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THE MOLECULAR BIOLOGY OF METAL ION TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Transition metals such as iron, copper, manganese, and zinc are essential nutrients. The yeast *Saccharomyces cerevisiae* is an ideal organism for deciphering the mechanism and regulation of metal ion transport. Recent studies of yeast have shown that accumulation of any single metal ion is mediated by two or more substrate-specific transport systems. High-affinity systems are active in metal-limited cells, whereas low-affinity systems play the predominant roles when the substrate is more abundant. Metal ion uptake systems of cells are tightly controlled, and both transcriptional and posttranscriptional regulatory mechanisms have been identified. Most importantly, studies of *S. cerevisiae* have identified a large number of genes that function in metal ion transport and have illuminated the existence and importance of gene families that play related roles in these processes in mammals.

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INTRODUCTION

When medieval cartographers mapped an uncharted region, they would label it "Here there be dragons." Until recently, the field of metal ion transport in eukaryotic organisms was filled with dragons. Thanks to the many researchers studying the baker's yeast *Saccharomyces cerevisiae*, however, a wealth of new information about metal ion metabolism in all eukaryotes is now available.

Transition metals such as iron, copper, manganese, and zinc are essential nutrients that play critical roles in a variety of biochemical processes. For example, iron and copper readily donate and accept electrons and are important cofactors in electron transport and in many redox-active metalloenzymes. Zinc, which under physiological conditions is not redox active, is a catalytic component of more than 300 enzymes and in many proteins plays critical structural roles. The ability of an organism to accumulate metal ions from its diet is critical to its health. As many as two billion people suffer from malnutrition due to deficiencies of micronutrients such as metal ions. Furthermore, several genetic diseases in humans—including hereditary hemochromatosis, acrodermatitus enteropathica, Wilson disease, and Menkes disease—are caused by mutations that alter metal ion transport.

Despite their nutritional importance, metal ions, when overaccumulated, can have toxic affects. When the intracellular level of a metal ion reaches an excess, the metal can inhibit critical processes, for example by competing with other metal ions for enzyme-active sites and other important biological ligands. Excess iron or copper can also generate reactive oxygen species that degrade DNA, proteins, and lipids. Because metal ions are essential, all organisms have uptake mechanisms to accumulate these substrates from their diet. Because these same metal ions can be toxic, regulatory systems are present to control this uptake.

Early biochemical studies suggested that uptake of several metal ions by *S. cerevisiae* was mediated by a single multisubstrate system (22, 44, 89, 110). In contrast, more recent genetic studies show that baker's yeast has multiple, substrate-specific systems for the uptake of any single metal ion. When a metal ion is in short supply, high-affinity systems have been found to provide it. These systems are tightly regulated and increase in activity in metal-limited cells. This regulation is mediated by metal-responsive transcriptional regulators that increase expression of the transporter genes when their respective substrate is limiting. Each metal ion also has one or more low-affinity uptake systems,

which supply the metal ion when it is abundant. Finally, metal ions are compartmentalized into different subcellular organelles (e.g. Golgi, mitochondria, etc), so intracellular transport systems are also required. A large number of yeast genes (Table 1) implicated in metal ion transport or its regulation are discussed in this review.

IRON

Iron Uptake

Although iron is abundant in nature, the metal is most commonly found as the virtually insoluble Fe^{3+} hydroxide $Fe(OH)_3$. Thus, iron-uptake systems require strategies to solubilize Fe^{3+}. Many organisms use siderophores, low-molecular-weight molecules that are secreted by bacteria, by some fungi, and by plants, which can solubilize Fe^{3+} for uptake by siderophore-specific transport systems. In *S. cerevisiae*, a reductive mechanism is used. Extracellular Fe^{3+} is reduced to the more-soluble Fe^{2+} by reductases embedded in the cell's plasma membrane. The Fe^{2+} generated is then the substrate for two different uptake systems, a high-affinity system expressed in iron-limited cells and a low-affinity system active in iron-replete cells. Viable mutant strains have been generated that are defective in both high- and low-affinity uptake, indicating that additional, as yet uncharacterized uptake pathways are also present in *S. cerevisiae* (34).

PLASMA MEMBRANE Fe^{3+} REDUCTASES Many studies have demonstrated that *S. cerevisiae* can reduce extracellular Fe^{3+} in a variety of chelate complexes (23, 73, 74, 106, 131). The role of Fe^{3+} reduction in iron uptake was first proposed when it was observed that yeast accumulate Fe^{2+} at a faster rate than Fe^{3+} and that Fe^{3+} reductase activity is induced in iron-limited cells (74). Furthermore, heme-deficient mutants (which lack the necessary cytochromes for reductase activity, see below) were defective for Fe^{3+} reduction as well as for accumulation of iron when supplied as Fe^{3+} but not when supplied as Fe^{2+} (2, 73).

The *FRE1* gene, which was identified because null mutants have reduced Fe^{3+} reductase activity (27), encodes a component of the plasma membrane Fe^{3+} reductase. The FRE1 protein has a potential signal sequence at its amino terminus and contains several potential transmembrane domains (28). Moreover, this protein shares strong sequence similarity (62%) with the $gp91^{phox}$ subunit of cytochrome b_{558}, the phagocyte respiratory burst oxidase (111). FRE1 also contains the sequences in $gp91^{phox}$ implicated in binding the essential cofactors flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH). Cytochrome b_{558} transfers electrons across the phagosome membrane to O_2 to generate a superoxide anion, O_2^-. The superoxide anion acts as an antimicrobial agent in the phagocyte, killing microbes

Table 1 *Saccharomyces cerevisiae* genes implicated in metal ion transport and its regulation

Gene	Proposed function	Subcellular location
Iron		
AFT1	Iron-responsive transcriptional regulator	Nuclear
FET3	High-affinity system multicopper ferroxidase	Plasma membrane
FET4	Low-affinity Fe^{2+} transporter	Plasma membrane
FET5	Unknown (*FET3* homolog)	Unknown
FRE1	Fe^{3+}/Cu^{2+} reductase	Plasma membrane
FRE2	Fe^{3+}/Cu^{2+} reductase	Plasma membrane
FRE3–FRE7	Unknown (*FRE1* and *FRE2* homologs)	Unknown
FTH1	Unknown (*FTR1* homolog)	Unknown
FTR1	High-affinity system iron transporter	Plasma membrane
UTR1	Potential Fe^{3+}/Cu^{2+} reductase subunit	Cytoplasmic
YFH1	Frataxin homolog, mitochondrial iron metabolism	Mitochondria
Copper		
ATX1	Copper chaperone	Cytoplasmic
CCC2	Intracellular copper transporter	Post-Golgi
COX17	Copper chaperone	Cytoplasmic
CTR1	High-affinity Cu^{+} transporter	Plasma membrane
CTR2	Unknown (*CTR1* and *CTR3* homolog)	Unknown
CTR3	High-affinity Cu^{+} transporter	Plasma membrane
FRE1	Fe^{3+}/Cu^{2+} reductase	Plasma membrane
FRE2	Fe^{3+}/Cu^{2+} reductase	Plasma membrane
LYS7	Copper chaperone	Cytoplasmic
MAC1	Copper-responsive transcriptional activator	Nuclear
PCA1	Unknown (*CCC2* homolog)	Unknown
SCO1	Unknown, cytochrome *c* oxidase assembly	Mitochondria
SCO2	Unknown, cytochrome *c* oxidase assembly	Mitochondria
Manganese		
ATX2	Potential Mn^{2+} transporter	Golgi
BSD2	Unknown (alters SMF1 and SMF2 activity)	Endoplasmic reticulum
CCC1	Potential Mn^{2+} transporter	Golgi
PMR1	Mn^{2+}/Ca^{2+} P-type ATPase transporter	Golgi
SMF1	High-affinity Mn^{2+} transporter	Plasma membrane
SMF2	Unknown (*SMF1* homolog)	Unknown
SMF3	Unknown (*SMF1* homolog)	Unknown
HUM1	Mn^{2+}, Ca^{2+}/H^{+} exchange transporter	Vacuolar
Zinc		
COT1	Intracellular Zn^{2+}/Co^{2+} transporter	Intracellular, unknown
ZAP1	Zinc-responsive transcriptional activator	Nuclear
ZRC1	Intracellular Zn^{2+} transporter	Intracellular, unknown
ZRT1	High-affinity Zn^{2+} transporter	Plasma membrane
ZRT2	Low-affinity Zn^{2+} transporter	Plasma membrane

contained within the phagosome. Although the substrate of cytochrome b_{558} is O_2, the substrate of the yeast enzyme is Fe^{3+} (71, 115).

