A Copper-sensing Transcription Factor Regulates Iron Uptake Genes in *Schizosaccharomyces pombe*\(^*\)

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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-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’-TGGTC(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.

\(^1\) The abbreviations used are: CuRE, copper-response element; BCS, bathecuproindisulfonate; 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 metallo-thionein 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<sup>5</sup> 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 Ah1 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 Ah1, 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 Fc reductases from S. cerevisiae and the mammalian gp91<sub>phox</sub> subunit of cytochrome b<sub>558</sub> is required at the cell surface to reduce Fe<sup>3+</sup> (41, 42). Once reduced, Fe<sup>2+</sup> is taken up by a permease/oxidase complex, Fpl1/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 Cuf1 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<sup>+</sup> 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<sup>c</sup> can1-1 leu1-32 ade6-M210 ura4-D18), the cuf1<sup>Δ</sup> disruption strain SPY1 (h<sup>c</sup> can1-1 leu1-32 ade6-M210 ura4-D18 cuf1<sup>Δ</sup> ura4<sup>Δ</sup>), and the cuf1<sup>Δ</sup> disruption strain MPY2 (h<sup>c</sup> can1-1 leu1-32 ade6-M210 ura4-D18 cuf1<sup>Δ</sup> ura4<sup>Δ</sup>), and the cuf1<sup>Δ</sup> disruption strain MY4 (h<sup>c</sup> can1-1 leu1-32 ade6-M210 ura4-D18 cuf1<sup>Δ</sup> ura4<sup>Δ</sup>). The functional ura4<sup>Δ</sup> cassette for gene disruption was isolated by PCR from pUR18 (46). The puc1<sup>Δ</sup>urad<sup>Δ</sup> disruption plasmid was constructed by the insertion of the BspEII-BglII ura4<sup>Δ</sup> cassette, thereby replacing the complete cuf1<sup>Δ</sup> ORF by the urad<sup>Δ</sup> gene and leaving 388 and 476 bp each side of the cuf1<sup>Δ</sup> locus for homologous recombination. The pctr4<sup>Δ</sup>urad<sup>Δ</sup> disruption plasmid was made by ligating a 499-bp Atp7I-BspEI fragment of the 5′ region of ctr4<sup>+</sup> and a 660-bp BglII-NotI 3′-flanking fragment of ctr4<sup>+</sup> to 5′ and 3′ ends of urad<sup>Δ</sup>.

**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 yeast copper homeostatic transcription factor and high affinity copper transporter. In S. pombe, the cuf1<sup>Δ</sup> disruption plasmid was constructed by the insertion of the ORF SPE31A2.11c found in S. pombe chromosome I cosmid c11A2. The ctr4<sup>Δ</sup> gene was discovered corresponding to the ORF SPC1393.10 found in S. pombe chromosome III cosmid c1393. From the cosmid c11A2, we performed a series of subcloning experiments using Smal and BamHI to obtain a 4.2-kilobase pair genomic fragment encompassing the cuf1<sup>Δ</sup> gene. This large fragment was isolated and ligated into the pUC18 plasmid, giving rise to pSPcuf1 S-B/4.2. To generate pSpcetr4<sup>Δ</sup>B-E/1.6, primers were made corresponding to the position -737 with respect to A of the ATG of the ctr4<sup>Δ</sup> 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 <sup>32</sup>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<sup>Δ</sup>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 bluescript SK<sup>+</sup>. To generate the pSK<sup>Δ</sup>plasmid, a 151-bp DNA fragment, which hybridizes to the S. pombe act1<sup>Δ</sup> gene positions +507 to +658, was also amplified and cloned into the bluescript SK<sup>+</sup> vector. The plasmid pSkScwZ used was described previously (28).

**SOD Activity Assay**—The S. cerevisiae isogenic strains 50.L4 (MATa gal1 trpl-1 his leu2-3,112 ura3-50) and DTY116 (MATa gal1 trpl-1 his leu2-3,112 ura3-50 sod1<sup>Δ</sup>Trp1<sup>Δ</sup>) were grown in YPD, while the S. pombe isogenic strains FY254 and SPY1 in YES media (51). SOD activity was measured at 30°C and the absorbance was conducted 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 stained with 2.5 mM nitroblue 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 pSPcuf1<sup>Δ</sup>GFP, an insert delineated by the NruI site located at position 977 with respect to A of the ATG of the cuf1<sup>Δ</sup> gene and the stop codon including a NotI site was isolated. The fragment was swapped for an identical DNA region, except for the NotI site, comprising the pSPcuf1<sup>Δ</sup>S-B/4.2 plasmid, creating pSPcuf1<sup>Δ</sup>NotI S-B/4.2. Subsequently, the gfp gene on a 750-bp NotI fragment (52) was cloned into pSPcuf1<sup>Δ</sup>NotI S-B/4.2. A similar strategy was utilized for generating the pSPctr4<sup>Δ</sup>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 (cuf1Δ), expressing either cuf1<sup>Δ</sup> or cuf1<sup>+</sup> 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. Cells 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<sub>2</sub>, 1 mM EDTA, 2.5 mM ZnSO<sub>4</sub>, 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 a<sup>32</sup>P-labeled fio1<sup>Δ</sup> 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 (39).

