

# Zap1p, a Metalloregulatory Protein Involved in Zinc-Responsive Transcriptional Regulation in *Saccharomyces cerevisiae*

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**Zinc ion homeostasis in *Saccharomyces cerevisiae* is controlled primarily through the transcriptional regulation of zinc uptake systems in response to intracellular zinc levels. A high-affinity uptake system is encoded by the *ZRT1* gene, and its expression is induced more than 30-fold in zinc-limited cells. A low-affinity transporter is encoded by the *ZRT2* gene, and this system is also regulated by zinc. We used a genetic approach to isolate mutants whose *ZRT1* expression is no longer repressed in zinc-replete cells, and a new gene, *ZAP1*, was identified. *ZAP1* encodes a 93-kDa protein with sequence similarity to transcriptional activators; the C-terminal 174 amino acids contains five C<sub>2</sub>H<sub>2</sub> zinc finger domains, and the N terminus (residues 1 to 706) has two potential acidic activation domains. The N-terminal region also contains 12% histidine and cysteine residues. The mutant allele isolated, *ZAP1-I<sup>up</sup>*, is semidominant and caused high-level expression of *ZRT1* and *ZRT2* in zinc-replete cells. This high-level expression is the result of a mutation that substitutes a**

**<sup>2</sup>H<sub>2</sub> zinc finger motif first identified in transcription factor IIIA (TFIIIA) (18).**

**Although it is essential for many different cellular functions, zinc can also be toxic. When the intracellular zinc level rises to some critical level, the metal can interfere with vital processes, perhaps by competing with other metal ions for enzyme active sites, transporter proteins, and other biologically important ligands. The delicate balance of intracellular zinc is accomplished through precise homeostatic regulation mediated by a number of mechanisms. These include binding of the metal by cytoplasmic macromolecules such as metallothioneins (14) and phytochelatins (27), zinc storage in intracellular compartments (3, 20, 25), and transport of the metal out of the cell (26).**

**The primary control point for zinc ion homeostasis is the regulation of zinc uptake across the plasma membrane. Little is known about the mechanism and regulation of zinc uptake in mammals and plants. In *Saccharomyces cerevisiae*, zinc accumulation is mediated by three or more different uptake systems. One system has a high affinity for zinc [apparent *K<sub>m</sub>* of 10 nM free Zn(II)] and is active in zinc-limited cells (38). The *ZRT1* gene is required for high-affinity uptake; recent studies suggested that this gene encodes the transporter of this system. A second pathway for zinc uptake has a lower affinity for**

substrate [apparent *K<sub>m</sub>* = 100 nM free Zn(II)] and is active in zinc-replete cells. The *ZRT2* gene encodes the transporter of this system (39). A *zrt1 zrt2* mutant strain, which lacks both the high- and low-affinity systems, is viable, indicating the presence of one or more additional uptake pathways.

Both the high- and low-affinity zinc uptake pathways are regulated by zinc. The activity of the high-affinity system is induced more than 30-fold in response to zinc-limiting growth conditions (38). Our studies indicated that this rise is due to increased transcription of the *ZRT1* gene in response to an intracellular pool of zinc. The low-affinity system was also found to be regulated by zinc, although that analysis did not distinguish between transcriptional and posttranscriptional mechanisms of regulation (39). These observations suggested that *ZRT1* and, perhaps, *ZRT2* are regulated by one or more transcriptional regulatory proteins whose activities are controlled by intracellular zinc pools. To identify genes that regulate zinc-responsive gene expression, we used a genetic scheme for selecting mutants of *S. cerevisiae* with altered zinc-responsive *ZRT1* transcription. This report describes the analysis of one gene that plays a critical role in the regulation of zinc uptake. We have called this gene *ZAP1* (for zinc-responsive activator protein).

## MATERIALS AND METHODS

**Yeast strains.** The yeast strains used are listed in Table 1. Unless indicated otherwise, all strains were generated during this study. Strains of opposite mating types designed for selection of mutants by use of a conditional *HIS3* marker were generated as follows. A 456-bp deletion removing most of the *HIS3* open reading frame (ORF; *his3Δ::TRP1*) was generated in strains DY1457 and DEY1502 by the  $\gamma$ -deletion method (30) by transforming these cells with *EcoRI*-linearized plasmid pKO-HIS3 (see below for plasmid descriptions). ZHY4 and ZHY5 were generated from the resulting strains by integrating a *ZRT1-HIS3* fusion gene on plasmid YIpZRT1-HIS3 into the *NcoI* site of the *ura3-52* locus. A *ZAP1* deletion mutant (*zap1Δ::TRP1*) was generated by the  $\gamma$ -deletion method by digesting plasmid pKO-ZAP1 with *EcoRI* and transforming the linearized plasmid into a diploid strain generated by mating DY1457 with DEY1502. ZHY6 is a haploid segregant of the resulting diploid transformant, and ZHY7 is a haploid segregant

