Yap5 Is an Iron-Responsive Transcriptional Activator That Regulates Vacuolar Iron Storage in Yeast

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The transporter Ccc1 imports iron into the vacuole, which is the major site of iron storage in fungi and plants. CCC1 mRNA is destabilized under low-iron conditions by the binding of Cth1 and Cth2 to the 3′ untranslated region (S. Puig, E. Askeland, and D. J. Thiele, Cell 120:99–110, 2005). Here, we show that the transcription of CCC1 is stimulated by iron through a Yap consensus site in the CCC1 promoter. We identified YAP5 as being the iron-sensitive transcription factor and show that a yap5Δ strain is sensitive to high iron. Green fluorescent protein-tagged Yap5 is localized to the nucleus and occupies the CCC1 promoter independent of the iron concentration. Yap5 contains two cysteine-rich domains, and the mutation of the cysteines to alanines in each of the domains affects the transcription of CCC1 but not DNA binding. The fusion of the Yap5 cysteine-containing domains to a GAL4 DNA binding domain results in iron-sensitive GAL1-lacZ expression. Iron affects the sulfhydryl status of Yap5, which is indicative of the generation of intramolecular disulfide bonds. These results show that Yap5 is an iron-sensing transcription factor and that iron regulates transcriptional activation.

Iron is an essential nutrient required by all eukaryotes. In high concentrations, however, iron can be toxic, necessitating tight control over its concentration within cells. Multicellular organisms can transfer iron between cell types; however, multicellular and single-cell eukaryotes do not have an excretory mechanism to dispose of iron. Iron homeostasis results from the ability to regulate iron acquisition or to store iron once it is absorbed. The ability to store iron makes it available for preserving cytosolic iron levels. Iron-dependent changes in iron. Iron-dependent changes in

MATERIALS AND METHODS

Yeast strains and culture conditions. Wild-type strain DY150 (W303 background) and its deletion strains were used in most experiments. The ccc1Δ strains were described previously (14). Strains with specific deletions (cht1Δ, cht2Δ, chl1Δ, whi2Δ, and yap5Δ) were generated in the DY150 background by PCR amplifying the KanMX4 deletion cassettes from specific yeast strains in the homozygous diploid deletion collection (Research Genetics, Stanford, CA). Deletion strains of yap1Δ-yap5Δ were obtained from the BY4741 (S288C background) haploid collection. The glr1Δ and trr1Δ strains were obtained from Dennis Winge (University of Utah). Complete minimal (CM) medium was composed of yeast nitrogen base, dextrose, and the required amino acids. Low-iron medium was made by adding 80 μM bathophenanthroline disulfonate, and high-iron medium resulted from the addition of the designated concentration of FeSO4 or ferrous ammonium sulfate. All experiments were performed a minimum of three times.

Plasmid construction. The CCC1-lacZ reporter construct was created by cloning 600 bp of the upstream region of CCC1 by PCR. The PCR fragment was placed in a Yep354 lacZ expression vector, which is a high-copy-number vector. For low-copy-number expression studies, we utilized reporter vector YCp pRW95-3lacZ, obtained from D. Stillman (University of Utah). Promoter tran-
cations were performed by using the following PCR primers: 5'-cgcggatccAAACATCATCGACAGAATG-3' (555 bp), 5'-cgcggatccGACACATGCGCCAGGTAAGACA-3' (496 bp), 5'-cgcggatccGACACATGCGCCAGGTAAGACAATGGC-3' (400 bp), 5'-cgcggatccTTCCGTTCCGTTGACAAATGCTC-3' (244 bp), 5'-cgcgagateCTAATATTTGCATACC-3' (221 bp), 5'-cgcggatccCATACCTTTCATGCTC-3' (199 bp), 5'-cgcggatccGACACATGCGCCAGGTAAGAC-3' (136 bp), and 5'-cgcggatccGACACATGCGCCAGGTAAGAC-3' (135 bp). The first upstream Yap binding site in the ccci promoter region was mutated by site-directed mutagenesis using the QuikChange kit from Stratagene (La Jolla, CA) with two primers: 5'-GCGCATTTCTCCTAATATTACAACATACCTTTCATGCTC-3' and 5'-GCAATAGGAGAGGTATGTTCTCGTTAATATTGCATTAATGGC-3' (260 bp). The second Yap binding site in the ccci promoter region was mutated by using primer 5'-cgcggatccTTCCGTTCCGTTGACAAATGCTC-3' and 5'-cgcggatccGACACATGCGCCAGGTAAGAC-3' (135 bp). The PCR product of 367 bp including the Yap binding sites in the promoter region was mutated by using primer 5'-GCGCATTTCTCCTAATATTACAACATACCTTTCATGCTC-3' and 5'-GGAATGTCTCACAATTTGCATACC-3' (260 bp). The PCR fragments were subcloned into a high-copy-number pTF63 vector or a low-copy-number vector (pRS416) obtained from Dennis Winge (University of Utah).

