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Repression of Choline Kinase by Inositol and Choline in *Saccharomyces cerevisiae*

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The regulation of choline kinase (EC 2.7.1.32), the initial enzyme in the CDP-choline pathway, was examined in *Saccharomyces cerevisiae*. The addition of *myo*-inositol to a culture of wild-type cells resulted in a significant decrease in choline kinase activity. Additional supplementation of choline caused a further reduction in the activity. The coding frame of the choline kinase gene, *CKI*, was joined to the carboxyl terminus of *lacZ* and expressed in *Escherichia coli* as a fusion protein, which was then used to prepare an anti-choline kinase antibody. Upon Western (immuno-) and Northern (RNA) blot analyses using the antibody and a *CKI* probe, respectively, the decrease in the enzyme activity was found to be correlated with decreases in the enzyme amount and mRNA abundance. The molecular mass of the enzyme was estimated to be 66 kilodaltons, in agreement with the value predicted previously from the nucleotide sequence of the gene. The coding region of *CKI* was replaced with that of *lacZ*, and *CKI* expression was measured by assaying β-galactosidase. The expression of β-galactosidase from this fusion was repressed by *myo*-inositol and choline and derepressed in a time-dependent manner upon their removal. The present findings indicate that yeast choline kinase is regulated by *myo*-inositol and choline at the level of mRNA abundance.

The cooperation of *myo*-inositol (inositol) and choline in the control of phospholipid synthesis in the yeast *Saccharomyces cerevisiae* was first demonstrated for the enzymes in the phosphatidylethanolamine methylation pathway (38). Thereafter, a number of enzymes involved in the synthesis of phospholipids were shown to be subject to regulatory changes in response to inositol and choline. These changes include inhibition of phosphatidylserine synthase (20), induction of phosphatidate phosphatase (28), and inactivation or degradation of phosphatidylglycerophosphate synthase (10), but a more general response is the coordinate repression of phospholipid-synthesizing enzymes, such as CDP-diacylglycerol synthase (13, 14), phosphatidylserine synthase (1, 5, 13, 22, 31), phosphatidylserine decarboxylase (6), phosphatidylethanolamine methyltransferase (22, 35–37), phospholipid methyltransferase (38), and inositol-1-phosphate synthase (7, 8, 12). The activities of these enzymes are considerably reduced when cells are cultured in the presence of inositol alone, but maximum reductions occur in the presence of both inositol and choline. The decreases in the activities have been correlated with decreases in the enzyme amounts in the cases of CDP-diacylglycerol synthase (13), phosphatidylserine synthase (13, 31), and inositol-1-phosphate synthase (8). Furthermore, in the case of phosphatidylserine synthase (1), inositol-1-phosphate synthase (12), phosphatidylethanolamine methyltransferase, and phospholipid methyltransferase (23; T. Kodaki, unpublished results), it has been shown that decreases in mRNA abundance are associated with changes in enzyme activities and amounts.

In the present study, we investigated the effects of inositol and choline on the CDP-choline pathway, whose regulation had not previously been extensively studied for *S. cerevisiae*. We show here that the addition of inositol and choline to the growth medium caused repression of the initial enzyme, choline kinase. The other two enzymes, cholinephosphate cytidylyltransferase and cholinephosphotransferase, were not significantly affected. Using the choline kinase gene, *CKI* (16), we prepared a β-galactosidase–choline kinase fusion protein and raised an anti-choline kinase antibody. Western (immuno-) blot analysis demonstrated reduction of the enzyme amount occurring in response to inositol and choline. This reduction was correlated with a decrease in mRNA abundance by Northern (RNA) blot analysis. The present report is the first demonstration of the control of the CDP-choline pathway in yeast and shows that choline kinase is coordinately regulated with other key enzymes in phospholipid synthesis.