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TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Full genotype
DY1457 <sup>a</sup>	Wild type	<i>MAT<math>\alpha</math> ade6 can1 his3 leu2 trp1 ura3</i>
DEY1502	Wild type	<i>MAT<math>\alpha</math> ade2 can1 his3 leu2 trp1 ura3</i>
ZHY1 <sup>b</sup>	<i>zrt1</i>	<i>MAT<math>\alpha</math> ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2</i>
ZHY2 <sup>c</sup>	<i>zrt2</i>	<i>MAT<math>\alpha</math> ade6 can1 his3 leu2 trp1 ura3 zrt2::HIS3</i>
ZHY3 <sup>c</sup>	<i>zrt1 zrt2</i>	<i>MAT<math>\alpha</math> ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3</i>
ZHY4	<i>his3<math>\Delta</math> ZRT1-HIS3</i>	<i>MAT<math>\alpha</math> ade6 can1 his3<math>\Delta</math>::TRP1 leu2 trp1 ura3::ZRT1-HIS3 URA3</i>
ZHY5	<i>his3<math>\Delta</math> ZRT1-HIS3</i>	<i>MAT<math>\alpha</math> ade2 can1 his3<math>\Delta</math>::TRP1 leu2 trp1 ura3::ZRT1-HIS3 URA3</i>
M18	<i>his3<math>\Delta</math> ZRT1-HIS3 ZAP1-1<sup>up</sup></i>	<i>MAT<math>\alpha</math> ade6 can1 his3<math>\Delta</math>::TRP1 leu2 trp1 ura3::ZRT1-HIS3 URA3 ZAP1-1<sup>up</sup></i>
ZHY6	<i>zap1<math>\Delta</math></i>	<i>MAT<math>\alpha</math> ade6 can1 his3 leu2 trp1 ura3 zap1<math>\Delta</math>::TRP1</i>
ZHY7	<i>ZAP1-1<sup>up</sup></i>	<i>MAT<math>\alpha</math> ade6 can1 his3 leu2 trp1 ura3 ZAP1-1<sup>up</sup></i>

<sup>a</sup> Strain obtained from D. Stillman, University of Utah.

<sup>b</sup> Reference 38.

<sup>c</sup> Reference 39.

of a diploid obtained by mating M18 with DEY1502. All plasmid integration and deletion events were confirmed by Southern hybridization analysis.

**Yeast methods and genetic analysis.** *Escherichia coli* and yeast transformations, plasmid rescue, generation of diploid strains, sporulation, and tetrad dissection were performed by standard procedures. A liquid zinc-limiting medium (LZM) was prepared in the same manner as LIM (7) except that the ZnSO<sub>4</sub> in LIM was replaced by 10  $\mu$ M FeCl<sub>3</sub> in LZM. Synthetic defined (SD) medium (29), supplemented with the necessary auxotroph supplements and either 2% glucose or 2% galactose, was also used. For some experiments, SD medium was made zinc limiting by adding 1 mM EDTA and the stated concentration of zinc.

**Mutant selection and cloning of the ZAP1-1<sup>up</sup> allele.** Approximately 10<sup>6</sup> ZHY4 cells were plated onto each of 30 agar plates containing SD medium supplemented with 2% glucose, 2 mM ZnCl<sub>2</sub>, and all necessary auxotroph supplements except histidine. 3-Aminotriazole (30 mM; Sigma Chemical Co.) was also added to inhibit the low level of His3p expressed by the *ZRT1-HIS3* fusion gene during growth on this medium. After 4 days of incubation at 30°C, 30 independent colonies (i.e., one colony from each plate) arising from the background of nongrowing cells were isolated. One of these isolates was the M18 strain. To clone the *ZAP1-1<sup>up</sup>* allele, a plasmid library was constructed from M18 genomic DNA. This DNA was partially digested with *Sau3A*, and 4- to 10-kb fragments were isolated by sucrose gradient fractionation and ligated into the *Bam*HI site of YEp351 (16). ZHY4 was transformed with the M18 genomic library and selected for histidine prototrophy on the same selective medium used for mutant isolation. Two plasmids (pZH4 and pZH5) were isolated from 10<sup>5</sup> total transformants, and the endpoints of their inserts were determined by DNA sequencing.

**Plasmid construction.** Plasmid pKO-HIS3 was constructed as follows. Two-step overlapping PCR (17) was used with template YCp407 (31) to generate a *Bam*HI-*Kpn*I fragment containing an *Eco*RI site flanked on one side by the 203 bp immediately upstream of the *HIS3* start codon and on the other side by the carboxy-terminal 202 bp of the *HIS3* ORF and 148 bp of 3' flanking DNA. This fragment was then inserted into *Bam*HI- and *Kpn*I-digested pRS304 (30). To construct plasmid YlpZRT1-HIS3, a fragment containing bases -706 to -1 (the first base of the ATG initiation codon is designated as position +1) was generated by PCR with pMC5 (39) as the template. This fragment was then digested with *Sac*I and *Bam*HI and inserted into pCM105 (a gift of A. Dancis, National Institutes of Health). The sequence of the *ZRT1-HIS3* junction was verified by DNA sequencing as CAAATATCAAAAAGGAATT(GGATCC)ATGACA, with the *ZRT1* sequences followed by the *Bam*HI site (in parentheses) and the ATG of the *HIS3* gene (underlined). To generate the *zap1 $\Delta$*  deletion allele, two-step overlapping PCR was used with DY1457 genomic DNA as the template to generate a 620-bp *Bam*HI-*Kpn*I fragment containing an *Eco*RI site flanked on one side by bases -271 to -1 upstream of the *ZAP1* start codon and on the other side by the 343 bp of flanking DNA immediately downstream of the stop codon. This fragment was then inserted into *Bam*HI- and *Kpn*I-digested pRS304 to generate pKO-ZAP1. Plasmid pSK<sup>+</sup>ZAP1 was constructed by amplifying a 2.6-kb fragment containing the *ZAP1* ORF with primers bearing either a *Sal*I or a *Sac*I site at their 5' ends. The resulting fragment was digested with *Sac*I and *Sal*I and ligated into pBluescript SK(+). A 1-kb PCR fragment containing bases -1047 to +3 of the *ZRT2* gene was generated with primers containing either a *Sal*I or a *Hind*III site at their 5' ends. The fragment was digested with *Sal*I and *Hind*III and inserted into *Sal*I- and *Hind*III-digested YEp353 (23) to generate pZRT2-lacZ. Similarly, a PCR fragment containing bases -1111 to +3 of the *ZAP1* gene was generated with primers containing either *Eco*RI or *Bam*HI sites at their 5' ends. This fragment was inserted into *Eco*RI- and *Bam*HI-digested YEp353 (23) to generate YEpZAP1-lacZ. Frame-shift mutations were introduced into pZH5 by digesting the plasmid with either *Bss*HII or *Mlu*I and filling in the ends with the Klenow fragment of DNA polymerase I prior to religation. These mutations were confirmed by DNA sequencing.

