Iron Activates In Vivo DNA Binding of *Schizosaccharomyces pombe* Transcription Factor Fep1 through Its Amino-Terminal Region

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In *Schizosaccharomyces pombe*, the iron sensor Fep1 mediates the transcriptional repression of iron transport genes in response to high concentrations of iron. On the other hand, *fep1* expression is downregulated under conditions of iron starvation by the CCAAT-binding factor Php4. In this study, we created a *fep1Δ php4Δ* double mutant strain where expression of *fep1* was disengaged from its iron limitation-dependent repression by Php4 to examine the effects of iron on constitutively expressed functional *fep1*-*GFP* and *TAP-fep1* alleles and their gene products. In these cells, Fep1-green fluorescent protein was invariably localized in the nucleus under both iron-limiting and iron-replete conditions. Using chromatin immunoprecipitation assays, we found that Fep1 is associated with iron-responsive promoters in vivo. Chromatin binding was iron dependent, with a loss of binding observed in the presence of low iron. Functional dissection of the protein revealed that the N-terminal 241-residue segment that includes two consensus Cys2/Cys2-type zinc finger motifs and a Cys-rich region is required for optimal promoter occupancy by Fep1. Within this segment, a minimal module encompassing amino acids 60 to 241 is sufficient for iron-dependent chromatin binding. Using yeast one-hybrid analysis, we showed that the replacement of the repression domain of Fep1 by fusing the activation domain of VP16 to the chromatin-binding fragment of amino acids 1 to 241 of Fep1 converts the protein from an iron-dependent repressor into an iron-dependent transcriptional activator. Thus, the repression function of Fep1 can be replaced with that of a transcriptional activation function without the loss of its iron-dependent DNA-binding activity.

Because iron readily gains and loses an electron, it is an essential cofactor for a wide variety of cellular enzymes, including metalloproteins that are involved in the transfer of electrons (11, 40). Iron-dependent enzymes are essential in numerous biochemical processes, such as respiration, the tricarboxylic acid cycle, photosynthesis, and nitrogen fixation (15). However, this same redox active property of iron makes it potentially toxic. Indeed, iron has the potential to generate toxic oxygen radicals via the Fenton reaction (15). Thus, in order to maintain sufficient but not excessive concentrations of iron, organisms have evolved with regulated mechanisms for ensuring the balance between essential and toxic iron levels.

Unicellular organisms have developed efficient means to regulate iron uptake (4, 18, 37). Studies of yeasts have shown that several genes known to encode components of the iron transport machinery are regulated at the transcriptional level; they are induced under conditions of iron starvation and repressed under conditions of iron repletion (19). In the model organism *Schizosaccharomyces pombe*, it has been shown that downregulation of the iron transport machinery is directed by Fep1, a member of the GATA protein family of transcription factors (22). Consistently, we determined that Fep1 recognizes and binds to consensus GATA sequences found in the region upstream of their target genes, including the promoters of genes encoding components of the reductive (e.g., *fpl1*+, *fpl2*+, and *fpl3*+) and nonreductive (e.g., *str1*+, *str2*+, and *str3*+) iron transport systems (32, 33).

The N-terminal 241-amino-acid region of Fep1 contains two Cys2/Cys2-type zinc finger motifs, denoted ZF1 and ZF2. In addition, there is a highly conserved region, termed the cysteine-rich region, located between the zinc fingers. The sequence of the N terminus of Fep1 is highly similar to the N termini of other fungal GATA-type transcriptional repressors of iron-responsive genes, including Urbs1 (from *Ustilago maydis*), SRE (from *Neurospora crassa*), SREA (from *Aspergillus nidulans*), Sfu1 (from *Candida albicans*), and Sre1 (from *Histoplasma capsulatum*) (2, 3, 6, 14, 16, 24, 30, 42). In vitro binding studies demonstrated that recombinant proteins containing residues 1 to 241 of Fep1 (1-241) or 116 to 374 of Sre1, expressed in and purified from bacteria, specifically interact with GATA elements in an iron-dependent manner (6, 34). For Fep1, it was shown that ZF2 alone is sufficient for DNA binding in vitro (35). For SRE and Sre1, although mutations in either zinc finger motif led to a significant loss in DNA binding, functional dissection of their DNA-binding domains indicated that inactivation of ZF2 resulted in a more severe defect (6, 41). The critical requirement of ZF2 for DNA binding is reminiscent of Urbs1 in *U. maydis* where the second zinc finger alone is necessary and sufficient for specific binding to DNA (3). However, as opposed to Urbs1, the transcription factors Fep1 and SRE require both ZF1 and ZF2 for normal iron-dependent downregulation of target gene expression (16, 35).

As mentioned above, a hallmark feature of these fungal iron-regulating GATA transcription factors is the presence of a conserved 27-residue segment containing four invariant Cys...
residues that is positioned between the two zinc finger motifs (12). Specific mutations converting the Cys residues to Ala resulted in transcription factors with significantly lower affinities for DNA (6, 35). Mutation of these residues in Fep1 resulted in a reduction in its DNA binding affinity which correlated with a loss in its ability to repress target gene expression (35). Interestingly, it was observed that during the purification of recombinantly expressed SRE, Fep1, and Sre1 DNA binding domains by affinity chromatography, the wild-type proteins which possess the ZF1, Cys-rich, and ZF2 motifs were reddish-brown in color, presumably due to the presence of protein-bound iron (6, 16; B. Pelletier and S. Labbé, unpublished data). This assumption was recently confirmed by experiments showing that ferric iron was directly associated with purified recombinant Sre1 (6). In contrast, when the conserved Cys residues of the SRE, Fep1, and Sre1 Cys-rich regions were mutated to alanines, the mutant recombinant peptides exhibited no distinctive color during purification. Consistent with this observation, the Sre1 protein with mutations in the Cys-rich region contained much less iron than the wild-type Sre1 (6). It has also been shown that wild-type Sre1 is associated with zinc ions (1.6 equivalent per monomer). Disruption of either ZF1 or ZF2 motifs resulted in the reduction of associated zinc to about half that of the normal concentration found in the wild-type protein (6). As previously reported for SRE (16), zinc-chelated Sre1 failed to bind to GATA elements (6). However, since neither SRE nor Sre1 responded to exogenous zinc via enhanced downregulation of target gene transcription, it is likely that the zinc plays mainly a structural role in the fungal iron-regulating GATA transcription factors.

Genes encoding iron-regulatory GATA-type transcriptional repressors are either constitutively expressed (e.g., Ure31 and SRE) or differentially expressed as a function of iron availability (e.g., SREA, Fep1, and Sre1) (6, 14, 27). Transcript levels of SREA, fep1+, and SRE1 are induced under iron-replete conditions and repressed under conditions of iron starvation. Downregulation of fep1+ gene expression in response to iron limitation occurred only upon treatment with a permeant iron chelator (27).

