Aerobic organisms possess efficient systems for the transport of copper. This involves transporters that mediate the passage of copper across biological membranes to reach essential intracellular copper-requiring enzymes. In this report, we identify a new copper transporter in Schizosaccharomyces pombe, encoded by the ctr6\(^+\) gene. The transcription of ctr6\(^+\) is induced under copper-limiting conditions. This regulation is mediated by the cis-acting promoter element CuSE (copper-signaling element) through the copper-sensing transcription factor Cuf1. An S. pombe strain bearing a disrupted ctr6\(^\Delta\) allele displays a strong reduction of copper,zinc superoxide dismutase activity. When the ctr6\(^+\) gene is overexpressed from the thiamine-inducible nmt1\(^+\) promoter, the cells are unable to grow on medium containing exogenous copper. Surprisingly, this copper-sensing growth phenotype is not due to an increase of copper uptake at the cell surface. Instead, copper delivery across the plasma membrane is reduced. Consistently, this results in repressing ctr4\(^+\) gene expression. By using a functional ctr6\(^+\) epitope-tagged allele expressed under the control of its own promoter, we localize the Ctr6 protein on the membrane of vacuoles. Furthermore, we demonstrate that Ctr6 is an integral membrane protein that can trimerize. Moreover, we show that Ctr6 harbors a putative copper-binding Met-X-His-Cys-X-Met motif in the amino terminus, which is essential for its function. Our findings suggest that under conditions in which copper is scarce, Ctr6 is required as a means to mobilize stored copper from the vacuole to the cytosol.

Acquisition of copper is crucial for aerobic life, because this element is an essential component of enzymes of primary metabolism (1, 2). Despite this vital role, too much copper in the cell can be detrimental, because in the presence of oxygen, copper can catalyze the production of cell-damaging hydroxyl radicals (3, 4). To balance the need for copper and its potentially harmful effects, living organisms have developed various specialized pathways of copper transport and distribution (5–8).

The use of bakers' yeast Saccharomyces cerevisiae as a model organism has provided fundamental information of copper homeostasis in eukaryotic cells (6, 8–10). For high affinity copper transport into S. cerevisiae cells, Cu\(^{2+}\) is reduced to Cu\(^+\) by the Fre1 and Fre2 cell surface reductases (11–15). Following reduction, copper ions are specifically transported across the plasma membrane by two distinct transporters, Ctr1\(^1\) (16–18) and Ctr3 (19, 20). Ctr1 is characterized by the presence of eight copies of the consensus sequence Met-X\(_3\)-Met-X-Met in its amino-terminal extracellular domain (16, 17), whereas Ctr3 is rich in Cys residues with 11 cysteines found throughout the protein but lacks the Met-clustered motif. Although the eight Met-X\(_3\)-Met-X-Met motifs found in Ctr1 play an important role in copper uptake when cells are grown under copper starvation conditions, the last methionine (amino acid residue 127) of the Met-X\(_3\)-Met-X-Met motif 8 is essential for Ctr1 function (16). Likewise, a Met-X\(_3\)-Met motif (residues 256–260) within the second transmembrane domain of Ctr1 was also identified as essential for copper transport (16). Despite the fact that the S. cerevisiae Ctr3 protein exhibits a limited overall sequence homology to Ctr1, it has been demonstrated that Ctr3 bears a similar Met-X\(_3\)-Met motif (residues 185–189) within its second transmembrane domain (16). This enables Ctr3 to transport copper across the plasma membrane in conjunction with other critical residues such as Cys-16 within the amino-terminal portion, Cys-48 and Cys-51 within the first transmembrane domain, and Cys-199 found into the third transmembrane domain of the protein (16, 19). Once inside the cell, free copper ions are virtually undetectable (21). In fact, copper ions are transiently associated with small copper-binding proteins, so-called copper chaperones, that possess the ability to distribute copper to specific intracellular destinations (22). To date, three distinct copper chaperones Atx1 (23, 24), CCS (also termed Lys\(_7\)) (25), and Cox17 (26–29) have been identified and found to deliver copper to the secretory compartment (into the Fet3 multicopper oxidase (30) via the intracellular copper transporter Ccc2 (31)), cytosolic copper,zinc-SOD1, and mitochondria (into the cytochrome c oxidase presumably with the aid of Sco1 (32–34) and Cox11 (35, 36) proteins), respectively. Consistent with their function in discrete pathways of intracellular...
fomer distribution, mutations in any one of the copper chaperone genes gives rise only to specific defects in its respective pathway (22). In addition to the high affinity copper transporter and copper chaperones, a gene denoted CTR2 (37) has been characterized that encodes a putative copper transporter located predominantly in the vacuolar membrane (38). Although Ctr2 may mobilize intracellular copper stores, its precise mechanism of action has not been ascertained.

The early molecular mechanisms of copper acquisition in Schizosaccharomyces pombe differ from those of S. cerevisiae. Two proteins, Ctr4 and Ctr5, form a two-component copper-transporting complex at the cell surface (39, 40). This association between Ctr4 and Ctr5 appears to be critical for protein maturation and secretion of the heteroprotein complex to the transporting complex at the cell surface (39, 40). This association between Ctr4 and Ctr5 appears to be critical for protein maturation and secretion of the heteroprotein complex to the transporting complex at the cell surface (39, 40). This association between Ctr4 and Ctr5 appears to be critical for protein maturation and secretion of the heteroprotein complex to the transporting complex at the cell surface (39, 40).