MATERIALS AND METHODS

Yeast strains and culture. Wild-type strains X2180-1A (**a** *SUC2 mal mel gal2 CUP1*) and X2180-1B (α *SUC2 mal mel gal2 CUP1*) were provided by the Yeast Genetic Stock Center (University of California). AH22 (**a** *leu2-3 leu2-112 his4-519 can1*) and C5014-2B (**a** *ura3 arg9 hom3 his1*) were obtained from N. Gunge (Kumamoto Institute for Technology). D302-2 (α *leu2-3 leu2-112 his4-519 can1*) and D302-3C (**a** *leu2-3 leu2-112 his4-519 can1*) were derived from a cross of AH22 with X2180-1B. D395-2 (α *leu2-3 leu2-112*) was derived from a cross of D302-2 with X2180-1A. Strain 2014 (**a** *leu2-3 leu2-112 his4-519 can1 cki*::*LEU2*) was constructed previously (16) and used as the choline kinase gene disruptant. D448-2 (α *ura3 can1*) was derived from a cross of C5014-2B with D395-2. D451-3 (**a** *leu2-3 leu2-114 ura3 can1*) was constructed by crossing D448-2 with D302-3C. The composition of inositol-depleted minimal medium (M-i) was as described previously (37). Inositol-supplemented minimal medium (M2i) was prepared by supplementing M-i medium with 2 μg of inositol per ml. Yeast cells were grown aerobically at 30°C. Cell growth was monitored by measuring the A_{550} in a test tube (diameter, 18 mm) with a Hitachi 101 spectrophotometer.

Preparation of yeast cell extracts and fractionation. Yeast cells were grown in 250 ml of M-i medium, supplemented as

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indicated, and harvested at the mid-logarithmic phase by centrifugation at 7,600 × *g* for 10 min. The cells were washed sequentially with water and 50 mM Tris hydrochloride buffer (pH 7.5) containing 1 mM EDTA and 20% (vol/vol) glycerol (GTV buffer) and then suspended in 1 to 2 ml of the same buffer. The cells were disrupted by vortexing them vigorously with 2.5 g of glass beads (diameter, 0.3 mm) for 30 s five times at 4°C and then centrifuging them at 4,000 × *g* for 10 min. The cell extracts thus obtained were further centrifuged at 100,000 × *g* for 60 min to obtain the cytosolic and membrane fractions. The membrane fraction was suspended in 0.2 to 0.5 ml of GTV buffer.

Enzyme assays. Cell extracts, the cytosolic fraction, and the membrane fraction were used for assaying cholinephosphate cytidylyltransferase (EC 2.7.7.15), choline kinase, and cholinephosphotransferase (EC 2.7.8.2), respectively. The assays were performed at 30°C as described previously (16, 17, 30). β-Galactosidase was assayed at 30°C by measuring the increase in A_{420} with *o*-nitrophenyl-D-galactoside as the substrate after the cells had been permeabilized by treatment with chloroform and sodium dodecyl sulfate, as described by Miller (27). Units were calculated by the formula $1{,}000 \times [\Delta A_{420}/(tv \times A_{550})]$, where t = reaction time in minutes, $\Delta A_{420} = A_{420}$ at time t with cells $- A_{420}$ at time t without cells, v = the volume (in milliliters) of culture used for the assay, and A_{550} was measured in an 18-mm test tube.

Construction of choline kinase-overproducing plasmid pCK1D$_2$. pCK1D is a 2μm DNA-based plasmid carrying one copy of the yeast *CKI* gene (16), from which a 2.7-kilobase-pair (kb) *Pst*I-*Hin*dIII fragment containing *CKI* was excised, end repaired with the Klenow fragment, and then ligated into the *Sma*I site of another pCK1D. The obtained plasmid, pCK1D$_2$, containing two tandem copies of *CKI* with the same orientation, was transformed into D395-2 yeast cells.

