Studies have shown the fundamental contribution of the yeast vacuole as a site for storage and detoxification of metals. Whereas the transmembrane proteins responsible for iron transport into and out of the vacuole have been identified in *Saccharomyces cerevisiae*, less information is available concerning the mobilization of vacuolar iron stores in *Schizosaccharomyces pombe*. In this study, we report the identification of a gene designated abc3* that encodes a protein which exhibits sequence homology with the ABCC subfamily of ATP-binding cassette transporters. The transcription of abc3* is induced by low concentrations of iron but repressed by high levels of iron. The iron-mediated repression of abc3* required a functional fep1* gene. Chromatin immuno-precipitation assays showed that Fep1 associates with the abc3* promoter in vivo, in an iron-dependent manner. Microscopic analyses revealed that a functional Abc3-green fluorescent protein localizes to the membrane vacuole when iron levels were low. Abc3 was required for growth in low-iron medium in the absence of the transport system mediated by Fio1 and Fip1. abc3Δ cells exhibited increased levels of expression of the *frp1*'-encoded ferric reductase, suggesting a loss of Fep1 repression and, consequently, the activation of Fep1-regulated genes. When abc3* was expressed using the *nmt1* promoter system, its induction led to a reduced transcriptional activity of the *frp1*'-gene. Because *S. pombe* does not possess vacuolar membrane-localized orthologs to *S. cerevisiae* Fth1, Fet5, and Smf3, our findings suggested that Abc3 may be responsible for mobilizing stored iron from the vacuole to the cytosol in response to iron deficiency.

All eukaryotes require iron for survival. The ability of this transition metal to exist in two different redox states makes it an essential component of the active centers of many enzymes and electron transporters (23). For instance, DNA synthesis, cell cycle progression, and energy-generating respiratory chain and electron transporters (23). For instance, DNA synthesis, an essential component of the active centers of many enzymes.

**abc3** Encodes an Iron-Regulated Vacuolar ABC-Type Transporter in *Schizosaccharomyces pombe*™

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Received 7 September 2009/Accepted 2 November 2009

Studies have shown the fundamental contribution of the yeast vacuole as a site for storage and detoxification of metals. Whereas the transmembrane proteins responsible for iron transport into and out of the vacuole have been identified in *Saccharomyces cerevisiae*, less information is available concerning the mobilization of vacuolar iron stores in *Schizosaccharomyces pombe*. In this study, we report the identification of a gene designated abc3* that encodes a protein which exhibits sequence homology with the ABCC subfamily of ATP-binding cassette transporters. The transcription of abc3* is induced by low concentrations of iron but repressed by high levels of iron. The iron-mediated repression of abc3* required a functional fep1* gene. Chromatin immuno-precipitation assays showed that Fep1 associates with the abc3* promoter in vivo, in an iron-dependent manner. Microscopic analyses revealed that a functional Abc3-green fluorescent protein localizes to the membrane vacuole when iron levels were low. Abc3 was required for growth in low-iron medium in the absence of the transport system mediated by Fio1 and Fip1. abc3Δ cells exhibited increased levels of expression of the *frp1*'-encoded ferric reductase, suggesting a loss of Fep1 repression and, consequently, the activation of Fep1-regulated genes. When abc3* was expressed using the *nmt1* promoter system, its induction led to a reduced transcriptional activity of the *frp1*'-gene. Because *S. pombe* does not possess vacuolar membrane-localized orthologs to *S. cerevisiae* Fth1, Fet5, and Smf3, our findings suggested that Abc3 may be responsible for mobilizing stored iron from the vacuole to the cytosol in response to iron deficiency.

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Although *S. pombe* vacuoles may serve as an important site for intracellular iron stores, transport of iron into and out of the vacuole has not been investigated in detail in fission yeast. The second pathway of iron uptake in *S. pombe* relies on the transport of iron-siderophore chelates, a process that primarily involves the hydroxamate-type siderophore ferrichrome (39, 52).

A critical issue for cells is the absolute requirement of being able to control iron concentrations in order to be able to rapidly respond to changes in extracellular iron levels. Exposure of *S. pombe* to elevated concentrations of iron is sensed by the GATA-type transcriptional repressor Fep1 (26, 38). An important response to Fep1 activation is the downregulation of the genes encoding the components of the high-affinity iron transport machinery, including *frp1*, *fio1*, and *fip1* (38, 50).

Based on genomic profiling studies, it has been proposed that Fep1 and its orthologs are also required for the iron-regulated expression of the genes encoding the proteins involved in iron-sulfur cluster formation, in compartmentalization, and in the utilization of inorganic iron (22, 50). Once activated, Fep1 binds to the 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 the iron concentration is limited inside the cell, Fep1 dissociates from the chromatin, thereby allowing transcription of its target genes to take place (21, 40).

Analysis of iron-regulated gene expression in *S. pombe* using DNA microarrays has identified several uncharacterized genes that are transcriptionally activated in response to iron deprivation (34). Although the roles of these genes remain unclear, their iron starvation-dependent induction suggests that they possess iron-related functions. One example of these genes is *SPBC359.05*, which is also called *abc3* (20). This gene encodes a putative transmembrane protein that exhibits sequence homology with the ABC subfamily of ATP-binding cassette (ABC) transporters (9, 10, 15, 37). Members of the ABCC subfamily have a typical ABC “core” region, consisting of two homologous halves. Each half contains a membrane spanning domain that is connected to the cytoplasm via a linker region (L1). A hallmark of the ABCC transporters is the presence of an additional N-terminal extension (NTE) that contains five putative transmembrane spans (MSD0) that are connected to the ABC core domain by a hydrophilic region (L0). A previous study has shown that *S. pombe* cells lacking a functional *abc3* gene are sensitive to cerulenin, an antibiotic that inhibits the biosynthesis of fatty acids (20). Using direct fluorescence microscopy, the expression of a green fluorescent protein (GFP)-tagged form of Abc3 transformed in wild-type *S. pombe* cells suggests that Abc3 may localize to the vacuole membrane, although neither its cellular localization nor its role within the cell has been firmly characterized (20). *S. pombe* is one of the yeast species that does not have any homologs of *S. cerevisiae* proteins Fet5, Fth1, and Smt3 (16). However, as shown for the maintenance of the concentration of intracellular copper (6), the fission yeast vacuole may contribute to the overall iron metabolism of the cell.