Two proteins, Ctr4 and Ctr5, form a two-component copper-dependent manner. A deletion of the cis-acting promoter element, denoted CuSE (Cu-signaling element) (43), results in a significant reduction of copper, zinc-SOD1 activity. Cells overexpressing ctr6Δ are unable to grow on medium containing exogenous copper. Surprisingly, this copper toxicity phenotype resulting from ctr6Δ overexpression is not due to an increase of copper uptake. Instead, the cell surface copper transporter activity is reduced, and consistently the steady-state levels of the ctr4Δ mRNA are diminished. By using a ctr6Δ-HA epitope-tagged allele, which retains wild-type function, we have localized Ctr6-HA to the vacuolar membrane when cells are grown under conditions of low copper availability. Interestingly, we show that the amino terminus of Ctr6 harbors a Met-XHis-Cys-X-Met-X-Met sequence, which is essential for its intracellular copper transport activity. Furthermore, we demonstrate that Ctr6 is an integral membrane protein, which can assemble into a homotrimeric complex. Taken together these results suggest that under copper scarcity, Ctr6 may serve to mobilize intravacuolar stores of copper in fission yeast.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions—** S. pombe strains used in this study were the wild type FY435 (h+ his7-366 leu1-32 ura4Δ18 ade6-M210) (43), the ctr6Δ (h+ his7-366 leu1-32 ura4Δ18 ade6-M210 cte6Δ::ura4Δ), and ctr6Δ ctr4Δ double mutant (h+ his7-366 leu1-32 ura4Δ18 ade6-M210 cte6Δ::hisG cte4Δ::ura4Δ) disruption strains. S. pombe cells were grown in yeast extract plus supplements or under selection in Edinburgh minimal medium (EMM) with necessary auxotrophic requirements (44). Liquid cultures of single colony purified yeast cells were grown to mid-logarithmic phase ( ÄOD600 of 1.0) at 30 °C, and copper starvation or copper repletion was carried out by adding the indicated amount of BCS or CuSO4 to the medium. After 2 h, samples were taken for mRNA analyses (49), three plasmids for making antisense RNA probes were utilized. The plasmids pSlocZ and pSRact1 were used previously (40, 50). The plasmid pSKctr6Δ was constructed by inserting a 213-bp BamHI-EcoRI fragment of the ctr6Δ cDNA into the same sites of the pBluescript II SK. The antisense RNA hybridizes to the first 173 ribonucleotides of the ctr6Δ transcript. To assess the ability of the Ctr6 copper transporter, the plasmid pSPctr6Δ-546lacZ containing the ctr6Δ promoter region up to −546 from the start codon of the ctr6Δ gene in addition to the Escherichia coli lacZ gene was created. The plasmid was constructed via three-piece ligation by simultaneously introducing the EcoRI-Stul fragment of Yepl57R (51) and the BamHI-EcoRI fragment from the ctr6Δ promoter containing 546-bp of the 5′-noncoding region and the first 11 codons of the ctr6Δ gene into the BamHI-SmaI cut pSP1 vector (52). Furthermore, the plasmid pSKctr6Δ-546 containing nucleotides from position −546 to position +53 with respect to the start codon of the ctr6Δ ORF was created to introduce mutations in the CuSEs (positions −210 to +201; positions −196 to −187) by site-directed mutagenesis. Precisely, the oligonucleotide 5′-caaaatggcattacagttgatattcagttgccgc-3′ (letters that are underlined represent multiple point mutations in the CuSEs) was used in conjunction with pSKctr6Δ-546 and the Chameleon mutagenesis kit (Stratagene, La Jolla, CA). The DNA sequence of the mutant promoter was verified by dideoxy sequencing and then used to replace the equivalent wild type ctr6Δ promoter in pSPctr6Δ-546.

**Disruption of the S. pombe ctr6Δ Gene—** A functional ura4Δ cassette was isolated from pUR18 (53) by PCR. The primers were designed to create Smal and BglII sites to the beginning and the end of the ura4Δ genetic marker, respectively. After digestion at these sites, the ura4Δ fragment was inserted to replace the ctr6Δ ORF, leaving 710 and 438 bp each side of the ctr6Δ locus for homologous recombination, creating pctr6Δ::ura4Δ. The gene disruption fragment (5′-ctgGur4Δ-3′) was generated by restriction endonuclease digestion using unique flanking sites (BanHI and Asp718) and then transformed into the S. pombe FY435 strain by electroporation (54). The allele status of the disrupted locus was verified using Southern blotting and diagnostic Asp718 and BamHI probe (49). Three plasmids for making antisense RNA probes were utilized. The plasmids pSlocZ and pSRact1 were used previously (40, 50). The plasmid pSKctr6Δ was constructed by inserting a 213-bp BamHI-EcoRI fragment of the ctr6Δ cDNA into the same sites of the pBluescript II SK. The antisense RNA hybridizes to the first 173 ribonucleotides of the ctr6Δ transcript. To assess the ability of the Ctr6 copper transporter, the plasmid pSPctr6Δ-546lacZ containing the ctr6Δ promoter region up to −546 from the start codon of the ctr6Δ gene in addition to the Escherichia coli lacZ gene was created. The plasmid was constructed via three-piece ligation by simultaneously introducing the EcoRI-Stul fragment of Yepl57R (51) and the BamHI-EcoRI fragment from the ctr6Δ promoter containing 546-bp of the 5′-noncoding region and the first 11 codons of the ctr6Δ gene into the BamHI-SmaI cut pSP1 vector (52). Furthermore, the plasmid pSKctr6Δ-546 containing nucleotides from position −546 to position +53 with respect to the start codon of the ctr6Δ ORF was created to introduce mutations in the CuSEs (positions −210 to +201; positions −196 to −187) by site-directed mutagenesis. Precisely, the oligonucleotide 5′-caaaatggcattacagttgatattcagttgccgc-3′ (letters that are underlined represent multiple point mutations in the CuSEs) was used in conjunction with pSKctr6Δ-546 and the Chameleon mutagenesis kit (Stratagene, La Jolla, CA). The DNA sequence of the mutant promoter was verified by dideoxy sequencing and then used to replace the equivalent wild type ctr6Δ promoter in pSPctr6Δ-546.

**SOD Enzymatic Activity and Sod1 Δ′ mRNA Analysis—** The S. pombe isogenic strains FY435 (wild type), DBY31 (ctr6Δ), and DBY11 (ctr6Δ ctr4Δ) were grown in yeast extract plus supplements (YES) medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium. Copper treatment of yeast strains was conducted as described previously (51). Once treated, cultures were divided in half and grown in liquid YES medium.
activity by standard in-gel assay with micro blue tetrazolium staining (40). Spectrophotometric determination of SOD activity was also performed from these protein extracts by measuring the inhibition of the reduction rate of cytochrome c by SOD, which acts as a reactive oxygen species produced from the xanthine-xanthine oxidase system (56). The other half of each cell of culture was stored at −80 °C until total RNA was extracted as described previously (50). For analysis of sod−1 gene expression by Northern blot, a 545-bp genomic DNA fragment from S. pombe FY435 was isolated by PCR using primers that corresponded to the start and stop codons of the sod−1 gene. The PCR product purification, 32P-labeling, and hybridization were performed as described above.