Partial purification of yeast choline kinase. Unless otherwise stated, all procedures were carried out at 0 to 4°C. Yeast strain D395-2 harboring pCK1D$_2$ was cultured in M2i medium at 30°C to the early stationary phase, harvested by centrifugation at 7,600 × *g* for 10 min, washed with distilled water, and then frozen at −20°C until used. The cells (25 g [wet weight]) were suspended in 80 ml of GTV buffer containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (GTV-PMSF buffer). The cells were disrupted by shaking them with glass beads (diameter, 0.3 mm) in a Vibrogen cell mill (Edmund Buhler, Tübingen, Federal Republic of Germany) followed by centrifugation at 10,000 × *g* for 30 min. The supernatant (80 ml) was applied to a DEAE-Sepharose column (2.5 by 10 cm) which had been equilibrated with GTV-PMSF buffer. The column was washed with one bed volume of the same buffer and then eluted with a 300-ml linear gradient of NaCl (0 to 0.5 M) in the same buffer. The enzyme was eluted between 0.1 and 0.2 M NaCl, and precipitated by 70% saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min, dissolved in GTV-PMSF buffer, dialyzed against 2 liters of the same buffer overnight, and then loaded onto a heparin-Sepharose column (1.8 by 9 cm) which had been equilibrated with GTV-PMSF buffer. The column was washed with one bed volume of the same buffer and then eluted with an 80-ml linear gradient of NaCl (0 to 0.7 M) in the same buffer. The enzyme was eluted from the column between 0.1 and 0.3 M NaCl and was precipitated by 70% saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min, dissolved in GTV-PMSF buffer, dialyzed against 1 liter of the same buffer overnight, and then adsorbed to a hydroxyapatite column (1.8 by 4.5 cm) which had been equilibrated with GTV-PMSF buffer. The column was washed with 3 bed volumes of 10 mM potassium phosphate buffer (pH 7.4) containing 0.2 mM PMSF and 20% (vol/vol) glycerol. The enzyme was eluted with an 80-ml linear gradient of potassium phosphate buffer (10 to 400 mM, pH 7.4) containing 0.2 mM PMSF and 20% (vol/vol) glycerol. The enzyme was eluted as a single peak between 50 and 150 mM potassium phosphate. The overall purification was 19-fold, the yield being 16%. The final specific activity was 5.8 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, which was about 600-fold higher than that of the 100,000 × *g* supernatant of wild-type strain X2180-1B.

Preparation of a β-galactosidase–choline kinase fusion protein and immunization of a rabbit. Plasmid pGK1, a fusion gene encoding amino acid residues 153 to 582 of choline kinase preceded by *E. coli* β-galactosidase, was constructed by cloning the 1.9-kb *Bgl*II-*Hin*dIII fragment of pCK1D (16) into pUR289 (33) which had been treated with *Bam*HI and *Hin*dIII. *E. coli* JM103 transformed with pGK1 was grown to an A_{550} of 0.5 in Luria broth (2) at 37°C and then treated with 0.75 mM isopropyl-D-thiogalactoside for 2 h to facilitate the production of the *lacZ-CKI* fusion protein. The fusion protein was extracted from the *E. coli* cells by the method of Germino et al. (9) and then separated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A portion of the gel was then cut out and stained with Coomassie brilliant blue to locate the 170-kilodalton (kDa) fusion protein. The fusion protein was electrophoretically eluted from the rest of the gel and dialyzed against 10 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl. A rabbit was immunized with 0.1 mg of the protein emulsified with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). The rabbit was boosted every 3 weeks three times with 50 μg of the fusion protein in Freund incomplete adjuvant each time.

