

Regulation of the *Saccharomyces cerevisiae* CK1I-encoded Choline Kinase by Zinc Depletion*

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In the yeast *Saccharomyces cerevisiae*, the CK1I-encoded choline kinase catalyzes the committed step in the synthesis of phosphatidylcholine via the CDP-choline branch of the Kennedy pathway. Analysis of a P_{CK1I} -*lacZ* reporter gene revealed that CK1I expression was regulated by intracellular levels of the essential mineral zinc. Zinc depletion resulted in a concentration-dependent induction of CK1I expression. This regulation was mediated by the zinc-sensing and zinc-inducible transcriptional activator Zap1p. A purified Zap1p probe interacted with two putative UAS_{ZRE} sequences (ZRE1 and ZRE2) in the CK1I promoter. Mutations of ZRE1 and ZRE2 to a nonconsensus UAS_{ZRE} attenuated the induction of CK1I expression in response to zinc depletion. A UAS_{INO} element in the CK1I promoter was responsible for stimulating CK1I expression, but this element was not involved with the regulation by zinc depletion. The induction of CK1I expression in zinc-depleted cells translated into increased choline kinase activity *in vitro* and *in vivo*, and an increase in phosphatidylcholine synthesis via the Kennedy pathway.

Recent studies have uncovered a novel relationship between the control of zinc homeostasis and the regulation of membrane phospholipid synthesis in the yeast *Saccharomyces cerevisiae* (1). Zinc is an essential mineral for the growth and metabolism of *S. cerevisiae*; it serves as a cofactor for hundreds of enzymes and it is a structural component of several transcription factors (2–4). The membrane function of zinc transport plays a central role in controlling the cellular levels of zinc (5). Zinc transporters are found in the plasma membrane (e.g. Zrt1p, Zrt2p, and Fet4p) (6–8), and membranes of the endoplasmic reticulum (Msc2p, Zrg17p) (4, 9), mitochondria (Mrs3p, Mrs4p) (10), and vacuole (e.g. Zrt3p, Cot1p, Zrc1p) (11–14). In controlling the cytoplasmic levels of zinc, some transporters mediate zinc influx (e.g. Zrt1p, Zrt2p, Fet4p, Cot1p, and Zrc1p), whereas some transporters mediate zinc efflux (e.g. Zrt3p) (5, 15).

The zinc transporters are regulated at the level of gene expression, and indeed, the most highly regulated transporter is

Zrt1p (5, 6, 16). The expression of Zrt1p is induced for increased zinc uptake when the cytoplasmic levels of zinc are limiting (6). The increase in Zrt1p expression is dependent on the zinc-sensing and zinc-inducible transcriptional activator Zap1p, which interacts with a UAS_{ZRE} sequence in the ZRT1 promoter to activate transcription (6, 17, 18). Conversely, when the cytoplasmic levels of zinc are high, the expression of Zrt1p is reduced to attenuate zinc uptake (6). Reduced Zrt1p expression is due to the loss of Zap1p activation of ZRT1 expression (18). Moreover, when the cytoplasmic levels of zinc are limiting, Zrt1p is a stable protein, and when the levels of zinc are high, Zrt1p is removed from the plasma membrane by endocytosis and is proteolytically degraded in the vacuole (19). Mechanisms to attenuate zinc uptake are important because an excess amount of cellular zinc is toxic (2).

Phospholipids are major components of cellular membranes. Because of their amphipathic nature, they are responsible for the membrane bilayer structure (20). In addition, phospholipids serve as precursors for the synthesis of complex membrane macromolecules (21–25), they are reservoirs of second messengers (26), and they regulate the structure, topography, and function of membrane transporters (27–34). Whereas little is known about the role of phospholipids in the structure and/or function of zinc transporters, it is known that the synthesis of membrane phospholipids in *S. cerevisiae* is coordinately regulated with the expression of zinc transporters in response to zinc depletion (1).

In *S. cerevisiae*, the major membrane phospholipid PC² is synthesized (*de novo*) from the phospholipid precursor phosphatidate by the complementary CDP-DAG and Kennedy (CDP-choline branch) pathways (Fig. 1) (35, 36). When wild type cells are depleted for zinc, the activities of the CDP-DAG pathway enzymes PS synthase, PS decarboxylase, and the PE and phospholipid methyltransferases are repressed (37). On the other hand, zinc depletion causes the induction of PI synthase activity (37, 38). The regulation of the PS synthase and PI synthase enzymes in response to zinc depletion occurs at the level of transcription. The repression of *CHO1*, which encodes PS synthase, is mediated by the phospholipid synthesis repressor Opi1p (37), whereas the induction of *PIS1*, which encodes PI synthase, is mediated by Zap1p (38). Opi1p attenuates transcription of *CHO1* (and other UAS_{INO}-containing genes in the

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² The abbreviations used are: PC, phosphatidylcholine; CDP-DAG, CDP-diaclylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; UAS_{INO}, upstream activating sequence inositol-responsive element; UAS_{ZRE}, upstream activating sequence zinc-responsive element; GST, glutathione S-transferase.

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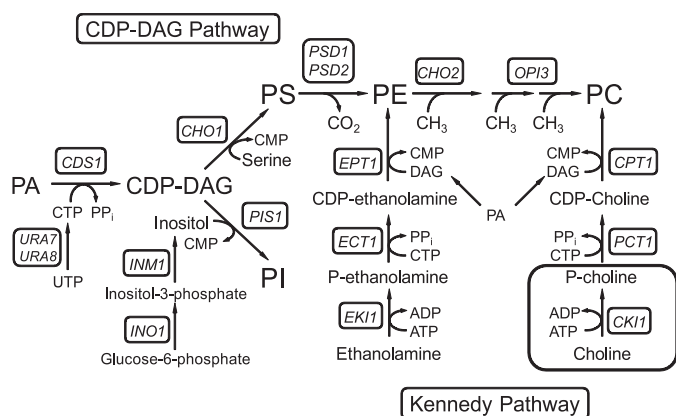


FIGURE 1. **Phospholipid synthesis in *S. cerevisiae*.** The pathways shown for the synthesis of phospholipids include the relevant steps discussed throughout the article. The genes encoding enzymes responsible for the reactions in the pathways are indicated in the figure. The reaction catalyzed by the *CKI1*-encoded choline kinase enzyme is highlighted in the box. *P*-choline, phosphocholine; *P*-ethanolamine, phosphoethanolamine.

pathway) by interaction with Ino2p, a component of the transcriptional activator Ino2p-Ino4p complex that interacts with a UAS_{INO} in the promoter (1, 36). Zap1p induces transcription of *PIS1* by interaction with UAS_{ZRE} sequences in the *PIS1* promoter (38). The transcriptional regulation of the PS synthase and PI synthase enzymes translates into a decrease in the cellular PE content³ and an increase in the cellular PI content, respectively (37). The reciprocal regulation of these CDP-DAG branch point enzymes is part of an overall mechanism by which the synthesis of PI is coordinately regulated with the synthesis of phospholipids by way of the CDP-DAG pathway (1, 39, 40).