Strains mutated in the FRE1 gene have reduced Fe^{3+} reductase activity and are unable to grow under iron-limiting conditions. Although defective for uptake of Fe^{3+}, *fre1* mutants can accumulate Fe^{2+} (27, 39). Surprisingly, *fre1* mutants retain a low level of iron-regulated Fe^{3+} reductase activity (5, 28), dependent on a second gene, *FRE2* (46). *FRE2* encodes a protein related to FRE1; these proteins share 24.5% amino acid sequence identity. Analysis of the *S. cerevisiae* genome sequence also detected five additional genes (*FRE3–FRE7*) that are closely related to *FRE1* and *FRE2* (DR Winge, personal communication). Their roles in iron metabolism or the metabolism of other metal ions (e.g. copper, see below) are unknown. In addition to the membrane-bound reductases, excreted compounds may also contribute to Fe^{3+} reduction under some growth conditions (46, 75).

Initial attempts to purify an active Fe^{3+} reductase from yeast plasma membranes were unsuccessful, perhaps because multiple subunits are required for activity. A partially purified sample of plasma membrane Fe^{3+} reductase was characterized, and consistent with the relationship of FRE1 and FRE2 with $gp91^{phox}$, this crude preparation required NADPH and FAD for activity (72). More-recent studies indicated that FRE1 is a plasma membrane flavocytochrome; FRE1 is glycosylated and enriched in plasma membrane preparations (71). Yeast plasma membranes have an absorbance spectrum of a *b*-type cytochrome similar to that of cytochrome b_{558}; the magnitude of this absorbance correlated with the level of Fe^{3+} reductase activity and the level of FRE1 (71, 115). Plasma membrane FAD levels also showed a correlation with FRE1 expression level (115).

The *UTR1* gene encodes a potential cytoplasmic subunit required for Fe^{3+} reductase activity (4). A *utr1* null mutant retains only 5% of the wild-type Fe^{3+} reductase activity. Overexpression studies also indicated a role for UTR1 in Fe^{3+} reductase activity; overexpression of either FRE1 or UTR1 alone failed to increase Fe^{3+} reductase activity, whereas overexpressing both proteins resulted in a fivefold increase in activity (71). The predicted product of the *UTR1* gene is hydrophilic, which suggests that this protein is soluble and cytoplasmic. However, no similarity exists between UTR1 and the cytoplasmic p47 and p67 subunits of the phagocyte cytochrome b_{558}, so the role of UTR1 in Fe^{3+} reductase activity is unclear.

LOW-AFFINITY IRON UPTAKE A low-affinity Fe^{2+} uptake system is responsible for iron accumulation in iron-replete cells (34, 39). This system has an apparent K_m of approximately 30 μM Fe^{2+}. The low-affinity system is specific for Fe^{2+} over Fe^{3+} but may be capable of transporting other metal ions as well (34). This is suggested by the observation that Fe^{2+} uptake is inhibited by high

concentrations of Ni^{2+}, Cd^{2+}, Co^{2+}, and Cu^{2+}. Moreover, overexpressing the low-affinity system transporter (FET4, see below) made cells hypersensitive to these other metals (32). Thus, the low-affinity iron uptake system may also play a relevant role in the uptake of other metals.

The *FET4* gene encodes the transport protein of the low-affinity system. This gene was cloned because its overexpression suppresses the growth defect of a yeast mutant defective for high-affinity uptake (see below) (34). Overexpression of FET4 increased low-affinity uptake whereas a disruption mutation eliminated this activity. FET4 contains six potential transmembrane domains, is an integral membrane protein, and is localized to the plasma membrane (33). Furthermore, mutations affecting potential Fe^{2+} ligands in the transmembrane domains of FET4 significantly altered the apparent K_m and/or V_{max} of the low-affinity system. This protein is unique and has no significant similarity to any other genes contained in the current (i.e. 10/97) sequence databases.

HIGH-AFFINITY IRON UPTAKE When iron becomes limiting, a second uptake system is induced that has a 200-fold higher affinity for iron (apparent $K_m = 0.15\ \mu M\ Fe^{2+}$) (39). Although the low-affinity system may be capable of transporting other metal ions, the high-affinity system is exquisitely specific for Fe^{2+}. Insight into the molecular mechanism of high-affinity uptake was first provided by the identification of the *FET3* gene (7). Cells mutant in the *FET3* gene grow poorly on iron-limited media and are defective for high-affinity iron uptake. The *FET3* gene is not required for low-affinity uptake activity, nor is the *FET4* gene required for high-affinity activity. This indicates that these pathways of iron accumulation are separate (7, 34).

FET3 encodes a protein with remarkable similarity to a family of enzymes known as multicopper oxidases (7, 31); these include laccase, ascorbate oxidase, and ceruloplasmin. Multicopper oxidases carry out a variety of different functions, including wound healing in plants (14), lignin degradation by fungi (3), and copper resistance in bacteria (18). Despite this functional diversity, these proteins all share an identical characteristic: They catalyze four single-electron oxidations of substrate, an action that is followed by a four-electron reduction of O_2 to generate two molecules of H_2O. This activity requires four to six copper atoms that are bound by a group of highly conserved ligands, which are present in these proteins as well as in FET3. FET3 also has a signal sequence located at its amino terminus and a carboxy-terminal transmembrane domain. These observations suggest that FET3 is an integral membrane protein whose oxidase catalytic domain is located on the cell surface, a fact that has been confirmed (31, 133).

The sequence of FET3 and the effects of *FET3* mutations on high-affinity uptake suggest that copper-dependent oxidation is an important step in this

process. This hypothesis has been supported in several ways. Copper-deficient cells also have low levels of high-affinity iron uptake activity (7, 29). FET3 has a copper-dependent oxidase activity in vitro (133) that is required in vivo for a mitochondrial-independent O_2 consumption that also requires Fe^{2+} (31). The importance of copper in iron uptake was also demonstrated by analyses of genes required for intracellular copper transport. *CCC2* encodes a P-type ATPase transporter that delivers copper to FET3 in a post-Golgi compartment of the secretory pathway (43, 133), and the *ATX1* gene encodes a cytoplasmic copper-binding protein that delivers copper to *CCC2* (80, 81) (see below). Although *ccc2* and *atx1* mutants show no defect in plasma membrane copper uptake or in their intracellular level of copper, they are both defective for high-affinity iron uptake. Copper incorporation into FET3 also requires the product of the *VPS41* gene, which normally plays a role in the trafficking of proteins in the post-Golgi–vacuolar pathway (104). The iron uptake defect in *vps41* mutants may result from mislocalization of the CCC2 protein.