**Zinc uptake and  $\beta$ -galactosidase assays.** Zinc uptake assays were performed as described previously for iron uptake (6) except that <sup>65</sup>ZnCl<sub>2</sub> (Amersham) and LZM prepared without EDTA (LZM-EDTA) were substituted for <sup>59</sup>FeCl<sub>3</sub> and LIM-EDTA, respectively. Cells were incubated for 5 min in LZM-EDTA plus 20  $\mu$ M <sup>65</sup>Zn, collected on glass fiber filters (Schleicher & Schuell), and washed with 10 ml of ice-cold SSW (1 mM EDTA, 20 mM trisodium citrate, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 5 mM MgSO<sub>4</sub>, 1 mM NaCl, pH 4.2); cell-associated radioactivity was measured by liquid scintillation counting.  $\beta$ -Galactosidase activity was assayed in cells harvested at an optical density at 600 nm of 0.5 to 2.0 as described by Guarente (12); activity was expressed as follows: (change in absorbance at 420 nm  $\times$  1,000)/(minutes  $\times$  milliliters of culture  $\times$  optical density of the culture at 600 nm).

**RNA isolation and Northern blot analysis.** Total RNA was isolated, denatured, separated by agarose gel electrophoresis (6  $\mu$ g per lane), and analyzed by Northern blotting. The probe used was the 1-kb *Bam*HI-*Sal*I fragment of pSK<sup>+</sup>ZRT1 (38) or an actin fragment labeled with <sup>32</sup>P (Amersham) by the random priming method (9). The *ZRT2* and *ZAP1* probes were generated with the Riboprobe system (Promega) with *Sal*I-digested pSK<sup>+</sup>ZRT2 (39) and *Bam*HI-digested pSK<sup>+</sup>ZAP1, respectively, as the template.

## RESULTS

**Isolation of mutants with altered ZRT1 expression.** To identify genes whose products regulate *ZRT1* transcription, we used a genetic screening method to isolate mutants with an elevated level of *ZRT1* transcription during growth on a zinc-replete medium (Fig. 1). The design of this screening method was similar to a scheme used to isolate yeast mutants with altered iron-responsive gene expression (5). The *ZRT1* promoter is active in zinc-limited cells but inactive in zinc-replete cells. We inserted this promoter upstream of the coding region of the *HIS3* gene, which encodes an enzyme required for histidine biosynthesis. The *ZRT1-HIS3* fusion gene was integrated into the genome of a *his3 $\Delta$*  mutant to generate the strain ZHY4. Because of the zinc-responsive expression of the *ZRT1* promoter, ZHY4 cells grow without added histidine on zinc-limiting medium but not on zinc-replete medium (Fig. 2A).

Thirty spontaneous His<sup>+</sup> strains were isolated from ZHY4 cultures. The phenotype of M18, the His<sup>+</sup> strain characterized in this report, is shown in Fig. 2. Each of the 30 isolates was crossed with an isogenic strain of the opposite mating type (ZHY5), and the His<sup>+</sup> phenotype segregated in a 2:2 ratio in the asci obtained from these diploid strains. Thus, the His<sup>+</sup> phenotype in each strain is caused by a single-gene chromosomal mutation. To determine if the His<sup>+</sup> phenotype was due to *trans*-acting effects on the *ZRT1* promoter rather than, for example, mutations within the promoter of the *ZRT1-HIS3* fusion, zinc uptake activity was also assayed in the mutant strains. For all 30 strains, zinc uptake activity was increased from 3- to 10-fold (data not shown and Fig. 2B). To determine if these mutations were dominant or recessive, each of the heterozygous mutant diploids was assessed for histidine prot-

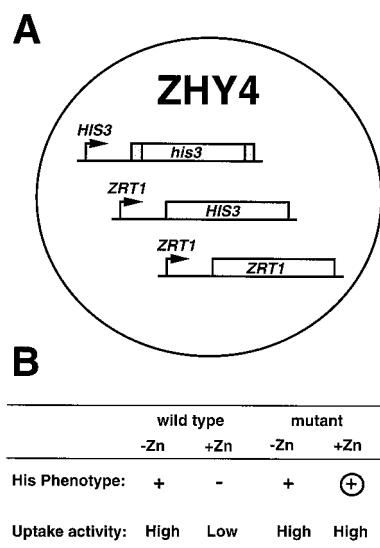


FIG. 1. The method of genetic selection used for isolating mutants with altered *ZRT1* expression. (A) Relevant genotype of strain ZHY4. ZHY4 bears a deletion removing the ORF of the chromosomal *HIS3* gene. The *ZRT1* promoter (bases -706 to -1; the first base of the ATG initiation codon is designated as position +1) was fused to the *HIS3* ORF, which retained its own translation initiation codon (ATG). This *ZRT1-HIS3* fusion gene was then inserted at another site in the genome (*URA3*). The wild-type *ZRT1* gene is also present in ZHY4. (B) Histidine auxotrophy and zinc uptake phenotypes of the wild-type ZHY4 and mutant derivatives in zinc-limited (-Zn) and zinc-replete (+Zn) media. Mutants were isolated based on their His<sup>+</sup> phenotype on zinc-replete media (circled).

otrophy on zinc-replete plates. Twenty-nine of the 30 strains were recessive (data not shown), whereas the M18 strain was semidominant (Fig. 2A). This semidominance was also apparent in zinc uptake assays. The uptake rate in the M18/+ diploid, although lower than the rate measured in the M18 haploid, was still significantly higher than the wild-type diploid rate ( $P < 0.01$ ) (means  $\pm$  standard deviations,  $6.1 \pm 0.1$  and  $1.1 \pm 0.1$  pmol/min/ $10^6$  cells, respectively) (Fig. 2B).