Two novel transcription factors that are critical for downregulating expression of genes encoding iron-using proteins under iron-limiting conditions have been identified in S. pombe and A. nidulans (17, 26). S. pombe Ph4 is an important subunit of the fission yeast CCAAT-binding protein complex (25, 26). In response to iron starvation, Ph4 is synthesized and interacts with the Php2/Php3/Php5 heterotrimer to mediate repression of genes encoding iron-using proteins, including the gene fep1+ (26, 27). When iron is abundant, Php4 fails to act as a repressor. fep1+ transcription is therefore derepressed. Once made, Fep1 associates to GATA-type regulatory sequences made, Fep1 associates to GATA-type regulatory sequences bound by iron, Fep1 binds to its target DNA sequence within the region encompassing the Cys-rich domain and ZF2 (amino acids 60 to 241) constitutes the minimal module for iron-dependent binding of Fep1 to chromatin at the fio1+ promoter, while truncation of ZF1 (1-59Δ) led to a decrease in its binding ability. Using yeast one-hybrid analysis, we showed that the VP16 active domain fused to the N-terminal segment 1-241 of Fep1 is able to activate transcription in response to iron. Collectively, our results reveal that Fep1 1-241 constitutes a module that, upon activation by iron, can bind to GATA sequences in vivo. Furthermore, depending upon the nature of the iron-based acids fused to the Fep1 1-241 segment, the new hybrid protein can potentially trigger a positive or negative iron-dependent transcriptional regulatory response.

MATERIALS AND METHODS

Yeast strains and media. The genotypes of the S. pombe strains used in this study were as follows: FY435 (h+ his7-366 leu1-32 ura4-D18 ade6-M210), phpΔ (h+ his7-366 leu1-32 ura4-D18 ade6-M210 phpΔ::KAN1), fep1Δ (h+ his7-366 leu1-32 ura4-D18 ade6-M210 tep1::ura4+), and tep1Δ phpΔ (h+ his7-366 leu1-32 ura4-D18 ade6-M210 tep1::KAN1 pep4::loxP::loxp). Yeast cells were grown to exponential phase at 30°C in yeast extract plus supplements (225 mg/liter of adenine, histidine, leucine, uracil, and lysine) (1) with or without the addition of 250 mM 2,2'-dipyridyl (Dp), 250 mM bathophenanthroline disulfonic acid (BPS), and 100 or 250 μM FeCl3. Edinburgh minimal medium (1) lacking specific amino acids required for plasmid selection was also used when yeast strains were transformed with nonintegrative plasmids. Yeast transformations and manipulations were carried out using standard techniques (1).

Plasmids. Plasmids pJK-1478fep1+, pJK-1478fep1+GFP, pJK-1478TAPfep1+, pJK-1478TAPfep1+M241, pJK-1478TAPfep1+M318, and pJK-1478TAPfep1+M359 were constructed by molecular strategies described previously (35). The full-length coding region of fep1+ was isolated from the plasmid pJK-1478TAPfep1+ using the BamHI and PstI restriction enzymes and subcloned into the same restriction enzyme sites of pBluecript SK (Stratagene, La Jolla, CA), generating pSKnullfep1+. To create truncations from the N-terminal end of Fep1, the fep1+ gene starting at codons 60, 130, and 242 was amplified by PCR from the plasmid pSKnullfep1+ using primers designed to initiate an initiator codon at the 5' end and unique BamHI and PstI sites at the termini of the upstream and downstream DNA fragments, respectively. The PCR products obtained were digested with BamHI and PstI and exchanged with an identical DNA region into the plasmid pJK-1478fep1+ using primers that were used to amplify the initial fep1+ gene from the plasmid pSKnullfep1+. The purpose of the DNA fragment exchange into plasmid pSKnullfep1+ was to facilitate the validation of the integrity of each construct by DNA sequencing. pJK-1478TAPfep1+ plasmid derivatives were denoted pJK-1478TAPfep1-99A, pJK-1478TAPfep1-129A, and pJK-1478TAPfep1-241Δ. An identical DNA cloning strategy was used to generate plasmids pJK-1478TAPfep1::C85A/C88A and pJK-1478TAPfep1::R184A/R185A/D186A/E187A, except that the initial DNA products were obtained from templates pJK-1478fep1::C85A/C88A and pJK-1478fep1::R184A/
Tris-borate-EDTA gels. PCR signals were quantitated by Phosphorimager scanning and normalization to the input DNA reaction and the internal intergenic control primer pair (to correct for PCR efficiency and background signal). All experiments were conducted at least three times, and each experiment yielded similar results. Primers that span fio1′ and str1 promoter regions that include functional GATA boxes (32, 33) were used for PCR analysis. Primers were designated by the name of the gene promoter followed by the position of their 5′ ends relative to the translation initiation codon as follows: fio1-m4a (5′-CACCGGGTAGTTGGCAACAGCGGGGGGGAACATGCCC-3′), fio1-a-2 (5′-GATAGGACAGTGTGGCCCTGGTTGTGTTCTACTTGG-3′), str1-a-5 (5′-GTATGACCTGCTATGATTTCTTGCGTACAC-3′), str1-b-25 (5′-CTTAAACCTTACTGAGGAAAGG-3′), Intergenic-cf3600000a-5′ (5′-GGCGAAGGCCATCGAACATCTCAAGTC-3′).

Preparation of S. pombe extracts and Western blot analysis. Protein extracts were prepared from aliquots of cell cultures that were taken after their incubation in the presence of 250 μM Dip or 250 μM FeCl3 but just before their treatment with 1% formaldehyde, which was used for ChIP analyses. Harvested cells were washed two times in ice-cold water. Cells were resuspended in cold extraction buffer (200 mM Tris-HCl, pH 8.0, 400 mM NaCl, 10 mM MgCl2, 10 mM EDTA, 10% glycerol, 7 mM [N-mersaptic acid, and 1 mM phenethyl-sulfonyl fluoride) containing a complete protease inhibitor cocktail (P8340; Sigma) and lysis by vortexing with an equivalent volume of glass beads (G-8772; Sigma) for 10 min at 4°C. The beads were removed by centrifugation, and the lysate was quantitated by the Bradford assay (5). Equal amounts of each sample were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) for Western blot analysis.

