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Biochemistry

The yeast *ZRT1* gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation

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ABSTRACT **The yeast *Saccharomyces cerevisiae* has two separate systems for zinc uptake. One system has high affinity for substrate and is induced in zinc-deficient cells. The second system has lower affinity and is not highly regulated by zinc status. The *ZRT1* gene encodes the transporter for the high-affinity system, called Zrt1p. The predicted amino acid sequence of Zrt1p is similar to that of Irt1p, a probable Fe(II) transporter from *Arabidopsis thaliana*. Like Irt1p, Zrt1p contains eight potential transmembrane domains and a possible metal-binding domain. Consistent with the proposed role of *ZRT1* in zinc uptake, overexpressing this gene increased high-affinity uptake activity, whereas disrupting it eliminated that activity and resulted in poor growth of the mutant in zinc-limited media. Furthermore, *ZRT1* mRNA levels and uptake activity were closely correlated, as was zinc-limited induction of a *ZRT1–lacZ* fusion. These results suggest that *ZRT1* is regulated at the transcriptional level by the intracellular concentration of zinc. *ZRT1* is an additional member of a growing family of metal transport proteins.**

Zinc is an integral cofactor of many proteins and is indispensable to their catalytic activity and/or structural stability (1). Moreover, zinc is a ubiquitous component of enzymes involved in transcription and of accessory transcription factors, the zinc finger proteins, that regulate gene expression (2). Because of the many roles this metal plays in cellular biochemistry, zinc is an essential nutrient for all organisms. Despite this importance, very little is known about the molecular mechanisms cells use to obtain zinc. No transporter genes involved in zinc uptake (i.e., influx transporters) have been isolated from any organism. Recently, genes have been identified whose products are responsible for detoxifying intracellular zinc by transporting the metal from the cytoplasm to the cell exterior or into intracellular compartments (i.e., efflux transporters). These genes include the closely related eukaryotic genes, *COT1*, *ZRC1*, and *Znt-1* (3–5). While important for zinc detoxification, these genes do not appear to play a role in zinc uptake.

The yeast *Saccharomyces cerevisiae* provides an excellent model system in which to study zinc uptake in a eukaryotic cell. Biochemical assays of zinc uptake in yeast indicated that this process was transporter-mediated—i.e., uptake was dependent on time, temperature, and concentration and required metabolic energy (6–8). In this report, the presence of two separate zinc uptake systems in *S. cerevisiae* is demonstrated. One system has high affinity for zinc, and its activity markedly increases in zinc-limited cells. The second system has a lower affinity for zinc and is not highly regulated by zinc availability. We have characterized a gene, *ZRT1* (for zinc-regulated transporter), identified because of its significant similarity to *IRT1*, an Fe(II) transporter gene from the plant *Arabidopsis thaliana* (9). Our results indicate that Zrt1p is the zinc transporter protein of the high-affinity uptake system. To our knowledge, *ZRT1* is the first influx zinc transporter gene from any organism to be characterized at the molecular level, and it is a member of a growing family of metal transport proteins identified in fungi, nematodes, plants, and humans.

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MATERIALS AND METHODS

Yeast Strains and Culture Conditions. Strains used were DY1457 (*MATα ade6 can1 his3 leu2 trp1 ura3*) and ZHY1 (*MATα ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2*). Yeast were grown in standard culture media (SD and YPD; see ref. 10) supplemented with necessary auxotrophic requirements and either 2% glucose or 2% galactose. A zinc-limiting medium (low-zinc medium, LZM) was prepared in the same manner as low-iron medium (LIM) (11) except that $ZnSO_4$ in LIM was replaced with 10 μM $FeCl_3$ in LZM. Cell number in liquid cultures was determined by measuring the optical density of cell suspensions at 600 nm (OD_{600}) and converting to cell number with a standard curve.