**Cloning of the *ZAP1* gene.** We cloned the mutated gene of the M18 isolate by virtue of its semidominant phenotype. A plasmid library was generated from M18 genomic DNA and transformed into ZHY4 (*his3 $\Delta$ ZRT1-HIS3*), and transformants were screened for histidine prototrophy. Two plasmids, pZH4 and pZH5, were isolated that conferred both the His<sup>+</sup> phenotype and increased uptake activity (Fig. 3). Sequence analysis of the inserts of these two plasmids indicated that they were overlapping genomic fragments from yeast chromosome X (11) and that each contained two ORFs of unknown function, YJL055W and YJL056C. A 4-bp frameshift mutation introduced into the *Bss*HIII site of YJL056C eliminated both the His<sup>+</sup> and uptake phenotypes, whereas a similar mutation introduced into the *Mlu*I site of YJL055W had no effect. Based on the results described in this report, we have designated YJL056C as the *ZAP1* gene (for zinc-responsive activator protein). Three different experiments confirmed that *ZAP1* is the mutated gene in the M18 isolate. First, a genetic marker (*LEU2*) was inserted adjacent to the wild-type *ZAP1* locus in ZHY5. This inserted gene did not segregate independently of the M18 mutation; i.e., when the spores of 10 asci from the ZHY5 *ZAP1::LEU2*  $\times$  M18 diploid were examined, the His<sup>+</sup> phenotype segregated 2:2 in each, and all His<sup>-</sup> spores were also Leu<sup>+</sup>. Second, a wild-type genomic clone of the *ZAP1* gene was isolated from a ZHY4 genomic library by colony

hybridization. The wild-type and M18 mutant *ZAP1* alleles were integrated in a single copy into the genome of a *zap1* deletion mutant (see below). In these transformants, the M18 allele showed a fivefold-higher uptake rate than the strain transformed with the wild-type allele (data not shown). Finally, we sequenced the entire *ZAP1* ORF from M18 and found that it differed from the wild-type sequence in ZHY4 by a single base substitution (see below). Because of its effects on *ZRT1* expression, we have designated the M18 mutant allele *ZAP1-1<sup>up</sup>*. Surprisingly, we also noted that ZHY4 transformed with a multicopy plasmid bearing the wild-type *ZAP1* allele also showed the His<sup>+</sup> and increased-uptake phenotypes in zinc-replete cells. Thus, overexpression of wild-type Zap1p may also cause constitutive *ZRT1* expression.

**Amino acid sequence of Zap1p.** The predicted amino acid sequence of wild-type Zap1p is 880 amino acids in length and has a molecular mass of 93 kDa. Zap1p has several features expected in a transcriptional activator protein (Fig. 4). First, the carboxy-terminal 174 amino acids (residues 707 to 880) contain five zinc finger domains of the TFIIIA type, i.e., C-X<sub>2-4</sub>-C-X<sub>12</sub>-H-X<sub>3-5</sub>-H. This region has several basic amino acids and a predicted isoelectric point (pI) of 9.9. The predicted pI of the full-length protein is 6.4. We also noted the presence of a sixth potential zinc finger in this region with the noncanonical sequence C-X<sub>2</sub>-C-X<sub>12</sub>-Q-X<sub>3</sub>-C (amino acids 752 to 772). Amino acids 1 to 706 contain two regions rich in acidic residues (aspartate and glutamate) that could be transcription activation domains. One region (amino acids 190 to 331) is 142 amino acids in length and has a predicted pI of 4.4. The second region (amino acids 603 to 703) is 101 amino acids in length and has a predicted pI of 4.6. There is also an asparagine-rich domain (residues 402 to 480; 27%) and a serine- and threonine-rich region (residues 482 to 564; 29%), and such domains have been noted in other transcription factors (2, 10, 24). Amino acids 529 to 532 (-KNRR-) are similar to a consensus nuclear localization signal (1).

Previous results suggested that the *ZRT1* gene is controlled by an intracellular pool of zinc (38). The simplest hypothesis for this regulation is that Zap1p activity is controlled by zinc

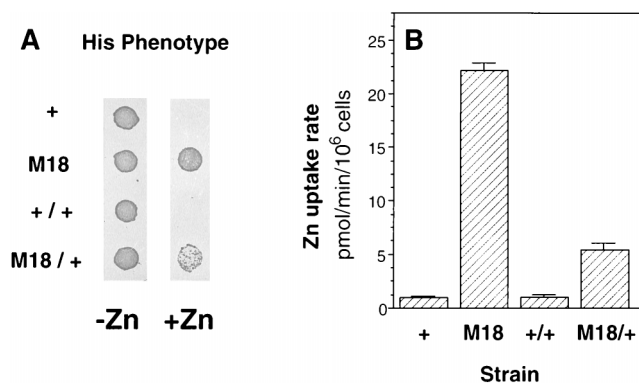


FIG. 2. Phenotypic properties of the *ZAP1-1<sup>up</sup>* allele. (A) Effects on histidine auxotrophy. Haploid strain ZHY4 (+), its M18 derivative (M18), and diploid strains obtained by mating ZHY5 with either ZHY4 (+/+) or M18 (M18/+ ) were evaluated for histidine auxotrophy. The semisolid medium consisted of SD supplemented with 2% glucose, all necessary auxotroph supplements except histidine, 30 mM 3-aminotriazole, and either 1 mM EDTA, 50  $\mu$ M ZnCl<sub>2</sub> (zinc limiting [-Zn]), or 2 mM ZnCl<sub>2</sub> (zinc replete [+Zn]). Approximately 200 cells of each strain were inoculated in a 5- $\mu$ l volume, and the plates were incubated at 30°C for 3 days prior to photography. (B) Effects on zinc uptake rate. The same strains as used for the study shown in panel A were grown to exponential phase in SD medium and assayed for the rate of zinc uptake. The error bars represent 1 standard deviation ( $n = 4$ ).