In the present study, we determined that *abc3* is regulated at the level of gene transcription, and its iron-dependent regulated expression requires a proximal GATA-type cis-acting element and a functional *fep1* gene. Using a chromatin immunoprecipitation (ChIP) approach, we show that Fep1 occupies the *abc3* promoter in the presence of high levels of iron, whereas iron deficiency results in a loss of Fep1 occupancy at the *abc3* promoter. Using an *abc3*-GFP allele that retained wild-type function, we found that Abc3-GFP is localized to the vacuole membrane when iron levels are low but become undetectable upon exposure to iron. Cell fractionation experiments revealed that Abc3 is an integral membrane protein. The loss of Abc3 resulted in an elevated transcriptional activity of the *frp1* gene. In contrast, permanent expression of *abc3* lowered the steady-state levels of the *frp1* transcript. Taken together, these results strongly suggest that under iron-limiting conditions, Abc3 could serve to transport iron from the vacuole to the cytoplasm.

**MATERIALS AND METHODS**

**Strains and media.** The *S. pombe* strains used in the present study were the wild-type FY435 (*h* + his7-366 leu1-32 ade4-Δ18 ade6-M210) and eight isogenic mutant strains: *abc3Δ* (*h* + his7-366 leu1-32 ade4-Δ18 ade6-M210 abc3Δ::KAN), *pcl1Δ* (*h* + his7-366 leu1-32 ade4-Δ18 ade6-M210 pcl1Δ::KAN), *fep1Δ* (38), *cvi1Δ* (6), *fep1Δ pfcpΔ* (21), *abc3Δ* *cvi1Δ* (*h* + his7-366 leu1-32 ade4-Δ18 ade6-M210 abc3Δ::KAN creΔ::ura4+), *fio1Δ fip1Δ* (38), and *abc3Δ fio1Δ fip1Δ* (*h* + his7-366 leu1-32 ade4-Δ18 ade6-M210 abc3Δ::loxP fio1::fip1::KAN). All *S. pombe* cells were cultured in either yeast extract plus supplements (YES) or selective Edinburgh minimal medium (EMM) lacking the specific amino acids required for integrative or nonintegrative plasmid selection (1). Unsupplemented EMM contained 74 nM iron, unless otherwise stated. Liquid cultures were seeded to an M 2,2×10^7 cell/ml density and were cultured at 30°C in the presence of high levels of iron, unless otherwise stated. Yeast transformations and manipulations were carried out by using standard techniques (1).

**Plasmids.** A BamHI-EcoRI PCR-amplified fragment derived from the *abc3* promoter containing 584 bp of the 5′-noncoding region and the first 10 codons of the *abc3* gene was introduced into the BamHI-EcoRI-digested YEp357R vector (35). The *abc3*′ promoter region was isolated from YEp357R *Rab7Δ*::584ura4 Vector via digestion with BamHI and Bsu36I. It was then swapped for the *abc3*′ promoter region isolated from YEp357R *Rab7Δ*::584ura4 Vector via digestion with BamHI and Bsu36I, and the resulting plasmid was named pSKprom-abc3::584ura4. This plasmid was introduced into the BamHI-EcoRI-digested YEp357R plasmid in the presence of high levels of iron, unless otherwise stated. Yeast transformations and manipulations were carried out by using standard techniques (1).
Apal sites at the 5' and 3' termini, respectively, of the GFP gene. The resulting DNA fragment was used to clone the GFP gene into the pSPLabc3+ plasmid to which XhoI and Apal restriction sites had previously been introduced by PCR and were placed immediately before the abc3+ stop codon. For this particular construct, named pSPLabc3+-GFP, the XhoI-Apal GFP-encoded fragment was placed in frame with the C-terminal region of Abc3. The nmt1+ 41X promoter (13) up to position –1178 from the initiator codon of the nmt1+ gene was isolated by PCR and then swapped to replace the Stsl-Apel3+ promoter fragment in either pSPLabc3+ or pSPLabc3+-GFP. The resulting plasmids were named pSP-Labc1178 (20) and pSP-Labc3+ (20), except that the cells were not resuspended in distilled water prior to resuspension in SEM for microscopic analysis. Briefly, the cells were harvested and resuspended in EMM containing 16 μM FM4-64 for 30 min at 30°C. The cells were pelleted, resuspended in fresh EMM, and incubated at 30°C for an additional 90 min. They were then placed on microscope slides and viewed on a Nikon Eclipse E800 epifluorescence microscope (Nikon, Melville, NY) equipped with a Hamamatsu ORCA-ER cooled charge-coupled device camera (Hamamatsu, Bridgewater, NJ). The samples were analyzed using ×1,000 magnification with the following filters: 465 to 495 nm (GFP) and 510 to 560 nm (FM4-64). The cells fields shown in the present study are representative of a minimum of five independent experiments.

Preparation of S. pombe extracts, Western blot analysis, and spectrophotometric methods using BPS-citric acid. Protein extracts were prepared from logarithmic-phase cells that were grown in standard EMM or taken after their incubation in the presence of either 50 μM Dp or 50 μM FeCl₃. The harvested cells were washed in HEGNₐ buffer (20 μM HEPES [pH 7.9], 1 mM EDTA, 10% glycerol, 100 mM NaCl) and lysed with glass beads using the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and a complete protease inhibitor mixture (P3540; Sigma-Aldrich, St. Louis, MO). The cells were broken by using a FastPrep FP120 instrument (Bio 101; Thermo Electron Corp., Milford, MA; twice for 45 s at 4°C, with cooling intervals of 1 min in an ice bath). The resulting lysates were centrifuged at 100,000 × g for 30 min at 4°C. The supernatant was kept at 4°C, whereas the pellet fraction was resuspended in 0.2 ml of buffer A (phosphate-buffered saline [pH 7.4], 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, and the above-mentioned protease inhibitors) and incubated on ice for 30 min. Alternatively, the pellet fraction was resuspended and left untreated or was adjusted to 0.1 M NaCl, 1% Triton X-100. After incubation on ice, the pellet fraction was resuspended and centrifuged at 100,000 × g for 30 min at 4°C. Both supernatant and pellet fraction were resuspended in 2X sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris-HCl [pH 6.8], 0.1 mM EDTA, 15% SDS, 0.01% bromophenol blue, and 150 mM dithiothreitol) containing 8 M urea and 4% β-mercaptoethanol, unless otherwise indicated. After a 30-min incubation at 37°C, the samples were resolved by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie brilliant blue staining, or they were used for Western blot analysis. Monoclonal anti-GFP (clone B-2; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal anti-HA (clone F-7; Santa Cruz Biotechnology) antibodies were used for protein expression analysis of the Abc3-GFP and Ctr6-HA fusion proteins, respectively. A monoclonal anti-PCNA antibody (clone PC10; Sigma-Aldrich) was used to detect PCNA as an internal control for soluble proteins. For measuring intracellular iron, the BPS-based spectrophotometric assay was carried out as described previously (45).

