THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 51, Issue of December 17, pp. 36252–36260, 1999

Printed in U.S.A.

A Copper-sensing Transcription Factor Regulates Iron Uptake Genes in *Schizosaccharomyces pombe**

(Received for publication, September 7, 1999, and in revised form, September 24, 1999)

Simon Labbé‡§, Maria M. O. Peña‡¶, Alexandra R. Fernandes‖, and Dennis J. Thiele‡ ‡‡**

From the ‡Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606 and ‖Centro de Engenharia Biologica e Quimica, Instituto Superior Tecnico, Lisbon 1046-001, Portugal

Copper and iron serve essential functions as catalytic co-factors in a wide variety of critical cellular enzymes. Studies in yeast have demonstrated an absolute dependence upon copper acquisition for proper assembly and function of the iron transport machinery. We have cloned genes for a high affinity copper transporter (Ctr4) and copper-sensing transcription factor (Cuf1) from *Schizosaccharomyces pombe*. Interestingly, the primary structure of Ctr4 and a putative human high affinity copper transport protein, hCtr1, suggests that they are derived from a fusion of the functionally redundant but structurally distinct Ctr1 and Ctr3 copper transporters from *Saccharomyces cerevisiae*. Furthermore, although Cuf1 activates *ctr4*$^+$ gene expression under copper starvation conditions, under these same conditions Cuf1 directly represses expression of genes encoding components of the iron transport machinery. These studies have identified an evolutionary step in which copper transport modules have been fused, and describe a mechanism by which a copper-sensing factor directly represses expression of the iron uptake genes under conditions in which the essential copper co-factor is scarce.

The mechanisms by which organisms accumulate, sense and respond to nutrients is a critical aspect of cellular growth control. Copper and iron are two metal ions that are essential for the growth of all organisms, serving as redox active co-factors for a wide variety of cellular enzymes including mitochondrial cytochromes, multi-copper ferroxidases, and superoxide dismutases (1–4). Furthermore, as a consequence of chemical reactions between copper or iron and oxygen, these same metals generate reactive oxygen species that damage nucleic acids, proteins and membranes (5). When copper and iron accumulate in excess or are abnormally mobilized, a number of disease states are manifest such as Menkes and Wilson diseases and anemia, disorders of copper and iron acquisition, respectively, and hemochromatosis, an iron overload condition that is one of the most common inherited disorders affecting people of Northern European genealogy (6–9). Therefore, it is critical for cells to maintain sufficient levels of copper and iron to carry out essential biochemical reactions, but regulatory mechanisms must be in place to prevent these metals from accumulating in excess.

The bakers' yeast, *Saccharomyces cerevisiae*, is an excellent model system to understand the function and regulation of fundamental mechanisms for copper and iron uptake and distribution in eukaryotic cells. Biochemical and genetic experiments have shown that copper and iron ions are reduced by cell surface Fe^{3+}/Cu^{2+} reductases, encoded by the *FRE* genes (10–13). Cu^+ is transported into *S. cerevisiae* cells via two plasma membrane high affinity copper transporters, Ctr1 (14) and Ctr3 (15). The Ctr1 and Ctr3 proteins are functionally redundant; while cells expressing either Ctr1 or Ctr3 are fully competent for high affinity copper transport, *ctr1*Δ *ctr3*Δ cells exhibit copper starvation phenotypes including the inability to grow on nonfermentable carbon sources, lack of measurable high affinity iron uptake activity, and defective copper/zinc SOD activity (14, 15). Interestingly, Ctr1 and Ctr3 are structurally distinct proteins, with Ctr1 harboring several potential copper binding motifs (M-X_2-M-*X*-M) in the amino-terminal extracellular domain while Ctr3 lacks these motifs, but has an abundance of cysteine residues throughout the protein. Three yeast copper chaperone proteins have been identified, Atx1 (16, 17), CCS (18), and Cox17 (19), that deliver copper from the cytosol to a late secretory compartment, cytosolic copper/zinc superoxide dismutase, and mitochondria, respectively. Copper is delivered into the lumen of the Golgi/endosome by the Ccc2 protein (20), a P-type ATPase similar to those known to be mutated in Menkes and Wilson disease (21–25), where it is incorporated into the Fet3 multi-copper ferroxidase (26). Together with the Ftr1 iron permease, copper-metallated Fet3 traverses the secretory pathway to generate an active iron transport complex at the plasma membrane (7, 27).

Many of the genes involved in copper or iron transport are transcriptionally regulated by copper or iron availability (4). *CTR1*, *CTR3*, *FRE1*, and *FRE7* are induced by copper starvation and repressed by copper repletion. This regulation involves *cis*-acting copper-responsive elements (CuREs),[1] found in each of these promoters, with the consensus sequence 5′-TTTGC(T/G)C(A/G)-3′, and a copper-sensing transcription factor Mac1 (13, 28–30). *In vivo* footprinting has demonstrated that Mac1 is bound to CuRE elements under conditions of copper scarcity

* This work was supported in part by National Institutes of Health Grant RO1 GM41840 (to D. J. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF175404 and AF175405.

§ Recipient of a Centennial fellowship from the Medical Research Council of Canada.

¶ National Institutes of Health postdoctoral fellow; supported by National Institutes of Health Grant F32 GM18089.

** Participant in an international thesis training program between the United States and Portugal.

‡‡ Burroughs Wellcome Toxicology Scholar. To whom correspondence should be addressed. Tel.: 734-763-5717; Fax: 734-763-4581; E-mail: dthiele@umich.edu.

[1] The abbreviations used are: CuRE, copper-response element; BCS, bathocuproinedisulfonate; BPS, bathophenanthrolinedisulfonic acid; bp, base pair(s); Ctr, copper transporter; Cuf1, copper factor 1; ORF, open reading frame; SOD, superoxide dismutase; PCR, polymerase chain reaction; GFP, green fluorescent protein; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

and is not bound under conditions of copper sufficiency (28). Furthermore, Mac1 undergoes copper-responsive intra-molecular interactions that modulate *trans*-activation function (31). Mac1 has homology with the amino-terminal 40 amino acids of the minor groove DNA binding domain found in the Ace1/Amt1 copper-activated DNA-binding proteins that activate metallothionein gene expression, but little homology outside of this region (32, 33). In the Mac1 carboxyl-terminal region reside two cysteine-rich repeats, REP-I and REP-II, that have been demonstrated to function in copper sensing (34, 35). The *MAC1^{up1}* allele, one of several dominant alleles with mutations in REP-I, encodes a protein unable to sense copper and, as a consequence, is constitutively bound to CuREs and strongly constitutively activates expression of *CTR1*, *CTR3*, and *FRE1/7* (13, 28). Like genes involved in copper transport, *FET3*, *FTR1*, and many other iron homeostatic genes are regulated as a function of iron availability through conserved *cis*-acting promoter elements by the Aft1 iron-sensing transcription factor (36, 37). Indeed, the interdependence of the iron uptake pathway on copper availability is also underscored by the observations that *FRE1* is regulated by copper and iron and the *ATX1* and *CCC2* genes, encoding copper trafficking proteins, are regulated by iron and Aft1, but not copper (10, 16, 32, 37).

The fission yeast *Schizosaccharomyces pombe* is phylogenetically distinct from *S. cerevisiae*, exhibiting several features more similar to mammalian cells. For example, *S. pombe* utilizes pathways involved in stress-responsive signal transduction that are conserved in humans but not in *S. cerevisiae* (38–40). Moreover, little is known about trace metal ion homeostasis in fission yeast. In *S. pombe* three components involved in the high affinity iron uptake system have been identified. Frp1, which shares amino acid sequence similarity with the Fre reductases from *S. cerevisiae* and the mammalian gp91^{phox} subunit of cytochrome b_{558}, is required at the cell surface to reduce Fe^{3+} (41, 42). Once reduced, Fe^{2+} is taken up by a permease/oxidase complex, Fip1/Fio1, and transported into the cell, as occurs with the orthologous Fet3/Ftr1 complex in *S. cerevisiae* (43). To gain further insight into the structure and regulation of proteins involved in copper homeostasis, we have identified *S. pombe* genes encoding a copper-responsive transcription factor and a high affinity copper transporter. Interestingly, the *S. pombe* Cuf1 copper-sensing transcription factor is composed of modules from both the Mac1 and Ace1/Amt1 families of copper metalloregulatory transcription factors (44, 45). Likewise, the *S. pombe* high affinity copper transporter, Ctr4, has no functionally redundant counterpart and resembles a fused version of the *S. cerevisiae* Ctr1 and Ctr3 copper transport proteins. Surprisingly, we show that the Cuf1 protein, which normally serves to activate expression of the *ctr4*$^+$ gene under conditions of copper scarcity, directly represses the expression of iron transport genes under the same conditions. These studies in *S. pombe* provide insight into the evolution of copper transport protein structure in eukaryotes and into the mechanisms by which cells couple copper availability with the expression of a functional iron transport complex.

