Regulation of the PIS1-encoded Phosphatidylinositol Synthase in Saccharomyces cerevisiae by Zinc*

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In the yeast Saccharomyces cerevisiae, the mineral zinc is essential for growth and metabolism. Depletion of zinc from the growth medium of wild type cells results in changes in phospholipid metabolism, including an increase in phosphatidylinositol content (Iwanyshyn, W. M., Han, G.-S., and Carman, G. M. (2004) J. Biol. Chem. 279, 21976–21983). We examined the effects of zinc depletion on the regulation of the PIS1-encoded phosphatidylinositol synthase, the enzyme that catalyzes the formation of phosphatidylinositol from CDP-diacylglycerol and inositol. Phosphatidylinositol synthase activity increased when zinc was depleted from the growth medium. Analysis of a zrt1Δ zrt2Δ mutant defective in plasma membrane zinc transport indicated that the cytoplasmic levels of zinc were responsible for the regulation of phosphatidylinositol synthase. PIS1 mRNA, its encoded protein Pis1p, and the β-galactosidase activity driven by the P_{PIS1}-lacZ reporter gene were elevated in zinc-depleted cells. This indicated that the increase in phosphatidylinositol synthase activity was the result of a transcriptional mechanism. The zinc-mediated induction of the P_{PIS1}-lacZ reporter gene, Pis1p, and phosphatidylinositol synthase activity was lost in zap1Δ mutant cells. These data indicated that the regulation of PIS1 gene expression by zinc depletion was mediated by the zinc-regulated transcription factor Zap1p. Direct interaction between glutathione S-transferase (GST)-Zap1p_{687–880} and a putative upstream activating sequence (UAS) zinc-responsive element in the PIS1 promoter was demonstrated by electrophoretic mobility shift assays. Mutations in the UAS zinc-responsive element in the PIS1 promoter abolished the GST-Zap1p_{687–880} DNA interaction in vitro and abolished the zinc-mediated regulation of the PIS1 gene in vivo. This work advances understanding of phospholipid synthesis regulation by zinc and the transcription control of the PIS1 gene.

Phosphatidylinositol (PI) is the third most abundant phospholipid in the cellular membranes of the yeast Saccharomyces cerevisiae (1–3), and it is essential for the growth and metabolism of this model eukaryote (4–6). In addition to being a major structural component of the membrane, PI serves as the precursor for sphingolipids (7, 8), the D-3, D-4, and D-5 phosphoinositides (3, 9–12), and glycosylphosphatidylinositol anchors (13, 14) (Fig. 1). Several of these PI-derived lipids and their metabolic products are prominent signaling molecules in S. cerevisiae and in higher eukaryotes that contribute to essential physiological functions (12, 15–20).

The enzyme responsible for the synthesis of PI in S. cerevisiae is the essential PIS1-encoded PI synthase (CDP-diacylglycerol:myo-inositol 3-phosphatidylinositol transferase, EC 2.7.8.11) (6, 21–23). This endoplasmic reticulum-associated (24) enzyme catalyzes the formation of PI and CMP from CDP-diacylglycerol and inositol (25) (Fig. 1). The regulation of PI synthase activity in vivo is largely governed by the availability of its substrates inositol and CDP-diacylglycerol (26–28). Cellular inositol levels are controlled by expression of the INO1 gene encoding inositol-3-phosphate synthase and by inositol supplementation (26–28). The levels of CDP-diacylglycerol are controlled through its utilization by the PI synthase enzyme itself and the competing activity of PS synthase (26–29) (Fig. 1). PS synthase catalyzes the committed step in the synthesis of PC via the CDP-diacylglycerol pathway (27) (Fig. 1). Indeed, the coordinate regulation of the PI synthase and PS synthase enzymes is part of an overall mechanism by which the synthesis of PI is coordinately regulated with the synthesis of PC (3, 27, 30–34).

Zinc is an essential nutrient required for the growth and metabolism of S. cerevisiae and of higher eukaryotes (35). It is a cofactor for hundreds of enzymes (e.g. alcohol dehydrogenase, carbonic anhydrase, proteases, RNA polymerases, superoxide dismutase) (35) and a structural constituent of many proteins (e.g. transcription factors, chaperones, lipid-binding proteins) (36, 37). Zinc deficiency in rats is associated with oxidative damage to DNA, lipids, and proteins (38), and in humans, it is manifested by defects in appetite, cognitive function, embryonic development, epithelial integrity, and immune function (39). Despite its essential nature, zinc is toxic to cells when accumulated in excess amounts (35).