RESULTS

Molecular architecture of S. pombe Abc3. There are 11 genes that encode putative proteins related to the ABC family of transporters in S. pombe (20). Analysis of iron-regulated gene expression using DNA microarrays has identified abc3+ as one of the affected genes (34, 50). Because the transcript levels of abc3+ (SPBC359.05) showed increases of expression in response to iron deficiency (34), we hypothesized that the gene product (Abc3) could be part of a mechanism that was activated under conditions of iron deficiency. The hypothesis was tested by isolating the gene for further analysis. abc3+ encodes a polypeptide composed of 1,465 amino acid residues with a predicted molecular mass of 166.5 kDa. Abc3 is 42% sequence identical and 56% sequence similar to S. cerevisiae Ycf1, a prototypical member of the ABCB subfamily of ABC transporters (58). Similarly to Ycf1 and the other members of the ABCB subfamily, Abc3 possesses an ABC core domain that consists of two homologous halves, each half containing a membrane-spanning domain (MSD) with six putative transmembrane spans, and an NBD (Fig. 1). The two halves of Abc3 are connected by a putative cytosolic loop (L1). The Abc3 N-terminal portion strongly resembles the unique NTE that is a hallmark of proteins belonging to the subgroup ABCB (31). The Abc3 NTE is very hydrophobic, harboring five predicted
transmembrane helices, in addition to a cytoplasmic linker region (L0). Three conserved motifs (614GkvGaGK620 [Walker A], 704LSGGQ708 [signature], and 726LLD728 [Walker B] for NBD1; 1260GrtGaGK1266 [Walker A], 1364SsGQ1367 [signature], and 1384LLLDE1388 [Walker B] for NBD2) are present within the NBD domains. These motifs may participate in the binding and hydrolysis of nucleotides such as ATP to energize the transport process. Sequence alignment analysis of the amino acid residues that compose the NBD1 and NBD2 of Abc3 and Ycf1 indicated that the spacing of the conserved motifs (Walker A, signature, and Walker B) in Abc3 was highly similar to that of Ycf1 (data not shown). Furthermore, Abc3 residues 614 to 620 (Walker A), 704 to 708 (signature), and 726 to 728 (Walker B) for NBD1, as well as residues 1260 to 1266 (Walker A), 1364 to 1367 (signature), and 1384 to 1388 (Walker B) for NBD2, were 90% identical to the Walker A, signature, and Walker B motifs found in Ycf1. Collectively, these observations strongly suggested that abc3 encodes an ABC protein of the ABCB subfamily similar to Ycf1.

**abc3 transcript was induced under conditions of iron deficiency and negatively regulated by iron through Fep1.**

Our gene expression profiling data suggested that abc3 gene expression was downregulated in the presence of iron (34). To independently verify the microarray data (34), S. pombe wild-type strain was left untreated or treated with Dip (250 μM) or FeCl3 (100 μM). In the presence of the iron chelator Dip, abc3 mRNA levels were increased ~17- to 20-fold compared to the low basal levels observed in the untreated cells (Fig. 2). In contrast, under iron-replete conditions, the transcript levels of abc3 were repressed and remained approximately equal to those observed in the untreated cells (Fig. 2). The abc3 increases in transcript levels observed under conditions of iron deficiency paralleled those observed by DNA microarray analysis (16.9-fold) (34). The results of the dependency of abc3 gene expression on iron concentrations suggested that the iron-regulatory transcriptional repressor Fep1 could play a role in abc3 gene regulation. This possibility was investigated by using an isogenic fep1 deletion strain of S. pombe. The results showed that the levels of abc3 mRNA were constitutive and unresponsive to cellular iron status in the yeast mutant (Fig. 2), suggesting that the iron-mediated repression of abc3 occurred through the activity of Fep1. Furthermore, these results revealed that wild-type abc3 gene was regulated by iron in a manner identical to that of iron uptake genes such as frp1, fio1, and fip1 (5, 27, 38).

According to the ABC transporter classification system (10), S. pombe Abc3 is a member of the ABCB subfamily. In fission yeast, three other genes, namely, abc1, abc2, and abc4, encode putative ABC transporters related to the ABCB subfamily (20). To examine whether they were differentially regulated as a function of iron availability, we investigated the profiles of expression of abc1, abc2, and abc4 by RNA protection assays (Fig. 2). The hmt1 gene (36) that encodes a member of the ABCB subfamily was also analyzed in RNase...
protection experiments. The results showed that the steadystate mRNA levels of abc1+, abc2+, abc4+, and hmt1+ in the wild-type strain were not regulated by iron depletions (Dip, 250 μM) or iron abundance (FeCl3, 100 μM) (Fig. 2). There were no significant changes in the levels of abc1+, abc2+, abc4+, and hmt1+ transcription in treated cells compared to the basal levels in untreated cells. To further examine whether abc1+, abc2+, abc4+, and hmt1+ transcription was controlled by Fep1, a fep1Δ null strain was grown in either the absence or the presence of either 250 μM Dip or 100 μM FeCl3. As shown in Fig. 2, the fep1Δ mutant had no significant effect on the expression of the above-mentioned genes. The bulk of the data led us to conclude that, among the five ABC-encoded transporter genes tested, only abc3+ was regulated by iron through the activity of the transcription factor Fep1.