**Fluorescence microscopy.** Fluorescence microscopic analysis was performed as described previously (35) except that liquid cultures were seeded to an A600 of ~0.5 and grown to exponential phase (A600 of ~1.0) and then treated with 250 μM Dip or 100 μM FeCl3 for 90 min. Furthermore, 4′,6-diamidino-2-phenylindole (DAPI; D-8417; Sigma) was used for DNA staining instead of Hoechst 33342. Fluorescence and differential interference contrast images of the cells were obtained on an Eclipse E800 epifluorescent microscope (Nikon, Melville, NY) equipped with an ORCA-ER digital cooled camera (Hamamatsu, Bridgewater, NJ). The samples were analyzed using ×1 magnification with the following filters: 465 to 495 nm (green fluorescence protein [GFP]) and 340 to 380 nm (DAPI).

**One-hybrid system.** Saccharomyces cerevisiae strain CM3260 (Mata tpi1-63 leu2-3,112 can1-100 his3-609 ara1-52) (9) was used for one-hybrid analysis. Cells were grown in a modified synthetic minimal medium containing 83 mg/liter tryptophan, 83 mg/liter histidine, and 30 mg/liter lysine, 2% dextrose, 50 mM MOPS buffer [2-(N-morpholino)ethanesulfonic acid, pH 6.1], and 0.67% yeast nitrogen base minus neutral and iron (MP Biomedicals, Solon, OH). To generate the reporter plasmid pCM22GATA-IacZ, a 297 bp BglII-XhoI PCR-amplified DNA segment from the fio1′ promoter that contains two iron-responsive GATA sequences (positions ~800 to ~795 and ~777 to ~772) was inserted into the BglII and XhoI sites of plasmid pM64 (23). Plasmid PVP16-1Fep1241 was created as follows. The DNA sequence encoding the N-terminal region of Fep1 (residues 1 to 241) was isolated by PCR from pJK1478fep1′ (35) and cloned into the BamHI and Asp718 sites of pVP16 (34). A similar strategy was used to generate plasmid pVP′-1Fep1241′R184A/R185A/D186A/E187A (instead of pJK1478fep1′) (35). A second combination of primers that contained BamHI and PstI restriction sites was used to PCR amplify the first 241 codons of fep1′. The PCR fragment was then inserted into the BamHI-PstI sites of the p524ID plasmid (25) creating a vector that expresses the first 241 residues of Fep1′ (Fep1′Δ241). Linearized cultures of prototrophic transformed cells were seeded to an A600 density of 0.5 and then grown to exponential phase (A600 of 1.0). At log phase, cultures were treated with 250 μM Dip or 100 μM FeCl3 for 90 min. β-Galactosidase activity was determined as described previously (43). Expression of VP16-1Fep1241 and VP16-6Fep1241.
RESULTS

Fep1-GFP is localized in the nucleus of fep1Δ php4Δ double mutant cells under conditions of both low and high levels of iron. As determined in a previous study (27) and shown in Fig. 1A, treatment of wild-type cells for 90 min with the permeant iron chelator Dip (250 μM) resulted in a decrease in the steady-state level of fep1Δ mRNA by ~3.5-fold compared to the basal levels observed in untreated cells. On the other hand, treatment of cells with the nonpermeant iron chelator BPS (250 μM) or with iron (100 μM) for the same incubation time had no significant effect on fep1Δ transcript levels (Fig. 1A). Importantly, we previously showed that the downregulation of fep1Δ expression in response to iron limitation by Dip required the CCAAT-binding transcription factor Php4 (27). To begin to understand the mechanism by which Fep1 occupies the promoter of target genes in vivo, we developed a biological system that allows us to unlink iron-dependent behavior of Fep1 protein from its transcriptional regulation by Php4. We first eliminated the Php4-dependent downregulation of fep1Δ mRNA levels to ensure constitutive expression of Fep1 regardless of iron status. The php4Δ locus was inactivated by insertional deletion to create a php4Δ mutant strain (Fig. 1A). The fep1Δ open reading frame in this strain was replaced with the kanMX2 genetic marker to create a fep1Δ php4Δ double mutant strain. This strain was then transformed with integrative plasmids harboring a fep1Δ, fep1Δ-GFP, or TAP-fep1Δ allele that was subsequently expressed under the control of the fep1Δ promoter upon integration into the genome. fep1Δ php4Δ double mutant cells expressing these alleles exhibited fep1Δ mRNA levels that were unregulated by iron or iron starvation (Fig. 1A and B). We examined the effect of intracellular iron levels on the subcellular localization of Fep1 protein fused to GFP (Fep1-GFP) expressed in this strain. As shown in Fig. 1C, Fep1-GFP fluorescence was detected in the nuclei of cells expressing the fusion allele under both iron-limited and iron-replete conditions. Fep1-GFP fluorescence colocalized with the DNA-staining dye DAPI, which was used as a marker to stain the nucleus. While no fluorescence was observed in cells that were integrated with the empty vector, the fep1Δ php4Δ double deletion strain expressing GFP alone displayed a pattern of fluorescence throughout the cytoplasm and nuclei of cells (Fig. 1C).

To test whether insertion of TAP and GFP interfered with Fep1 function, the untagged (fep1Δ) and tagged (TAP-fep1Δ and fep1Δ-GFP) coding sequences were separately integrated into fep1Δ php4Δ mutant cells. After their isolation, the integrants were analyzed for their abilities to repress fio1Δ mRNA levels in response to iron. As shown in Fig. 2, when TAP-Fep1 and Fep1-GFP were expressed in fep1Δ php4Δ mutant cells, they functionally conferred iron-dependent repression of fio1Δ expression in a fashion similar to that of the wild-type Fep1 protein. However, the absence of Fep1 resulted in sustained expression of the fio1Δ mRNA levels that were unresponsive to iron (Fig. 2). Collectively, these results show that we have established a biological system where functional alleles of fep1Δ are constitutively expressed and whose gene products are localized within the nuclei of cells under both low and high iron concentrations.

