Shu1 Is a Cell-surface Protein Involved in Iron Acquisition from Heme in Schizosaccharomyces pombe*

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Background: Organisms have evolved diverse strategies to scavenge extracellular iron.
Results: The iron-regulated cell-surface Shu1 protein serves as a novel component for heme acquisition.
Conclusion: This study is the first report to show that a member of Archaeomycetes can acquire exogenous heme.
Significance: Shu1 is a noncanonical heme-binding protein possessing a unique role.

Iron is an essential metal cofactor that is required for many biological processes. Eukaryotic cells have consequently developed different strategies for its acquisition. Until now, Schizosaccharomyces pombe was known to use reductive iron uptake and siderophore-bound iron transport to scavenge iron from the environment. Here, we report the identification of a gene designated shu1 that encodes a protein that enables S. pombe to take up extracellular heme for cell growth. When iron levels are low, the transcription of shu1 is induced, although its expression is repressed when iron levels rise. The iron-dependent down-regulation of shu1 requires the GATA-type transcriptional repressor Fep1, which strongly associates with a proximal promoter region of shu1 in vivo in response to iron repletion. HA4-tagged Shu1 localizes to the plasma membrane in cells expressing a functional shu1-HA allele. When heme biosynthesis is selectively blocked in mutated S. pombe cells, their ability to acquire exogenous hemin or the fluorescent heme analog zinc mesoporphyrin IX is dependent on the expression of Shu1. Further analysis by absorbance spectroscopy and hemin-agarose pulldown assays showed that Shu1 interacts with hemin, with a Kd of ~2.2 μM. Taken together, results reported here revealed that Shu1 possesses an unexpected pathway for heme assimilation, which may also serve as a source of iron for cell growth.

Eukaryotic cells are committed to acquire iron because it is an essential component of the active center of several proteins that are associated with fundamental processes (1–3). Iron is generally found in forms that are biologically unavailable; therefore, organisms have developed diverse strategies to scavenge it from their environment. Although two strategies, namely reductive iron uptake and siderophore-bound iron transport, have been mostly studied in fungi, heme is known to be an important source of iron. However, cellular components that are required for heme assimilation and iron acquisition from heme are still poorly understood (4, 5). One reason may be the fact that Saccharomyces cerevisiae, one of the most widely adopted yeast, is unable to transport exogenous heme with high affinity (6).

Heme is constituted of a protoporphyrin IX structure in which one atom of iron is coordinated by the imidazole rings (7). Typically, heme serves as a prosthetic group for many enzymes that are intimately linked to essential cellular functions such as respiration, antioxidative protection, and synthesis and degradation of several metabolites (8). Heme biosynthesis and heme uptake are part of two different means to acquire heme in several organisms. Heme biosynthesis has been extensively studied (7). It involves eight enzymes that are located either in mitochondria or the cytoplasm, depending where their specific action occurs along the biosynthetic pathway. In Schizosaccharomyces pombe, these eight enzymes are respectively encoded by the following genes: hem1, hem2, hem3, ups1, hem12, hem13, hem14, and hem15. Hem1 (or δ-aminolevulinate acid synthase) catalyzes the biosynthesis of δ-aminolevulinate (ALA) from succinyl-CoA and glycine. ALA is then used by a second enzyme (Hem2 or porphobilinogen synthase) of the biosynthetic pathway toward heme biosynthesis (involving six additional steps). In the absence of Hem1, biosynthesis of heme is blocked. The hem1Δ deletion is lethal for S. pombe (9), unless exogenous ALA is provided, allowing heme biosynthesis from step 2 to proceed. An additional way to maintain hem resulting from step 2 to proceed. An additional way to maintain hem1Δ cells alive is to add exogenous heme (heme chloride), thereby fostering cells to use their heme uptake system. The hem1Δ mutant strain can thus be used to selectively block heme biosynthesis by controlling growth medium composition (absence of ALA), thereby allowing the possibility of investigating the mechanism of exogenous heme acquisition.

A number of fungal proteins containing a common in fungal extracellular membrane (CFEM) domain have been reported to bind heme and facilitate its transport. A canonical eight-Cys-containing CFEM domain corresponds to PXC(A/G)X2CX2X8–12CX1–X(T/D)X2–3CX3–4CX3–4CX15–16C (where X

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2 The abbreviations used are: ALA, δ-aminolevulinate; Dip, 2,2′-dipyridyl; GPI, glycylosphatidylinositol; YES, yeast extract plus supplements; ZnMP, zinc (II) mesoporphyrin IX; PCNA, proliferating cell nuclear antigen; qPCR, quantitative PCR; CFEM, common in fungal extracellular membrane.
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Yeast Strains and Media—Genotypes of strains used in this study are described in Table 1. All strains were maintained on YES medium containing 0.5% yeast extract, 3% glucose, and 225 mg/liter of adenine, histidine, leucine, uracil, and lysine. In addition, hem1Δ deletion strains were supplemented with ALA (200 μM) under nonselective growth conditions. An alternative way to maintain hem1Δ cells alive in the absence of ALA was to add exogenous hemin (heme chloride, 0.075 mM), thereby fostering cells to use their heme uptake system instead of using their own heme biosynthesis pathway. Strains used for plasmid integration were grown in synthetic Edinburgh minimal medium in which specific amino acids were absent as required to promote a chromosomal integration event. In the case of gene expression profile analysis, S. pombe liquid cultures were seeded to an A600 of ~0.5, allowed to grow to exponential phase (A600 of ~0.9), and left untreated or were treated with 2,2′-dipyridyl (Dip, 250 μM) or FeCl₃ (100 μM) for 3 h in each case. For monitoring cell growth, pre-cultures of cells were carried out in YES medium containing 250 μM Dip to chelate any trace of inorganic iron and, at the same time, fostering Shu1 expres-

is any amino acid residue). In Candida albicans, two CFEM proteins, Rbt5 and Rbt51 (also denoted Pga10), have been shown to bind hemin and hemoglobin (13). However, only deletion of RBT5 (rbt5Δ) (not rbt51Δ) affects heme-iron acquisition, indicating that Rbt5 plays a major role for C. albicans growth in the absence of heme as a sole source of iron. In contrast, growth defect due to the absence of Rbt5 could be restored by adding increasing concentrations of hemin (or hemoglobin), suggesting the existence of additional cellular components or mechanism(s) for acquisition of heme (14). Genome sequence of C. albicans has revealed other genes encoding CFEM-related proteins, including Pga7, Csa1, Csa2, and Ssr1. In the case of Pga7, its inactivation causes a greater growth defect phenotype than an rbt5Δ mutant in the presence of hemin or hemoglobin as a sole iron source (14). Although Pga7 exhibits a higher affinity for hemin than Rbt5, hemin can restore growth in the presence of hemin as a sole source of iron. In contrast, growth defect due to the absence of Rbt5 could be restored by adding increasing concentrations of hemin (or hemoglobin), suggesting the existence of additional cellular components or mechanism(s) for acquisition of heme (14).