Plasmids and DNA Manipulations. *Escherichia coli* and yeast transformations were performed by using standard methods (12, 13). Plasmids constructed are diagrammed in Fig. 1. A fragment bearing the *ZRT1* open reading frame was prepared by the polymerase chain reaction (PCR), using primers derived from the *ZRT1* sequence with either *Bam*HI (primer 3) or *Sal* I restriction sites (primer 4) added to their 5′ ends (Fig. 1; primer 3, 5′-CGGATCC/ATGAGCAACGTTACTACG-3′; and primer 4, 5′-TACGCGTCGAC/TTAAGCCCACTTACCGAT-3′; the slash indicates the beginning of the *ZRT1* sequence in each primer). The resulting fragment was inserted into pBluescript II SK(+) (Stratagene, La Jolla, CA) to generate pSK$^+$ZRT1. A *Pst* I fragment containing the *LEU2* gene was prepared as described (14) and inserted into pSK$^+$ZRT1 to generate pZH2. This plasmid contains the *zrt1* disruption mutation *zrt1::LEU2*. Plasmid pZH2 was digested with *Bam*HI and *Sal* I and used to transform DY1457 to replace the chromosomal locus by single-step gene transplacement (15). The resulting strain, ZHY1, was confirmed to contain the *zrt1::LEU2* mutation by Southern blot analysis (data not shown). Because ZHY1 grows more slowly than the wild-type strain on media containing metal chelators, a plasmid (pMC5) containing a genomic *ZRT1* fragment was isolated from a genomic library (16) by complementation (17) of the growth defect displayed by ZHY1 on YPD + 200 μM bathophenanthroline disulfonate (Sigma). The 2.2-kb *Sac* I–*Hin*dIII fragment from pMC5 containing the genomic *ZRT1* gene was subcloned in pRS316 (18) to generate pMC5-HS. The *Bam*HI–*Sal* I fragment generated with primers 3 and 4 was also inserted into pRS316-GAL1 (19) to generate pOE1. A PCR fragment containing bases −706 to +3 of *ZRT1* (the first base of the ATG initiation codon is designated as position +1) was generated with primers 1 and 2 (Fig. 1; primer 1, 5′-GGAATTC/GAAGGCAAGAGTATTTCAGAC-3′; primer 2, 5′-CGGGATC/CATAATTCCTTTTTTGATATTTG-3′;

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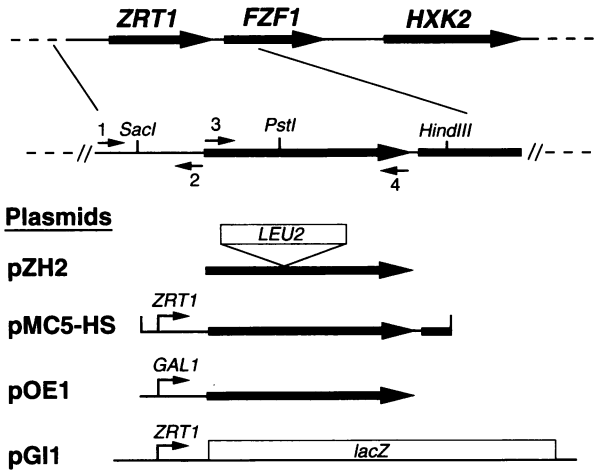

FIG. 1. The chromosomal region of the *ZRT1* gene and plasmids constructed for these studies. The open reading frames on chromosome VII are indicated by large arrows. The locations of the relevant restriction sites in this region are indicated, and small arrows numbered 1–4 represent the primers used in plasmid construction. The promoters in the plasmids are identified by arrows labeled either *ZRT1* or *GAL1*.

the slash indicates the beginning of the *ZRT1* sequence in each primer). This PCR fragment was digested with *Eco*RI and *Bam*HI and inserted into the yeast integrating vector YIp353 (20) to generate pGI1. This plasmid contains a fusion between the *ZRT1* upstream flanking sequences, 5′ untranslated region, and initiation methionine residue and the *E. coli lacZ* gene. Plasmid pGI1 was then digested with *Nco* I and used to transform DY1457 and ZHY1 to integrate the plasmid at the *URA3* locus (15). The plasmid pHYC3 contains *HIS4* promoter elements fused to *lacZ* (21). Data base comparisons were performed with the National Center for Biotechnology Information data bases by using BLAST (22), and topology analysis was performed by using the TOP-PREDII program (23).