Fep1 binds to target gene promoters in vivo in an iron-dependent manner. To determine if Fep1 is constitutively associated with the fio1Δ promoter, we used a ChIP method in which proteins were cross-linked in vivo to DNA that they are associated with using formaldehyde (21). Cell lysates were prepared from fep1Δ php4Δ cells in which a functional fep1Δ gene containing a TAP tag immediately after the initiator codon (TAP-Fep1) was returned by integration. The chromatin was prepared and sonicated to an average size of 500 bp. DNA segments cross-linked to TAP-Fep1 were isolated by immunoprecipitation with an anti-mouse IgG that bound to two IgG-binding units of protein A encoded within the TAP tag. To identify the DNA sequences bound to TAP-Fep1, the cross-links were reversed and the DNA was analyzed by quantitative PCR using primer sets specific to the fio1Δ or str1Δ promoter regions that are known to be required for iron-mediated repression of gene expression (32, 33). An intergenic region on chromosome II (contig location 3860292 to 3860402) devoid of open reading frames and GATA-type cis-acting sequences was used as a control for unregulated and nontranscribed DNA. Cells expressing untagged or TAP-tagged fep1Δ alleles (Fig. 3) were precultured in the presence of the iron chelator Dip (100 μM) to preclude iron-dependent downregulation of target gene expression. Cells were harvested from cultures at logarithmic phase, washed, and resuspended in the same medium containing either Dip (250 μM) or FeCl3 (250 μM) for 90 min. The results from ChIP analysis showed that when cells were treated with iron (250 μM FeCl3), TAP-Fep1 occupied the fio1Δ and str1Δ promoters at high levels (Fig. 3A and B). Its association was iron-dependent, exhibiting 8.5-fold-higher levels of fio1Δ promoter DNA immunoprecipitated with anti-mouse IgG antibodies when the chromatin was isolated from cells grown in the presence of iron (250 μM FeCl3) than from cells that were cultured in the presence of Dip (Fig. 3A).

Likewise, anti-mouse IgG antibodies immunoprecipitated 41.6-fold more TAP-Fep1 associated with the str1Δ promoter DNA in cells grown in the presence of 250 μM FeCl3 than in cells cultivated under conditions of iron starvation (Fig. 3B). In contrast, low levels of fio1Δ and str1Δ promoter were immunoprecipitated in iron-starved cells grown in the presence of 250 μM Dip (Fig. 3A and B). These levels were similar to the background signals observed when ChIP was performed in a strain expressing untagged Fep1 (Fig. 3A and B). These observations are consistent with results showing strong induction of fio1Δ (Fig. 2) and str1Δ (33) mRNA levels during iron starvation, suggesting that under these conditions, binding of TAP-Fep1 repressor to the promoters of these genes occurred at the background level (Fig. 3A and B).

To verify whether the TAP-Fep1 fusion protein was expressed under conditions of both low (250 μM Dip) and high (250 μM FeCl3) levels of iron, fission yeast nuclei that contain unbound nuclear proteins and proteins cross-linked to chromatin by formaldehyde were analyzed by immunoblotting (see Fig. S1 in the supplemental material). Irrespective of whether the proteins were cross-linked to chromatin or not, TAP-Fep1 was clearly produced under both iron-limiting and iron-replete
conditions (see Fig. S1 in the supplemental material). Furthermore, we found that IgG-Sepharose beads attached to both cross-linked and un-cross-linked Fep1 because TAP-Fep1 was detected in the immunoprecipitates in the absence or presence of iron (see Fig. S1 in the supplemental material). However, as expected, no immunoprecipitate was observed in fep1Δ php4Δ cells expressing the untagged fep1+ allele (see Fig. S1 in the supplemental material). Taken together, these results indicate...
that under iron-replete conditions, Fep1 is strongly associated with its target promoters in vivo and dissociates from these promoters in response to iron starvation.

Under conditions of high levels of iron, preexisting Fep1 molecules associate with the \textit{fio1} promoter in vivo. To determine the effect of iron on preexisting Fep1 molecules, we utilized the \textit{nmt1} inducible/repressible promoter system (28) that allowed us to extinguish the biosynthesis of TAP-Fep1 and subsequently examine the abilities of preexisting TAP-Fep1 molecules to associate with chromatin in response to iron. First, we determined if TAP-Fep1 can be expressed from the \textit{nmt1} expression system without affecting its function. \textit{fep1} cells expressing a TAP-fep1 allele under the control of the \textit{nmt1} promoter (10) were grown for 18 h in the absence of thiamine and in the presence of the iron chelator Dip (100 \textmu M) to an \textit{A}_{600} of 0.9. This step allowed the biosynthesis of TAP-Fep1 and its nuclear sequestration (Fig. 1C). Subsequently, the cells were harvested, washed, and resuspended in the same medium without Dip. After the addition of thiamine (15 \textmu M) to repress further synthesis, the cells were treated with Dip (250 \textmu M) or iron (250 \textmu M) for 30 and 60 min before cross-linking with formaldehyde. At the indicated time points, aliquots of each culture were removed for analyses of steady-state mRNA levels. In the presence of thiamine and iron, preexisting TAP-Fep1 clearly repressed \textit{fio1} mRNA levels compared to the levels in cells grown under iron-limiting conditions in the presence of Dip (Fig. 4A). Similarly, iron-dependent downregulation of \textit{fio1} gene expression was observed when the \textit{nmt1} \textit{X}-TAP-fep1 allele was constitutively expressed in the absence of thiamine (Fig. 4A), whereas no regulation by iron was observed in a \textit{fep1} strain (Fig. 4A).