Genome-wide studies have identified a gene (SPAC1F8.02c), denoted shu1+, whose transcript is induced or repressed as a function of iron depletion or repletion (29, 30). Here, we show that iron-mediated repression of shu1+ transcription required GATA-type elements and a functional fep1+ gene. Using a shu1+::HA₄ allele that retained wild-type function, microscopic analyses revealed that HA₄-tagged Shu1 is localized at the plasma membrane when iron levels are low. When a hem1Δ shu1Δ mutant strain was incubated in the absence of ALA and in the presence of hemin, cells were unable to grow unless an untagged shu1+ or HA₄-tagged shu1+ allele was re-integrated and expressed in this mutant strain. This result revealed that Shu1 allowed the hem1Δ shu1Δ mutant to regain its capacity to acquire hemin. As expected, cells expressing Shu1 were able to take up zinc mesoporphyrin IX (ZnP), a heme analog, a heme analog. Results showed that Shu1 binds to hemin-agarose and exhibits an equilibrium dissociation constant (K₅) value of ~2.2 μM for hemin. Taken together, these results led to the interpretation that Shu1 supports iron acquisition from extracellular heme under low iron conditions.

EXPERIMENTAL PROCEDURES

Two pathways of iron acquisition have so far been identified in S. pombe (22). The first pathway consists of a ferrireductase and a ferroxidase-permease complex for high affinity elemental iron uptake (23). The ferrireductase Fpr1 reduces Fe³⁺ to Fe²⁺ ions prior to uptake through transport by the Fio1-Fip1 heteromeric complex. The second pathway consists of the production and uptake of siderophores. The siderophore synthetase Sib1 and L-ornithine N²-oxygenase Sib2 participate in ferrichrome synthesis, whereas Str1, Str2, and Str3 are known or predicted to function as siderophore-iron transporters (24–26). A common hallmark of frp1+, fio1+, fip1+, sib1+, sib2+, str1+, str2−, and str3+ genes is the fact that they are regulated at the transcriptional level as a function of iron availability. Their expression is induced under conditions of iron starvation, whereas repression of these genes occurs under iron-replete conditions. The iron-dependent repression of frp1+, fio1+, fip1+, sib1+, sib2+, str1+, str2−, and str3+ transcription relies on the presence of the GATA-type transcriptional repressor Fep1 (24, 25, 27). Once activated under conditions of elevated iron, Fep1 binds to chromatin-containing DNA sequences known as GATA elements (5’- (A/T)GATA(A/T)-3’), which are found in the promoters of its target genes. Conversely, when iron levels are low, Fep1 fails to bind chromatin and that results in strong activation of transcription of genes encoding iron uptake proteins (28).

Other yeasts such as Candida parapsilosis and Paracoccidioides brasiliensis use Rbt5-like proteins to acquire heme (18, 19). In the case of Cryptococcus neoformans, the extracellular heme-binding glycoprotein Cig1 is required for exogenous heme uptake (20). cig1Δ mutant cells consistently exhibit delayed growth when heme is the sole source of iron. However, Cig1 is not a member of the CFEM family of proteins because it lacks the typical canonical eight-Cys-containing CFEM domain found in other members of this family. Nevertheless, downstream cellular components of Cig1 that participate in heme uptake may be common to those found in C. albicans because Vps23, a component of the C. neoformans ESCRT system, is also required for heme acquisition (21).

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sition. During pre-cultures of cells, 200 μM ALA was added to ensure cell growth until mid-logarithmic phase (for all indicated strains). At this point, the indicated strains were washed twice, and then cell suspensions were diluted 1000-fold in YES containing 10 μM Dip, 0.075 μM heme but in the absence of ALA (unless otherwise stated). Cell growth was then re-started and monitored at each of the indicated times.

**DNA Constructs**—To generate the pKS-5′ UTR-fep1′-loxP-kanMX6-loxP-fep1′3′UTR plasmid, a 3,123-bp NotI-EcoRV PCR-amplified DNA segment containing the fep1′ locus starting at −1,528 from the translational initiator codon up to the stop codon was inserted into the NotI and EcoRV sites of pKsloxp-kanMX6-loxP. Similarly, a 650-bp SalI-Asp718 PCR-amplified DNA segment containing the 3′-UTR region of the fep1′ locus was inserted downstream of the second loxP sequence using the SalI and Asp718 sites of pKsloxp-kanMX6-loxP. Once generated, the 5′ UTR-fep1′-loxP-kanMX6-loxP-fep1′3′UTR cassette was isolated using NotI and Asp718 digestion and was then integrated at the chromosomal locus of fep1′, which had previously been disrupted with the ura4+ gene (27). The TAP-fep1′ allele was constructed as described (31), and its integration at the chromosomal locus of fep1′ was performed by homologous recombination using a similar strategy as described above. Two BamHI-EcoRI PCR-amplified fragments containing 200- and 117-bp, respectively, of the promoter region of shu1′ were inserted into a YEp357R vector (32). The two promoter regions were then isolated from YEp357Rshu1′-200lacZ and YEp357Rshu1′-117lacZ, respectively, through digestion with BamHI and AatII. The promoter fragments were then swapped for the equivalent DNA restriction fragment in pSPflo1′-884lacZ (27) to generate pSPshu1′-200lacZ and pSPshu1′-117lacZ, respectively. Plasmid pSPshu1′-200lacZ was used to introduce mutations in all three GATA boxes (positions −122 to −127, −131 to 136, and −136 to −141 relative to the A of the ATG codon of shu1′) (CCTGTC instead of (A/T)GATA) using the overlap extension method (33). To create wild-type and mutant pSPshu1′-195lacZ fusion plasmids, four high performance liquid chromatography-purified complementary oligonucleotides were annealed pairwise (wild-type strands 1 + 2 and mutated strands 3 + 4) to form double-stranded DNAs. The resulting double-stranded DNAs containing either three consensus GATA-binding sites or three mutated sites were then amplified by PCR. Because of the fact that the primers contained NotI and SpeI restriction sites, the two purified PCR-amplified fragments were digested with these enzymes and inserted immediately upstream of the minimal zym1′ promoter gene fused to lacZ in pSP1zym1′-367lacZ (30).

