A widespread transposable element masks expression of a yeast copper transport gene

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The trace element copper (Cu) is essential for cell growth. In this report we describe the identification of a new component of the high-affinity Cu transport machinery in yeast, encoded by the *CTR3* gene. Ctr3p is a small intracellular cysteine-rich integral membrane protein that restores high-affinity Cu uptake, Cu, Zn superoxide dismutase activity, ferrous iron transport, and respiratory proficiency to strains lacking the *CTR1* (Cu transporter 1) gene. In most commonly used *Saccharomyces cerevisiae* laboratory strains, expression of *CTR3* is abolished by a Ty2 transposon insertion that separates the *CTR3* promoter from the transcriptional start sites by 6 kb. In strains that do not possess a Ty2 transposon at the *CTR3* locus, expression of *CTR3* is repressed by copper and activated by copper starvation. In such strains inactivation of both *CTR1* and *CTR3* is required to generate lethal copper-deficient phenotypes. Although Ctr1p and Ctr3p can function independently in copper transport, the expression of both proteins provides maximal copper uptake and growth rate under copper-limiting conditions. These results underscore the importance of mobile DNA elements in the alteration of gene function and phenotypic variation.

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The trace element copper (Cu) is an essential cellular redox component required for catalysis by a wide variety of enzymes such as Cu, Zn superoxide dismutase (Cu, Zn SOD), lysyl oxidase, cytochrome *c* oxidase, and dopamine β hydroxylase (Linder 1991). Ironically, the same redox-active nature of Cu renders it extremely cytotoxic, attributable to its ability to engage in chemical reactions that generate hydroxyl radicals. This radical causes lipid peroxidation, nucleic acid cleavage, and direct protein damage (Halliwell and Gutteridge 1984; Stadtman and Oliver 1991). Therefore, it is critical that all cells acquire sufficient Cu for essential biochemical reactions, yet prevent the accumulation of Cu to toxic levels.

Yeast cells have provided a tractable genetic system in which to identify and analyze components that make up the cellular Cu homeostatic machinery. In response to high levels of exogenous Cu, the baker's yeast *Saccharomyces cerevisiae* activates transcription of the metallothionein genes *CUP1* and *CRS5*, which encode low molecular weight, cysteine-rich, Cu-binding/detoxification proteins (Culotta et al. 1994; Zhu et al. 1995). Cu-responsive gene activation occurs through *cis*-acting Cu-responsive elements and the *trans*-acting Cu-activated

metalloregulatory transcription factor Acelp (Zhu et al. 1995). Under low environmental Cu conditions yeast cells accumulate Cu by the reduction of Cu (II) to Cu(I), catalyzed by the extracellular Fe(III)/Cu(II) reductases Fre1p and Fre2p, encoded by the *FRE1* and *FRE2* genes, respectively (Dancis et al. 1992; Georgatsou and Alexandraki 1994; Hassett and Kosman 1995). Cu enters the cell through a high-affinity transport system, an important component of which is encoded by the *CTR1* gene (Dancis et al. 1994b). The Ctr1 protein is localized to the plasma membrane and contains 11 putative Cu-binding motifs (Met-X₂-Met) that resemble those found in prokaryotic Cu homeostasis proteins (Cha and Cooksey 1991; Odermatt et al. 1993; Dancis et al. 1994b). In response to elevated extracellular Cu levels, expression of both the *CTR1* and *FRE1* genes is repressed, as would be expected for loci encoding proteins that have important roles in high-affinity Cu transport (Dancis et al. 1994a; Hassett and Kosman 1995).

Elegant studies have uncovered an important link between Cu availability and Fe transport in *S. cerevisiae*. Yeast strains harboring a deletion of the *CTR1* gene (*ctr1Δ*) are defective in ferrous iron uptake, attributable to a failure to provide Cu to a multi-Cu Fe oxidase, Fet3p (Askwith et al. 1994; Dancis et al. 1994b). Fet3p is structurally similar to the human serum Cu-binding protein

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ceruloplasmin and is required for high-affinity iron accumulation (Askwith et al. 1994; De Silva et al. 1995; Stearman et al. 1996). Furthermore, *ctr1Δ* strains display a number of other phenotypes associated with Cu deficiency, including defective high-affinity Cu transport, poor growth on low Cu medium, deficient Cu, Zn SOD activity with a concomitant sensitivity to oxidative stress, inability to grow on respiratory carbon sources, and an inability to activate *CUP1* gene transcription through the Ace1p Cu metalloregulatory factor, except at very high exogenous Cu concentrations (Dancis et al. 1994a,b).

Although little is known about the complexity and organization of prokaryotic or eukaryotic Cu transporters, many ion transporters are composed of a complex array of polypeptides that are either directly involved in ion transport or that modify the function of the transport machinery (Catterall 1995; Vulpe and Packman 1995). To identify additional components of the Cu transport machinery, we isolated mutants that allow yeast cells to dispense with the Ctr1 protein, yet maintain high-affinity Cu uptake. One such mutation, *CTR3^s*, restores high-affinity Cu and Fe transport, growth on nonfermentable carbon sources, Cu, Zn SOD activity and other phenotypes associated previously with the loss of Ctr1 protein. The Ctr3 protein is an intracellular membrane protein, the expression of which is up-regulated in response to copper starvation and repressed by exogenous Cu. Interestingly, expression of the *CTR3* gene is abrogated in the large majority of commonly used strains of *S. cerevisiae*, through a transposon insertion within the *CTR3* promoter. Yeast cells that express both the *CTR1* and *CTR3* genes have a distinct growth advantage, when Cu is limiting, over cells expressing either one of these Cu transporters alone. These results demonstrate that the presence of mobile DNA elements within the genome of many widely used laboratory strains of yeast provide an important mechanism for phenotypic variation under specific physiological conditions.

Results

A mutation in *CTR3* restores high-affinity Cu transport

Yeast strains harboring an inactivated *CTR1* gene exhibit a spectrum of phenotypes, including the inability to grow on nonfermentable carbon sources, which can be corrected by the addition of Cu to the growth medium (Dancis et al. 1994b). To identify additional components of the yeast high-affinity Cu transport system, we used ethylmethane sulfonate (EMS) mutagenesis to isolate yeast cells that can respire in the absence of the Ctr1 protein. The *CTR1* gene was inactivated in the *S. cerevisiae* strain SEY6210 (*CTR1 CTR3*) to generate a *ctr1Δ* strain (*ctr1Δ CTR3*) and this strain was treated with EMS. Of 5×10^8 colonies that survived the EMS mutagenesis, one mutant designated *CTR3^s*, was isolated which grew on YPE medium [ethanol as sole carbon source] (Fig. 1A; *ctr1Δ CTR3^s*). This mutant is also resistant to oxidative stress, initiated by the superoxide-

