced at *SMF3* position +6 (with respect to the start codon) in the Smf3-HA expressing plasmids pMP043 and pMP054 (for pMP051 and pMP058 respectively) [14], and the plasmids were digested with *BamHI* and *XbaI* to excise the upstream non-coding region of *SMF3*. This was replaced with *SMF1* nt -202 to +195 amplified by PCR using primers engineered with terminal *XbaI* and *BamHI* sites. The resulting plasmids harboured *SMF1* upstream nt -202 to +195 fused in-frame with *SMF3* nt +6 to the stop codon. The integrity of all the plasmids was confirmed by DNA sequencing.

To construct the *aft1Δ::LEU2* plasmid, two *AFT1*-containing fragments that span nt -914 to -384 and +311 to +1723 were removed from vector AFT1up1-313 by digestion with *HindIII* and *XhoI*, and with *BamHI* and *HindIII* respectively. The restriction fragments were then ligated in a trimolecular reaction to the *XhoI* and *BamHI* sites of the *LEU2* integrating vector pRS305 [18]. The resulting plasmid, pLJ176, was linearized with

HindIII and used to delete chromosomal *AFT1* gene nt -384 to +311.

SMF3-lacZ promoter fusions were constructed either in the background of plasmid pLGA178 [19] or Yep357R [20], both 2 μm , *URA3 lacZ* expression vectors. To create a *SMF3-lacZ* fusion in pLGA178, *SMF3* upstream non-coding sequences were amplified by PCR using primers engineered with *XhoI* and *BamHI* restriction sites at positions -533 and +5. The PCR product was then digested with these enzymes and ligated into *XhoI*- and *BamHI*-digested pLGA178, creating pMP065. Plasmid pLJ060 is a derivative of pMP065 in which the potential Aft1p binding site from -361 to -353 was altered by site-directed mutagenesis (QuikChange; Stratagene) from CGCACCCT to GCTGCAGT resulting in vector pLJ060. To generate the construct containing *SMF3* nt -348 to -247 fused to the *CYC1* core promoter, this region of *SMF3* was PCR amplified introducing *SalI* and *XbaI* sites; the PCR product was then ligated at these same sites into vector pNB404 [21], resulting in pLJ053. The series of 5' truncation derivatives of the *SMF3* promoter were created in the background of vector Yep357R. For this, *SMF3* upstream non-coding sequences were amplified by PCR with *XbaI* and *KpnI* restriction sites engineered at positions -528 and +15 respectively. The PCR product was digested with *XbaI* and *KpnI*, and ligated at these sites in-frame with *lacZ* in YEp357R, creating pMP060. *XbaI* sites were then introduced at *SMF3* positions -452, -331 and -256. The mutant plasmids were then digested with *XbaI* and self-ligated to generate a set of nested 5' deletion truncations of *SMF3* called pMP061, pMP062 and pMP063 (for truncations at positions -452, -331 and -256 respectively).

Biochemical assays

Western-blot and indirect immunofluorescence microscopy analyses were conducted precisely as described previously [14]. Northern-blot analysis utilized a *SMF3* ^{32}P -radiolabelled probe spanning +418 to +1419. Total RNA was prepared and 10 μg of RNA was subjected to formaldehyde gel electrophoresis as previously described [14], yet a distinct hybridization and washing protocol was followed. Specifically, an aqueous 'Church buffer' [22] was used in place of the earlier Denhardt's/formamide solution [23], and more stringent washing conditions (0.2 $\times$ SSC and 65 $^{\circ}\text{C}$, rather than 1 $\times$ SSC; where 1 $\times$ SSC corresponds to 0.15 M NaCl/0.015 M sodium citrate) were employed. These modifications proved effective in reducing background and improving specific detection of *SMF3* RNA without interference from the homologous *SMF1* and *SMF2* transcripts (confirmed through studies of *SMF3* mRNA in *smf3Δ*, *smf1Δ* and *smf2Δ* strains).