**PCR amplification of the shu1′ gene** was performed using primers designed to generate XmaI and SacII restriction sites at the upstream and downstream termini of the open reading frame, respectively. The PCR product was digested with XmaI and SacII and cloned into the corresponding sites of pBPad6+ (34), generating plasmid pBPshu1′. Subsequently, the shu1′ promoter region from position −1317 upstream of the initiator codon of shu1′ was isolated by PCR amplification and was then inserted into pBPshu1′ at the Apal and XcmI sites. This pBP-shu1′ derivative was named pBP-1317shu1′, and it allowed expression of shu1′ under the control of its own promoter, once integrated in the genome. An 18-bp Stul-BspEl linker was inserted in-frame within the shu1′ gene at position +75 relative to the first nucleotide of the initiator codon. The linker was introduced by the overlap extension method. The insertion generated 6 extra amino acid residues following the Asn residue at position 25 (Asn25-Arg-Pro-Asp-Thr-Ser-Gly-Glu26), within a predicted hydrophilic region. We used the restriction sites Stul and BspEl created within shu1′ to swap the linker region with four copies of the HA epitope. To generate the HA4 DNA fragment, a short DNA region of pSkctr6′-HA4 (35) (harboring four copies of the HA tag) was isolated by PCR using primers that contained Stul and BspEl sites. The fragment was digested and cloned into the Stul-BspEl-digested pBP-1317shu1′-S-B linker vector. The resulting plasmid was named pBP-1317shu1′-HA4. Plasmid pBP-1317shu1′-HA4 was used to introduce mutations in the coding sequence of shu1′. Codons corresponding to Cys87 and Cys92 were replaced with Ala residues. These site-specific mutations were created by a PCR overlap extension method. The resulting plasmid was designated pBP-1317shu1′C87A/C92A-HA4. This plasmid was used as a template to substitute the codon corresponding to

**TABLE 1**

| Strain   | Genotype                                      | Source   |
|----------|-----------------------------------------------|----------|
| FY435    | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210        | 27       |
| fep1Δ    | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 fep1Δ  | 27       |
| fep1Δ TAP-fep1′ | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 TAP-fep1′-KAN′ | This study |
| TMY1     | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 hem1Δ-KAN′ | This study |
| TMY2     | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 hem1Δ-loxP shu1Δ-KAN′ | This study |
| TMY3     | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 fipl:loxP fio1-loxP hem1Δ-KAN′ | This study |
| TMY4     | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 hem1Δ-loxP shu1Δ-loxP abc3Δ-KAN′ | This study |
| TMY5     | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 hem1Δ-loxP shu1Δ-KAN′ shu1′-Δade6′ | This study |
| TMY6     | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 hem1Δ-loxP shu1Δ-KAN′ shu1′-Δade6′ | This study |
| TMY7     | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 hem1Δ-loxP shu1Δ-loxP abc3Δ-KAN′ shu1′-4Δade6′ abc3′-GFP::leu′ | This study |
| JSY22    | h+ his7-366 leu1-32 ura4-1Δ8 ade6-M210 ctr4Δ-loxP ctr5Δ-KAN′ ctr4′-GFP::ade6′ cts5::Myc1::leu′ | 34       |
Cys\textsuperscript{72} with an Ala codon. The PCR overlap extension strategy was repeated to generate pBP-1317shu1C72A/C87A/C92A-HA\textsubscript{3}. The shu1 mutant allele containing site-specific C72A/C87A/C92A/C101A mutations was created using a similar approach, except that the plasmid pBP-1317shu1C72A/C87A/C92A-HA\textsubscript{3} was used as a template.

**RNA Extraction and Analysis**—Total RNA was isolated using a hot phenol method as described previously (36). RNase protection assays were carried out as described previously (30). Plasmids pKSlacZ and pSKact1\textsuperscript{+} (37) were used to produce antisense RNA probes, allowing the detection of steady-state levels of lacZ and act1\textsuperscript{+} mRNAs, respectively. pKshu1\textsuperscript{+} was constructed by inserting a 192-bp BamHI-EcoRI fragment of the shu1\textsuperscript{+} gene into the same restriction sites in pBluescript (Stratagene). The antisense RNA hybridizes to the region between positions +49 and +241 downstream of the A of the initiation codon of shu1\textsuperscript{+}. 32P-Labeled antisense shu1\textsuperscript{+} RNA was generated using the BamHI-linearized plasmid pKshu1\textsuperscript{+}, [α-\textsuperscript{32}P]UTP, and T7 RNA polymerase as described previously (30).

**ChiP Assays**—fep1Δ php4Δ cells expressing untagged or TAP-tagged fep1\textsuperscript{+} alleles were pre-cultured in the presence of the iron chelator Dip (100 μM) to prevent iron-dependent activation of Fep1 and, consequently, repression of target gene expression. Cells were harvested from cultures (100 ml) at logarithmic phase, washed, and resuspended in the same medium containing either FeCl\textsubscript{3} (250 μM) or Dip (250 μM) for 3 h. The ChiP method was carried out essentially as described previously (38). Quantification of immunoprecipitated DNA was performed by real time PCR (qPCR) using primers that spanned a proximal shu1\textsuperscript{+} promoter region that includes three GATA-type elements. As positive controls, primers that encompassed a fio1\textsuperscript{+} promoter region that contained two functional GATA-boxes (27) were also used for qPCR analysis. TAP-tagged Fep1 density at the shu1\textsuperscript{+} or fio1\textsuperscript{+} promoter was calculated as the enrichment of the specific genomic shu1\textsuperscript{+} or fio1\textsuperscript{+} promoter region relative to an 18 S ribosomal DNA coding region in which no GATA box is present. Primers were designated by the name of the gene promoter followed by the position of their 5’ ends relative to that of the translational initiation codon: shu1-179 (5’-CAACTCTAATCAATTTGAGGGATAGTCTG-3’), shu1-80 (5’-GCCATCTTATATGATCTGGAAAATTTAATGAATTAAG-3’), fio1-830 (5’-CCACCTTCTTCAGGCGATCTG-3’), and fio1-741 (5’-GTCGGAGITGGTGTCCACTTGG-3’). Two primers derived from an 18 S ribosomal DNA coding region were used as internal background controls: 18S-5’a (5’-CAGCTTGGGTGATAACGTCC-3’) and 18S-5’b (5’-AGCCCATCAGGGCTCCTACTA-3’). Each qPCR experiment was performed in triplicate, and all ChiP experiments were repeated at least three times using independent chromatin preparations.

**Direct and Indirect Immunofluorescence Microscopy—Mid-logarithmic hem1Δ shu1Δ cells expressing Shu1-HA\textsubscript{3} or Shu1C72A/C87A/C92A-HA\textsubscript{3} were subjected to microscopic analysis. After two washes with water to remove ALA, cells were incubated in the presence of hemin (0.075 μM) and then treated with Dip (250 μM) or FeCl\textsubscript{3} (100 μM) for 3 h. To detect Shu1-HA\textsubscript{3} in intact cells, they were fixed by adding formaldehyde immediately at the end of the experimental treatments. In the case of spheroplast preparations, cells were treated with cell wall lytic zymolase for 90 min prior to fixation with formaldehyde. Fixed cells or spheroplasts were adsorbed on poly-L-lysine-coated (0.1%) multwall slides as described previously (34). Following a 30-min block with TNB (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA, 0.02% sodium azide), cells were incubated with an anti-HA antibody (F-7) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250 in TNB. After an 18-h incubation period at 4 °C, cells were washed with TNB and incubated for 4 h with a goat anti-mouse Alexa Fluor 546-labeled IgG antibody (Invitrogen) diluted 1:250 in TNB. In the case of ctrl4Δ ctrl5Δ cells expressing Ctr4-GFP and Ctr5-Myc\textsubscript{12}, they were incubated in the presence of the copper chelator BCS (100 μM) for 3 h. Subsequent steps were carried out as described above in the case of Shu1-HA\textsubscript{3}, except that a monoclonal anti-Myc antibody (clone 9E10, Roche Diagnostics) was used as a primary antibody.