To ensure that the iron responsiveness of the \textit{fio1} gene

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Integration of the \textit{fep1}, \textit{fep1}-GFP, or TAP-fep1 alleles in \textit{fep1} \textit{php4} mutant cells restores iron-dependent downregulation of \textit{fio1} expression. A \textit{fep1} \textit{php4} double mutant strain was transformed with pJK-1478\textit{fep1}, pJK-1478\textit{fep1}-GFP, or pJK-1478TAP\textit{fep1}. Total RNA from cultures treated with Dip (250 \textmu M) or FeCl$_3$ (100 \textmu M) (Fe) was isolated and analyzed by RNase protection assays. Steady-state levels of \textit{fio1} and \textit{act1} mRNA (indicated with arrows) were determined. As additional controls, a \textit{fep1} single mutant strain was transformed with an empty vector (vector alone), and the parent FY435 (WT) was used as a positive control for repression of \textit{fio1} gene expression under iron-replete conditions. The results shown are representative of three independent experiments.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{\textit{fio1} and \textit{str1} promoters are bound by TAP-Fep1 in an iron-dependent manner. (A) ChIP analysis was performed on the \textit{fio1} promoter in cells carrying an integrated untagged or TAP-tagged \textit{fep1} allele. \textit{fep1} \textit{php4} cells were precultivated in the presence of 100 \textmu M Dip and grown to an \textit{A}_{600} of ~1.0. Cells were then washed and incubated for 90 min in the presence of 250 \textmu M Dip or 250 \textmu M FeCl$_3$ (Fe). The top band is the \textit{fio1} specific signal, whereas the lower band is an internal background control from a nontranscribed region (intergenic region). The lower panel shows a graphic representation of the quantitation of three independent ChIP assays, including the experiment depicted in the upper panel. Signals are expressed as relative binding (%) and were calculated as a percentage of the highest value obtained. (B) ChIP analysis on the \textit{str1} promoter was performed as described for panel A. The graphic (bottom) shows a quantitation of three independent ChIP experiments. Input, input chromatin; IP, immunoprecipitated chromatin.}
\end{figure}
FIG. 4. Elevated iron levels induce iron-dependent association of nuclear TAP-Fep1 with chromatin. (A) Total RNA was prepared from aliquots of cultures that were grown in thiamine-free (−vit) or thiamine-replete (+vit) medium containing either Dip (250 μM) or FeCl3 (250 μM) (Fe) for 30 and 60 min. RNase protection assays were carried out on extracted RNA samples to determine fio1 and act1 mRNA levels. As additional controls, steady-state mRNA levels of fio1 and act1 were also analyzed in strains expressing (fep1) or lacking the fep1 allele (fep1Δ). (B) As described for panel A, RNA samples were examined by RNase protection assays for steady-state levels of fep1 and act1 mRNAs. (C) Total protein extracts were prepared from aliquots of cultures and then analyzed by immunoblotting using either mouse anti-IgG or anti-PCNA (as an internal control) antibody. TAP-Fep1 isolated from a fep1Δ null strain expressing a functional TAP-fep1 allele was used as a standard and a positive control (pJK-1478TAP-fep1). (D) Cells harboring a fep1Δ deletion were transformed with pJK-1178nmt181X-fep1 or pJK-1178nmt181X-TAP-fep1 and grown in thiamine-free medium containing 100 μM Dip for 18 h. Cells (at an A600 of 1.0) were then transferred to thiamine-replete medium (to repress further TAP-Fep1 protein synthesis) and grown further in the presence of 250 μM Dip or 250 μM FeCl3 for 60 min. Following sonication, purified chromatin was immunoprecipitated with anti-mouse IgG antibodies, and a specific region of the fio1 promoter was analyzed by PCR to monitor Fep1 occupancy at the promoter under conditions of both low and high levels of iron (top). Quantitation of anti-IgG immunoprecipitated chromatin is also shown (bottom). Binding activity values are the averages of triplicate determinations ± the standard deviation. The values are expressed as percentages of the largest amount of chromatin measured.
transcription was due to the preexisting TAP-Fep1 and not due to a newly synthesized TAP-Fep1 arising from a pool of stable mRNA, total RNA was prepared from cells at the time points (30 and 60 min) used in the experiments described above and analyzed by RNase protection assay. The results showed that the fep1 + mRNA was undetectable after 60 min of incubation in the presence of thiamine (Fig. 4B) compared to its levels observed in cells grown without thiamine (Fig. 4B). As controls, fep1 + mRNA steady-state levels were probed in an isogenic wild-type strain and in a fep1Δ mutant strain in response to iron availability (Fig. 4B). To ensure that the TAP-Fep1 fusion protein was synthesized when expressed under the control of the regulatable nth1 + 8IX promoter, aliquots of whole-cell extracts were analyzed by immunoblot assays using anti-IgG and anti-PCNA antibodies. We observed that regardless of the cellular thiamine or iron status, the TAP-Fep1 protein was present at comparable levels (Fig. 4C).

To assess the effect of iron on the association of preexisting Fep1 molecules with chromatin, we determined their presence on the fio1 + promoter in chromatin prepared from cells grown under both iron-limiting and iron-replete conditions. Under conditions of low iron levels, TAP-Fep1 did not immunoprecipitate a significant amount of fio1 + promoter sequence compared to the intergenic region reference (Fig. 4D). However, under iron-replete conditions, TAP-Fep1 strongly immunoprecipitated the fio1 + promoter DNA compared to the intergenic region reference (Fig. 4D). As a control, untagged TAP1 immunoprecipitated the background levels of the fio1 + promoter region. Taken together, these results strongly suggest that iron regulates the occupation of promoters by preexisting Fep1 molecules localized in the nucleus and does not require the synthesis of new Fep1 protein.

Cys-rich region and two zinc finger motifs in Fep1 are required for maximal binding to chromatin and its ability to mediate iron-responsive gene regulation. The primary amino acid sequence of Fep1 shows that it has a Cys-rich region, which contains four invariant Cys residues (amino acids 70, 76, 85, and 88), that is also present in other characterized GATA-type iron regulators (12). Moreover, the Cys-rich region is found between two GATA-type zinc finger motifs, denoted ZF1 (amino acids 12 to 60) and ZF2 (amino acids 172 to 220) (Fig. 5A). To determine if specific amino acid residues within these motifs play a role in the ability of Fep1 to interact with chromatin in vivo, we created five mutant derivatives of TAP-Fep1, denoted TAP-59ΔFep1, TAP-129ΔFep1, TAP-241ΔFep1, TAP-Fep1-C85A/C88A, and TAP-Fep1-R184A/R185A/D186A/E187A. These proteins are schematically diagrammed in Fig. 5A. fep1Δphp4A cells expressing an integrated TAP-59Δfep1 allele lacking the ZF1 motif were grown under conditions of low levels of iron (100 μM Dip) and then further maintained under iron-limiting conditions (250 μM Dip) or switched to conditions of high levels of iron by supplementing the medium with 250 μM FeCl3. As shown in Fig. 5B, when ZF1 of Fep1 was absent, the frequency by which TAP-59ΔFep1 occupied the fio1 + promoter decreased by 68% compared to that of wild-type TAP-Fep1 under iron-replete conditions. However, analogous to the wild-type protein, the TAP-59ΔFep1 mutant did not associate with chromatin in iron-starved cells (Fig. 5B). Further deletions to positions Leu30 (TAP-129ΔFep1) and Asp242 (TAP-241ΔFep1) completely abolished the ability of TAP-Fep1 to bind to chromatin irrespective of cellular iron status (Fig. 5C and D). To determine their role in the interaction of TAP-Fep1 with chromatin in response to iron in vivo, we mutated the Cys85 and Cys88 residues located within the Cys-rich intervening region. The results from ChIP experiments showed that the TAP-Fep1-C85A/C88A mutant failed to associate with the fio1 + promoter sequence, indicating that both Cys residues are necessary for TAP-Fep1 to associate with DNA (Fig. 5E). We also tested the role of the Arg184, Arg185, Asp186, and Glu187 residues, which are known to nullify the role of ZF2 in Fep1 when mutated (35). Similar to the TAP-Fep1-C85A/C88A mutant, TAP-Fep1-R184A/R185A/D186A/E187A immunoprecipitated only background levels of chromatin from cells grown under both iron-limiting and iron-replete conditions (Fig. 5F), indicating that ZF2 is necessary for chromatin binding in vivo.

