Zinc-Regulated Genes in *Saccharomyces cerevisiae* Revealed by Transposon Tagging

Daniel S. Yuan

Division of Pediatric Gastroenterology and Nutrition, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287-2631

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ABSTRACT

The biochemistry of human nutritional zinc deficiency remains poorly defined. To characterize in genetic terms how cells respond to zinc deprivation, zinc-regulated genes (*ZRG*'s) were identified in yeast. Gene expression was probed using random *lacZ* reporter gene fusions, integrated by transposon tagging into a diploid genome as previously described. About half of the genome was examined. Cells exhibiting differences in *lacZ* expression on low or moderate (~0.1 *vs.* 10 μM) zinc media were isolated and the gene fusions were sequenced. Ribonuclease protection assays demonstrated four- to eightfold increases for the RNAs of the *ZAP1*, *ZRG17* (*YNR039c*), *DPP1*, *ADH4*, *MCD4*, and *YEF3B* genes in zinc-deficient cells. All but *YEF3B* were shown through reporter gene assays to be controlled by a master regulator of zinc homeostasis now known to be encoded by *ZAP1*. *ZAP1* mutants lacked the flocculence and distended vacuoles characteristic of zinc-deficient cells, suggesting that flocculation and vacuolation serve homeostatic functions in zinc-deficient cells. *ZRG17* mutants required extra zinc supplementation to repress these phenotypes, suggesting that *ZRG17* functions in zinc uptake. These findings illustrate the utility of transposon tagging as an approach for studying regulated gene expression in yeast.

ZINC is one of the principal trace elements in biology, with structural or enzymatic roles in hundreds of proteins (Vallee and Falchuk 1993). Zinc finger proteins are especially numerous in eukaryotic genomes and play many roles in protein-DNA or protein-RNA interactions (Berg and Shi 1996). The enzymatic repertoire of zinc is extraordinary and includes many important phosphatases and metalloproteinases (Lippard and Berg 1994). Zinc also functions in a large number of oxidoreductases and transferases (Vallee and Falchuk 1993), and additional roles for zinc in cysteinyl transfer and methylation reactions are now known (Matthews and Goulding 1997).

Many studies, dating back to 1869, have confirmed the importance of zinc in nutrition (reviewed in Vallee and Falchuk 1993). In humans, zinc deficiency has gradually come to be recognized as a clinically significant and common form of malnutrition, particularly in chronically ill patients and in the Third World (Aggett and Comerford 1995; Bhutta *et al.* 1999). The clinical manifestations of zinc deficiency are diverse, with effects on immune function, epithelial integrity, appetite, cognitive function, and embryonic development (Walsh *et al.* 1994). Unfortunately it remains unclear how zinc deficiency relates to any of these changes at a biochemical level, even though a large variety of correlations have been made (*e.g.*, Shankar and Prasad 1998).

Studies of the biochemistry of zinc deficiency have been attempted for many years. "Throughout the period of discovery of zinc enzymes, there has been a diligent search for alterations of their activities in organs and tissues of zinc-deficient animals. The results have been almost uniformly disappointing" (Vallee and Falchuk 1993, p. 81). Various biochemical indicators of zinc status continue to be studied in a research setting (*e.g.*, Grider *et al.* 1990; Licastro *et al.* 1996; Beck *et al.* 1997). However, there still appears to be no way of predicting which zinc proteins are affected most by zinc deprivation. A promising new approach involves the identification of novel genes whose expression is regulated by zinc status. In particular, subtractive hybridization and differential display techniques have been used to identify several zinc-regulated genes in rats rendered zinc deficient or zinc replete through dietary manipulation (Shay and Cousins 1993; Blanchard and Cousins 1996). It is not yet clear why or how these genes are regulated by zinc status, however.

This article describes a novel application of yeast genetics to the problem of identifying zinc-regulated genes. The original impetus for this study was the emerging realization that many features of iron and copper metabolism are conserved between yeast and mammals (reviewed in Askwith and Kaplan 1998; Culotta *et al.* 1999), and indeed this conservation is now being extended to zinc (Eide 1997). In this study, zinc-regu-

Address for correspondence: WBSB 713, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205-2185. E-mail: dyuan@jhmi.edu

lated genes in yeast were identified on a quasi-genomic scale using an existing transposon tagging technique (Burns *et al.* 1994). A total of 17 genes were found to be differentially regulated at least 10-fold, as assessed by randomly generated translational fusions with the *lacZ* reporter gene. Five were subsequently shown to be regulated by the same transcription factor. One of these appears to encode a novel protein with a role in zinc uptake. These findings provide new starting points for characterizing the biochemical effects of zinc deficiency in eukaryotic cells.

MATERIALS AND METHODS

Plasmids and yeast strains: See Tables 1 and 2, respectively.

Preparation of yeast growth media: Glassware was scrubbed with Alconox detergent and acid washed before use; plasticware was used without further treatment. A defined growth medium lacking added zinc, iron, copper, phosphate, dextrose, and amino acids was prepared as a custom-made powder (Bio101, Vista, CA; following Dancis *et al.* 1994). All but the dextrose, zinc, and selectable markers were added back with the aid of heating to 70° to restore the composition of standard defined medium. The resulting solution (prepared as a 5× stock solution) was cooled to 40°, sterilized by filtration, and stored in the dark at room temperature for up to 1 year. Stock solutions of glucose (dextrose), MES buffer (2-[*N*-morpholino]ethanesulfonic acid hemisodium salt; Sigma, St. Louis), and nutritional supplements were treated with Chelex-100 resin and sterilized by filtration. Solid medium was prepared with 10 g/liter agarose (Biochemika grade; Fluka, Buchs, Switzerland), autoclaved for 15 min, and cooled to 50°. This was supplemented with 2% w/v glucose, 50 mm MES, nutritional supplements, and the indicated amounts of zinc (as zinc sulfate) immediately before dispensing in petri dishes. Liquid medium was prepared without agarose and refiltered instead of autoclaved.

YPD growth medium was prepared as described (Sherman 1991) and synthetic defined medium was prepared from yeast nitrogen base, glucose, and supplements as recommended (Bio101), except that glucose was added as a separately autoclaved or filtered stock solution after the medium was cooled to 50°.

Yeast transformation with the transposon insertion library: Cells of the diploid strain YPH274 (Sikorski and Hieter 1989) were transformed to leucine prototrophy with *Not*I-digested DNA that had been amplified once (Maxiprep; QIAGEN, Valencia, CA) from pool 21 of a library of transposon insertions (Burns *et al.* 1994), graciously provided by the laboratory of Michael Snyder (Yale University). The digested DNA was used without heat inactivation or further purification. A high-efficiency transformation protocol was used routinely (Gietz and Woods 1994; http://www.umanitoba.ca/faculties/medicine/units/human_genetics/gietz/Trafo.html). Cells were washed twice with low-zinc medium before plating on low-zinc medium lacking added leucine. Using 3.4 μg digested DNA with 25 pooled aliquots of cells, ~3500 colonies on each of 23 Leu-selective plates were obtained, indicating a transformation efficiency of $\sim 2 \times 10^4$ colonies/μg digested DNA.

Colony assays for zinc-regulated *lacZ* activity: Each transformation plate was replica plated to (1) a nylon membrane (Biotrans, 1.2 μm, 82-mm circles; ICN, Costa Mesa, CA) laid on a low-zinc plate, (2) a second membrane laid on low-zinc plates supplemented with 10 μmol/liter zinc sulfate, and (3) a master YPD plate. Velveteen squares used for replica-plating were scrubbed clean by hand, machine washed in hot water with chlorine bleach and a commercial laundry detergent, rinsed in hot water for three cycles, dried in a clothes dryer, and autoclaved in foil. To obtain the best replica fidelity, both the source and destination plates were allowed to dry out to ~80% of their former thickness before use, and the velveteen was underlaid with two circles cut from gel blotting paper (GB004; Schleicher and Schuell, Keene, NH). For the genetic screen, nylon membranes were boiled in 1 mm EDTA before rinsing with water and autoclaving, although similar results were obtained later using untreated membranes.