Live cell imaging of fluorescent ZnMP accumulation was performed using liquid cultures that were seeded to an A\textsubscript{600} of ~0.5. Mid-logarithmic phase cultures were then incubated in ALA-deficient medium containing 250 μM Dip or 100 μM FeCl\textsubscript{3} for 3 h. The cultures were subsequently exposed to ZnMP (2 μM) for 90 min. ZnMP accumulation was stopped by adding 5 volumes of ice-cold 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). After centrifugation, cells were resuspended in ice-cold 2% BSA in PBS and were examined by fluorescence microscopy using a ×1,000 magnification. Fluorescence and differential interference contrast images (Nomarski) of cells were obtained using a Nikon Eclipse E800 epifluorescent microscope (Nikon, Melville, NY) equipped with a Hamamatsu ORCA-ER digital cooled camera (Hamamatsu, Bridgewater, NJ). Fields of cells shown in this study correspond to a minimum of five independent experiments.

**Preparation of S. pombe Extracts, Western Blot Analysis, and Hemin-Agarose Pulldown Assays—hem1Δ shu1Δ cells expressing the wild-type shu1\textsuperscript{+}-HA\textsubscript{3} or mutant shu1-1C72A/C87A/C92A/C101A-HA\textsubscript{3} allele were grown to mid-logarithmic phase in the presence of ALA. At this point, cells were washed twice to remove ALA and then incubated in the presence of Dip (250 μM) or FeCl\textsubscript{3} (100 μM) for 3 h. After treatments, cells were lysed with glass beads using a RIPA buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS, 8 μm urea, 0.1 mM Na\textsubscript{2}VO\textsubscript{4}, 1 mM phenylmethylsulfon fluoride, 1 mM dithiothreitol, and a complete protease inhibitor mixture (Sigma; P8340). Cells were broken using a FastPrep-24 instrument (MP Biomedicals). Lysates were ultracentrifuged at 100,000 × g for 30 min at 4 °C. The supernatant containing soluble proteins was set aside, whereas the pellet fraction was resuspended in a buffer consisting of 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, and the mixture of protease inhibitors. Once resuspended, the pellet fraction was incubated on ice for 30 min and then recentrifuged at 100,000 × g for 30 min at 4 °C. The supernatant fraction that contained dissolved membrane proteins was used for Western blot analysis or hemin-agarose pulldown assays. In the case of pulldown assays with hemin-agarose, proteins (~50 μg) were incubated with...
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500 μl of hemin-agarose beads, and the suspensions were mixed end-over-end for 20 min at 25 °C. The beads were centrifuged, and unbound material was kept on ice. The beads were washed three times with 1 ml of buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% Triton X-100. The beads were transferred to a fresh microtube before the last wash. Immunoprecipitates and unbound material were resuspended and mixed, respectively, with 50 μl of SDS loading buffer and heated for 5 min at 95 °C. Samples were resolved by electrophoresis on 9-% SDS-polyacrylamide gels. The following antibodies were used for Western blotting analysis: monoclonal anti-HA (clone B-5-1-2) and monoclonal anti-PCNA antibody PC10 (Sigma). Following incubation with primary antibodies, membranes were washed and incubated with the appropriate horse-radish peroxidase-conjugated secondary antibodies (Amer sham Biosciences). Relevant proteins were revealed using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

**Purification of Shu1 Expressed in Escherichia coli**—A DNA fragment containing shu1<sup>+</sup> codons 21–200 was amplified by PCR, purified, and inserted in-frame in pET28a at NcoI and XhoI restriction sites. When expressed in E. coli, this form of Shu1 (21–200) showed hydrophilic behavior and was soluble. The first N-terminal 20 amino acid residues of Shu1 are predicted to correspond to a signal peptide that is overall hydrophobic, whereas the last 26 amino acid residues correspond to a short transmembrane domain (or pro-peptide). Removal of these regions yielded a protein that had the required properties for purification. A similar strategy was used to create a pET28a recombinant vector expressing Shu1<sub>C72A/C87A/C92A/C101A</sub> (sequence 21–200) mutant protein, except that the plasmid pBP-1317/Shu1C72A/C87A/C92A/C101A was used as a template for PCR amplification. Fresh transformants of E. coli BL21(DE3)-containing plasmid pETShu1(21–200) or pETShu1C72A/C87A/C92A/C101A (21–200) were grown to an A<sub>600</sub> of 0.5. At this growth phase, the cells were induced with isopropyl β-D-thiogalactopyranoside (0.4 mM) for 2 h at 18 °C in the presence of ethanol (2%). Cells were broken up by sonication in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% sucrose, 5 mM imidazole, 50 μg/ml lysozyme, and 1% Triton X-100) containing a mixture of protease inhibitors (P8340; Sigma). Protein extracts were incubated for 2 h at 4 °C with a suspension (2 ml) of nickel-nitrilotriacetic acid-agarose beads. Wild-type or mutant Shu1-His<sub>6</sub>(21–200) protein bound to the beads were eluted stepwise with buffer B (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol) containing 75, 200, 500, and 1000 mM imidazole. Samples (200 mM imidazole eluate fractions) containing Shu1(21–200) were dialyzed to remove imidazole (down to 5 mM) and processed for an additional purification on the same type of affinity resin.

**Absorbance Spectroscopy**—A stock solution of hemin (5 mM) was prepared by dissolution in NaOH (0.1 m). Following filtration, the solution was diluted (1:1,000), and hemin concentration was determined at 385 nm using an extinction coefficient of 58,400 liters·mol<sup>−1</sup>·cm<sup>−1</sup>. Association of proteins with heme was determined by adding increasing amounts of a protein (purified wild-type or mutant Shu1, 0–7 μM) to hemin (10 μM) in 40% dimethyl sulfoxide buffered with 20 mM HEPES, pH 7.4. Differences in absorption spectra over a range of 350–600 nm were recorded using a DU730 spectrophotometer (Beckman Coulter). Increases in absorbance at the Soret peak served to monitor formation of the protein-heme complex. Heme binding curves were generated to represent changes in absorbance at the Soret peak as a function of protein concentrations. Data were analyzed using the GraphPad Prism version 6.04 software.

**RESULTS**

**Fep1 Represses shu1<sup>+</sup> Gene Expression in Response to Iron**—Genome profiling studies have revealed that the SPAC1F8.02c gene (also denoted shu1<sup>+</sup>) is transcriptionally regulated at the level of its transcription in relationship with iron availability (29, 30). shu1<sup>+</sup> mRNA levels are induced under conditions of iron starvation and repressed under iron-replete conditions. To further determine whether Fep1 played a role in iron-mediated repression of shu1<sup>+</sup> transcription, we deleted fep1<sup>+</sup> (<i>fep1</i>Δ) cells were transformed with an empty integrative plasmid (vector alone) or integrative plasmids encoding fep1<sup>+</sup> and TAP-fep1<sup>+</sup> alleles. Total RNA prepared from culture aliquots was assayed by RNase protection assays. The steady-state levels of shu1<sup>+</sup> and act1<sup>+</sup> mRNAs are indicated with arrows. B, graphic representation of the quantification of results of three (n = 3) independent RNase protection assays, including the experiment shown in A. The histogram values represent the averages ± S.D. of triplicate determinations.
(Fig. 1). Collectively, the results showed that Fep1 is required to repress shu1+ gene expression in response to iron repletion.