Zinc Uptake and β-Galactosidase Assays. Zinc uptake assays were performed as described previously for iron uptake (24) except that $^{65}ZnCl_2$ (Amersham) and LZM–EDTA were substituted for $^{59}FeCl_3$ and LIM–EDTA. Cells were incubated at 30°C with ^{65}Zn for 5 min, filtered, and washed with 10 ml of ice-cold SSW (11). Cell-associated radioactivity was measured by liquid scintillation counting. Kinetic values were derived by using KINETASYST software (IntelliKinetics, Princeton). Zinc accumulation was measured in cells grown in LZM supplemented with 10 μM $^{65}ZnCl_2$ plus nonradioactive zinc to the indicated final concentration. Aliquots (0.5 ml) were filtered and washed with 10 ml of ice-cold SSW, and radioactivity was measured by liquid scintillation counting. β-Galactosidase activity was assayed as described by Guarente (25).

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from yeast (10), denatured, separated by agarose gel electrophoresis (6 μg per lane), and analyzed by Northern blotting (12). Equal loading of RNA in each lane was confirmed by staining the gel with acridine orange. Probes used were the *ZRT1 Bam*HI–*Sal* I insert of pSK$^+$ZRT1 and *ACT1* labeled with ^{32}P (Amersham) by the random priming method (26). Densitometric scanning was performed by using a Sierra Scientific charge-coupled device camera and IMAGE 1.4 software (National Institutes of Health, Bethesda).

RESULTS

Identification of *ZRT1*. *ZRT1* was identified fortuitously in our recent studies of iron uptake in plants. An Fe(II) transporter gene, *IRT1*, was cloned from *A. thaliana* by functional expression in yeast (9). Comparing the predicted Irt1p amino acid sequence against the current sequence data bases indicated that *IRT1* belongs to a family of closely related genes of unknown function, including two additional genes in *A. thaliana* and genes in rice, *Caenorhabditis elegans*, and humans. This comparison also identified two closely related open reading frames of unknown function from *S. cerevisiae*. We have designated one of these two yeast genes *ZRT1* for zinc-regulated transporter. The sequence of the open reading frame corresponding to *ZRT1* (GenBank accession no. P32804) was originally obtained during sequence analysis of a portion of the yeast genome (27). In this analysis, it was determined that *ZRT1* is located on chromosome VII immediately adjacent to the *FZF1* gene (Fig. 1) and is predicted to encode a protein of 376 amino acids. We have found that Zrt1p is 30% identical and 50% similar (i.e., identities plus conservative substitutions) to Irt1p (Fig. 2). A model of Zrt1p membrane topology suggested the presence of eight potential transmembrane domains located in positions in the amino acid sequence nearly identical to those predicted for Irt1p.

Irt1p contains an amino acid sequence, (-HG-)$_4$, that could be a metal-binding domain (9). A similar sequence was also found in Zrt1p (Fig. 2), in which three of the four histidines are conserved but the fourth potential ligand is unclear. A histidine located approximately 30 amino acids toward the carboxyl terminus may contribute to metal binding. In both Irt1p and Zrt1p, this histidine-rich domain is found in a large loop between transmembrane domains 3 and 4. Topological predictions based on the "positive-inside" rule (23) suggested that in both proteins this loop is located on the cytoplasmic surface of the membrane.