The observation that FET3 contains only a single transmembrane domain suggested that this protein may be only one subunit of a heteromeric protein complex. A potential second subunit of the high-affinity system is the product of the *FTR1* gene (116), which is also required for activity of this system. *FTR1* encodes a protein of 404 amino acids with an amino-terminal signal sequence and six potential transmembrane domains, a feature consistent with its proposed role as a transporter. FTR1 shares no amino acid similarity to other known metal transporters. Genes similar to *FTR1*, but of unknown function, were identified in the genomes of *Schizosaccharomyces pombe* and *Bacillus subtilis* (116). Evidence for a role for FTR1 as the permease subunit of the high-affinity system was provided by mutational analyses (116). Specifically, mutations were generated that deleted a series of glutamates, in repeats of EXXE, located at the carboxyl terminus. Also, either or both of the glutamates in a sequence found within a predicted transmembrane domain, REGLE, were replaced with alanine residues. All these mutations eliminated Fe^{2+} uptake activity. In marked contrast to the effects of an *ftr1* deletion mutation (see below), however, FET3 in these strains was properly localized to the plasma membrane. Interestingly, the REGLE sequence is conserved in the FTR1-related proteins as well as in the ferritin iron storage protein, where it is thought to line an iron-selective pore in the protein (121).

FTR1 is also necessary for movement of FET3 through the secretory pathway to the plasma membrane. This was first indicated by the observation that the *ftr1* deletion mutant had markedly reduced FET3 oxidase activity (116). Oxidase activity could be restored by adding copper in vitro, which suggests that the defect was in copper loading of FET3. This copper-reconstituted protein, however, was incorrectly glycosylated, indicating that in the absence of FTR1,

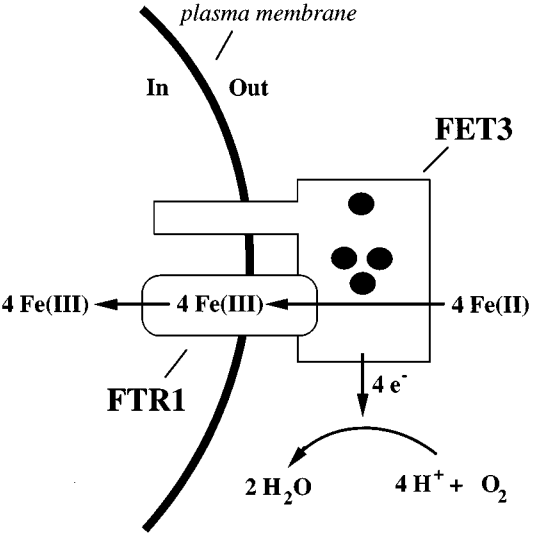

Figure 1 Proposed mechanism of iron uptake by a FET3/FTR1 oxidase/permease complex. Fe^{2+} is generated by the activity of the Fe^{3+} reductases FRE1 and FRE2. This Fe^{2+} is oxidized to Fe^{3+} by the FET3 ferroxidase subunit of the high-affinity transporter complex and then passed to the FTR1 permease subunit for transport into the cell. Sequential oxidation of 4 Fe^{2+} atoms is followed by a single four-electron reduction of O_2 to $2H_2O$. (*black circles*) Copper atoms bound by FET3.

FET3 is mislocalized within the secretory pathway. Similarly, it was found that in the absence of FET3, FTR1 is retained in the endoplasmic reticulum. These results suggested that assembly of a FET3/FTR1 complex is required for either protein to proceed through the secretory system to the plasma membrane.

The oxidase permease model of high-affinity iron uptake One model of how FET3 and FTR1 mediate Fe^{2+} uptake is described in Figure 1. In this model, Fe^{2+} produced by the Fe^{3+} reductases is oxidized to Fe^{3+} by the FET3 multicopper oxidase. The Fe^{3+} product is then transferred directly to an Fe^{3+} binding site on the FTR1 permease and, following a conformational change in the permease, delivered to the cytoplasm. This hypothesis is especially intriguing given previous studies on another multicopper oxidase, ceruloplasmin. Ceruloplasmin is a mammalian serum protein that can also oxidize Fe^{2+} to Fe^{3+} (96), and many studies have linked it to iron transport. Copper-deficient swine had lowered levels of ceruloplasmin and decreased transport of iron from intestinal mucosal cells and macrophages into the blood plasma (70). Iron transport increased when either copper or ceruloplasmin was injected into these copper-deficient

animals. More recently, a genetic defect in humans was identified in which a mutation in the ceruloplasmin gene results in the apparent absence of circulating ceruloplasmin (54, 132). This mutation also causes anemia and low serum-iron levels. Thus, the ceruloplasmin and FET3 multicopper oxidases have been implicated in the movement of iron across cellular membranes. It is unclear how these oxidases mobilize iron in opposite directions, i.e. how FET3 transports iron into cells while ceruloplasmin transports iron out of cells. One explanation may lie with the characteristics of the particular transport protein with which the oxidase interacts. For example, although FET3 is coupled to an Fe^{3+} transporter, ceruloplasmin may work in conjunction with an Fe^{2+} transporter and cause oxidation of the substrate to Fe^{3+} following its exit from the cell as Fe^{2+}.

A second oxidase/permease complex may also be present in *S. cerevisiae*. Homologs of both *FET3* and *FTR1* [*FET5* (36) and *FTH1* (116), respectively] are present in the *S. cerevisiae* genome. Both *FET5* and *FTH1* genes are regulated by iron, and their mRNA levels increase severalfold in iron-limited cells (36, 130). Furthermore, FET5 is a glycosylated integral membrane Fe^{2+} oxidase that, when overexpressed, can suppress the iron uptake defect of a *fet3* mutation (36). It is unclear what role FET5 and FTH1 play in the transport of iron across the plasma membrane or, perhaps, across the membrane of an intracellular compartment.

REGULATION Iron uptake in *S. cerevisiae* is tightly regulated in response to the availability of iron in the growth medium. For example, transfer of growing cells from an iron-rich to an iron-limiting medium causes a greater than 30-fold increase in Fe^{3+} reductase and high-affinity uptake activities (27, 39). The primary level of this control is transcriptional. Iron-limited growth increases the level of mRNA synthesized from the *FRE1*, *FRE2*, *FET3*, *FTR1*, *FET5*, and *FTH1* genes (7, 28, 36, 46, 130). Iron also controls the transcription of the copper transport genes *CCC2* and *ATX1* (see below), further demonstrating their importance to iron uptake by delivering copper to FET3 (81, 130). Regulation of these genes is mediated by the iron-responsive transcription factor AFT1, which activates their expression in iron-limited cells (16, 81, 128, 130).

The low-affinity Fe^{2+} uptake system is also iron regulated but in an AFT1-independent manner (16, 33). The V_{max} of the low-affinity system increases approximately fourfold in iron-limited cells compared with iron-replete cells (33). This result is consistent with an increased number of transporter proteins on the plasma membrane; the level of FET4 protein shows a similar increase in iron-limited cells. Because *FET4* mRNA levels do not change in response to iron limitation (16), increased FET4 activity in response to iron occurs through an unknown posttranscriptional mechanism. *FET4* mRNA levels are repressed

in cells grown on respired carbon sources (16), but the mechanism of this regulation has not been determined.

Intracellular Iron Transport

Most eukaryotes store intracellular iron in ferritin. Despite an initial report to the contrary (105), *S. cerevisiae* does not produce this protein. Rather, the major iron storage compartment in yeast is the vacuole (105), the lysosome-like organelle that also stores a variety of other ions (62). Vacuolar iron transport systems have been detected biochemically (10), but the genes responsible for this activity have not been identified.