Iron-dependent Repression of shu1+ mRNA Levels Requires GATA Sequences—We then sought to identify putative GATA cis-acting elements that could serve as Fep1-binding sites within the shu1+ promoter region. Eight GATA boxes containing the sequence (A/T)GATA(A/T) were identified within a promoter region 932 bp long. Three GATA boxes aligned exactly with an extended GATA-type sequence, ATC(A/T)GATA(A/T), that is known to mediate the strongest Fep1-dependent repression response (25). Among them, two elements were arranged as a direct repeat of ATC(A/T)-GATA(A/T)-GATA(A/T) between positions −136 to −141 and was oriented in the opposite way (inverted) relative to the transcriptional direction of shu1+. The findings that these three putative regulatory sequences were adjacent (in comparison with the others that were spread out along the promoter) led us to examine a region of the shu1+ promoter up to −200 (from the initiator codon) that included them. This promoter segment was fused upstream of and in-frame to the lacZ gene in a reporter plasmid. Results showed that cells harboring wild-type −200shu1+-lacZ fusion reporter were able to induce lacZ mRNA expression under conditions of iron starvation (−33-fold) (Fig. 2, B and C). In contrast, lacZ mRNA was barely detected under basal or iron-replete conditions. To investigate whether the three putative GATA boxes could control lacZ gene expression as a function of iron availability, we inserted multiple point mutations that simulated changes known to abolish binding of Fep1 to GATA boxes (27). Mutation of base pairs within the −127AGATA−122 (box 1), −136AGATA−131 (box 2), and −130TGATA−141 (box 3, inverted) elements (GCCGTC instead of (T/A)GATA) reduced the overall magnitude of lacZ expression. Furthermore, there was a complete lack of iron responsiveness of the reporter gene (Fig. 2, B and C). At the moment, the reason for an overall diminution of lacZ expression is unknown. One possibility is that mutation of GATA elements (in this promoter context) may negatively affect the ability of putative adjacent elements to be effective in activating the reporter gene expression. As an additional control, the shu1+ promoter up to −117 from the initiator codon, which excludes the three GATA elements, was also fused to the lacZ gene reporter. Similarly to mutant −200shu1+-lacZ fusion (M1), lacZ transcript levels from cells harboring −117shu1+-lacZ fusion exhibited no changes in response to variations in iron levels (Fig. 2, B and C).

Based on the findings that the integrity of GATA elements located in the proximal shu1+ promoter region was essential to trigger iron repression of −200shu1+-lacZ fusion, we examined whether these elements could regulate a heterologous reporter gene in an iron-dependent manner. A short DNA segment derived from the shu1+ promoter (positions −195 to −93) was inserted in its natural orientation upstream of the minimal promoter of the zym1+ gene fused to lacZ (30). This −195zym1+-lacZ fusion repressed lacZ mRNA expression under basal (untreated) and iron-replete conditions (Fig. 3). In contrast, under conditions of iron starvation, lacZ mRNA expression was induced (−4−5-fold) compared with the transcript levels detected in the case of control (untreated) or iron-treated cells (Fig. 3). When GATA elements were mutated (M2 mutant), sustained and constitutive expression of lacZ mRNA was observed, irrespective of cellular iron status (Fig. 3). In the absence of the short DNA segment derived from the shu1+ promoter (positions −195 to −93), low but constitutive expression of lacZ mRNA was detected in cells transformed with a plasmid containing only the zym1+ minimal promoter (Fig. 3).
The presence of the minimal promoter may explain why low levels of lacZ mRNA were detected under all conditions tested. Taken together, these results clearly showed that the proximal promoter region of shu1+ contains functional GATA-type elements, which are required for the transcriptional repression of shu1+ in response to iron.

Fep1 Interacts with the Shu1+ Promoter in Vivo in an Iron-replete Manner—In previous studies, we had created a fep1Δ php4Δ double mutant strain in which the expression of a functional TAP-fep1+ allele was disengaged from its transcriptional regulation by Php4, therefore ensuring its constitutive expression irrespective of the cellular iron status (28). Taking advantage of this mutant, we used a ChIP approach to test whether TAP-Fep1 interacted with the shu1+ promoter in vivo. Results showed that when cells were treated with iron, TAP-Fep1 occupied the shu1+ promoter at high levels with an ~6.5-fold enrichment relative to an 18 S ribosomal DNA coding region in which no GATA box was present. ChIP data were calculated as values of the largest amount of chromatin measured (fold enrichment). Results are shown as the averages ± S.D. of a minimum of three (n = 3) independent experiments. ChIP analysis was also performed on the fio1+ promoter under the same conditions. The fio1+ promoter region −858 to −753 (relative to the initiator codon of the fio1+ gene) included functional GATA boxes. This analysis was performed as a control experiment because it is known that the fio1+ promoter is bound by Fep1 in an iron-dependent manner.

FIGURE 3. Proximal shu1+ promoter GATA elements are sufficient to regulate a heterologous reporter gene in an iron-dependent manner. A, scheme of a short 102-bp shu1+ promoter region that harbors three GATA sequences, including two containing 9 bp (5′-ATC(A/T)GATA(A/T)-3′) (empty boxes 1 and 2) and one containing 6 bp (5′-(A/T)GATA(A/T)-3′) (hatched box 3). This shu1+ DNA fragment was inserted upstream of a minimal promoter of the zym1+ gene fused to lacZ. Filled boxes (M2) represent mutant versions (5′-GCGTC-3′) of GATA sequences. As a control, a third plasmid (vector alone) contained only the minimal zym1+ promoter fused to lacZ. B, wild-type cells harboring the indicated heterologous reporter plasmid were left untreated (−) or were treated with Dip (250 μM) or FeCl3 (50 μM) for 3 h. Total RNA was isolated and steady-state mRNA levels of lacZ and act1+ (indicated with arrows) were analyzed by RNase protection assays. C, RNase protection analyses were quantified based on three (n = 3) independent experiments. Data are shown as the averages of triplicate determinations ± S.D.