For measurements of β -galactosidase activity, cells were grown overnight in SD/LF medium, supplemented as needed with either 100 μM bathophenanthroline-disulphonic acid (BPS) or 1.6 μM FeCl_2 . Cultures of duplicate transformants were harvested, washed in Z buffer (85 mM Na_2HPO_4 , 45 mM NaH_2PO_4 , 10 mM KCl and 1 mM MgSO_4) and cell lysates were obtained by glass bead homogenization. β -Galactosidase activity was then measured using *o*-nitrophenyl β -D-galactopyranoside as the substrate, and results were converted into Miller units [24].

The start site of transcription for *SMF3* was determined in strain AA255 transformed with the *SMF3-lacZ* expression vector pMP065. Total RNA prepared from cells grown in MDM was subjected to primer extension analysis using the Promega system according to the manufacturer's instructions. RNA (35 μg) was mixed with a 5' ^{32}P -radiolabelled primer spanning *SMF3* nt +5

to -25 . Denaturation proceeded at 95°C for 5 min, followed by annealing at 42°C for 1 h. Reverse transcriptase (Promega) was added and the reaction was incubated for 30 min at 42°C . The nucleic acid samples were resolved by electrophoresis on an 8% (w/v) polyacrylamide/7 M urea sequencing gel and were subjected to autoradiography.

RESULTS

Discerning the manganese and iron regulatory regions of *SMF1* and *SMF3*

SMF1 is subject to down-regulation by manganese, whereas *SMF3* is not [14]. To demonstrate that the coding region of *SMF1* is all that is required for this regulation, we constructed a *SMF3-SMF1* fusion in which the upstream non-coding region of *SMF1* was removed and replaced with the *SMF3* promoter (nt -530 to $+3$). The Smf1 polypeptide in this case also harboured an HA epitope tag [25], as described previously [13], to facilitate protein immunodetection. As seen in the Western blots shown in Figures 1(A) and 1(B), Smf1-HA expressed from the *SMF3* promoter in the *SMF3-SMF1* construct exhibited the same regulation pattern as native Smf1p. Protein levels were greatly enhanced in *bsd2* mutants (Figure 1A) and were reduced in cells treated with manganese (Figure 1B). Therefore the manganese and Bsd2p regulation of *SMF1* requires only the coding sequence and not the 5' untranslated region of this gene.

The most obvious difference in the coding regions of *SMF1* and *SMF3* is an approx. 60-residue N-terminal extension that is absent in Smf3p [14]. To test whether this sequence accounts for manganese regulation, a fusion construct was obtained in which the first 61 amino acids of Smf1p were fused in frame to the N-terminus of Smf3p also containing an HA-epitope tag [14]. As seen in Figure 1(A), the fusion protein expressed from *SMF1-SMF3* accumulated to high levels under metal replete conditions, yet was not significantly affected by mutations in *BSD2*. Furthermore, this chimaeric protein showed no regulation by manganese (Figure 1B). Therefore the N-terminal extension of Smf1p by itself cannot confer regulation by Bsd2p and manganese.

We have also tested whether the Smf1p N-terminus can localize Smf3p to the plasma membrane when cells are starved of manganese, as is normally seen with Smf1p [12–14]. Localization of the fusion protein expressed from *SMF1-SMF3* was examined by immunofluorescence microscopy using a *pep4* mutant strain defective in vacuolar proteases. The *pep4* mutant facilitates detection of any protein that would be degraded in the vacuolar lumen (as with Smf1p during manganese replete conditions [13]). We found that the bulk of the fusion protein expressed from *SMF1-SMF3* does not localize to the vacuolar lumen or to the plasma membrane, but exhibits a vacuolar rim localization, even under conditions of metal starvation (Figure 1C). This is precisely the same localization pattern we have reported for native Smf3p [14]. Together with the results of Figures 1(A) and 1(B), we