The YAP5 carboxyl-terminal hemagglutinin (HA) epitope tag was generated by PCR utilizing a 12× HA-containing plasmid (5). YAP5 amino-terminal green fluorescent protein (GFP)- or His6-tagged plasmids were cloned into a high-copy-number vector pYP63 or a low-copy-number vector (pRS416) obtained from D. Stillman (University of Utah). All these epitope-tagged YAP5 genes were regulated by the YAP5 promoter, as the YAP5 open reading frame and 600 bp upstream of the ATG were cloned by PCR. Mutation of Yap5 cysteines to alanines, or cysteines to aspartic acids, was generated by PCR. A double-fusion PCR technique was used to modify the fragment between the Ptst1 site and the stop site. The GAL4 DNA binding domain (DBD) was fused to the YAP5 activation cysteine-rich domain (CRD) by cloning a PCR fragment from YAP5 that had been truncated and that included amino acid 115 to the stop codon into vector pMA424 (16) using the primers 5'-GAAGTGATGTCGACACATGCCAC-3', 5'-AACATCATCGACGAATG-3', and 5'-GCCGTTAGCAGTTGTT-3'. The primer for calmodulin and Galactosidase assay.

Cell extracts were prepared using a modification of the Northern blot analysis. The cells were homogenized, and vacuoles were isolated, solubilized, and examined by Western blot analysis. The Western blots were probed with a polyclonal antibody directed against Ccc1 or a monoclonal antibody against carboxypeptidase Y (CPY), followed by peroxidase-conjugated goat anti-rabbit IgG or peroxidase-conjugated goat anti-mouse IgG. (B) Wild-type cells were transformed with a plasmid containing a MET3-regulated ccci-FLAG construct. Cells were grown overnight in iron-replete or iron-limited medium in the absence of methionine, which permits the expression of MET3-ccc1. Cells were harvested, and the levels of Ccc1-FLAG or CPY were determined by Western blotting as described above (A). (C) mRNA was isolated from cells grown overnight in iron-replete or iron-limited medium, and cci1 or act1 mRNA was analyzed by Northern blotting.

All experiments were performed a minimum of two times and on average were performed three to four times.

Results

In order to determine how Ccc1 is regulated, we measured the levels of vacuolar Ccc1 in cells grown in iron-replete or iron-limited medium. Wild-type (WT) and cth1Δ cath2Δ cells were grown overnight in iron-replete or iron-limited medium. (A) Cells were homogenized, and vacuoles were isolated, solubilized, and examined by Western blot analysis. The Western blots were probed with a polyclonal antibody directed against Ccc1 or a monoclonal antibody against carboxypeptidase Y (CPY), followed by peroxidase-conjugated goat anti-rabbit IgG or peroxidase-conjugated goat anti-mouse IgG. (B) Wild-type cells were transformed with a plasmid containing a MET3-regulated ccci-FLAG construct. Cells were grown overnight in iron-replete or iron-limited medium in the absence of methionine, which permits the expression of MET3-ccc1. Cells were harvested, and the levels of Ccc1-FLAG or CPY were determined by Western blotting as described above (A). (C) mRNA was isolated from cells grown overnight in iron-replete or iron-limited medium, and ccc1 or act1 mRNA was analyzed by Northern blotting.

RESULTS

Ccc1 levels are controlled by mRNA transcript levels. To determine how Ccc1 is regulated, we measured the levels of vacuolar Ccc1 in cells grown in iron-replete or iron-limited medium. Vacuoles isolated from cells grown in iron-restricted medium had no detectable Ccc1, while vacuoles isolated from cells grown in iron-replete medium showed abundant Ccc1 (Fig. 1). Previously, we showed that Ccc1-FLAG could suppress the high-iron sensitivity of ccc1Δ cells, indicating that the epitope-tagged protein is competent to transport iron (14). To determine if Ccc1 is regulated at the protein level, we expressed Ccc1-FLAG with a carboxyl-terminal FLAG epitope under the control of a regulated promoter (MET3). The cells were grown in either high- or low-iron medium, without methionine, to permit the expression of Ccc1-FLAG. There was little difference in the amount of Ccc1-FLAG in cells grown under either iron-replete or iron-limited conditions (Fig. 1). Interestingly, we observed that the cth1Δ strain was more sensitive to iron-depletion than the wild-type strain (Fig. 1B), suggesting that iron does not affect protein stability.