EXPERIMENTAL PROCEDURES

Strains—The *S. pombe* strains used in this study were the wild-type FY254 (*h$^-$ can1–1 leu1–32 ade6-M210 ura4-D18*), the *cuf1*Δ disruption strain SPY1 (*h$^-$ can1–1 leu1–32 ade6-M210 ura4-D18 cuf1Δ::ura4$^+$*), and the *ctr4*Δ disruption strain MPY2 (*h$^-$ can1–1 leu1–32 ade6-M210 ura4-D18 ctr4Δ::ura4$^+$*). The functional *ura4*$^+$ cassette for gene disruption was isolated by PCR from pUR18 (46). The p*cuf1*Δ*::ura4*$^+$ disruption plasmid was constructed by the insertion of the *Bsp*EI-*Bgl*II *ura4*$^+$ cassette, thereby replacing the complete *cuf1*$^+$ ORF by the *ura4*$^+$ gene and leaving 388 and 476 bp each side of the *cuf1*$^+$ locus for homologous recombination. The p*ctr4*Δ*::ura4*$^+$ disruption plasmid was made by ligating a 499-bp *Asp*718-*Bsp*EI fragment of the 5′ region of *ctr4*$^+$ and a 660-bp *Bgl*II-*Not*I 3′-flanking fragment of *ctr4*$^+$ to the 5′ and 3′ ends of *ura4*$^+$.

S. pombe Homologues of Bakers' Yeast Copper Uptake System—The *S. pombe* Cuf1 and Ctr4 proteins were identified from the Sanger Center *S. pombe* genome data base based upon sequence homologies with the *S. cerevisiae* Mac1 (32) and Ctr3 (15) amino acid sequences, respectively. The *cuf1*$^+$ gene was identified to the ORF *SPAC31A2.11c* found in *S. pombe* chromosome I cosmid c31A2. The *ctr4*$^+$ gene was discovered corresponding to the ORF *SPCC1393.10* found in *S. pombe* chromosome III cosmid c1393. From the cosmid c31A2, we performed a series of subcloning experiments using *Sma*I and *Bam*HI to obtain a 4.2-kilobase pair genomic fragment encompassing the *cuf1*$^+$ *gene*. This latter fragment was isolated and ligated into the pSP1 vector (47), giving rise to pSP*cuf1* S-B/4.2. To generate pSP*ctr4*$^+$B-E/1.6, primers were made corresponding to the position -737 with respect to A of the ATG of the *ctr4*$^+$ gene and the end of that ORF, respectively. The expected 1.6-kilobase pair fragment was obtained by PCR using *S. pombe* FY254 genomic DNA.

RNA Analysis Methods—Northern blot analyses were carried out according to Schleicher & Schuell's protocol using random-primed ^{32}P-labeled DNA probes. Experiments regarding the mRNA steady-state levels of *FET3* and *ACT1* were carried out using the bakers' yeast strain and experimental conditions as described previously (28). To conduct the RNase protection analyses as described previously (48), two plasmids for making antisense RNA probes were created. The pSK*ctr4*$^+$ plasmid was made by the insertion of the 189-bp fragment (positions +413 to +602) isolated by PCR from *S. pombe* (FY254) genomic DNA into the pBluescript SK+. To generate the pSK*act1*$^+$ plasmid, a 151-bp DNA fragment, which hybridizes to the *S. pombe act1*$^+$ gene positions +507 to +658, was also amplified and cloned into the pBluescript SK+ vector. The plasmid pKS*lacZ* used was described previously (28).

SOD Activity Assay—The *S. cerevisiae* isogenic strains 50.L4 (*MAT*α *gal1 trp1–1 his leu2–3,-112 ura3–50*) (49) and DTY116 (*MAT*α *gal1 trp1–1 his leu2–3,-112 ura3–50 sod1*Δ*::TRP1*) (50) were grown in YPD, while the *S. pombe* isogenic strains FY254 and SPY1 in YES media (51). Copper treatment of yeast strains was conducted as described previously (15). The cells were washed with the lysis buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA) in presence of protease inhibitors. Once harvested, the cells were resuspended in 100 μl of lysis buffer, and then disrupted with an equivalent volume of glass beads. Typically, 1% of Triton X-100 was added to the lysate and the mixture incubated on ice for 10 min. Equal concentrations of protein extracts were analyzed on a 10% native polyacrylamide gel. To detect the SOD activity, the gel was soaked in 2.5 mM nitro blue tetrazolium and then subjected to the developing solution (36 mM potassium phosphate, 2.8 mM TEMED, 28 μM riboflavin (pH 7.8)) for 20 min.

Protein Epitope Tagging and Microscopy—To generate the pSP*cuf1*$^+$GFP, an insert delineated by the *Nru*I site located at position −977 with respect to A of the ATG of the *cuf1*$^+$ gene and the stop codon including a *Not*I site was isolated. The fragment was swapped for an identical DNA region, except for the *Not*I site, comprised into the pSP*cuf1*$^+$S-B/4.2 plasmid, creating pSP*cuf1*$^+$*Not*I S-B/4.2. Subsequently, the *gfp* gene on a 750-bp *Not*I fragment (52) was cloned into pSP*cuf1*$^+$*Not*I S-B/4.2. A similar strategy was utilized for generating the pSP*ctr4*$^+$GFP plasmid. To observe GFP fusion proteins by microscopy, the cells were grown in EMM media (51) under conditions of low copper availability. A 4-μl culture aliquot was prepared and examined as described previously (53). Calcofluor white M2R (Sigma) staining was carried out as described previously (54).

Electrophoretic Mobility Shift Assay—Different cultures of SPY1 (*cuf1*Δ), expressing either *cuf1*$^+$ or *cuf1*$^+$GFP were grown to early log-phase and then control, copper-treated (100 μM) or BCS-treated (100 μM) cultures were incubated for 1 h. Cell extracts were prepared as described previously (55) except for the composition of the extraction buffer (20 mM HEPES (pH 7.9), 10% glycerol, 75 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Electrophoretic mobility shift assay was conducted using 1× binding buffer that contained 12.5 mM HEPES (pH 7.9), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 2.5 μM $ZnSO_4$, 0.6 mM DTT, 0.05% Nonidet P-40, and 6% glycerol. Typically, 15 μg of protein of whole extract was incubated for 15 min at 25 °C with 1 ng of ^{32}P-end-labeled *fio1*$^+$ DNA fragment and 0.5 μg of salmon sperm DNA. When indicated, the reactions were pre-incubated in presence of anti-GFP antibodies (1 μg) for 15 min at 25 °C in absence of the probe. The samples were analyzed on a 1.5% agarose gel as described previously (55).

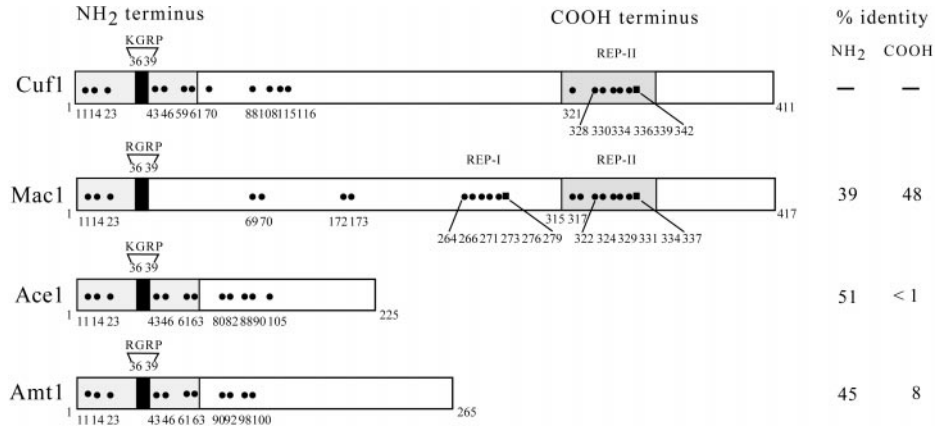

FIG. 1. **Primary structural and functional domain comparison of two classes of copper metalloregulatory transcription factors involved in copper metabolism.** The nutritional copper-sensing protein Cuf1 (*S. pombe*) exhibits 51% and 45% sequence identity with the amino-terminal 63 and 62 amino acid residues that are part of the copper-activated DNA-binding domains of the toxic copper sensors Ace1 (*S. cerevisiae*) and Amt1 (*C. glabrata*), respectively. While Cuf1 also shows 48% sequence identity with the REP-II domain at the carboxyl terminus of the nutritional copper sensor Mac1 (*S. cerevisiae*). The amino acid sequence numbers refer to the position relative to the first amino acid of the protein. The *dots* (●) indicate positions of cysteine residues. The *squares* (■) depict histidine residues that are required for function in Mac1. The *black-shaded regions* indicate the location of the (R/K)GRP motif that is part of a conserved minor groove binding domain (33).