64Cu Uptake Measurements—S. pombe cells were grown to mid-log phase prior to uptake experiments. At A600 of 0.2, the cells were harvested and washed twice with citrate buffer (50 mM sodium citrate, pH 6.5, 5% glucose) as described previously (17). Radioactive copper (250 μCi/μg of 64Cu in the form of 64CuCl2, in 0.1 mM HCl) was produced at the 64Cu production facility at the Sherbrooke PET Center. 64CuCl2 was added to 2 ml of cells to a final concentration of 2 μM 64Cu Uptake Measurements. The cells were grown to early log phase. After no treatment or incubation in the presence of either CuSO4 (100 μM) or BCS (100 μM) for 9 h as described previously (47), the cells were fixed by adding formaldehyde (methanol-free) (Polysciences, War- rington, PA) to 3.7%. Fixed cells were harvested and washed with 0.1 M potassium phosphate, pH 6.5, containing 1.2 μM sorbitol. Cells were spheroplasted as the above-described procedure and adsorbed to poly- lysine-coated multwell slides. After a 30-min block with TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% bovine serum albumin), cells were incubated with anti-HA antibody (F-7) and anti-GST antibody (Z-5) diluted 1:200 in TBS. After a 2-h reaction, cells were washed with TBS and incubated for 1 h with the appropriate secondary antibodies as follows: goat anti-mouse Alexa-Red conjugate or goat anti-rabbit Alexa- Green conjugate (Molecular Probes, Eugene, OR) both diluted 1:500 in TBS. Cells were observed with an Olympus BX60 epifluorescent microscope (Olympus America, Melville, NY). To localize GST-Ptc4, S. pombe cells were co-transformed with pcr6− HA4 and pDS473a GST-pcd4−. To generate this latter plasmid, the pcd4− gene was isolated by PCR from S. pombe FY435 genomic DNA using primers that corresponded to the start and stop codons of the pcd4− ORF (62). The PCR product obtained was digested with BsmHI and SmalI and cloned into the corresponding sites of pDS473a vector (63).

RESULTS

Ctr6 Is a Putative Member of the Ctr Transporter Family—Protein data base searches from the S. pombe Genome Project (64) revealed an ORF (SPBC23G7.16) encoding a putative copper transporter related to the Ctr transporter family (1). This was supported by the following observations. First, the amino terminus of this putative transporter harbors a Met-X-His-Cys-X-Met motif (residues 9–14) that contains only one difference, which is a cysteine (fourth position) instead of a methionine residue to be identical to the Met-X2- Met-X-Met motifs identified in the Ctr transporter family as potential copper ion-binding motifs (1). Second, the Ctr4 (40) and Ctr5 (39) proteins that form a two-component copper transporting complex at the cell surface of S. pombe, the SPBC23G7.16-encoded protein contains three transmembrane regions according to TOP-PRED II analysis (65). Third, the overall sequence homology with the S. pombe Ctr4 (32% identity in 140-amino acid overlap) and Ctr5 (27% identity in 138-amino acid overlap) proteins was noteworthy, especially within the putative transmembrane spanning domains.
CuSEs play a role in copper transport proteins in fission yeast. To ascertain if the copper-specific transcriptional repression of the $\text{ctr6}^+$ gene was transcriptionally regulated by copper availability via the Cuf1 copper-sensing transcription factor, we ascertained whether the $\text{ctr6}^+$ gene was transcriptionally regulated by copper availability via the Cuf1 copper-sensing transcription factor. As shown in Fig. 2a, the $S.\text{pombe ctr6}^+$ mRNA expression in a wild type strain was repressed (~6-fold) when cells were exposed to 100 $\mu$M CuSO$_4$ and derepressed (~3-fold) as compared with the basal levels when cells were grown in the presence of the copper chelator BCS. Furthermore, using isogenic strains harboring a wild type $\text{cuf1}^+$ gene and an insertionally inactivated $\text{cuf1}$ allele, we found that the copper-dependent regulation of $\text{ctr6}^+$ mRNA required the copper sensor Cuf1 (Fig. 2b). Indeed, in the absence of Cuf1, although a low level of $\text{ctr6}^+$ mRNA was still observed, its expression was clearly unregulated by cellular copper status. Interestingly, within the $\text{ctr6}^+$ promoter region up to ~546 from the start codon of the $\text{ctr6}^+$ ORF, two copies of a repeated sequence, 5'-D(T/A)DDHGCCTGD-3' (D = A, G or T; H = A, C, or T), termed CuSE (42), were found at positions ~210 to ~201 and ~196 to ~187. It is important to note that CuF1 factor directly interacts with CuSEs to mediate transcriptional copper regulation of the $\text{ctr4}^+$ and $\text{ctr5}^+$ genes, which encode high affinity copper transport proteins in fission yeast. To ascertain if the CuSEs play a role in $\text{ctr6}^+$ regulation by copper, we fused 546-bp of the 5'-noncoding region and the first 11 codons of $\text{ctr6}^+$ in-frame with the E. coli lacZ gene. $\text{ctr6}^+$-lacZ expression from the reporter plasmid was down-regulated in the presence of copper (~7-fold) and up-regulated in the presence of BCS (~3-fold) (Fig. 3). When we inserted multiple point mutations that mimic changes known to abolish binding of Cuf1 to CuSEs in both elements within the $\text{ctr6}^+$ promoter, a low and constitutive basal level of expression was observed (Fig. 3). In fact, there was a complete lack of either down- or up-regulation of the $\text{ctr6}^+$-lacZ fusion. Taken together, these data show that...
ctr6\(^+\) is regulated at the transcriptional level through CuSEs in a copper- and CuF1-dependent manner.