Another important destination for intracellular iron is mitochondria, where it is used for heme synthesis or assembly of nonheme iron proteins. Although little is known about the transport proteins responsible for iron uptake into the mitochondria, recent studies have established an exciting parallel between the control of mitochondrial iron accumulation in yeast and humans. This parallel comes from studies of the gene responsible for Friedreich's ataxia (FRDA), a human neurodegenerative disease, and its yeast homolog, *YFH1*. FRDA is an autosomal recessive genetic disorder affecting approximately 1 person out of 50,000. The disease is characterized by progressive gait and limb ataxia, hypertrophic cardiomyopathy, and an increased incidence of diabetes mellitus (35). Respiratory deficiency due to mitochondrial dysfunction was proposed to be a contributing factor to FRDA pathophysiology because other diseases known to affect mitochondria share many of these same clinical attributes (9).

The human gene responsible for FRDA was isolated by positional cloning (15). Its protein product, called frataxin, is a 210–amino acid protein that is similar to proteins of unknown function from *Caenorhabditis elegans* (49%) and *S. cerevisiae* (31%). Several observations suggested a link between frataxin and mitochondrial function. The human, nematode, and yeast proteins each contain a potential mitochondrial signal sequence. Human frataxin is expressed at its highest levels in tissues that are rich in mitochondria (66). Furthermore, both YFH1 and FRDA proteins localize to mitochondria (8, 66, 127). Yeast cells in which the *YFH1* gene was deleted had reduced respiratory O_2 consumption and rapidly lost their ability to grow on respired carbon sources (8, 41, 66, 127). This defect correlated with a high rate of mitochondrial DNA loss. The pivotal link between frataxin and iron metabolism was made when it was found that mitochondria in *yfh1* mutants accumulate approximately tenfold more iron than do the mitochondria of wild-type cells (8, 41). These results suggest that mitochondrial iron deposition could be responsible for the etiology of FRDA, i.e. DNA loss may occur as a result of iron-catalyzed oxidative damage. It remains to be seen what specific role frataxin plays in mitochondrial iron metabolism. It may act as a negative regulator of mitochondrial iron uptake or as a positive regulator

of mitochondrial iron efflux. Frataxin may also play a role in the utilization of iron in mitochondria for heme biosynthesis and/or iron-sulfur cluster formation.

COPPER

Copper Uptake

In *S. cerevisiae*, copper is a cofactor in Cu/Zn–superoxide dismutase, cytochrome *c* oxidase, and the FET3 and FET5 multicopper oxidases. Copper also activates the ACE1 transcription factor, which then activates transcription of the *CUP1* and *CRS5* copper metallothionein genes. Copper uptake in this yeast, like iron uptake, is mediated by separate high- and low-affinity systems. Also like the iron uptake systems, high-affinity copper uptake requires plasma membrane reductases to reduce Cu^{2+} to Cu^{+}. In fact, this reduction is mediated by the same plasma membrane reductases, FRE1 and FRE2 (47, 55), that are involved in iron uptake. Once reduced to Cu^{+}, the ion is taken up by separate high-affinity transporter proteins encoded by the *CTR1* and *CTR3* genes. High-affinity copper uptake is energy-dependent and specific for Cu^{+} over other metal ions (29, 30, 78). This process is also saturable with an apparent K_m of 1–4 μM copper (29, 30, 77, 78). Cu^{+} uptake and K^{+} efflux were coupled and had a 1:2 stoichiometry (30). This observation suggested that high-affinity Cu^{+} uptake occurs via a $Cu^{+}/2K^{+}$ antiport mechanism. A third, lower-affinity system for copper uptake has also been detected in yeast (29), but neither the biochemical properties nor the gene(s) responsible for this activity is known. Evidence for low-affinity uptake of copper by endocytosis has also been obtained (81).

Because of copper's role in iron uptake, the *CTR1* gene was originally identified in a genetic screen for yeast mutants defective for high-affinity iron uptake (29). Further study demonstrated the role of *CTR1*'s protein product in high-affinity copper uptake. The CTR1 protein is 406 amino acids long with three potential transmembrane domains and is found in the plasma membrane. Moreover, CTR1 can form multimeric complexes with itself in vivo that may represent the functionally active form of the transporter (26). The amino-terminal 128 amino acids of CTR1 are rich in methionine and serine residues and contain several potential copper binding sites. Specifically, there are three 19–amino acid repeats and 11 shorter motifs that each contain the sequence Met-X_2-Met. The possible role of these sequences in copper transport is unclear, but their importance is supported by the observation that similar motifs are also found in bacterial copper-resistance proteins, e.g. CopA of *Pseudomonas syringae* (18), the CopB protein of *Enterococcus hirae* (90), and the CutE (109) protein of *Escherichia coli*.

In addition to CTR1, some yeast strains have a second high-affinity uptake system for copper that requires the *CTR3* gene (64). CTR3 encodes a protein,

with 241 amino acids and three potential transmembrane domains, that shows some sequence similarity to CTR1. Although initially localized to an intracellular organelle (64), more recent results have shown a plasma membrane location for CTR3 (DJ Thiele, personal communication) that is more consistent with its proposed role as a copper-uptake transporter. Surprisingly, *CTR3* is disrupted by a transposon insertion in most commonly studied laboratory strains of *S. cerevisiae*. In the few strains where *CTR3* is expressed, both it and *CTR1* make significant contributions to copper accumulation in copper-limited cells.

Finally, an additional *S. cerevisiae* gene, *CTR2*, may play a role in copper metabolism. *CTR2* was identified because its product has sequence similarity to the COPT1 copper transporter (23% identity and 38% similarity) (see below) of the plant *Arabidopsis thaliana* (59). A mutation in *CTR2* does not alter copper requirements (even in a *ctr1 ctr3* strain), indicating that *CTR2* probably does not encode the low-affinity copper transporter. Nonetheless, *ctr2* mutants are more resistant to copper, and cells overexpressing *CTR2* are more sensitive to copper overload, which suggests that this gene does play a role in copper accumulation or intracellular compartmentalization.

REGULATION The high-affinity copper transporters CTR1 and CTR3 are induced in copper-limited cells (26, 64). Moreover, the FRE1 Fe^{3+}/Cu^{2+} reductase is also regulated by copper status independent of its regulation by iron and AFT1 (55). [The FRE2 Fe^{3+}/Cu^{2+} reductase is not regulated by copper despite the ability of this enzyme to reduce that ion for uptake (47).] Copper-responsive regulation of *CTR1*, *CTR3*, and *FRE1* expression occurs at the transcriptional level and is mediated by a copper-responsive transcriptional activator encoded by the *MAC1* gene (47, 50, 55, 57, 67, 129). A second mechanism for regulating copper uptake in yeast is at the posttranslational level. Although the CTR1 protein is stable in copper-limited cells, copper treatment causes the protein to be degraded rapidly through an unknown mechanism that does not require endocytosis (94). This system may provide additional protection from copper toxicity by preventing metal overaccumulation during acute copper exposure.