Because the mutations described above greatly reduced (68%) or abolished the ability of TAP-Fep1 to bind to chromatin, we examined the steady-state level of fio1 + mRNA in the presence of iron and compared that to steady-state levels under basal or iron-deprived (Dip) conditions. As shown in Fig. 6A, fep1Δphp4A cells expressing the various mutant alleles (TAP-59Δfep1, TAP-129Δfep1, TAP-241Δfep1, TAP-fep1-C85A/C88A, and TAP-fep1-R184A/R185A/D186A/E187A) exhibited sustained expression of the fio1 + mRNA levels that were unaffected by variations in intracellular iron levels (Fig. 6A). These results suggest that the mutants were inactive and therefore incapable of directing iron-mediated repression of fio1 + transcription. To ensure that all of the mutant proteins were stably expressed, we isolated total protein extracts from cells expressing these proteins that were grown under conditions of low or high levels of iron. Analysis of the extracts by immunoblotting using an antibody against IgG showed that all the TAP-Fep1 fusion proteins used in the experiments described above were consistently detected under conditions of both low and high levels of iron (Fig. 6B). With the exception of the TAP-241Δfep1 mutant, all the Fep1 mutant derivatives harboring N-terminal deletions or point mutations were localized in the nucleus as previously reported (35), thereby eliminating the possibility that the loss of function was due to protein mislocalization. Based on these collective results, we conclude that the two zinc finger motifs, ZF1 and ZF2, and the Cys-rich region, in particular the Cys85 and Cys88 residues within this motif, are necessary for maximum promoter occupancy and subsequently iron-mediated repression of the wild-type fio1 + promoter by the Fep1 transcription factor.

N-terminal region encompassing the first 241 amino acids of Fep1 is sufficient for its iron-dependent binding to chromatin but insufficient for transcriptional repression of fio1 + transcription. To gain additional insight into other domains of Fep1 that may be essential for its association with chromatin, truncations were created from the C-terminal end of the TAP-Fep1 fusion protein (Fig. 7A). A first allele (TAP-fep1359) was created by deleting the last 205 codons of Fep1, whereas a second one (TAP-fep1241) was generated by removing the last 323 codons of the transcription factor. Both alleles were separately integrated and expressed in fep1Δphp4A cells. Chromatin was prepared from cells treated with the iron chelator Dip (250 μM) or incubated in the presence of iron (250 μM FeCl3). When cells expressing wild-type Fep1 or TAP-fep1359
FIG. 5. The Cys-rich region and ZF2 of Fep1 are sufficient for in vivo promoter occupancy, but both zinc fingers and the Cys-rich region are required for maximal Fep1 binding to chromatin. (A) Schematic representations of the wild-type and mutant derivatives of TAP-Fep1 protein. ZF1 and ZF2 (black boxes) are the N- and C-terminal GATA-type zinc finger motifs, respectively. Between the two zinc finger motifs, a conserved amino acid sequence, denoted the Cys-rich region, is depicted with four cysteine (C) residues. The point mutations in the TAP-Fep1-R184A/R185A/D186A/E187A mutant are marked with alanine (A) residues. The white rectangle indicates the location of the TAP tag. The amino acid sequence of TAP-Fep1 is numbered relative to its initiator codon. (B to F) Cells were precultured in the presence of 100 μM Dip. Logarithmic-phase cultures of cells were then incubated in the presence of Dip (250 μM) or FeCl₃ (250 μM) (Fe) for 90 min. ChIP analysis was carried out using cells expressing the indicated TAP-tagged proteins as described for panel A. For each panel (B to F), results of ChIP analysis on the *fio1* promoter were quantified based on three independent experiments and illustrated as described in the legend to Fig. 3.
were grown in the presence of Dip, anti-IgG antibodies immunoprecipitated background levels of DNA, suggesting that both proteins did not stably associate with the fio1/H11001 promoter under these conditions (Fig. 7B). However, under the same conditions of low levels of iron, we observed that TAP-1Fep1241 occupied the fio1/H11001 promoter at a low but significant level of frequency (15.2%) compared to TAP-Fep1 (Fig. 7C). In contrast, when cells were grown in the presence of iron, TAP-Fep1 occupied the fio1/H11001 promoter at maximum level (100%) (Fig. 7B and C). Although the truncated TAP-1Fep1359 exhibited a reduced ability to bind to chromatin (33%) compared to the full-length protein, the presence of iron clearly triggered an increase in its binding to the fio1/H11001 promoter (Fig. 7B). Similarly, we found that elevated iron levels triggered the association of TAP-1Fep1241 with the fio1+ promoter, although its level of occupancy was 27.8% lower than that of the wild-type protein (Fig. 7C).

Expression of the wild-type TAP-Fep1 fusion protein restored iron-mediated repression of fio1+ transcription (Fig. 7D). In contrast, although the truncated alleles TAP-1Fep1359 and TAP-1Fep1241 were clearly expressed in fep1Δ php4Δ mutant cells under both iron-replete and iron-depleted conditions (Fig. 7E) and localized to the nucleus (data not shown), neither protein was capable of directing iron-dependent down-regulation of fio1+ gene expression (Fig. 7D). Taken together, these results revealed that while both truncated mutants of TAP-Fep1 were capable of iron-mediated binding to chromatin in vivo, they were insufficient for repression of fio1+ gene expression during growth under conditions of high levels of iron.

N-terminal 60 to 241 amino acid residues of Fep1 constitute a minimum module that is sufficient for the weak binding of Fep1 to chromatin in vivo. Based on the results described above indicating that the Fep1 N-terminal region containing the two zinc finger motifs and the Cys-rich region is capable of interacting with chromatin in vivo, we further truncated both mutant proteins to eliminate residues 1 to 59, encompassing ZF1 (Fig. 8A), to determine if the loss of these residues will abolish their abilities to bind to chromatin. fep1Δ php4Δ cells expressing the wild-type TAP-fep1+ or the mutant TAP60fep1359 or TAP60fep1241 allele were incubated under conditions of both low and high levels of iron. As previously shown, maximum