Analysis of abc3+ promoter sequences required to repress gene expression under basal and iron-replete conditions. The fact that Fep1 was necessary for the repression of abc3+ transcription under basal and iron-replete conditions led us to investigate whether the abc3+ promoter region up to -584 from the start codon of the abc3+ ORF harbored GATA elements. Six copies of the repeated sequence 5′-(A/T)GATA(A/T)-3′ were identified at positions -115 to -120, -197 to -202, -391 to -396, -405 to -410, -418 to -423, and -511 to -516. To determine whether the GATA sequences played a role in the abc3+ regulation by iron, we further examined two regions of the abc3+ promoter that were fused upstream of and in-frame to the lacZ gene in pSP1-lacZ. The first region contained the promoter up to -584 from the initiator codon of the abc3+ gene, whereas the second region harbored a shorter promoter segment up to -239. lacZ mRNA expression from these two plasmids was analyzed by RNase protection assays. The results showed that both fusion promoters were able to repress lacZ mRNA expression in the presence of iron (Fig. 3). Plasmids pSP1abc3+-584lacZ and pSP1abc3+-239lacZ were repressed -7- and -12-fold, respectively, compared to their levels of expression in iron-starved cells. Unexpectedly, the overall magnitude of iron-regulated expression of the lacZ mRNA was slightly higher when the abc3+ promoter was further deleted to position -239 (Fig. 3). As expected, fio1+/-884lacZ (38) and str1+-966lacZ (39) (asayed as controls) were derepressed (~56- and ~6-fold, respectively) after treatment with Dip (250 μM) (Fig. 3). In contrast, their expression was downregulated under basal or iron-replete conditions. Our data did not permit explanation of why the overall magnitude of the iron limitation-dependent activation of both fio1+-884lacZ and str1+-966lacZ gene expression was ~1.5 orders of magnitude greater than that for the abc3+-lacZ fusion derivatives. However, the abc3+-lacZ fusion plasmids were undoubtedly regulated in response to iron levels (Fig. 3).

To gain further insight into the mechanism by which abc3+ expression was regulated by iron, and because of the observa-
steady-state levels of lacZ mRNA under both basal and iron-replete conditions were slightly increased by 1.4- and 1.8-fold, respectively, above the levels observed in cells treated with Dip, the reason for such expression profile is unknown (Fig. 4). When the abc3′ lacZ transcript levels were very low, with no dramatic change as a function of iron availability (Fig. 4).

Based on the findings that the integrity of the second GATA element located between positions −120 and −115 was essential in triggering the iron repression of the abc3′ lacZ fusion, we examined whether this sequence could regulate a heterologous reporter gene in an iron-dependent manner. A short DNA segment derived from the abc3′ promoter (positions −239 to −56) was inserted in its natural orientation upstream of the minimal promoter of the CYC1 gene fused to lacZ in pCF83. The fact that the upstream region of lacZ in pCF83 contains the CYC1 minimal promoter may explain why low levels of lacZ transcript were detected from cells transformed with the plasmid alone. This promoter fusion was able to downregulate lacZ mRNA expression under both standard (untreated) and iron-replete conditions. In contrast, under conditions of iron deprivation, lacZ mRNA expression was induced (~5- to 6-fold) compared to the transcript levels detected with either control (untreated) or iron-exposed cells (Fig. 5). When the first GATA element (positions −202 to −197) was mutated and the second one (positions −120 to −115) was left unchanged (wild-type), the steady-state levels of lacZ mRNA were decreased by ~4- to 5-fold under basal and iron-replete conditions compared to the levels observed in iron-starved cells (Fig. 5). When the first GATA element was unaltered and the second one was mutated, iron-dependent downregulation of lacZ mRNA was compromised in a manner similar to that observed for the abc3′-239M2-lacZ mutant (Fig. 4 and 5). When both GATA elements were mutated, a sustained and constitutive level of lacZ mRNA was observed regardless of the iron status (Fig. 5). Taken together, these results were consistent with the interpretation that the proximal promoter region of abc3′ contained a single functional GATA-type element, −120TGATAT−115, which was required for the transcriptional repression of abc3′ in response to iron.

Iron triggered the association of Fep1 with the abc3′ promoter in vivo. In previous studies, we reported that the fusion of TAP to the N terminus of Fep1 does not interfere with the latter’s function (21). Furthermore, we created a fep1Δ php4Δ double-mutant strain in which the expression of TAP-fep1 was disengaged from its transcriptional regulation by Php4, thereby ensuring its constitutive expression irrespective of the iron status (21). Taking advantage of this mutant, we used a ChIP method (21) to test whether the TAP-Fep1 fusion protein associates with the abc3′ promoter in vivo. fep1Δ php4Δ mutant cells expressing either untagged or TAP-tagged fep1Δ alleles were precultivated in the presence of the iron chelator Dip (100 μM) to prevent any iron-dependent activation of Fep1 and, consequently, downregulation of target gene expression. Logarithmic-phase cells were harvested, washed, and resuspended in the same medium containing Dip (250 μM) or FeCl3 (250 μM) for 90 min. The cells were then fixed by formaldehyde treatment and chromatin was prepared to an average size of 500 bp. DNA fragments cross-linked to TAP-Fep1 were isolated by immunoprecipitation with an anti-
mouse IgG that bound to the TAP tag. To determine the DNA sequences bound to TAP-Fep1, the cross-links were reversed, and DNA was analyzed by quantitative PCR using primer sets specific for the 

$abc3$ promoter region encompassing the cis-acting element $5'-(T/A)GATA(T/A)-3'$, whereas the white ones represent the mutant versions ($5'-GC CGTC-3'$). The hatched arrow represents the lacZ reporter gene. The nucleotide numbers refer to the positions of the GATA boxes relative to that of the $abc3$ initiator codon. (B) Steady-state levels of lacZ mRNA from both the wild type (WT) and mutant GATA fusions (M1, M2, and M1+M2) were analyzed in the absence (−) or the presence of Dip (250 μM) or FeCl$_3$ (Fe, 100 μM) for 90 min. The lacZ and act1 mRNA levels are indicated with arrows. NS, nonspecific signal. (C) Quantitation of the lacZ levels after the treatments shown in panel B. The values shown are the means of three replicates ± the standard deviations.