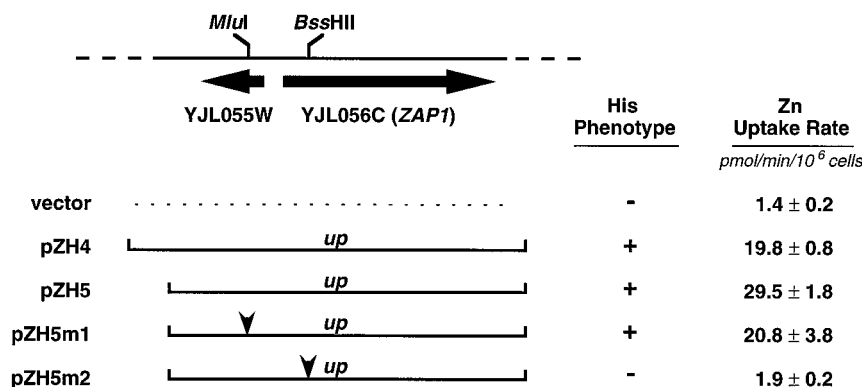


FIG. 3. Cloning and mapping of the *ZAP1* gene. The two ORFs on chromosome X contained in pZH4 and pZH5 (YJL055W and YJL056C) are indicated by the large arrows. The *MluI* and *BssHII* restrictions sites used to generate frameshift mutations in pZH5m1 and pZH5m2, respectively, are shown. The endpoints of the cloned fragments are indicated; the small arrowheads indicate the locations of the frameshift mutations, and “up” designates the presence of the *ZAP1-1<sup>up</sup>* allele on the fragment. Wild-type (ZHY4) transformants were assayed for histidine auxotrophy and zinc uptake rate as described in the legend to Fig. 2. Mean uptake rates are given ± 1 standard deviation ( $n = 4$ ).

binding directly to the protein and Zap1p contains a large number of potential zinc ligands. In addition to the zinc finger domains and the acidic residues described above, amino acids 1 to 706 are highly enriched in cysteine and histidine residues (~12%). This observation suggests that zinc binding in this region may alter Zap1p function, and this hypothesis is supported by our characterization of the *ZAP1-1<sup>up</sup>* allele. DNA sequencing of the wild-type *ZAP1* and *ZAP1-1<sup>up</sup>* alleles demonstrated that the only difference between these two alleles is the replacement of a cysteine in the wild-type protein by a serine in *ZAP1-1<sup>up</sup>* (Fig. 4). The mutated cysteine is at position 203, within one of the potential activation domains.

**Effect of *ZAP1* alleles on *ZRT1* expression.** To characterize more directly the effects of the *ZAP1-1<sup>up</sup>* allele on *ZRT1* transcription, we assessed the zinc responsiveness of *ZRT1* expression by zinc uptake assays,  $\beta$ -galactosidase activity generated from a *ZRT1-lacZ* reporter gene, and Northern blotting (Fig. 5). In the wild type, as was observed previously, zinc uptake activity,  $\beta$ -galactosidase activity, and *ZRT1* mRNA levels were high in zinc-limited cells and low in zinc-replete cells. In contrast, *ZRT1* expression in an isogenic *ZAP1-1<sup>up</sup>* strain was fully induced in both zinc-limited and zinc-replete cells. Thus, the *ZAP1-1<sup>up</sup>* mutation interferes with the zinc-dependent repression of the *ZRT1* promoter but does not prevent full induction of the *ZRT1* gene.

To further examine Zap1p's role in *ZRT1* regulation, we constructed a *zap1* $\Delta$  disruption mutation (*zap1* $\Delta::TRP1$ ) in which the entire *ZAP1* ORF was deleted from its chromosomal site. A haploid strain bearing this mutation was viable, indicating that *ZAP1* is not an essential gene. Zinc uptake activity, *ZRT1-lacZ* expression, and *ZRT1* mRNA levels were very low in zinc-limited as well as zinc-replete *zap1* $\Delta$  cells (Fig. 5). Thus, the *ZAP1* gene is required for transcription of the *ZRT1* gene in response to zinc-limiting growth conditions.

**Effect of *ZAP1* alleles on zinc-limited growth.** We predicted that if *ZAP1* plays an important role in controlling zinc uptake activity, then the *zap1* $\Delta$  allele would alter the level of zinc required for these cells to grow. Growth of isogenic wild-type, *ZAP1-1<sup>up</sup>*, and *zap1* $\Delta$  cells was examined in a zinc-limiting medium, LZM, supplemented with a range of zinc concentrations. No difference between the wild-type and *ZAP1-1<sup>up</sup>* strains in the growth response to zinc was observed (Fig. 6A). This observation is consistent with the normal maximal level of *ZRT1* expression observed in the *ZAP1-1<sup>up</sup>* cells. In contrast,

the *zap1* $\Delta$  strain required significantly more zinc for growth than did wild-type cells. Maximum growth of the wild-type and *ZAP1-1<sup>up</sup>* strains was achieved with 10  $\mu$ M zinc, whereas the *zap1* $\Delta$  strain required 1 mM zinc to attain its maximum growth yield. LZM is zinc limiting because of the presence of 1 mM EDTA, a high-affinity zinc chelator. Thus, the *zap1* $\Delta$  mutant is only able to grow in LZM with a concentration of added zinc sufficient to saturate the metal buffering capacity of the EDTA.

Surprisingly, the zinc requirement of the *zap1* $\Delta$  strain was higher than that of an isogenic *zrt1* mutant. To compare the zinc requirements of *zap1* $\Delta$  and *zrt1* mutants under more controlled metal buffering conditions, we examined zinc requirements in LZM-EDTA (Fig. 6B). LZM-EDTA is less zinc limiting than LZM at any given concentration of total zinc because citrate, the predominant chelator in LZM-EDTA, binds the metal with lower affinity than EDTA. While the *zrt1* mutant underwent the maximum number of cell divisions in LZM-EDTA with as little as 0.5  $\mu$ M zinc (data not shown), the *zap1* $\Delta$  strain required 500  $\mu$ M zinc to do so. Thus, the *zap1* $\Delta$  mutant strain requires at least 1,000 times more zinc for growth than does the *zrt1* mutant. A *zrt1 zrt2* double mutant, lacking both the high- and low-affinity uptake systems, required only slightly more zinc (750  $\mu$ M) than the *zap1* $\Delta$  mutant to achieve its maximum growth yield.