It may be that the carboxyl-terminal epitope prevented protein degradation or that the protein was not regulated at the level of degradation but at the level of mRNA. To test these possibilities, we examined ccc1 mRNA levels from wild-type and cth1Δ cath2Δ strains grown in iron-replete or iron-limited
medium. In iron-replete medium, CCC1 mRNA levels were similar in wild-type and cth1Δ cth2Δ cells (Fig. 1C). Little CCC1 mRNA was detected in wild-type cells grown in iron-limited medium. Puig et al. previously demonstrated that CCC1 mRNA could be regulated by changes in half-life through the binding of Cth1 and Cth2 to the 3′ untranslated region (19). We observed an increase in CCC1 transcripts in cth1Δ cth2Δ cells grown under low-iron conditions compared to wild-type cells grown in low iron. The deletion of both CTH1 and CTH2 did not restore CCC1 mRNA levels to that of wild-type cells grown in iron-replete medium, confirming observations described previously by Puig et al. (19). The inability of the double-deletion strain to restore message levels to that of the iron-replete wild type was also seen at the protein level (c.f. Fig. 1A). These results lead to two conclusions: Ccc1 levels reflect mRNA levels, and CCC1 message levels are not regulated solely by Cth1 and Cth2.

Transcription of CCC1 is iron regulated. To examine whether the transcription of CCC1 was subject to regulation, we generated a lacZ reporter construct driven by 600 bp of the CCC1 promoter. This region encompasses the complete intergenic region between CCC1 and the 5′-distal gene MSL3. The ability of the reporter construct to respond to iron was measured in wild-type and cth1Δ cth2Δ cells. The addition of iron resulted in the increased expression of CCC1-lacZ (Fig. 2A). The absolute level of reporter expression was lower in cth1Δ cth2Δ cells, but the relative changes in expression were similar to those of wild-type cells. The change in reporter construct activity mirrors changes in mRNA levels, although the absolute amount of the mRNA level depends on the expression of Cth1 and Cth2 (Fig. 2A). We note that cth1Δ cth2Δ cells grown in iron-containing medium have a lower level of CCC1 mRNA than do wild-type cells. If the degradation of CCC1 mRNA was prevented by the deletion of CTH1 and CTH2, then the level of mRNA in cth1Δ cth2Δ cells would be expected to be higher. These results suggest that CCC1 mRNA levels may be responsive to factors other than Cth1 or Cth2. The increase in CCC1 transcripts was rapid, occurring within 2 h of the addition of iron (Fig. 2B). These results indicated that the transcription of CCC1 was affected by iron, independent of the effect of Cth1 and Cth2 on message stability.

The deletion of CCC1 leads to increased cytosolic iron, as the transport of iron into the vacuole is severely impaired (14). If CCC1 transcription is regulated by cytosolic iron, then an increased level of cytosolic iron should lead to the increased expression of the CCC1-lacZ reporter construct. Wild-type and ccc1Δ cells, transformed with a high-copy reporter construct, were grown in iron-limited or iron-replete medium, and CCC1-lacZ activity was measured. Cells grown in iron-limited medium showed low levels of expression of the reporter construct, and the deletion of CCC1 further reduced that expression (Fig. 2C). Conversely, when ccc1Δ cells were incubated in iron-replete medium, there was enhanced expression of the CCC1-lacZ reporter construct. Similar results were obtained if cells were transformed with a low-copy-number plasmid containing the reporter construct (data not shown). Thus, the lack of Ccc1 results in increased cytosolic iron and consequently increased expression of β-galactosidase.

The overexpression of CCC1 was shown to reduce cytosolic Mn2+, suggesting that Ccc1 may not be specific for iron but that it can transport other transition metals (13). Cells transformed with a high-copy-number CCC1-lacZ plasmid were incubated in CM medium containing different levels of transition metals (Fig. 2D). The concentrations of Cu2+, Co2+, and Cd2+ were limited by the toxicity of the metals, but no effect on CCC1-lacZ was seen at concentrations of these metals that ranged from nontoxic to subtoxic. The addition of iron led to the increased expression of CCC1-lacZ; no effect was seen with either Zn2+ or Mn2+. The addition of H2O2 to the medium had no effect on the transcription of the reporter construct. Similar results were obtained with a low-copy-number plasmid containing the reporter construct (data not shown). These results indicate that the transcription of CCC1 is specific for iron and not for oxidant damage, supporting the view that increased cytosolic iron is essential for the transcriptional activation of CCC1.