Figure 1. *CTR3^s* suppresses phenotypes associated with a *ctr1* deletion. (A) Strains SEY6210 (*CTR1 CTR3*), SKY1 (*ctr1Δ CTR3*), SKY3 (*ctr1Δ CTR3^s*), and SKY12 (*ctr1Δ CTR3^s Δ*) were grown on YPD, YPE, and YPD containing 100 μM menadione. The YPD and YPD–menadione plates were photographed after 2 days incubation at 30°C and the YPE plate was photographed after 4 days. (B) ⁶⁴Cu uptake in strains SEY6210, SKY1, and SKY3. ⁶⁴Cu uptake (*V*_{max}) was calculated from radioactive isotope accumulated during 3-min incubations, under high-affinity transport conditions. ⁵⁹Fe uptake rate was determined from radioactive isotope accumulated during a 20-min incubation. Cell-surface reductase activities were calculated from the absorbance change associated with the reduced forms of each metal. SOD activity, determined by inhibition of superoxide anion generation, is shown as total SOD activity and Cu, Zn SOD activity. Error bars represent the standard deviation for three independent experiments.

generating drug menadione and grew at high oxygen concentrations (Fig. 1A; data not shown). A diploid generated from a cross between the *CTR3^s* suppressor strain

and a strain of the opposite mating type bearing a *ctr1Δ* disruption grew on YPE, demonstrating that the *CTR3^s* mutation acts in a dominant fashion. Sporulation of the diploid and genetic analysis of 10 complete tetrads demonstrated that the *CTR3^s* suppressor mutation segregates as a single Mendelian locus (data not shown).

Because *ctr1Δ* strains possess a number of Cu-remedial phenotypes, we ascertained whether the *CTR3^s* allele is capable of suppressing all of the phenotypes associated previously with *ctr1Δ* strains (Dancis et al. 1994a,b). As shown in Figure 1B, inactivation of the *CTR1* gene, in strain SEY6210, resulted in a >90% reduction in high-affinity ⁶⁴Cu transport (cf. SEY6210 to SKY1). In the *ctr1Δ CTR3^s* strain (SKY3) high-affinity Cu transport was restored to ~50% that of the wild-type starting strain, a level sufficient for growth on respiratory carbon sources (Fig. 1A). ⁵⁹Fe uptake, almost undetectable in the *ctr1Δ* mutant, was restored to wild-type levels in cells harboring the *CTR3^s* allele (Fig. 1B). Additionally, cells bearing the *CTR3^s* allele (SKY3) have nearly wild-type levels of Cu, Zn SOD activity, whereas the *ctr1Δ* strain, as observed previously by Dancis et al. (1994), was devoid of measurable Cu, Zn SOD activity (Fig. 1B). Therefore, although the *CTR3^s* mutation restores the high-affinity Cu uptake rate to 50% that of the wild-type starting strain, this level of uptake provides sufficient Cu for high-affinity Fe uptake, for normal Cu, Zn SOD activity, and growth in the presence of the superoxide anion-generating drug menadione. Moreover, although the *ctr1Δ* mutation increases the activity of the Cu/Fe reductase more than twofold, presumably as a consequence of Cu and Fe starvation, reductase activity in the *CTR3^s* background is reduced nearly to that found in the wild-type parental strain (Fig. 1B). The inability to lower reductase activity completely to wild-type levels may be attributable to a partial Cu starvation, as the *ctr1Δ CTR3^s* strain only restores Cu transport to 50% the level of the starting strain. Taken together, these results and those described below establish that the *CTR3^s* mutation restores high-affinity Cu transport to a rate that is sufficient to provide Cu for normal biochemical and growth functions in a *ctr1Δ* strain.

Isolation and characterization of CTR3

Based on the dominant nature of the *CTR3^s* mutation, we isolated the *CTR3^s* locus by constructing a single-copy DNA library from a partial *Sau3A* digestion of genomic DNA from the *ctr1Δ CTR3^s* strain SKY3 (Ausubel et al. 1987). The *ctr1Δ* parental strain was transformed directly with the ligation mixture and transformants were replica-plated to YPE medium. Plasmid DNA was isolated from two YPE⁺ transformants by retrieval into *Escherichia coli* cells, and retransformation of the *ctr1Δ* strain demonstrated that the YPE⁺ phenotype was plasmid-dependent. Restriction enzyme digestion demonstrated that these two plasmids contained nonidentical genomic DNA fragments that shared a 3.5-kb *XbaI* DNA fragment. This fragment was further digested and DNA fragments were subcloned into the single-copy plasmid

PRS315 (Sikorski and Hieter 1989). In vivo complementation analysis demonstrated that a 1.4-kb *HpaI*–*XbaI* DNA fragment harbored the full suppression activity of the *CTR3^s* allele (data not shown).

DNA sequence analysis of the 1.4-kb *HpaI*–*XbaI* fragment revealed a 723-nucleotide-long open reading frame (ORF) with 100% sequence identity to an ORF, of unknown function, identified in the *S. cerevisiae* genome sequencing project of chromosome XII (M. Johnston, unpubl. ORF L9931.6). The *CTR3^s* ORF encodes a polypeptide of 241 amino acids with a predicted molecular mass of 27.5 kD (Fig. 2A). This coding region is essential for *CTR3^s* function because disruption of the ORF abolishes the suppression of the respiratory defect, oxidative stress sensitivity (Fig. 1A; *ctr1Δ CTR3^sΔ*) and other phenotypes (data not shown) associated with the *ctr1Δ* mutation. Several features of the Ctr3p sequence are noteworthy. Ctr3p contains two potential asparagine-linked glycosylation sites and two potential membrane-spanning regions identified using the TMPred program (Hofmann and Stoffel 1993) (Fig. 2A). Furthermore, Ctr3p contains 11 cysteine residues, three pairs of which are in potential metal coordination arrangements Cys–Cys or Cys–X₂–Cys (Winge et al. 1985), however, at present it is unknown whether these residues interact directly with metals. A direct alignment of the amino acid sequences of Ctr3p with Ctr1p only identified a short stretch of similarity between the first seven amino-terminal residues of Ctr3p (MNMGGSS) and a sequence (MNMGGSS) of Ctr1p four residues from the amino terminus. Comparison of Ctr3p with sequences currently in the data base revealed homology (25% identity, 53% similarity) with Ctr2p, a protein of 189 amino acids identified as a yeast homolog to a putative Cu transport protein, COPT1, of *Arabidopsis thaliana* (Kampfenkel et al. 1995). The potential role of Ctr2p-mediated Cu uptake in yeast cells, however, has not yet been ascertained.