Despite the fact that the CDP-DAG pathway activities are repressed in response to zinc depletion, this growth condition does not have a significant effect on the cellular PC content (37). Because of the complementary nature of the phospholipid synthesis pathways in *S. cerevisiae*, we hypothesized that the decrease in CDP-DAG pathway enzyme activities is compensated by an increase in PC synthesis via the CDP-choline branch of the Kennedy pathway (Fig. 1). We focused our studies on the regulation of *CKI1*-encoded choline kinase, the enzyme that catalyzes the committed step in the pathway (Fig. 1). Indeed, choline kinase is a highly regulated enzyme that is controlled by transcriptional (41–43) and post-translational (44, 45) mechanisms to regulate PC content in *S. cerevisiae*. We found that the expression of *CKI1* was induced by zinc depletion, and that the mechanism for this regulation involved the zinc-regulated transcriptional activator Zap1p. Moreover, this transcriptional regulation translated into increased choline kinase activity and PC synthesis via the Kennedy pathway.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade. Growth medium supplies and yeast nitrogen base lacking zinc sulfate were purchased from Difco and BIO 101 Inc., respectively. Clontech was the supplier of the YeastmakerTM transformation kit. The QuikChange site-directed mutagenesis kit was pur-

chased from Stratagene. Sigma was the source of ampicillin, aprotinin, benzamidine, bovine serum albumin, choline, phosphocholine, CDP-choline, leupeptin, *O*-nitrophenyl β -D-galactopyranoside, pepstatin, phenylmethylsulfonyl fluoride, and IGEPAL CA-630. Avanti Polar Lipids was the source of phosphatidylcholine. Silica Gel 60 thin layer chromatography plates were from EM Science. Protein assay reagents, electrophoretic reagents, and acrylamide solutions were purchased from Bio-Rad. Radiochemicals and scintillation counting supplies were purchased from PerkinElmer Life Sciences and National Diagnostics, respectively. Genosys Biotechnology, Inc. prepared oligonucleotides for PCR and electrophoretic mobility shift assays. ProbeQuant G-50 columns were purchased from Amersham Biosciences. Liqui-Nox detergent was from Alconox, Inc.

Strains and Growth Conditions—The strains used in this work are listed in Table 1. Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete medium (46) containing 2% glucose at 30 °C. The appropriate amino acids of synthetic complete medium were omitted for selection purposes. Zinc-free medium was synthetic complete medium (46) prepared with yeast nitrogen base lacking zinc sulfate. To deplete internal stores of zinc (47), the following routine was followed. Cells were first grown for 24 h in synthetic complete medium containing 1.5 μ M zinc sulfate. Saturated cultures were diluted into zinc-free medium at an initial concentration of 1×10^6 cells/ml, and grown for 24 h. Cultures were then diluted to 1×10^6 cells/ml and grown in zinc-free medium containing 0 or 1.5 μ M zinc sulfate. The growth medium for the inositol auxotrophic *ino2Δ* and *ino4Δ* mutants (48) was supplemented with 75 μ M inositol. Plasmid maintenance and amplification were performed in *Escherichia coli* strain DH5 α . *E. coli* cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (100 μ g/ml) was added to bacterial cultures that carried plasmids. For growth on plates, yeast and bacterial media were supplemented with 2 and 1.5% agar, respectively. Yeast cell numbers in liquid medium were determined spectrophotometrically at an absorbance of 600 nm. Exponential phase cells were harvested at a density of 1.5×10^7 cells/ml. Glassware were washed with Liqui-Nox, rinsed with 0.1 mM EDTA, and then rinsed several times with deionized distilled water to remove zinc contamination.

DNA Manipulations, PCR, and Site-directed Mutagenesis—Standard methods were used for isolation and manipulation of DNA (49). PCR were optimized as described previously (50). Site-specific mutations in plasmids were generated with the QuikChange site-directed mutagenesis kit. All mutations were confirmed by DNA sequencing.

Plasmid Constructions—The plasmids used in this work are listed in Table 1. The pKSK11 plasmid contains the *CKI1* gene promoter fused to the coding sequence of the *lacZ* gene of *E. coli*. This plasmid was constructed by replacing the *CRD1* gene promoter in pSD90 (a plasmid based on YEp357R) with the *CKI1* gene promoter sequence at the SphI/KpnI sites. The *CKI1* promoter was obtained by PCR (primers, 5'-TCAGCATG-CCTGCAGATATGAATTCCATAGG-3' and 5'-CGAGGTAC-CCCTGGACGTGATTCTTGAC-3') using strain W303-1A genomic DNA as the template. The PCR primer used in the

³ The decrease in PE content is attributed to reductions in both PS synthase and PS decarboxylase activities (37).

TABLE 1
Strains and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristics	Source or Ref.
<i>E. coli</i> DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 <i>deoR recA1 endA1 hdR17</i> (r _k ⁻ m _k ⁺) <i>phoA supE44 l⁻ thi-1 gyrA96 relA1</i>	49
<i>S. cerevisiae</i> W303-1A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	77
DY1457	<i>MATα ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52</i>	78
ZHY6	<i>MATa ade6 can1-100oc his3 leu2 ura3 zap1Δ::TRP1</i>	78
ZHY3	<i>MATα ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 zrt1Δ::LEU2 zrt2Δ::HIS3</i>	79
SH304	<i>MATa his3Δ200 leu2Δ1 trp1Δ63 ura3-52 opi1Δ::LEU2</i>	S. A. Henry
SH303	<i>MATa his3Δ200 leu2Δ1 trp1Δ63 ura3-52 ino2Δ::TRP1</i>	S. A. Henry
SH307	<i>MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 ino4Δ::LEU2</i>	S. A. Henry
Plasmid		
pSD90	P _{CRD1} -lacZ reporter gene containing the <i>CRD1</i> promoter with <i>URA3</i>	W. Dowhan
pKSK11	P _{CKI1} -lacZ reporter gene containing the <i>CKI1</i> promoter with <i>URA3</i>	This work
pCK-ZRE1	Derivative of pKSK11 with mutations in ZRE1	This work
pCK-ZRE2	Derivative of pKSK11 with mutations in ZRE2	This work
pCK-ZRE1,2	Derivative of pKSK11 with mutations in ZRE1 and ZRE2	This work
pCK-UASino	Derivative of pKSK11 with mutations in the UAS _{INO} element	This work
pDg2	P _{CYCI-ZRE} -lacZ reporter gene containing the UAS _{ZRE} element with <i>URA3</i>	17

forward direction corresponds to -308 bp to the start codon, and the primer used in the reverse direction corresponds to +23 bp to the start codon. The correct orientation of the *CKII* promoter was confirmed by restriction enzyme digestion.