Figure 1 Metal regulation of Smf fusion constructs

(A) The wild-type (WT) strain AA255 or isogenic *bsd2* Δ mutant XL115 were transformed with either the *SMF3-SMF1* plasmid pMP059 (Smf1-HA under the control of the *SMF3* promoter) or the *SMF1-SMF3* plasmid pMP054 (*SMF1* nt -202 to $+195$ fused to the coding region of Smf3-HA). Cells were grown in SD medium to stationary phase and lysates were prepared and analysed by Western blotting as described previously [14] using an anti-HA antibody. (B) Strain AA255 was transformed with the designated plasmids and was grown to stationary phase in MDM supplemented with either $10\ \mu\text{M}$ MnSO_4 (+Mn) or $10\ \mu\text{M}$ $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (+Fe), or MDM without metal supplementation ($-\text{Fe/Mn}$). Lysates were prepared and analysed by Western blotting as in (A). Plasmids utilized: *SMF1*, Smf1-HA expressing plasmid pSF10 [13]; *SMF3*, Smf3-HA expression plasmid pMP054 [14]; *SMF3-SMF1* and *SMF1-SMF3* are as described in (A). (C) The *SMF1-SMF3* plasmid pMP051 was transformed into the *pep4* Δ strain XL126. Cells were grown overnight in MDM containing no metal additions and were fixed and subjected to immunofluorescence microscopy using an anti-HA antibody and a secondary antibody conjugated to FITC as described previously [14]. Vacuoles were visualized as indentations using Nomarski (DIC) optics.

Figure 2 Northern-blot analysis of *SMF3* gene expression

The indicated strains were grown overnight to confluency in MDM that was supplemented where indicated with 10 μ M $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (+Fe). Total RNA was isolated and subjected to Northern-blot analysis as described in the Materials and methods section. The blot was sequentially hybridized with probes to *SMF3* and to *ACT1* as a loading control. Strains utilized: wild-type (WT), YPH250; *aft1* null, YPH250 Δ .

conclude that the Smf1p N-terminal extension is not responsible for the degradation of Smf1p when manganese is abundant, or Smf1p stabilization at the cell surface when manganese is sparse. Instead, sequences within the internal coding region of Smf1p must be responsible for manganese regulation.

The *SMF1*–*SMF3* and *SMF3*–*SMF1* fusion constructs were also used to study regulation of Smf3p by iron. As shown in Figure 1(B), the fusion protein expressed from *SMF1*–*SMF3* was not down-regulated by iron, even though the full Smf3p coding region was intact. By comparison, Smf1–HA expressed from the *SMF3* promoter (in the *SMF3*–*SMF1* fusion) exhibited strong down-regulation by iron, similar to the level seen with Smf3–HA (Figure 1B), but unlike the weak iron regulation of Smf1–HA (Figure 1B and [13]). In fact, *SMF3*–*SMF1* exhibited the metal regulation properties of both *SMF1* and *SMF3*, i.e. moderately repressed by manganese and very strongly repressed by iron. Therefore, while the response to manganese involves the internal protein coding region of *SMF1*, the response to iron involves the upstream non-coding region of *SMF3*.

Iron regulation of *SMF3* mRNA

Since the upstream non-coding region of *SMF3* confers iron regulation, it seemed likely that *SMF3* is regulated at the transcriptional level. Originally, we failed to detect changes in *SMF3* transcript levels with iron starvation [14]. However, with our current detection system for *SMF3* mRNA (see the Materials and methods section) we now consistently observe an increase in abundance of the message during iron starvation (Figure 2). Therefore iron regulation of *SMF3* occurs, at least in part, through changes in *SMF3* mRNA levels.