Recent studies have revealed that the synthesis of phospholipids in S. cerevisiae is influenced by zinc deficiency (40). In particular, PI synthase activity is elevated in zinc-depleted cells, whereas several enzyme activities (e.g. PS synthase, PE decarboxylase, PE methyltransferase, and phospholipid methy- ltransferase) in the CDP-diacylglycerol pathway for PC synthesis are reduced in response to zinc depletion (40). The regulation of these activities by zinc availability contributes to alterations in the cellular levels of the major membrane phospholipids PI (elevated) and PE (reduced) (40). For the PS synthase enzyme, the reduction in activity in response to zinc depletion is controlled at the level of transcription through the

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* The abbreviations used are: PI, phosphatidylinositol; GST, glutathione S-transferase; HA, hemagglutinin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; UASINO, upstream activating sequence inositol-responsive element; UASZRE, UAS zinc-responsive element.
Regulation of Phosphatidylinositol Synthase by Zinc

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade. Growth medium supplements were from Bio-Rad. Ampicillin (100 µg/ml) was added to bacterial cultures that contained plasmids. Yeast and bacterial media were supplemented with 2% and 1.5% agar, respectively, for growth on plates. Glassware were washed with Liqui-Nox, rinsed with 0.1 M EDTA, and then rinsed several times with deionized distilled water to prevent zinc contamination.

**DNA Manipulations and Amplification of DNA by PCR**—Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligation were performed by standard methods (42). Conditions for the amplification of DNA by PCR were optimized as described previously (44). Transformation of yeast (45) and *E. coli* (42) were performed using standard protocols.

**Construction of Plasmids**—Plasmid pWM11 contains a 2.2-kb DNA fragment for the *PIS1* gene with sequences for an HA epitope tag inserted after the start codon. Genomic DNA prepared from strain W303-1A was used as a template to produce a 5′-fragment of *PIS1HA* (primers 5′-CCCCCCCCCTGGAATTCGATGCAAATAG-3′ and 5′-AGCTAGTCGACTGCGTATGGCAGATGAGTCTAAGC-3′) and a 3′-fragment of *PIS1HA* (primers 5′-TACCGTACGATATGCGTATGGCCATGGAGTCTAAGCTG-3′ and 5′-CGTCTGATTCCGCGGAGCAGGAGTCTGAGGCTC-3′). The 5′- and 3′-fragments of *PIS1HA* were digested with *NheI* and *AatII*, respectively, and ligated into the *NheI* and *AatII* sites of pRS416 to generate the plasmid pWM11. The Stratagene QuikChange site-directed mutagenesis kit was utilized according to the manufacturer's instructions to generate plasmids pPZM1–pPZM3. These plasmids were derivatives of pMA109 and contained mutations in *UASINO*, *UASINSO*, and *UASRE* of the *PIS1* promoter.

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from yeast strains (46, 47), resolved by agarose gel electrophoresis (48), and then transferred to Zeta Probe membranes by vacuum blotting. The *CMI1* and *CMD1* probes were labeled with [α-32P]dCTP using the NEBlot random primer labeling kit, and unincorporated nucleotides were removed using ProbeQuant G-50 columns. Prehybridization, hybridization with the probes, and washes to remove nonspecific binding were carried out according to the manufacturer's instructions. Autoradiography of the radiolabeled mRNAs were acquired by phosphorimaging analysis.

**Anti-PI Synthase Antibodies and Immunoblotting**—The peptide sequence AALILADNDAKNANE (residues 201–215 at the C-terminal end of the deduced amino acid sequence of *PIS1*) was synthesized and used to raise antibodies in New Zealand White rabbits by standard procedures at Bio-Synthesis, Inc. The IgG fraction was isolated from the antiserum using protein A-Sepharose CL-4B (49). SDS-PAGE (50) using 10% slab gels and the transfer of proteins to polyvinylidene difluoride membranes (51) were performed as described previously. The membrane was probed with 12.5 µg/ml purified anti-PI synthase IgG fraction. Mouse monoclonal anti-HA antibodies were used at a dilution of 1:1,000. Goat anti-rabbit and anti-mouse IgG-alkaline phosphatase conjugates were used as secondary antibodies at a dilution of 1:5,000. The PI synthase protein (*PIS1p*) was detected using the enhanced chemifluorescence Western blotting detection kit, and the signals were acquired by FluorImaging. The relative density of the signal was analyzed using ImageQuant software. Immunoblot signals were in the linear range of detectability.