The presence of a Ty2 transposon eliminates CTR3 expression

Although *CTR3^s* is a dominant mutation, we observed no sequence differences in the ORF between our genomic DNA isolate and the sequence reported in the yeast genome project for this locus derived from the most commonly used wild-type *S. cerevisiae* laboratory strain, S288C (Mortimer and Johnston 1986). However, comparison of the sequence of these loci in the promoter regions revealed that in S288C, a 6-kb Ty2 transposon is present 105 bp upstream of the initiator AUG of the *CTR3* ORF (Fig. 2B; Ty2::CTR3 sequence). In the cloned *CTR3^s* gene, the majority of the Ty2 element was absent, with only 25 nucleotides derived from the 3' terminus of the Ty2 δ element, flanked by a repeated 5'-CTTCT-3' sequence present (Fig. 2B; *CTR3^s* sequence). Immediately upstream and downstream of these 25 nucleotides, the sequence of the *CTR3^s* locus is identical to that described for S288C for at least several hundred nucleotides, demonstrating that the only mutation at this locus is the absence of most of the Ty2 element. As de-

Figure 2. *Ctr3* protein sequence and *CTR3* allele structure. (A) Predicted amino acid sequence of *Ctr3p*. The 11 cysteines and 2 histidines are in bold. Asterisks indicate the two putative asparagine-glycosylation sites and the boxed residues are predicted transmembrane regions. (B) Wild-type and mutant *CTR3* loci. DNA sequence Ty2::*CTR3* corresponds to the allele present in S288C and SEY6210 in which the putative TATA box (underscored) is displaced ~6 kb by the Ty2 element. The *CTR3^S* DNA sequence corresponds to the *ctr1Δ* suppressor mutation (strain SKY3) in which the Ty2 element has been excised leaving 25 bp of the δ element (boxed sequence) and shifting the TATA element adjacent to the *CTR3* ORF. The *CTR3* DNA sequence is the allele present in BR10 that does not have a Ty2 element at this locus. The overlined sequences show the site of insertion of the Ty2 element. Bold and thin arrows represent major and minor transcriptional start sites, respectively, and mark the direction of transcription. The numbers below the arrows indicate the positions of these start sites upstream from the ATG of the ORF.

scribed below, both the SEY6210 and SKY1 (*ctr1Δ*) strains harbor a Ty2 at the same position within *CTR3* as is found in S288C. The Ty2 insertion into the *CTR3* promoter region is in the same orientation as the *CTR3* gene, and is predicted to separate the putative promoter and TATA box from the ORF by 6 kb. Although this TATAAA sequence has not been demonstrated to function in *CTR3* gene transcription, this is the only TATA box consensus sequence (Hahn et al. 1989) within 200 bp upstream of the *CTR3* ORF. Additionally, primer extension analysis identified the major start sites for *CTR3^S* transcription in the *ctr1Δ CTR3^S* strain to positions -57 and -53 with respect to the AUG that initiates this ORF, with four minor start sites upstream of these (Fig. 2B; sequence *CTR3^S*). These correspond to positions 84 and 88 nucleotides downstream of the proposed TATAA element and are consistent with this element serving as the functional *CTR3* TATA box. These *CTR3* transcriptional start sites are slightly shifted in a strain for which

there is no sequence evidence for a *CTR3*-associated Ty2 element (Fig. 2B; sequence *CTR3*), suggesting that the residual 25 nucleotides of the Ty2 sequence downstream of the TATAAA sequence in the *CTR3^S* gene have shifted the start sites for transcription (Fig. 2B; sequence *CTR3*). Taken together, these results strongly suggest that the presence of the Ty2 element in the *CTR3* promoter abrogates *CTR3* transcription because of the separation of the promoter from the transcriptional initiation sites by 6 kb. Excision of the Ty2 element, through an unknown mechanism, results in the juxtaposition of the promoter and putative TATA element to the transcription initiation sites.

The insertion of transposable elements adjacent to ORFs is well established to modulate flanking gene expression in prokaryotes and eukaryotes (Roeder and Fink 1983; Kleckner 1989; Boeke and Sandmeyer 1991). Therefore, we examined the structure of the *CTR3* promoter in the isogenic wild-type parental strain SEY6210,

the *ctr1Δ* strain SKY1, and the *ctr1Δ CTR3^s* strain SKY3 and compared the steady-state levels of *CTR3* mRNA in these backgrounds by RNA blotting. As shown in Figure 3, *CTR3* mRNA levels were undetectable in both SEY6210 and SKY1 and there was no change when cells were either treated with Cu, the Cu chelator bathocuproine disulfonate (BCS), or the Cu/Fe chelator bathophenanthroline disulfonate (BPS). The weak background hybridization band represents cross-reactivity with the 18S ribosomal RNA, slightly larger than *CTR3* mRNA. In contrast, in the isogenic *CTR3^s* strain SKY3, *CTR3* mRNA levels were detectable in control cultures, repressed by the addition of Cu concentrations as low as 1 μ M and derepressed $\sim$ 20-fold, as determined by phosphorimager analysis, when cells were treated with the copper and iron/copper chelators BCS and BPS (Fig. 3; *ctr1Δ CTR3^s*). As expected, *CUP1* mRNA levels were induced by Cu in a dose-dependent manner in the *CTR1* wild-type strain but were only induced with high Cu levels in the *ctr1Δ* strain. Consistent with the restoration of high-affinity Cu transport in the *ctr1Δ CTR3^s* strain, *CUP1* mRNA levels were induced in a Cu dose-dependent manner (Fig. 3). Therefore, the *CTR3^s* mutation permits expression of the *CTR3* locus, strongly suggesting that insertion of the Ty2 element prevents transcription of *CTR3* and that excision of the Ty2 transposon restores gene expression and Cu regulation.

To determine whether a correlation exists between the absence of a Ty element within the *CTR3* locus and *CTR3* mRNA expression, we analyzed several common laboratory strains of *S. cerevisiae* for the presence of Ty2 adjacent to *CTR3* and measured *CTR3* mRNA levels (Fig. 4). Of 15 laboratory strains examined by Southern blotting and PCR, only three had no Ty element associated with *CTR3*. The data in Figure 4 show such an analysis for five different strains, two of which lacked the Ty2 element [SKY3(*CTR3^s* mutant) and BR10] and three of which had the Ty2 located within the *CTR3* promoter region (SEY6210, SKY1, S288C). The presence of an *Xba*I restriction site in the Ty2 element permitted a convenient analysis; strains with a Ty2 element in the *CTR3*

Figure 3. *CTR3* mRNA is expressed from the *CTR3^s* allele and regulated by cellular Cu status. Strains SEY6210 (*CTR1 CTR3*), SKY1 (*ctr1Δ CTR3*) and SKY3 (*ctr1Δ CTR3^s*), were grown to early log-phase in YPD media. CuSO₄ (0, 1, 10, and 100 μ M), 100 μ M BCS (C), or 100 μ M BPS (P) were added and after a 3-hr incubation at 30°C total RNA was isolated. Shown is an RNA blot of *CTR3*, *CUP1*, and *ACT1* mRNA steady-state levels indicated by arrows. Results shown are representative of three independent experiments.