***ZRT1* Is Required for Zinc-Limited Growth.** To examine the function of *ZRT1*, we constructed a disruption mutation, *zrt1::LEU2*, by inserting the *LEU2* gene into the center of *ZRT1* (Fig. 1). This *zrt1* disruption allele was then introduced

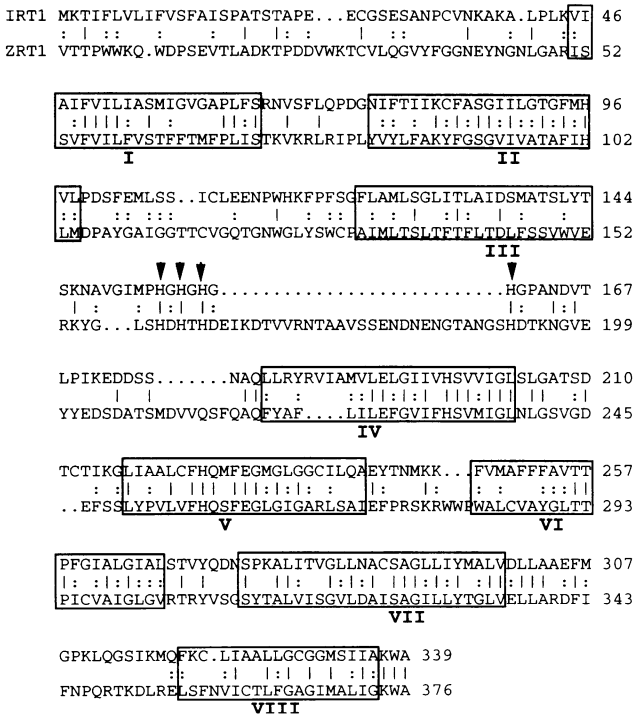

FIG. 2. Alignment of the Irt1p and Zrt1p amino acid sequences. Amino acids were aligned by using the Genetics Computer Group BESTFIT program (28) and are numbered on the right. The first three amino acids of Zrt1p (MSN) are not shown. Bars indicate positions of amino acid identity and colons indicate positions of conservative substitutions (29). Potential transmembrane domains in Irt1p and Zrt1p are boxed and numbered I–VIII, and histidines in the potential metal-binding domains are indicated by arrows.

into a haploid yeast strain. The resulting mutant was viable, indicating that *ZRT1* is not an essential gene. Northern blot analysis failed to detect *ZRT1*-related mRNA in this mutant strain, indicating that the disruption allele was unlikely to retain any residual function (data not shown). Despite its resemblance to the Irt1p iron transporter, Zrt1p does not play a role in iron uptake in yeast. No defect was observed in iron uptake in the *zrt1* mutant (data not shown). However, this mutant strain did not grow in an iron-limiting medium (LIM). Because of the high EDTA concentration in LIM (1 mM), this medium is expected to have low available levels of other metals that are bound tightly by this chelator. We tested if supplements of other metals improved growth of the *zrt1* mutant in LIM. Adding 500 μM Co, Cu, Fe, Mg, or Mn to LIM had no effect on *zrt1* growth (data not shown), but adding 500 μM zinc greatly enhanced growth of this mutant strain. To study this effect further, we developed a low-zinc medium, LZM, in which we could limit cell growth by zinc deficiency and examine the growth response of the wild-type and *zrt1* mutant strains to increasing levels of supplemented zinc. While growth of the wild-type strain in LZM without zinc supplement was severely inhibited, adding as little as 10 μM zinc allowed this strain to go through its maximum number of seven cell divisions over a 16-hr period (Fig. 3). Mutant *zrt1* cells attained this same maximum number of cell divisions only with zinc supplements of 750 μM or more—i.e., a 75-fold increase in the zinc requirement of the *zrt1* mutant compared with the wild type. This growth defect could be complemented fully by the plasmid pMC5-HS (Fig. 1), a genomic clone of the *ZRT1* gene, indicating that the phenotype resulted from loss of *ZRT1* function and not because the mutation affected the nearby *FZF1* gene on chromosome VII.