VACUOLAR TRANSMEMBRANE PROTEIN Abc3 reduced cerulenin cytotoxicity and contributed to iron metabolism. It has been shown that inactivation of Abc3 increased the sensitivity of *S. pombe* cells to cerulenin (20). Although the molecular basis of this growth inhibition remains unclear, we took advantage of this phenotype to determine whether insertion of GFP interfered with Abc3 function. As shown in Fig. 7A, the abc3Δ mutant strain exhibited an increased sensitivity to cerulenin compared to the wild-type strain. Whereas the wild-type strain showed a 77% inhibition of growth in medium containing 1 μg of cerulenin/ml, the abc3Δ mutant was 7-fold more sensitive to cerulenin. When the abc3Δ disruptant was transformed with the wild-type or GFP epitope-tagged abc3 allele, cell resistance was restored to 184 to 226% of the wild-type starting strain (Fig. 7A). The Abc3-GFP fusion protein possessed Abc3 activity that was comparable to that of the wild-type (untagged) Abc3 protein (Fig. 7A). We next sought to determine the subcellular location of active Abc3-GFP in response to changing environmental iron concentrations. As shown in Fig. 7B, Abc3-GFP fluorescence was detected in the vacuole membranes of cells expressing the fusion allele under iron-limited conditions. Abc3-GFP fluorescence colocalized with the vacuole-staining dye FM4-64, which was used as a marker to stain the vacuolar membrane. Consistent with the iron-dependent downregulation of the $abc3$ gene expression, Abc3-GFP fluorescence levels were strongly reduced in cells grown under
basal or elevated (100 μM) iron concentrations. Whereas there was an absence of fluorescence in cells that had been trans-
formed with an empty vector, the \( \text{abc3} \) deletion strain ex-
pressing GFP alone displayed a pattern of fluorescence that was distributed throughout the cytoplasm and nuclei of the cells (data not shown). These results led us to conclude that Abc3 functions at the vacuole membrane under conditions of iron deficiency.

The observation that \( \text{abc3} \) transcription responded to low iron concentrations suggested that the Abc3 protein was in-
volved in iron metabolism and that its expression was required under iron-limiting conditions. To begin to address this ques-
tion, we created a set of isogenic strains that contained disrup-
tions in genes known to play a role in iron transport. Gene de-
letions were introduced in the parental strain to disrupt the \( \text{fio1} \) and \( \text{fip1} \) genes that encode the cell surface oxidase-
permease-based iron transport system in \( \text{S. pombe} \) (5). Subse-
quently, we inactivated the \( \text{abc3} \) locus in the context of a \( \text{fio1} \) \( \text{fip1} \) double mutant background. We tested whether these mutations affected the ability of cells to grow on media containing the iron chelator Dip. Although the \( \text{abc3} \Delta \) single mutant cells exhibited no obvious defects, the deletion of \( \text{abc3} \) in the \( \text{fio1} \Delta \text{fip1} \Delta \) background resulted in a more severe growth defect than was observed with the \( \text{fio1} \Delta \text{fip1} \Delta \) double mutant strain (Fig. 7C). Thus, a role for Abc3 in mobilizing iron became apparent only when the Fio1-Fip1 iron transport system was missing. As a control, the wild-
type parental strain was able to grow on medium containing 135 or 150 μM Dip.

If Abc3 functions by exporting Fe, the rationale is that total cell Fe should increase in cells with \( \text{abc3} \) deleted. To determine total cell Fe concentrations in wild-type versus \( \text{abc3} \Delta \) null cells, we used a BPS-based spectrophotometric assay for quantita-
tive measurement of iron (45). The wild-type cells exhibited a total Fe concentration of 0.11 g/mg of protein. Interestingly, the \( \text{abc3} \) and \( \text{fep1} \) mutant strains displayed a total cell Fe concentration of 0.16 and 0.21 g/mg of protein, respectively, which was 1.5 and 1.9 times higher than the wild-type strain (Fig. 8A). The total cell Fe content results revealed that Abc3 plays a role in Fe efflux. Deletion of \( \text{abc3} \) blocked Fe efflux, triggering Fe accumulation within the cells. For the \( \text{fep1} \) mutant strain, the quantitative data revealed that \( \text{fep1} \) cells accumulate Fe in excess of the physiological requirement (Fig. 8A). These results were fully consistent with the fact that in the...
absence of Fep1, there is lack of transcriptional repression of
genes encoding components of the high-affinity Fe uptake ma-
chinery (38). We also measured total cell Fe content in an
*S. pombe* pcl1/H9004 disruption strain (33). As observed for the vacu-
olar Fe importer Ccc1 in *S. cerevisiae* (28), deletion of the
cell1/H11001 gene in *S. pombe* rendered cells sensitive to Fe compared
to the wild-type strain (33; data not shown). Consistent with
this, we observed that *S. pombe* cells lacking the putative vac-
uolar Fe importer Pcl1 contained 0.04 g/mg of protein, which
is three times weaker than the wild-type strain (Fig. 8B).

Previous studies in *S. cerevisiae* have shown that, during the
transition from growth on glucose to growth on a nonferment-
able carbon source, the vacuolar iron stores are redistributed
within the cell and contribute to iron-requiring processes such
as mitochondriogenesis (46). To test whether *abc3Δ* cells dis-
played a lower efficiency in making the switch from nonrespi-
ratory to respiratory metabolism, cells were first grown on
and then incubated (90 min) in the presence of 250 μM Dip or 50 μM FeCl3 (Fe). Chromatin
was immunoprecipitated with anti-mouse IgG antibodies, and a spe-
cific region of the *abc3* promoter was analyzed by PCR to determine
Fep1 occupancy. The top band represents the *abc3*-specific signal,
whereas the lower band is an internal background control derived from
a nontranscribed region (intergenic region). (B) Quantitation of the
PCR products obtained from anti-IgG immunoprecipitated (IP) chromatin.
The results are representative of three independent experi-
ments. The signals are expressed as the relative binding (%) and were
calculated as percentages of the largest amount of chromatin mea-
sured. Input, input chromatin; IP, immunoprecipitated chromatin.