A FAMILY OF EUKARYOTIC COPPER TRANSPORTERS Functional expression cloning in yeast mutants defective for copper uptake has been instrumental in identifying copper transport proteins from both animals and plants. This method involves constructing a cDNA library from an organism of interest in a yeast expression vector and screening this library for clones that can complement a yeast mutant phenotype. The *Arabidopsis COPT1* gene was isolated in this manner because its expression in yeast complements a *ctr1 ctr3* mutant for growth on copper-limiting media (59). A human gene, *hCTR1*, was cloned in a similar fashion from HeLa cells (137). Overexpression of *COPT1* and *hCTR1* in yeast caused hypersensitivity to copper in the medium. Therefore, although

copper uptake was not directly examined in these studies, the available data strongly suggest that *COPT1* and *hCTR1* encode copper transport proteins. Human *hCTR1* is expressed in many tissues, which suggests a ubiquitous role for the hCTR1 protein in copper transport in human cells.

COPT1 and hCTR1 are related proteins (56% similarity), which are also related to yeast CTR1. For example, COPT1 and CTR1 share 49% sequence similarity. Also like CTR1, COPT1 and hCTR1 have three potential transmembrane domains and have the methionine- and serine-rich domains at their amino-terminal ends. COPT1 (169 amino acids) and hCTR1 (190 amino acids) are significantly smaller than CTR1 (406 amino acids), mostly because of deletions at their carboxy-terminal ends. Interestingly, it has been recently demonstrated that the carboxy-terminal 78 amino acids of CTR1 are not required for function of this transporter (94). These proteins are the founding members of a larger family of related transporters. Additional members of this family have already been identified in the current databases as expressed sequence tags from humans, rats, and mice (137). Thus, copper transport in all eukaryotes may be mediated by this family of proteins.

Intracellular Copper Transport

Upon entering the cell, copper is quickly bound by intracellular copper ligands (76, 77) and is then delivered to cytoplasmic proteins such as Cu/Zn superoxide dismutase. If copper levels are high, the metal is also bound by the metallothioneins encoded by the *CUP1* (13, 60) and *CRS5* (24) genes, whose expression is activated by the copper-binding ACE1 transcriptional regulator (24, 120). In addition to these cytoplasmic and nuclear ligands, intracellular transporters move copper into cellular compartments like the mitochondria for cytochrome *c* oxidase assembly and the secretory pathway for FET3 assembly.

Recent studies have clarified how copper is partitioned to different proteins and into different compartments within the cell. An example alluded to earlier is the role of CCC2 in delivering cytoplasmic copper to FET3 in a post-Golgi compartment of the secretory system (43, 133). CCC2 is a member of the P-type ATPase family, whose members transport a large variety of cations. Among these proteins, CCC2 bears the greatest similarity to the human Menkes (MNK) and Wilson disease (WD) transporters (133). Both MNK and WD are intracellular transporters that pass copper from the cytoplasm into the secretory system. In the human liver, this copper transport is mediated by the WD protein (12, 119). The copper is then excreted into the bile or loaded into ceruloplasmin for secretion into the blood plasma. The MNK protein exports copper from other tissues, including the intestinal mucosa (19, 123). Thus, mutations in the *MNK* gene block the absorption of dietary copper.

CCC2 shares 31% amino acid identity to WD and 29% identity to MNK. Furthermore, CCC2 contains two putative metal-binding motifs (MTCXXC)

at its amino terminus; this same sequence is also found in six copies in the amino termini of both MNK and WD. This motif was originally identified in a bacterial mercury-binding protein, MerP; the two cysteines were critical for metal binding by this protein (113). Although the exact role of this motif in the P-type ATPases is not clear, it may serve as a binding site for cytoplasmic copper prior to transport (102). A second P-type ATPase in *S. cerevisiae*, closely related to CCC2, is encoded by the *PCA1* gene (103), but its potential function in copper transport has not been examined.

INTRACELLULAR COPPER CHAPERONES Given the potential toxicity of copper, it is unlikely that the free metal ion exists at high levels in the cytoplasm. We now know that copper is delivered to specific proteins within the cell (e.g. Cu/Zn superoxide dismutase, CCC2, etc) through separate and distinct pathways. This delivery is mediated by soluble cytoplasmic proteins called copper chaperones (Figure 2). These proteins bind copper after it enters the cell and subsequently donate the bound copper to their corresponding recipient proteins. For example, ATX1 is the copper chaperone that delivers copper specifically to the CCC2 P-type ATPase. ATX1 is a small protein (73 amino acids) that contains one of the MTCXXC metal-binding motifs described previously (80). Consistent with its proposed role in copper trafficking, ATX1 is soluble and localizes to the cytoplasm (81). *atx1* deletion mutants are unable to grow on iron-limited media because of impaired iron uptake activity. This defect can be suppressed by treating the cell with high copper, which suggests that copper delivery to FET3 is defective in the *atx1* mutant. Indeed, Klomp et al (63) showed that incorporation of copper into FET3 in vivo was defective in the *atx1* mutant. ATX1 is not required for incorporating copper into SOD1 or cytochrome *c* oxidase (81), indicating the ATX1 plays a specific role in delivering copper to the iron uptake system. This specificity of function is further demonstrated by the observation that *ATX1* mRNA levels are coordinately regulated with the components of the high-affinity iron uptake system through the AFT1 transcriptional regulator (81).

Copper binding by ATX1 in vitro has recently been demonstrated where copper was bound as Cu^{+} and not as Cu^{2+} (102). Cu^{+} binding was found in either a two-coordinate Cu-thiolate complex or a three-coordinate complex containing two thiol ligands and a third, unidentified ligand. A copper-dependent, protein-protein interaction between ATX1 and the amino terminus of CCC2 (where the two MTCXXC sequences are located) was also demonstrated. These results suggest that a copper-exchange mechanism exists in which ATX1 directly donates cytoplasmic Cu^{+} to CCC2 prior to transport. This mechanism would allow for diffusion-driven movement of copper from one site to another in the cell, perhaps by passing copper progressively from weaker to stronger binding sites.

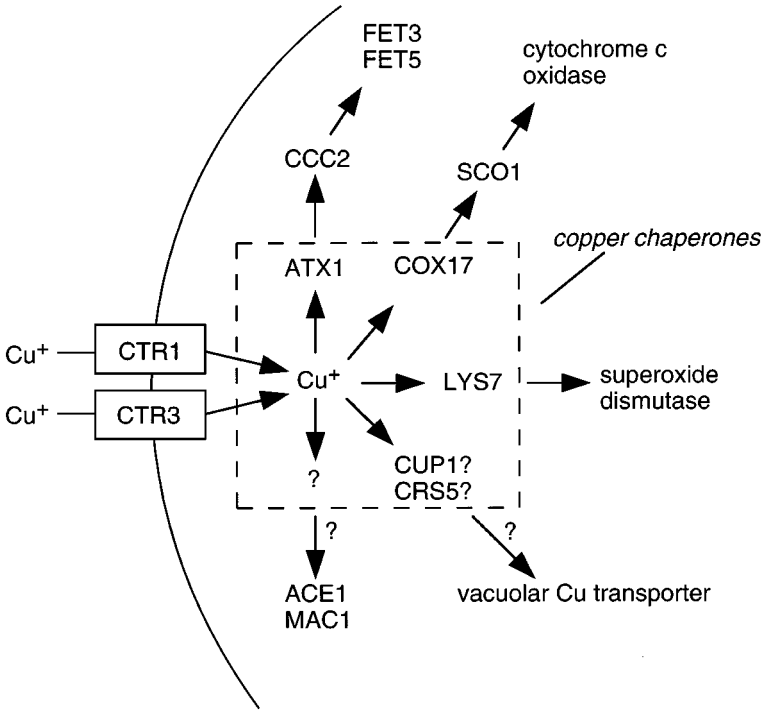

Figure 2 Proposed role of copper chaperone proteins in the partitioning of cytoplasmic copper. Cu^{+} enters the cell through one of the copper uptake systems. Upon entering the cytoplasm, the copper is bound by a cytoplasmic chaperone and then delivered to the appropriate target protein. It is unknown if copper chaperones deliver copper to either the ACE1 or MAC1 copper-responsive transcriptional activators or to a vacuolar copper transporter. Likewise, a role for the CUP1 or CRS5 copper metallothioneins as copper chaperones is speculative.