After 24 hr of incubation at 30° to elicit the color differences associated with zinc status (see Figure 2B), membranes were transferred to a surgical clamp and dipped twice for 10 sec each into liquid nitrogen to permeabilize the cells (Matchmaker protocol; Clontech Laboratories, Palo Alto, CA). The frozen membranes were gently thawed over a small flame, laid on agar plates containing X-gal (Burns *et al.* 1994) with 10 mm sodium azide, and incubated at 30° in a closed bag for up to 4 wk. The pink or orange pigmentation associated with the *ade2* genetic marker in zinc-treated cells faded completely

TABLE 1

Plasmids

Designation	Description	Reference
pDirect	Vector for ligation-independent cloning (LIC) in *E. coli*	Clontech Laboratories
pRS416	*URA3* centromeric yeast-*E. coli* vector	Sikorski and Hieter (1989)
pDY195	*ZAP1* (−283 to +2952) cloned in pDirect	This study
pDY233	*zap1::URA3* disruption construct derived from pDY195	This study
pDY276	*zap1*Δ*::URA3* deletion construct derived from pDY195	This study
YEp368R	Vector for *lacZ* fusion constructs; 2μ, *LEU2*	Myers *et al.* (1986)
pDY269	Vector for *lacZ* fusion constructs; *CEN6, TRP1*	This study
pDY297	*ZAP1 (ZRG10)* (−800 to +4) fused to *lacZ* in pDY269	This study
pDY277	*ADH4 (ZRG5)* (−800 to +244) fused to *lacZ* in pDY269	This study
pDY350	*MCD4 (ZRG16)* (−800 to +4) fused to *lacZ* in pDY269	This study
pDY273	*DPP1 (ZRG1)* (−800 to +4) fused to *lacZ* in pDY269	This study
pDY272	*DPP1 (ZRG1)* (−353 to +4) fused to *lacZ* in pDY269	This study
pDY275	*DPP1 (ZRG1)* (−800 to +718) fused to *lacZ* in pDY269	This study
pDY274	*DPP1 (ZRG1)* (−353 to +718) fused to *lacZ* in pDY269	This study

TABLE 2

Yeast strains

Designation	Pertinent genotype or description	Reference
Parental strains		Sikorski and Hieter (1989)
YPH252	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*	
YPH274	*MAT***a**/α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*	
Clones from the genetic screen		
See Table 3		
ZAP1 and *zap1* disruption strains		
DYY1083	*zap1::URA3* (from pDY233) in YPH252	This study
DYY1198	*zap1*Δ*::URA3* (from pDY276) in YPH252	This study
DYY637	*ZAP1/zap1::URA3* (from pDY233) in *MAT***a**/α *ZRG1/ZRG1-lacZ* (DYY515)	This study
DYY641	*MAT*α *ZRG1-lacZ ZAP1* (spore 3D from DYY637)	This study
DYY640	*MAT*α *ZRG1-lacZ zap1::URA3* (spore 3C from DYY637)	This study
DYY1200	*ZRG10(ZAP1)-lacZ* plasmid (pDY297) in YPH252	This study
DYY1187	*ZRG10(ZAP1)-lacZ* plasmid (pDY297) in *zap1::URA3* (DYY1083)	This study
DYY1118	*ZRG5-lacZ* plasmid (pDY277) in YPH252	This study
DYY1119	*ZRG5-lacZ* plasmid (pDY277) in *zap1::URA3* (DYY1083)	This study
DYY1242	*ZRG16-lacZ* plasmid (pDY350) in YPH252	This study
DYY1243	*ZRG16-lacZ* plasmid (pDY350) in *zap1*Δ*::URA3* (DYY1198)	This study
DYY1179	*ZRG17-242* (spore 1B from DYY754)	This study
DYY1171	*URA3* vector (pRS416) in *zrg17-242* (DYY1179)	This study
DYY1172	*zap1::URA3* (from pDY233) in *zrg17-242* (DYY1179)	This study
DYY1202	*ZRG1* (−800 to +4)-*lacZ* plasmid (pDY273) in YPH252	This study
DYY1185	pDY273 in *zap1::URA3* (DYY1083)	This study
DYY1201	*ZRG1* (−353 to +4)-*lacZ* plasmid (pDY272) in YPH252	This study
DYY1184	pDY272 in *zap1::URA3* (DYY1083)	This study
DYY1199	*ZRG1* (−800 to +718)-*lacZ* plasmid (pDY275) in YPH252	This study
DYY1134	pDY275 in *zap1::URA3* (DYY1083)	This study
DYY1203	*ZRG1* (−353 to +718)-*lacZ* plasmid (pDY274) in YPH252	This study
DYY1186	pDY274 in *zap1::URA3* (DYY1083)	This study
ZRG17 disruption strains		
DYY1206	*MAT*α *zrg17-242* (spore 1D from DYY754)	This study
DYY617	*MAT*α *zrg17-531* (spore 3C from DYY505)	This study

after a day of incubation and did not interfere with color development. The sodium azide was added to inhibit artifacts due to bacterial overgrowth and had no effect on yeast-associated *lacZ* activity.

Isolation of cells differentially expressing *lacZ* activity: After development of the X-gal color for 2 wk, the membranes were photographed using Ektapan (Kodak, 4162) film and the resulting pairs of negatives were superimposed with a slight offset and examined by eye against a clear incandescent lightbulb. By applying strips of removable tape, the negatives were readily scanned for colonies putatively exhibiting differential *lacZ* expression. Colonies on the master plate were located by comparison with prints from the negatives. To purify the clones and document zinc-regulated *lacZ* expression, clones of interest were dispersed into 1 ml of low-zinc, Leu-selective medium and 1 μl of this suspension spread as sectors on another low-zinc, Leu-selective plate. After colonies reached full size, the replica-plating procedure was repeated. About 70% of the clones exhibited perceptible differences in X-gal color in the two growth conditions and were kept for further study.

Identification of sequences upstream of genomic *lacZ* insertions by inverse PCR: Yeast genomic DNA was prepared from each purified clone essentially as described but at 1/10 scale (Philippsen *et al.* 1991). Templates for inverse PCR were prepared by digesting aliquots of 200 ng genomic DNA (2 μl) with 0.4 units *Nla*III or *Nsp*I enzyme in its recommended reaction buffer (New England Biolabs, Beverly, MA) in a total volume of 50 μl (giving a DNA concentration of 4 μg/ml) for 1 hr at 37°. After heat inactivation at 65°, the mixture was supplemented with ATP (to 1 mm), KCl (to 30 mm to promote intramolecular circularization; Dugaiczyk *et al.* 1975), and 0.5 units T4 DNA ligase (GIBCO BRL, Grand Island, NY) and incubated overnight at 14°. A 10-μl aliquot of this reaction mixture was diluted fivefold with 8 standard units of KlenTaq1 enzyme (Ab Peptides, St. Louis), components of the KlenTaq1 enzyme buffer without magnesium, deoxynucleotides, and 50 pmol each of the oligonucleotides *lacZ*-5′INV (GGCGATTA AGTTGGGTAACGCCAGGG, directed retrograde toward upstream promoter sequences) and *lacZ*-3′INV (CCGACTACA CAAATCAGCGA, directed anterograde toward the first *Nla*III restriction site in the *lacZ* coding sequence). After 40 cycles of PCR, reaction mixtures were cleaned up with PCR Select II columns (5 Prime → 3 Prime, Boulder, CO) and analyzed by agarose gel electrophoresis. PCR products were sequenced using a cycle sequencing kit (GIBCO BRL) and a 96-well

thermal cycler (MJ Research, Watertown, MA) in conjunction with the oligonucleotides *lacZ*-5′SEQ (CGTTGTAAAACGAC GGGATCCCCCT; Burns *et al.* 1994) or *lacZ*-5′INV (see above). The sequences obtained were matched to yeast sequences in GenBank or the Saccharomyces Genome Database using the BLAST program (Altschul *et al.* 1990).

Preparation of RNA probes for ribonuclease protection assays: Sequences spanning the lacZ fusion sites for each zinc-regulated gene (*ZRG*) were amplified from genomic DNA samples corresponding to each *ZRG* clone using the *lacZ* oligonucleotide GGGAAAGCCGGCtaatacgactcactatagggATTAAG TTGGGTAACGCCAGGGT (T7 promoter in lowercase letters and *lacZ* sequences underlined) and a *ZRG* oligonucleotide (with CCCGAGCTC preceding sequences from each *ZRG*) designed to amplify a fragment of defined length (150 bases for *ZRG1*, 160 bases for *ZRG2*, etc.). Cycle numbers were optimized for each reaction to avoid saturation. All products were pure and of the expected size except for the *ZRG12* fragment, which was gel purified to remove a smaller contaminant. Fragments for *ZRG*'s 1, 2, 4, 6, 10, and 17 were digested with *Ngo*MIV and *Sac*I for directional cloning in pRS416, which was digested with the same enzymes to excise the endogenous T7 promoter. The resulting plasmids were validated by sequencing, linearized with *Sac*I, and purified for *in vitro* transcription. Fragments for the other *ZRG*'s were used directly without cloning. Fragments for *TDH3* were synthesized with the oligonucleotides GGGAAAGCCGGCtaatacgactcactatagg gATGGTAGAGTAACCGTATTCG (T7 promoter sequence in lowercase letters and *TDH3* sequences underlined) and CCCGAGCTCCCTCTGACTTCTTGGGTGAC, designed to amplify 120 bases near the 3′ end of the *TDH3* coding sequence.