Figure 4. A Ty2 element prevents *CTR3* expression in many yeast strains. (A) Southern blot analysis for the presence or absence of the *CTR3*-associated Ty2 element. Genomic DNA isolated from *S. cerevisiae* strains SEY6210, SKY1, SKY3, S288C, and BR10 was digested with *Xba*I and probed with ³²P-labeled DNA derived from the *CTR3*-coding region. The 3.0-kb fragment indicates absence of the Ty2 element and the 2.1-kb fragment indicates the presence of a Ty2 element adjacent to the *CTR3*-coding region. (B) PCR confirmation of the Ty2 status within the *CTR3* locus. Synthetic DNA oligonucleotides representing a sequence from the Ty2 element and the *CTR3* ORF were used in PCR procedures. The presence of a 0.85-kb PCR product indicates the presence of a Ty2 element within the *CTR3* promoter. The 1.4-kb DNA PCR product was produced from synthetic DNA oligonucleotides designed to amplify the *FRE1* gene and included in the PCR as a positive control. (C) The five strains that were analyzed in A and B were grown in YPD to early log phase. Cu (100 μ M), BCS (C, 100 μ M), or nothing (0) was added, and the cells were incubated for 3 hr after which total RNA was isolated. Shown is an autoradiograph of an RNA blot of *CTR3* and *ACT1* mRNA steady-state levels, indicated with arrows.

promoter gave rise to a 2.1-kb band by Southern blotting, whereas strains without the Ty2 element give a 3.0-kb DNA fragment (Fig. 4A). To verify that the size differences observed by Southern blotting were attributable to a Ty2 insertion rather than another polymorphism, PCR analysis was carried out using one primer specific to the *CTR3*-coding region and a second primer that specifically hybridizes with Ty2 DNA (Fig. 4B). The amplification of the expected 0.85-kb PCR product using genomic DNA from the strains that were Ty2 positive by Southern blotting, but not those that were negative, confirmed the presence of the Ty2 element in the *CTR3* promoter of these three strains. The 1.4-kb fragment corresponds to the product from primers included as a positive control for PCR (Fig. 4B). Expression from the *CTR3* locus in the same strains was examined by RNA blotting. These experiments demonstrated that the strains lacking a *CTR3*-associated Ty2 element, SKY3 and BR10, express detectable *CTR3* mRNA under control growth condi-

tions (Fig. 4C). *CTR3* mRNA expression in these strains is repressed in cultures exposed to 100 μ M Cu (Fig. 4, lanes 100) and highly induced in cultures starved for Cu by treatment with the Cu chelator BCS (Fig. 4, lanes C). *CTR3* mRNA was not detected under any of these conditions in the strains demonstrated to harbor a Ty2 element in the *CTR3* promoter (SEY6210, SKY1, and S288C). Therefore, a strict correlation exists between the absence of the *CTR3*-associated Ty2 element and *CTR3* mRNA expression. We have also examined several *S. cerevisiae* strains isolated from human lung, peritoneal fluid, bile, and blood (McCusker et al. 1994) and found no Ty2 element associated with the *CTR3* locus, suggesting that these clinically isolated strains express the *CTR3* gene (data not shown).

Expression and subcellular location of Ctr3p

The Ctr1p is a plasma membrane-associated protein with limited sequence homology to prokaryotic Cu homeostasis proteins (Cha and Cooksey 1991; Odermatt et al. 1993). Ctr3p restores high-affinity Cu transport to *ctr1* Δ strains, yet exhibits no strong sequence homology to Ctr1p. Therefore, to begin to characterize Ctr3p, we carried out experiments to determine the Ctr3p subcellular location. The Ctr3 protein was tagged at the carboxyl terminus with three tandem repeats of the influenza virus hemagglutinin epitope (HA) by site-directed mutagenesis of the *CTR3*^s gene. *S. cerevisiae* strain SKY1 (*ctr1* Δ) transformed with a single-copy plasmid carrying the *CTR3-HA* gene grew on YPE medium, indicating that the Ctr3-HA protein is functional in high-affinity Cu uptake (data not shown). This strain was grown in the absence or presence of exogenous Cu and equal amounts of protein extract subjected to SDS-PAGE and Western blotting using the anti-HA mAb 12CA5 (Niman et al. 1983; Kolodziej and Young 1991). This analysis demonstrated the presence of a major polypeptide species slightly larger than 30 kDa, consistent with

the expected size of Ctr3-HA as predicted by the sequence of the ORF (Fig. 5A). A second higher molecular weight polypeptide species of low abundance was observed in these extracts that may represent a multimeric or posttranslationally modified form of the polypeptide, or incompletely denatured Ctr3-HA polypeptide. Consistent with the regulation of *CTR3* mRNA levels, the Ctr3-HA protein levels were dramatically reduced in cells grown for 1.5 hr in the presence of 100 μ M CuSO₄ (Fig. 5A).

The primary structure of Ctr3p predicts that this protein contains two potential membrane-spanning regions (Fig. 2A). To ascertain whether Ctr3p is a membrane protein, Ctr3-HA was expressed on a single-copy plasmid in a yeast strain (RSY620) deficient in vacuolar proteases because of a *pep4* gene disruption. Extracts prepared from this strain were fractionated by centrifugation and analyzed by Western blotting (Fig. 5B). The majority of epitope-tagged Ctr3 protein was detected in the 10,000g pellet (P₁₀) and the 105,000g pellet (P₁₀₀) with smaller amounts in the crude 450g pellet (P_{0.5}). No Ctr3-HA was detected in the soluble fraction from the 105,000g spin (S₁₀₀). An identical fractionation was carried out in the presence of 0.1 M Na₂CO₃ (pH 11), which linearizes membrane structures releasing nonintegral membrane proteins into the soluble fraction (Fujiki et al. 1982). Kar2 protein, an endoplasmic reticulum (ER) lumen protein that is undetectable in the S₁₀₀ fraction in the absence of Na₂CO₃, was released into the S₁₀₀ fraction by the Na₂CO₃ treatment (Fig. 5B). However, no CTR3-HA was observed in the Na₂CO₃ S₁₀₀ fraction, demonstrating that Ctr3p is an integral membrane protein (Fig. 5B).