**Preparation of Cell Extracts and Protein Determination**—Cell extracts were prepared as described previously (52). Cells were suspended in 50 mM Tris-maleate buffer, pH 7.0, containing 1 mM EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin. Cells were disrupted by homogenization with chilled glass beads (0.5-mm diameter) using a Biospec Products Mini-Bead-Beater-8. Samples were homogenized for 10-1 min bursts followed by a 2-min cooling between bursts at 4°C. The cell extract (supernatant) was assayed for protein content by the method of Lowry et al. (53).
The culture was harvested, and the resulting pellet was resuspended in 50 mM Tris-HCl, pH 7.0, 3 mM EDTA, and 2.5 mM dithiothreitol. It was dialyzed against phosphate-buffered saline containing 10% glycerol to remove any non-specific adsorption. A unit of PIS1 activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min.

Electrophoretic Mobility Shift Assays—DNA binding assays were performed by annealing 30 µM complementary single-stranded oligonucleotides in a total volume of 0.1 ml containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA. The reaction mixtures were incubated for 5 min at 50 °C in a heat block and then for 2 h in the heat block that was turned off. Annealed oligonucleotides were designed to contain a 5'-overhanging end, and they were labeled by incorporating [32P]dATP to the ends. Annealed oligonucleotides (100 pmol) were incubated with 5 units of Klenow fragment and [32P]dATP (400–800 Ci/nmol) for 30 min at room temperature. Labeled oligonucleotides were purified from unincorporated nucleotides using ProbeQuant G-50 spin columns.

Formation of the protein-DNA complexes was for 15 min at room temperature in a total volume of 10 µl containing 1 pmol of labeled oligonucleotide. The samples were resolved on a 5% PAGE gel in 0.5xTBE buffer at 100 V for 45 min. Gels were dried onto blotting paper, and the radioactive signals were visualized by phosphorimaging analysis.

TABLE I

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<th>Strain or plasmid</th>
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Plasmids

- pWM1: HA-tagged PIS1 gene ligated into the XmaI/XbaI sites of pRS416
- pRS416: Single copy E. coli/yeast shuttle vector containing URA3
- pPi14: PIS1 gene on a multicopy plasmid with a 3' overhanging end, and they were labeled with [32P]dATP
- pGST-687: E. coli expression plasmid for recombinant GST-Zap1p687-880 fusion protein
- pMA109: PpDRlacz reporter plasmid containing the PIS1 promoter with UASZRE
- pPZM1: Derived of pMA109 with mutations in UASZRE
- pPZM2: Derived of pMA109 with mutations in UASZRE
- pPZM3: Derived of pMA109 with mutations in UASZRE

Effect of the zrt1::TRP1 zrt2::LEU2 double mutant (56). This mutant lacks both the high affinity (Zrt1p) and low affinity (Zrt2p) plasma membrane zinc transporters that are primarily responsible for regulating the cytoplasmic levels of zinc in S. cerevisiae (56, 57). For this and subsequent experiments, the growth medium lacked inositol and choline supplementation to exclude the regulatory effects that these phospholipid precursors have on phospholipid synthesis (3, 27, 30, 31). As described previously (40), depletion of zinc from the growth medium of wild type cells caused a 2-fold increase in the expression of PI synthase activity (Fig. 2). The level of PI synthase activity in the zrt1::TRP1 zrt2::LEU2 mutant grown in the presence of zinc was similar to that expressed in the wild type control cells.
cells that were depleted for zinc (Fig. 2). This result indicated that the intracellular levels of zinc were responsible for regulating the expression of PI synthase activity.

**Effect of Zinc Depletion on the Expression of PI Synthase Protein and PIS1 mRNA**—Antibodies were generated against a peptide sequence found at the C-terminal end of the PI synthase protein. These antibodies recognized a protein with a subunit molecular mass of 24 kDa, the predicted size of the PIS1 gene product (6). To confirm the identity of this 24-kDa protein as the PI synthase protein, an immunoblot experiment was performed using a cell extract derived from wild type cells that overexpressed the PIS1 gene on a high copy plasmid. Consistent with the overexpression of the PIS1 gene, the amount of the 24-kDa protein that was recognized by the anti-PI synthase antibodies was elevated ~7-fold. As a further confirmation, an immunoblot experiment was performed using a cell extract from wild type cells that expressed the PIS1HA gene on a single copy plasmid. The anti-PI synthase antibodies recognized both the native and HA-tagged versions of the PI synthase protein. HA-tagged PI synthase migrated with a molecular mass of 25 kDa because of the HA epitope. The identity of the HA-tagged PI synthase protein was confirmed by immunoblot analysis using anti-HA antibodies.