Yap5 is responsible for the iron-dependent transcription of CCC1. To investigate the transcriptional regulation of CCC1, we subcloned the promoter region to identify cis-acting elements. A 600-bp sequence between the 5′ end of the CCC1 open reading frame and the start of the adjacent gene was placed in front of a lacZ reporter construct, and the plasmid was transformed into wild-type cells. There was little expression of β-galactosidase from a reporter lacking the CCC1 promoter sequence (data not shown). The insertion of the CCC1 promoter sequence, however, resulted in a fivefold increase in CCC1-lacZ expression over background levels upon the addition of iron (Fig. 3A). There was a dramatic decrease in expression in the absence of the 46 bp from the region of residues −244 to −199. Examination of this 46-bp region showed the presence of a Yap consensus binding site (9). Mutation of nucleotides in the Yap site showed that this site is both necessary and sufficient for the iron-regulated expression of the CCC1-lacZ reporter construct. There was a second upstream Yap consensus site, but the truncation of this site had no effect on CCC1-lacZ expression.

FIG. 2. Expression of a CCC1-lacZ reporter construct is regulated by iron. (A) Cells (wild type [WT] and cth1Δ cth2Δ) were transformed with a reporter plasmid containing 600 bp of the CCC1 promoter region fused with the lacZ gene. Cells were incubated for 3 h in increasing concentrations of iron, harvested, and assayed for β-galactosidase activity and cell protein. For this and all subsequent β-galactosidase measurements, the data are reported as activity in nmol product/min/mg protein. CCC1 and ACT1 mRNAs were analyzed by Northern blot. CCC1 or ACT1 mRNA band intensity was quantified by image analysis, and the ratio of the two is presented. (B) Wild-type and cth1Δ cth2Δ cells were incubated in CM medium supplemented with 500 µM FeSO4. At the selected times, cells were harvested, β-galactosidase activity was determined, and activity was normalized for protein. (C) Wild-type and ccc1Δ cells, transformed with a CCC1-lacZ reporter plasmid, were incubated for 2 h with or without 500 µM FeSO4. Cells were harvested, and β-galactosidase activity was determined. (D) Wild-type cells transformed with a CCC1-lacZ reporter plasmid were incubated in CM medium with 500 µM Fe2+, 1.0 mM Zn2+, 1.0 mM Mn2+, 100 µM Co2+, 200 µM Cu+, 25 µM Cd2+, or 0.0025% H2O2 for 2 h. Cells were harvested, and β-galactosidase activity was determined.
Members of the YAP transcription family are responsible for much of the cellular response to oxidative stress. For example, Yap1 is activated by oxidative stresses such as H$_2$O$_2$ (12); however, the addition of 0.0025% H$_2$O$_2$ did not lead to the expression of CCC1-lacZ (Fig. 2D). These results suggest that Yap1 is not responsible for the transcription of CCC1. Previously, we showed that the deletion of CCC1 led to decreased growth on high-iron medium (14). To define which member of

FIG. 3. Yap5 is responsible for the iron-dependent transcription of CCC1. (A) Selected regions of the CCC1 promoter or mutated regions of the CCC1 promoter sequence were cloned into lacZ reporter constructs, and the constructs were transformed into wild-type cells grown in iron-limited medium. Iron (500 μM FeSO$_4$) was added to the medium for 2 h, cells were harvested, β-galactosidase activity was determined, and activity was normalized for protein (yellow bars with associated numbers). The data are presented as the change in β-galactosidase activity. The green ovals represent consensus YAP binding sites, and the × indicates the site of mutations. The 46-bp region is highlighted with a red bracket. (B) Wild-type (WT) and YAP deletion strains in the BY4741 haploid deletion collection were spotted in serial dilutions on medium containing high concentrations of iron. The cells were grown for 2 days at 30°C. (C) Wild-type and yap5Δ cells (haploid W303 background) were transformed with a plasmid containing a CCC1-lacZ reporter construct. Cells grown in iron-limited medium were incubated with (black bars) or without (white bars) 500 μM FeSO$_4$ for 2 h, β-galactosidase activity was determined, and activity was normalized for protein.
the Yap family is involved in the iron regulation of *CCC1*, we examined the iron sensitivity of BY4741 strains deleted for specific *YAP* genes (Fig. 3B). In the presence of high iron, only *yap5Δ* grew poorly. The poor-growth phenotype of the *yap5Δ* strain could be suppressed by the transformation of cells with a *MET3*-regulated *CCC1* plasmid (data not shown). This result is consistent with the view that the lack of Ccc1 expression was responsible for the iron-sensitive phenotype (14). To determine if *YAP5* affected the expression of *CCC1*, we generated a *YAP5* deletion in a *W303* background and examined the effect of the deletion on the iron-induced transcription of a *CCC1-lacZ* reporter construct. The deletion of *YAP5* prevented the iron-induced expression of β-galactosidase activity (Fig. 3C).