To investigate the cellular location of Ctr3-HA, indirect immunofluorescence microscopy was performed using the mAb 12CA5 to detect HA-tagged Ctr3p and anti-myc monoclonal antibody to detect myc-tagged Ctr1p. These experiments were performed in strain SLY4, which expresses both the *CTR1* and *CTR3* genes,

Figure 5. Ctr3p is an integral membrane protein biosynthetically regulated by Cu. (A) Strain SKY1, transformed with plasmid pCTR3-C-HA/315, was grown to early log phase in SC-leucine media. Cells were harvested, resuspended in YPD media, and incubated at 30°C for 1 hr. Cu (100 μ M) was added to half the culture, and the Cu-treated (+) and control (-) cultures were incubated for 1.5 hr at 30°C, 300 rpm. Protein extract (30 μ g) from each culture was fractionated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose for Western blot analysis with 12CA5 anti-HA antibody. Immunoreactive protein was detected by chemiluminescence and exposure to Hyperfilm. Molecular mass markers are indicated at right, and the position of Ctr3-HA is indicated with an arrow. (B) Strain RSY620 (*pep4* Δ) was transformed with pCTR3-C-HA/315 and grown in a similar manner to that described for SKY1 above, but without the addition of Cu. Cell extracts, prepared in the absence or presence of 0.1 M Na₂CO₃ at pH 11, were fractionated by centrifugation to give three pellets, P_{0.5} (450g), P₁₀ (10,000g), and P₁₀₀ (105,000g), and a 105,000g supernatant S₁₀₀. Protein (30 μ g) from each fraction was separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose for Western blot analysis of Ctr3-HA and Kar2p using the corresponding antibodies. The positions of the Kar2 and Ctr3-HA proteins are indicated with arrows.

Figure 6. Ctr3-HAp is an intracellular protein associated with the vesicular structures. Strain SLY4 (*CTR1 CTR3*) transformed with plasmid pCTR3-C-HA/315 or p352-myc (*CTR1-myc*) was grown overnight in SC-selective media and switched to YPD media for 3 hr to induce gene expression. Fixed cells were incubated with anti-HA monoclonal antibody to detect Ctr3-HAp (A) and anti-Kar2p antisera (C) or anti-myc monoclonal antibody to detect Ctr1-mycp (B). FITC or rhodamine red-conjugated secondary antibodies was used to visualize the primary antibody-protein complexes. DAPI staining visualized nuclear and mitochondrial DNA (D). The same cell fields are shown in A, C, and D. (E) Ctr3-HAp location in strain SKY1 (*ctr1Δ*); (F) DAPI staining in the same field of cells as in E. (G) Field of five SLY4 cells transformed with control plasmid pRS315 and treated identically to cells in A.

transformed with either pCTR3-C-HA/315 or p352-myc (for Ctr1-mycp expression). The intracellular punctate pattern of Ctr3-HAp (Fig. 6A), combined with the biochemical fractionation data, suggests that Ctr3-HAp is associated with vesicular components of the cell. Focusing through the planes of the cell confirmed the intracellular, as opposed to peripheral, location of Ctr3-HAp. In cells with small anuclear buds there was an enrichment of Ctr3-HAp in the bud and at the bud interface with the mother cells. This is a common feature of proteins localized to the ER, Golgi, and secretory vesicles (Preuss et al. 1992). We did not observe Ctr3-HAp at the plasma membrane, suggesting that unlike Ctr1p, which has been shown previously to be located at the plasma membrane (Fig. 6B; Dancis et al. 1994b), Ctr3p is not concentrated at this site. Double immunofluorescence labeling carried out with antibodies to Kar2p, which is located in the ER, and Kex2p, which is a trans-Golgi protein (Rose et al. 1989; Redding et al. 1991), indicated that Ctr3-HAp is not concentrated solely in either of these compartments, though there is partial overlap of some of the Ctr3p punctate spots with the Kar2p protein (Fig. 6, cf. A and C; data not shown). These same experiments, carried out in the *ctr1* deletion strain SKY1, showed similar Ctr3-HAp punctate staining, indicating that Ctr1p does not influence Ctr3p localization (Fig. 6E). In addition, DAPI staining (Fig. 6D,F) showed that Ctr3-HAp is not enriched in the nucleus or mito-

chondria. Together, the dispersed punctate pattern of intracellular staining, the integral membrane nature of Ctr3p, and Ctr3p primary sequence features (see Discussion) are suggestive of a protein that is associated with the yeast secretory/vesicular system.

Ctr1p and Ctr3p function requires Cu(II) reduction

Although Ctr1p and Ctr3p both function in high-affinity Cu uptake, these proteins are structurally dissimilar and are concentrated in distinct subcellular locations. To determine whether these two proteins may have similar requirements for high-affinity Cu uptake, we tested the dependence on *FRE1*, encoding a cell-surface Cu/Fe reductase, for Ctr1p and Ctr3p function in Cu transport. The data in Figure 7 demonstrate, as observed previously (Hassett and Kosman 1995), that Ctr1p-mediated high-affinity Cu transport is decreased ~90% in the *fre1Δ* strain as compared with the isogenic wild-type strain SEY6210. Similarly, ~70% of the Ctr3p-mediated high-affinity Cu transport is abolished in the absence of *FRE1*. Therefore, the Ctr1 and Ctr3 proteins require the *FRE1*-encoded Cu/Fe reductase for efficient high-affinity Cu uptake, suggesting strongly that both components are involved in the transport of reduced Cu (Cu I).

Contributions of Ctr1p and Ctr3p-Cu transport and cell growth

Our results suggest that in a strain in which both the *CTR1*- and *CTR3*-Cu transport components are normally active, elimination of either system alone may not eliminate high-affinity Cu transport. To test this hypothesis, we created single and double deletions of the

Figure 7. Ctr3p function in high-affinity Cu uptake requires a cellular Cu reductase. The *FRE1* gene encoding a Cu/Fe reductase was insertionally inactivated in the isogenic yeast strains SEY6210 (*CTR1 CTR3*), SKY1 (*ctr1Δ CTR3*), and SKY3 (*ctr1Δ CTR3s*). These strains were grown in YPD to early log-phase and high-affinity ^{64}Cu uptake was measured as described in Materials and methods. Solid bars indicate the ^{64}Cu uptake rate in *FRE1* cells; shaded bars indicate the ^{64}Cu uptake rate in *fre1Δ* cells. Results are representative of two experiments; error bars indicate the standard deviation of samples analyzed in triplicate.

CTR1 and *CTR3* genes in strain BR10, which expresses both the *CTR1* and *CTR3* genes because of a lack of the Ty2 element in the *CTR3* promoter (Fig. 4B). As shown in Figure 8A, high-affinity Cu transport is reduced to ~50% that of the parental strain by deletion of the *CTR1* gene and to ~75% that of the parental strain attributable to a deletion of the *CTR3* gene. This decrease in the rate of Cu uptake in the *ctr1Δ* or *ctr3Δ* cells did not render these strains defective for growth on YPE agar (Fig. 8A). When both the *CTR1* and *CTR3* genes were insertionally inactivated, these cells failed to carry out significant levels of high-affinity Cu transport and, as a consequence, failed to grow on YPE agar (Fig. 8A). To assess the potential contributions of Ctr1p and Ctr3p to cell growth more quantitatively, the rate of growth in YPE medium for the isogenic parent strain (*CTR1 CTR3*), *ctr1Δ* and *ctr3Δ* strains was measured in YPE broth (Fig. 8B). Under these conditions, no significant differences in the growth rate of either of the three strains was observed. If, however, Cu was limited in YPE broth by the addition of the Cu-chelator BCS, then the growth rate of the strains possessing either only Ctr1p or Ctr3p was decreased significantly as compared with the isogenic wild type expressing both proteins (Fig. 8C). This was most apparent at 72 hr when the wild-type (*CTR1 CTR3*) strain is at mid-log phase but the two deletion strains (*ctr1Δ* or *ctr3Δ*) are only at the entry to log-phase. Therefore, under standard laboratory growth conditions, either Ctr1p or Ctr3p will suffice for respiratory growth; however, under copper-limiting conditions both proteins are required for maximal growth rate. These data clearly establish that both Ctr1p and Ctr3p have important physiological roles in high-affinity Cu transport and cell growth.