RNA probes labeled with [α-^{32}P]CTP were synthesized at 1/4 scale with 10 μM total CTP using an *in vitro* transcription kit (Maxiscript; Ambion, Austin, TX). The *TDH3* probe was synthesized with 500 μM total CTP. Probes of validated length were gel purified as recommended (RPA III; Ambion) except that the elution step was performed in 1/2 volume and with two freeze-thaw cycles to hasten elution.

Ribonuclease protection assays (RPAs): Total yeast RNA was prepared from matched low- and high-zinc cultures (100 ml) of the parental diploid strain, YPH274. The protocol used was chosen to allow the concurrent isolation of small RNAs (Wise 1991). RPAs were carried out with a kit (RPA III; Ambion). For each *ZRG*, 10 μg of total RNA from low-zinc or high-zinc cells and 10,000 cpm each of the purified *ZRG* and *TDH3* probes were precipitated and solubilized in hybridization buffer. A negative control containing the same probes but no added RNA was prepared for each *ZRG*. Hybridization was carried out at 42° for 12–16 hr. Unhybridized RNAs were digested with RNase A/T1 as directed except that all samples were kept at 15° and 10 μg Torula yeast RNA was added to the RNase solution just before mixing with the negative controls. After 60 min, all samples were processed for electrophoresis and separated on denaturing polyacrylamide gels (Novex, Encinitas, CA). A 10-bp DNA ladder was used as standard (GIBCO BRL end labeled using T4 polynucleotide kinase); RNA size was determined to be the DNA size plus 8%. After drying the gels onto filter paper, signals were quantitated by phosphorimaging (Storm; Molecular Dynamics, Sunnyvale, CA), using the negative controls to determine the level of background signal in adjacent bands.

Cloning and disruption of the *ZAP1* gene: The *ZAP1* open reading frame and 5′ and 3′ flanking sequences (283 and 309 bases, respectively) were cloned as a PCR product into the bacterial cloning vector pDirect (Clontech Laboratories, Palo Alto, CA), designed for ligation-independent cloning (Aslanidis and de Jong 1990), as directed. Genomic DNA from strain YPH252 was used as the PCR template. This yielded pDY195. A *ZAP1* disruption construct was prepared from pDY195 by inserting the *URA3* gene flanked by Klenow-blunted *Hin*dIII sites (prepared from plasmid B728, from T. Donahue and M. Cigan, National Institutes of Health) in reverse orientation into the *Msc*I site of *ZAP1*, 16 codons into the open reading frame. This yielded pDY233. The fragment released by digestion with *Cla*I and *Not*I was used to transform yeast to uracil prototrophy in synthetic defined medium. Transformants were validated by colony PCR using *ZAP1* and *URA3* oligonucleotides flanking the 5′ end of the transforming fragment. A *ZAP1* deletion construct was created and validated as for pDY233 except that *ZAP1* sequences lying between *Msc*I and *Nsi*I (containing all but the first 16 codons of the open reading frame) were removed by digestion with these enzymes and treatment with T4 DNA polymerase, and the *URA3* fragment was prepared by digestion with *Eco*RI and blunting with Klenow fragment. The resulting plasmid, pDY276, was used as for pDY233. Correct integration at the 3′ end was also verified in the same manner as at the 5′ end.

Cloning of synthetic promoter-*lacZ* fusion constructs: A centromeric *lacZ* reporter vector, pDY269, was prepared from the high-copy number *lacZ* reporter vector, YEp368R (Myers *et al.* 1986), by subcloning the *lacZ* gene and flanking sequences into the *TRP1*-marked plasmid pRS414 (Sikorski and Hieter 1989) and inserting the ligation-independent cloning site of pDirect (see above) into the *Sma*I site upstream of the *lacZ* gene. The construction and use of this vector will be described in detail elsewhere. DNA fragments of interest were synthesized by PCR from YPH252 (Sikorski and Hieter 1989) genomic DNA. Oligonucleotides were designed to create an in-frame fusion of yeast sequences containing a putative promoter and an initiation codon with the promoterless *lacZ* gene lacking a translation initiation codon. Ligation-independent cloning (Aslanidis and de Jong 1990) was accomplished by direct transformation of competent yeast with a mixture of suitably digested vector and insert fragments. Colonies growing on tryptophan-free medium were screened directly for zinc-regulated *lacZ* expression using the replica-plating techniques described for the genetic screen (above), maintaining selection for the *TRP1* plasmid. Representative colonies were cloned for further analysis.

Plasmid rescue: Yeast cells containing plasmids of interest were grown to saturation in 20-ml cultures of synthetic defined medium with the appropriate selectable markers. Pelleted cells were digested for at least 1 hr at 37° with 100 μg Zymolyase 100-T spheroplasting enzyme (ICN), in 1 ml of a buffer containing 1.2 M sorbitol, 40 mM sodium phosphate, pH 7.0, 0.5 mM magnesium chloride, and 0.2% v/v 2-mercaptoethanol. After centrifugation at 1000 × *g* for 2 min, spheroplasts were subjected to a plasmid miniprep protocol (QIAprep; QIAGEN). The eluate was used to transform *Escherichia coli*.

Quantitative assay of zinc-regulated *lacZ* expression: Cells from relatively fresh plates were washed twice in low-zinc medium and used to inoculate paired 10-ml cultures to calculated optical densities of 0.02 or 0.002 OD_{600}/ml. Zinc was then added (100 μmol/liter zinc sulfate) to the second culture. Cells were typically in late exponential growth phase at the end of a 24-hr growth period at 30° on a rotary shaker. Longer culture times were used as needed. After chilling to 0°, cells were collected in microcentrifuge tubes and stored in a buffer containing 5% glucose and 50 mM sodium citrate, pH 6.5. Measurements of *lacZ* activity were as described (Guarente 1983) except that 10 mM sodium azide was included. In control experiments this amount of azide had no effect on *lacZ* activity. OD_{600} values were corrected for nonlinearities but not for zinc-dependent effects (1 OD_{600} of diploid cells corresponded to 33.5×10^6 or 38.8×10^6 cells ($\pm 1 \times 10^6$) for cells grown in

low- or high-zinc medium, respectively). Results are expressed as Miller units (1000 × OD_{420}/OD_{600}/min; Miller 1972). Where presented, means and standard deviations represent measurements from six to eight aliquots of the same culture; otherwise means of duplicate aliquots are given.

Determination of cellular phenotypes affected by zinc: Cells were prepared as for quantitative *lacZ* assays except as noted.

Pigmentation: Cells were grown from 0.02 OD_{600}/ml in 1-ml cultures for 40–48 hr and transferred to a microtiter plate for photography.

Flocculence: Cells were grown as for cell pigmentation assays and vigorously swirled without rotating the plate, then allowed to settle for ~2 min before photography.

Vacuolization: Cells were grown as indicated and suspended at room temperature in glucose-citrate buffer. Representative fields of cells were photographed with a digital camera under Nomarski optics at ×1600.

RESULTS

Validation of a low-zinc growth medium: Methods for depleting cells of zinc are fundamental to studies of zinc deficiency. A low-zinc growth medium for this purpose was prepared by omitting the 1.3 μM zinc sulfate that is present in standard defined medium (Sherman 1991) and taking precautions against zinc contamination (see materials and methods). Cells with the *ade2* genetic marker lost their usual coloration (Weisman *et al.* 1987) when grown in this medium (Figure 1), providing a useful indicator of zinc status. Subtle darkening was evident with cells grown in medium containing 100 nmol/liter added zinc, suggesting that concentration of bioavailable zinc in the low-zinc medium was on the order of 0.1 μM. Cells were still able to grow in medium containing concentrations of zinc up to 1000 μM (Figure 1), although growth was initially delayed at 1000 μM and above. The ability of these cells to tolerate ambient zinc concentrations spanning the range 0.1–1000 μM demonstrated that robust mechanisms exist for zinc homeostasis in these cells.