In the results of the experiment described in Fig. 6A, we also noted that while the *zrt1* mutant divided more than three times in extremely zinc-limiting media (e.g., 1  $\mu$ M Zn), the *zap1* $\Delta$  strain divided only once. This observation suggests that at the time of inoculation into the zinc-limiting medium, the *zap1* $\Delta$  strain had lower pools of available intracellular zinc than did the *zrt1* mutant strain.

**Effect of *ZAP1* alleles on *ZRT2* expression.** The higher-level zinc requirement of the *zap1* $\Delta$  strain relative to the *zrt1* mutant was unexpected if the sole function of Zap1p is to activate transcription of *ZRT1*. Therefore, one or more additional genes might require Zap1p for their expression, and a likely candidate was the *ZRT2* gene. On Northern blots, *ZRT2* mRNA was much less abundant than *ZRT1* mRNA; the use of probes with higher specific activities and longer exposure times was required to detect *ZRT2* mRNA. This is consistent with the observation that low-affinity uptake activity is less than 5% of fully induced high-affinity activity (38). Like *ZRT1*, *ZRT2* showed zinc-responsive regulation in *ZAP1* wild-type cells (Fig. 7A). *ZRT2* mRNA was approximately threefold more



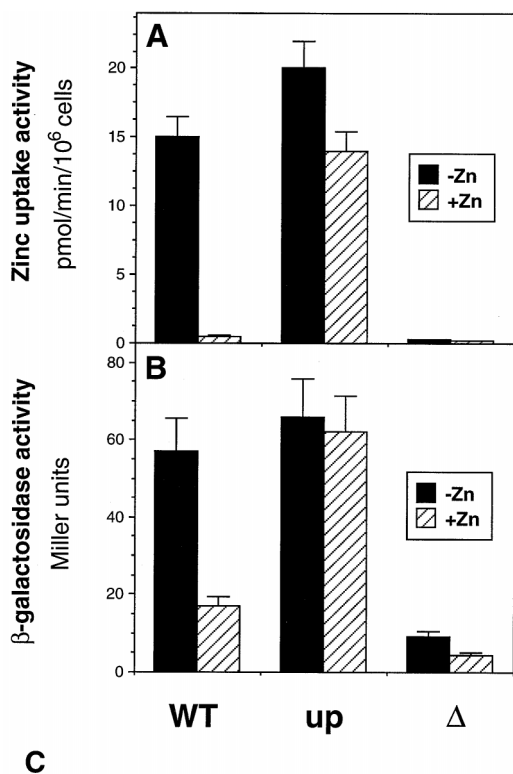


FIG. 5. Effect of *ZAP1* alleles on expression of the *ZRT1* gene. The strains used were the wild type (WT; DY1457), *ZAP1-1<sup>up</sup>* (up; ZHY7), and *zap1Δ* (Δ; ZHY6), each of which had been transformed with a *ZRT1-lacZ* fusion gene on plasmid pG11 (38). The cells were grown to exponential phase in SD medium supplemented with 2% glucose and all necessary auxotroph supplements either with (zinc limiting [-Zn]) or without (zinc replete [+Zn]) 1 mM EDTA and 50  $\mu$ M ZnCl<sub>2</sub>. These cells were then assayed for high-affinity uptake activity at 1  $\mu$ M <sup>65</sup>Zn (A),  $\beta$ -galactosidase activity (B), and *ZRT1* mRNA levels by Northern blotting (C). Actin (*ACT1*) mRNA levels were also determined to confirm equal loading of RNA in the lanes. The error bars in panels A and B represent 1 standard deviation ( $n = 4$ ).

the *ZAP1* gene may itself be regulated by zinc. To assess this regulation more quantitatively,  $\beta$ -galactosidase activity generated by a *ZAP1-lacZ* reporter gene was measured in wild-type cells (Fig. 7C). These results demonstrated that the *ZAP1* gene is also transcriptionally regulated in response to zinc; *ZAP1-lacZ* expression was approximately threefold higher in zinc-limited cells than in zinc-replete cells. Moreover, in cells grown under more stringent zinc-limiting conditions (i.e., SD with glucose and 1 mM EDTA), *ZAP1-lacZ*  $\beta$ -galactosidase activity was almost 50-fold higher than in zinc-replete cells ( $270 \pm 23$  versus  $5.7 \pm 0.2$  Miller units, respectively). Zinc-responsive regulation of *ZAP1*, like that of *ZRT1* and *ZRT2*, appears to be mediated by Zap1p. *ZAP1-lacZ* expression in *ZAP1-1<sup>up</sup>* cells was no longer zinc responsive, being elevated under both sets of culture conditions (Fig. 7C). Furthermore, expression was

markedly reduced in both zinc-limited and zinc-replete *zap1Δ* cells. These data suggest that *ZAP1* controls the activity of its own promoter in response to zinc.

## DISCUSSION

The *ZAP1* gene encodes an important component of the regulatory system that controls zinc uptake in *S. cerevisiae*. Zinc status had been previously noted to alter expression of the high- and low-affinity uptake systems, and we found that Zap1p regulates the transcription of the *ZRT1* and *ZRT2* genes in response to zinc availability. This protein may regulate other genes as well. Yuan and Klausner (37) recently identified 16 genes in addition to *ZRT1* and *ZRT2* whose mRNA levels increase in zinc-limited cells. This group included genes involved in phosphate and carbon source utilization (*PHO13* and *ADH4*), the control of amino acid biosynthesis (*MET30*), and intracellular cation transport (*PMR2* and *ZRC1*). While the zinc responsiveness of these genes may be caused by indirect effects of zinc status, it is exciting to speculate that Zap1p plays a global role in the regulation of zinc homeostasis and the metabolic response to zinc limitation. Zinc-responsive transcription of *ZRC1* is particularly intriguing because this gene encodes a protein thought to transport zinc from the cytoplasm into an unknown organelle (20).