Discussion

Cu is an essential biochemical cofactor that is required for a wide variety of enzymatic reactions. In this work we have used classical yeast extragenic suppression approaches to identify a new gene, *CTR3*, involved in high-affinity Cu transport. In the absence of the plasma membrane-associated high-affinity Cu transport protein Ctr1, yeast cells that express Ctr3p suppress all of the Cu deficiency-based phenotypes that occur as a result of inactivation of the *CTR1* gene. Furthermore, the analysis of yeast strains in which either *CTR1*, *CTR3*, or both genes have been insertionally inactivated clearly demonstrates that either *CTR1*- or *CTR3*-mediated high-affinity Cu transport suffices for growth under standard laboratory conditions. The inactivation of both *CTR1* and *CTR3* in such strains is required to generate all of the Cu deficiency phenotypes associated with a *CTR1* gene deletion in a strain lacking a *CTR3*-encoded Cu transport protein (Dancis et al. 1994a,b). Both the *CTR1* and *CTR3* gene products have physiologically important roles in high-affinity Cu transport and cell growth.

It is intriguing that the yeast genome encodes two structurally unrelated proteins that function in high-affinity

Figure 8. Both Ctr1p and Ctr3p have important physiological roles in high-affinity Cu transport and cell growth. (A) Three isogenic yeast strains and one congenic strain, [BR10 (WT), SKY52 (*ctr1Δ CTR3*), SKY44 (*CTR1 ctr3Δ*), and SKY46 (*ctr1Δ ctr3Δ*)] were spotted at a density of 2000 cells/10 μ l onto YPD and YPE agar media and incubated at 30°C for 2 and 4 days, respectively. The high-affinity ⁶⁴Cu uptake rate was measured in these strains, grown in YPD broth, as described in Materials and methods. The Cu uptake values are represented as a horizontal bar with the standard deviation indicated for triplicate measurements. Growth of strains BR10 (WT, □), SKY52 (*ctr1Δ CTR3*, ◇), and SKY44 (*CTR1 ctr3Δ*, ○) was assessed in YPE liquid cultures in the absence (B) or presence (C) of 100 μ M BCS. All cultures were started at an OD₆₀₀ of 0.02; optical density measurements represent the mean of three independent cultures in C.

finity Cu transport. On one hand, the *CTR1*- and *CTR3*-mediated Cu transport activities are similar in the Cu-dependent biochemical processes they support, the requirement of both for Cu reductase activity for function in Cu transport, the membrane association of Ctr1p and Ctr3p, and the regulation of gene expression in response to fluctuations in cellular Cu status. However, these two proteins are distinct with respect to their primary sequences, subcellular distribution, and apparent functional independence, as ascertained by gene deletion studies and lack of direct interaction in coimmunoprecipitation experiments (data not shown). Subcellular fractionation experiments and indirect immunofluorescence microscopy demonstrate that Ctr3p is an integral membrane protein associated with intracellular vesicu-

lar structures. In support of these experimental data, the Ctr3p carboxyl terminus contains two structural features associated with proteins that are retained in the ER/Golgi network. First, the carboxy-terminal sequence of Ctr3p, RKCCCL, is similar to the yeast ER retention signal (Basic/Basic/X/X) (Nilsson et al. 1989; Jackson et al. 1990; Townsley and Pelham 1994), and our data suggest that a fraction of Ctr3p colocalizes with Kar2p, which is located in the ER. Second, Ctr3p also contains the sequence FCYEPL, which conforms to an element of the consensus (Y/F)X(Y/F)XX(I/L), which, in turn, has a critical role in Golgi retention for both the Kex2 protease and dipeptidyl aminopeptidase of *S. cerevisiae* (Wilcox et al. 1992; Nothwehr et al. 1993). The integral membrane nature of Ctr3p and the abundance of punctate staining that does not strictly colocalize to either the ER or *trans*-Golgi, is suggestive of a protein that might be associated with other Golgi compartments or larger secretory/endosomal vesicles as well (Franzusoff et al. 1991).

How might an integral membrane protein localized to intracellular vesicular structures function in high-affinity Cu transport? One possible mechanism is that Ctr3p functions in an endocytic pathway of copper uptake distinct from the Ctr1p pathway. There is biochemical evidence for two Cu transport pathways in mammalian cells, one for unbound Cu and a second endocytic process for ceruloplasmin-bound Cu, though the cellular components involved in these pathways have not been identified (Vulpe and Packman 1995). As part of an endocytic Cu transport pathway in yeast, Ctr3p may rapidly recycle to and from the plasma membrane from intracellular vesicles. The *S. cerevisiae* Ste6 protein has been shown to move to and from the membrane as a function of α -factor stimulation (Kuchler et al. 1993; Kolling and Hollenberg 1994). Although we do not detect Ctr3p concentrated at the plasma membrane like Ctr1p, either a very transient association or rapid protein turnover, as suggested by the Cu-mediated down-regulation of Ctr3p (Fig. 5A), could preclude our detection of Ctr3p at the cell surface. The analysis of Ctr3p function in an *end4* mutant background did not reveal any defect in Cu transport or cell growth (data not shown); however, this mutant does have residual endocytic transport function (Raths 1993). In both yeast and mammalian cells it is clear that there are at least two distinct pathways of endocytosis (Helenius et al. 1983; Riezman 1985). Alternatively, Ctr3p may function to release Cu to the cytosol from intracellular vesicles, thereby increasing the rate of endocytic Cu uptake. It is also possible that Ctr3p has a less direct role in the same high-affinity Cu transport pathway as Ctr1p. Ctr3p could serve as a chaperone to facilitate the trafficking of other protein components, that might operate with Ctr1p, through the secretory network to the plasma membrane. Such specific chaperone roles have been found for Hsp47, a collagen-specific chaperone, and receptor-associated protein (RAP), a chaperone for the low-density lipoprotein (LDL) receptor-related protein (Bu et al. 1995; Nagata 1996). Consistent with our Cu transport measurements in a *CTR1 ctr3 Δ* strain, these components could be inefficiently de-

livered to the plasma membrane but their delivery may be enhanced by Ctr3p. In cells lacking Ctr1p, the action of Ctr3p in augmenting proper Cu transport complex assembly at the plasma membrane could allow a significant level of high-affinity Cu transport. Clearly, our data demonstrate that the expression of both Ctr1p and Ctr3p is required for the most efficient high-affinity Cu transport activity and for normal cell growth under conditions in which copper is required, yet limiting.