![FIG. 6. Fep1 binds to the *abc3*+ promoter in vivo in an iron-de-
pendent manner. (A) ChIP analysis of the *abc3*+ promoter in fep1Δ
php4Δ cells harboring an integrated untagged or TAP-tagged fep1+ allele. The cells were precultured in the presence of 100 μM Dip,
allowed to grow to an A600 of 1.0, washed and then incubated (90
min) in the presence of 250 μM Dip or 250 μM FeCl3 (Fe). Chromatin
was immunoprecipitated with anti-mouse IgG antibodies, and a spe-
cific region of the *abc3* promoter was analyzed by PCR to determine
Fep1 occupancy. The top band represents the *abc3*-specific signal,
whereas the lower band is an internal background control derived from
a nontranscribed region (intergenic region). (B) Quantitation of the
PCR products obtained from anti-IgG immunoprecipitated (IP) chromatin.
The results are representative of three independent experi-
ments. The signals are expressed as the relative binding (%) and were
calculated as percentages of the largest amount of chromatin mea-
sured. Input, input chromatin; IP, immunoprecipitated chromatin.

![FIG. 7. Vacuolar localization of a functional *ABC3*-GFP fusion pro-
tein and the contribution of *ABC3* to cell growth under low-iron con-
ditions. (A) *abc3Δ* mutant cells were transformed with the indicated plasmids and their growth was measured in unmodified (control) Edin-
burgh minimal medium or that supplemented with cerulenin (1 μg/ml).
Total growth relative to that determined in the absence of ceru-
lenin (percent control growth) was evaluated by turbidimetry at A600.
Each point represents the average of triplicates ± the standard devi-
ation. (B) Expression of *abc3*+ in transformed *abc3Δ* cells. Exponentially
growing cells (2 × 10^6 cells) were incubated in the absence (−) or the presence of Dip (50 μM) or FeCl3 (Fe, 50 μM) for 16 h at 30°C.
Cells were analyzed by direct fluorescence microscopy for GFP. FM4-64 staining visualized the vacuolar membranes, and Nomarski
optics were used to examine cell morphology. (C) The indicated *S.
pombe* strains were streaked on EMM iron-poor media containing
either 135 or 150 μM Dip and then incubated for 9 days at 30°C in
order to test for cell viability. The strains were also streaked on EMM
medium lacking Dip (control) and were incubated at 30°C for 4 days.
*WT*, isogenic wild-type strain.
Effects of deletion and expression of abc3<sup>+</sup>

Expression and membrane association of Abc3. The iron-dependent regulated expression of abc3<sup>+</sup>, and its vacuolar detection under iron-limiting conditions, prompted us to examine the Abc3-GFP protein levels in both untreated cells and cells incubated under conditions of low and high levels of iron. abc3<sup>+</sup>-GFP and ctr6<sup>−</sup>-HA<sub>4</sub> (6) fusion genes expressed under the control of their own promoters were cotransformed to an abc3Δ ctr6Δ double mutant disruption strain. The abc3Δ ctr6Δ mutant strain coexpressing the untagged abc3<sup>+</sup> and ctr6<sup>−</sup>-HA<sub>4</sub> alleles was used in parallel experiments. Cells coexpressing either the abc3<sup>+</sup>-GFP and ctr6<sup>−</sup>-HA<sub>4</sub> alleles or the abc3<sup>−</sup> and ctr6<sup>−</sup>-HA<sub>4</sub> alleles were grown in the presence of the iron chelator Dip. Membrane fractions collected after ultracentrifugation were treated with Triton X-100, and the supernatants were fractionated by electrophoresis. The results of immunoblotting with an antibody directed against GFP showed that Abc3-GFP was detected after 16 h of treatment (Fig. 9A and data not shown). Consistent with the regulation of abc3<sup>+</sup> mRNA levels, the Abc3-GFP protein levels were markedly reduced in cells grown for 16 h under basal and high-iron conditions. Immunoblot analyses of the Triton X-100-solubilized cell membranes were also carried out with an anti-HA antibody. In this case, results showed that Ctr6-HA<sub>4</sub> was detected after 16 h in cultures containing Dip or iron, as well as under untreated (basal) conditions (Fig. 9A).

The primary amino acid sequence of Abc3 suggests that it is integrated into cellular membranes. This possibility was investigated in the following manner. Cell membranes were obtained by ultracentrifugation of whole-cell extracts of cells grown under iron-limiting conditions. Soluble and detached peripheral membrane proteins present in the supernatants were precipitated, resuspended, and left untreated before analysis by immunoblot assays. The pellet fraction was resuspended and left untreated or was adjusted to 0.1 M Na<sub>2</sub>CO<sub>3</sub> or 1% Triton X-100, and then centrifuged at 100,000 × g. The results showed that in the absence of any treatment, Abc3-GFP and Ctr6-HA<sub>4</sub> proteins were not detected in the supernatant fractions but only in the pellet fractions (Fig. 9B). An identical protein pattern was observed when the procedure had been carried out in the presence of 0.1 M Na<sub>2</sub>CO<sub>3</sub>, which is known to linearize membrane structures, releasing nonintegral membrane proteins into the soluble fraction. In the presence of Triton X-100, a nonionic detergent that solubilizes membranes, Abc3-GFP and Ctr6-HA<sub>4</sub> were released from the membrane and were detected in the supernatant fractions, indicating that Abc3-GFP was an integral membrane protein as previously reported in the case of Ctr6-HA<sub>4</sub> (6). On the other hand, the soluble PCNA protein was only found in the supernatant fraction.

Effects of deletion and expression of abc3<sup>+</sup> on the transcriptional regulation of the frp1<sup>+</sup> ferrireductase gene. The observation that abc3<sup>+</sup> was part of the transcriptional program that cells use to respond to low iron levels suggested that this