The expression of the PI synthase protein was analyzed by immunoblotting to examine the mechanism by which PI synthase activity was regulated in response to zinc depletion. Zinc depletion resulted in a nearly 2-fold increase in the amount of the PI synthase protein compared with cells grown with zinc (Fig. 3A). This indicated that the increase in PI synthase activity was a result of an increase in enzyme level.

We next examined the level of PIS1 mRNA to determine whether the increase in enzyme content was caused by an increase in gene expression. CMD1 mRNA (encodes calmodulin) was measured in this analysis as a loading control because its expression level is not affected by zinc availability (58, 59).

Northern blot analysis of total RNA isolated from exponential phase cells showed that the relative amount of PIS1 mRNA in zinc-depleted cells was almost 2-fold greater compared with that found in cells grown with zinc (Fig. 3B). These results indicated that a transcriptional mechanism was responsible for the regulation of PI synthase in zinc-depleted cells.

**Effect of Zinc Depletion on the Expression of β-Galactosidase Activity in Cells Bearing the P_{pyn}−lacZ Reporter Gene**—The analysis of PIS1 expression was facilitated by the use of plasmid pMA109, which bears a P_{pyn}−lacZ reporter gene where the expression levels of β-galactosidase activity are dependent on transcription driven by the PIS1 promoter (60). To examine further the effect of zinc depletion on the expression of the PIS1 gene, we measured β-galactosidase activity from wild type cells bearing plasmid pMA109 which were grown with various concentrations of zinc. Reduction for zinc in the growth medium resulted in a dose-dependent increase in β-galactosidase activity (Fig. 4). The activity found in cells grown in the absence of zinc was 3.5-fold greater than the activity in cells grown in the presence of 1.5 μM zinc (Fig. 4). Concentrations of zinc above 1.5 μM did not result in a further reduction in β-galactosidase activity.

**Effects of the ino2Δ, ino4Δ, and opt1Δ Mutations on the Regulation of PI Synthase by Zinc Depletion**—The PI synthase enzyme is found at a branch point in phospholipid synthesis where it competes with another enzyme, PS synthase, for the common liponucleotide substrate CDP-diacylglycerol (27). Unlike PIS1, the expression of the PS synthase gene (CHO1) is repressed in wild type cells when zinc is depleted from the growth medium (40). The regulation of PS synthase expression by zinc depletion is mediated through a UAS_{INO} element in the CHO1 promoter and by the positive transcription factors Ino2p and Ino4p and the negative transcription factor Opi1p (40). Because the PIS1 promoter contains a UAS_{INO} element (60) and the synthesis of PI and PS is regulated coordinately in S. cerevisiae (3, 27, 27, 30, 31), we questioned whether the regulation of PI synthase expression by zinc depletion was...
mediated by Ino2p, Ino4p, and Opi1p. To address this question, PI synthase activity was measured in ino2Δ, ino4Δ, and opi1Δ mutant cells that were grown in the presence and absence of zinc. In all three regulatory mutants, the PI synthase enzyme was elevated in response to zinc depletion similar to that observed in wild type cells (data not shown). These results indicated that the induction of PI synthase in zinc-depleted cells was not mediated by Ino2p, Ino4p, and Opi1p.