Our observation that *CTR3* gene transcription in most laboratory strains examined is eliminated by an insertion of a transposable element in the *CTR3* promoter is consistent with previous observations in bacteria and yeast (Kleckner 1989; Boeke and Sandmeyer 1991). It is well established that transposable elements preferentially insert in the 5'-flanking regions of genes and either inactivate or modify the expression of the flanking loci (Eibel and Philippsen 1984; Boeke 1989). The yeast *his4-917* allele contains a spontaneously inserted Ty2 element that displaces the TATA box, thereby inhibiting *HIS4* gene transcription (Roeder et al. 1980). The insertion of a Ty2 element in the *CTR3* promoter has a similar consequence and excision of the Ty2 element restores *CTR3* gene expression and high-affinity Cu transport. The nature of the *CTR3* Ty2 insertion is in agreement with the current model that Ty elements inserted with the same orientation for expression as the target gene displace the normal regulatory sequences without substituting Ty-mediated regulation (Boeke and Sandmeyer 1991).

An important ramification of the effect of Ty element insertion on gene expression involves the current yeast sequencing project. The entire yeast genome has now been sequenced. The next step in this project is the systematic deletion of ORFs coupled with phenotype screening (Wach et al. 1994). Based on our results with *CTR3*, and previous observations of the effect of transposons on gene expression, the ORF disruption experiments must be interpreted carefully. It is quite possible that many of the phenotypic differences observed in yeast strains are caused by the presence (or absence) of the Ty class of mobile DNA elements in the genome. Inspection of the entire yeast genome sequence reveals that in strain S288C there are six Ty2 elements with 98% or greater identity with the Ty2 at the *CTR3* locus. This suggests that the expression of other genes, and consequently yeast cell phenotypes, might be altered by this class, as well as other Ty elements. Recent studies of mice with a disrupted epidermal growth factor receptor (EGFR) gene demonstrated that embryonic lethality varied in a manner dependent on the mouse genetic background (Sibilia and Wagner 1995; Threadgill et al. 1995). The nature of this genetic variation is not clear, however, it is possible that the retrotransposon-like short or long interspersed elements (SINEs and LINEs, respectively) are involved in shaping the genome of these highly inbred mouse strains (Deininger 1989; Hutchinson et al. 1989). This work, demonstrating that a Ty2 element suppresses the expression of an important Cu transport gene, underscores the critical role that trans-

possible elements have in shaping genome evolution and function.

Materials and methods

Yeast strains and plasmids

Yeast strains used in this study are listed in Table 1. The *ctr1::URA3* allele was constructed by deleting a 1.5-kb DNA fragment encoding 82% of the *CTR1* ORF and 5' untranslated region, and replacing it with the *URA3* genetic marker. The *ctr1::TRP1* allele was constructed by replacing a 0.9-kb DNA fragment encoding the 145 amino-terminal amino acids and 0.45 kb of the 5' untranslated region with the *TRP1* genetic marker. The *pctr3::LEU2* disruption plasmid was constructed by ligating a *Bgl*II-*LEU2* fragment, isolated from Yep13, into the *Bgl*II site of the *CTR3* gene in pCTR3/S-X/315. *pctr3::LEU2* was digested with *Pvu*II and the DNA products were transformed into yeast cells to generate the *ctr3::LEU2* allele by homologous recombination. The *ctr3⁺::URA3* and *ctr3::TRP1* alleles were constructed by integration of the respective genetic markers into the *Bgl*II site of the *CTR3* gene. These disruptions rendered the *CTR1* and *CTR3* genes nonfunctional as assessed by the absence of growth on YPE media. Strain SKY46 (*ctr1::URA3 ctr3::TRP1*) was obtained by sporulation of a diploid derived from a cross of SKY1 and SKY44. The *fre1::LEU2* allele was constructed by deletion of 800 bp of *FRE1* DNA and replacement with the *LEU2* gene as described (Dancis et al. 1992). The allele status of each of these loci in all strains generated in this study was verified using Southern blotting and diagnostic PCR. The *CTR3* allele in strain BR10 lacks the Ty2 element and sequence analysis of the *CTR3* promoter from this strain shows no evidence for prior Ty2 insertion (Fig. 2B).

Growth media and genetic methods

Yeast cells were grown, unless otherwise stated, in YPD (1% yeast extract, 2% bacto-peptone, and 2% dextrose), YPE, or SC

(synthetic complete), which are described elsewhere in detail (Sherman et al. 1986). Yeast cells were transformed with DNA using lithium acetate (Ito et al. 1983) and plasmids were rescued by lysis of yeast spheroplasts and enriching for plasmid DNA on a Qiagen-tip 100 (Qiagen, Inc., Chatsworth, CA). Mating of yeast cells, EMS mutagenesis, sporulation of diploids, and tetrad dissection used standard methods (Sherman et al. 1986). All DNA and RNA manipulation and analyses were carried out as described previously (Ausubel et al. 1987).

Ctr3p epitope tagging

A *Not*I site was generated in pCTR3/H-X/SK immediately after the *CTR3* carboxy-terminal leucine, using a Muta-gene kit (Bio-Rad) for in-frame insertion of the HA tag. This plasmid expresses a functional HA-tagged Ctr3p protein as assessed by complementation of a *ctr1Δ* allele in SKY1. Plasmid 352-*myc*, which encodes the *myc*-tagged Ctr1 protein, was a generous gift from Andrew Dancis (National Institutes of Health, Bethesda, MD).