COX17 appears to be a copper chaperone that delivers copper to the mitochondria for cytochrome *c* oxidase assembly (48). *cox17* mutants fail to assemble a functional cytochrome *c* oxidase complex. These cells express normally all subunits of the complex, indicating that COX17 is required for a posttranslational step in assembly. This defect is suppressed by high copper, which suggests a defect in copper transport into the mitochondria. Superoxide dismutase activity is normal in the cox17 strain, showing that this mutation does not cause major perturbations in copper metabolism. The COX17 protein product, which bears no obvious sequence similarity to ATX1, is a cysteine-rich protein (10%) of approximately 8 kDa. Cell fractionation experiments demonstrated that COX17 is both soluble and cytoplasmic. As was the case with

ATX1, copper binding by COX17 in vitro has been demonstrated (DR Winge, personal communication). A mitochondrial copper transporter that could serve as the recipient for copper delivered by COX17 has not yet been identified. SCO1 and SCO2, both integral membrane proteins found in the mitochondrial inner membrane, may also participate in the transfer of copper from COX17 to cytochrome *c* oxidase (49).

A third copper chaperone in yeast, LYS7, is required to incorporate copper into Cu/Zn superoxide dismutase (SOD1) (25). LYS7 is larger (249 amino acids) than either ATX1 or COX17, but it also contains an MTCXXC copper-binding motif. *lys7* deletion mutants contain normal levels of SOD1 protein but fail to incorporate copper into the enzyme. This copper delivery defect is specific to SOD1; no defect was observed in delivery of copper to cytochrome *c* oxidase, to FET3, or to the ACE1 copper-responsive transcription factor that controls *CUP1* and *CRS5* expression. As was the case with ATX1 and COX17, LYS7 is also a soluble, cytoplasmic protein.

It seems likely that additional copper chaperones exist in the cell. Excess copper is probably stored in the vacuole (37, 124), which suggests that a copper chaperone, perhaps even the CUP1 or CRS5 metallothioneins, could deliver copper to a vacuolar copper transporter. Furthermore, copper chaperones could deliver copper to the copper-responsive transcriptional activators, ACE1 and MAC1. One intriguing feature of copper chaperones is the specificity they show with respect to the particular target proteins to which they deliver copper. A likely hypothesis for this specificity is that unique protein-protein interactions occur between the chaperone and the recipient during copper delivery. Copper chaperones may also play a very active role in regulating the distribution of cellular copper, i.e. controlling expression of a particular chaperone would influence the partitioning of cytoplasmic copper. For example, the induction of ATX1 by iron-limiting growth conditions (81) suggests that increasing the level of this chaperone redirects copper to the secretory pathway.

Copper chaperone proteins appear to function in copper trafficking in human cells as well as in yeast. Human homologs of ATX1, COX17, and LYS7 (i.e. HAH1, hCOX17, and CCS, respectively) have been identified by database analysis (25, 63) and by functional expression cloning (1). Expressing these proteins in their corresponding mutant restores copper trafficking and suppresses the associated phenotypic defects. For example, expression of HAH1 in an *atx1* mutant strain restores copper delivery to FET3 and high-affinity iron uptake (63). The human proteins share considerable similarity to their yeast counterparts; ATX1 and HAH1 share 47% amino acid sequence identity, COX17 and hCOX17 share 32% identity, and LYS7 and CCS share 28% identity. Although the tissue specificity of hCOX17 expression is not yet known, both HAH1 and CCS are expressed in a wide variety of human cell types. This suggests that these proteins play fundamental roles in cellular copper handling.

MANGANESE

Manganese Uptake

In yeast, manganese is a cofactor of several enzymes, including mitochondrial superoxide dismutase (107) and arginase (51). Surprisingly, although a role for Ca^{2+} in the control of yeast cell cycle progression is widely accepted, Mn^{2+} can completely replace Ca^{2+} as a mediator of this process (84). Many early studies of Mn^{2+} accumulation in yeast focused on uptake of the substrate at extremely high concentrations (i.e. >1 mM) and, therefore, only examined low-affinity systems (88, 91, 93, 110). Low-affinity Mn uptake was competitively inhibited by Mg^{2+}, which suggests that this ion is also a substrate for Mn^{2+} transport systems. A study of Mn^{2+} uptake at lower substrate concentrations (i.e. <100 μM) detected two separate uptake systems, a high-affinity system with an apparent K_m of 0.3 μM and a low-affinity system with a K_m of 60 μM (45). The *SMF1* gene encodes a plasma membrane protein that is probably the transporter of the high-affinity system. When overexpressed, this gene suppresses the effect of mutational defects in Mn^{2+}-dependent processes, i.e. cell cycle control (117) and mitochondrial protein processing (125). These results suggest that increased *SMF1* expression aided manganese accumulation. Supporting this hypothesis, uptake assays demonstrated that *SMF1* overexpressing cells had elevated rates of high-affinity Mn^{2+} uptake activity (117). Moreover, an *smf1* mutant grew poorly on Mn^{2+}-limiting media and had reduced high-affinity activity. Two other genes in *S. cerevisiae*, *SMF2* (125) and *SMF3* (118), encode proteins that are closely related to SMF1. SMF2 has recently been linked to Co^{2+} uptake (83).

REGULATION Transcriptional regulation of Mn^{2+} accumulation in yeast has not been examined. The *BSD2* gene may play a posttranscriptional role in controlling metal ion accumulation by SMF1 and SMF2 (82). *bsd2* mutants overaccumulate Mn^{2+} and Co^{2+} in SMF1- and SMF2-dependent processes, respectively (83). Thus, BSD2 normally exerts a negative control over the SMF1 and SMF2 metal transporters. This influence is apparently indirect, given that the BSD2 protein is an integral membrane protein found in the endoplasmic reticulum rather than on the plasma membrane (83).

THE ROLES OF NRAMP PROTEINS IN EUKARYOTIC METAL ION TRANSPORT The yeast SMF proteins are members of a family of transporters found in many eukaryotic organisms. The first known member of this family, mouse Nramp1 (natural resistance-associated macrophage protein), is required for resistance to infection by pathogenic bacteria such as *Mycobacterium bovis* and *Leishmania donovani* (122). Resistance to these pathogens requires macrophage phagocytosis. Once the bacterium is engulfed within the phagosome, the macrophage

produces a burst of reactive oxygen and nitrogen species that kill the pathogen. This ability to kill intracellular pathogens is compromised in mice expressing mutant Nramp1. Genes encoding *Nramp1* homologs have been cloned from humans as well as other mammals. A second gene, *Nramp2*, is also found in mammals (52), and additional Nramp-related transporters have been identified in birds, insects, and plants (17).

Nramp proteins contain 10–12 potential transmembrane domains, and all members share extensive sequence conservation (i.e. >40% similarity and >25% identity) (17). These proteins also contain a short sequence motif often found in prokaryotic and eukaryotic membrane proteins. This motif was originally identified in bacterial traffic ATPase transporters and was proposed to couple peripheral ATP-binding proteins to the transporters (56). However, the function of this domain in Nramp proteins is unknown.