These results indicate that Yap5 is responsible for the iron-induced transcription of *CCC1* mRNA.

**Yap5 is constitutively localized to the nucleus.** To determine the effect of iron on Yap5, we examined the behavior of epitope-tagged Yap5. Yap5 with a carboxyl-terminal HA epitope was capable of suppressing the iron toxicity of a *yap5Δ* strain; however, the expression of *CCC1-lacZ* was constitutive and iron independent (data not shown). Since Yap5-HA was constitutively active, we examined the effect of Yap5 containing other epitopes. Amino-terminal His or amino-terminal GFP-Yap5 constructs complemented the high-iron toxicity of the *yap5Δ* strain and led to the iron-dependent expression of *CCC1-lacZ* activity (Fig. 4A and B). There was, however, a
FIG. 5. Expression of \textit{CCCI} is dependent on the cysteines in the Yap5 CRD. (A) Comparison of the architectures of Yap1 and Yap5. The architecture of Yap1 is abstracted from data described previously (17, 24). Yap5 has two CRDs in which the cysteines are conserved in all species.
slightly lower level of β-galactosidase at 10 μM iron in cells expressing GFP-Yap5 than in those expressing His-Yap5, suggesting that the larger GFP epitope may affect the sensitivity of the protein to iron. Iron increased the transcriptional activity of the His-Yap5 construct but did not increase the amount of His-Yap5 (Fig. 4A). We note a consistent decrease in His-Yap5 protein levels at high iron concentrations. GFP-Yap5 expressed by a high-copy-number plasmid was localized to the nucleus independent of iron levels (Fig. 4B). The fluorescence signal from a low-copy-expressed GFP-Yap5 was too low to be detected. However, as shown below, the data suggest that a low-copy GFP-Yap5 is constitutively localized to the nucleus.

The best-studied Yap family member is Yap1, which contains two CRDs that surround a nuclear export sequence (Fig. 5A) (for a review, see reference 10). Oxidizing agents directly or indirectly generate disulfides within or between CRDs. The generation of disulfides affects protein structure, resulting in the occlusion of the nuclear export sequence and the nuclear accumulation of Yap1. We have not identified a nuclear export sequence in Yap5, and our data suggest that Yap5 is constitutively localized to the nucleus. The structure of Yap5 is similar to that of Yap1 in that Yap5 contains two CRDs. The aminoterminal CRD of Yap5 contains four cysteines, and the carboxyl-terminal CRD contains three cysteines. The seven cysteine residues within the CRDs of Yap5 are conserved in all species of Saccharomyces. The two CRDs in Yap5 are separated by 37 amino acids, while the two CRDs in Yap1 are separated by 283 amino acids. Mutation of either single or multiple cysteines to alanine in the amino-terminal CRD reduced CCC1-lacZ activity by 70% (Fig. 5B). Mutation of a single cysteine or all of the cysteines to alanine in the carboxy-terminal CRD lowered CCC1-lacZ activity by approximately 40%. Mutation of all amino- and carboxyl-terminal CRD cysteines resulted in a complete loss of transcription activity. All Yap5 mutants were expressed at wild-type levels and were localized to the nucleus (data not shown). Mutation of the cysteines in the CRD affected the ability of Yap5 to prevent high-iron toxicity (Fig. 5C). Mutation of the cysteines in each domain led to a differential response to iron toxicity when expressed in yap5Δ cells. Amino-terminal Yap5 cysteine mutants supported growth on high iron better than vector alone but not as well as the carboxyl-terminal cysteine Yap5 mutants. The compound Yap5 mutant (N4C3) with mutations in both amino- and carboxyl-terminal CRDs was unable to provide any resistance to high-iron toxicity. These results suggest that the cysteines in the CRD are required for Yap5 function and that the two CRDs may have a modular effect.