We observed that the *ZAP1* promoter is also zinc regulated, and the effects of *ZAP1* alleles on *ZAP1-lacZ* expression indicate that this regulation is mediated by Zap1p itself. These

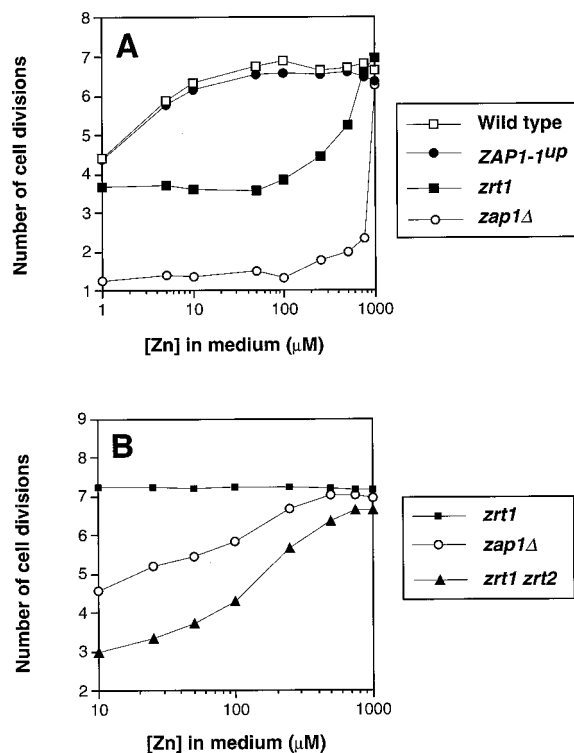


FIG. 6. Effect of *ZAP1* alleles on zinc-limited growth. Wild-type (DY1457), *ZAP1-1<sup>up</sup>* (ZHY7), *zap1Δ* (ZHY6), *zrt1* (ZHY1), and *zrt1 zrt2* (ZHY3) strains were grown to stationary phase in SD medium plus glucose, washed in LZM, and reinoculated into either LZM (A) or LZM-EDTA (B) supplemented with the indicated concentrations of ZnCl<sub>2</sub>. These cultures were then grown for 16 h at 30°C prior to cell number determination. The number of cell divisions is plotted against the total zinc concentration of the medium. Each value is the mean of four separate cultures, and the standard deviation of each was less than 10% of the corresponding mean.

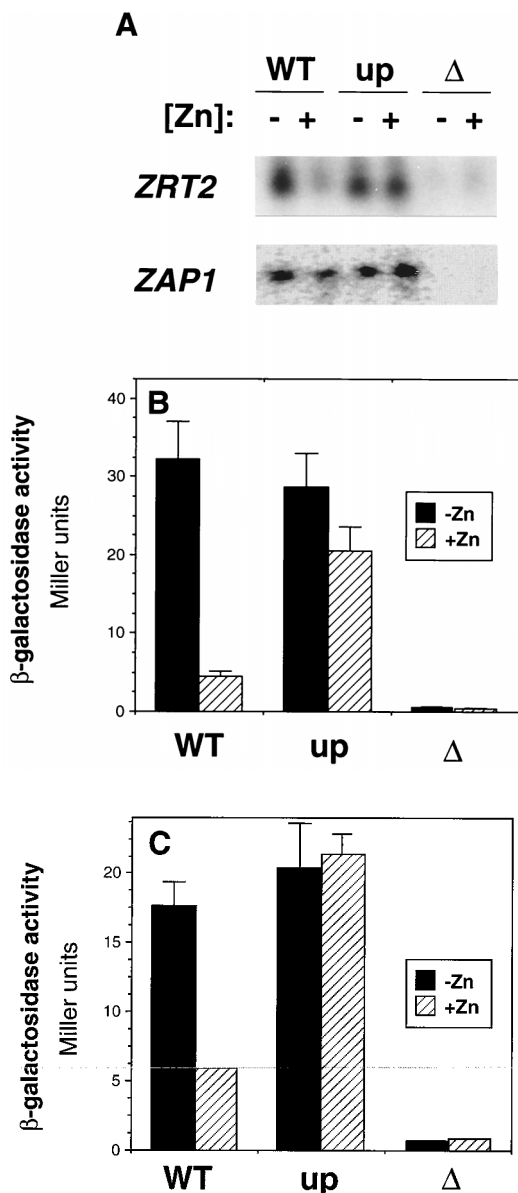


FIG. 7. Effect of *ZAP1* alleles on expression of the *ZRT2* and *ZAP1* genes. The strains and growth conditions were as described in the legend to Fig. 5. (A) *ZRT2* and *ZAP1* mRNA levels were determined by Northern blotting. Equal loading of RNA in each lane was confirmed beforehand by acridine orange staining of the agarose gel (data not shown). The image of the *ZAP1* blot was obtained with a Bio-Rad model GS-363 molecular imaging system. These strains were transformed with pZRT2-lacZ, a plasmid bearing a reporter gene in which the *ZRT2* promoter was fused to the *lacZ* gene (B), or with YEpZAP1-lacZ bearing a *ZAP1*-lacZ reporter fusion (C). These cells were also grown as described in the legend to Fig. 5 except that uridine was not included in the medium to allow selection of the plasmid. Cells were harvested in exponential phase and assayed for  $\beta$ -galactosidase activity. Mean values are given, and the error bars represent 1 standard deviation.

data suggest the existence of a positive autoregulatory mechanism in the control of zinc homeostasis. By this scenario, when zinc-replete cells become zinc limited, synthesis of Zap1p is induced, and Zap1p, in turn, increases the expression of other target genes. This type of regulatory circuitry would allow a rapid, amplified response to changes in Zap1p activity under zinc-limiting conditions.