Plasmid pCK-UASino is a derivative of pKSK11, in which the UAS_{INO} element (39) sequence (5'-TATTCACAT-3') in the *CKII* promoter was mutated to a nonconsensus sequence 5'-TATTT-TTTTT-3'. Mutagenesis was performed with the QuikChange site-directed mutagenesis kit using plasmid pKSK11 as the template and the mutagenic primers 5'-CTTGTTCTTTGTTCTTT-ATGGTATAAATTTTTTTTGTGCTCTACCGTTTTTCTT-GTCGGCCAGC-3' and 5'-GCTGGGCCGACAAGAAAAAC-GGTAGAGCACAAAAAATATTATACCATAAAGAACA-AAGAACAAG-3'. Plasmids pCK-ZRE1 (primers, 5'-CTAAGC-GATTGGTAACCAAAAAAAAAAAGAACCACCAAC-3' and 5'-GTTGGTGGTGTCTTTTTTTTTTTTGGTTACCA-ATCGCTTAC-3') and pCK-ZRE2 (primers, 5'-CAGATCGTT-CTCTTGTCTTTGAAAAAAAAAAAAATAATATTCACA-TGGTGCTCTAC-3' and 5'-GTAGAGCACCATGTGAAT-ATTATTTTTTTTTTTCAAAGAACAAGAGAACGAT-CTG-3') were also derivatives of pKSK11, in which the putative UAS_{ZRE} element sequences ZRE1 and ZRE2 in the *CKII* promoter were mutated, respectively, to a nonconsensus sequence 5'-AAAAAAAAAAA-3'. Plasmid pCK-ZRE1,2 is a derivative of pKSK11 where both ZRE1 and ZRE2 were mutated to the nonconsensus sequence. pCK-ZRE1,2 was constructed by PCR amplification of plasmid pCK-ZRE1 using the primers for the construction of pCK-ZRE2. After PCR amplifications, all samples were digested with DpnI to eliminate the template DNA. The plasmids were amplified in *E. coli*, purified, and sequenced to confirm the mutations in the *CKII* promoter. Transformation of yeast (51, 52) and bacteria (49) with plasmids were performed as described previously.

Electrophoretic Mobility Shift Assays—Double-stranded oligonucleotides (Table 2) were prepared by annealing 25 μ M complementary single-stranded oligonucleotides in a reaction mixture (0.1 ml) containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. The annealing reactions were incubated for 5 min at 100 °C in a heat block, and then kept in the

TABLE 2
Oligonucleotides used for electrophoretic mobility shift assays

Element	Annealed oligonucleotides ^a
ZRE1	5'-GTAACCTCCTTCACTTTAGAAca-3' 3'-CATTGGAGGAAGTGAATCCTTGA-5'
ZRE2	5'-TCTTTGTTCTTTATGGTATAaata-3' 3'-AGAAACAAGAAATACCATATTAT-5'
ZRE3	5'-GGTTAAATCTCGAAGAGACAGaa-3' 3'-CCAATTTAGAGCTTCTCTGTCTT-5'

^a Underlined sequences are putative ZRE sites. The lower case letters indicate the nucleotides filled with the Klenow fragment.

heat block for another 2 h after it had been turned off. The annealed oligonucleotides (100 pmol), which had a 5' overhanging end, were labeled with [α -³²P]dATP (400–800 Ci/nmol) and Klenow fragment (5 units) for 30 min at room temperature. The labeled oligonucleotides were separated from unincorporated nucleotides by gel filtration using ProbeQuant G-50 spin columns. Purified recombinant GST-Zap1p^{687–880} (38) was incubated with 1 pmol of radiolabeled DNA probe (2.0 \times 10⁵ cpm/pmol) for 15 min at room temperature in a total volume of 10 μ l. The reaction buffer contained 10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, 0.025 mg/ml poly(dI-dC)·poly(dI-dC), 0.2 mg/ml bovine serum albumin, 0.04% IGEPAL CA-630, and 10% glycerol. Following incubation, the reaction mixture was resolved on a 6% polyacrylamide gel (1.5-mm thickness) in 0.5 \times Tris borate-EDTA buffer at 100 V for 45 min. Gels were dried onto blotting paper, and the radioactive signals were visualized by phosphorimaging analysis.

Preparation of Cell Extracts and Protein Determination—All steps were performed at 5 °C. Yeast cells were disrupted with glass beads with a Mini BeadBeater-16 (Biospec Products) in 50 mM Tris malate 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 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 5 μ g/ml pepstatin (53). Glass beads and cell debris were removed by centrifugation at 1,500 \times g for 10 min, and the supernatant was used as the cell extract. Protein concentration was determined by the Coomassie Blue dye binding assay of Bradford (54) using bovine serum albumin as the standard.

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Enzyme Assays—Choline kinase activity was measured in a reaction mixture that contained 67 mM glycine-NaOH buffer (pH 9.5), 5 mM [*methyl*- ^{14}C]choline (2,000 cpm/nmol), 5 mM ATP, 10 mM MgSO_4 , 1.3 mM dithiothreitol, and enzyme protein in a final volume of 60 μl (55). The radiolabeled product, phosphocholine, was separated from the radiolabeled substrate by precipitation of choline as a reineckate salt (56). A unit of choline kinase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of phosphocholine/min. β -Galactosidase activity was measured for 5 min at room temperature by following the release of *o*-nitrophenol from *o*-nitrophenyl β -D-galactopyranoside at 410 nm. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 1 mM MgCl_2 , 100 mM 2-mercaptoethanol, 3 mM *o*-nitrophenyl- β -D-galactopyranoside, and enzyme in a total volume of 0.1 ml. A unit of β -galactosidase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of *o*-nitrophenol/min. Specific activity was defined as units/mg of total cellular protein. All enzyme assays were conducted in triplicate, and the average standard deviation of the assays was $\pm 5\%$. Enzyme reactions were linear with time and protein concentration.

Labeling and Analysis of CDP-Choline Pathway Intermediates and PC—Exponential phase cells were labeled for five to six generations with 10 μM [*methyl*- ^{14}C]choline (0.2 $\mu\text{Ci}/\text{ml}$). The CDP-choline pathway intermediates and PC were extracted from whole cells by a chloroform:methanol:water extraction, followed by separation of the aqueous and chloroform phases (57). The aqueous and chloroform phases that contained the CDP-choline pathway intermediates and PC, respectively, were dried *in vacuo* and then dissolved in 100 μl of methanol:water (1:1, v/v) and 100 μl of chloroform, respectively. The CDP-choline pathway intermediates and PC were subjected to TLC on silica gel plates using the solvent systems methanol, 0.6% sodium chloride, ammonium hydroxide (10:10:1, v/v) and chloroform, pyridine, 88% formic acid, methanol, water (60:35:10:5:2, v/v), respectively. The positions of the labeled compounds on chromatograms were determined by phosphorimaging and compared with standards. The amount of each labeled compound was determined by liquid scintillation counting and normalized based on cell number.