Cysteines are potential metal binding amino acids. No iron was found bound to Yap5 when Yap5 was isolated from 59Fe-labeled cells (data not shown). To further test whether the cysteines bound metals, we mutated the cysteines to aspartic acid, as the carboxyl group should bind positively charged transition metals. These mutations did not lead to increased transcriptional activity compared to the corresponding alanine substitution (Fig. 5D).

**Binding of Yap5 to the CCC1 promoter is iron independent, but transcriptional activation is iron dependent.** The data suggested that Yap5 constitutively localizes to the nucleus but induces the transcription of CCC1 in an iron-dependent manner only. We determined if iron affected the ability of Yap5 to occupy the CCC1 promoter by CHIP. GFP-Yap5 expressed from a high-copy-number (data not shown) or low-copy-number plasmid occupied the CCC1 promoter in an iron-independent manner, as iron-limited and iron-replete cells showed similar amounts of GFP-Yap5 at the CCC1 promoter, supporting the view that Yap5 is constitutively localized to the nucleus (Fig. 6A). To confirm that the growth conditions employed affected cellular iron, we examined the binding of the low-iron-sensing transcription factor Aft1 to one of its targets, the FET3 promoter. Aft1 binding to the FET3 promoter was reduced under high-iron conditions (Fig. 6B), confirming our previously reported results (5). Since iron regulates CCC1-lacZ expression, we conclude that iron affects the transcriptional activation of Yap5 but does not affect binding to DNA. Based on these data, we examined whether Yap5 cysteine-to-alanine mutants could bind to the CCC1 promoter. The GFP-Yap5(N4C3) mutant with all cysteines mutated (N1234C123 equals N4C3) was chosen because it was unable to induce the expression of CCC1-lacZ. CHIP analysis showed that GFP-Yap5(N4C3) bound to the CCC1 promoter independent of iron.

If GFP-Yap5(N4C3) can occupy the CCC1 promoter but is unable to induce transcription, we predict that it should function as a dominant negative. To test this prediction, we transformed wild-type cells containing the CCC1-lacZ expression plasmid with a control vector, GFP-YAP5 or GFP-YAP5(N4C3), and measured iron-dependent β-galactosidase activity. Wild-type cells expressed similar increased amounts of β-galactosidase activity. Wild-type cells transformed with a control vector or GFP-YAP5 expressed similar increased amounts of β-galactosidase in the presence of iron (Fig. 6C). In contrast, wild-type cells transformed with the GFP-YAP5(N4C3) plasmid showed dramatically reduced levels of expression of β-galactosidase in the presence of iron.

If the binding of Yap5 to DNA is iron insensitive but transcription is iron sensitive, then we expect that the Yap5 activation domain would act on heterologous DNA binding pro-
The DNA binding domain of GAL4 was fused to a truncation version of YAP5 lacking the endogenous DNA binding domain but containing the two CRDs. This construct (Gal4-tYap5) was transformed into cells that contained a GAL1-lacZ expression vector, which contained a Gal4 binding site. The addition of iron led to a dose-dependent increase in β-galactosidase activity (Fig. 6D). The expression of the reporter construct was specific for iron, as the addition of Cu, Zn,
Mn, or H$_2$O$_2$ had no effect (data not shown). No expression of GAL1-lacZ activity was seen in cells transformed with a construct containing a GAL4 binding domain and the CRDs from Yap5(N4C3). As a further control, we used a fusion protein between Gal4 and the activation domain of the zinc-responsive transcription factor Zap1, which has been shown to transcriptionally activate the GAL1-lacZ reporter construct in response to zinc (1). The Gal4-Zap1 (tZap1) chimeric protein did not induce the transcription of the GAL1-lacZ construct in response to iron. Western analysis of transformed cells showed equivalent levels of chimeric Gal4-tYap5, Gal4-tYap5(N4C3), and Gal4-tZap1. We conclude that iron leads to the transcriptional activation of Yap5 but does not affect its DNA binding activity.

Transcriptional activation is dependent on cysteines in the Yap5 CRD. We attempted to examine the sulfhydryl status of epitope-tagged Yap5 by electrophoretic mobility assays. The migration behavior of epitope-tagged Yap5 in native gels or after cysteine derivitization was difficult to resolve by Western blot analysis (data not shown). We took advantage of the Gal4-tYap5 chimeric protein to determine if iron affected the cysteine status in the CRD. Cells expressing Gal4-tYap5 were grown in high- or low-iron medium, and the migration of Gal4-tYap5 was examined by SDS-PAGE. In the absence of the reducing agent DTT, Gal4-tYap5 extracted from high-iron cells showed two bands, while Gal4-tYap5 extracted from low-iron-grown cells showed one band (Fig. 6E). In contrast, only one band was seen when the samples were treated with high concentrations of DTT. We also examined the effect of DTT on the electrophoretic mobility of Gal4-tYap5(N4C3) and Gal4-tZap1 extracted from control and iron-treated cells. While there is evidence for more than one electrophoretic form of each protein, there was no iron-dependent change in those forms.