RESULTS

The S. pombe Copper Metalloregulatory Transcription Factor Cuf1—Although there is a large evolutionary gap between *S. pombe* and *S. cerevisiae*, almost as large as that separating both yeasts from animals, in general, *S. pombe* genes and proteins exhibit greater similarity to those from mammalian cells than do *S. cerevisiae* genes (56, 57). Therefore, we sought to identify genes encoding components of the copper homeostasis machinery in *S. pombe* to gain insight about structure-function relationships of copper balancing proteins in higher organisms. Analysis of genomic DNA sequences from the *S. pombe* Genome Project revealed an open reading frame (ORF) (*SPAC31A2.11c*) related to two previously identified groups of copper metalloregulatory transcription factors: the Ace1 and Amt1 proteins from *S. cerevisiae* and the *Candida glabrata*, respectively, involved in copper detoxification via induction of metallothionein gene transcription (58–60), and the Mac1 protein from *S. cerevisiae* that regulates transcription of genes involved in copper acquisition (13, 28, 32). We denote this *S. pombe* protein as Cuf1 (Cu factor 1) (Fig. 1). Interestingly, the amino-terminal 61 amino acids of Cuf1 display 51% and 45% identity to the amino-terminal 63 and 62 amino acids of the *S. cerevisiae* Ace1 and *C. glabrata* Amt1 proteins, respectively. Within this region of Cuf1 lies a zinc coordination domain that constitutes a minor groove DNA binding domain also found in Mac1 (33); Cuf1 also harbors extended homology (amino acid residues 41–61) to a region similar to Ace1, known to be involved in copper cluster formation and copper-inducible DNA binding to metal response elements in *S. cerevisiae* metallothionein gene promoters (44, 45). In support of the notion that the Cuf1 amino-terminal domain is more similar to that found in Ace1 and Amt1 than Mac1, we have demonstrated that Cuf1 can partially substitute for Ace1, but not Mac1 function in *S. cerevisiae*.[2] Although residues 1–61 of Cuf1 display lower identity to this same region of Mac1 (39%), Cuf1 possesses a cysteine-rich domain near its carboxyl terminus with strong identity to a similar domain (residues 321–345) that plays a critical role in copper ion sensing in Mac1, conserving the five Cys and one His residue as well as additional flanking and intervening amino acids (31, 35). Therefore, Cuf1 bears strong similarity to domains found in the Ace1/Amt1 class, as well as the Mac1 class of copper metalloregulatory transcription factors.

To understand the role of Cuf1 in *S. pombe*, the *cuf1*$^+$ locus was insertionally inactivated by deletion and replacement with the *S. pombe ura4*$^+$ gene (61). *cuf1*$^+$ mRNA was found to be moderately abundant in wild type cells and not regulated by copper addition or starvation, and absent in *cuf1*Δ cells as determined by RNA blotting (Fig. 2*A*). Although we detected no obvious defect in copper resistance in *cuf1*Δ cells as compared with isogenic *cuf1*$^+$ wild type cells (data not shown), *cuf1*Δ cells are completely defective in utilizing the respiratory carbon source glycerol for growth, with only slight growth defects observed when cells are grown on glucose (Fig. 2*B*). The glycerol growth defect could be corrected either by returning a wild type copy of the *cuf1*$^+$ gene expressed from a plasmid, or by the addition of $CuSO_4$ to the growth medium at concentrations of at least 25 μM (Fig. 2*B*). To test whether *cuf1*Δ cells display other phenotypes that are known to be associated with copper deficiency in *S. cerevisiae* cells, copper/zinc SOD activity was assayed from whole cell extracts using native enzyme polyacrylamide gel electrophoresis from wild type and *cuf1*Δ cells grown either under control conditions or in the presence of 100 μM $CuSO_4$. Although copper/zinc SOD activity was detected in *cuf1*$^+$ cells under both conditions, this activity was detected only in *cuf1*Δ cells supplemented with $CuSO_4$ (Fig. 2*C*). Moreover, *cuf1*$^+$ deletion cells are defective in growth under iron starvation conditions (Fig. 2*D*), presumably due to their inability to provide copper to the multi-copper ferroxidase Fio1, essential for iron transport in *S. pombe* (43). Furthermore, this defect in high affinity iron uptake is associated with the integrity of the copper transport machinery since an identical phenotype is observed when cells harbor an inactivated *ctr4*$^+$ gene (*ctr4*Δ), encoding a high affinity copper permease in fission yeast, as described below (Fig. 2*D*). Taken together, the observed defects in respiration, copper/zinc SOD activity, and iron acquisition indicate that *cuf1*Δ cells are copper- and iron-starved, and suggest that Cuf1 plays a crucial role in copper and iron transport, rather than copper detoxification in *S. pombe*.

Since Cuf1 resembles two distinct classes of copper metalloregulatory transcription factors and plays an important role in copper acquisition, we localized the protein in *S. pombe* cells to understand how it functions in copper homeostasis. A *cuf1*$^+$-*GFP* fusion allele was constructed that fully complements the *cuf1*Δ glycerol growth defect (Fig. 3*A*). As shown in Fig. 3*B*, the Cuf1-GFP fusion protein localizes to the *S. pombe* nucleus as

[2] S. Labbé and D. J. Thiele, unpublished data.

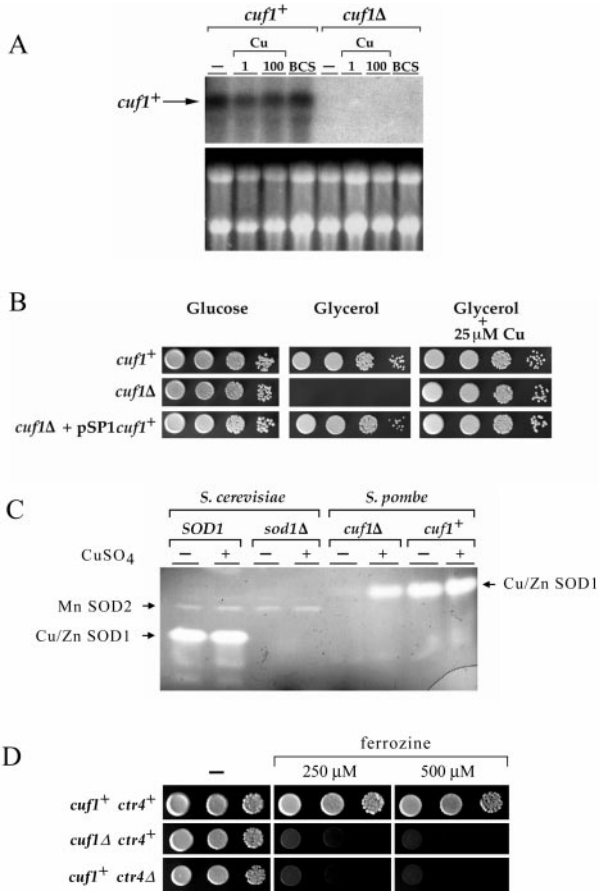

FIG. 2. **Disruption of the *cuf1*$^+$ gene gives rise to defects linked with copper and iron deficiency.** *A*, the steady-state levels of *cuf1*$^+$mRNA in wild type strain FY254 are unaffected by either exogenous $CuSO_4$ (1, and 100 μM) or BCS (100 μM). No *cuf1*$^+$ transcript was observed in the disruption strain (*cuf1*Δ). *Bottom panel* shows ethidium bromide-stained ribosomal RNAs as a control. *B*, *cuf1*Δ cells are defective for respiratory growth. The growth is remediated by exogenous $CuSO_4$ (25 μM) or a plasmid-borne copy (*cuf1*Δ + pSP1*cuf1*$^+$) of the wild type *cuf1*$^+$ gene. Strains spotted onto YES-glucose and YES-glycerol media were incubated at 30 °C for 3 and 7 days, respectively. *C*, an *S. pombe* strain bearing the disrupted *cuf1*Δ allele displays a deficiency in copper/zinc SOD1 activity (*right side*), unless exogenous $CuSO_4$ (100 μM) (+) is added. As a control (*left side*) a wild-type strain of *S. cerevisiae* exhibits high levels of copper/zinc SOD1 activity, whereas the *sod1*Δ disruption strain lacks such activity, even in presence of exogenous copper. Manganese SOD2 activity detected in bakers' yeast is indicated (*Mn SOD2*). *D*, strains harboring either an inactivated *cuf1*$^+$ or *ctr4*$^+$ gene are defective in iron accumulation as shown by their inability to grow on medium containing the iron chelator ferrozine (250 and 500 μM).

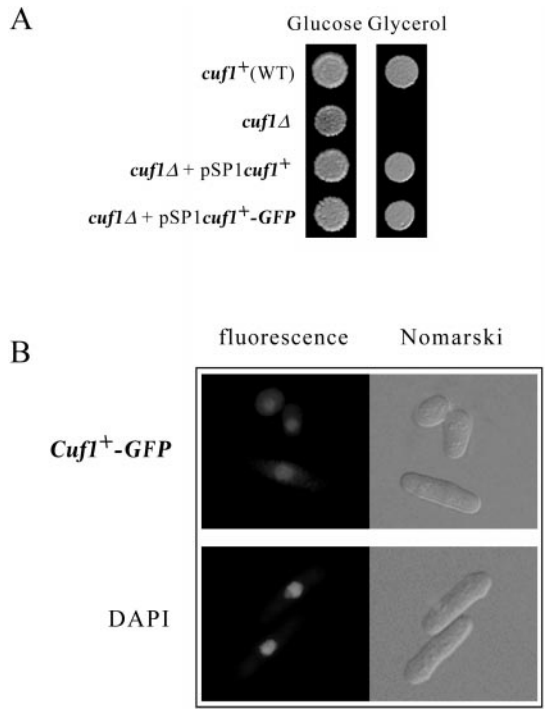

FIG. 3. **Cuf1 is a nuclear protein.** *A*, the Cuf1-GFP fusion protein complements the *cuf1*Δ growth defect on nonfermentable carbon sources (glycerol) in a manner indistinguishable from the unadulterated Cuf1 protein. Cultures grown to identical optical density were spotted (3000 cells/5 μl) onto YES-glucose and YES-glycerol media and incubated at 30 °C for 3 and 7 days, respectively. *B*, representative cells from Cuf1-GFP cultures grown at 30 °C for 16 h in the presence of BCS (100 μM) were visualized using the appropriate filter, and then 4,6-diamidino-2-phenylindole staining to determine the location of the nucleus. The cells were also viewed by Nomarski microscopy to examine cell morphology (on the *right*).

shown by staining with 4,6-diamidino-2-phenylindole, a marker for nuclear and mitochondrial DNA. Furthermore, no change in the localization of Cuf1-GFP fusion protein was apparent based on cellular copper status (data not shown). Thus, the results described here are consistent with Cuf1 serving as a nuclear transcription factor required for expression of genes that are critical for copper acquisition.