Effects of the zap1Δ Mutation on the Regulation of PI Synthase by Zinc Depletion-Zap1p is a positive transcription factor that is expressed maximally in zinc-depleted cells and repressed in zinc-replete cells (61). Zap1p directly regulates UASZRE-containing genes (e.g. ZRT1, ZRT2, ZRT3, ZRC1, PET4, DPPI) whose expression is induced by zinc depletion (43, 58, 62–64). Inspection of the PIS1 promoter revealed that it contains sequences that bear resemblance to the consensus UASZRE (see below). Accordingly, we questioned whether the regulation of PI synthase expression by zinc was dependent on Zap1p function. In the first set of experiments, the zap1Δ mutant bearing the P_{PIS1-lacZ} reporter gene was grown in the presence and absence of zinc followed by the measurement of β-galactosidase activity. In contrast to wild type cells, zinc depletion did not result in the induction of β-galactosidase activity (Fig. 5A). In a second set of experiments, PI synthase protein and activity levels were measured in cell extracts derived from zap1Δ mutant cells grown in the presence and absence of zinc. Unlike wild type cells, the depletion of zinc from the growth medium of the zap1Δ mutant did not result in elevated levels of PI synthase protein (Fig. 5B) and activity (Fig. 5C). These results indicated that the zinc-mediated regulation of PIS1 expression was dependent on the Zap1p transcription factor.

Interactions of GST-Zap1p687–880 with Putative UASZRE Sites in the PIS1 Promoter—We sought evidence that Zap1p mediates the regulation of PIS1 expression in response to zinc depletion by direct interaction with the PIS1 promoter. The PIS1 promoter contains three putative UASZRE sites (UASZRE1, UASZRE2, and UASZRE3) with sequences that resemble the consensus UASZRE sequence for Zap1p binding (Fig. 6A). Electrophoretic mobility shift assays were performed with labeled oligonucleotides containing the putative UASZRE sites using recombinant GST-Zap1p687–880 purified from E. coli. Zap1p687–880 contains the UASZRE binding domain (amino acids 687–880) of Zap1p (65). Of the three probes, the oligonucleotide containing UASZRE3 showed the strongest interaction with GST-Zap1p687–880 (Fig. 6B). The interaction of GST-Zap1p687–880 with UASZRE1 was ~20-fold lower compared with UASZRE3, whereas an interaction with UASZRE2 was hardly detectable (Fig. 6B). The interaction of GST-Zap1p687–880 with UASZRE1 was examined further using the same assay. The formation of the GST-Zap1p687–880-UASZRE1 complex was dependent on the concentration of GST-Zap1p687–880 (Fig. 7A). In addition, the unlabeled UASZRE1 probe competed with the labeled probe for binding to GST-Zap1p687–880 in a dose-dependent manner (Fig. 7B). Moreover, this interaction was abolished when the UASZRE1 sequence was mutated (M1) to a nonconsensus sequence (Fig. 7C). When the UASZRE3 sequence was mutated (M2) to the consensus UASZRE, the extent of interaction with GST-Zap1p687–880 was 10-fold greater than the interaction with the wild type UASZRE3 sequence (Fig. 7C).

Effects of Mutations in the Putative UASZRE Elements in the PIS1 Promoter on the Zinc-mediated Regulation of PIS1 Expression—The effects of mutations in UASZRE1, UASZRE2, and UASZRE3 in the PIS1 promoter on the zinc-mediated regulation of PIS1 expression was examined. P_{PIS1-lacZ} reporter genes were constructed with mutations in each of the three putative UASZRE elements. For each element, the core sequences were changed to the nonconsensus sequence of 5′-CAATTTCCAATTT-3′. Cells bearing the wild type or mutant P_{PIS1-lacZ} reporter
FIG. 7. Interactions of GST-Zap1p<sup>687–880</sup> with UAS<sub>ZRE</sub><sup>3</sup>. Samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotide (2.5 × 10<sup>5</sup> cpm/pmol) with the sequence for UAS<sub>ZRE</sub><sup>3</sup> in the P<sub>INS1</sub> promoter were incubated with recombinant GST-Zap1p<sup>687–880</sup>. A, the experiment was performed with 0, 0.15, 0.3, and 0.6 μg of recombinant GST-Zap1p<sup>687–880</sup> and 0, 25, 50, and 100 pmol of unlabeled oligonucleotide with the sequence for UAS<sub>ZRE</sub><sup>3</sup>. C, the experiment was performed with 0.6 μg of recombinant GST-Zap1p<sup>687–880</sup> and sequences for wild type (WT) and mutated forms of UAS<sub>ZRE</sub><sup>3</sup>. The wild type UAS<sub>ZRE</sub><sup>3</sup> sequence was mutated from 5'-ACCTGAGAGGT-3' to the nonconsensus sequence 5'-CAATCCCAAT-3' (M1) and to a consensus sequence 5'-ACCTTGAAGGT-3' (M2). Interaction of GST-Zap1p<sup>687–880</sup> with the labeled oligonucleotides was determined by electrophoretic mobility shift assay using a 6% polyacrylamide gel. The data shown are representative of two independent experiments.