FIG. 8. Iron accumulates in abc3Δ mutant cells. (A) The wild-type (WT), fep1Δ, and abc3Δ strains were grown in YES medium to the exponential phase (A<sub>600</sub> of ~1.0). The cells were harvested, and the iron content was determined by the BPS-based spectrophotometric method (45). The values indicated are the averages of triplicate measurements ± the standard deviations. (B) Logarithmic-phase cultures of the isogenic wild-type (WT), abc3Δ, and pcl1Δ strains were grown in EMM containing 0.74 g of iron. The cell lysates were prepared from each culture and analyzed using the BPS-based spectrophotometric assay for quantitative measurement of iron. The values of total iron concentration shown are the means of three replicates ± the standard deviations. (C) An S. pombe strain bearing a disrupted abc3Δ allele was transformed with pJK148 (plasmid alone, abc3Δ) or pJK148abc3<sup>+</sup> (abc3<sup>+</sup>). For step 1, cultures were grown in YES medium containing glucose and Dip (150 μM). For step 2, cells were washed in water, and equivalent amount of each culture was streaked onto fermentable (glucose) and nonfermentable (ethanol/glycerol) agar media, and incubated at 30°C for 4 and 7 days, respectively. WT, wild-type strain.
protein was required during iron deficiency. Based on the fact of the vacuolar localization of Abc3, we reasoned that Abc3 could be important for providing iron to the cell from intracellular stores and that its activity could lead to the activation of Fep1. Under conditions of activation, Fep1 negatively regulates several genes, including those encoding components of the high-affinity iron transport machinery. To test how Abc3 expression influences cellular iron-dependent regulation, we investigated whether the levels of frp1+ transcript were increased in cells harboring an inactivated abc3+ gene. Cells were grown to early logarithmic phase in Edinburgh minimal basal medium. The data showed that the expression of frp1+ was increased ~2.8-fold compared to the basal level of frp1+ transcripts detected in wild-type cells (abc3+) (Fig. 10A). When the abc3Δ mutant was transformed with the wild-type abc3+ allele, frp1+ mRNA levels decreased ~1.8-fold under the basal levels observed in the wild-type starting strain (Fig. 10A). We concluded that deletion of the abc3+ gene (abc3Δ) increased the steady-state levels of frp1+ mRNA, suggesting a function for Abc3 in providing iron to the cell from intracellular stores. Its absence would lead to activation of the expression of the plasma membrane uptake machinery through the downregulation of the iron-sensing transcription factor Fep1.

To further investigate the ability of the cell to downregulate the transcription of frp1+ as an indicator of Abc3 activity, we utilized the nmt1+ inducible/repressible promoter system (32). The expression of abc3+ or abc3+-GFP under the control of the nmt1+ 41X promoter (13) permitted the repression of the synthesis of Abc3 in the presence of iron (10 μM), thereby ensuring intracellular accumulation of iron. Subsequently, the cells were harvested, washed, and resuspended in the same medium in the absence of iron. After induction for 60 min, the ability of Abc3 or Abc3-GFP to generate an iron-mediated signal that fostered repression of frp1+ transcription was analyzed. As shown in Fig. 10C, cells expressing abc3+ or abc3+-GFP triggered downregulation of the frp1+ mRNA levels (~3- to 4-fold). In contrast, treatment of the cells with thiamine to repress Abc3 synthesis induced the upregulation of frp1+ mRNA which exhibited steady-state levels similar to that observed in abc3Δ null cells. Taken together, the results strongly suggested that iron levels were compromised sufficiently in abc3Δ mutant cells to induce the activation of the frp1+ gene. In contrast, when abc3+ was induced under the control of the nmt1+ promotor, a larger pool of labile iron may become available and activate Fep1, which in turn downregulates the frp1+ transcription levels.

**DISCUSSION**

Members of the ABC protein superfamily include transporters that are involved in the translocation of a wide variety of substrates across membranes (15). ABC transporters are classified into seven subfamilies (ABCA to ABCG) based on conserved sequences within their amino acid sequences (10). *S. pombe* has 11 putative ABC transporters, four of which (Abc1, Abc2, Abc3, and Abc4) are members of the ABCB subfamily (20). A previous report has localized a GFP-tagged form of Abc1 to the endoplasmic reticulum and the Abc2-GFP, Abc3-GFP, and Abc4-GFP fusion proteins to the vacuolar membrane (20).

The amino acid identities between Abc3 and Abc1, Abc2, and Abc4 are 23.6, 62.8, and 22.8%, respectively, while the amino acid similarities are 39.7, 74.7, and 38.3%, respectively. Although Abc2 and Abc3 exhibit the highest percentage of amino acid identity and similarity, it has been shown that these two proteins are functionally distinct with respect to vacuolar accumulation of glutathione-conjugated compounds, including both the adenine biosynthetic intermediate phosphoribosylaminomimidazole and monochlorobimane (20). In addition, although Abc2 and Abc4 possess much less homology (23.7% identity; 40% similarity), the two proteins share similar functions in vivo, and differ considerably from Abc3 and Abc1 (20). Given the unrelated localizations of Abc3 and Abc1 and the lack of common phenotypes associated with their respective gene deletions (20), the probability that Abc3 and Abc1 share a biological role is low.

As would be expected for genes regulated by Fep1, putative
Fep1 consensus binding sites were found in the abc3+ promoter. The removal of four GATA boxes from the 5’ end of the abc3+ promoter had no apparent effect on either the iron- or the Dip-regulated expression of the abc3+/lacZ fusion gene. The overall magnitude of the response was found to be even higher when the DNA between positions 584 and 239 was deleted. From there, we used two independent reporter gene assays, one using the endogenous abc3+ minimal promoter and the second using a heterologous CYC1 minimal promoter. Both approaches revealed that the integrity of the GATA sequence located between positions –120 to –115 was essential to the iron-dependent repression of abc3+. Thus, abc3+ is the second Fep1-regulated gene to be the subject of negative transcriptional regulation via the presence of a single GATA element, the first reported example being str1+ (39). When iron is in excess in Ustilago maydis, siderophore transporter gene expression is negatively regulated at the transcriptional level by Urbs1 (2, 3). Similarly to Fep1, Urbs1 has two Cys2/
Cys$_2$-type zinc fingers located within its DNA-binding domain. Analogous to the situation for Fep1, in vitro DNA-binding assays have shown that Urbs1 can specifically interact with a single GATA element (2). However, as opposed to Fep1, Urbs1 requires the presence of two GATA boxes for its in vivo function (2). These observations may indicate differences in the use of amino acids that serve to interact with DNA between Fep1 and Urbs1. Alternatively, identification and characterization of additional target genes in *U. maydis* may reveal that some of them are negatively regulated through binding to Urbs1 to a single GATA element. A sequence comparison between many functional GATA boxes found that a variation occurred within the consensus cis-acting 5'-ATCTGATA(A/T)-3' element of target gene promoters that respond to Fep1.

We have observed that when the GATA element contained the following sequence, 5'-ATCTGATA -183-3' element of *fio1* +1, the 5'-193ATCACGATA -193-3' and the 5'-160ATCTGATA -160-3' elements of *php4* +4, and the 5'-123ATCTGATA -123-3' element of *abc3* +13. Furthermore, other groups have also observed that the extended 5'-ATCTGATA(A/T)-3' motif was significantly over-represented in target gene promoters that responded to *U. maydis* Urbs1 and *A. fumigatus* SreA, two fungal iron-responsive GATA factors (12, 51).