Data Analyses—The Student's *t* test (SigmaPlot software) was used to determine statistical significance, and *p* values < 0.05 were taken as a significant difference.

RESULTS

Effect of Zinc Depletion on the Expression of *CKII*—In this and in subsequent experiments, cells were grown in medium that lacked inositol supplementation to obviate the regulatory effects that inositol has on phospholipid metabolism (36, 40, 58). Cells were grown in a defined zinc-free medium that contained 1.5 μM zinc sulfate. This concentration of zinc was equivalent to that found in standard synthetic growth media (46). To confirm that our growth conditions depleted the intracellular level of zinc, we made use of a $\text{P}_{\text{CYC1-ZRE}}\text{-lacZ}$ reporter gene assay that is sensitive to the labile pool of intracellular zinc (11, 17). In this assay, when the total intracellular concentration

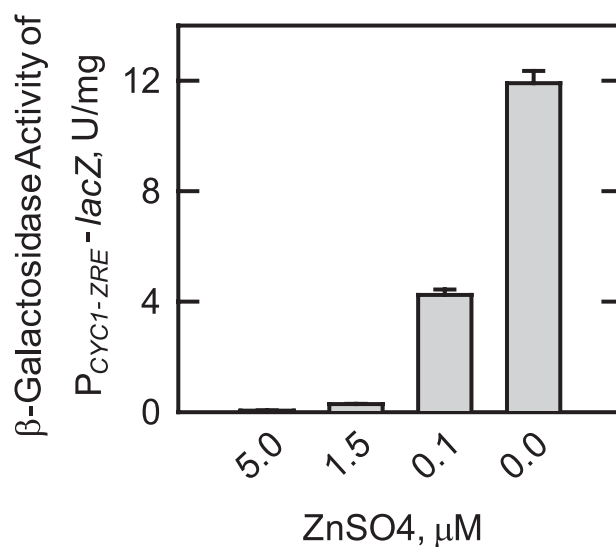


FIGURE 2. Effect of zinc depletion on the expression of β -galactosidase activity in wild type cells bearing the $\text{P}_{\text{CYC1-ZRE}}\text{-lacZ}$ reporter gene. Wild type cells bearing the $\text{P}_{\text{CYC1-ZRE}}\text{-lacZ}$ reporter plasmid pDg2 were grown to the exponential phase of growth in the presence of the indicated concentrations of ZnSO_4 . Cell extracts were prepared and assayed for β -galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments \pm S.D.

of zinc is limiting (~ 10 pmol/ 10^6 cells),⁴ the transcriptional activity of Zap1p is up-regulated, and this results in elevated levels of β -galactosidase activity (11, 18). As described previously (11), the β -galactosidase activity of wild type zinc-depleted cells was greatly induced when compared with cells that were grown with zinc (Fig. 2).

The effect of zinc depletion on the expression of the *CKII* gene was examined by use of a $\text{P}_{\text{CKII}}\text{-lacZ}$ reporter gene. This reporter gene was constructed by fusing the *CKII* promoter in-frame with the coding sequence of the *E. coli lacZ* gene. Thus, the expression of β -galactosidase activity was dependent on transcription driven by the *CKII* promoter. Wild type cells bearing the $\text{P}_{\text{CKII}}\text{-lacZ}$ reporter gene were grown to the exponential phase in the absence or presence of increasing concentrations of zinc. Following growth, cell extracts were prepared and used for the assay of β -galactosidase activity. The depletion of zinc from the growth medium resulted in a concentration-dependent increase in *CKII* expression (Fig. 3). The β -galactosidase activity in zinc-depleted cells was 7-fold greater than the activity found in cells grown with 1.5 μM zinc. *CKII* expression was not further affected by 5 μM zinc.

To confirm that the zinc-mediated regulation of *CKII* expression was due to the intracellular level of zinc, $\text{P}_{\text{CKII}}\text{-lacZ}$ reporter gene activity was examined in a *zrt1 Δ zrt2 Δ* double mutant that was grown in the presence of zinc. This mutant lacks both the high (*Zrt1p*) and low (*Zrt2p*) affinity plasma membrane zinc transporters and contains a low cytoplasmic level of zinc even when the growth medium contains a standard amount of zinc (6, 7). The *zrt1 Δ zrt2 Δ* double mutant exhibited a high level of β -galactosidase activity that was similar to that shown for wild type cells grown without zinc (Fig. 4). Thus, the

⁴ It is technically difficult to quantify the labile pool of zinc (11).

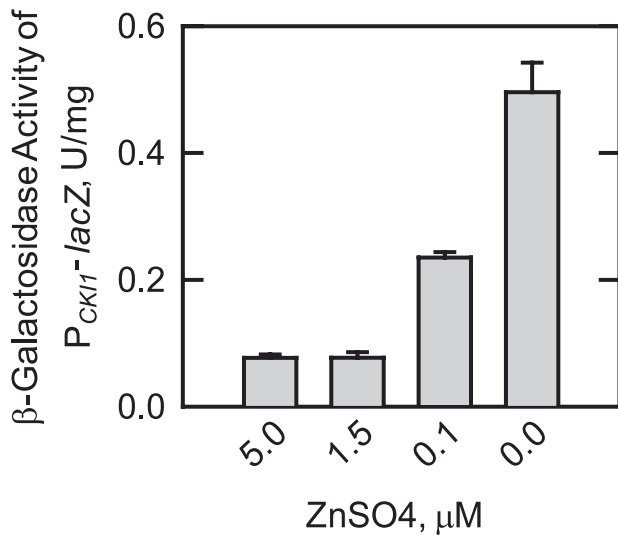


FIGURE 3. Effect of zinc depletion on the expression of β -galactosidase activity in wild-type cells bearing the P_{CK11} -lacZ reporter gene. Wild type cells bearing the P_{CK11} -lacZ reporter plasmid pKSK11 were grown to the exponential phase of growth in the presence of the indicated concentrations of $ZnSO_4$. Cell extracts were prepared and assayed for β -galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments \pm S.D.

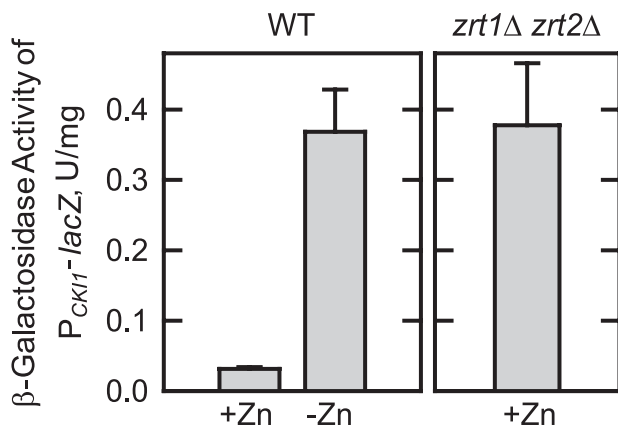


FIGURE 4. Effect of $zrt1\Delta$ $zrt2\Delta$ mutations on the expression of the P_{CK11} -lacZ reporter gene. Wild type (WT) and $zrt1\Delta$ $zrt2\Delta$ mutant cells bearing the P_{CK11} -lacZ reporter plasmid pKSK11 were grown to the exponential phase of growth in the absence and presence of $1.5 \mu M$ $ZnSO_4$. 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 \pm S.D.

induction of *CK11* expression was mediated by a low intracellular level of zinc.