In *S. cerevisiae*, a wide array of iron homeostasis genes are known to be regulated at the mRNA level by the iron regulatory transcription factor Aft1p [26,27]. *SMF3* contains in its upstream non-coding region, a pair of Aft1p consensus binding sequences at positions –431 to –423, and –361 to –353. However, in our previous studies, the Smf3–HA protein was still induced by iron deprivation and repressed by iron in yeast mutants for *AFT1* [14]. As can be seen in Figure 2, the *aft1* Δ yeast mutant also exhibited iron-repression of *SMF3* at the mRNA level. These

Figure 3 Iron regulation of a *SMF3*–*lacZ* reporter

Strain BY4741 was transformed with the *SMF3*–*lacZ* expression plasmid pMP065 (top) or the same plasmid containing a mutation in the putative Aft1p binding sequence at position –361 (marked by 'X'; bottom). Cells were grown to stationary phase in SD/LF medium that was supplemented where indicated with either 100 μ M BPS or 1.6 μ M FeCl_2 . Cell lysates were prepared and measurements of β -galactosidase activity were made as described in the Materials and methods section. Results represent the means of four independent trials with two separate transformants ($\pm$ S.D.). Boxes represent putative binding sites for Aft1p in the *SMF3* gene promoter.

studies would suggest that Aft1p alone is not critical for iron regulation of *SMF3*.

SMF3 promoter sequences responsible for iron regulation

To examine more closely the gene sequences involved in iron regulation of *SMF3* we utilized a *lacZ* reporter system. *SMF3* nt –533 to +5 were fused to the coding sequence of bacterial *lacZ* such that expression could be monitored by β -galactosidase measurements. When expressed in yeast cells, LacZ reporter activity was strongly induced in cells treated with the iron chelator BPS and was repressed in cells treated with iron (Figure 3). Iron repression of this construct was observed whether cells were grown in SD/LF medium (as in Figure 3) or in MDM (results not shown).

We previously noted a consensus sequence for Aft1p binding at position –431 in the *SMF3* promoter [14]. We mutated this sequence in a *SMF3*–*lacZ* fusion and found no discernable effect on iron regulation (results not shown). In addition to this site, there is a second candidate binding site for Aft1p at position –361. As seen in Figure 3, mutation of this site from GCACCC to GCTGCA resulted in an overall decrease in expression of *SMF3*–*lacZ*. Iron regulation was still apparent, but the degree of induction with BPS compared with iron-treated cells was reduced by approx. 4-fold. Therefore the consensus Aft1p binding site at position –361 contributes to *SMF3* promoter activity, but is not the sole element required for iron regulation.

To begin to search for the iron regulatory sequences for *SMF3*, a series of 5' promoter truncations were constructed. The truncations examined are shown in Figure 4(A). The full-length promoter extends 5' to position –528, with respect to the start codon. The series of truncation derivatives contained deletions to positions –452, –331 and –256. The –528 and –452 constructs contain both of the putative Aft1p sites, while the remaining two truncations lack these sites (Figure 4A). All contain a putative TATA box and the start site for *SMF3* transcription, approximately 60 bp upstream of the translational start site (as defined by primer extension analysis; see the Materials and methods section). These constructs were assayed for LacZ reporter activity in the SD/LF medium and in the same medium supplemented with either BPS or iron.

As seen in Figure 4(A), deletion to –452 had no major effect on expression levels or iron regulation, whereas deletion to –331 caused a significant increase in overall expression of the *SMF3*–*lacZ* fusion. This 5' –331 construct lacking both of the Aft1p

Figure 4 Promoter sequences that mediate iron regulation of *SMF3*

Strain BY4741 was transformed with the indicated *SMF3-lacZ* expression plasmids and β -galactosidase measurements were made as described in Figure 3. (A) Plasmids tested: —528, pMP060; —452, pMP061; —331, pMP062; —256, pMP063. (B) Plasmid pLJ053 was tested, in which *SMF3* nt —348 to —247 were fused to the core promoter of *CYC1*. Boxes represent putative Aft1 sites and arrows mark the start site and direction of transcription.

consensus sequences still exhibited iron regulation, however, the fold induction with BPS compared with iron treatment was compromised (40-fold compared with 10-fold with the —528 and —331 constructs respectively). Lastly, the deletion to —256 resulted in a complete loss of induction by iron depletion (Figure 4A). Therefore iron regulation of *SMF3* involves two regions of the promoter: nt —452 to —331, harbouring the putative Aft1 sites; and nt —331 to —256, lacking potential sites for Aft1.