Effects of Deletion and Overexpression of ctr6\(^+\) on Cell Growth and Regulation of Cell Surface Copper Transporter—To understand the role of Ctr6, we inactivated the ctr6\(^+\) locus by deletion and replacement with the S. pombe G418 resistance marker. Whereas the ctr6\(^\Delta\) mutant cells exhibited no obvious defect to use respiratory carbon sources (e.g. glycerol) or to grow on medium containing an iron chelator (e.g. BPS, ferrozine) (data not shown), the copper,zinc-SOD1 activity in ctr6\(^\Delta\) cells was strongly diminished as compared with wild type cells (Fig. 4, A and B). As observed for Ctr6, deletion of the ctr4\(^\Delta\) gene (ctr4\(^\Delta\)) dramatically lowered copper,zinc-SOD1 activity, whereas the ctr6\(^\Delta\) ctr4\(^\Delta\) double disruptant was devoid of measurable activity (Fig. 4, A and B). In all cases, loss of endogenous SOD1 activity was repaired to \(\sim 40\% - 85\%\) that of the wild type starting strain by the addition of exogenous copper (Fig. 4, A and B). Importantly, under low basal copper conditions (Fig. 4, C and D), the sod1\(^\Delta\) transcript levels remained virtually unchanged and clearly visible in all isogenic strains used, whereas under the same conditions, inactivation of the ctr6\(^\Delta\) and ctr4\(^\Delta\) genes resulted in an \(\sim 6\)- and \(\sim 19\)-fold reduction in SOD activity, respectively (Fig. 4B). These data clearly suggest a physiological and post-translational function for Ctr6 and Ctr4 in copper delivery to copper-zinc-SOD1. Although in S. pombe the sod1\(^\Delta\) mRNA expression was found to increase \(\sim 2\)-fold with the addition of exogenous CuSO\(_4\) to the growth medium, the effect of the disruptions (ctr6\(^\Delta\), ctr4\(^\Delta\), and ctr6\(^\Delta\) ctr4\(^\Delta\)) should be mainly considered under low copper conditions, because copper transport proteins become critical for cell function only under these conditions. Furthermore, the fact that the reduction in SOD activity in ctr6\(^\Delta\), ctr4\(^\Delta\), and ctr6\(^\Delta\) ctr4\(^\Delta\) strains is largely reversed by addition of exogenous copper clearly implicates Ctr6 and Ctr4 in copper metabolism.

Interestingly, when the ctr6\(^+\) gene was overexpressed from the thiamine-inducible nmt1\(^+\) promoter, the cells were hypersensitive to copper and unable to grow on medium containing 100 \(\mu\)M CuSO\(_4\) (Fig. 5A). Furthermore, this phenotype appeared to be highly copper-specific because among 10 different metal ions, CuSO\(_4\), AgNO\(_3\), HgCl\(_2\), CdCl\(_2\), FeCl\(_3\), NH\(_4\)Fe(SO\(_4\))\(_2\), CoCl\(_2\), Pb(C2H\(_3\)O\(_2\))\(_2\), MnCl\(_2\), and ZnCl\(_2\), tested at many concentrations, only copper and silver, a metal that is electronically similar to the reduced form of Cu\(^2\)+, gave rise to that growth defect (Fig. 5A and data not shown). To ascertain whether this copper toxicity phenotype resulting from ctr6\(^+\) overexpression was due to an increase of copper uptake, we measured \(^{64}\)Cu transport. Surprisingly, as shown in Fig. 5B, activation of the ctr6\(^+\) gene resulted in an \(\sim 60\% - 70\%\) reduction in the high affinity \(^{64}\)Cu transport. Consistently, in the ctr6\(^\Delta\) strain overexpressing the wild type ctr6\(^+\) gene, the steady-state levels of the ctr4\(^\Delta\) mRNA were strongly diminished \((\sim 12\)-fold) as compared with the levels observed in the same strain (ctr6\(^\Delta\)) harboring either the plasmid alone or a mutated ctr6 allele (Fig. 5C). This diminution of the ctr4\(^\Delta\) steady-state mRNA levels was particularly striking under
copper-limiting conditions (Fig. 5C, medium under copper-limiting conditions due to the presence of the copper chelator BCS, Fig. 5B). These data may suggest a copper re-distribution within the cell, perhaps because of a release of copper from intracellular organelle(s). To confirm that the copper toxicity phenotype was linked with the overexpression of \( \text{ctr}^{+} \), a mutant version of the gene was created. Precisely, site-directed mutagenesis was used to convert the methionine (Met-9) and histidine (His-11) codons to that encoding alanine. Although the \( \text{ctr}6^{+} \) mutant localized properly into vacuoles, \( \text{ctr}4^{+} \) was only found into the supernatant fraction. In the presence of Na\(_2\)CO\(_3\), and then re-fractionated at 100,000 g, no fluorescence was observed in cells expressing the Ctr6-HA\(_4\) fusion protein when overproduced.

Subcellular Location of Ctr6—To begin to ascertain the mechanism by which Ctr6 functions in copper mobilization in \( S. \text{pombe} \), we conducted experiments to determine the Ctr6 subcellular location. Ctr6 was tagged by inserting four tandem repeats of the HA epitope within a predicted hydrophilic loop region located between the first and second transmembrane domains of the protein. A \( \text{ctr}6^{+} \) mutant strain transformed with a plasmid harboring the \( \text{ctr}^{+} \)-HA\(_4\) gene gave rise to the above-mentioned phenotypes (Fig. 5) as observed for the wild type \( \text{ctr}^{+} \) gene, indicating that the Ctr6-HA\(_4\) protein is functional. This strain was grown without treatment or was incubated in the presence of either CuSO\(_4\) (100 \( \mu \text{M} \)) or BCS (100 \( \mu \text{M} \)). Protein extracts were prepared, and the Ctr6-HA\(_4\) fusion protein was enriched by immunoprecipitation using equal amounts of protein extract with anti-HA F-7 antibody. Immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting. As shown in Fig. 6A, a polypeptide species of \(-22\) kDa was detected, in keeping with the expected size of Ctr6-HA\(_4\) fusion protein (21.4 kDa) as predicted by its primary DNA sequence. Consistent with the regulation of \( \text{ctr}^{+} \) mRNA steady-state levels, the Ctr6-HA\(_4\) protein levels were dramatically reduced in cells grown in the presence of 100 \( \mu \text{M} \) CuSO\(_4\) (Fig. 6A).