Effect of the $zap1\Delta$ Mutation on the Zinc-mediated Regulation of *CK11* Expression—The *CK11* promoter contains putative UAS_{ZRE} sequences (see below) that are potential binding sites for the Zap1p transcription factor (17). Accordingly, we questioned whether the induction of *CK11* expression by zinc depletion was dependent on Zap1p function. To address this question, the P_{CK11} -lacZ reporter gene activity was examined in $zap1\Delta$ mutant cells that were grown in the presence and absence of zinc. The β -galactosidase activity of $zap1\Delta$ mutant cells grown with zinc was similar to the activity found in wild type cells grown with zinc (Fig. 5). However, when cells were grown without zinc, the β -galactosidase activity in the $zap1\Delta$ mutant was 4.4-fold lower than that found in the wild type

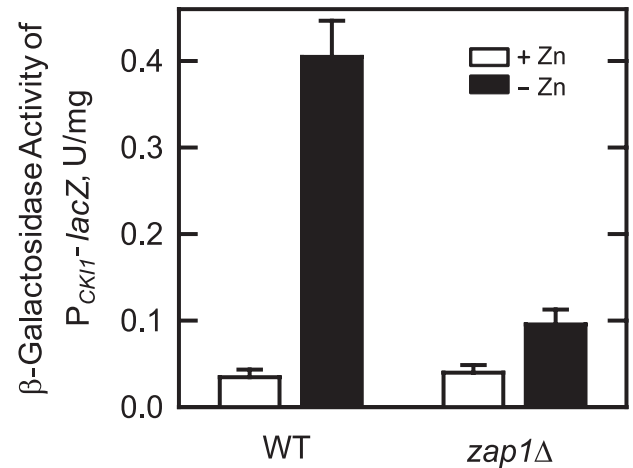


FIGURE 5. Effect of the $zap1\Delta$ mutation on the expression of the P_{CK11} -lacZ reporter gene in response to zinc depletion. Wild type (WT) and $zap1\Delta$ mutant cells bearing the P_{CK11} -lacZ reporter plasmid pKSK11 were grown to the exponential phase of growth in the absence and presence of $1.5 \mu M$ $ZnSO_4$. Cell extracts were prepared and assayed for β -galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments \pm S.D.

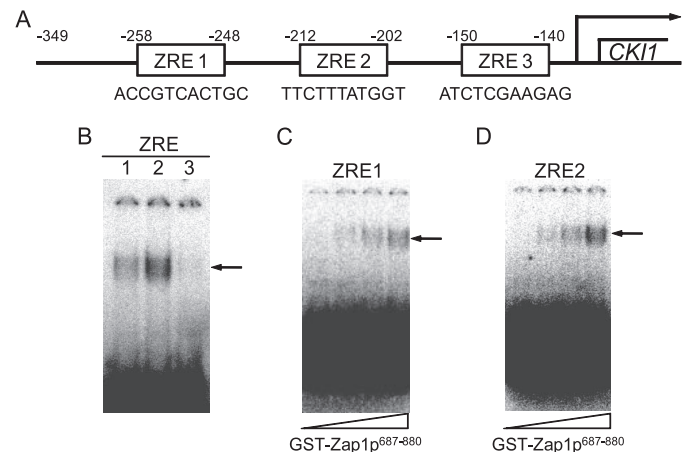


FIGURE 6. Interactions of GST-Zap1p⁶⁸⁷⁻⁸⁸⁰ with putative ZRE sequences in the *CK11* promoter. A, the locations and sequences of the putative ZRE sites in the *CK11* promoter are shown in the figure. B, samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotides (2.0×10^5 cpm/pmol) with sequences for ZRE1 (lane 1), ZRE2 (lane 2), and ZRE3 (lane 3) in the *CK11* promoter were incubated with $0.5 \mu g$ of purified recombinant GST-Zap1p⁶⁸⁷⁻⁸⁸⁰. The ZRE1 (C) and ZRE2 (D) radiolabeled probes were incubated with 0, 0.15, 0.3, and $0.5 \mu g$ of recombinant GST-Zap1p⁶⁸⁷⁻⁸⁸⁰. Interaction of GST-Zap1p⁶⁸⁷⁻⁸⁸⁰ 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. The arrow indicates the position of the GST-Zap1p⁶⁸⁷⁻⁸⁸⁰-ZRE complex.

control (Fig. 5). Thus, the $zap1\Delta$ mutation attenuated the induction of *CK11* expression when cells were depleted for zinc.

Binding of Zap1p to Putative UAS_{ZRE} Sequences in the *CK11* Promoter—There are three putative UAS_{ZRE} sequences in the *CK11* promoter for Zap1p binding (Fig. 6A). The percent identities of ZRE1, ZRE2, and ZRE3 to the consensus UAS_{ZRE} sequence (ACCTTNAAGGT) (17) are 64, 73, and 64%, respectively. We questioned whether Zap1p would bind to labeled oligonucleotides containing these sequences using the electrophoretic mobility shift assay. Purified recombinant GST-Zap1p⁶⁸⁷⁻⁸⁸⁰ (amino acids 687–880 of the Zap1p binding domain (59)) interacted with ZRE1 and ZRE2, but not with

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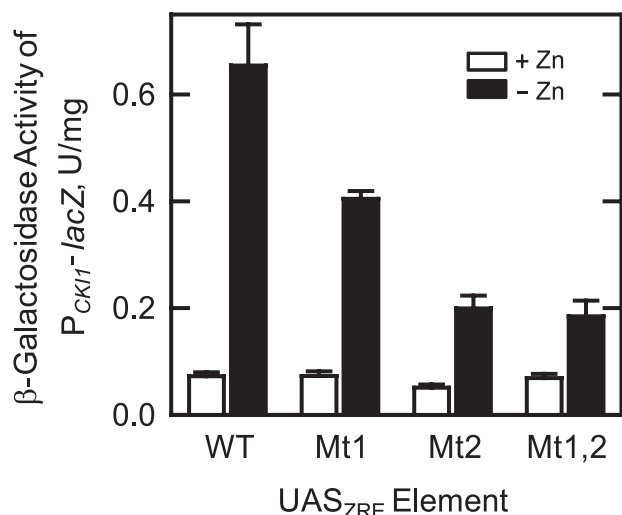


FIGURE 7. Effects of mutations in ZRE1 and ZRE2 in the *CKII* promoter on the expression of the P_{CKII} -lacZ reporter gene in response to zinc depletion. Wild type cells bearing the P_{CKII} -lacZ reporter plasmids pKSK11 (WT), pCK-ZRE1 (Mt1, mutation of ZRE1), pCK-ZRE2 (Mt2, mutation of ZRE2), and pCK-ZRE1,2 (Mt1,2, mutations of ZRE1 and ZRE2) were grown to the exponential phase of growth in the absence and presence of 1.5 μ M ZnSO₄. Cell extracts were prepared and assayed for β -galactosidase activity. Each data point represents the average of triplicate determinations from two independent experiments \pm S.D.