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 metabolism (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 Ace1Amt1 class, as well as the Mac1 class of copper metalloregulatory transcription factors.

To understand the role of Cuf1 in S. pombe, the cuf1p’ locus was insertionally inactivated by deletion and replacement with the S. pombe ura4 gene (61). cuf1p’ mRNA was found to be moderately abundant in wild type cells and not regulated by copper addition or starvation, and absent in cuf1Δ cells as determined by RNA blotting (Fig. 2A). Although we detected no obvious defect in copper resistance in cuf1Δ cells as compared with isogenic cuf1’ wild type cells (data not shown), cuf1Δ 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. 2B). The glycerol growth defect could be corrected either by returning a wild type copy of the cuf1p’ gene expressed from a plasmid, or by the addition of CuSO4 to the growth medium at concentrations of at least 25 μM (Fig. 2B). To test whether cuf1Δ 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 cuf1Δ cells grown either under control conditions or in the presence of 100 μM CuSO4. Although copper/zinc SOD activity was detected in cuf1Δ cells under both conditions, this activity was detected only in cuf1Δ cells supplemented with CuSO4 (Fig. 2C). Moreover, cuf1Δ deletion cells are defective in growth under iron starvation conditions (Fig. 2D), 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 (ctr4Δ), encoding a high affinity copper permease in fission yeast, as described below (Fig. 2D). Taken together, the observed defects in respiration, copper/zinc SOD activity, and iron acquisition indicate that cuf1Δ 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 cuf1GFP fusion allele was constructed that fully complements the cuf1Δ glycerol growth defect (Fig. 3A). As shown in Fig. 3B, the Cuf1-GFP fusion protein localizes to the S. pombe nucleus as

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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 observed in the disruption strain (cuf1Δ). Without Cuf1 protein, the cells were not able to grow on medium containing the iron chelator ferrozine (250 μM) or a plasmid-borne copy (cuf1Δ + pSP1cuf1+) 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 cuf1Δ allele displays a deficiency in copper/zinc SOD1 activity (right side), unless exogenous CuSO4 (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 sod1Δ 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).

Intersection between Iron and Copper Homeostatic Pathways

**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 CuSO4 (I, and 100 μM) or BCS (100 μM). No cuf1+ transcript was observed in the disruption strain (cuf1Δ). Bottom panel shows ethidium bromide-stained ribosomal RNAs as a control. **B**, cuf1Δ cells are defective for respiratory growth. The growth is remediated by exogenous CuSO4 (25 μM) or a plasmid-borne copy (cuf1Δ + pSP1cuf1+) 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 cuf1Δ allele displays a deficiency in copper/zinc SOD1 activity (right side), unless exogenous CuSO4 (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 sod1Δ 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).

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 (ctr4Δ) 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. 4B). Second, a functional Ctr4-GFP fusion protein is localized to the S. pombe plasma membrane (Fig. 4, C and D). Third, ctr4Δ strain cells are defective in iron transport (Fig. 2D). 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. 5A). Taken together, these data are consistent with Ctr4 functioning as a transcription factor required for expression of genes involved in 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. 4A). 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-X2-Met-X2-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 carboxy-terminal 65% of the protein, with very high identity within the two regions which encompass putative transmembrane domains (Fig. 4A). 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.

**Fig. 3.** Cuf1 is a nuclear protein. **A**, the Cuf1-GFP fusion protein complements the cuf1Δ growth defect on nonfermentable carbon sources (glycerol) in a manner indistinguishable from the unaltered 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).
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. 5B, 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 *cuf1Δ* cells under all conditions (Fig. 5B). Taken together, the convergence of *cuf1Δ* and *ctr4Δ* 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 Fe³⁺ and Cu²⁺ 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 *cuf1Δ* cells grown under copper or iron starvation or replete conditions. As shown in Fig. 6A for wild type strains, under conditions of iron adequacy but low copper availability, very low levels of *frp1* mRNA are observed. In response to 100 μM CuSO₄, 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. 6B (left panel) *fip1* mRNA is also dramatically induced by 100 μM CuSO₄ and, as expected, induced by treatment with the iron chelator BPS. Remarkably, deletion of the *cuf1* gene (*cuf1Δ*) significantly elevated basal levels of *fip1* and *fio1* mRNA and eliminated further induction by 100 μM CuSO₄ (Fig. 6B, right panel). A slight (−2-fold) reduction of *fip1* and *fio1* mRNA levels was observed in the *cuf1Δ* strain in response to copper administra-
The *cuf1* gene is required for copper-dependent regulation of *ctr4* gene expression. A, strains FY254 (*ctr4*) and MPY2 (*ctr4Δ*) were treated with CuSO4 (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 (*cuf1Δ*) cultures were incubated in the absence (−) or presence of CuSO4 (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.