FIG. 6. The presence of both zinc finger motifs and the Cys-rich region of Fep1 is necessary for iron-mediated repression of fio1+ expression. (A) fep1Δ php4Δ cells transformed with the indicated integrative constructs (as described in the legend to Fig. 5A) were grown under iron starvation conditions (250 μM Dip), were untreated (−), or were treated with 250 μM FeCl3 (Fe) for 90 min. After extraction of total RNA, the fio1+ and act1+ steady-state mRNA levels (indicated with arrows) were analyzed by RNase protection assays. Results shown are representative of three independent experiments. (B) Whole-cell extracts were prepared from fep1Δ php4Δ cells expressing the wild-type (WT) TAP-Fep1 fusion protein and its mutant derivatives. For simplicity, two culture conditions (250 μM Dip and 250 μM FeCl3) are shown for each fusion derivative because the protein steady-state levels detected were constant under basal (untreated) growth conditions. The samples were analyzed by immunoblotting using either anti-IgG or anti-PCNA (for internal control) antibody.
FIG. 7. The N-terminal 241 amino acid residues of Fep1 interact with chromatin in an iron-dependent manner. (A) Schematic diagram of the C-terminal truncations of the TAP-Fep1 fusion protein containing ZF1, ZF2, and the Cys-rich region (CCCC). The LLIII motif (residues 522 to 536) is part of a predicted coiled-coil region involved in intermolecular interaction between Fep1 molecules. Amino acids 405 to 541 represent the minimal region required for the association between Fep1 and its corepressor Tup1. The amino acids are numbered relative to the first amino acid of the Fep1 protein. (B) ChIP analysis was performed on the fio1 promoter in fep1Δ php4Δ cells expressing the wild-type TAP-Fep1 or the truncated protein TAP-Fep1359 (1-359). Each PCR contained a specific primer pair from the fio1 promoter and a control primer pair from a nontranscribed region (intergenic region). The histogram (bottom) represents quantification of three independent ChIP assays, including the experiment shown in the upper part of this panel. (C) ChIP assays were carried out as described for panel B, except that cells were expressing the truncated TAP-1Fep1241 (1-241) fusion protein. The histogram (bottom) is a graphic representation of quantification of three independent ChIP experiments, including the experiment depicted at the top of this panel. Input, input chromatin; IP, immunoprecipitated chromatin. (D) Total RNA from cultures grown in the absence (–) or presence of 250 μM Dip and 250 μM FeCl₃ (Fe) was isolated and analyzed. Shown is a representative RNase protection assay of fio1 and act1 mRNA steady-state levels. NS, nonspecific signal. (E) Protein extracts were prepared from aliquots of cultures used as described for panel D and then analyzed by immunoblotting using either anti-IgG or anti-PCNA (as an internal control) antibody.
FIG. 8. Minimal N-terminal region of Fep1 required for a weak interaction with DNA in vivo. (A) Schematic illustration of truncated versions of the TAP-Fep1 fusion protein. The sequence motifs illustrated have been described in the legend to Fig. 7. (B to C) ChIP analysis was conducted on the fio1" promoter in fep1Δ php4Δ cells carrying the wild-type TAP-fep1" or the mutant TAP-60Fep1359 or TAP-60Fep1241 allele. Cells were grown to logarithmic phase in the presence of Dip (100 μM), washed, and then incubated in the presence of 250 μM Dip or 250 μM FeCl3 (Fe) for 90 min at 30°C. PCR analysis was performed on total sheared chromatin (input) and immunoprecipitated (IP) chromatin. Each PCR contained a specific primer pair for the fio1" promoter (fio1") and a control primer pair from a nontranscribed region (intergenic region). The bottom of panels B and C shows quantitation of PCR products obtained from three independent ChIP assays (for each allele). (D) fep1Δ php4Δ cells were incubated in the absence (−) or presence of 250 μM Dip or 250 μM FeCl3. After extraction of total RNA, the fio1" mRNA levels were analyzed by RNase protection assay with actin (act1") as an internal control. Results shown are representative of three independent experiments. (E) Immunoblotting for TAP-Fep1, TAP-60Fep1359, and TAP-60Fep1241 was performed on samples incubated in the presence of Dip (250 μM) or FeCl3 (250 μM). Immunoblotting for PCNA on the same samples was carried out as a loading control.
levels of fio1+ promoter DNA were immunoprecipitated with TAP-Fep1 by anti-IgG antibodies from cells grown in the presence of iron (Fig. 8B and C). Under the same conditions, only 36% and 27% of the fio1+ promoter were immunoprecipitated with either TAP-Fep1359 or TAP-Fep1241, respectively (Fig. 8B and C). As expected, for all three alleles examined, no significant amount of fio1+ promoter DNA was immunoprecipitated when cells were cultured in the presence of the iron chelator Dip (Fig. 8B and C). Curiously, the removal of ZF1 had a negligible effect on the ability of 66Fep1359 to interact with chromatin, because both TAP-1Fep1241 and TAP-2Fep1359 showed similar levels of promoter occupancy (33% and 36%) compared to that of the full-length TAP-Fep1 (Fig. 7B and 8B). On the other hand, deletion of ZF1 strongly affected the ability of TAP-60Fep1241 to bind to DNA, as indicated by its reduced level of occupancy (45.2%) of the fio1+ promoter compared to that of TAP-1Fep1241 (Fig. 7C and 8C). Consistent with the results that TAP-60Fep1359 and TAP-66Fep1241 have an equivalent or even lower level of promoter occupancy compared to that of TAP-1Fep1359 and TAP-1Fep1241, we found that neither mutant (TAP-60Fep1359 or TAP-66Fep1241) was capable of directing normal iron-dependent repression of fio1+ transcription (Fig. 8D). The reduced level of promoter occupancy by these mutants was not due to a lack of protein expression since both proteins were clearly expressed as confirmed by immunoblot analysis (Fig. 8E). Taken together, these data suggest that while the C-terminal zinc finger motif, ZF2, and the Cys-rich region are sufficient for binding to chromatin in vivo, the presence of the N-terminal zinc finger ZF1 strengthens the association of Fep1 with DNA.

**Fusion of VP16 to the first 241 amino acids of Fep1 activates gene expression in an iron-dependent manner.** Based on our previous data (34, 35) and the results described here thus far, we propose a mechanism wherein under iron-replete conditions, Fep1 interacts with the GATA elements on the promoters of target genes through its N-terminal 241-amino-acid segment. To further establish the function of this region, we carried out one-hybrid experiments with chimeric proteins containing the transactivation domain of VP16 fused to the wild-type Fep1 1-241 or the mutant Fep1241-R184A/R185A/D186A/E187A (Fig. 9). As controls, we used constructs expressing the VP16 transactivation domain alone or Fep1 1-241 alone (Fig. 9). To assess the ability of Fep1 1-241 to act as an exportable iron-dependent DNA binding cassette, we separately expressed all the constructs described above in *S. cerevisiae*, an organism that does not possess an ortholog of the *S. pombe* Fep1, thereby providing an excellent background for its heterologous characterization. In addition, we cotransformed these cells with a reporter plasmid harboring two consensus Fep1 binding sites that were introduced upstream of the minimal promoter of the *iso-1-cytochrome c* (*CYC1*) gene, which was fused to the reporter gene *lacZ*. To quantitate the amount of expression of the gene *lacZ*, we measured β-galactosidase activity in cultures of the doubly transformed cells. The results shown in Fig. 9B reveal that VP16-1Fep1241 induced the reporter gene expression in the presence of iron as indicated by approximately 14-fold-higher β-galactosidase activity in cells expressing this fusion protein. In contrast, VP16-1Fep1241 did not stimulate β-galactosidase production in the presence of the iron chelator Dip. Regardless of the iron levels in the culture medium, no significant β-galactosidase activity was detected in cells transformed with VP16 alone, Fep1 1-241 alone, or VP16-1Fep1241-R184A/R185A/D186A/E187A (Fig. 9B). To ascertain that the VP16-1Fep1241 or the VP16-1Fep1241-R184A/R185A/D186A/E187A protein was expressed in *S. cerevisiae* cells, total protein extracts from cells transformed with the plasmids expressing the indicated molecules were analyzed by immunoblotting. As shown in Fig. 9C, all the proteins tested in the one-hybrid assay were efficiently expressed; therefore, the loss of iron-dependent production of β-galactosidase in cells expressing VP16-1Fep1241-R184A/R185A/D186A/E187A was not due to the lack of protein expression. We have been unable to detect the presence of the VP16 transactivation domain alone, perhaps due to its low predicted molecular mass of ~8 kDa. Taken together, these results reveal that when Fep1 1-241 is heterologously expressed in *S. cerevisiae*, it dictates DNA binding activity as a consequence of intracellular iron concentrations, while the nature of the amino acids fused to this conserved N-terminal region dictates the ability of the transcription factor to positively regulate target gene transcription.