ZRE3 (Fig. 6B). The interaction of GST-Zap1p^{687–880} with ZRE2 was 2.5-fold greater when compared with ZRE1. The specificity of GST-Zap1p^{687–880} binding to ZRE1 and ZRE2 was examined further. The formation of the GST-Zap1p^{687–880}-ZRE1 and GST-Zap1p^{687–880}-ZRE2 complexes was dependent on the concentration of GST-Zap1p^{687–880} (Fig. 6, C and D, respectively). Moreover, unlabeled ZRE1 and ZRE2 probes competed with their labeled probe counterparts for GST-Zap1p^{687–880} interactions, and mutations in ZRE1 and ZRE2 to a nonconsensus UAS_{ZRE} sequence abolished interactions with GST-Zap1p^{687–880} (data not shown).

Effects of ZRE1 and ZRE2 Mutations on the Zinc-mediated Regulation of *CKII* Expression—We sought evidence that the induction of *CKII* in response to zinc depletion was dependent on the UAS_{ZRE} sequences in the *CKII* promoter. The ZRE1 and ZRE2 sequences within the P_{CKII} -lacZ reporter gene were mutated to the nonconsensus UAS_{ZRE} sequence 5'-AAAAAAAAAAAA-3'. Cells bearing the wild type or mutant P_{CKII} -lacZ reporter genes were grown in the presence and absence of zinc; cell extracts were prepared and assayed for β -galactosidase activity. The ZRE1 and ZRE2 mutations caused the attenuation of *CKII* induction in response to zinc depletion (Fig. 7). The β -galactosidase activity of zinc-depleted cells bearing the P_{CKII} -lacZ reporter gene with the ZRE1 and ZRE2 mutations was 1.6- and 3.2-fold lower, respectively, when compared with cells bearing the control reporter gene. These results supported the conclusion that the zinc-mediated regulation of *CKII* expression was dependent on ZRE1 and ZRE2. These results were consistent with the electrophoretic mobility shift assays that indicated that the regulation was primarily governed by ZRE2. We considered the possibility that the full extent of induction was dependent on interaction of Zap1p with ZRE1 and ZRE2 together. However, the effects of the ZRE1 and ZRE2 mutations on the zinc-mediated regulation of *CKII* expression

were not additive or synergistic. The β -galactosidase activity in zinc-depleted cells bearing the reporter gene with the ZRE1 and ZRE2 mutations made in combination was the same as that found in cells bearing the reporter gene with the ZRE2 mutation alone (Fig. 7).

Effects of *opi1* Δ , *ino2* Δ , and *ino4* Δ Mutations, and a UAS_{INO} Element Mutation in the *CKII* Promoter on the Zinc-mediated Regulation of *CKII* Expression—The *CKII* promoter contains a UAS_{INO} element (–189 to –197) that is four bases away from ZRE2. The UAS_{INO} element is a binding site for the transcriptional activator Ino2p-Ino4p complex (60) that is responsible for maximum expression of phospholipid synthesis UAS_{INO}-containing genes (36, 40, 58, 61). Expression of the UAS_{INO}-containing genes is balanced by the Opi1p repressor (62), which attenuates transcription through its interaction with Ino2p (36, 40, 58, 61, 63). Given the close proximity of ZRE2 with the UAS_{INO} element in the *CKII* promoter, we questioned whether the functions of Opi1p, Ino2p, or Ino4p affected the zinc-mediated regulation of *CKII* expression. As expected, the expression of the P_{CKII} -lacZ reporter gene was elevated (2.6-fold) in the *opi1* Δ mutant and reduced (5-fold) in the *ino2* Δ and *ino4* Δ mutants (Fig. 8A). Thus, Ino2p and Ino4p played a positive role in *CKII* expression and Opi1p played a negative role in expression. The *opi1* Δ mutation did not have a major effect on the induced expression of the P_{CKII} -lacZ reporter gene in response to zinc depletion (Fig. 8A), indicating that Opi1p did not play a role in the zinc-mediated regulation of *CKII*. However, the depletion of zinc from the growth medium of the *ino2* Δ and *ino4* Δ mutants did not result in the induction of the P_{CKII} -lacZ reporter gene (Fig. 8A). This indicated that Ino2p and Ino4p might play a role in the zinc-mediated regulation of *CKII*.

The inositol auxotrophic *ino2* Δ and *ino4* Δ mutants grew very slow in synthetic medium despite the supplementation of the medium with 75 μ M inositol. The depletion of zinc from this growth medium further reduced the growth of the mutants. We were concerned that the repressive effects of inositol supplementation on *CKII* expression (41) and the added stress of zinc depletion on the growth of the mutants may have affected the zinc-mediated regulation of *CKII* expression. Accordingly, we utilized another approach to examine whether the UAS_{INO} element played a role in the zinc-mediated regulation of *CKII*. The UAS_{INO} element within the P_{CKII} -lacZ reporter gene was mutated to a nonconsensus sequence (5'-TATTTTTTTT-3'), and the plasmid was expressed in wild type cells that were grown in the presence and absence of zinc. The β -galactosidase activity of cells bearing the mutant reporter gene was reduced (9.5-fold with zinc and 5-fold without zinc) when compared with the activity of cells bearing the wild type reporter gene (Fig. 8B). This result was consistent with the notion that the UAS_{INO} element was required for maximum expression of *CKII*. The depletion of zinc from the growth medium resulted in a 10-fold induction of β -galactosidase activity driven by the reporter gene with the UAS_{INO} mutation (Fig. 8B). These results supported the conclusion that the UAS_{INO} element was not involved with the zinc-mediated regulation of *CKII*.