The *ZAP1-1<sup>up</sup>* mutation interferes with the zinc-dependent

shutoff of the *ZRT1*, *ZRT2*, and *ZAP1* promoters. This defect is consistent with either a loss-of-function mutation in a transcriptional repressor or a gain-of-function mutation in a transcriptional activator protein. The available data strongly suggest that *ZAP1* encodes an activator. First, *ZAP1* expression is increased in zinc-limited cells. Second, the *ZAP1-1<sup>up</sup>* allele is dominant, a property consistent with this allele producing a protein that activates transcription in zinc-replete cells even in the presence of wild-type Zap1p. Overexpressing the wild-type allele from a high-copy-number plasmid also increased expression of the *ZRT1* promoter in zinc-replete cells. Finally, the *zap1Δ* deletion mutation caused low-level expression of *ZRT1*, *ZRT2*, and *ZAP1* in either zinc-limited or zinc-replete cells; i.e., Zap1p is required for zinc-responsive expression of these genes.

Features of Zap1p also support its proposed role as a transcriptional activator. For example, the carboxy terminus of the protein has five C<sub>2</sub>H<sub>2</sub> zinc finger domains. First identified in TFIIIA (15), this motif has since been found in literally hundreds of DNA-binding proteins. Zap1p also has two regions that are likely to be transcriptional activation domains. Yeast activation domains are typified by an abundance of acidic residues (13). We cannot yet rule out the possibility that Zap1p is part of a signal transduction system that communicates cellular zinc status to other proteins that regulate transcription. However, preliminary results strongly favor a direct role of Zap1p in this regulation; in vitro-synthesized Zap1p can bind to sites in the *ZRT1* promoter in a sequence-specific manner (7a).

Intracellular zinc somehow inhibits transcriptional activation by Zap1p, and we propose that Zap1p plays a dual role in this regulation, acting both as the primary zinc "sensor" and as a transcriptional activator. Zinc sensing might involve direct binding of the metal ion to Zap1p, which would then inhibit the protein's ability to activate transcription. To understand this process, we must distinguish between structural and regulatory zinc binding. Structural zinc binding probably occurs in some or all of the zinc finger domains of Zap1p. This binding is likely to be of high affinity to allow zinc finger formation and DNA binding even when intracellular zinc levels are low. Regulatory zinc binding, i.e., the metal-protein interaction that controls transcriptional activation by Zap1p, could involve lower-affinity sites whose occupancy would vary with changing intracellular zinc levels. For example, regulatory zinc binding in the amino-terminal region of the protein could lead to the formation of an inactive conformation (i.e., intramolecular repression) or interaction with an inhibitor protein (i.e., intermolecular repression) that represses Zap1p function. Alternatively, as some investigators have proposed for other zinc finger proteins (4, 32), zinc binding in the finger domains could regulate metal-responsive gene expression. Regulatory zinc binding by Zap1p may occur in a subset of the zinc finger domains. In zinc-limited cells, high-affinity fingers would bind zinc and be responsible for binding of Zap1p to sites in its target promoters. In zinc-replete cells, low-affinity fingers would form and cause binding of Zap1p to other sites in the genome, thus titrating the activator protein away from the *ZRT1*, *ZRT2*, and *ZAP1* promoters.

Further studies will be required to assess these various models, but the data gathered thus far are very suggestive. First, amino acids 1 to 706 include several cysteine and histidine residues; many of them may play a role in regulatory zinc binding. The mutation in the *ZAP1-1<sup>up</sup>* allele alters one of these potential ligands, a cysteine at position 203 that is located within one of the two potential activation domains. This mutation interferes with Zap1p's ability to be repressed by zinc and could interfere directly with regulatory zinc binding. Sec-

ond, several recessive mutations were isolated in our genetic screen. These may be loss-of-function mutations in the gene that encodes the inhibitor protein proposed in the intermolecular-repression model described above.

Although Zap1p is the first transcription factor known to increase expression in response to zinc limitation, other zinc-responsive factors have been identified. The SmtB protein, which represses metallothionein expression in the cyanobacterium *Synechococcus* sp. strain PCC7942, is a helix-turn-helix protein whose DNA binding ability is inhibited by zinc binding to the protein (8, 22). Zinc-induced expression of metallothionein genes in mammals involves the binding of one or more metal-responsive transcription factors (e.g., MTF-1 or ZRF) to sites (metal-regulated enhancer elements) in the metallothionein gene promoters (21, 34). MTF-1 has been cloned, and its product bears features similar to those of Zap1p, including C<sub>2</sub>H<sub>2</sub> zinc finger domains and transcriptional activation domains (2). However, while MTF-1 increases transcription in response to elevated zinc levels, Zap1p activity is repressed by the metal. How MTF-1 and Zap1p dictate opposite transcriptional responses to zinc is an interesting paradox that may soon be resolved.

The Zap1p zinc regulator exhibits many similarities to the iron-responsive Aft1p (35) and copper-responsive Mac1p (19) proteins of yeast. Each of these proteins is a transcriptional activator that increases expression of uptake systems when the concentration of its respective metal ion is limiting in the environment. All three proteins contain potential DNA binding domains, and DNA binding has been demonstrated for Aft1p (36). Furthermore, dominant alleles of each have been isolated that cause constitutive expression of their target genes. These "up" mutations alter amino acids that are potential metal ligands and suggest that these residues are involved in regulatory metal binding interactions that control the transcriptional activity of the protein. The functional similarity of Aft1p, Mac1p, and Zap1p suggests a central theme for transcriptional regulators that control metal uptake in *S. cerevisiae* and, perhaps, other eukaryotes.

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