An S. pombe Copper Transporter with Properties of Both Ctr1 and Ctr3—Based on the properties of Cuf1 and the hypothesis that it activates target genes involved in copper acquisition, we searched the unassigned *S. pombe* genome data base for ORFs with homology to either the Ctr1 or Ctr3 high affinity copper transporters of *S. cerevisiae*. A single ORF (*SPCC1393.10*) of 289 codons, which we have denoted Ctr4, was identified that bears homology to Ctr1 at its amino terminus (38% identity) and Ctr3 at the carboxyl terminus (41% identity) (Fig. 4*A*). Like both Ctr1 and Ctr3, Ctr4 is predicted (using the TopPred II analysis) to possess two or three transmembrane domains, an extracellular hydrophilic amino terminus and a cytosolic tail. The amino terminus of Ctr4 harbors five copies of the sequence Met-X_2-Met-*X*-Met, a putative extracellular copper binding motif found eight times in the amino-terminal region of Ctr1 and absent in Ctr3. Furthermore, Ctr4 exhibits strong identity to Ctr3 from residues 111 to 248, which encompasses the carboxyl-terminal 65% of the protein, with very high identity within the two regions which encompass putative transmembrane domains (Fig. 4*A*). Based on these observations, it appears that the *S. pombe* Ctr4 protein represents a fusion between the *S. cerevisiae* Ctr1 and Ctr3 proteins. Indeed, inspection of the sequence of a putative human copper transporter, hCtr1 (62), strongly suggests that it is also structurally related in its amino terminus to *S. cerevisiae* Ctr1, but in its carboxyl terminus to Ctr3. Furthermore, both the *S. pombe* Ctr4 and human Ctr1 proteins exhibit more overall sequence homology to *S. cerevisiae* Ctr3 than to Ctr1.

A role for *S. pombe* Ctr4 in high affinity copper transport was confirmed by four experimental results. First, as observed for Cuf1, deletion of the *ctr4*$^+$ gene (*ctr4*Δ) renders *S. pombe* cells defective for growth on respiratory carbon sources that can be rescued with either copper supplementation or by providing a plasmid-borne copy of the *ctr4*$^+$ gene (Fig. 4*B*). Second, a functional Ctr4-GFP fusion protein is localized to the *S. pombe* plasma membrane (Fig. 4, *C* and *D*). Third, *ctr4*Δ strain cells are defective in iron transport (Fig. 2*D*). Fourth, as found for the *CTR1* and *CTR3* genes in *S. cerevisiae*, the *S. pombe ctr4*$^+$ gene expression is repressed by copper and induced by copper limitation using the Cu^+ chelator BCS (Fig. 5*A*). Taken together, these data are consistent with Ctr4 functioning as a

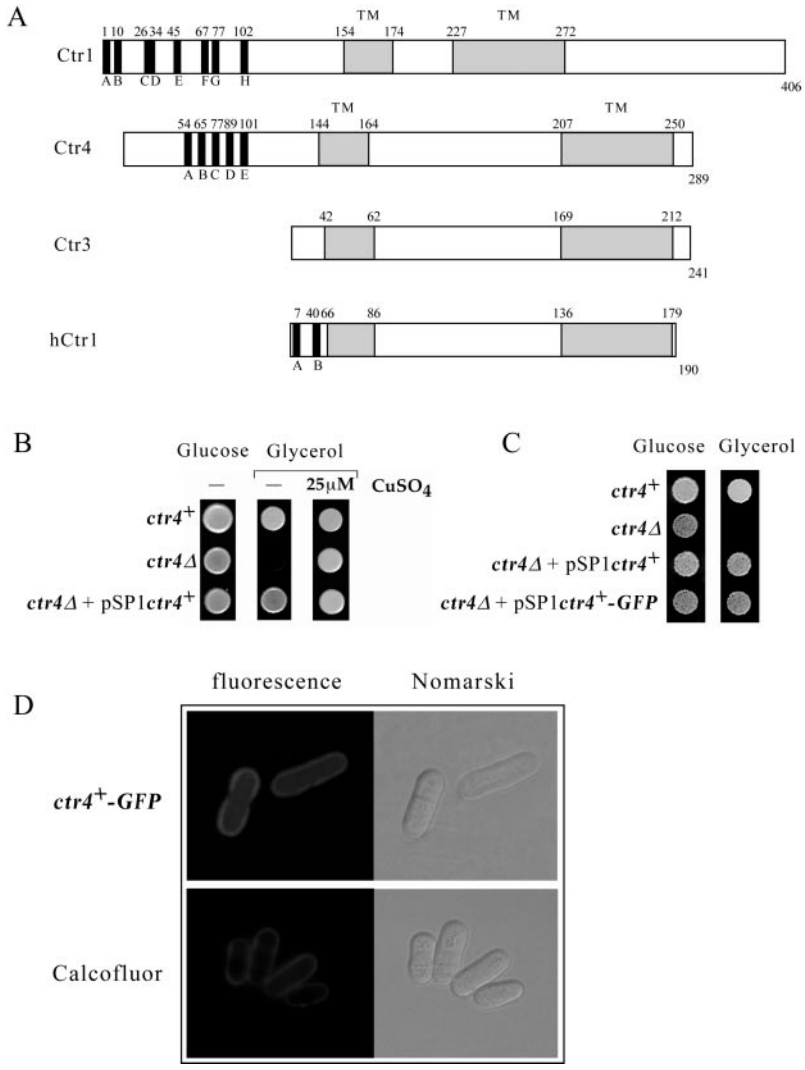

FIG. 4. **Primary structural comparison of bakers' yeast and human high affinity copper transporters with *S. pombe* copper transporter.** *A*, the amino-terminal region (45–106) of Ctr4 exhibits 38% identity to Ctr1. Similarly, hCtr1 (1–46) shows 28% identity to Ctr4. The motif Met-X_2-Met-*X*-Met is depicted by the *filled rectangles* (also indicated with *capital letters*). The carboxyl terminus of Ctr4 (111–248) resembles the *S. cerevisiae* Ctr3, as shown by the score of 41% sequence identity. *TM*, putative transmembrane domain. *B*, growth defect phenotype on nonfermentable carbon source associated with the insertionally inactivated *ctr4*Δ allele was analyzed as described in Fig. 2*B*. *C*, *ctr4*Δ cells express a functional Ctr4-GFP fusion protein as assessed by complementation of a *ctr4*Δ allele (*ctr4*Δ + pSP1*ctr4*$^+$*-GFP*) in a similar manner as specified for the Cuf1-GFP fused protein in Fig. 3*A* except that the cultures were spotted at a density of 300 cells/5 μl. *D*, representative cells from *ctr4*$^+$*-GFP* cultures grown and examined under the same conditions as detailed in Fig. 3*B* for the Cuf1-GFP fused protein except that calcofluor staining was used to delineate the cell periphery.

high affinity copper transport protein in *S. pombe*. Unlike the redundancy of copper transport functions provided by Ctr1 and Ctr3, encoded by separate genes in *S. cerevisiae*, deletion of the *ctr4*$^+$ gene is sufficient to confer a copper starvation phenotype to *S. pombe* cells.

Based on our assignment of Cuf1 as a nutritional copper-sensing transcription factor, and the findings that deletion of either the *cuf1*$^+$ or *ctr4*$^+$ genes leads to defects observed in copper-deficient cells, we ascertained if *ctr4*$^+$ expression is under the control of copper and Cuf1. As shown in Fig. 5*B*, wild type cells express detectable basal levels of *ctr4*$^+$ mRNA that are repressed by copper and induced by copper starvation with the Cu^+ chelator BCS, but not under iron starvation conditions (with iron chelator BPS). Moreover, *ctr4*$^+$ mRNA is absent in *cuf1*Δ cells under all conditions (Fig. 5*B*). Taken together, the convergence of *cuf1*Δ and *ctr4*Δ phenotypes, and the absolute dependence of *ctr4*$^+$ expression on Cuf1, strongly argue that Cuf1 and Ctr4 represent a copper-sensing and uptake system in *S. pombe*.