Effect of Zinc Depletion on Choline Kinase Activity, and on the Incorporation of Choline into PC via the Kennedy Pathway—We sought evidence that the transcriptional regulation of *CKII*

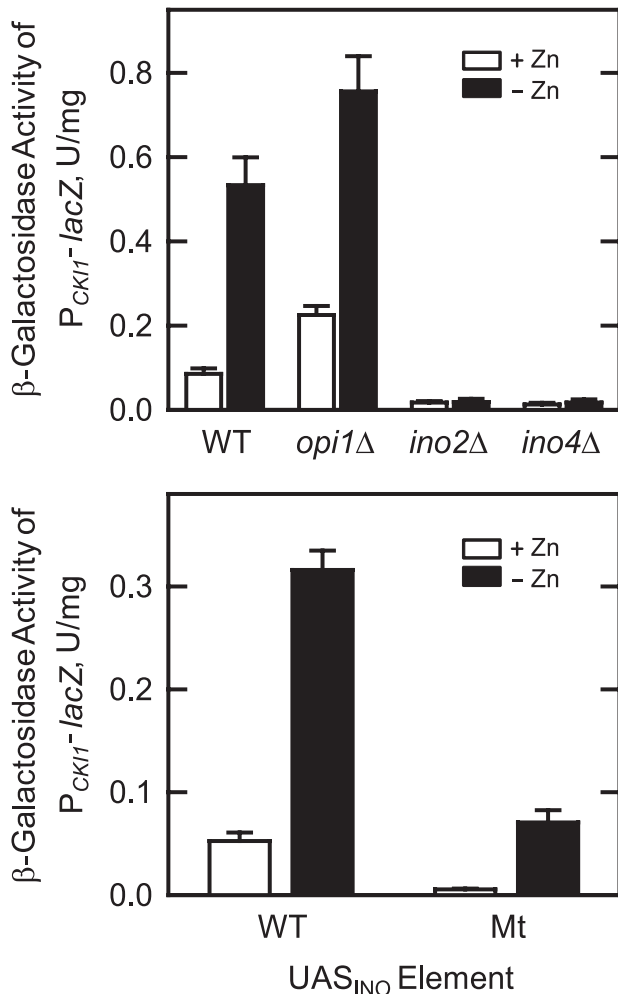


FIGURE 8. Effects of the *opi1* Δ , *ino2* Δ , and *ino4* Δ mutations, and a mutation in the UAS_{INO} element in the *CKI1* promoter on the expression of the P_{CKI1} -lacZ reporter gene in response to zinc depletion. Wild type (WT), *opi1* Δ , *ino2* Δ , and *ino4* Δ cells bearing the P_{CKI1} -lacZ reporter plasmid pKSK11 (A), and wild type cells bearing the P_{CKI1} -lacZ reporter plasmids pKSK11 (WT) and pCK-UASino (Mt, mutation of UAS_{INO}) (B) were grown to exponential phase of growth in the absence and presence of 1.5 μ M ZnSO₄. The growth medium for the *ino2* Δ and *ino4* Δ mutants was supplemented with 75 μ M inositol. 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 \pm S.D.

in response to zinc depletion translated into changes in choline kinase activity. Wild type cells were grown in the presence and absence of zinc, cell extracts were prepared, and choline kinase activity was measured by following the incorporation of [*methyl*-¹⁴C]choline into phosphocholine. The specific activity of choline kinase in zinc-depleted cells was 2.7-fold greater when compared with the activity from cells grown with zinc (Fig. 9). This level of induced choline kinase activity is not as great as that observed for the induction of P_{CKI1} -lacZ reporter gene activity (Fig. 5). This difference may be attributed to the stable nature of lacZ fusion proteins that are not subject to proteolytic turnover when expressed in yeast (64). We also examined the expression of choline kinase activity in *zap1* Δ mutant cells. This analysis showed that the induction of choline kinase activity that occurred in wild type cells in response to zinc depletion was precluded by the *zap1* Δ mutation (Fig. 9). These results

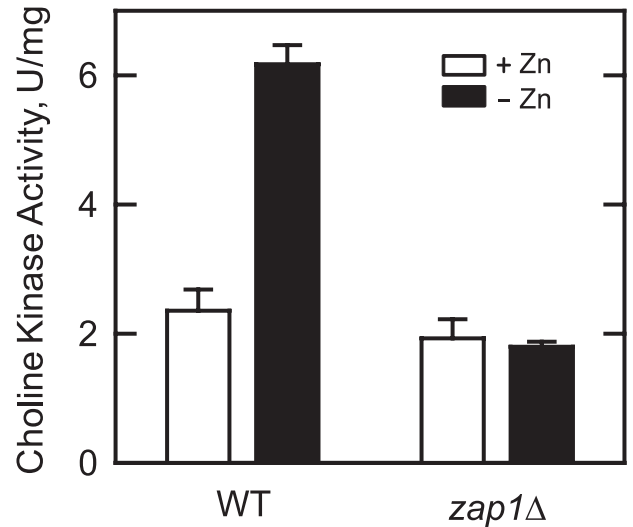


FIGURE 9. Effect of the *zap1* Δ mutation on choline kinase activity in response to zinc depletion. Wild type (WT) and *zap1* Δ mutant cells were grown to the exponential phase of growth in the absence and presence of 1.5 μ M ZnSO₄. Cell extracts were prepared and assayed for choline kinase activity. Each data point represents the average of triplicate determinations from two independent experiments \pm S.D.

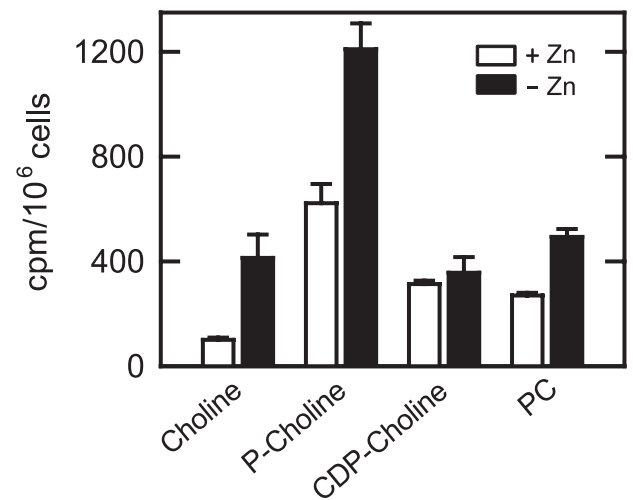


FIGURE 10. Effect of zinc depletion on the composition of the CDP-choline pathway intermediates and on PC. Wild type cells were grown to the exponential phase of growth in the absence or presence of 1.5 μ M ZnSO₄. The cells were labeled for five to six generations with [*methyl*-¹⁴C]choline (0.2 μ Ci/ml). The CDP-choline pathway intermediates and PC were extracted and analyzed by thin-layer chromatography. The values reported were the average of four separate experiments \pm S.D.

provided further evidence for the role of Zap1p in the regulation of *CKI1* expression in response to zinc.

We questioned whether the induction of choline kinase activity by zinc depletion was reflected *in vivo*. To address this question, zinc-depleted cells were labeled to steady-state with [*methyl*-¹⁴C]choline followed by the extraction and analysis of phosphocholine by TLC. The amount of label incorporated into phosphocholine in zinc-depleted cells was 2-fold greater when compared with cells grown in the presence of zinc (Fig. 10). The choline label was also incorporated into CDP-choline and the end product of the Kennedy pathway PC. Whereas zinc depletion did not affect the steady-state level of CDP-choline, it caused a 1.8-fold

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increase in the level of PC that was derived from CDP-choline (Fig. 10). That CDP-choline was not affected might be a reflection that it is a rate-limiting intermediate in the Kennedy pathway. Zinc depletion also caused a 4-fold increase in the cellular content of free choline (Fig. 10).