There is growing evidence that Nramp proteins are a family of metal ion transporters. Studies of yeast SMF1 demonstrate the role of this protein in Mn^{2+} uptake. In *Drosophila melanogaster*, the *malvolio* (*mvl*) gene is required for flies to discriminate between food containing or lacking sugar (108): Mutant *mvl* flies feed with equal frequency from these two food supplies, whereas wild-type flies prefer the sugar-laden diet. Dietary Mn^{2+} or Fe^{2+} supplements restored normal taste behavior whereas Ca^{2+}, Mg^{2+}, and Zn^{2+} failed to reverse the mutant phenotype (S Orgad, H Nelson, D Segal, N Nelson, submitted for publication). The role of Mn^{2+} and/or Fe^{2+} in taste perception is not clear, but these results suggest that Mvl is a metal transporter required for this process to occur normally.

A third example of the role of Nramp proteins in metal ion transport comes from recent analyses of mammalian members of the family. The rat *Nramp2* gene was cloned because it confers increased Fe^{2+} uptake when expressed in *Xenopus* oocytes (53). Injection of mRNA from the duodenum of iron-deficient rats into oocytes generated iron-uptake activity severalfold higher than that achieved with mRNA from iron-replete animals. The uptake activity observed in injected oocytes showed biphasic kinetics, which suggests the presence of high- and low-affinity systems with apparent K_m values of 10 and 85 μM. The cDNA responsible for the high-affinity activity was isolated and found to be the rat *Nramp2* gene. Nramp2 is also capable of transporting other metal ions in addition to Fe^{2+}, including Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, and Ni^{2+}. This broad substrate specificity led to the designation of rat *Nramp2* as DCT1 (divalent cation transport gene 1). Cation transport mediated by Nramp2/DCT1 was rheogenic, voltage dependent, and H^+-coupled. These results suggested that the electrochemical H^+ gradient that exists across the plasma membrane of eukaryotic cells is a major driving force for cation uptake by Nramp2/DCT1 and probably the other Nramp proteins as well.

Nramp2/*DCT1* is expressed in a wide variety of tissues (52, 53). In the intestine, this mRNA was most abundant in the duodenum and less abundant in the jejunum, ileum, and colon. Within the duodenum, expression was highest in enterocytes located in the duodenal crypt and lower segment of the villi. This pattern of expression corresponds well with the major sites of dietary iron absorption and suggests a role for Nramp2/DCT1 in this process. *Nramp2*/*DCT1* mRNA was also detected in other tissues, including kidney, liver, heart, and brain. In all tissues examined, expression was increased in rats fed an Fe-deficient diet (53). It was proposed that this regulation was mediated by the posttranscriptional regulation of message stability. This hypothesis was based on the presence of a consensus iron-responsive element (IRE) in the 3′ untranslated region of the *Nramp2*/*DCT1* mRNA. IREs found in the 3′ untranslated region of the transferrin receptor (Tf R) mRNA are binding sites for a protein, the IRE binding protein (IRE-BP) (61). IRE-BP binding in iron-deficient cells prevents Tfr mRNA degradation and leads to increased synthesis of Tf R and increased iron accumulation. The presence of a potential IRE in the 3′ untranslated region of *Nramp2*/*DCT1* mRNA suggests that this gene is coordinately regulated with TfR.

Genetic evidence also strongly supports a role for Nramp2/DCT1 in iron absorption. The mouse homolog of *Nramp2* was identified by positional cloning as the gene responsible for microcytic anemia (*mk*) (40). Homozygous *mk*/*mk* mice have microcytic hypochromic anemia due to defects in intestinal absorption and erythroid iron metabolism. Based on these effects, it now appears that Nramp2/DCT1 is the transport protein responsible for the intestinal absorption of dietary ionic iron (and perhaps other metals) and its subsequent distribution throughout the body.

In contrast to *Nramp2*/*DCT1*, *Nramp1* is expressed only in reticuloendothelial cells. Although the function of Nramp1 is not known, the role of other Nramp family members in metal transport suggests some exciting possibilities. For example, the phagosomes of macrophages with impaired Nramp1 function may generate fewer bactericidal oxygen radicals because of a cellular defect in the uptake of a metal ion, perhaps iron, across the plasma membrane.

Intracellular Manganese Transport

Many intracellular Mn^{2+} transporters were identified initially because of their roles in Ca^{2+} transport, but subsequent studies suggested their involvement in Mn^{2+} compartmentalization as well. One such transporter is PMR1, a P-type ATPase that is closely related to the Ca^{2+} ATPases of mammalian sarcoplasmic reticulum (112). PMR1 localizes to a Golgi-like compartment (6) and probably transports Mn^{2+} as well as Ca^{2+} into the secretory system (68). The *CCC1* gene may also transport Mn^{2+} and Ca^{2+} into the Golgi (42, 69). *CCC1*'s product has

five potential transmembrane domains and is an integral membrane protein that localizes to the Golgi. The ATX2 protein is also found in a Golgi-like compartment and may transport Mn^{2+} in the opposite direction to that of PMR1 and CCC1 (79). ATX2 appears to be a transport protein; it is an integral membrane protein containing seven to eight potential transmembrane domains.

The *HUM1* gene encodes a Ca^{2+} transporter now implicated in Mn^{2+} uptake into the vacuole. HUM1 is related to mammalian Na^{+}/Ca^{2+} exchangers and is required for H^{+}/Ca^{2+} exchange in purified vacuolar vesicles (101). In some genetic backgrounds, *hum1* mutants are hypersensitive to Mn^{2+} and cells overexpressing HUM1 show increased Mn^{2+} tolerance (101). These results suggest that HUM1 plays a role in the uptake of Mn^{2+} into the lumen of the vacuole, presumably to store and detoxify the metal ion. The acidic pH of the vacuolar lumen probably provides the driving force for this uptake (10, 92).

ZINC

Zinc Uptake

Zinc plays an amazing number of critical roles in the cellular biochemistry. Analysis of the *S. cerevisiae* genome sequence indicated that almost 3% of all yeast proteins (i.e. >150 of approximately 6000 total genes) contain potential zinc binding domains (DJ Eide, unpublished observation). Of these, 109 yeast genes encode transcriptional regulators containing either the C_2H_2 zinc finger domain (11) or the C_6 zinc cluster motif (114). Zinc uptake in *S. cerevisiae* is time, temperature, and concentration dependent and is saturable (44, 85, 126). Kinetic studies of zinc uptake by cells grown with different amounts of zinc in the medium suggest the presence of at least two uptake systems. One system has a high-affinity for zinc with an apparent K_m of 1 μM Zn^{2+} and is active in zinc-limited cells (134). The second system has a lower affinity for zinc (apparent K_m of 10 μM Zn^{2+}) and is detectable in zinc replete cells (135).

The *ZRT1* gene encodes the transporter protein of the high-affinity system (134). The level of *ZRT1* mRNA correlated with activity of the high-affinity system. Overexpressing ZRT1 increased high-affinity uptake, whereas disrupting the *ZRT1* gene eliminated high-affinity activity and resulted in poor growth of the mutant on zinc-limiting media. In similar studies, it was determined that the *ZRT2* gene encodes the transporter of the low-affinity uptake system (135). More recently, the ZRT1 protein has been found to be glycosylated and localized to the plasma membrane of the cell (M Broderius, D Eide, unpublished observation). Additional, as yet uncharacterized zinc uptake systems are also present in *S. cerevisiae*, as demonstrated by the observation that the *zrt1 zrt2* mutant is viable (135).