FIGURE 4. Fep1 interacts with the shu1+ promoter in vivo under iron-replete conditions. ChIP analysis was performed on the shu1+ promoter in fep1Δ php4Δ cells carrying an integrated untagged or TAP-tagged fep1+ allele. Chromatin was immunoprecipitated, and a region of the shu1+ promoter (positions −179 to −80) was analyzed by qPCR to determine Fep1 occupancy. Binding of TAP-Fep1 to the shu1+ promoter was calculated as the enrichment of a specific shu1+ promoter region relative to an 18 S ribosomal DNA coding region in which no GATA box was present. ChIP data were calculated as values of the largest amount of chromatin measured (fold enrichment). Results are shown as averages ± S.D. of a minimum of three (n = 3) independent experiments. ChIP analysis was also performed on the fio1+ promoter under the same conditions. The fio1+ promoter region −858 to −753 (relative to the initiator codon of the fio1+ gene) included functional GATA boxes. This analysis was performed as a control experiment because it is known that the fio1+ promoter is bound by Fep1 in an iron-dependent manner.
predictions, the first 20 amino acids of Shu1 correspond to a signal peptide for targeting to the secretory pathway. The last 26 amino acid residues may correspond to a short transmembrane region or pro-peptide. Shu1 is also predicted to be a GPI-anchored protein in which Ser199 is the GPI attachment site (39). Shu1 was successfully tagged (without loss of function) by
inserting four tandem repeats of the HA epitope within a predicted hydrophilic region located between amino acid residues 25 and 26. To determine the cellular location of Shu1-HA4, indirect immunofluorescence microscopy was performed using anti-HA antibody. When *S. pombe* *hem1Δ shu1Δ* cells expressing the *shu1Δ-HA3* allele were incubated under low iron, in the absence of ALA and in the presence of hemin (0.075 μM), Shu1-HA3 fluorescence was detected at the cell periphery (Fig. 5B). In contrast, the fluorescence signal was absent when cells expressing Shu1-HA4 were exposed to high levels of iron (Fig. 5B). To determine whether Shu1-HA4 was incorporated into the cell wall or attached to the plasma membrane, the cell wall of iron-starved Shu1-HA4 cells was enzymatically removed prior to fluorescent antibody labeling. Under these conditions, spheroplasted cells expressing Shu1-HA4 exhibited a clear plasma membrane pattern of localization (Fig. 5C). Conversely, the fluorescence signal was lost when spheroplasts were prepared from cells grown in the presence of iron (Fig. 5C). Cells expressing the Ctr5-Myc12 protein, which is known to localize in the plasma membrane under low copper conditions (34), was used as a control in parallel experiments (Fig. 5B). As observed in the case of Shu1-HA4, spheroplasts from cells expressing Ctr5-Myc12 exhibited a clear plasma membrane pattern of localization (Fig. 5C).

We next analyzed Shu1-HA4 protein levels in untreated, iron-starved, or iron-replete cells. *shu1Δ-HA3* and *abc3Δ-GFP* (40) fusion alleles expressed under the control of their own promoters were co-transformed in a *hem1Δ shu1Δ abc3Δ* mutant strain. Cultures grown to mid-logarithmic phase were washed to remove ALA followed by incubation in the presence of hemin (0.075 μM). The cells were then left untreated or exposed to dip or iron for 3 h. Cell membranes were obtained by ultracentrifugation of whole-cell extracts and were treated with Triton X-100 to release membrane proteins. Results of immunoblotting with an antibody directed against HA showed that Shu1-HA3 was detected in cells grown under iron-limiting conditions (Fig. 5D). In contrast, and consistent with the regulation of *shu1Δ* transcript levels, Shu1-HA4 protein levels were undetectable under basal and high iron conditions (Fig. 5D).

Similar results were obtained using an antibody directed against GFP because it is known that the expression of Abc3 (or Abc3-GFP) is induced by iron deficiency but repressed under basal and iron-replete conditions (Fig. 5D) (40). Taken together, the results suggested that Shu1 is a plasma membrane protein induced by iron deficiency.

**Shu1 Is Required for Assimilation of Exogenous Hemin**—Heme biosynthesis and heme uptake represent two different ways to acquire heme in several organisms. Although heme biosynthesis has been extensively studied, there is relatively few studies concerning its uptake in eukaryotic cells. One reason may be that it is difficult to dissociate its transport from on-going biosynthesis. To address this challenge, we generated a strain in which the *hem1Δ* gene encoding the first enzyme of the heme biosynthetic pathway was disrupted (*hem1Δ*). In the absence of Hem1, biosynthesis of heme was blocked. Results showed that *hem1Δ* deletion was lethal for *S. pombe* (Fig. 6A), unless exogenous ALA was added, allowing heme biosynthesis starting at the second step of the biosynthetic pathway. In the presence of ALA, *hem1Δ* or *hem1Δ shu1Δ* cells exhibited similar growth compared with wild-type strain (Fig. 6A). An additional way to maintain *hem1Δ* cells alive was to add exogenous hemin (heme chloride), thereby fostering cells to use their own heme uptake system (Fig. 6B). This second approach (*hem1Δ* + hemin) selectively blocked heme biosynthesis, allowing study of heme acquisition. To investigate whether Shu1 was involved in heme acquisition, *hem1Δ shu1Δ* cells were incubated in the absence of ALA and in the presence of heme. Under these conditions, *hem1Δ shu1Δ* cells exhibited poor growth as compared with *hem1Δ* cells containing an endogenous *shu1Δ* gene or *hem1Δ shu1Δ* cells in which functional untagged *shu1Δ* and HA4-tagged *shu1Δ* alleles were reintegrated (Fig. 6B). After 68 h, the *hem1Δ shu1Δ* strain displayed ~6–8-foldless growth compared with strains that expressed functional *shu1Δ* alleles (Fig. 6B). To further define the role of Shu1 in heme versus iron acquisition, *hem1Δ fip1Δ* *fio1Δ* cells were incubated in the absence of ALA and in the presence of heme. Under these conditions, *hem1Δ fip1Δ fio1Δ*...
Shu1 Is Required for Assimilation of the Heme Analog ZnMP—To further elucidate the role of Shu1 in heme assimilation, we investigated the presence of Shu1 in cells that had been grown under iron-replete conditions. A strain lacking the wild-type hem1 allele was preincubated for 3 h under iron-deficient or iron-replete conditions. Cells were then incubated for 90 min in the presence of ZnMP (2 μM) under low iron conditions. Under low iron conditions, hem1Δ shu1Δ mutant cells generated a high intracellular ZnMP fluorescence signal that was predominantly located in the cytosol and that was, in most cells, largely excluded from the nucleus (Fig. 7A). In contrast, deletion of the shu1 allele in a strain lacking hem1Δ (hem1Δ shu1Δ mutant) dramatically lowered the ZnMP fluorescent signal as compared with a hem1Δ single mutant expressing an endogenous shu1 allele or a hem1Δ shu1Δ mutant in which a wild-type shu1 allele was re-integrated (Fig. 7A). There was an absence of ZnMP fluorescent signal in hem1Δ shu1Δ, hem1Δ shu1Δ, or hem1Δ shu1Δ + shu1Δ cells when they had been preincubated under high levels of iron (Fig. 7B). Thus, under low iron conditions, the Shu1 protein plays a physiological function in taking up heme as suggested by its ability to mediate uptake of ZnMP.