Cu and Fe uptake

Yeast cells were grown to early log phase in YPD media. The cells were harvested by centrifugation and washed twice in metal uptake/metal reductase (UR) buffer [0.2 M MES (4-morpholinepropanesulfonic acid) containing 5% (wt/vol) glucose and citrate (20 mM) at pH 6.0]. The cells were resuspended in UR buffer at the original density of 1×10^7 to 2×10^7 cells/ml or $A_{660} = 1.0$. Metal radionucleotide [plus nonradioactive metal ion as needed for a given (metal)_{total}] was equilibrated in 1 ml of UR buffer containing, unless noted, 1 mM dihydroascorbic acid. The equilibrated metal solution was added to 6 ml of yeast cells and samples were removed at 2 and 5 min, for ^{64}Cu uptake, or at 20 min, for ^{59}Fe uptake. Cell samples were quenched in 5 ml of ice-cold 10 mM EDTA in 0.1 M Tris succinate at pH 6.0, and washed by filtration through nitrocellulose filters (0.45 μm Gelman) with a total of 20 ml of quench solution. The filters were

Table 1. *S. cerevisiae* strains used in this work

Strain	Genotype	Source
SEY6210	<i>MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Scott Emr
SKY1	<i>MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ctr1::URA3</i>	this study
W303-1A	<i>MATα SUC2 ade2-1 can1-100 his3-11,115 leu2-3,112 trp1-1 ura3-1</i>	Yeast Genetic Stock Center
SKY2	<i>MATα SUC2 ade2-1 can1-100 his3-11,115 leu2-3,112 trp1-1 ura3-1 ctr1::URA3</i>	this study
SKY3	<i>MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 <i>lys2-801</i> <i>svy2-Δ9</i> ctr1::URA3 CTR3^s</i>	this study
SKY12	<i>MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ctr1::URA3 ctr3^s::LEU2</i>	this study
S288C	<i>MATα SUC2 mal mel gal2 CUP1</i>	Yeast Genetic Stock Center
BR10	<i>MATα gal1 trp1-1 his3 ade CUP1</i>	Brian Rymond
SKY44	<i>MATα gal1 trp1-1 his3 ade CUP1 ctr3::TRP1</i>	this study
SKY46	<i>MATα ctr1::URA3 ctr3::TRP1 lys2-801 his3</i> (spore clone from SKY1 × SKY44 cross)	this study
SKY51	<i>MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ctr1::TRP1</i>	this study
SKY52	<i>MATα gal1 trp1-1 his3 ade CUP1 ctr1::TRP1</i>	this study
SKY61	<i>MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 fre1::LEU2</i>	this study
SKY62	<i>MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ctr1::URA3 fre1::LEU2</i>	this study
SKY63	<i>MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ctr1::URA3 CTR3^sfre1::LEU2</i>	this study
Y55	<i>HO lys5</i>	John McCusker
DTY14	<i>MATα thr4</i>	Gary Fabian
SLY4	<i>MATα gal1 trp1-1 his3 ade CUP1-10 leu2::hisG ura3::kan^r</i>	this study
RSY620	<i>MATα ade2 leu2-3,-112 his3 trp1 ura3 pep4::TRP1</i>	Randy Schekman

counted in an LKB CompuGamma counter that corrected the values for isotope decay. Data for ^{64}Cu uptake was derived from the difference between the 2- and 5-min counts. ^{59}Fe uptake was taken as the value for the 20-min sample directly, because unlike ^{64}Cu uptake, there is no nonspecifically absorbed cell-associated radionucleotide at $t=15$ sec (Hassett and Kosman 1995). Kinetic constants for ^{64}Cu uptake were obtained by direct nonlinear fit of the data to the Michaelis–Menten equation using the program UltraFit (Biosoft). ^{59}Fe uptake was performed under $V_{\max}/K_m$ conditions (0.2 mM) and the results given as the velocity at this Fe concentration (Eide et al. 1992).

Protein and enzyme assays

Protein concentrations in cell extracts were determined using the Pierce BCA kit following the manufacturer's instructions. Total superoxide dismutase and Cu, Zn SOD activities were determined colorimetrically, by measuring the inhibition of nitrite formation from hydroxylamine-O-sulfonic acid in the absence or presence of KCN (Oyanagui 1984). Cupric and ferric reductase activities were determined by the change in absorbance attributable to formation of Cu(I) with bathocuproine disulfonate and Fe(II) with bathophenanthroline disulfonate (Lesuisse and Labbe 1989; Hassett and Kosman 1995). Ctr3-HAP and Kar2p detection was carried out by Western blotting as described (Ausubel et al. 1987).

Cellular fractionation

The *pep4* delete strain RSY620 transformed with pCTR3–C–HA/315 was grown in SC–leucine overnight, subcultured into YPD, and grown for a further 2 hr at 30°C to induce Ctr3–HAP synthesis. Spheroplasts were generated from the harvested cells and cell extracts were prepared in the absence or presence of 0.1M Na_2CO_3 at pH 11 (Fujiki et al. 1982). The extracts were centrifuged to obtain three pellet fractions of 450g, 10,000g, and 105,000g, plus a 100,000g supernatant, $P_{0.5}$, P_{10} , P_{100} , and S_{100} , respectively. The presence of Ctr3–HAP and Kar2p in these fractions was assessed by Western blotting.

Indirect double-labeling immunofluorescence

These methods were based on those described by Franzusoff et al. (1991). For localization of Ctr3–HAP and Kar2p, yeast strain SLY4 or SKY1 transformed with single-copy pCTR3–C–HA/315 or control vector pRS315 was grown overnight in SC–leu media. Cells were harvested, resuspended in YPD to an $\text{OD}_{600} = 1.5$ and incubated at 30°C for 3 hr to induce Ctr3–HAP expression under conditions of low Cu availability. Formaldehyde (4.0% final concentration) and potassium phosphate buffer at pH 6.5 (0.1 M final concentration) were added directly to the growing cells. After 40 min, the fixed cells were harvested and washed with 0.1 M potassium phosphate at pH 6.5, containing 1.2 M sorbitol (KPS). Spheroplasts were formed by incubating the cells with 1% β -mercaptoethanol and 0.25 mg/ml of Zymolyase T100 in KPS for 40 min at 30°C. Spheroplasts were washed in KPS and adsorbed to polylysine-coated multiwell slides. Cells were incubated with anti-HA antibody (12CA5) and the anti-Kar2p antibody (a generous gift from R. Fuller, University of Michigan Medical School, Ann Arbor) diluted 1:300 and 1:100, respectively, in PBST containing 10 mg/ml of BSA (PBST-B). All primary and secondary antibodies, except anti-Kar2p, were presorbed against yeast spheroplasts transformed with control vectors. After an overnight incubation at 4°C, cells were washed with PBST and incubated for 2 hr with the appropriate secondary antibodies, goat-antimouse IgG- and

IgM-conjugated fluorescein (BMB), and/or anti-rabbit IgG-conjugated rhodamine (BMB) diluted 1:500 in PBST-B. Cells were then washed in mounting solution containing DAPI (4,6-diamidino-2-phenyl-indole), and phenylamine diamine was added to each well. To localize Ctr1–myc, SLY4 was transformed with p352–myc (a generous gift from Andrew Dancis, National Institutes of Health, Bethesda, MD). The transformed cells were grown overnight in SC–uracil, and then treated identically to those described above, except that anti-myc antibodies were used. Cells were observed using a Zeiss Axioskop photomicroscope with filters for observing fluorescein, rhodamine, and DAPI fluorescence. Fields of cells were photographed with Kodak TMAX 400 print film pushed to ASA 1600 in development. The negatives were digitized to CD-ROM and optimized for contrast and sharpness using Adobe Photoshop software.

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