***ZRT1* Is Required for High-Affinity Zinc Uptake.** To determine if *ZRT1* plays a role in zinc uptake, we first characterized the biochemical properties of this process in wild-type cells. These experiments indicated that ^{65}Zn uptake in our assay system is transporter-mediated; this process is time-, temperature-, and energy-dependent (data not shown). At 30°C, zinc accumulation was linear with time for up to 5 min, after which the uptake rate decreased, and little accumulation was detected with cells incubated at 0°C or starved for glucose for 1 hr prior to assay. The rate of zinc uptake was concentration-dependent and saturable (Fig. 4). The Michaelis–Menten kinetic properties differed, depending on the medium in which the cells were grown prior to assay. Zinc-replete cells had an apparent K_m of 10 ± 1 μM and V_{max} of 2 pmol/min per 10^6 cells (Fig. 4*A*). In zinc-limited cells, the apparent K_m was 1 ± 0.1 μM and the V_{max} was 80 pmol/min per 10^6 cells (Fig. 4*B*). Thus, uptake activity in zinc-limited cells had a markedly lower

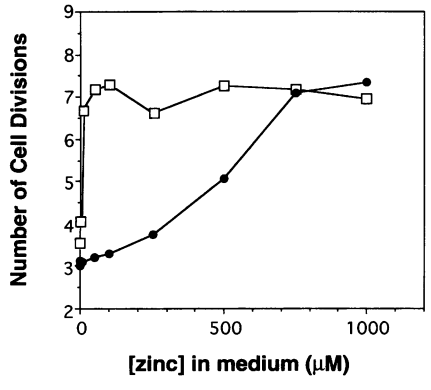

FIG. 3. *ZRT1* is required for zinc-limited growth. Wild-type (DY1457, □) and *zrt1* mutant (ZHY1, ●) cells were inoculated into LZM supplemented with the indicated amount of $ZnSO_4$ and grown for 16 hr prior to cell number determination. Shown are the mean values of three experiments.

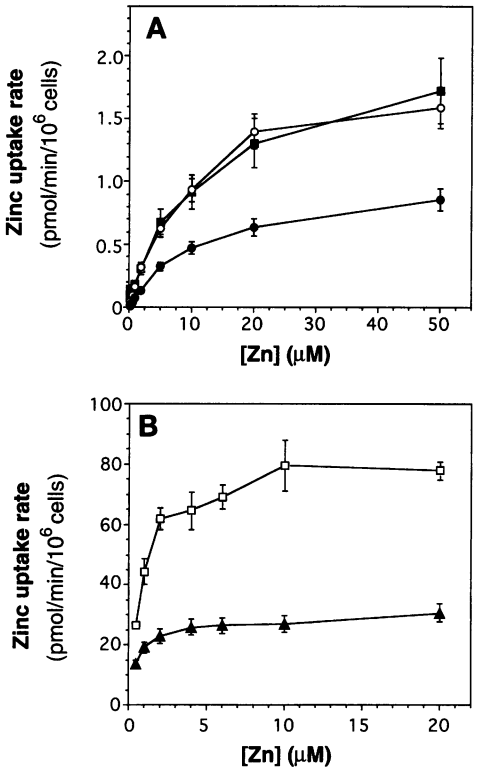

FIG. 4. *ZRT1* is required for high-affinity zinc uptake. Wild-type (DY1457, squares) and *zrt1* mutant (ZHY1, circles) cells were grown to exponential phase in zinc-limited (open symbols) and zinc-replete (closed symbols) media and assayed for zinc uptake rate over a range of $ZnSO_4$ concentrations. Zinc-limited media were LZM + 10 μM zinc for the wild type and LZM + 500 μM zinc for the mutant. Zinc-replete conditions were LZM + 1000 μM for both strains. These conditions were selected on the basis of the experiment described in Fig. 6. ZHY1(pOE1) cells (triangles) were grown in zinc-replete SD/galactose medium. Shown are the mean values of two experiments each performed in duplicate; error bars indicate ±1 SD.

apparent K_m and higher V_{max} than the activity observed in zinc-replete cells. These results suggest the presence of two zinc uptake systems in yeast, a high-affinity system induced by zinc limitation and a low-affinity system active in zinc-replete cells.