Identification of genes differentially regulated by zinc status: In 1994 a procedure for identifying differentially expressed genes on a genomic scale in yeast was described (Burns *et al.* 1994; Goffeau 1994). This procedure involves the random insertion of the *lacZ* gene into the yeast genome for use as reporter gene fusion constructs. Specifically, yeast genomic DNA is subjected to transposition of a *lacZ::LEU2::bla* cassette, flanked at either end by inverted repeats of the 38-base mini-Tn*3* transposon (m-Tn*3*), in *E. coli* (Figure 2A). The resulting library of random *lacZ* insertions is then digested with *Not*I to release the yeast genomic DNA from its vector and is used to transform a *leu2/leu2* diploid yeast strain to leucine prototrophy. It is expected that the yeast genomic sequences flanking the *lacZ::LEU2::bla* cassette will mediate integration of the cassette into one copy of the diploid yeast host genome. In a minority of cases, the *lacZ* gene (lacking an initiation codon) will be inserted in frame within a translated open reading frame, resulting in *lacZ* activity driven by the upstream promoter. Transformants exhibiting regulated *lacZ* activity

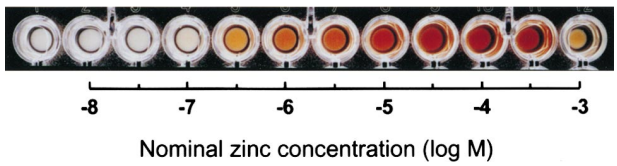

Figure 1.—Pigmentation phenotypes in zinc-deficient cells. See materials and methods. Wild-type cells (YPH252) were grown to stationary phase in 1-ml cultures containing the indicated nominal concentrations of zinc cultures. Cells were photographed after transfer to a microtiter plate.

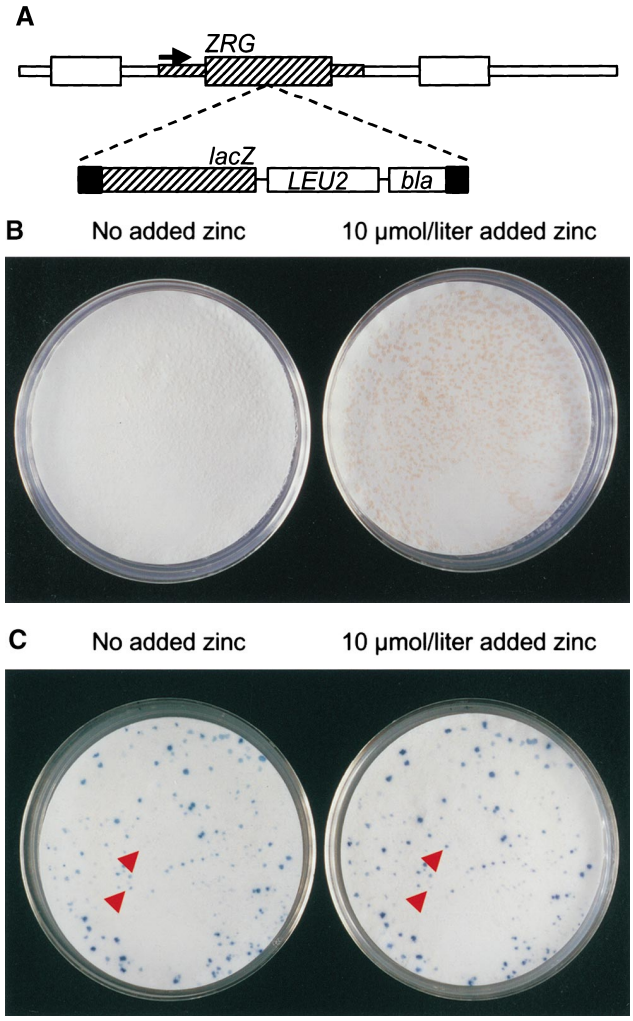

Figure 2.—Illustration of the genetic screen for *ZRG*s. (A) Creation of a *ZRG-lacZ* fusion construct by insertion of a modified Tn*3* transposon into yeast genomic DNA. The arrow schematizes the *ZRG* promoter (adapted from Burns *et al.* 1994). (B) Zinc-dependent pigmentation. Shown is a representative pair of replicas grown on low-zinc (no added zinc) or moderate-zinc (10 μmol/liter added zinc) medium. (C) Zinc-dependent *lacZ* activity. Shown are colonies from the same plates in B after cell permeabilization in liquid nitrogen and incubation with the *lacZ* substrate X-gal at 30° for 2 wk. Arrowheads highlight colonies displaying differences in *lacZ* activity.

can then be studied to identify the gene fused to *lacZ*.

The efficiency of this procedure was improved through a number of technical modifications (see MATERIALS AND METHODS). One modification was to assess differential *lacZ* expression by replica-plating colonies onto pairs of plates, rather than by streaking colonies out individually. Thus, only a few hours were needed to process the 80,000 transformants in this study. A second modification was to sequence the *lacZ* insertion sites in individual clones by amplifying them directly by PCR from circularized genomic DNA fragments, rather than by cloning them through plasmid rescue. Two transformations and two plasmid preparations were saved in this way for each of the 100+ transformants that were later analyzed.

To apply this procedure to the identification of zinc-regulated genes, transformants were first grown on low-zinc (~0.1 μM) medium and then replica plated in succession to low-zinc and moderate-zinc (10 μM) media. Growth of cells on the low-zinc solid medium resulted in loss of pigmentation (Figure 2B), consistent with the loss of pigmentation observed in cells grown in low-zinc liquid medium (Figure 1). *lacZ* reporter gene activity was detected in ~8000 colonies, or ~10% of transformants, similar to the fractions reported elsewhere (BURNS *et al.* 1994; ERDMAN *et al.* 1998). Of these 8000 colonies, 105, or ~1%, exhibited visibly different levels of *lacZ* expression when subcloned and reassayed. The fact that 99% of the *lacZ* fusions examined in this genetic screen were not obviously affected by a 100-fold range in ambient zinc concentrations (Figure 2C) was reassuring, in view of a previous report describing markedly decreased protein content in zinc-deficient cells (OBATA *et al.* 1996). Because many zinc-dependent enzymes are involved in RNA biosynthesis, including RNA polymerase II (*e.g.*, THURIAUX and SENTENAC 1992), it was conceivable that extreme zinc deprivation would result in global decreases in *lacZ* expression. That this was not observed suggested that zinc deprivation has specific effects on gene expression, at least in its early stages.

The 105 clones expressing zinc-regulated *lacZ* activity were analyzed by sequencing the *lacZ* insertion sites in these cells and measuring levels of *lacZ* expression after growth in low-zinc (~0.1 μM) or high-zinc (100 μM) liquid media. Unambiguous identification of the *lacZ* insertion sites was achieved by BLAST searches in GenBank in almost every case. The only exceptions were one clone that contained two independent *lacZ* insertion sites and two clones in which the *Nla*III site used for fragmenting genomic DNA was situated within a few bases of the *lacZ* cassette. In all identified clones the *lacZ* gene was situated in the same reading frame as upstream portions of the disrupted gene, as defined by the presence of an upstream ATG initiation codon without an interposed termination codon.

Assessment of the genetic screen: The number of independent *lacZ*-positive colony transformants examined in this genetic screen, ~8000, compared favorably with the ~6000 genes in the *Saccharomyces cerevisiae* genome. As a first approximation Poisson statistics would imply that at least half of the ~6000 genes were examined. Some examples of saturation of the genetic screen were also found: *ZRG17* was isolated 6 times as 5 different insertions (Table 3); *ENA1/PMR2*, found as a cluster of 5 highly similar genes in the genome of strain S288C (an ancestor of the parental strain used in this study; WIELAND *et al.* 1995), was isolated 9 times as 5 different insertions (regulated weakly; not shown); and the TyA gene in the Ty1 retrotransposon, represented by ~33 copies in the genome (KIM *et al.* 1998), was isolated 31 times in 17 different insertions (also regulated weakly, not shown). Together, these observations suggest that roughly half of the genes in the genome were examined.

Analysis of *ZRG* expression by ribonuclease protection assays: Clones exhibiting a 10-fold or greater range of *lacZ* expression were arbitrarily chosen for further study. The 17 *ZRG*'s represented by these clones are listed in Table 3.