We tested whether the sequences surrounding —331 to —256 in fact contain an iron-responsive upstream activating sequence (UAS). A chimaeric promoter was constructed in which *SMF3* nt —348 to —247 were fused to the core promoter (TATA box and start site for transcription) for the *S. cerevisiae* *CYC1* gene. As seen in Figure 4(B), this region of the *SMF3* promoter conferred strong iron-regulated expression to the *CYC1-lacZ* reporter. Therefore sequences contained within —348 to —247 appear to harbour a novel UAS for iron regulation of *SMF3*.

Aft1p and Aft2p transcription factors

While these studies were in progress, a second iron regulatory factor was identified for *S. cerevisiae*. This factor, known as Aft2p, bears significant homology to Aft1p and also confers iron regulation to a variety of yeast genes involved in iron homeostasis [28,29]. Although our studies with *aft1Δ* strains have helped to exclude Aft1p as the only regulator of *SMF3* (Figure 2 and [14]), it remained possible that Aft2p was involved. We therefore monitored iron regulation of *SMF3-lacZ* in *aft1Δ*, *aft2Δ* and *aft1Δaft2Δ* double-null strains. For these experiments, strains were grown in either SD/LF medium with no iron or the same medium supplemented with iron. BPS treatment was omitted because *aft1Δaft2Δ* strains fail to grow under severe iron depletion conditions (results not shown and [28,29]). Even without the addition of BPS, we could observe a strong induction of *SMF3-lacZ* in wild-type cells that were not treated with iron

Figure 5 Effects of deleting *AFT1* and *AFT2* on *SMF3* promoter activity

The indicated strains were transformed with the pMP065 *SMF3-lacZ* expression plasmid and were grown in SD/LF medium supplemented with 1.6 μ M FeCl_2 where indicated. β -Galactosidase measurements were made as described in Figure 3. Strains utilized: wild-type (WT), BY4741; $\Delta aft1 \Delta aft2$, MP127.

(Figure 5), and such iron regulation was still evident in strains lacking either *AFT1* or *AFT2* (results not shown). However, in the *aft1Δaft2Δ* double mutant, induction of *SMF3-lacZ* by iron limitation was reduced to only 2–3-fold (Figure 5). These studies demonstrate that at least one of the Aft factors is needed for efficient expression and iron regulation of *SMF3*. However, the residual iron regulation in the *aft1Δaft2Δ* strain is suggestive of an additional tier of *SMF3* gene control.

DISCUSSION

The goal of the present study was to begin to understand the differential regulation of yeast Nramp metal transporters by manganese and by iron. *S. cerevisiae* Smf1p is regulated primarily by manganese, whereas Smf3p levels are controlled by iron. Through promoter and protein-domain swapping experiments, we demonstrate that the sequences for iron regulation of *SMF3* lie within the upstream non-coding region of the gene. By comparison, the manganese regulation of *SMF1* does not involve 5' promoter sequences, or the N-terminal extension of Smf1p that is lacking in Smf3p. Instead, sequences internal to the Smf1 protein serve as a target for regulation by Bsd2p and manganese. Although the nature of this protein sequence is not known, it should be common to Smf1p and Smf2p, but absent in Smf3p. By amino acid sequence alignment, the three yeast Nramp transporters show vast overall homology [14]. Yet there are some noteworthy differences. For example, the cytosolic loops between transmembrane segments TM6 and TM7 and between TM10 and TM11 are somewhat smaller in Smf3p compared with Smf1p and Smf2p [14]. Further protein-domain swapping experiments should help to discern the differential effects of manganese on Smf1p and Smf2p compared with Smf3p.