Zinc uptake assayed in *zrt1* mutant cells grown in zinc-limiting and zinc-replete media displayed only low affinity activity (Fig. 4*A*, ○ and ●, respectively). The apparent K_m in each case was 10 ± 1 μM and the V_{max} was 1–2 pmol/min per 10^6 cells. Expressing *ZRT1* from the *GAL1* promoter (pOE1; Fig. 1) in zinc-replete cells resulted in high-affinity uptake activity (apparent K_m of 0.6 ± 0.1 μM) with a V_{max} of 30 pmol/min per 10^6 cells (Fig. 4*B*). No high-affinity activity was observed in these cells grown in glucose, in which the *GAL1* promoter is not expressed, or in vector-only control cells grown in galactose or glucose (data not shown). These results demonstrate that the *ZRT1* gene is both necessary and sufficient for high-affinity system activity but is not required for low-affinity system activity.

Regulation of *ZRT1* mRNA Levels by Zinc. The observation that zinc-limited wild-type cells possess *ZRT1*-dependent uptake activity absent from zinc-replete cells suggested that the *ZRT1* gene could be regulated by zinc. To test this hypothesis, we measured *ZRT1* mRNA levels and zinc uptake activity in cells grown in a range of zinc concentrations. To provide a simpler means of assessing *ZRT1* expression, we also constructed a fusion between the *ZRT1* promoter and 5′ untranslated region and the *E. coli lacZ* gene, which encodes β-galactosidase (pGI1; Fig. 1). *ZRT1* mRNA was regulated in a

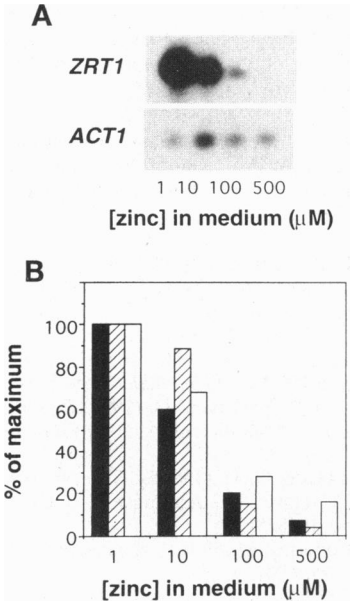

FIG. 5. Regulation of the *ZRT1* gene and zinc uptake. Wild-type (DY1457) cells bearing pGI1 were grown to exponential phase in LZM supplemented with different concentrations of $ZnSO_4$. (*A*) Northern blot analysis of *ZRT1* and actin (*ACT1*) mRNA levels. (*B*) Correlation between mRNA levels, uptake activity, and β-galactosidase activity. The *ZRT1* mRNA levels were determined by densitometric scanning and are normalized to the total RNA loaded in each lane (closed bars), and zinc uptake (assayed at 1 μM ^{65}Zn, hatched bars) and β-galactosidase activities (open bars) were measured. Shown are the mean values of two experiments each performed in duplicate. The standard deviation within each experiment was less than 10% of the corresponding mean.