**DISCUSSION**

The toxic yet essential nature of iron in biological systems demands that cells maintain iron levels within homeostatic boundaries. In fungal cells, iron uptake is tightly regulated in response to the availability of iron in the growth medium. For instance, transfer of growing cells from an iron-limiting to an iron-rich medium causes a strong inhibition of high-affinity iron uptake systems (4, 36). In *S. pombe*, iron-mediated repression of iron uptake genes is controlled by the transcription factor Fep1. We propose a model of regulation by Fep1 wherein the presence of excess iron triggers its binding to GATA elements within the promoters of target genes, where together with its corepressors it represses target gene expression. This model is based largely on in vitro results showing that the binding of recombinant MBP-Fep1, MBP-Sfu1 (32, 34), and MBP-Sre1 (6) to DNA is dependent on the presence of iron. In the current study, we created a system where expression of TAP-Fep1 is constitutive and unlinked from its iron limitation-dependent downregulation by Phy4. We used this system to isolate DNA segments that TAP-Fep1 normally associates under conditions of high levels of iron using a ChIP technique, thereby providing us with a snapshot of the in vivo associations between Fep1 and the promoters of its target genes. Using this method, we show that the transcription factor is associated with the fio1+ and str1+ regulatory regions under iron-replete conditions. Importantly, after 90 min of exposure to iron, the binding of Fep1 to iron-responsive promoters was concomitant with the repression of fio1+ and str1+ gene expression (33) (Fig. 2). Conversely, when cells were incubated in the presence of Dip, the association of Fep1 with the same promoters occurred at the background level and paralleled the induction of fio1+ and str1+ gene expression (Fig. 2 and data not shown). These results are consistent with a model in which Fep1 is either completely bound to or dissociated from the promoter in the presence or absence of iron, respectively.

Our results indicate that iron is a necessary requirement for the recruitment of Fep1 to promoters in vivo. How might this
occur? Activation of Fep1 function may occur from direct iron binding. The detection of iron in purified *H. capsulatum* Sre1 (6) and the discovery of a UV-visible spectrum that is distinctive of iron-binding proteins for SRE (16) are consistent with the notion that these GATA transcription factors may act as direct iron sensors. Alternatively, Fep1 may be activated through the action of other proteins that may specifically turn on its function via protein-protein interactions or through post-translational modifications. If this is the case, then either Fep1 or the partner protein could contain an iron-binding regulatory site that might control the association between the proteins.

When we studied the effect of intracellular iron levels on the subcellular localization of Fep1, we carried out these experiments using a wide range of different conditions, including the use of different iron chelators (permeant and nonpermeant) at different concentrations. We also used different times of treatment (from minutes to several hours of incubation). Fep1-GFP fluorescence was always detected in the nuclei of cells under
conditions of both low and high levels of iron. This suggests that if Fep1 acts as a direct iron sensor, incorporation of iron into Fep1 may occur during its cytosolic translation or after its nuclear translocation. To gain insight into this process, we utilized the regulatable nmt1 promoter system (28), which allowed us to induce the synthesis of TAP-Fep1 in the presence of Dip. After accumulation of a nuclear pool of TAP-Fep1, thiamine was added to repress further protein synthesis, and the cells were treated with iron to examine its effect on the ability of nuclear TAP-Fep1 to associate with chromosomal DNA. Our results showed that the presence of elevated iron can be transmitted to the transcription factor within the nucleus. Likewise, we determined that cycloheximide treatment did not impair the iron activation of Fep1 (M. Jbel, A. Mercier, and S. Labbé, unpublished data). What are the possible mechanisms by which iron communicates with the nuclear pool of Fep1? First, it is possible that a soluble carrier may deliver iron to the nucleus to activate Fep1. Recently, a cytosolic iron chaperone, denoted PCBP1, has been identified in human cells (39). Iron bound by PCBP1 is available to ferritin, contributing to its metallation. It remains to be determined if a similar chaperone exists for specific delivery of iron into the nucleus. Second, it is possible that a sensor may exist on the cell surface that can detect elevated concentrations of extracellular iron and communicate this information through a signal transduction pathway to Fep1 within the nucleus. The binding of iron to Fep1 may represent the final step prior to its association with DNA only in the presence of excess intracellular iron. In S. cerevisiae, when iron is elevated a homeostatic mechanism operates at the posttranscriptional level. It implicates the production of iron-sulfur clusters and the formation of an as-yet-unidentified compound that is exported from mitochondria and may possibly be imported into the nucleus. A proposed model suggests that in response to elevated concentrations of iron, the unidentified compound accumulates in the nucleus and causes the iron-induced repression of iron transport gene expression (8, 31, 38). Such a compound may exist in S. pombe and stimulate the inhibitory function of Fep1.

Although the precise mechanism by which Fep1 directs iron-mediated regulation of transcription remains to be defined, the results from this study clearly demonstrate that Fep1 is a modular protein with an autonomous DNA-binding domain. Beyond simple DNA recognition, the Fep1 N-terminal DNA-binding domain (amino acids 1 to 241) can also directly or indirectly sense iron. The domain by itself has the ability to bind to GATA elements in vivo in an iron-dependent manner and indirectly sense iron. The domain by itself has the ability to bind to GATA elements in vivo in an iron-dependent manner and indirectly sense iron.

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