The iron regulation of *SMF3* occurs at the mRNA level and seems quite complex. On the one hand, there is clearly a role for the Aft1p and Aft2p iron regulatory factors. There are two potential Aft binding sites in the *SMF3* promoter, and mutating one of these (at site —361), or deleting the entire region surrounding both sites (5' deletion to —331), resulted in a reduction of iron regulation. And in an *aft1Δaft2Δ* double-null strain, iron regulation of the *SMF3* promoter was dramatically reduced, although not eliminated. These effects are consistent with recent microarray experiments in which *SMF3*, but not *SMF1* or *SMF2*, was up-regulated in strains expressing constitutively active alleles of either Aft1p (C. Phillpott, personal communication) or Aft2p [28]. Yet, in apparent contradiction of these findings, we noted that a *SMF3* promoter lacking the Aft1 consensus sites (5' deletion to —331) still exhibited substantial

iron regulation. In fact, *SMF3* nt –348 to –247 harbour an apparently strong UAS for iron, with no notable binding sites for the Aft factors.

The *trans*-acting factor(s) that recognizes the putative iron UAS at –348 to –247 is not known. One possibility is that it might be Aft1p or Aft2p. However, we consider this possibility unlikely, as this region is devoid of any Aft-like binding sites [27,29], and we observed a consistent, albeit much reduced, level of iron regulation in the *aft1Δaft2Δ* null mutant. Furthermore, the *FET4* gene of yeast, encoding a cell surface iron transporter, also appears to be regulated by iron in a non-Aft1 manner [30]. As an alternative possibility, the –348 to –247 region of *SMF3* may be recognized by a factor other than Aft1p and Aft2p. The ‘non-Aft’ factor itself may fall under Aft control because iron regulation is so greatly diminished in the *aft1Δaft2Δ* strain. A yeast genetic screen should prove invaluable for identifying this factor. In addition to this complicated regulation by iron, we recently noted that *SMF3* is regulated by oxygen via a separate set of regulatory factors and promoter sequences (L. T. Jensen and V. C. Culotta, unpublished work). The tight regulation of *SMF3* by iron atoms and the redox status of the cell provide further evidence that this particular member of the Nramp family has evolved to function primarily in iron homeostasis.

We are indebted to members of Dr Judith Bender's laboratory (Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, U.S.A.) for assistance with Northern-blot analysis and to Professor Dennis Winge (Department of Medicine and Biochemistry, University of Utah Health Science Center, Salt Lake City, UT, U.S.A.) for helpful discussions, as well as the AFT1up1-313 plasmid. This work was supported by the Johns Hopkins University National Institutes of Health Sciences (JHU NIEHS) Center and by National Institutes of Health grant ES 08996 (awarded to V.C.C.). M.E.P. was supported by an Environmental Protection Agency Star (EPA STAR) Fellowship (U915646).