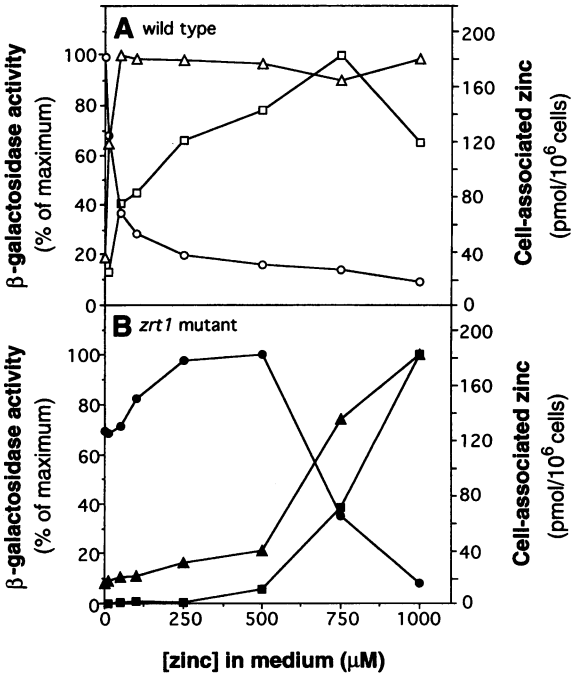

FIG. 6. Effects of the *zrt1* mutation on *ZRT1* regulation and cell-associated zinc levels. Wild-type (DY1457, open symbols) and *zrt1* mutant (ZHY1, closed symbols) cells transformed with either pGI1 (circles) or pHYC3 (the *HIS4–lacZ* fusion) (triangles) were inoculated into LZM supplemented with the indicated level of $ZnSO_4$, grown for 16 hr, and assayed for β-galactosidase activity. In a parallel experiment, these strains were grown for 16 hr in LZM media containing tracer amounts of ^{65}Zn (squares). Cells were harvested, and cell-associated zinc was measured. Shown are the mean values of two experiments each performed in duplicate. The standard deviation within each experiment was less than 10% of the corresponding mean.

zinc-dependent manner; zinc-limited cells had 10-fold more *ZRT1* mRNA than did zinc-replete cells (Fig. 5*A*). Uptake activity of the high-affinity system closely correlated with *ZRT1* mRNA levels, and the *ZRT1–lacZ* fusion was regulated in an identical manner (Fig. 5*B*). The close correlation between *ZRT1* expression levels and zinc uptake activity supports the hypothesis that *ZRT1* encodes the high-affinity transporter. Furthermore, these results suggest that the *ZRT1* gene is regulated at the transcriptional level by zinc and that the *ZRT1–lacZ* fusion accurately reflects that regulation.

The *ZRT1–lacZ* fusion allowed us to compare *ZRT1* regulation in wild-type and *zrt1* mutant cells grown over a range of zinc concentrations. In the wild-type strain, β-galactosidase activity was highest in zinc-limited cells and decreased with increasing zinc concentrations in the medium (Fig. 6*A*). To test if zinc status alters β-galactosidase activity per se, cells bearing a *HIS4–lacZ* fusion were also assayed. *HIS4* encodes a histidine biosynthetic enzyme and is dependent on the *GCN4* leucine zipper protein for expression (30). This promoter fusion in wild-type cells generated β-galactosidase activity that correlated closely with the strain's growth response to zinc (Fig. 3). Therefore, the repressive effects of zinc on β-galactosidase activity were not caused by zinc toxicity or negative effects of zinc excess on the activity of this enzyme. To estimate the size of the intracellular zinc pool in these cells and determine its relationship to *ZRT1* expression, we measured the cell-associated zinc levels in cells grown in LZM containing ^{65}Zn. The decrease in *ZRT1*-dependent β-galactosidase activity coincided with an increase in cell-associated zinc.

In the *zrt1* mutant strain, *ZRT1–lacZ* expression remained at its maximum level in cells grown with much higher concentrations of zinc in the medium than wild type (Fig. 6*B*). Thus, the *zrt1* mutant required more zinc in the medium to repress *ZRT1* expression than did wild type cells. *HIS4*-dependent β-galactosidase activity was similar to the growth response of this strain to zinc as well. Finally, although the response of the *ZRT1–lacZ* fusion to extracellular zinc levels was very different in the wild type and mutant, the response to cell-associated zinc levels was unaffected. For example, approximately equal levels of cell-associated zinc were present in wild-type cells grown in LZM + 50 μM zinc and *zrt1* mutant cells grown in LZM + 750 μM zinc, and these cells also had similar levels of *ZRT1* expression. These data suggest that the *ZRT1* gene is regulated by intracellular zinc pools and that, although the amount of zinc required in the medium to supply these pools is greatly altered in the mutant, the regulatory system that controls ZRT1 expression in response to pool size is unaffected.