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 CuSO4 (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 by arrows. B, cultures from strains FY254 (*cuf1*) and SPY1 (*cuf1Δ*) 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 *frp1*, *fio1*, and *act1* (denoted by arrows) in regard to the *cuf1* allele status.
DNA binding experiments were conducted using extracts derived from <i>S. pombe</i> <i>cuf1</i><sup>Δ</sup> wild type and <i>cuf1Δ</i> cells, and from <i>cuf1Δ</i> cells expressing a functional Cuf1-GFP fusion protein. As shown in Fig. 8A by a representative electrophoretic mobility shift assay, the wild type 271-bp <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> promoter fragment forms a strong protein-DNA complex when extracts are prepared from <i>cuf1</i><sup>Δ</sup> wild type cells grown under copper starvation conditions (BCS) and this complex is diminished to background levels when cells are grown in the presence of 100 μM CuSO<sub>4</sub>, prior to extract preparation. Furthermore, a complex of slowly 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 anti-

**Fig. 7.** The <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> promoter TTTGTC elements confer copper starvation-mediated repression to the minimal promoter <i>CYC1-lacZ</i>. A, schematic representation of the <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> 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 <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> ORF. The four upper boxes (□) shown in the common promoter region indicate that the TTTGTC elements are oriented toward the <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> gene, while the two lower boxes depict the elements oriented toward the <i>cuf1</i>-<i>Δ</i> 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 <i>CYC1</i> gene fused to <i>lacZ</i>. B, <i>CYC-lacZ</i> fusions 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 into <i>FET3</i><sup>Δ</sup> strains and analyzed for the <i>lacZ</i> mRNA steady-state levels status. <i>cuf1Δ</i> 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 (<i>MAC1</i>), <i>mac1Δ</i>, and <i>MAC1<sup>Δ</sup></i> strains were treated for 1 h in the presence of CuSO<sub>4</sub> (0, 1, and 10 μM) or BCS (100 μM). Shown is an RNA blot of <i>FET3</i> and <i>ACT1</i> 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 32P-labeled 271-bp <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> promoter fragment used with whole cell extracts prepared from <i>SPY1</i> cells (<i>cuf1Δ</i>) expressing either the pSP1 vector alone (−), no Cuf1 (<i>cuf1Δ</i>), wild-type Cuf1 (<i>cuf1</i>), or Cuf1-GFP (<i>cuf1</i>-GFP) as their sole source of Cuf1. BCS-treated cells expressing either <i>cuf1</i><sup>Δ</sup> or <i>cuf1</i>-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 <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> 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).

**Fig. 8A.** Schematic representation of the 271-bp <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> 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 <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> ORF. The four upper boxes (□) shown in the common promoter region indicate that the TTTGTC elements are oriented toward the <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> gene, while the two lower boxes depict the elements oriented toward the <i>cuf1</i>-<i>Δ</i> 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 <i>CYC1</i> gene fused to <i>lacZ</i>. B, <i>CYC-lacZ</i> fusions 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 into <i>FET3</i><sup>Δ</sup> strains and analyzed for the <i>lacZ</i> mRNA steady-state levels status. <i>cuf1Δ</i> 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 (<i>MAC1</i>), <i>mac1Δ</i>, and <i>MAC1<sup>Δ</sup></i> strains were treated for 1 h in the presence of CuSO<sub>4</sub> (0, 1, and 10 μM) or BCS (100 μM). Shown is an RNA blot of <i>FET3</i> and <i>ACT1</i> mRNA steady-state levels indicated by arrows, respectively.

serum was added to the binding reactions in supershift experiments. As shown in Fig. 8A, the <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> 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 <i>S. pombe</i> Cuf1 protein, which activates copper transporter gene expression under conditions of low copper availability, directly represses the expression of the <i>flip</i><sup>1</sup>-<i>flip</i><sup>1</sup> and <i>frp1</i>-<i>Δ</i> iron uptake genes under these same conditions through the TTTGTC elements found within these promoters.

**DISCUSSION**

Our results in <i>S. pombe</i> 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 <i>S. cerevisiae</i> 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-
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. 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′-TTTGC(T/G)C(A/G)-3′) differ from the CuRE element consensus sequence found in the CTR1, CTR3, FRE1, and FRE7 genes in S. cerevisiae (5′-TTTGTC(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 (MAC1upp) 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-X₆-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. 4A). 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 cuf4+ gene (ctr4Δ) 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, the 5′-TTTGC(T/G)C(A/G)-3′ was sufficient to cause a specific mutation of the first repeated element in Mac1 expression by the Mac1up1 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 fip1+, 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 Mac1upp protein, known to bind DNA in vivo even in the presence of copper. Since the iron uptake genes are indubitably 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.
Intersection between Iron and Copper Homeostatic Pathways

The regulation of iron uptake genes in both S. cerevisiae and S. pombe by copper-sensing transcription factors provides a means to 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 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.

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