ZRT1 and ZRT2 share 44% sequence identity and 67% similarity. They each contain eight potential transmembrane domains and have a similar predicted membrane topology in which the amino- and carboxy-terminal ends of the protein are located on the outside surface of the plasma membrane. These proteins are also similar in sequence and predicted topology to the IRT1 iron transporter from *Arabidopsis* (38). Expression of the *IRT1* gene in yeast suppresses the iron-limited growth defect of a *fet3 fet4* mutant. Our current hypothesis is that IRT1 is an Fe^{2+} transporter that takes up iron from the soil, a proposal that is consistent with the observation that yeast expressing IRT1 possess a novel Fe^{2+} uptake activity. Moreover, in *Arabidopsis*, *IRT1* mRNA is expressed in roots and is induced by iron-limiting growth conditions. In addition to sharing sequence similarity and numbers of potential transmembrane domains, ZRT1, ZRT2, and IRT1 each have a potential metal-binding domain between transmembrane domains three and four that is predicted to be cytoplasmic. For example, in ZRT1, this sequence is HDHTHDE, and in IRT1, this motif is HGHGHGH. Although the function of this motif is unknown, its conserved location in these three proteins and its potential for metal binding suggest that it plays an important role in metal ion uptake or its regulation.

Through DNA sequence database comparisons and additional expression cloning studies, it is clear that these three metal ion transporters are members of another family of proteins found in a diverse array of eukaryotic organisms (ML Guerinot, DJ Eide, unpublished observation). This family is referred to collectively as the ZIP family (ZRT, IRT-like protein). Sixteen ZIP members have been identified, including eight in plants (seven from *Arabidopsis* and one from rice), two in *S. cerevisiae* (i.e. ZRT1 and ZRT2), four in nematodes, and two in humans. All but two of these proteins contain the putative metal-binding domain described above. Four of the *Arabidopsis* proteins, ZIP1-4, may be involved in zinc transport (ML Guerinot, DJ Eide, unpublished observation). Expressing ZIP1, ZIP2, or ZIP3 in yeast confers increased Zn^{2+} uptake activity with distinct biochemical properties. Furthermore, *ZIP1*, *ZIP3*, and *ZIP4* mRNAs are induced in zinc-limited plants. Based on the studies of IRT1, ZRT1, ZRT2, and ZIP1-4, it seems likely that the other proteins in this family are also metal ion transporters.

REGULATION Zinc uptake in yeast is controlled at the transcriptional level in response to intracellular zinc levels. The high-affinity system is induced more than 30-fold in zinc-limited cells and results from increased transcription of the *ZRT1* gene (134). The low-affinity system is also regulated through the control of *ZRT2* transcription (136). Regulation of these genes in response to zinc is mediated by the product of the *ZAP1* gene (136). *ZAP1* is likely to encode a transcriptional activator; the carboxy-terminal end of the protein contains five

C_2H_2 zinc finger domains, and the amino terminus has two potential activation domains. ZAP1 was also found to regulate its own transcription through a positive autoregulatory mechanism. This type of regulatory circuitry would allow a rapid, amplified response to changes in zinc levels and ZAP1 activity under progressively zinc-limiting conditions.

Intracellular Zinc Transport

Two potential intracellular zinc transporters have been identified in *S. cerevisiae*. These transporters are encoded by the *ZRC1* and *COT1* genes. *ZRC1* was isolated as a suppressor of zinc toxicity, i.e. overexpression of *ZRC1* results in zinc resistance (58). A *zrc1* mutation was later found to increase sensitivity to lipid hydroperoxides and decrease glutathione levels by approximately 40% (65). The relationship between these phenotypes and zinc is unknown. The *COT1* gene was isolated in a similar fashion to *ZRC1*, i.e. as a suppressor of cobalt toxicity, but was later found to confer zinc resistance as well (20, 21). Disruption of either *ZRC1* and *COT1* resulted in greater sensitivity to zinc, further supporting the role of these genes in zinc compartmentalization.

ZRC1 and COT1 are closely related proteins (60% identity) of approximately 440 amino acids and six to seven potential transmembrane domains. The physiological roles of these transporters are still unclear. Neither *ZRC1* nor *COT1* are essential genes, and a *zrc1 cot1* mutant is also viable. Thus, these two genes do not together provide a function essential for growth. Neither protein appears to catalyze zinc efflux from the cell. The subcellular location of ZRC1 is not known, but COT1 was enriched in mitochondrial preparations (21), which suggests that accumulation of zinc into this organelle may be important for sequestering and detoxifying zinc.

ZRC1 and COT1 belong to yet another family of metal transport proteins, referred to as the CDF (cation diffusion facilitator) family (87, 100). In all, 13 members of this family have been identified in prokaryotes and eukaryotes. Analyses of other CDF proteins lend support to the role of the yeast proteins in metal transport. For example, one member of this family, the CzcD protein of the prokaryotic *Alcaligenes eutrophus*, confers resistance to zinc as well as cobalt and cadmium (86). Three mammalian CDF proteins, ZnT-1, ZnT-2, and ZnT-3, are zinc transporters. ZnT-1 is a zinc efflux transporter in the plasma membrane (99). ZnT-2 is found in the membrane of an acidic endosomal/lysosomal compartment and may play a role in zinc sequestration (97). ZnT-3 is expressed only in the brain and testis and is most abundant in the neurons of the hippocampus and the cerebral cortex (98). ZnT-3 is localized to synaptic vesicle membranes, which suggests that this protein transports zinc into this compartment. The similarity of these proteins with ZRC1 and COT1 supports the hypothesis that the yeast proteins detoxify excess zinc by

transporting the metal into an intracellular vesicular compartment. In an intriguing point of similarity with ZIP proteins, the eukaryotic CDF proteins contain a histidine-rich region with the sequence $(HX)_n$, where $n = 3–6$. As is the case of the ZIP proteins, this domain is predicted to be cytoplasmic, and its function is unknown.

SUMMARY AND PERSPECTIVE

The transport systems responsible for metal ion accumulation in *S. cerevisiae* have been well-characterized thanks to the sophisticated genetic and molecular biological techniques available to study this organism. An overview of the recent findings in this field illuminates several consistent themes that will undoubtedly apply to metal ion transport in mammals. For example, one general feature of metal ion uptake in yeast is the presence of two or more relatively substrate-specific transport systems for the uptake of any single metal ion. High-affinity systems are active in metal-limiting conditions, whereas low-affinity systems play an important role when the substrate is more abundant. A second important theme coming from yeast studies is that metal ion uptake systems are tightly regulated through both transcriptional and posttranscriptional mechanisms of regulation. Third, and perhaps most importantly, these studies have helped us recognize the existence and importance of new protein families that appear to play a role in these processes in all eukaryotes. These families include Fe^{3+} /Cu^{2+} reductases like FRE1 and FRE2, the copper transporting P-type ATPases, and the Nramp and ZIP metal transport proteins. The new area of metal ion trafficking via cytoplasmic chaperone proteins is another exciting example of the conservation of both proteins and processes between yeast and mammals.

Despite these recent successes, many important questions remain. For instance, we know little about the biochemical mechanisms of metal ion uptake used by the transporter proteins that have been identified. A good example is how iron transport is mediated by the oxidase/permease systems in yeast and in humans. Having cloned genes in hand will allow detailed structure/function analyses to be carried out on these proteins and other transporters as well. In addition, although we now have several clues about how metal ions are compartmentalized within the cell, we are far from having a detailed understanding of these processes. Another burgeoning area of research is assigning physiological roles to the growing number of metal ion transport proteins that are being identified in mammals and plants. Only through combined nutritional, biochemical, molecular, and genetic approaches will we be able to fully decipher the function of these transporters in the whole organism. These studies should also lead to an understanding of the underlying causes of various human genetic diseases of metal metabolism.

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