To assess the contribution of transcriptional regulation (or other mechanisms affecting RNA abundance) to the regulated expression of *ZRG-lacZ* fusions, ribonuclease protection assays (RPAs) were undertaken for each of the *ZRG*'s. The total RNA used in these experiments was derived from cells grown in the same low- or high-zinc media as for the quantitative *lacZ* assays. RNA was isolated using a procedure developed to ensure the concurrent isolation of small molecular weight species (WISE 1991). The internal standard chosen was *TDH3* (encoding glyceraldehyde-3-phosphate dehydrogenase), a gene that was fortuitously isolated in the genetic screen as a strongly expressed control that was minimally sensitive to zinc status (1270 and 770 Miller units in low- and high-zinc media, respectively; *TDH3* was disrupted by the modified transposon after codon 6).

Of the 17 *ZRG*'s, *ZRG*'s 1, 5, 7, 10, 16, and 17 were most clearly regulated by zinc at the level of RNA expression (Figure 3). These were induced 3.8-, 7.6-, 5.4-, 5.7-, 8.7-, and 6.6-fold, respectively, in zinc-deficient cells relative to *TDH3*. The *ZRG7* data provide the first evidence that *ZRG7* (*YEF3B*) is in fact expressed (*cf.* MAURICE *et al.* 1998; SARTHY *et al.* 1998). *ZRG6* and *ZRG14* RNAs were induced in zinc excess as expected in one experiment, but for unknown reasons this was not reproducible in a second experiment (not shown).

Of the remaining *ZRG*'s, RNAs for *ZRG*'s 8, 11, 12, 13, and 15 were induced 1.9-, 3.6-, 1.9-, 1.9-, and 2.0-fold, respectively, in zinc-deficient cells relative to *TDH3* (Figure 3). Of the four *ZRG*'s encoding unusual open reading frames, *ZRG2* RNA (68 codons) was barely detectable. (In Figure 3 the *ZRG2* hybridization was carried out without *TDH3* probe, and imaging thresholds were decreased 10-fold.) *ZRG3* RNA (9 codons) could not be confidently assessed due to unexplained and variable

TABLE 3

***ZRG*'s identified in the genetic screen**

ZRG	Other names (or bases 5′ of *lacZ*)	Amino acids retained/total[a]	Clone designation	*lacZ* activity: Zinc concentration (μM) ~0.1	100	Ratio	Function (from Hodges *et al.* 1999)
ZRG1	*DPP1*	*237/289	DYY515	8.6	0.01	~500	Diacylglycerol pyrophosphate phosphatase (Toke *et al.* 1998)
ZRG2	XV 43169−	*9, 9/68	DYY507	0.41	0.00	~100	—
ZRG3	XVI 607360+	*3/9	DYY763	2.68	0.02	~100	—
ZRG4	XVI 121698+	*4/12	DYY764	1.37	0.01	~100	—
ZRG5	*ADH4*	*81, 81/465	DYY723	15.8	0.27	60	Alcohol dehydrogenase type IV (Drewke and Ciriacy 1988)
ZRG6	*OYE3*	*67/400	DYY510	0.68	45.	1/60	Old yellow enzyme (Stott *et al.* 1993)
ZRG7	*YEF3B*	*523/1044	DYY750	24.2	0.54	50	Translational elongation factor EF-3B (Sarthy *et al.* 1998; Maurice *et al.* 1998)
ZRG8	YER033c	*389/1076	DYY504	0.34	0.01	30	—
ZRG9	XII 235509−	*16/25	DYY741	2.00	0.07	25	—
ZRG10	*ZAP1*	*619, 619/880	DYY506	0.45	0.02	25	Zinc-regulated DNA binding protein (Zhao and Eide 1997)
ZRG11	*MET30*	*289/640	DYY731	0.57	0.02	25	F-box protein involved in sulfur metabolism and protein ubiquitination (Thomas *et al.* 1995; Bai *et al.* 1996)
ZRG12	*DFG16*	*175, 175/619	DYY759	0.26	0.01	25	Protein involved in cell wall maintenance (Mosch and Fink 1997)
ZRG13	*THO2*	*234/1597	DYY752	0.26	0.00	25	Protein required for RNA pol II transcription (Piruat and Aguilera 1998)
ZRG14	*TSA1*	*30, 30/196	DYY720	1.0	20.5	1/20	Thioredoxin peroxidase (Netto *et al.* 1996)
ZRG15	*ECM7*	*303/448	DYY508	0.56	0.02	25	Protein involved in cell wall maintenance (Lussier *et al.* 1997)
ZRG16	*MCD4*	*581, 581, 581, 581/919	DYY509	15.1	1.50	10	Protein required for GPI anchor synthesis (Gaynor *et al.* 1999)
ZRG17	*YNR039c*	*242, 285, 285, 442, (*)531, 568/605	DYY754, (DYY505)	7.25	0.70	10	—

[a] (*) Clone assayed.

excesses in probe length, suggesting secondary structure in the probe; an A-rich sequence (43/51 bases) lies within the expected probe. *ZRG4* RNA (12 codons) was undetectable. Interestingly, however, *ZRG9* RNA (25 codons, antiparallel to the *STU2* open reading frame) was clearly detected (Figure 3), with a 2.3-fold induction in zinc-deficient cells.

Identification of the *ZRG10* gene product as a regulator of zinc-regulated genes: During the course of these studies, Zhao and Eide (1997) described the *ZAP1* gene, encoding a transcriptional activator of the *ZRT1* and *ZRT2* genes and also of *ZAP1* itself. *ZRT1* and *ZRT2* had been shown previously to encode high- and low-affinity zinc uptake transporters whose expression is highly regulated by zinc (Zhao and Eide 1996a,b). In zinc-deficient cells expression of *ZRT1* and *ZRT2* is elevated, consistent with a role for these transport proteins in maintaining cellular zinc homeostasis in the face of fluctuations in nutritional zinc bioavailability. The *ZAP1* protein functions *in vitro* as a DNA-binding protein that binds specifically to a degenerate 11-base motif [zinc regulatory element (ZRE)], ACCYYNAAGGT, in the promoters of the *ZRT1*, *ZRT2*, and *ZAP1* genes (Zhao *et al.* 1998).

ZRG10 was found to be identical to *ZAP1*. This information enabled a search for ZREs in promoter sequences of the *ZRG*'s. ZRE-like sequences (ACCTTNAAGGT, with one allowed mismatch in the underlined bases) were identified within the upstream 800 bases of *ZRG*'s 1, 5, 10, 16, and 17. Two approaches were used to determine the role of *ZAP1* in regulating *ZRG*'s. In the first, haploid *ZRG-lacZ* strains were prepared as for *ZRG17* (see above) and the effects of *ZAP1* disruption on regulated *lacZ* expression were determined. In the second, the *ZRG-lacZ* fusion construct was prepared synthetically. Promoter fragments adjacent to an initiation codon were fused to the *lacZ* gene in a centromeric (low-copy-number) plasmid and introduced into *ZAP1*

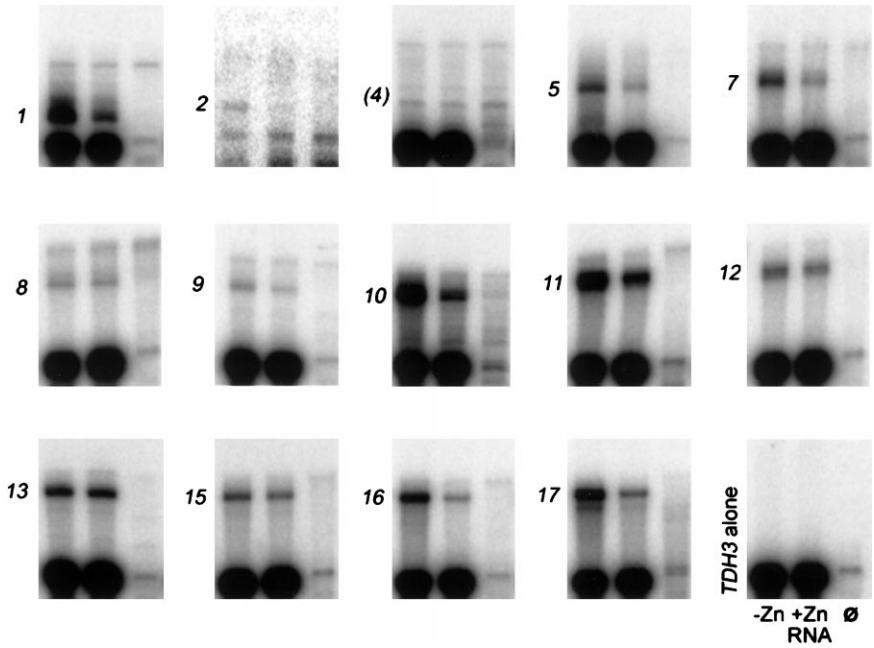

FIGURE 3.—Ribonuclease protection assays of zinc-regulated *ZRG* expression. The indicated radiolabeled *ZRG* probes (top bands) and a radiolabeled *TDH3* probe (lower bands) were used in ribonuclease protection assays to probe RNA from zinc-deficient cells (left lane), RNA from zinc-replete cells (center lane), or no hybridizing RNA (right lane). Numerals denote the expected location of the hybridization signal for the *ZRG* indicated.

null cells or their wild-type counterparts. This second approach was used when haploid mutants could not be used due to issues of conceptual clarity (*ZRG10/ZAP1*) or cell viability (*ZRG16/MCD4*).