Schizosaccharomyces pombe Heme Acquisition

To gain insight into the nature of key amino acid residues that were required for the heme binding properties of Shu1, four Cys residues (positions 72, 87, 92, and 101) within the CXXC region of Shu1 were replaced with alanine residues. When expressed in hem1Δ shu1Δ cells that had been washed to remove ALA (mid-log phase) and then incubated in the presence of Dip (3 h), the Shu1-C72A/C87A/C92A/C101A-HA4 mutant protein was located at the cell surface as observed in the case of wild-type HA-tagged Shu1 protein (Fig. 8, B and C). Membrane fractions were solubilized (Triton X-100) and pulldown assays using hemin-agarose were performed. Results showed that Shu1-C72A/C87A/C92A/C101A-HA4 was primarily detected in the unbound fraction, suggesting a low affinity for hemin (Fig. 8B). Only a very minor fraction of the mutant protein (Shu1-C72A/C87A/C92A/C101A-HA4) was detected with hemin-agarose beads (Fig. 8B). Consistent with iron-mediated repression of shu1-C72A/C87A/C92A/C101A-HA4 mRNA levels (under the control of shu1 promoter), Shu1-C72A/C87A/C92A/C101A-HA4 was not detected in membrane protein fractions that were prepared from iron-treated cells (Fig. 8, B and C). Taken together, these results showed that Shu1 interacts with hemin and that Cys72, Cys87, Cys92, and Cys101 residues or at least some subset of these residues are important for the binding to hemin.

Purified Shu1 Binds Hemin—Hemin-agarose pulldown experiments of S. pombe extracts could not rule out the possibility that interaction of Shu1 and hemin could be mediated by a protein partner. To determine whether Shu1 interacted directly with hemin, we investigated the ability of purified wild-type and mutant Shu1 produced in E. coli to associate with hemin-agarose beads. The region of Shu1 corresponding to residues 21–200 was fused to the His6 tag. A mutant version of Shu1 (21–200) in which Cys72, Cys87, Cys92, and Cys101 were substituted by Ala residues was also generated and fused to His6. The two fusion proteins (wild-type and mutant) were solubilized (Triton X-100) and pulldown assays using hemin-agarose were performed. Results showed that the wild-type version of Shu1 (21–200) was retained on hemin-agarose beads, then incubated in the presence of Dip (250 μM) or iron (100 μM) for 3 h. Whole extracts from these cells were prepared, and cell membranes were obtained by ultracentrifugation. Soluble proteins in the supernatants were precipitated and kept on ice before analysis by immunoblot assays (Fig. 8A). Pellet fractions were treated with Triton X-100 (1%) and then re-fractionated by performing a second ultracentrifugation. Treatment with Triton X-100 released Shu1-HA4, and the extract was treated with hemin-agarose or agarose beads (control). Results showed that Shu1-HA4 expressed in iron-starved cells was strongly retained on hemin-agarose beads (Fig. 8A, bound). Only a weak signal of Shu1-HA4 was detected in the flow-through fraction (Fig. 8A, unbound). In contrast, Shu1-HA4 was primarily found in the unretained fraction (flow-through) when agarose beads (control) were used (Fig. 8A). As expected, no Shu1-HA4 protein was retained on hemin-agarose beads when extracts had been prepared from cells grown under iron-replete conditions (Fig. 8A). Soluble PCNA was only detected in the first supernatant fraction of extracts prepared from cells grown in the presence of Dip or iron (Fig. 8A).
with almost no protein found in the flow-through (unbound) fraction (Fig. 9A). In contrast, the mutant form of Shu1 (containing C72A/C87A/C92A/C101A substitutions) accumulated primarily in the flow-through (unbound) fraction, and only traces were retained by hemin-agarose beads (Fig. 9A). Additional evidence that Shu1 bound hemin was obtained by spectroscopy. Absorbance spectra of addition of increasing concentrations of purified Shu1(21–200) (0–7 μM) to a fixed concentration of hemin (10 μM) were recorded. In the absence of Shu1(21–200) (hemin alone), results showed an absorption peak corresponding to 388–391 nm. After addition of Shu1, maximum absorption shifted to 407 nm, and absorbance at this peak (Soret peak) increased as a function of increasing concentrations of Shu1 (Fig. 9B). In contrast, similar experiments performed with mutated Shu1 did not show a shift of absorbance of 388/391 nm to 407 nm. Furthermore, the intensity of the peak did not show significant changes as a function of an increasing concentration of Shu1-C72A/C87A/C92A/C101A (Fig. 9B). Analysis of the data yielded a value for the constant of dissociation ($K_D$) of $2.2 \times 10^{-6}$ M. In contrast, lack of interaction between Shu1-C72A/C87A/C92A/C101A and hemin did not allow a $K_D$ value to be determined (Fig. 9C). The His$_6$ tag did not play a role in the observed heme binding by Shu1 because the mutant form of Shu1 (Shu1-C72A/C87A/C92A/C101A, which also contained the His$_6$ tag) failed to bind hemin with high affinity. Collectively, these results provided additional evidence that Shu1 interacts directly with hemin without the need for an additional partner.

**DISCUSSION**

Heme and hemoglobin represent an important source of iron in mammals. Heme is also essential for growth of pathogenic fungi such as *C. albicans*, *C. parapsilosis*, and *C. neoformans*, which have evolved mechanisms to acquire iron from this source (12). In the cases of *C. albicans* and *C. parapsilosis*, a group of proteins involved in this process are members of the CFEM family (14, 18). These CFEM proteins are thought to be anchored to the cell wall or attached to the plasma membrane of fungal cells through putative GPI structures. Expression of CFEM2, CFEM3, CFEM4, and CFEM6 in *C. parapsilosis* as well
as RBT5, RBT51, and PGA7 in C. albicans is transcriptionally induced under low iron conditions (14, 18). In C. albicans, the proposed model involves at least two CFEM-type proteins, Rbt5 and Pga7. These two proteins act as heme receptors and cooperate in a protein-protein relay mechanism, which presumably promotes an internalization event of heme via endocytosis. Once internalized, heme is degraded by an intracellular heme oxygenase or is delivered by way of an as yet undefined endocytic pathway into vacuoles for storage or processing (14, 17). It has been proposed that the role of CFEM proteins in iron acquisition from heme is likely to be conserved between C. albicans and C. parapsilosis (18). In C. neoformans, the extracellular glycoprotein Cig1 binds heme and supports iron acquisition from this prosthetic compound (iron-protoporphyrin) (20). Analogous to RBT5/S1 and PGA7 genes in C. albicans, transcript levels of CIG1 are strongly induced under conditions of iron starvation (44, 45). However, unlike Rbt5/S1 and Pga7 proteins, Cig1 does not contain a CFEM domain or any known conserved heme-binding motif (20). Therefore, its property as a hemophore remains to be established.