Cuf1 Represses Expression of Iron Uptake Genes under Copper Starvation—In bakers' yeast a clear mechanistic link exists between copper availability and iron acquisition and mobilization in at least two respects. The Fet3 protein is a multi-copper ferroxidase essential for assembly of an iron transport complex at the plasma membrane with the Ftr1 iron permease. Accordingly, *S. cerevisiae* cells that are copper-deficient or unable to deliver copper to the lumen of the Golgi/endosome are unable to assemble an active iron-transport complex at the plasma membrane and, as a consequence, are iron-starved. Furthermore, the Fre1 protein is an Fe^{3+} and Cu^{2+} reductase that functions in both copper and iron uptake. While *FET3* and *FTR1* gene expression is regulated by iron availability through the iron-sensing transcription factor Aft1, the *FRE1* gene is regulated by copper and iron availability (7, 13, 28). To ascertain if any of the genes known to be involved in iron uptake in *S. pombe* are regulated by copper availability, we carried out RNA blotting experiments with isogenic wild type and *cuf1*Δ cells grown under copper or iron starvation or replete conditions. As shown in Fig. 6*A* for wild type strains, under conditions of iron adequacy but low copper availability, very low levels of *frp1*$^+$ metalloreductase mRNA are observed. In response to 100 μM $CuSO_4$, a dramatic increase in *frp1*$^+$ mRNA was observed relative to basal levels; however, copper starvation with BCS treatment did not alter *frp1*$^+$ mRNA abundance. This same observation was true in two independent wild type *S. pombe* strains, GYP4 and FY254. Moreover, as shown in Fig. 6*B* (*left panel*) *fip1*$^+$ iron permease and *fio1*$^+$ multi-copper ferroxidase mRNA is also dramatically induced by 100 μM $CuSO_4$ and, as expected, induced by treatment with the iron chelator BPS. Remarkably, deletion of the *cuf1*$^+$ gene (*cuf1*Δ) significantly elevated basal levels of *fip1*$^+$ and *fio1*$^+$ mRNA and eliminated further induction by 100 μM $CuSO_4$ (Fig. 6*B*, *right panel*). A slight (~2-fold) reduction of *fip1*$^+$ and *fio1*$^+$ mRNA levels was observed in the *cuf1*Δ strain in response to copper administra-

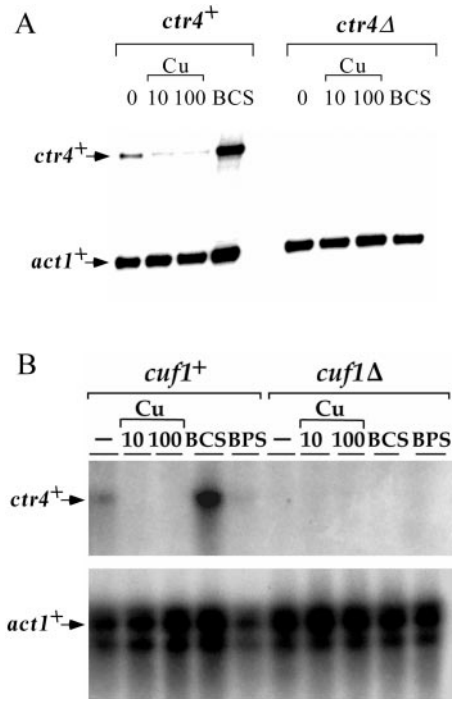

FIG. 5. **The *cuf1*$^+$ gene is required for copper-dependent regulation of *ctr4*$^+$ gene expression.** *A*, strains FY254 (*ctr4*$^+$) and MPY2 (*ctr4*Δ) were treated with $CuSO_4$ (0, 10, and 100 μM) or BCS (100 μM) for 1 h followed by total RNA isolation and RNase protection analysis of *ctr4*$^+$ and *act1*$^+$ mRNA steady-state levels (indicated by *arrows*). *B*, FY254 (*cuf1*$^+$) and SPY1 (*cuf1*Δ) cultures were incubated in the absence (−) or presence of $CuSO_4$ (10 and 100 μM), the copper chelator BCS (100 μM), or the iron chelator BPS (100 μM) for 1 h at 30 °C, and then total RNA was isolated. Shown is an RNA blot of *ctr4*$^+$ and *act1*$^+$ mRNA steady-state levels indicated by *arrows*, respectively.

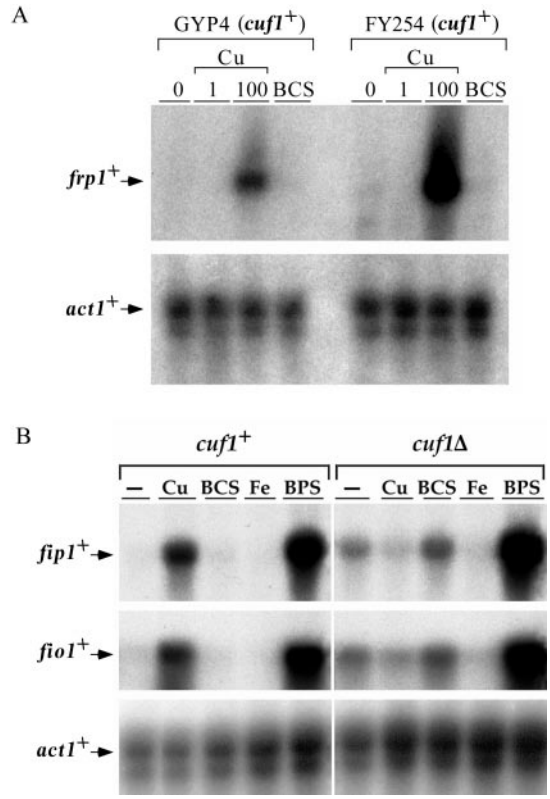

FIG. 6. **Iron transport genes are transcriptionally repressed under conditions of low copper availability in a Cuf1-dependent manner.** *A*, two different genetic backgrounds of fission yeast strains (GYP4 and FY254) harboring wild type *cuf1*$^+$ alleles were grown in the presence of $CuSO_4$ (0, 1, and 100 μM) or under copper starvation conditions (100 μM BCS) for 1 h at 30 °C. The steady-state mRNA levels of *frp1*$^+$ and *act1*$^+$ are indicated with *arrows*. *B*, cultures from strains FY254 (*cuf1*$^+$) and SPY1 (*cuf1*Δ) treated with control (−), copper (100 μM), BCS (100 μM), iron (100 μM), and BPS (100 μM) were used to assess the mRNA steady-state levels of *fip1*$^+$, *fio1*$^+$, and *act1*$^+$ (denoted by *arrows*) in regard to the *cuf1* allele status.

tion, perhaps due to stimulation of intracellular iron levels sensed by an Aft1 ortholog in *S. pombe*. As a control, *fio1*$^+$ and *fip1*$^+$ mRNA levels were assayed from cells grown under iron-sufficient (iron) and iron starvation (BPS) conditions and as observed previously, the expression of these genes was induced by iron starvation and repressed by iron adequacy in both *cuf1*$^+$ and *cuf1*Δ backgrounds (43).

We tested whether Cuf1 directly represses expression of iron uptake genes in *S. pombe*, or whether this is an indirect response due to copper starvation in *cuf1*Δ strains and, hence, failure to provide copper to the iron transport complex resulting in iron starvation. The *fio1*$^+$-*fip1*$^+$ genes are divergently transcribed in *S. pombe* (43). Within this shared promoter region, we identified six copies of a repeated sequence, 5′-TTTGTC-3′, that is similar, although not identical, to the CuREs found in the *CTR1*, *CTR3*, *FRE1*, and *FRE7* genes in *S. cerevisiae* and bound by the Mac1 protein (13, 28) (Fig. 7*A*). To ascertain whether these elements play a direct role in *fio1*$^+$-*fip1*$^+$ regulation by Cuf1 and copper, we constructed *CYC1* minimal promoter-*lacZ* fusion genes harboring either a 271-bp fragment from the *fio1*$^+$-*fip1*$^+$ promoter that contains three TTTGTC elements, or the same fragment in which two of the three TTTGTC elements have been extensively mutagenized (GGGAGA instead of TTTGTC). As shown in Fig. 7*B* by a representative RNase protection assay, the wild type *fio1*$^+$-*fip1*$^+$-*CYC1-lacZ* fusion gene was induced approximately 4-fold by copper treatment, whereas the same fusion in which two of the three TTTGTC boxes have been mutated exhibits high constitutive basal levels of expression that are not further elevated by copper treatment. These data directly implicate the TTTGTC elements in repression of the *fio1*$^+$ and *fip1*$^+$ genes and in copper-mediated gene induction. Consistent with this notion, two inverted TTTGTC repeats are also found within the *frp1*$^+$ promoter (data not shown).

Interestingly, the promoter of *FET3* and other iron homeostatic genes in *S. cerevisiae* harbor similar repeated TTTGTC elements in their regulatory regions. To test whether this Cuf1-mediated repression mechanism may operate in *S. cerevisiae*, *FET3* mRNA levels were measured in isogenic wild type cells, *mac1*Δ cells, and cells bearing a dominant *MAC1*^{up1} mutation in which the Mac1 protein is unable to sense copper, constitutively binds to DNA instead of releasing in response to copper, and strongly activates expression of the *CTR1*, *CTR3*, and *FRE1* copper transport genes even in the presence of copper. As shown in Fig. 7*C*, in response to 1 or 10 μM $CuSO_4$ *FET3* mRNA levels are modestly, but significantly induced (approximately 4-fold). In marked contrast, *FET3* mRNA levels are dramatically elevated in the *mac1*Δ background under all conditions (by approximately 18-fold), and are super-repressed under all conditions in the *MAC1*^{up1} strain, when Mac1 protein is constitutively bound to CuREs (3-fold lower than the basal levels detected in the wild type strain). Therefore, both *S. pombe* and *S. cerevisiae* regulate expression of iron uptake genes in response to alterations in the availability of copper, a critical co-factor for the iron transport complex, through orthologous copper-sensing transcription factors. Although direct repression of *FET3* by Mac1 has not been established, based on our results with Cuf1, this is a likely mechanism.