DISCUSSION

Our analyses demonstrate that yeast has two zinc uptake systems. One system has a high affinity for substrate, is induced by zinc limitation, and is necessary for growth in zinc-limiting conditions. Although other roles are formally possible, we propose that the *ZRT1* gene encodes the transporter of this high-affinity system, and several lines of evidence support this hypothesis. First is the similarity between Zrt1p and Irt1p; Irt1p has been demonstrated to be an Fe(II) transporter and may also be capable of transporting zinc (9). Second, a mutation in the *ZRT1* gene eliminated high-affinity uptake activity and inhibited growth on zinc-limiting media. Third, overexpressing *ZRT1* increased activity of an uptake system that had an apparent K_m almost identical to that of the high-affinity system. These results indicate that *ZRT1* expression is both necessary and sufficient for high-affinity system

activity. We also found that high-affinity activity and *ZRT1* expression were closely correlated across a wide range of extracellular zinc concentrations. It is possible that Zrt1p is only one subunit of a heteromeric transporter complex, but this is unlikely, given that overexpression of *ZRT1* alone increased high-affinity activity. We have designated the second *IRT1* homolog in yeast *ZRT2* because of its similarity to *ZRT1*. Preliminary studies suggest that *ZRT2* encodes the low-affinity transporter.

ZRT1 is, to our knowledge, the first influx zinc transporter gene from any organism to be characterized at the molecular level. Neither Irt1p nor Zrt1p contain ATP-binding domains, suggesting that uptake is driven by indirect coupling to energy metabolism, perhaps through a gradient of another ion such as K^+ (6, 31). A group of histidine residues found in Irt1p was conserved in Zrt1p. This region may be a metal-binding domain, given that the imidazole ring nitrogens of histidine may serve as coordinating ligands for metal ions. In both proteins, this sequence is found in a loop region predicted to be on the cytoplasmic surface of the membrane. Similar histidine-rich sequences are also found in the three eukaryotic proteins implicated in zinc detoxification—i.e., Zrc1p, Cot1p, and Znt1p (3–5). In each case, the domain is predicted to be cytoplasmically located. This conservation suggests that the domain plays an important functional role in Irt1p and Zrt1p. For example, these histidines may serve as a means of feedback regulation of zinc transport. High intracellular zinc levels could result in binding of zinc by Zrt1p and reduce the activity of the transporter.

Zinc limitation induces activity of the high-affinity system. Because our results suggest that this system is regulated at the transcriptional level, it is tempting to speculate that a zinc finger DNA-binding protein may sense intracellular zinc levels to regulate *ZRT1* expression. However, we cannot rule out a mechanism that controls mRNA stability through sequence elements located in the 5′ untranslated region of the mRNA. Whatever the mechanism, the high-affinity system is clearly regulated in response to the intracellular zinc content. This is demonstrated by the fact that the *ZRT1–lacZ* fusion gene shows similar responses to cell-associated zinc levels in wild-type and *zrt1* mutants despite a 15-fold difference in their response to external levels of zinc. Thus, the regulatory system that controls *ZRT1* expression in response to intracellular zinc pools is unaffected in the *zrt1* mutant. We have also found that the *zrt1* mutant is not any more resistant to high extracellular zinc levels than are wild-type cells (unpublished result). This result is consistent with the low level of *ZRT1* expression observed in zinc-replete cells and demonstrates that the high-affinity uptake system does not play an important role in zinc toxicity.

All organisms require transition metals such as zinc and iron. Accumulation of these metals is transporter-mediated, yet little is known about these transporters or their molecular mechanisms of action. We have identified a zinc transporter gene from yeast that is a member of a growing family of similar genes found in organisms as diverse as fungi, plants, nematodes, and humans. The two members that have been examined experimentally, *IRT1* and *ZRT1*, have been shown to encode metal transport proteins. Therefore, it seems likely that other genes in this family play similar roles in metal uptake in the organisms in which they are found.

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