To determine if the difference in electrophoretic mobility could be ascribed to disulfide bond formation, we treated samples obtained from high- and low-iron-grown cells with AMS. This cysteine-modifying reagent binds to free sulfhydryls and changes the mobility of the protein. When treated with AMS, samples from both high- and low-iron-grown cells showed decreased mobility by SDS-PAGE. Samples from low-iron-grown cells, however, showed a greater shift in mobility than high-iron-grown cells, suggesting that more of their cysteines were available to be modified by AMS. These results suggest that iron changing the behavior of the cysteines is consistent with iron-induced disulfide formation. AMS treatment did not lead to any change in the Gal4-tYap5(N4C3) protein, suggesting that the cysteines were responsible for the change. The Gal4-tZap1 protein did show a change in AMS response, although the change was seen in the presence and not in the absence of iron, which differs from the Gal4-tYap5 protein. These results indicate that the change in AMS sensitivity cannot be ascribed to the Gal4 domain.

The change in cysteine behavior is not in response to an increase in oxidative stress: the iron-dependent activation of CCC1 transcription occurs anaerobically (data not shown), and the addition of H$_2$O$_2$ does not lead to CCC1 transcription (Fig. 2D). The deletion of TRR1 (thioredoxin reductase) or the deletion of GLR1 (glutathione oxidoreductase) also does not activate CCC1 transcription (data not shown). The deletion of either gene leads to increased oxidation and increased disulfide bond formation (21). Thus, decreased intracellular reductant levels or increased oxidant levels do not lead to the activation of CCC1 transcription, suggesting that the effect of iron is highly specific.

**DISCUSSION**

Vacuolar iron homeostasis is a dynamic process in which iron can be transported into and out of the vacuole. Iron transport out of the vacuole is accomplished by two different transport systems, both of which are homologues of cell surface iron transporters. Aft1 transcriptionally regulates the transporters responsible for iron transport into the cell or out of the vacuole in response to low cytosolic iron (23) or defective iron-sulfur cluster synthesis, a measure of low cytosolic iron (2). Iron transport into the vacuole is also a regulated process. The only identified vacuolar iron importer is Ccc1, and two different processes regulate the expression of CCC1 mRNA. Puig et al. previously identified the first process: under low-iron conditions, Cth1 and Cth2 bind to elements in the 3′ untranslated region of CCC1 mRNA, destabilizing the mRNA (19). Aft1 regulates the expression of Cth2, linking a response to low iron and regulation of vacuolar iron transport. Thus, when cells perceive that they are iron limited, transcripts for the vacuolar iron importer are destabilized, preventing reductions in cytosolic iron levels. Simultaneously, the activation of Aft1 leads to the expression of the vacuolar iron exporters Fet5/Fth1 and Smf3. As a result, iron export to the cytosol is increased, while vacuolar iron import is decreased. The second mode of CCC1 regulation is iron-dependent transcriptional activation. The expression of either CCC1 mRNA or a CCC1-lacZ reporter construct is increased in response to iron in both wild-type and cth1Δ cth2Δ cells. The ability to control CCC1 mRNA levels by increased transcription in response to high iron and then to destabilize those mRNA transcripts in response to low iron results in a tight regulation of vacuolar iron import (Fig. 7).

Our studies show that Yap5 is responsible for the high-iron induction of CCC1 mRNA, as the deletion of Yap5 results in the decreased expression of CCC1 and increased iron sensitivity. Yap1 is one of eight homologous YAP genes that are a distinct subgroup of the bZIP family of transcription factors similar to that of mammalian AP-1 (9). Yap1, the best-studied member of the yeast YAP family, is a transcription factor that confers resistance to oxidative stresses such as H$_2$O$_2$ and cadmium. Yap2 also controls the transcriptional response to cadmium (11). While Yap1 was shown to bind in vitro to a consensus 7-bp nucleotide sequence, Yap5 did not bind to that sequence in vitro, although genetic studies showed that it could weakly activate a Yap consensus-driven His construct (9). Our data show that one of the two consensus Yap sites in the promoter region of CCC1 responds to Yap5, as the mutation of that site decreases iron-dependent transcription. Comparison of the CCC1 promoter regions of different Saccharomyces species (Saccharomyces Genome Database) showed that the first Yap consensus site is not conserved. In contrast, there is remarkable sequence conservation starting downstream of the first Yap consensus site. This evolutionary conservation of sequence provides support for the function of the downstream