DISCUSSION

The synthesis of phospholipids is coordinately regulated with the expression of zinc transporters that control zinc homeostasis in *S. cerevisiae* (1). The depletion of zinc from the growth medium results in the induced expression of zinc transporters (e.g. Zrt1p, Zrt2p, Fet4p, and Zrt3p), and their transport functions increase the cytoplasmic levels of zinc (5, 15). At the same time, zinc depletion causes alterations in membrane phospholipid composition (e.g. an increase in PI and decreases in PE, phosphatidate, and diacylglycerol pyrophosphate) that are brought by changes in the expression of phospholipid synthesis enzyme activities (1, 37, 38, 65, 66). The PC content of cellular membranes is not altered by zinc depletion even though the enzyme activities responsible for its synthesis via the CDP-DAG pathway are repressed (37). In this study, we provided a mechanistic explanation for this observation. The decrease in PC synthesis via the CDP-DAG pathway was compensated by an increase in PC synthesis via the CDP-choline branch of the Kennedy pathway. Data indicated that the Zap1p-mediated induction of the *CKII*-encoded choline kinase played an important role in this regulation. This compensatory mechanism complements the Zap1p-mediated induction of the *EKII*-encoded ethanolamine kinase (Fig. 1) for increased PC synthesis in response to zinc depletion (65).

Analysis of P_{CKII} -*lacZ* reporter gene activity indicated that the expression of *CKII* was induced in response to zinc depletion, a growth condition that resulted in a limiting intracellular concentration of zinc (11, 18). That a limiting cellular zinc concentration was responsible for this regulation was confirmed by the induced expression of P_{CKII} -*lacZ* reporter gene activity in zinc-supplemented *zrt1Δ zrt2Δ* mutant cells that lack the major plasma membrane zinc transporters Zrt1p and Zrt2p (6, 7). The induction of P_{CKII} -*lacZ* reporter gene activity was precluded by the *zap1Δ* mutation. This indicated that *CKII* expression was mediated by Zap1p, a positive transcription factor that is induced in zinc-depleted cells and repressed in zinc-replete cells (18).

Zap1p interacts with a UAS_{ZRE} in the promoters of several genes to activate transcription when wild type cells are depleted for zinc. These include zinc transporter genes (e.g. *ZRT1*, *ZRT2*, *ZRT3*, and *FET4*) that control intracellular stores of zinc (5, 8, 12, 67, 68) and phospholipid synthesis genes (e.g. *DPPI*, *PIS1*, and *EKII*) that control membrane phospholipid composition (38, 47, 65). Electrophoretic mobility shift assays indicated that purified GST-Zap1p^{687–880} interacted with two of the three putative UAS_{ZRE} sequences (i.e. ZRE1 and ZRE2) within the *CKII* promoter. The *in vitro* interactions of GST-Zap1p^{687–880} with ZRE1 and ZRE2 were specific and could be abolished by mutations to a nonconsensus UAS_{ZRE} sequence. Moreover, mutations of ZRE1 and ZRE2 to a nonconsensus UAS_{ZRE} sequence attenuated the induced expression of the P_{CKII} -*lacZ* reporter gene activity when cells were depleted for zinc.

The Zap1p binding sites (i.e. ZRE1 and ZRE2) in the *CKII* promoter were not identical (64 and 73% identity, respectively) to the core consensus UAS_{ZRE} that is based on the promoters of *ZRT1*, *ZRT2*, and *ZAP1* (17). It is known that deviations from the core sequence reduce the interaction of Zap1p to the UAS_{ZRE} sequence (38, 65). This provides an explanation as to why the Zap1p-mediated induction of *CKII* was not as great as that observed for Zap1p-mediated induction of zinc transporter genes (e.g. *ZRT1*, *ZRT2*, and *ZRT3*) or for the phospholipid metabolism gene *DPPI* that contain UAS_{ZRE} sequences with high identity to the core sequence (6, 7, 11, 47). We speculate that differences in Zap1p binding efficiency based on the UAS_{ZRE} sequence provide a mechanism to control the relative induction of various genes in response to zinc depletion.

The UAS_{INO} element is found in several phospholipid synthesis genes in *S. cerevisiae* (35, 39, 40, 58, 61). It is the binding site for the transcriptional activator complex Ino2p-Ino4p that stimulates transcription of most UAS_{INO} -containing genes (36, 40, 58, 61).⁵ When exponential phase wild type cells are supplemented with inositol, or depleted for zinc, the repressor Opi1p interacts with Ino2p to attenuate transcription of UAS_{INO} -containing genes such as *CHO1* and *INO1* (1, 36). *CKII* contains a UAS_{INO} , and the work presented here using the P_{CKII} -*lacZ* reporter gene showed that this element was responsible for stimulating *CKII* expression. However, the UAS_{INO} element was not involved with the regulation of *CKII* expression in response to zinc depletion.

Choline kinase activity is the functional product of the *CKII* gene (55, 69). The Zap1p-mediated induction of *CKII* expression in response to zinc depletion translated into a 2.7-fold increase in choline kinase activity. This conclusion was confirmed by the loss of induced choline kinase activity in *zap1Δ* mutant cells. Moreover, the *in vitro* data showing the zinc-regulated induction of choline kinase activity was mirrored *in vivo*. The incorporation of labeled choline into phosphocholine was elevated by 2-fold in response to zinc depletion. In addition, the increase in *CKII* expression and choline kinase activity correlated with a 1.8-fold increase in the amount of PC that was synthesized from choline. Thus, the synthesis of PC via the Kennedy pathway was elevated in zinc-depleted cells, and choline kinase played an important role in this regulation. We also showed that zinc depletion caused an increase in the cellular choline content. This result might reflect an increase in choline transporter activity and/or an increase in the turnover of PC that was synthesized via the Kennedy pathway. Additional work will be required to address these hypotheses.

This work advances our understanding of the regulation of phospholipid synthesis in *S. cerevisiae* by zinc availability, and in particular, the transcriptional regulation of the *CKII*-encoded choline kinase. The importance of understanding choline kinase regulation is highlighted by the fact that the enzyme in mice is essential to embryonic development (α form (70)) and to normal muscular development (β form (71)). Moreover, unregulated levels of choline kinase play a role in the generation of human tumors by *ras* oncogenes

⁵ One exception is the *PIS1* gene, which contains a UAS_{INO} element but the element does not control *PIS1* expression (76).

(72–75). By catalyzing the committed step in the Kennedy pathway, choline kinase regulation governs PC content. Whether PC content *per se* or whether another membrane phospholipid(s) (e.g. PE and PI) regulates zinc transporter function to control zinc homeostasis are important questions that warrant further investigation.

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