Affinity purification of an anti-choline kinase antibody. Unless otherwise stated, all incubations were carried out at room temperature on a TAITEC R-30 mini rotary shaker (Taiyo Scientific, Tokyo, Japan). A nitrocellulose membrane (2 by 6 cm) was soaked in 3 ml of 75 mM potassium phosphate buffer (pH 7.4) containing 2.5 mg of the choline kinase obtained above and then incubated for 4 h. The membrane was transferred to 10 ml of 20 mM Tris hydrochloride buffer (pH 7.5)–0.15 M NaCl (TBS) containing 10% (wt/vol) dried milk (Yukijirushi, Sapporo, Japan) for 1 h and then rinsed with four changes of 10 ml of TBS for a total period of 20 min. The treated membrane was cut into 12 pieces (0.5 by 2 cm each), which were kept wet with TBS at 4°C until used. Four pieces were placed in 5 ml of 10-fold-diluted anti-choline kinase serum in TBS, incubated for 1 h, and then washed four times with TBS, from which the choline kinase antibody was eluted with 0.8 ml of 1 M propionic acid at 4°C. The eluate was immediately neutralized with 0.4 ml of 2 M Tris containing 3% (wt/vol) bovine serum albumin, dialyzed against 1 liter of TBS at 4°C for 3 h, and used immediately.

Immunoblotting. Cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel by the method of Laemmli (24). The protein bands were transferred to a nitrocellulose membrane (Hybond-C) with a Semi Dry Electroblotter (Sartorius, Göttingen, Federal Republic of Germany). The membrane was treated with 10% (wt/vol) dried milk in TBS to block nonspecific binding at 4°C overnight and then with the affinity-purified anti-choline kinase antibody diluted 1 to 2 for 1 h, followed by extensive rinsing with TBS and treatment for 1 h with the protein

A-peroxidase complex (Zymed Laboratories, San Francisco, Calif.) diluted 1 to 2,500. Immunoreactive proteins were located by staining with an Immunostaining HRP Kit IS-50B (Konica, Tokyo, Japan).

Northern blot analysis. Poly(A)$^+$ RNA was isolated as described by Kataoka et al. (19) from wild-type yeast X2180-1B cells cultured under the indicated conditions, separated by formaldehyde-agarose gel electrophoresis (26), and then transferred to a Biodyne A membrane (Nihon Pole, Tokyo, Japan). Hybridization was performed with multiprime-labeled probes according to the instruction manual of the manufacturer. The membrane was washed, dried, and then autoradiographed by using Fuji RX X-ray film.

Construction of *CKI-lacZ* fusion gene by replacing the coding region of *CKI* with that of *lacZ*. A yeast promoter detection vector, YEplacZ2, was constructed by ligating the 3.2-kb *Sal*I fragment of pMC1871 (34) containing a promoterless *lacZ* into the *Sal*I site of the YEpM7 yeast-*E. coli* shuttle vector. YEpM7, an analog of YEpM4 (29), contained the *E. coli* ampicillin resistance gene, the multicloning site of pUC18 (39), the yeast *LEU2* gene (32), and the 2μm replication origin (11) obtained from YEp13 (3), for a total nucleotide length of 5.65 kb. YEplacZ2 was digested with *Hin*dIII and then end repaired with the Klenow fragment. Into this gap was inserted the 0.4-kb *Hin*cII fragment of pUK1 (16) containing the 5′ flanking region as well as a short N-terminal sequence of *CKI*. Plasmid pCKZ1 thus obtained was transformed into D451-3 and used for studying *CKI* expression.

Materials. [α-^{32}P]CTP (400 Ci/mol), CDP-[*methyl*-^{14}C]choline (58 Ci/mol), a multiprime DNA labeling kit, and Hybond-C were purchased from Amersham International (Buckinghamshire, England). [*methyl*-^{14}C[choline chloride (52.0 Ci/mol) and phosphoryl[*methyl*-^{14}C]choline (55 Ci/mol) were products of Du Pont, NEN Research Products, Boston, Mass. T4 ligase and restriction endonucleases were obtained from Takara Shuzo (Kyoto, Japan). DEAE-Sepharose and hydroxyapatite (Hypatite C) were obtained from Pharmacia (Uppsala, Sweden) and Clarkson (Williamsport, Pa.), respectively. Heat-killed, Formalin-fixed *Staphylococcus aureus* cells (Staphylosorb) were purchased from Wako Pure Chemical (Osaka, Japan). Heparin-Sepharose was prepared by the method of Iverius (18).