Yeast studies have shown that the vacuole is an important storage compartment for metals, either as a means of detoxifying the cell or as a reservoir of metal that enables the cell to grow under metal-deficient conditions (6, 47, 48, 53, 54). In the case of *S. cerevisiae*, when the cells dispose of ample iron, Ccc1 mediates the import of iron into the vacuole (28). In the case of *S. pombe*, cell1 +1 encodes a putative ortholog of *S. cerevisiae* Ccc1. As is observed for Ccc1, the deletion of the cell1 +1 gene renders the cells sensitive to iron compared to the wild-type strain (33; data not shown). Two distinct mechanisms are used for vacuolar iron mobilization when *S. cerevisiae* cells respond to a shift from sufficient to low iron concentrations. One mechanism involves the NRAMP homologue Smf3, which localizes to the vacuolar membrane and helps to mobilize vacuolar stores of iron (44). The other mechanism involves the vacuolar membrane-resident Fth1/Fet5 complex, which transports stored iron out of the vacuole, resulting in a subsequent redistribution within the cell (60). In *S. pombe*, iron is mobilized in response to iron deficiency that has not been established and may respond differently. A lack of homologs to the *S. cerevisiae* Smf3, Fth1, and Fet5. In fission yeast, Pdt1 is the only protein homologous to the NRAMP family of metal transporters (59). Analysis of protein localization has shown that Pdt1 is detected in periphery of the nucleus and the cell perimier, suggesting a biological role in the endoplasmic reticulum and, perhaps, at the cell surface (59). Furthermore, genetic and functional studies have shown that Pdt1 and Pmr1 cooperatively regulate cell morphogenesis through an as-yet-uncharacterized manganese-dependent homoeostatic mechanism (30).

In the present study, our data do not allow us to establish a relationship between cerulinen and Fe homeostasis. However, it has been previously shown that cerulinen is an effective inhibitor of sterol synthesis in yeast (61). Interestingly, like *abc3* +1, the mRNA levels of genes involved in sterol synthesis increase upon Fe depletion (34). One can envision that in the absence of *Abc3*, less Fe is available for Fe-requiring proteins involved in sterol synthesis, making cerulinen more effective to inhibit sterol biosynthesis and then cell growth.

Given the fact that genetic studies have implicated the vacuole as a player in iron storage (28, 54), and assuming that vacuolar iron is present in a usable form, we suggest that *S. pombe* Abc3 is an intracellular transporter that mobilizes stores of iron from the organelle for redistribution throughout the cell. This model is supported by several experimental results. First, a functional Abc3-GFP fusion protein was localized to the membrane vacuole under iron-limiting conditions. Second, deletion of the *abc3* +1 gene led to elevated transcriptional activity of *frp1* +1 to presumably compensate for iron-poor conditions. Third, when *abc3* +1 was induced from the *nmt1* +1 promoter, there was inhibition of *frp1* +1 gene expression. Fourth, *abc3*Δ cells contained more total cell iron than wild-type cells. Fifth, in *S. pombe* cells lacking the high-affinity plasma membrane transporting complex Fio1 and Fip1, Abc3 was required for growth in low-iron medium. Sixth, the fact that *abc3* +1 was transcriptionally regulated by iron in the same direction as the genes encoding components of the high-affinity iron uptake machinery (e.g., *frp1* +1, *frp1* +1, and *fio1* +1) suggests a function for Abc3 in iron utilization as opposed to iron detoxification.

Fungi such as *Aspergillus nidulans*, *A. fumigatus*, and *U. maydis* do not possess Fth1 orthologs (16). As in the case of *S. pombe* Abc3, *A. nidulans* AtfH, *A. fumigatus* Afu3g03430 and Afu3g03670, and *U. maydis* Fer6 are all ABC-like proteins that are regulated at the level of gene transcription (12, 16, 51). Their loci are induced in iron-depleted cells and repressed in iron-replete cells. Although the protein localization of these putative ABC-type transporters has not yet been ascertained, the fission yeast *S. pombe* may represent an attractive model system for understanding the involvement of these proteins in *A. nidulans*, *A. fumigatus*, and *U. maydis* with respect to iron intracellular transport and homeostasis.

One feature of the members of the ABC subfamily of transporters that distinguishes them from other ABC transporters is their ability to transport substrates in the form of glutathione conjugates or complexes (17, 29). In the presence of excess iron, the vacuole is postulated to function as a storage compartment, preventing detrimental levels of iron accumulation in the cytosol (28). Intravacuolar iron may be bound in a bio-unavailable form such as Fe$^{3+}$ to polyphosphates or other molecules. In response to iron deficiency, Abc3 may mobilize stored iron either in an inorganic form or in the form of iron conjugates. This possibility remains speculative since a putative interaction between Abc3 and iron inorganic iron or organic iron conjugates is unknown.

It is interesting that our data concerning Abc3 are reminiscent of those observed in the case of the *IDI7* protein isolated from barley root cells, which is a member of the ABC superfamily of transporters (62). *IDI7* localizes to the vacuolar surface in plant cells that is known as the tonoplast. As observed in the case of Abc3, *IDI7* is expressed only in cells grown under conditions of iron deprivation. More recently, a third ABC transporter, named *NtPDR3*, has been found to be induced in iron-starved tobacco cells (11). Considering this information,
one can envision the possibility that a new group of ABC transporters, including Abc3, ID17, and NtPDR3, may be responsible for mobilizing intravacuolar stores of iron when cells face iron deprivation.

ACKNOWLEDGMENTS

We are grateful to Gilles Dupuis and William Home for critically reading the manuscript. We thank Jeff Beaudin for stimulating discussion and suggestions.

P.P. and M.I. are the recipients of studentships from the Foundation of Stars for Children’s Health Research and the Faculty of Medicine and Health Sciences of the Université de Sherbrooke, respectively. A.M. is the recipient of a studentship from Fonds de la Recherche en Santé du Québec (FRSQ). This study was supported by Natural Sciences and Engineering Research Council of Canada grant MOP-238238-01 to S.L. S.L. is the recipient of a Senior Investigator Scholarship from FRSQ.

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