The primary sequence of Ctr6 predicted that this protein was integrated into a cellular membrane. To investigate this, \( \text{ctr}^{+} \)-HA\(_4\) and \( \text{ctr}^{+} \)-FLAG\(_2\) fusion genes were co-transformed in a \( \text{ctr}6^{+} \) double mutant disruption strain. Whole cell extracts, prepared from cells grown under conditions of low copper availability, were subjected to ultracentrifugation at 100,000 \( \times \) g to collect membranes. The supernatant that contains soluble and detached peripheral membrane proteins was precipitated, washed with acetone, resuspended, and left untreated before analysis by Western blotting. The pellet fractions were resuspended and left untreated, or were adjusted to 0.2 M Na\(_2\)CO\(_3\) or 1% Triton X-100, and then re-fractionated at 100,000 \( \times \) g. As shown in Fig. 6B, in the absence of treatment, or in the presence of Na\(_2\)CO\(_3\), which dissociates peripheral but not integral membrane proteins from the membrane, Ctr6-HA\(_4\) and Ctr4-FLAG\(_2\) proteins were not detected into the supernatant fractions but only in the pellet fractions. Conversely, the PCNA protein, which is soluble, was only found into the supernatant fraction. In the presence of Triton X-100, a nonionic detergent that solubilizes membranes, both Ctr6-HA\(_4\) and Ctr4-FLAG\(_2\) proteins were detected in the pellet and supernatant fractions, implying that Ctr6-HA\(_4\) is an integral membrane protein as shown previously for Ctr4-FLAG\(_2\) (39) and reproduced here as a control.

To determine the cellular location of Ctr6-HA\(_4\), indirect immunofluorescence microscopy was carried out using anti-HA antibody. When \( S. \text{pombe} \) cells expressing the Ctr6-HA\(_4\) fusion protein were grown under copper starvation conditions, Ctr6-HA\(_4\) fluorescence appeared to localize in vacuole membranes (Fig. 7A). These organelles around which Ctr6-HA\(_4\) was detected appear as indentations by Nomarski optics (Fig. 7A, DIC). Conveniently, when \( \text{ctr}^{+} \)-HA\(_4\) was induced by copper removal, the number and size of the vacuoles decreased and became bigger, respectively, as a consequence of nutrient limitation (data not shown) (62), facilitating the Ctr6-HA\(_4\) localization. Importantly, the fluorescence was absent when \( \text{ctr}6^{+} \) mutant cells expressing the Ctr6-HA\(_4\) fusion protein were grown under copper-replete conditions (100 \( \mu \text{M} \) CuSO\(_4\)) (Fig. 7A). Furthermore, no fluorescence was observed in cells expressing the untagged \( \text{ctr}^{+} \) allele (data not shown). To further confirm the
Ctr6-HA₄ localization, cells were co-transformed with plasmids expressing both the Ctr6-HA₄ and GST-Ptc4 fusion proteins. The use of GST-Ptc4 fusion protein, which is known to localize to the vacuolar membrane (62), served as a positive control. As shown in Fig. 7B, double immunofluorescence labeling carried out with anti-HA and anti-GST antibodies revealed that Ctr6-HA₄ and GST-Ptc4 proteins were both visualized at the vacuolar membrane. Taken together, the copper-mediated repression of Ctr6-HA₄ protein levels, the integral membrane nature of Ctr6-HA₄, and the vacuolar membrane staining are suggestive of a mechanism whereby Ctr6 provides copper ions from the vacuole to cytosolic copper-requiring enzyme(s) when cells are grown under copper starvation conditions.

Identification of Amino-terminal Residues Necessary for Ctr6 Function—To gain insight into the mechanisms by which Ctr6 transport copper ions, we carried out a functional dissection of a potential metal-binding motif, Met-X-His-Cys-X-Met-X-Met (residues 9–16), within the amino-terminal region of Ctr6. Although a cysteine was found (fourth position) instead of a methionine to be identical to the Met-X₂-Met-X-Met motif identified in the Ctr transporter family as potential copper-binding motif, the chemical nature of cysteine with an external SH group may replace the methionine to coordinate copper. Recently, an elegant study (16) has demonstrated that a conserved methionine located 20 amino acid residues from the beginning of the first transmembrane domain in S. cerevisiae Ctr1 protein is essential for copper transport. Analogous to the situation described for Ctr1, the last methionine of the putative Met motif of Ctr6 was found 18 amino acid residues from the first transmembrane domain. Because of these observations, site-directed mutagenesis was used to convert codons encoding residues, which have the potential to bind copper, to codons encoding alanine (Fig. 8A). To assess the effects of these mutations on Ctr6 function, plasmids expressing the mutant proteins shown in Fig. 8A were transformed into an S. pombe ctr6Δ strain. As controls, subcellular localization was performed to ensure that the mutant proteins were produced and properly localized (Fig. 8B). For each mutant, we measured ⁶⁴Cu uptake. As shown in Fig. 8C, all three Ctr6 mutant proteins failed to diminish high affinity ⁶⁴Cu transport as compared with the reduction observed in the same strain (ctr6Δ) expressing wild type Ctr6. Furthermore, despite the fact that these mutant alleles were overexpressed in the ctr6Δ strain, the steady-state levels of ctr4⁻ mRNA were still robustly induced under copper deprivation conditions as opposed to the ctr4⁻ mRNA levels detected in the ctr6Δ mutant strain overexpressing the wild type ctr6⁺ allele (Fig. 8D). These results suggest that the methionine (Met-9, Met-14, and Met-16), histidine (His-11), and cysteine (Cys-12) residues, which compose the copper-binding motif, Met-X-His-Cys-X-Met (residues 9–16), within the amino-terminal region of Ctr6 are involved in the process of copper transport mediated by Ctr6. However, whether these
EGS, we noted that Ctr6-HA4 migrates as an ~22-kDa monomeric protein (Fig. 9), which is consistent with its predicted molecular mass of 21.4 kDa. As the EGS concentration was increased, the monomeric form of Ctr6-HA4 protein disappeared, with concomitant appearance of homodimeric (~44 kDa) and homotrimeric (~66 kDa) forms of Ctr6-HA4. Although only a very low level of Ctr6-HA4 homodimer was detectable, the Ctr6-HA4 homotrimer was clearly visible (Fig. 9). Taken together, these results strongly suggest that the Ctr6-HA4 protein forms a homotrimer as part of a copper transporter unit in the vacuolar membrane in fission yeast.