Expression of these *ZRG-lacZ* fusion constructs was strongly zinc regulated in cells with an intact *ZAP1* gene (Table 4). Cells lacking a functional *ZAP1* gene exhibited greatly decreased *lacZ* expression. In the case of *ZRG1*, restoration of *ZAP1* function via a centromeric plasmid containing *ZAP1* sequences completely restored the zinc-regulated expression of a *ZRG1-lacZ* fusion construct (not shown). Additionally, $MnSO_4$, $FeNH_4(SO_4)_2$, and $CuSO_4$ (100 μM) failed to repress *ZRG1-lacZ* expression in zinc-deficient cells to the same extent as $ZnSO_4$ (100 ± 1%, 91 ± 1%, and 87 ± 3%, respectively; *cf.* 0.1 ± 0.1%). These findings indicated that expression of *ZRG*'s 1, 5, 10, 16, and 17 requires *ZAP1*, as expected from the presence of ZREs in the promoters of these genes. Conversely, *lacZ* expression in various other haploid *ZRG-lacZ* strains (*ZRG*'s 2, 3, 8, 14, and 15) or synthetic *ZRG-lacZ* constructs (*ZRG*'s 6 and 11) was not visibly affected by disruption of *ZAP1*, and ZRE-like motifs were correspondingly absent from the upstream 2000 bases of these *ZRG*'s (not shown). (*ZRG7* remains to be tested; the clone from the genetic screen failed to sporulate.) Thus, the presence of ZRE-like consensus sequences in promoter sequences of the *ZRG*'s was sufficient to predict the *ZAP1*-dependent expression of those genes.

The discrepancy between the ~500-fold regulation of *ZRG1* in the genetic screen and the ~4-fold regulation of *ZRG1* in RPAs was investigated using a panel of synthetic *ZRG1-lacZ* fusions (Table 5). A centromeric plasmid construct containing 800 bases of *ZRG1* pro-

TABLE 4

***ZAP1* (*ZRG10*)-dependent expression of selected *ZRG*'s**

Strain	Construct fused to *lacZ*	*ZAP1* status	*lacZ* activity (units ± SD): Zinc concentration (μM) ~0.1	100
DYY641	*ZRG1* (to +718), integrated	Wild type	40.0 ± 2.0	0.1 ± 0.1
DYY640		Disrupted	0.2 ± 0.1	0.0 ± 0.0
DYY1200	*ZRG10* (−800 to +4), plasmid	Wild type	4.8 ± 0.1	0.1 ± 0.0
DYY1187		Disrupted	0.1 ± 0.0	0.0 ± 0.0
DYY1118	*ZRG5* (−800 to +244), plasmid	Wild type	3.3 ± 0.3	0.1 ± 0.0
DYY1119		Disrupted	0.1 ± 0.1	0.1 ± 0.0
DYY1242	*ZRG16* (−800 to +4), plasmid	Wild type	2.0 ± 0.1	0.1 ± 0.1
DYY1243		Disrupted	0.1 ± 0.2	0.0 ± 0.1
DYY1171	*ZRG17* (to +727), integrated	Wild type	4.3 ± 0.2	0.3 ± 0.1
DYY1172		Disrupted	0.7 ± 0.1	0.3 ± 0.1

TABLE 5

***ZAP1*-dependent regulation of truncated *ZRG1-lacZ* fusion constructs**

Strain	Construct fused to *lacZ*	*ZAP1* status	*lacZ* activity (units ± SD): Zinc concentration (μM) ~0.1	100
DYY1202	−800 to +4, plasmid	Wild type	6.8 ± 0.1	0.6 ± 0.1
DYY1185		Disrupted	0.1 ± 0.0	0.5 ± 0.1
DYY1201	−353 to +4, plasmid	Wild type	0.2 ± 0.1	0.5 ± 0.1
DYY1184		Disrupted	0.1 ± 0.1	0.3 ± 0.0
DYY1199	−800 to +718, plasmid	Wild type	3.7 ± 0.3	0.0 ± 0.1
DYY1134		Disrupted	0.0 ± 0.0	0.0 ± 0.0
DYY1203	−353 to +718, plasmid	Wild type	0.0 ± 0.1	0.1 ± 0.0
DYY1186		Disrupted	0.0 ± 0.1	0.0 ± 0.0

moter sequence and a start codon for the *lacZ* gene was modestly zinc regulated (~12-fold) and completely *ZAP1* dependent. This degree of regulation was comparable to the ~4-fold regulation observed in the RPAs, consistent with the fusion functioning as a transcriptional fusion. Expression was poor with just 353 bases of *ZRG1* promoter sequence fused to *lacZ*, consistent with exclusion of the single ZRE (at base –452) from this sequence. Stringent (>100-fold) zinc-regulated expression was reconstituted, however, by including coding sequences from *ZRG1* that were in the *lacZ* fusion from the genetic screen. This observation suggested that *ZRG1* coding sequences play a role in the regulation of the *ZRG1-lacZ* fusions isolated from the genetic screen.

Control of phenotypes of zinc deficiency by *ZAP1*: Two other phenotypes of zinc-deficient cells were observed in addition to zinc-dependent growth and loss of *ade2*-dependent pigmentation and zinc-dependent growth (Figure 1). First, cells grown in shaking cultures to stationary phase in low-zinc medium tended to flocculate. The flocs dispersed immediately when cells were resuspended in a glucose-citrate buffer. Minimal flocculence was observed in cells grown with added zinc (Figure 4A). Second, cells growing in low-zinc medium in exponential phase exhibited striking distension of the vacuole (Figure 4B). Vacuole size varied substantially from cell to cell but nonetheless appeared much larger than the dilated vacuoles that are commonly seen in cells in stationary phase or cells stored in water (Roberts *et al.* 1991). These drastic changes were also repressed by zinc supplementation (Figure 4B).

As noted above, *ZAP1* mutants have a defect in expression of the high- and low-affinity zinc uptake transporters encoded by *ZRT1* and *ZRT2*. They should be more zinc deficient than wild-type cells in conditions with limited zinc bioavailability, and indeed this has been demonstrated directly (Zhao and Eide 1997). It was therefore surprising to observe that *ZAP1* mutants grown in low-zinc medium exhibited neither flocculence (Figure 4A) nor vacuolization (Figure 4B). This finding suggested that flocculation and vacuolar distension are not merely passive metabolic consequences of zinc deficiency, but rather are part of an active cellular response to zinc deprivation. The loss of flocculence and vacuolization was not observed in haploid cells with disruptions of known *ZAP1*-regulated genes (other than *ZAP1* mutants themselves), *i.e.*, *ZRG1-lacZ*, *ZRG1* null, *ZRG5-lacZ*, or *ZRG17-lacZ* cells (not shown). [*ZRG16-lacZ* haploid strains were not viable, as reported for null mutants of this gene (Mondesert *et al.* 1997; Gaynor *et al.* 1999).] Therefore, the flocculence and vacuolization characteristic of zinc-deficient cells appears to be mediated by other *ZAP1*-regulated genes that have not yet been identified.