REFERENCES

- Cellier, M., Prive, G., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W. and Gros, P. (1995) Nramp defines a family of membrane proteins. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10089–10093
- Fleming, M. D., Romano, M. A., Su, M. A., Garrick, L. M., Garrick, M. D. and Andrews, N. C. (1998) Nramp2 is mutated in the anemic Belgrade (*b*) rat: Evidence of a role for Nramp2 in endosomal iron transport. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1148–1153
- Forbes, J. R. and Gros, P. (2001) Divalent-metal transport by NRAMP proteins at the interface of host–pathogen interactions. *Trends Microbiol.* **9**, 397–403
- Supek, F., Supekova, L., Nelson, H. and Nelson, N. (1996) A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5105–5110
- Kehres, D. G., Zaharik, M. L., Finlay, B. B. and Maguire, M. E. (2000) The NRAMP proteins of *Salmonella typhimurium* and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. *Mol. Microbiol.* **36**, 1085–1100
- Gunshin, H., Mackenzie, B., Berger, U. V., Gushin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L. and Hediger, M. A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature (London)* **388**, 482–488
- Chen, X. Z., Peng, J. B., Cohen, A., Nelson, H., Nelson, N. and Hediger, M. A. (1999) Yeast SMF1 mediates H⁺-coupled iron uptake with concomitant uncoupled cation currents. *J. Biol. Chem.* **274**, 35089–35094
- Thomine, S., Wang, R., Ward, J. M., Crawford, N. M. and Schroeder, J. I. (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4991–4996
- Curie, C., Alonso, J. M., Jean, M. L., Ecker, J. R. and Briat, J. F. (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochem. J.* **347**, 749–755
- Cohen, A., Nelson, H. and Nelson, N. (2000) The family of SMF metal ion transporters in yeast cells. *J. Biol. Chem.* **275**, 33388–33394
- Luk, E. and Culotta, V. C. (2001) Manganese superoxide dismutase in *S. cerevisiae* acquires its metal co-factor through a pathway involving the Nramp metal transporter, Smf2p. *J. Biol. Chem.* **276**, 47556–47562
- Liu, X. F. and Culotta, V. C. (1999) Mutational analysis of *Saccharomyces cerevisiae* Smf1p, a member of the Nramp family of metal transporters. *J. Mol. Biol.* **289**, 885–891
- Liu, X. F. and Culotta, V. C. (1999) Post-translational control of Nramp metal transport in yeast: role of metal ions and the *BSD2* gene. *J. Biol. Chem.* **274**, 4863–4868
- Portnoy, M. E., Liu, X. F. and Culotta, V. C. (2000) *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the Nramp family of metal transporters. *Mol. Cell. Biol.* **20**, 7893–7902
- Lin, S. J., Pufahl, R., Dancis, A., O'Halloran, T. V. and Culotta, V. C. (1997) A role for the *Saccharomyces cerevisiae* *ATX1* gene in copper trafficking and iron transport. *J. Biol. Chem.* **272**, 9215–9220
- Sherman, F., Fink, G. R. and Lawrence, C. W. (1978) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Dancis, A., Klausner, R. D., Hinnebusch, A. G. and Barriocanal, J. G. (1990) Genetic evidence that ferric reductase is required for iron uptake in *S. cerevisiae*. *Mol. Cell. Biol.* **10**, 2294–2301
- Sikorski, R. S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27
- Guarente, L. and Mason, T. (1983) Heme regulates transcription of the *CYC1* gene of *S. cerevisiae* via an upstream activation site. *Cell (Cambridge, Mass.)* **32**, 1279–1286
- Myers, A. M., Tzagoloff, A., Kinney, D. M. and Lusty, C. J. (1986) Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. *Gene* **45**, 299–310
- Bachhawat, N. (1995) Functional characterization of an inositol-sensitive upstream activation sequence in yeast. *J. Biol. Chem.* **270**, 25087–25095
- Church, G. M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991–1995
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., vols. 1–3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Giacomini, A., Corich, V., Ollero, F. J., Squartini, A. and Nuti, M. P. (1992) Experimental conditions may affect reproducibility of the β -galactosidase assay. *FEMS Microbiol. Lett.* **79**, 87–90
- Wilson, I. A., Niman, N. H., Houghten, R. A., Cherenon, A. R., Connolly, M. L. and Lerner, R. A. (1984) The structure of an antigenic determinant in a protein. *Cell (Cambridge, Mass.)* **37**, 767–778
- Yamaguchi-Iwai, Y., Dancis, A. and Klausner, R. D. (1995) AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *EMBO J.* **14**, 1231–1239
- Yamaguchi-Iwai, Y., Stearman, R., Dancis, A. and Klausner, R. D. (1996) Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. *EMBO J.* **15**, 3377–3384
- Rutherford, J., Ray, E., Brown, P. O. and Winge, D. R. (2001) A second iron-regulatory system in yeast independent of Aft1. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14322–14327
- Blaiseau, P., Lesuisse, E. and Camadro, J. (2001) Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. *J. Biol. Chem.* **276**, 34221–34226
- Dix, D., Bridgman, J., Broderius, M. and Eide, D. (1997) Characterization of the FET4 protein of yeast. Evidence for a direct role in the transport of iron. *J. Biol. Chem.* **272**, 11770–11777

Received 24 September 2001/1 November 2001; accepted 29 November 2001