**DISCUSSION**

Because of their property to promptly gain and lose electrons, copper ions are redox-active co-factors that serve as catalytic centers of numerous proteins involved in a variety of essential enzymatic processes (2, 68). Despite this crucial role, copper ions, when present in excess, can have detrimental effects due to their proclivity to engage in redox reactions or by competing with other metal ions for enzyme-active sites (4, 69). Thus, distinct pathways have evolved for the signaling, transport, trafficking, and sequestration of copper ions within cells to keep the delicate balance between essential and toxic levels (70).

In this study, we identified a novel *S. pombe* copper-responsive gene, termed *ctr6*+, which encodes a vacuolar membrane transporter. Like the *ctr4*+ and *ctr5*+ genes encoding the high affinity copper heteromeric transport complex at the cell surface in *S. pombe* (39), *ctr6*+ is activated at the transcriptional level in response to copper limitation by the Cuf1 nutritional copper-sensing transcription factor through the CuSE recognition sequence. Based on this observation that *ctr6*+ is transcriptionally regulated by copper in the same direction as the genes encoding components of the high affinity copper uptake machinery suggests a function for Ctr6 in copper utilization as opposed to copper detoxification. Given the fact that genetic studies have implicated the vacuole as playing a role for copper storage (58, 71, 72), and assuming that vacuolar copper is present in a usable form, we envision Ctr6 as an intracellular transporter to mobilize stores of copper from the organelle, thereby representing a specialized pathway by which copper could be distributed within cells (Fig. 10). The proposed model is supported by the fact that a deletion of the *ctr6*+ gene (*ctr6Δ*) results in a significant reduction of copper,zinc-SOD1 activity, suggesting a role for *S. pombe* Ctr6 in delivering copper to cytosolic copper-dependent enzyme(s) under conditions of copper scarcity. Furthermore, when Ctr6 was overexpressed from the thiamine-inducible *nmt1*+ promoter, the cells exhibited a copper-sensitive growth phenotype, which was not attributable to an increase of copper uptake. Consistently, in response to the action of Ctr6, there was loss of Cuf1-dependent activation of the cell surface copper transporter *ctr4*+ gene expression, which represents an additional argument indicating the increased of copper cellular levels within the cell.

Because some membrane proteins that function in the secretory pathway (e.g. endoplasmic reticulum/Golgi apparatus) or at the plasma membrane may be mis-localized to the vacuole when overexpressed (73), for subcellular localization of Ctr6, we used a functional epitope-tagged *ctr6*+ allele that was expressed under the control of its own promoter. This latter system ensured low levels of *ctr6*+ gene expression. Moreover, localization of Ctr6 to the vacuolar membrane was also observed using the disruption strain (*ctr6Δ*) in which a *ctr6*+·HA4 allele was re-integrated. To examine further the localization of Ctr6, we compared the labeling pattern of Ctr6-HA4 to the GST-Ptc4 fusion protein known to function in the vacuolar membrane (62). Indirect immunofluorescence microscopy dem-

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2 D. R. Bellemare, L. Shaner, K. A. Morano, and S. Labbé, unpublished data.
Onstrated that Ctr6 localizes on the membrane of vacuoles in a manner identical to that observed for GST-Ptc4. Interestingly, similar subcellular localization has been reported recently (38) for the *S. cerevisiae* Ctr2 protein. Analogous to the situation for Ctr2, Ctr6 was visualized surrounding the vacuole with points of concentration of the protein around the organelle. This observation further supports a possible common role for these two proteins in the mobilization and transmission of intracellular pools of copper to metalloenzymes.

Complementary to immunofluorescence localization, subcellular fractionation experiments demonstrate that Ctr6 is an integral membrane protein that is undetectable in soluble fractions, unless cell extracts were supplemented with Triton X-100, a detergent that solubilizes membrane structures (19). As demonstrated for the *S. cerevisiae* Ctr3 (19) and human Ctr1 (74), EGS cross-linking experiments revealed that Ctr6 can assemble as a trimer. Importantly, the homo-multimeric state of Ctr6 may be required to form a functional translocation path, which contains, in general, 6 /H1006 3 and up to 12 /H1006 2 transmembrane domains within transport proteins (67). Nine transmembrane domains from three Ctr6 molecules could be sufficient to form a pore by which copper can be translocated from the vacuole into the cytoplasm. The oligomeric state may also play a role in other functions of Ctr6, including its stabilization into the membrane structure, or interaction with the cytosolic domain of delivering copper proteins.

Based on computer algorithm analysis, the amino-terminal 33 amino acids of Ctr6 are predicted to be inside the vacuole. Within this region of Ctr6 lies a putative copper coordination motif, Met-X-His-Cys-X-Met-X-Met (residues 9–16), that may function in copper capture within the vacuole. This is supported by the observation that mutations in which the methionine (Met-9) and histidine (His-11) or cysteine (Cys-12) and methionines (Met-14 and Met-16), or all five of these residues, were substituted to alanine altered copper transport activity of Ctr6. The methionine and histidine residues at positions 9 and 11, respectively, when mutated (mutant M1), gave rise to a stronger alteration with respect to Ctr6 activity compared with the mutant M2 in which the cysteine and methionine residues at position 12, 14, and 16 were mutated. However, whether one residue contributes more than another one in copper transport must await a fine mapping dissection of each amino acid that could play a role in the handling of copper. Similarly to the situation for the *S. cerevisiae* Ctr1 and Ctr3, and human Ctr1 (16), Ctr6 contains in its second transmembrane domain a conserved Met-X<sub>3</sub>-Met motif (residues 111–115). Although we have not ascertained its function, this Met-X<sub>3</sub>-Met motif may play a critical function in copper translocation across the vacuolar membrane.