To test whether Cuf1 directly represses *fio1*$^+$ and *fip1*$^+$ expression through the TTTGTC elements in these promoters,

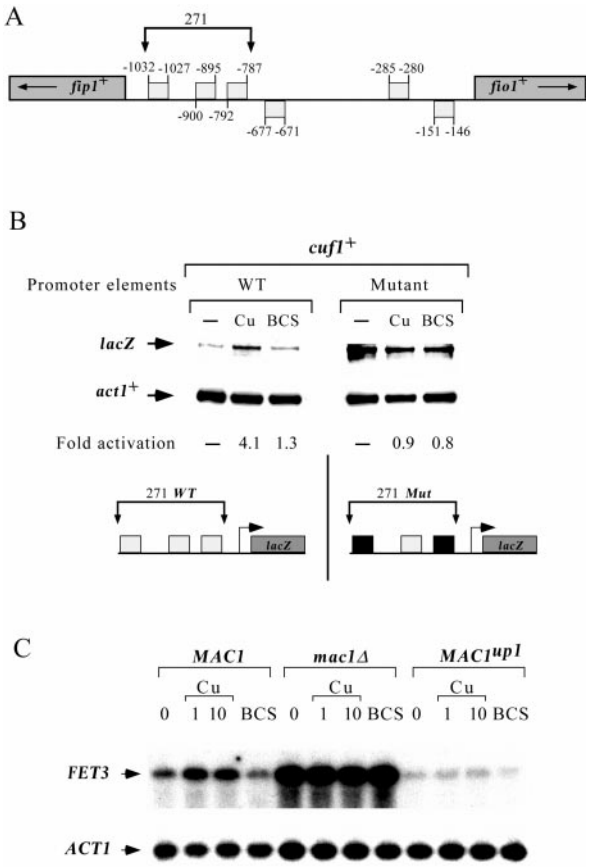

FIG. 7. **The *fip1*$^+$-*fio1*$^+$ promoter TTTGTC elements confer copper starvation-mediated repression to the minimal promoter *CYC1-lacZ*.** *A*, schematic representation of the *fip1*$^+$-*fio1*$^+$ intergenic promoter region within which lie six copies of the repeated sequence, TTTGTC. The nucleotide numbers refer to the position relative to the A of the start codon of the *fio1*$^+$ ORF. The *four upper boxes* (□) shown in the common promoter region indicate that the TTTGTC elements are oriented toward the *fio1*$^+$ gene, while the *two lower boxes* depict the elements oriented toward the *fip1*$^+$ gene. *Vertical arrows* represent the edges of the 271-bp promoter DNA fragment encompassing three *cis*-acting elements that were inserted in its natural orientation into the minimal promoter of the *CYC1* gene fused to *lacZ*. *B*, *CYC-lacZ* fusion genes containing the wild type 270-bp fragment (271 *WT*, □) and the same fragment in which two of the TTTGTC elements have been mutated (*271 Mut*, ■) were transformed in FY254 (*cuf1*$^+$) and analyzed for the *lacZ* mRNA steady-state levels status. *act1*$^+$ transcripts steady-state levels were also measured as control. Total RNA was isolated from untreated cultures (−) or cultures treated with copper (100 μM), and BCS (100 μM). *C*, the isogenic wild type (*MAC1*), *mac1*Δ, and *MAC1*^{up1} strains were treated for 1 h in the presence of $CuSO_4$ (0, 1, and 10 μM) or BCS (100 μM). Shown is an RNA blot of *FET3* and *ACT1* mRNA steady-state levels indicated by *arrows*, respectively.

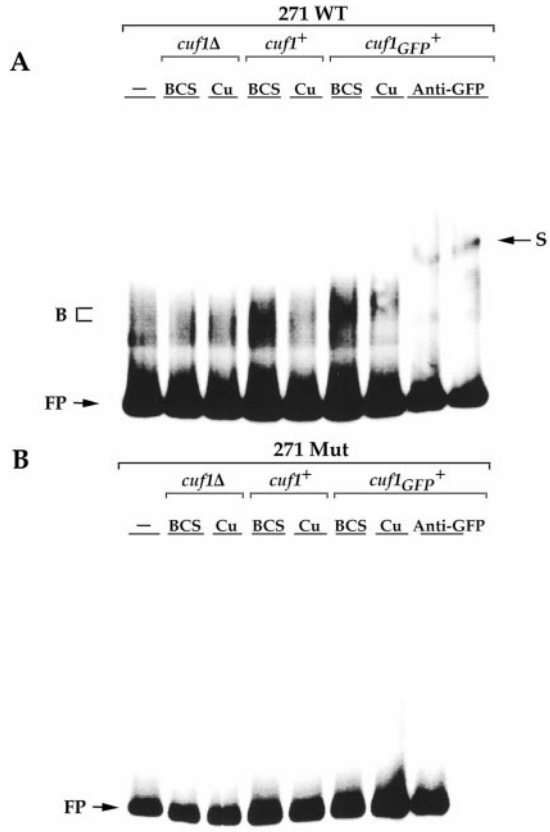

FIG. 8. **Cuf1 and Cuf1-GFP proteins expressed in fission yeast bind to the repeated TTTGTC elements.** *A*, representative electrophoretic mobility shift gel of the wild type ^{32}P-labeled 271-bp *fio1*$^+$ promoter fragment used with whole cell extracts prepared from SPY1 cells (*cuf1*Δ) expressing either the pSP1 vector alone (−), no Cuf1 (*cuf1*Δ), wild-type Cuf1 (*cuf1*$^+$), or Cuf1-GFP (*cuf1*$^+$-*GFP*) as their sole source of Cuf1. BCS-treated cells expressing either *cuf1*$^+$ or *cuf1*$^+$-*GFP* alleles exhibit a specific TTTGTC-protein complex (*B*), which is absent in extracts from cells treated with copper (100 μM). In the presence of anti-GFP either pre-incubated or added once the probe is mixed in the presence of the cell extracts, the protein-bound complex is shifted (*S*). *FP*, free probe DNA. *B*, representative electrophoretic mobility shift assay gel using the mutated *fio1*$^+$-*fip1*$^+$ 271-bp fragment. As shown, no DNA-protein complexes were observed when two of the three TTTGTC elements harbor multiple point mutations (GGGAGA instead of TTTGTC).

DNA binding experiments were conducted using extracts derived from *S. pombe cuf1*$^+$ wild type and *cuf1*Δ cells, and from *cuf1*Δ cells expressing a functional Cuf1-GFP fusion protein. As shown in Fig. 8*A* by a representative electrophoretic mobility shift assay, the wild type 271-bp *fio1*$^+$-*fip1*$^+$ promoter fragment forms a strong protein-DNA complex when extracts are prepared from *cuf1*$^+$ wild type cells grown under copper starvation conditions (BCS) and this complex is diminished to background levels when cells were grown in the presence of 100 μM $CuSO_4$ prior to extract preparation. Furthermore, a complex of slightly slower electrophoretic mobility formed on this DNA fragment when extracts were prepared from copper-starved cells expressing the Cuf1-GFP fusion protein, the levels of which were substantially diminished by growth in the presence of copper. Additionally, a protein-DNA complex of slower electrophoretic mobility was observed when anti-GFP antiserum was added to the binding reactions in supershift experiments. As shown in Fig. 8*B*, the *fio1*$^+$-*fip1*$^+$ promoter fragment in which two of the three TTTGTC elements were mutated did not form significant protein-DNA complexes with these same extracts under any of the conditions tested. Taken together, these data strongly suggest that the *S. pombe* Cuf1 protein, which activates copper transporter gene expression under conditions of low copper availability, directly represses the expression of the *fio1*$^+$, *fip1*$^+$, and *frp1*$^+$ iron uptake genes under these same conditions through the TTTGTC elements found within these promoters.