genes were grown in the presence and absence of zinc; cell extracts were prepared and assayed for β-galactosidase activity. The mutations in UAS<sub>ZRE</sub><sup>3</sup> in the reporter plasmid pPZM3 abolished the induction of β-galactosidase activity which was observed in zinc-depleted cells bearing the wild type P<sub>PIS1</sub>-lacZ reporter plasmid pMA109 (Fig. 8). Although the expression of the β-galactosidase activities found in cells bearing the reporter plasmids with mutations in UAS<sub>ZRE</sub><sup>1</sup> (pPZM1) and UAS<sub>ZRE</sub><sup>3</sup> (pPZM2) was somewhat attenuated, the PIS1 gene was still induced when cells were depleted for zinc (Fig. 8). These data indicated that the zinc-mediated regulation of PIS1 expression was mediated primarily by the UAS<sub>ZRE</sub><sup>3</sup> sequence in its promoter.

DISCUSSION

The yeast <i>S. cerevisiae</i> has the ability to cope with a variety of stress conditions (e.g. nutrient deprivation) by regulating the expression of enzyme activities including those involved in phospholipid synthesis (4, 27, 40, 42, 43, 66, 67). In particular, the stress condition of zinc depletion results in an increase in PI content which is attributed to elevated expression of PI synthase activity (40). Analysis of the zrt1Δ zrt2Δ mutant defective in the major plasma membrane zinc transporters Zrt1p and Zrt2p indicated that a decrease in the intracellular levels of zinc was responsible for the induction of PI synthase activity. That PIS1 mRNA, its encoded protein PIS1p, and the β-galactosidase activity driven by the P<sub>PIS1</sub>-lacZ reporter gene were elevated in zinc-depleted cells indicated that the increase in PI synthase activity was the result of a transcriptional mechanism.

The zinc-mediated induction of the P<sub>PIS1</sub>-lacZ reporter gene and PI synthase protein and activity was lost in zap1Δ mutant cells. These data indicated that the regulation of PIS1 gene expression by zinc was mediated by the Zap1p transcription factor. Zap1p is a zinc-sensing and zinc-inducible regulatory protein that binds to a UAS<sub>ZRE</sub> found in the promoter of zinc-regulated genes to drive their transcription (58, 61, 68–71). Zap1p plays a major role in regulating the intracellular levels of zinc in <i>S. cerevisiae</i> (61, 71). For example in zinc-depleted cells, Zap1p mediates increased expression and activity of the high affinity (Zrt1p) and low affinity (Zrt2p, Fet4p) zinc transporters in the plasma membrane and of the efflux zinc transporter Zrt3p in the vacuole membrane to elevate the cytoplasmic levels of zinc (56, 58, 62, 68, 71, 72).

The promoter of the PIS1 gene does not contain a consensus UAS<sub>ZRE</sub>: However, three putative UAS<sub>ZRE</sub> sites were identified in the PIS1 promoter by a motif search using the Vector NTI computer program. Electrophoretic mobility shift assays with DNA probes containing the putative UAS<sub>ZRE</sub> sites and purified recombinant GST-Zap1p<sup>687–880</sup> showed that UAS<sub>ZRE</sub><sup>3</sup> in the PIS1 promoter was required for GST-Zap1p<sup>687–880</sup> binding in vitro. Moreover, mutations in UAS<sub>ZRE</sub><sup>3</sup> to a nonconsensus sequence abolished the GST-Zap1p<sup>687–880</sup>-DNA interactions in vitro and abolished the induction of PIS1 gene expression (as reflected in β-galactosidase activity) in response to zinc depletion. A genomewide cDNA microarray analysis of gene expression identified 46 direct Zap1p target genes that are induced by zinc depletion (58). The PIS1 gene was not identified in that microarray study (58). This might be attributed to the relatively modest level of PIS1 induction (~2-fold) compared with the >10-fold inductions of other Zap1p target genes (e.g. ZRT1, DPP1) (43, 58). The differences between the magnitudes of induction of the PIS1 gene and other Zap1p target genes correlated with the relative binding efficiencies of GST-Zap1p<sup>687–880</sup> with the PIS1 promoter UAS<sub>ZRE</sub> sequence compared with this sequence mutated to a consensus UAS<sub>ZRE</sub> sequence. Notwithstanding, the 2-fold induction of the PIS1 gene in response to zinc depletion correlated with the ~2-fold increase in the PI content of yeast cells depleted for zinc (40). The steady-state composition of PI in <i>S. cerevisiae</i> is tightly regulated (~2–3-fold changes) (2, 3, 27). In this regard, we found that the expression of PI synthase did not respond to zinc depletion when the PIS1 gene was overexpressed from a plasmid.