In the presence of excess iron or copper ions, the vacuole has been proposed to play an important role to detoxify the cell, preventing their accumulation in the cytosol to toxic levels. Once inside the vacuole, perhaps, these metal ions could be bound under a bio-unavailable form as Fe<sup>3+</sup>/Cu<sup>2+</sup> to polyphosphates or other molecules. Conversely, when grown under copper starvation conditions, Ctr6 would mobilize stored copper from the vacuole to replenish the cytosol according to copper need. The similarity in the potency of silver in fostering the

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**Fig. 7.** Ctr6 localizes to the vacuolar membrane. *A*, *ctr6Δ* deletion strain expressing Ctr6-HA<sub>4</sub>, was grown to early logarithmic phase in Edinburgh minimal medium and incubated in the absence (−) or presence of BCS (100 μM) or CuSO<sub>4</sub> (100 μM). Cells were fixed, permeabilized, and labeled with anti-HA monoclonal antibody (Ab). DAPI staining visualized DNA and Nomarski microscopy was used to determine cell morphology. The indentations seen by Nomarski (DIC) represent the vacuoles. *B*, the vacuolar *S. pombe* Ptc4 (fused to GST without loss of function) (62) was expressed and viewed as a control.
copper-sensitive growth phenotype because of the expression of ctr6/H11001 and the electronic similarity of Ag/H11001 to Cu/H11001, but not Cu2/H11001, suggest that the intracellular copper transporter Ctr6 may pump Cu/H11001 rather than Cu2/H11001. This would suggest a role for a vacuolar membrane metalloreductase. So far, analysis of genomic DNA sequences from the S. pombe Genome project has revealed two open reading frames (SPBC1683.09C, denoted frp1/H11001, and SPBC947.05C) related to Cu2/H11001/Fe3/H11001 ion reductases found in S. cerevisiae. Although the frp1/H11001-encoded reductase can reduce Fe3/H11001 to Fe2/H11001 at the cell surface of fission yeast, its role in the metabolism of other metal ions (e.g. copper) is unknown. Regarding the second ORF, SPBC947.05C, its potential role in Fe3/H11001/Cu2/H11001 reductase activity is still uncharacterized. Finally, given the extended amino acid sequence homology between Ctr6 and all Ctr family members, especially within the regions that encompass the transmembrane do-

![FIG. 8. The amino-terminal Met-X-His-Cys-X-Met-X-Met motif is necessary for the copper transport activity of Ctr6. A, schematic representation of the Ctr6 protein tagged with four copies of the HA epitope. The primary sequence of the Met-X-His-Cys-X-Met-X-Met motif is shown below, and the putative copper-binding ligands are underlined. TM1-3, putative transmembrane domains. The amino acid numbers refer to the position relative to the first amino acid of the protein. The sequence of the mutations (M1, M2, and M3) in the Met-X-His-Cys-X-Met-X-Met motif are shown corresponding to the residues in the wild type Ctr6. B, representative cells from M1, M2, and M3 mutants of Ctr6. Cells from cultures grown in the presence of BCS (100 μM) were fixed, probed for the HA epitope, and viewed by epifluorescence. DAPI staining was used to determine the location of the nucleus. Shown are matched images of anti-HA-GAM-Alexa Red fluorescence and DAPI merged images. C, ctre Δ cells expressing the wild type ctre6-HA4 gene display a distinct 64Cu uptake rate to that observed with cells expressing ctre6-M1-HA4, ctre6-M2-HA4, and ctre6-M3-HA4 alleles. Cells were incubated with 2 μM 64Cu in citrate buffer (pH 6.5) (17) for 10 min. Copper uptake was quantitated and normalized to culture density and temperature-dependent transport. Error bars represent the S.D. for three independent experiments. D, ctre6 Δ strain, transformed with pctr6-HA4, pctr6-M1-HA4, pctr6-M2-HA4, and pctr6-M3-HA4, was grown under low copper conditions. Cultures were untreated or treated with CuSO4 (100 μM) or BCS (100 μM) for 1 h. Total RNA was prepared from culture aliquots. ctr4 and act1 mRNAs (arrows) were detected using RNase protection assays. Results shown are representative of three independent experiments.

![FIG. 9. Ctr6 multimerizes. Representative EGS cross-linking experiment of Triton X-100-solubilized cell lysates prepared from ctr6 Δ cells expressing Ctr6-HA4. After incubations with 0, 0.5, 1.0, 2.5, 3.0, and 5.0 mM EGS for 30 min at room temperature, the cross-linked complexes were immunoprecipitated, separated on 9% SDS-PAGE, and detected by immunoblotting. Monomeric (~22-kDa, 1 oval), dimeric (~44-kDa, 2 ovals), and trimeric (~66-kDa, 3 ovals) forms of Ctr6 were detected. M, reference marker.]
main, it will be interesting to determine what motif of the intracellular pool of labile copper, preventing futile expression of the ctr1 and ctr3 cell surface transport genes.