Evidence for a role for *ZRG17* in zinc uptake: To examine the function of the *ZRG17* gene, haploid disruption mutants were conveniently prepared from the *ZRG17* clones from the genetic screen by sporulating these heterozygous diploid cells and dissecting the products of meiosis. In liquid culture the *ZRG17* mutants were equally as flocculent as wild-type cells in low-zinc medium, but 10-fold higher concentrations of zinc were needed to repress flocculence (Figure 5A). Cells from mutants grown to stationary phase on YPD plates contained distended vacuoles, and vacuolar distension was ameliorated if the cells were grown with zinc supplementation (Figure 5B). These phenotypes resembled those of zinc-deficient cells and suggested that the *ZRG17* protein participates in zinc uptake, a function that is consistent with the identification of *ZRG17* as a *ZAP1*-dependent gene.

DISCUSSION

Transposon tagging as an approach for identifying differentially regulated genes: This study describes an application of transposon tagging to the identification of differentially regulated genes in yeast. Transposon tagging was first adapted from bacterial systems for application in yeast by several groups in the mid-1980s

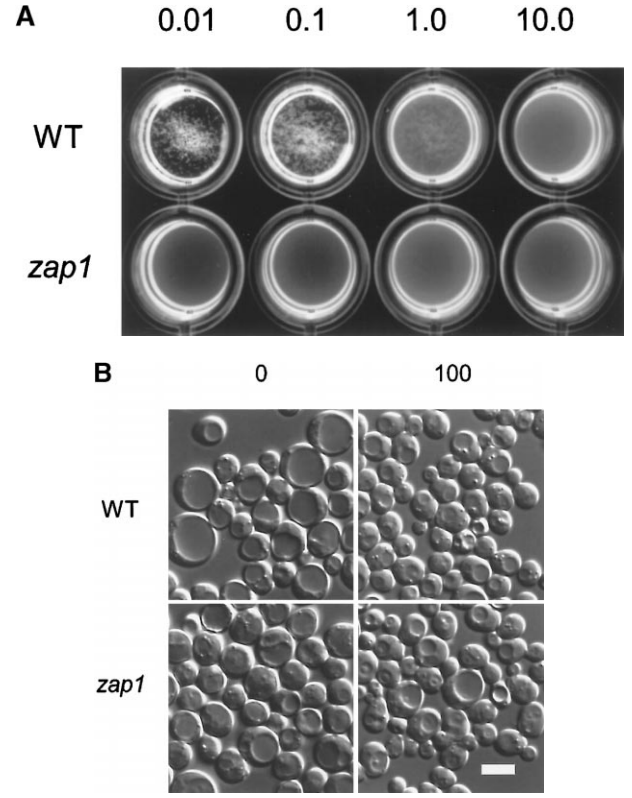

FIGURE 4.—*ZAP1*-dependent phenotypes of zinc-deficient cells. See MATERIALS AND METHODS. (A) Flocculence. Wild-type (YPH252) and congenic *zap1* null (DYY1198) cells were shaken for 2 days in low-zinc medium supplemented with the indicated amounts of zinc (μmol/liter) and photographed. (B) Vacuolization. Same as A except that cells were grown for 2 days in defined medium supplemented with the indicated amounts of zinc (μmol/liter) and examined with Nomarski optics. Bar, 5 μm.

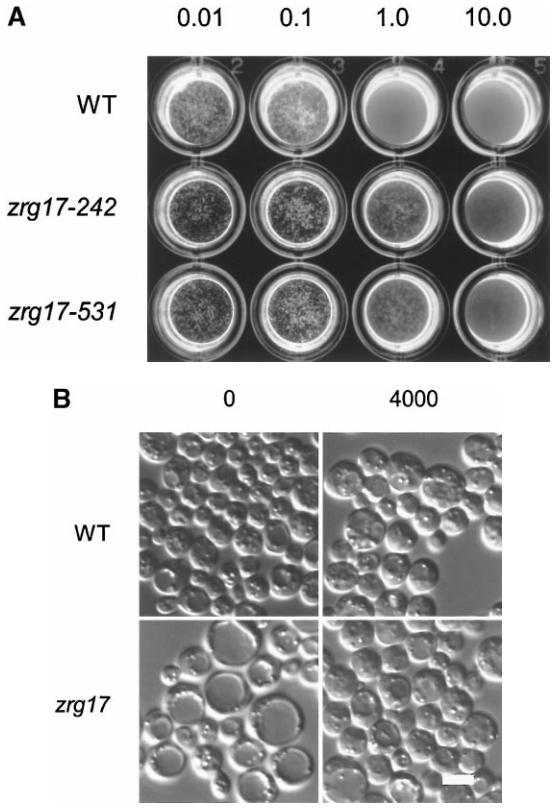

FIGURE 5.—Zinc-inhibitable phenotypes of *ZRG17* mutants. (A) Flocculence. Cells of strain YPH252, a *zrg17-242* haploid strain (DYY1206), and a *zrg17-531* haploid strain (DYY617) were grown and photographed as for Figure 4A. (B) Vacuolization. Cells of strain YPH252 and the haploid *zrg17* mutant strain *zrg17-242* (DYY1179) were grown to stationary phase on the same YPD or zinc-supplemented YPD plates. Samples were taken from colonies with comparable and visually indistinguishable regions of growth and photographed under Nomarski optics. Bar, 5 μm.

(RUBY and SZOSTAK 1985; SEIFERT *et al.* 1986; MCCAFFREY *et al.* 1987), and further useful refinements were introduced in 1994 that allowed studies of differential gene expression to be performed on a quasi-genomic scale (BURNS *et al.* 1994; DANG *et al.* 1994). However, there are still only a few studies of this type in the literature (for an excellent example, see ERDMAN *et al.* 1998).

Several newer methodologies for identifying differentially regulated genes have been applied recently on a large scale in yeast. These include microarray hybridization technology (DERISI *et al.* 1997), serial analysis of gene expression (SAGE; VELCULESCU *et al.* 1995, 1997), and two-dimensional gel electrophoresis of proteins combined with mass spectrometry (GODON *et al.* 1998). When compared with these methodologies, transposon tagging has some advantages that are illustrated in this study: (1) Transposon tagging uses only instrumentation found in any molecular biology laboratory; (2) levels of gene expression can be quantitated precisely with simple assays that have a dynamic range spanning almost four orders of magnitude; (3) reporter gene constructs are embodied in the clones isolated from the genetic screen as robust and renewable reagents; and (4) potential disruption alleles are conveniently prepared from the clones in the genetic screen by sporulation and dissection, facilitating the functional characterization of novel genes.

Mechanistic classification of the *ZRG*'s: The identification of multiple *ZRG*'s raises immediate questions about the mechanisms underlying zinc sensing and how such signals are coupled to the mechanisms of gene transcription and translation. In the case of the *ZAP1*-dependent genes, a partial answer is already at hand (ZHAO and EIDE 1997; ZHAO *et al.* 1998): The *ZAP1* protein may have dual roles as zinc sensor and DNA binding protein. While much remains to be learned about how zinc binds to this protein and how zinc binding is structurally coupled to interactions with zinc-response elements, it is clear that such properties will ultimately account for many of the observed phenotypes of zinc deprivation in these cells.

A challenge that remains to be addressed is to recon-

cile the 10- to 500-fold regulation observed with the *ZRG-lacZ* fusion constructs with the much more modest 2- to 8-fold regulation of the endogenous *ZRG* RNAs measured in carefully performed ribonuclease protection assays. In addition to this exaggerated degree of regulation, *lacZ* expression levels correlated rather poorly with RNA expression levels. For example, *ZRG10* and *ZRG13* RNA expression levels were much stronger than suggested by the corresponding *lacZ* expression levels, while the opposite was true for *ZRG2* and *ZRG4*. While *lacZ* fusion constructs have been used for many years in reporter gene assays of gene expression (Rose and Botstein 1983), there are several reasons why RNA abundance may correlate poorly with *lacZ*-encoded β-galactosidase activity:

1. β-Galactosidase is active only as a tetramer (Nichtl *et al.* 1998). At very low monomer concentrations it is possible that this enzyme activity may be disproportionately weak, thus amplifying the -fold regulation observed at the RNA level. However, this nonlinearity does not explain how the RNA is regulated to begin with.
2. Biochemical idiosyncrasies associated with the *lacZ* fusions (*e.g.*, transmembrane domains) could contribute to differences in *lacZ* expression levels. For example, *lacZ* activities varied 10-fold among the five different species of *ZRG17* clones (not shown).
3. The *ZRG-lacZ* fusions obtained in the genetic screen may be susceptible to post-transcriptional modes of regulation. Indeed, coding sequences in *ZRG1* appear to contribute substantially to the zinc-regulated expression of *ZRG1* (Table 5). It is pertinent to note that ubiquitination of the zinc uptake transporter encoded by *ZRT1* can be instigated by exposure of cells to zinc (Gitan *et al.* 1998).

