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 found 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...
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 afs2Δ 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 (afs1ΔA) and MP127 (afs1Δafs2Δ). The bsd2Δ strain XL115 and the pep4Δ mutant XL126 were derived from AA255 [12], and the isogenic YPH250 and YPH250Δaf1 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 MgSO4, 13 mM KCl, 2.0 mM CaCl2, 0.36 mM NaCl and 0.36 mM ZnCl2. When needed, 10 μM Fe(NH4)2(SO4)2 and/or 10 μM MnSO4 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 MgSO4, 1.4 mM CaCl2, 3.2 μM MnCl2, 2.9 μM ZnCl2 and 250 mM CuSO4.

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 afs1Δ::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 Xhol, 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 Yep535R [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 XhoI and KpnI restriction sites engineered with terminal positions 538 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 [32P]-radio labelled probe spanning +418 to +1491. 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× SSC and 65 °C, rather than 1× SSC; where 1× 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 FeCl3. Cultures of duplicate transformants were harvested, washed in Z buffer (85 mM Na2HPO4, 45 mM NaH2PO4, 10 mM KCl and 1 mM MgSO4) 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′ [32P]-radio labelled primer spanning SMF3 nt +5
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 chimera 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 bsd2Δ 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 μM MnSO₄ (+Mn) or 10 μM Fe(H₄N)₂(SO₄)₂ (+Fe), or MDM without metal supplementation (−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 pep4Δ 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.
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 null yeast mutant also exhibited iron-repression of SMF3 at the mRNA level. These studies would suggest that Aft1p alone is not critical for iron regulation of SMF3.

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 μM BPS or 1.6 μM FeCl3. 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 (+ S.D.). Boxes represent putative binding sites for Aft1p in the SMF3 gene promoter. 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 GCCACC 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
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 chimera promoter was constructed in which SMF3 nt −348 to −247 were fused to the core promoter of CYC1. 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), 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 S′ 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 Aft1Δfs mutants [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 Aft1Δfs 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.

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