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REFERENCES

1. Puig, S., and Thiele, D. J. (2002) Curr. Opin. Chem. Biol. 6, 171–180
2. Peña, M. M. O., Lee, J., and Thiele, D. J. (1999) J. Biol. Chem. 274, 12531–12536
3. Halliwell, B., and Gutteridge, J. M. (1992) FEBS Lett. 307, 108–112
4. Halliwell, B., and Gutteridge, J. M. (1984) Biochem. J. 219, 1–14
5. O’Halloran, T. V., and Colotta, V. C. (2000) J. Biol. Chem. 275, 25057–25060
6. Labbé, S., and Thiele, D. J. (1999) Trends Microbio. 7, 500–505
7. Eide, D. J. (1999) Annu. Rev. Nutr. 18, 441–469
8. Radisky, D., and Kaplan, J. (1999) J. Biol. Chem. 274, 4481–4484
9. Eide, D. J. (2000) Adv. Microb. Physiol. 43, 1–38
10. Daniels, A. (1998) J. Pediatr. 132, S24–S29
11. Daniels, A., Kuasr, R. D., Hinnebusch, A. G., and Barriocanal, J. G. (1990) Mol. Cell. Biol. 10, 2284–2291
12. Daniels, A., Roman, D. O., Anderson, G. J., Hinnebusch, A. G., and Kuasr, R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3869–3873
13. Georgatou, E., and Alexandraki, D. (1994) Mol. Cell. Biol. 14, 3065–3073
14. Hassett, R., and Kosman, D. J. (1995) J. Biol. Chem. 270, 128–134
15. Marti, L. J., Jensen, L. T., Simons, J. R., Keller, G. L., and Winge, D. R. (1998) J. Biol. Chem. 273, 23716–23721
16. Puig, S., Lee, J., Lau, M., and Thiele, D. J. (2002) J. Biol. Chem. 277, 26201–26206
17. Danèse, A., Yuan, D., Thiele, D. J. (2000) J. Biol. Chem. 275, 33244–33251
18. Knight, S. A., Labbé, S., Kwon, L. F., Kosman, D. J., and Thiele, D. J. (1996) Genes Dev. 10, 1917–1929
19. Rae, T. D., Schmidt, P. J., Pufahl, R. A., Colotta, V. C., and O’Halloran, T. V. (1999) Science 285, 805–808
20. Huffman, D. L., and O’Halloran, T. V. (2001) Annu. Rev. Biochem. 70, 677–701
21. Pufahl, R. A., Singer, C. P., Pearsio, K. L., Lin, S. J., Schmidt, P. J., Fahrni, C. J., Colotta, V. C., Penner-Hahn, J. E., and O’Halloran, T. V. (1997) Science 278, 853–856
22. Lin, S. J., Pufahl, R. A., Dancis, A., O’Halloran, T. V., and Colotta, V. C. (1997) J. Biol. Chem. 272, 9210–9220
23. Colotta, V., Klomp, L. W., Strain, J., Casareno, R. L., Krems, B., and Giltin, J. D. (1997) J. Biol. Chem. 272, 23469–23472
24. Heaton, D. N., George, G. N., Garrison, G., and Winge, D. R. (2001) Biochem. Soc. 40, 743–751
25. Heaton, D., Nittis, T., Srinivasan, C., and Winge, D. R. (2000) J. Biol. Chem. 275, 37582–37587
26. Beers, J., Glerum, D. M., and Tsagoloff, A. (1997) J. Biol. Chem. 272, 33191–33196
27. Glerum, D. M., Shtanko, A., and Tsagoloff, A. (1996) J. Biol. Chem. 271, 14504–14509
28. Carr, H. S., George, G. N., and Winge, D. R. (2002) J. Biol. Chem. 277, 31237–31242
29. Fujii, H., Li, Valentini, M., Hamer, A. G., and Hosler, J. P. (2000) J. Biol. Chem. 275, 619–623
30. Kampfenkel, K., Kushnir, S., Babiyak, E., Inze, D., and Van Montagu, M. (1995) J. Biol. Chem. 270, 28479–28486
31. Pertony, M. E., Schmidt, P. J., Rogers, R. S., and Colotta, V. C. (2001) Mol. Genet. Genom. 265, 873–882
32. Zhou, H., and Thiele, D. J. (2001) J. Biol. Chem. 276, 20528–20535
33. Labbé, S., Peña, M. M. O., Fernandes, A. R., and Thiele, D. J. (1999) J. Biol. Chem. 274, 36252–36260
34. Labbé, S., Beaudoin, J., Bellemare, D. R., and Pelletier, B. (2002) in Handbook of Copper Pharmacology and Toxicology (Massaro, E. J., ed.) pp. 571–587, Humana Press Inc., Totowa, NY
35. Beaudoin, J., and Labbé, S. (2001) J. Biol. Chem. 276, 14572–14580
36. Beznania, M., Forsburg, S. L., and Pollard, T. D. (1997) Mol. Cell. Biol. 8, 2683–2695
37. Alfa, C., Fantin, P., Hyams, J. M., Meckord, W., and Warbrick, E. (1993) in Experiments with Fission Yeasts: Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
38. Forsburg, S. L. (1995) Nucleic Acids Res. 23, 2955–2956
39. Maundrell, K. (1993) Gene (Amst.) 123, 127–130
40. Bellemare, D. R., Sancaglini, M., Beaudoin, J., and Labbé, S. (2001) Gene (Amst.) 273, 191–198
41. Pelletier, B., Beaudoin, J., Mukai, Y., and Labbé, S. (2002) J. Biol. Chem. 277, 22850–22854
42. Keogh, K. A., and Thiele, D. J. (1996) Mol. Cell. Biol. 16, 2724–2728
43. Labbé, S., Zhu, Z., and Thiele, D. J. (1997) J. Biol. Chem. 272, 15951–15958
44. Myers, A. M., Tsagoloff, A., Kinney, D. M., and Lusty, C. J. (1996) Gene (Amst.) 175, 299–310
45. Cottarel, G., Beach, D., and Deuschle, U. (1993) Curr. Genet. 23, 547–548
46. Barbet, N., Muriel, W. J., and Carr, A. M. (1992) Gene (Amst.) 114, 59–66
47. Prentice, H. L. (1992) Nucleic Acids Res. 20, 621
48. Knight, S. A., Tamia, R. T., Kosman, D. J., and Thiele, D. J. (1994) Mol. Cell. Biol. 14, 7792–7804
49. Crapo, J. D., McCord, J. M., and Fridovich, I. (1978) Methods Enzymol. 53, 350–354
50. McNab, D. S., Pak, S. M., and Guarente, L. (1997) BioTechniques 22, 1134–1139
51. Ho, S. N., Hunt, H. D., Horten, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
52. Kolodziej, P. A., and Young, R. A. (1991) Methods Enzymol. 194, 508–519
53. Fission Yeast Vacuolar Copper Transporter Ctr6
**Ctr6, a Vacuolar Membrane Copper Transporter in** *Schizosaccharomyces pombe*

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