Functional roles of *ZRG*'s in the *ZAP1* regulon: Perhaps the most important questions still to be answered involve the functions of the *ZAP1*-dependent genes—whether they play some unsuspected role in zinc homeostasis, or whether they function to maintain some biochemical process that is sensitive to zinc status.

ZRG17 mutants have mutant phenotypes suggestive of cellular zinc deficiency (Figure 5), suggesting that the *ZRG17* protein functions in zinc uptake. This idea is supported by the presence of seven potential transmembrane domains in the predicted protein and by a cluster of histidine residues admixed with acidic residues following the third potential transmembrane domain (not shown). The latter feature could represent a zinc-interacting domain, in view of the histidine clusters that are present in other zinc-transporting proteins (*e.g.*, Zhao and Eide 1996b). It is an open question why *ZRG17* mutants exhibited phenotypes of zinc deficiency in the presence of unmutated alleles of *ZRT1* and *ZRT2*, although it is noteworthy that the low-zinc growth medium used in the *ZRG17* studies was different from the pH 4.2 EDTA-containing medium used to characterize *ZRT1* and *ZRT2* (Zhao and Eide 1996b). Further characterization of the function of the *ZRG17* protein in the context of the physiologic functions of *ZRT1* and *ZRT2* is underway.

DPP1 (*ZRG1*) was the most highly regulated of all the *ZRG-lacZ* fusions in the genetic screen. Expression of *DPP1-lacZ* fusions was completely dependent on *ZAP1* function, suggesting a role in zinc homeostasis. The structure of the *DPP1* protein suggested a role in zinc transport, with six transmembrane domains and a highly conserved set of three histidine residues. Surprisingly, however, overexpression or deletion of *DPP1* (*ZRG1*) revealed no discernable phenotypes, zinc-related or otherwise, compared with controls (not shown). It is provocative to note that the *DPP1* protein has a known enzymatic activity, diacylglycerol pyrophosphate phosphatase; in fact, *DPP1* was first cloned after purification of this enzyme (Toke *et al.* 1998). There is a single report of cell aggregation, impaired mitotic cytokinesis, and abnormal cell shape in a *DPP1* mutant (Katagiri and Shinozaki 1998). These phenotypes recall the flocculence and vacuolization in zinc-deficient cells. It is unclear why they were not observed in any of the *DPP1* mutants in this study or elsewhere (Toke *et al.* 1998).

ADH4 (*ZRG5*) was also stringently regulated without an observable phenotype. The *ADH4* protein has been characterized as a zinc-dependent alcohol dehydrogenase that was thought to be minimally expressed if at all (Drewke and Ciriacy 1988). The induction of *ADH4* expression in low-zinc conditions suggests that this protein functions in these conditions as a backup to the strongly expressed alcohol dehydrogenase encoded by *ADH1*, but this has not been tested.

Finally, *MCD4* (*ZRG16*) encodes a protein required for the synthesis of glycosylphosphatidylinositol (GPI) anchors that mediate the cell-surface expression of various proteins (Gaynor *et al.* 1999). The fact that *MCD4* is an essential gene poses the challenge of explaining why zinc-supplemented cells and *ZAP1* null cells are viable, given the strong repression of this gene in those cells as gauged by *lacZ* fusion constructs. Perhaps residual levels of expression are sufficient to maintain cell viability. Interestingly, the *FLO1* protein, a cell-surface protein required for flocculence (see below), is believed to be GPI anchored before it is conjugated to carbohydrates in the cell wall (van der Vaart *et al.* 1996).

Prospects for identifying other genes in the *ZAP1* regulon: Because zinc is a required cofactor for hundreds of proteins throughout metabolism, it is not surprising that zinc deprivation elicits a variety of cellular phenotypes. However, it was unexpected that two of the most prominent phenotypes of zinc-deficient cells, flocculence and vacuolar dilatation, were missing or attenuated in *ZAP1* mutants. As noted earlier, zinc uptake is impaired in these mutants, so if these phenotypes

were consequences of zinc depletion in some zinc-dependent protein, exaggerated phenotypic expression should have been observed in *ZAP1* mutants. That the phenotypes were missing or attenuated indicates instead that these phenotypes are directly controlled by *ZAP1*.

Flocculation has been studied for many years due to its importance in the brewing industry (reviewed in Stratford 1992). As fermentation mixtures age, yeast cells form large aggregates or flocs that sediment, thereby effecting a separation of the yeast from the brewery product. Two types of flocculation have been described in *S. cerevisiae*, *FLO1* dependent and the New-Flo type, distinguished by their differing sensitivities to inhibition by glucose and other culture conditions. The flocculation observed in zinc-deficient cells in this study was evidently not inhibited by glucose, as glucose was present in the culture medium, and preliminary studies indicate that calcium can rescue the loss of flocculation observed when zinc-deficient cells are resuspended in a citrate buffer (not shown). These characteristics are therefore consistent with the *FLO1*-dependent type of flocculence that is observed in most laboratory strains (Stratford 1992). The *FLO1* gene encodes a recently characterized mannose-specific and divalent cation-dependent lectin that appears to promote flocculence through its ability to bind to mannosylated constituents of the cell wall (Kobayashi *et al.* 1998). Expression of the *FLO1* gene is known to be controlled by the general transcriptional repressors encoded by *TUP1* and *SSN6* (Teunissen *et al.* 1995), but little is known about other modes of regulation. A consensus ZRE is present at base –401 in the *FLO1* promoter, suggesting that *FLO1* is another *ZAP1*-dependent gene. A synthetic *FLO1* (–800 . . . +3) promoter fusion with the *lacZ* gene was in fact found to be expressed at very low levels (5% of wild type) in a *ZAP1* null mutant (not shown), possibly accounting for why *ZAP1* mutants do not flocculate. However, unexpectedly, *FLO1-lacZ* expression was induced rather than repressed in zinc-replete cells, even in *ZAP1* null cells (not shown). Understanding how *ZAP1* controls flocculence therefore requires a more thorough investigation that is beyond the scope of this article.

Progress in understanding the phenotype of vacuolar dilatation has been rapid recently with the discovery of a biochemically characterized yeast mutant exhibiting hugely dilated vacuoles. This mutant was originally discovered in a genetic screen for cells defective in mitotic cytokinesis (*cf.* the discussion of *DPP1*, above), but this latter phenotype was later shown to be secondary to the presence of the hugely dilated vacuole (Yamamoto *et al.* 1995). The gene affected in this mutant, *FAB1*, encodes a phosphatidylinositol-3-phosphate 5-kinase, suggesting that the novel phospholipid produced by this enzyme participates in post-Golgi, prevacuolar membrane trafficking (Gary *et al.* 1998). The similarity between the distended vacuoles observed in zinc-deficient cells and those in *FAB1* mutants may indicate that maintenance of cytosolic phosphatidylinositol-3,5-bisphosphate levels requires at least one *ZAP1*-dependent gene.

An important task that lies ahead is to develop a comprehensive list of the *ZAP1*-dependent genes. The promoters in the seven *ZAP1*-dependent genes identified so far all contain a sequence motif that closely resembles the consensus ZREs previously derived. Several other *ZRG*'s lacking a ZRE in their promoters were found to be expressed independently of *ZAP1*, suggesting that ZREs have predictive value in identifying *ZAP1*-dependent genes. The number of *ZAP1*-dependent genes is not known. Five *ZAP1*-dependent genes were identified here in a genetic screen that examined approximately half of the genes in the genome, suggesting that there are perhaps 10 *ZAP1*-dependent genes. However, a search for the sequence ACCTT NAAGGT in the *S. cerevisiae* genome (http://genome-www2.stanford.edu/cgi-bin/SGD/PATMATCH/nph-patmatch) revealed 9 genes containing this sequence within 500 bases of their putative start codons, including *ZRT1* and *MCD4*. The actual number of candidate *ZAP1*-regulated genes is considerably larger, since the ZRE recognized by the *ZAP1* protein is clearly somewhat degenerate (*e.g.*, for *DPP1*), and also since the promoters for some genes may be much longer than 500 bases (*e.g.*, Rupp *et al.* 1999). DNA microarrays (DeRisi *et al.* 1997) may provide a rapid experimental approach for testing which of these are in fact *ZAP1*-dependent genes.

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