Inositol, the water-soluble substrate of the PI synthase enzyme reaction, plays a major role in the regulation of phospholipid synthesis and composition in <i>S. cerevisiae</i> (2–5, 27). The addition of inositol to the growth medium of wild type cells causes an increase in the level of PI and a decrease in the levels of PS, PE, and PC (28, 52). The decreased levels of PS, PE, and PC are primarily the result of a repression mechanism that involves the positive transcription factors Ino2p and Ino4p, and the negative transcription factor Op1p, and a UAS<sub>INO3</sub> element found in the promoter of genes (i.e. CHO1, PSD1, CHO2, and OPI3) encoding the enzymes in the CDPA-diacylglycerol pathway for PC synthesis (3, 27, 30–32) (Fig. 1). The coordinate repression of the CDPA-diacylglycerol pathway enzymes by inositol requires the ongoing synthesis of PC (73, 74) and en-

FIG. 8. Effects of mutations in UAS<sub>ZRE</sub><sup>1</sup>, UAS<sub>ZRE</sub><sup>3</sup>, and UAS<sub>ZRE</sub><sup>5</sup> in the PIS1 promoter on the zinc-mediated regulation of β-galactosidase activity in cells bearing the P<sub>PIS1</sub>-lacZ reporter gene. Wild type cells bearing the indicated P<sub>PIS1</sub>-lacZ reporter plasmids were grown in the presence (1.5 μM) and absence of zinc. The UAS<sub>ZRE</sub><sup>1</sup>, UAS<sub>ZRE</sub><sup>3</sup>, and UAS<sub>ZRE</sub><sup>5</sup> sequences in the PIS1 promoter of plasmid pMA109 were mutated to the nonconsensus sequence 5'-CAATCCCAAT-3' in plasmids pPZM1, pPZM2, and pPZM3, respectively. Cell extracts were prepared and used for the assay of β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D.
hanced by the inclusion of choline in the growth medium (3, 27, 30–32). The increased level of PI in response to inositol/choline supplementation is not caused by increased expression of PIS1 mRNA (75) and the PI synthase enzyme (76). Transcription of the PIS1 gene is insensitive to inositol/choline, and it does not require the UASINO element in its promoter or the transcription factors Ino1p and Op1p (60). The regulation of PI synthase by inositol is the result of a biochemical mechanism (28). Given the low intracellular levels of inositol and the relatively high Km value for inositol, the synthesis of PI by the PI synthase enzyme is regulated by the availability of inositol (28). Moreover, inositol is an inhibitor of the PS synthase enzyme, and this regulation also contributes to the decrease in the synthesis of PS and ultimately PE and PC (28). These observations raised the suggestion that PI synthase is a constitutively expressed enzyme (3, 30, 31). However, as shown here, the level of PI synthase enzyme is regulated by zinc availability.

This is not the first study to show that the expression of the PIS1 gene is subject to transcriptional regulation. Anderson and Lopes (60) have shown that expression of PIS1 is regulated in response to growth medium carbon source. Compared with glucose, glycerol represses PIS1 expression, whereas galactose induces expression (60). The transcription factor Mcm1p mediates the glycerol-dependent repression of PIS1 gene expression, whereas the transcription factor Snl1p mediates the galactose-mediated induction of gene expression (60). The expression of the PIS1 gene is also regulated by oxygen availability (77). Gene expression is induced when cells are grown under anaerobic conditions and repressed under aerobic conditions. Repression is dependent on transcription factor Rpo1p and its binding site in the PIS1 promoter (77). Similar to that observed in cells deprived for zinc (40), a reduction in oxygen availability results in elevated levels of PI (77). The induction of PIS1 gene expression may represent one of the mechanisms by which cells cope with the stress conditions of zinc and oxygen deficiencies given that PI is a precursor to several lipid molecules (sphingolipids, phosphoinositides, and glycosylphosphatidylinositol anchors) that are essential to the growth and metabolism of this eukaryotic organism (3, 9–20).

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REFERENCES

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