In this study, S. pombe shu1+ transcript levels were induced in iron-starved cells. These observations were reminiscent of the iron-dependent transcriptional regulation of RBT5/S1, PGA7, CFEM2/3/4/6, and CIG1 genes involved in heme-iron acquisition in C. albicans, C. parapsilosis, and C. neoformans, respectively. Conversely, in cells undergoing transition from low to high iron, shu1+ gene expression was repressed by the iron sensor Fep1. The fact that Fep1 was required for iron-dependent repression of shu1+ suggested that cellular handling of iron and heme was closely interconnected, reinforcing the concept that exogenous heme provides an iron source for S. pombe cells. The existence of a gene in S. pombe that encodes a cell-surface heme-binding protein raises an intriguing question with respect to the advantage for S. pombe to be able to take up iron from heme as this yeast is not a pathogenic fungus for humans or warm-blooded organisms. S. pombe was originally isolated from East African millet beer, but subsequent natural strains have been found in various alcoholic beverages, cultivated fruits such as apples and grapes, and leaves used to produce tea (46–48). Although the natural ecology of S. pombe is not well understood, it is possible that yeast strains can pick up unbound prosthetic groups from degraded hemoproteins, which are released from decomposed organic matter. Laboratory S. pombe strains are almost all derivatives of strains 968, 972, and 975 that were isolated from an over-sulfurized sample of French wine (49). This finding suggests that these strains had been in contact with vining leaves and plants, which can liberate iron-porphyrin complexes (or heme-based molecules) following death and rupture of plant cells and the degradation of cellular hemoproteins. Direct heme uptake systems have been identified in other microorganisms that are not obligate microbes for animals. For instance, several marine bacteria have been found to be able to acquire exogenous heme from their environment such as lysed phytoplankton (50). It would...
be of interest to study a collection of S. pombe isolates to determine whether the pathway of heme-iron acquisition is conserved in relationship to their ability to grow in various environments.

Based on results reported here, S. pombe should be considered to have three distinct systems for iron acquisition. The first system takes up reduced iron through a heteroprotein complex that consists of the ferrooxidase Fio1 and the permease Fip1. The second system mediates the uptake of siderophore-bound iron through siderophore-specific transporters, including Str1 and Str2. The third system mediates Shu1-dependent iron assimilation from exogenous heme. Upon addition of the iron chelator Dip (250 μM), we observed that mRNA levels of fio1+ (or fip1+) and str1+ were rapidly induced within 30 min of treatment (38). This step was followed by a sustained induction of fio1+ and str1+ mRNA levels for at least 4 h. In the case of shu1+, under the same conditions, mRNA levels were detected at later times, being detected only 90 min after treatment. After 4 h of iron starvation-dependent induction, shu1+ mRNA levels were at their maximum levels. This observation suggests a temporal regulation of S. pombe iron acquisition systems, reflecting a source-dependent iron preference among distinct uptake systems that would favor transport of iron according to the following sequence: free iron ions, siderophore-bound chelates, and then heme. Although the pathway for heme acquisition in S. pombe remains to be elucidated, its activity is independent of the Fio1/Fip1 oxidase-permease complex because a hem1△fio1△mutant strain was able to grow at the same level as hem1△cells in the absence of ALA and in the presence of hemin as the sole source of iron.

Examination of global gene expression by DNA microarray analysis has shown that, under low iron conditions, hem3+ and ups1+ expression was decreased 3.2- and 1.7-fold, respectively, compared with the basal levels observed in a wild-type strain (30). hem3+ and ups1+ encode the third (hydroxymethylbilane synthase) and fourth (uroporphyrinogen decarboxylase) enzyme of the heme biosynthetic pathway, respectively. This finding reveals that iron deprivation negatively regulates heme biosynthesis. To counterbalance this response, fission yeast mitochondria were rapidly induced within 30 min of treatment. Upon addition of the iron chelator Dip (250 μM), we observed that mRNA levels of fio1+ (or fip1+) and str1+ were rapidly induced within 30 min of treatment (38). This step was followed by a sustained induction of fio1+ and str1+ mRNA levels for at least 4 h. In the case of shu1+, under the same conditions, mRNA levels were detected at later times, being detected only 90 min after treatment. After 4 h of iron starvation-dependent induction, shu1+ mRNA levels were at their maximum levels. This observation suggests a temporal regulation of S. pombe iron acquisition systems, reflecting a source-dependent iron preference among distinct uptake systems that would favor transport of iron according to the following sequence: free iron ions, siderophore-bound chelates, and then heme. Although the pathway for heme acquisition in S. pombe remains to be elucidated, its activity is independent of the Fio1/Fip1 oxidase-permease complex because a hem1△fio1△mutant strain was able to grow at the same level as hem1△cells in the absence of ALA and in the presence of hemin as the sole source of iron.

Microscopic and biochemical analyses have revealed that functional HA4-tagged Shu1 localized to the plasma membrane when iron levels are low. After enzymatic digestion of the cell wall, Shu1 was still located at the plasma membrane. Shu1 is predicted to contain a GPI anchor, which would serve to attach it to the cell membrane. In the case of Rbt5, its localization has been reported in plasma membrane and in the cell wall (13, 14). Although the localization of Pga7 is less defined, its cellular distribution suggests that the protein may be located more internally when compared with Rbt5 (14). Cellular localization of Cig1 or CFEM2, -3, -4, or -6 has not yet been reported. Although Shu1 contains seven Cys residues (positions 72, 87, 92, 101, 130, 172, and 214), their arrangement does not correspond to that of PXC(A/G)XXCX10–12CX11–13(X/T)DX14–15X16–17C that is found in the canonical eight-Cys-containing CFEM domains (10). However, four of these Cys residues (positions 72, 87, 92, and 101) exhibit an arrangement reminiscent of a partial CFEM domain, with a CX9–14CX4CX15–16C configuration in Shu1 as compared with a CX11CX4CX15–16C configuration in Rbt5 or Pga7. When these four Cys residues were mutated in Shu1 (Shu1-C72A/C87A/C92A/C101A), hem1△shu1△cells expressing the shu1-C72A/C87A/C92A/C101A allele were unable to grow in the absence of ALA and in the presence of hemin as a sole source of iron. This defect was not due to protein mislocalization because results showed that the HA4-tagged Shu1-C72A/C87A/C92A/C101A localized to the cell surface in a manner similar to that of wild-type protein. Pulldown assays using protein lysates prepared from cells expressing Shu1-C72A/C87A/C92A/C101A showed that substitutions of the four Cys residues strongly decreased Shu1 binding to hemin-agarose. Furthermore, this mutant did not possess significant ability to bind heme when subjected to absorbance spectroscopy. These results suggested that these Cys residues, or at least some of them, participate in heme coordination in Shu1. However, definition of their respective contribution will require structural studies of the hemoprotein.

It has been suggested that Rbt5 mediates heme endocytosis in C. albicans, which subsequently allowed heme to be delivered to the vacuole (17). Once in vacuoles, heme would undergo further degradation, resulting in the release of iron from protoporphyrin IX. This series of events would constitute a specialized pathway and make the vacuole an important site for intracellular iron stores. S. pombe is one of the yeast species that does not have heme oxygenase, thereby leaving open the possibility for the existence of an endocytic pathway for iron acquisition from heme (as reported in C. albicans). Future studies are required to determine the nature of the mechanisms by which Shu1 and potential partners operate with respect to heme assimilation as well as its utilization as a source of iron.

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