DISCUSSION

Our results in *S. pombe* have provided important information on the structural modularity of both copper ion-sensing transcription factors and copper ion transporters. Indeed, our studies clearly demonstrate that Cuf1 is functionally similar to Mac1 from *S. cerevisiae* in terms of its regulation of copper transporter gene expression and repression of iron transport genes. However, structurally Cuf1 has hallmarks of the Ace1/Amt1 proteins at its amino terminus that extend beyond the similarity observed between Mac1 and the Ace1/Amt1 amino-

terminal DNA binding domains. This similarity is further supported by the observations that Cuf1 will partially suppress an *ace1*Δ mutation, but not when the major target gene of Ace1, *CUP1*-encoded metallothionein, is deleted. Furthermore, in *ace1*Δ cells expressing Cuf1, the robust basal levels of *CUP1* mRNA are repressed approximately 3-fold in response to copper, consistent with the manner by which Cuf1 regulates *ctr4*$^+$ gene expression in *S. pombe* and the plausibility that Cuf1 directly binds to the *CUP1* promoter.[2] The possibility that the unique amino terminus of Cuf1 displays distinct DNA binding specificity from that of Mac1 is supported by the fact that the Cuf1 binding sites identified in the *fio1*$^+$-*fip1*$^+$ promoter region (5′-TTTGTC-3′) differ from the CuRE element consensus sequence found in the *CTR1*, *CTR3*, *FRE1*, and *FRE7* genes in *S. cerevisiae* (5′-TTTGC(T/G)C(A/G)-3′). Therefore, the network of hydrogen bonds made by the conserved Cuf1 amino-terminal minor groove DNA binding domain and the T-rich DNA sequence, while likely similar to those made by Mac1 and Ace1/Amt1, are likely to be highly specific for each protein within the more G-C rich core of their respective *cis*-acting DNA sequences. We also note that we have shown Cuf1 DNA binding in cell extracts to be responsive to copper levels, a feature associated with Ace1 and Amt1 in cell extracts, but one that has only been demonstrated for Mac1 *in vivo* (28).

The Cuf1 carboxyl terminus harbors a Cys-His repeat element (REP element) absent in Ace1/Amt1 but found duplicated in both Mac1 and Grisea of *Podospora anserina* (63, 64). Because a specific mutation of the first repeated element in Mac1 (*MAC1*^{up1}) generates a protein that is unable to repress target gene expression in response to copper, this element likely serves a copper-sensing function to homeostatically modulate expression of the *ctr4*$^+$ gene, and perhaps other genes, by Cuf1. Taken together, *S. pombe* has utilized copper-sensing domains from two distinct classes of copper metalloregulatory transcription factors for Cuf1, creating a chimeric protein from the phylogenetic standpoint.

The *S. pombe* Ctr4 protein appears to be a chimera of the *S. cerevisiae* Ctr1 and Ctr3 high affinity copper transport proteins. This is supported by the observation that the Ctr4 amino terminus harbors five copies of a putative extracellular copper binding domain (M-X_2-M-*X*-M) that is found in the amino termini of both *S. cerevisiae* and human Ctr1, but not in *S. cerevisiae* Ctr3. Furthermore, the carboxyl-terminal ~65% of Ctr4 has strong identity to Ctr3, especially within the transmembrane spanning domains, but little identity to Ctr1 in this segment. Since the transmembrane domains are likely to play crucial roles in guiding copper across the plasma membrane, this homology to Ctr3 may be very important in copper transport. Indeed, the analysis of human Ctr1, by domain structure, sequence identity, and phylogenetic tree formation using the Clustal method, demonstrates that like Ctr4, the human Ctr1 protein has homology to *S. cerevisiae* Ctr1 in the amino terminus (29%) and Ctr3 in the carboxyl terminus (31%) (Fig. 4*A*). Furthermore, hCtr1 is more closely related to *S. cerevisiae* Ctr3 over the entire length of the protein, than it is to Ctr1. Based on these structural features of Ctr4, we speculate that *S. pombe* may represent an early evolutionary phase at which the Ctr1 and Ctr3 proteins, encoded by distinct and functionally redundant genes in *S. cerevisiae*, may have fused into one protein. This idea is supported by our observation that, unlike the functionally redundant situation in *S. cerevisiae* with Ctr1 and Ctr3, deletion of the *S. pombe ctr4*$^+$ gene (*ctr4*Δ) is sufficient to confer a copper starvation phenotype. This would predict that inactivation of the mouse homologue of Ctr1, if a single copy gene, would be likely to generate a copper deficiency.

It has been well established that iron uptake and mobilization in mammals is tightly linked to copper availability (65, 66). Indeed, swine suffering from copper malnutrition exhibit several abnormalities in iron metabolism including the inability to absorb, mobilize, and utilize administered iron, and were diagnosed in an early study as anemic (67, 68). Moreover, failure to incorporate copper ions into the copper-containing ferroxidase ceruloplasmin gives rise in an unstable protein and a loss of the ferroxidase activity, which is essential for iron mobilization (69). Furthermore, a recent study has revealed the existence of a putative copper-dependent ferroxidase, hephaestin, which would transfer iron from the intestinal mucosal cells through the baso-lateral membrane into the blood stream in concert with other component(s), such as a putative iron transporter (9). The requirement for copper in iron transport has also been biochemically characterized in some mammalian cell lines (70). Recent studies in *S. cerevisiae* have uncovered the mechanisms that underlie this dependence, through the isolation and analysis of genes encoding high affinity copper transporters (Ctr1 and Ctr3), the intracellular copper chaperone Atx1, a TGN localized P-type ATPase copper pump (Ccc2), a chloride channel that facilitates copper loading onto Fet3 (Gef1) (71–73), and two subunits of the high affinity iron uptake complex (the Fet3 multi-copper ferroxidase and the Ftr1 iron permease). The high affinity copper transporters are essential to mediate the passage of copper ions across the plasma membrane, with mobilization of copper to the Ccc2 P-type ATPase by the Atx1 copper chaperone, whereby it is delivered to the lumen of the secretory compartment through the action of Ccc2. Within the late secretory pathway, four copper atoms are assembled with Fet3 (74, 75) with the assistance of the Gef1 chloride channel. The failure to incorporate copper into Fet3 is thought to lead to a misfolded and enzymatically inactive ferroxidase, which prevents the Fet3-Ftr1 high affinity iron transport complex from assembling at the plasma membrane, resulting in iron deficiency. Therefore, it is clear that copper must be provided for the iron transport/mobilization machinery to function. Previously, it was established that the *ATX1* copper chaperone gene is regulated by intracellular iron availability by the Aft1 iron sensor. In this work, we have demonstrated that at least three genes involved in iron uptake in *S. pombe*, and the *FET3* gene in *S. cerevisiae*, are transcriptionally regulated by the copper-sensing transcription factors Cuf1 and Mac1, respectively. Our data support a direct role for Cuf1 in repression of these genes since either inactivation of the *cuf1*$^+$ gene, or mutation of Cuf1 binding sites in the *fio1*$^+$-*fip1*$^+$ promoter region, gave a marked de-repression of the *fio1*$^+$-*fip1*$^+$ gene expression. We suggest based on these data and on *in vitro* DNA binding experiments, that Cuf1 represses *frp1*$^+$, *fio1*$^+$, *fip1*$^+$, and possibly other genes involved in iron uptake/mobilization, under conditions of copper scarcity, by binding to TTTGTC elements with their respective promoters. When copper is present at higher levels, Cuf1 de-represses gene expression through copper-sensing mechanisms that inactivate DNA binding and perhaps via other intramolecular conformational changes. This model is consistent with the demonstrated copper-responsive DNA binding of Cuf1 *in vitro* and Mac1 *in vivo* (28), and the marked repression of *FET3* expression by the *S. cerevisiae* Mac1^{up1} protein, known to bind DNA *in vivo* even in the presence of copper. Since the iron uptake genes are inducibly expressed in response to the iron chelator BPS, even under conditions of copper scarcity, we speculate that there may be functional interactions between the copper- and iron-sensing transcription factors. Further studies will elucidate how Cuf1 and Mac1 repress expression of the iron uptake genes and how these copper-responsive transcription factors may interact with iron-sensing transcription factors.

The regulation of iron uptake genes in both *S. cerevisiae* and *S. pombe* by copper-sensing transcription factors provides a means of tightly coordinating and fine tuning iron transport with respect to copper availability. Since the Fet3 multi-copper ferroxidase is a key component of the high affinity iron transport complex at the plasma membrane, futile expression of the iron transport genes, in the absence of sufficient concentrations of the copper co-factor, would be undesirable. Yeast cells have therefore evolved both repressive and activator functions for copper-sensing transcription factors to maintain the delicate balance of these two essential, yet toxic metal ions.

Acknowledgments—We greatly appreciate the critical reading of this manuscript by members of the Thiele laboratory. We are grateful to Jerry Kaplan for the expression vectors containing the *fio1*$^+$ and *fip1*$^+$ genes. We are indebted to Susan Forsburg and Christian Fankhauser for the *S. pombe* strain FY254 and the plasmid pCF83, respectively. We are grateful to Keith Koch for the *S. cerevisiae ace1*Δ strain KKY17. We thank Kun-Liang Guan for providing fission yeast strains. We also thank Bart Barrell, Rhian Gwilliam, and Valerie Wood for the cosmid c31A2 and helpful information regarding the Sanger data base.