**Figure 7.**
Yap5 occupies the levels do not change with iron levels. We also observed that the nucleus even in the absence of iron, and Yap5 protein a nuclear export sequence. Yap5 is constitutively localized to two sulfhydryl-rich domains, but these domains do not bracket activation of Yap1 target genes. The molecular architecture of to its accumulation in the nucleus and the transcriptional ac-
tivation of Yap1 target genes. The molecular architecture of Yap1 conformation, leading to its accumulation in the nucleus, where it is active as a transcription factor. Yap1 as a result of oxidation forms disulfide bonds, either intramolecular disulfides or disulfides with other proteins such as glutathione per-
oxidase (Gpx3) (8). These disulfides lead to a change in protein structure, obscuring the nuclear export signal on Yap1, leading to its accumulation in the nucleus and the transcriptional ac-
tivation of Yap1 target genes. The molecular architecture of Yap5 shows some analogy to Yap1. Yap5, like Yap1, contains two sulfhydryl-rich domains, but these domains do not bracket a nuclear export sequence. Yap5 is constitutively localized to the nucleus even in the absence of iron, and Yap5 protein levels do not change with iron levels. We also observed that Yap5 occupies the CCC1 promoter independent of iron, suggesting that iron regulates transcriptional activation as op-
posed to DNA binding activity and that the mutation of cys-
teines in the CRD does not affect promoter binding. The mutation of either one or most of the cysteines in the carboxyl-terminal domain has a milder effect on transcriptional activity than the mutation of cysteines in the amino-terminal domain, suggesting that the CRDs act as independent modules. It is interesting that the incubation of cells with cadmium, a cysteine binding transition metal, resulted in a decrease in Yap5 transcriptional activity (Fig. 2D).

Our data indicate that iron affects the status of the cysteine sulfhydryls. Cysteines can be iron binding residues, and it is possible that iron binding mediates a structural change in Yap5, leading to transcriptional activation. We found no evidence of iron binding to Yap5 and no difference in the transcriptional activity of Yap5 in which the cysteines were mu-
tated to alanine or to aspartic acid. Alanine will not bind metals; however, the metal binding ability of aspartic acid might lead to a constitutive response. Iron affects the migration of the Gal4-Yap5 chimeric protein in SDS-PAGE gels. The change in protein behavior after AMS treatment is consistent with iron-induced disulfide formation. The treatment of cells with H2O2 did not lead to CCC1-lacZ expression, suggesting that transcriptional activation required more than the simple oxidation of sulfhydryls. We recognize that while our data are consistent with disulfide bond formation, it is possible that there could be other modifications of cysteine sulfhydryls. Sev-
eral experiments indicate that the change in cysteines is not due to the nonspecific oxidation of sulfhydryls, as iron-depen-
dent activation of CCC1 transcription occurs anaerobically, and the addition of H2O2 or deletion of GRII or TRRI, which results in increased oxidation, does not activate CCC1 trans-
scription.

The finding that Yap5 can regulate CCC1 expression in response to high iron demonstrates the dynamic response of yeast to the redox-active transition metals iron and copper. Both low and high copper levels result in a transcriptional response regulating copper transport genes and copper storage genes (for a review, see reference 20). High levels of copper result in the transcription of metallothionein genes through the activation of Ace1.

Mac1 and Ace1 are copper binding proteins: the binding of copper activates Ace1, whereas the loss of copper activates Mac1. Our data show that iron homeostasis, like copper ho-
meostasis, is regulated by two different transcription factors, Aft1, which senses low iron and activates the transcription of iron acquisition systems, and Yap5, which senses high iron and activates transporters that affect iron storage. The regulation of vacuolar iron transport is critical for maintaining cytosolic iron levels. Our previous studies showed that the activity of Ccc1 could be affected by changes in mitochondrial iron ho-
meostasis (15). The data presented here indicate that the tran-
scription of CCC1 is responsive to iron. We also note that the level of CCC1 mRNA in cth1Δ cth2Δ cells grown in iron-
containing medium is lower than expected if CCC1 mRNA degradation is prevented. This observation suggests that there are other mechanisms that either modify the transcriptional response to iron or regulate the level of CCC1 mRNA.
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