REFERENCES

1. Eide, D. J. (1998) *Annu. Rev. Nutr.* **18,** 441–469
2. Andrews, N. C., Fleming, M. D., and Gunshin, H. (1999) *Nutr. Rev.* **57,** 114–123
3. Peña, M. M. O., Lee, J., and Thiele, D. J. (1999) *J. Nutr.* **129,** 1251–1260
4. Radisky, D., and Kaplan, J. (1999) *J. Biol. Chem.* **274,** 4481–4484
5. Santoro, N., and Thiele, D. J. (1997) in *Yeast Stress Responses*, eds. Hohmann, S., and Mager, W. H. (Springer-Verlag, Heidelberg), pp. 171–211
6. Askwith, C., and Kaplan, J. (1998) *Trends Biochem. Sci.* **23,** 135–138
7. Dancis, A. (1998) *J. Pediatr.* **132,** S24–29
8. Anderson, G. J., and Powell, L. W. (1999) *Lancet* **353,** 2089–2090
9. Vulpe, C. D., Kuo, Y. M., Murphy, T. L., Cowley, L., Askwith, C., Libina, N., Gitschier, J., and Anderson, G. J. (1999) *Nat. Genet.* **21,** 195–199
10. Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G., and Klausner, R. D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3869–3873
11. Georgatsou, E., and Alexandraki, D. (1994) *Mol. Cell. Biol.* **14,** 3065–3073
12. Hassett, R., and Kosman, D. J. (1995) *J. Biol. Chem.* **270,** 128–134
13. Martins, L. J., Jensen, L. T., Simons, J. R., Keller, G. L., and Winge, D. R. (1998) *J. Biol. Chem.* **273,** 23716–23721
14. Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Elde, D., Moehle, C., Kaplan, J., and Klausner, R. D. (1994) *Cell* **76,** 393–402
15. Knight, S. A., Labbé, S., Kwon, L. F., Kosman, D. J., and Thiele, D. J. (1996) *Genes Dev.* **10,** 1917–1929
16. Lin, S.-J., Pufahl, R. A., Dancis, A., O'Halloran, T. V., and Culotta, V. C. (1997) *J. Biol. Chem.* **272,** 9215–9220
17. Pufahl, R. A., Singer, C. P., Peariso, K. L., Lin, S. J., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E., and O'Halloran, T. V. (1997) *Science* **278,** 853–856
18. Culotta, V. C., Klomp, L. W. J., Strain, J., Casareno, R. L. B., Krems, B., and Gitlin, J. D. (1997) *J. Biol. Chem.* **272,** 23469–23472
19. Glerum, D. M., Shtanko, A., and Tzagoloff, A. (1996) *J. Biol. Chem.* **271,** 14504–14509
20. Yuan, D. S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., and Klausner, R. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 2632–2636
21. Bull, P. C., and Cox, D. W. (1994) *Trends Genet.* **10,** 246–252
22. Solioz, M., and Vulpe, C. (1996) *Trends Biochem. Sci.* **21,** 237–241
23. DiDonato, M., and Sarkar, B. (1997) *Biochim. Biophys. Acta* **1360,** 3–16
24. Petris, M. J., Mercer, J. F., and Camakaris, J. (1999) *Adv. Exp. Med. Biol.* **448,** 53–66
25. Schaefer, M., and Gitlin, J. D. (1999) *Am. J. Physiol.* **276,** G311–314
26. Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., and Kaplan, J. (1994) *Cell* **76,** 403–410
27. Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D., and Dancis, A. (1996) *Science* **271,** 1552–1557
28. Labbé, S., Zhu, Z., and Thiele, D. J. (1997) *J. Biol. Chem.* **272,** 15951–15958
29. Yamaguchi-Iwai, Y., Serpe, M., Haile, D., Yang, W., Kosman, D. J., Klausner, R. D., and Dancis, A. (1997) *J. Biol. Chem.* **272,** 17711–17718
30. Jensen, L. T., Posewitz, M. C., Srinivasan, C., and Winge, D. R. (1998) *J. Biol. Chem.* **273,** 23805–23811
31. Jensen, L. T., and Winge, D. R. (1998) *EMBO J.* **17,** 5400–5408
32. Jungmann, J., Reins, H. A., Lee, J., Romeo, A., Hassett, R., Kosman, D., and Jentsch, S. (1993) *EMBO J.* **12,** 5051–5056
33. Turner, R. B., Smith, D. L., Zawrotny, M. E., Summers, M. F., Posewitz, M. C., and Winge, D. R. (1998) *Nat. Struct. Biol.* **5,** 551–555
34. Graden, J. A., and Winge, D. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5550–5555
35. Zhu, Z., Labbé, S., Peña, M. M. O., and Thiele, D. J. (1998) *J. Biol. Chem.* **273,** 1277–1280
36. Yamaguchi-Iwai, Y., Dancis, A., and Klausner, R. D. (1995) *EMBO J.* **14,** 1231–1239
37. Yamaguchi-Iwai, Y., Stearman, R., Dancis, A., and Klausner, R. D. (1996) *EMBO J.* **15,** 3377–84
38. Shiozaki, K., and Russell, P. (1996) *Genes Dev.* **10,** 2276–2288
39. Wilkinson, M. G., Samuels, M., Takeda, T., Toone, W. M., Shieh, J. C., Toda, T., Millar, J. B., and Jones, N. (1996) *Genes Dev.* **10,** 2289–2301
40. Giga-Hama, Y. (1997) in *Fission yeast Schizosaccharomyces pombe: an attractive host for heterologous protein production*, eds. Giga-Hama, Y., and Kumagai, H. (Springer-Verlag, Heidelberg), pp. 3–28
41. Roman, D. G., Dancis, A., Anderson, G. J., and Klausner, R. D. (1993) *Mol. Cell. Biol.* **13,** 4342–4350
42. Anderson, G. J., Dancis, A., Roman, D. G., and Klausner, R. D. (1994) *Adv. Exp. Med. Biol.* **356,** 81–89
43. Askwith, C., and Kaplan, J. (1997) *J. Biol. Chem.* **272,** 401–405
44. Koch, K. A., Peña, M. M. O., and Thiele, D. J. (1997) *Chem. Biol.* **4,** 549–560
45. Winge, D. R. (1998) *Prog. Nucleic Acids Res. Mol. Biol.* **58,** 165–195
46. Barbet, N., Muriel, W. J., and Carr, A. M. (1992) *Gene* **114,** 59–66
47. Cottarel, G., Beach, D., and Deuschle, U. (1993) *Curr. Genet.* **23,** 547–548
48. Koch, K. A., and Thiele, D. J. (1996) *Mol. Cell. Biol.* **16,** 724–734
49. Thiele, D. J., Walling, M. J., and Hamer, D. H. (1986) *Science* **231,** 854–856
50. Tamai, K. T., Gralla, E. B., Ellerby, L. M., Valentine, J. S., and Thiele, D. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8013–8017
51. Moreno, S., Klar, A., and Nurse, P. (1991) *Methods Enzymol.* **194,** 795–823
52. Kim, J., and Hirsch, J. P. (1998) *Genetics* **149,** 795–805
53. Morano, K. A., Santoro, N., Koch, K. A., and Thiele, D. J. (1999) *Mol. Cell. Biol.* **19,** 402–411
54. Alfa, C., Fantes, P., Hyams, J., McLeod, M., and Warbrick, E. (1993) in *Experiments with Fission Yeasts: Laboratory Course Manual.* Cold Spring Harbor Laboratory Press (Cold Spring Harbor, New York), pp. 1–186
55. Liu, X. D., Liu, P. C., Santoro, N., and Thiele, D. J. (1997) *EMBO J.* **16,** 6466–6477
56. Hayles, J., and Nurse, P. (1992) *Annu. Rev. Genet.* **26,** 373–402
57. Chang, F., and Nurse, P. (1996) *Cell* **84,** 191–194
58. Fürst, P., Hu, S., Hackett, R., and Hamer, D. (1988) *Cell* **55,** 705–717
59. Thiele, D. J. (1988) *Mol. Cell. Biol.* **8,** 2745–2752
60. Zhou, P. B., and Thiele, D. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6112–6116
61. Grimm, C., Kohli, J., Murray, J., and Maundrell, K. (1988) *Mol. Gen. Genet.* **215,** 81–86
62. Zhou, B., and Gitschier, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7481–7486
63. Osiewacz, H. D., and Nuber, U. (1996) *Mol. Gen. Genet.* **252,** 115–124
64. Borghouts, C., and Osiewacz, H. D. (1998) *Mol. Gen. Genet.* **260,** 492–502
65. Kaplan, J., and O'Halloran, T. V. (1996) *Science* **271,** 1510–1512
66. Winzerling, J. J., and Law, J. H. (1997) *Annu. Rev. Nutr.* **17,** 501–526
67. Gubler, C. J., Lahey, M. E., Chase, M. S., Cartwright, G. E., and Wintrobe, M. M. (1952) *Blood* **7,** 1075–1092
68. Lee, G. R., Nacht, S., Lukens, J. N., and Cartwright, G. E. (1968) *J. Clin. Invest.* **47,** 2058–2069
69. Harris, Z. L., and Gitlin, J. D. (1996) *Am. J. Clin. Nutr.* **63,** 836S-841S
70. Yu, J., and Wessling-Resnick, M. (1998) *J. Biol. Chem.* **273,** 6909–6915
71. Greene, J. R., Brown, N. H., DiDomenico, B. J., Kaplan, J., and Eide, D. J. (1993) *Mol. Gen. Genet.* **241,** 542–553
72. Davis-Kaplan, S. R., Askwith, C. C., Bengtzen, A. C., Radisky, D., and Kaplan, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13641–13645
73. Gaxiola, R. A., Yuan, D. S., Klausner, R. D., and Fink, G. R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4046–4050
74. De Silva, D. M., Askwith, C. C., Eide, D., and Kaplan, J. (1995) *J. Biol. Chem.* **270,** 1098–1101
75. Hassett, R. F., Yuan, D. S., and Kosman, D. J. (1998) *J. Biol. Chem.* **273,** 23274–23282