RESULTS

Effects of inositol and choline on the activity level of choline kinase. In the course of enzymic studies, we noticed a considerable difference in choline kinase activity between cells grown in rich medium and those grown in minimal medium. Investigations in our laboratory (37, 38) and others (1, 5–7, 8, 12, 13, 22, 31, 35, 36) showed that in *S. cerevisiae* a number of enzymes involved in phospholipid synthesis are regulated in a coordinated manner in response to inositol and choline supplementation. Although our previous study suggested that yeast choline kinase is constitutive (37), we decided to reinvestigate the possibility that choline kinase is also under the control of inositol and choline.

The wild-type yeast was grown in M-i medium supplemented with different concentrations of inositol and choline and harvested at the mid-logarithmic phase, and then choline kinase activity was determined. The addition of inositol and choline to the culture medium significantly affected choline kinase (Table 1). The addition of inositol alone at final concentrations of 2 and 20 μg/ml caused 45 and 58% reductions in the activity, respectively, whereas choline alone at 20 μg/ml produced only a 14% reduction. The addition of a combination of inositol (20 μg/ml) and choline (20 μg/ml) resulted in a marked reduction in choline kinase activity (77%). Hence, inositol played a major role in the reduction in choline kinase activity, but choline decreased the activity further.

TABLE 1. Effects of inositol and choline on the choline kinase level in wild-type strain X2180-1B[a]

Addition (μg/ml)		Choline kinase activity (nmol · min^{-1} · mg^{-1})	% Decrease
Inositol	Choline		
0	0	22.0	0
2	0	12.1	45
20	0	9.2	58
0	20	18.9	14
2	20	8.4	62
20	20	5.1	77

[a] Cells were inoculated into 250 ml of M-i medium supplemented with the indicated concentrations of inositol and choline at an A_{550} of 0.01 and harvested at the mid-logarithmic phase. Choline kinase activity was determined as described in Materials and Methods.

Another notable finding was that the enzyme level varied with the growth phase of cultures, as has been shown for some other regulated phospholipid-synthesizing enzymes (15). The specific activity of choline kinase increased during the exponential phase, reached a maximum when the A_{550} of a culture was about 3 (for the absorbances of cultures, see Materials and Methods), and then decreased gradually. Furthermore, the response to inositol and choline was more significant in the exponential phase than it was in the stationary phase. The enzyme became less responsive when the A_{550} of a culture exceeded 2. This accounts for our failure to detect significant changes in choline kinase activity in response to inositol and choline supplementation in our previous study (37), in which we used stationary-phase cells.

The addition of 0.5 and 5 mM inositol to the assay mixture had virtually no effect on the partially purified enzyme, the activities being 102 and 103% of the control value, respectively. A 1:1 mixture of the extracts prepared from cells cultured in the presence and absence of inositol plus choline an average specific activity of choline kinase. Hence, the reduction in choline kinase activity observed here was not due to a direct effect of inositol on the enzyme, nor was it due to the production of an inhibitor in the supplemented cells.

We also examined the effects of inositol and choline on the other enzymes in the CDP-choline pathway. Wild-type cells were grown to the mid-logarithmic phase in the presence or absence of inositol and choline, and then the responses of cholinephosphate cytidylyltransferase and cholinephosphotransferase were compared with that of choline kinase. The activities of cholinephosphate cytidylyltransferase and cholinephosphotransferase were affected to much lesser extents by inositol and choline (Table 2).

Preparation of an anti-choline kinase antibody. We next tried to determine whether the observed changes in the choline kinase activity were associated with changes in the enzyme amount. As yeast choline kinase has not yet been purified to homogeneity, we used the previously cloned choline kinase gene, *CKI*, for the preparation of an anti-choline kinase antibody. To this end, we constructed a *lacZ-CKI* fusion gene, as shown diagrammatically in Fig. 1. A 1.9-kb *Bgl*II-*Hin*dIII fragment containing the N-terminus-truncated *CKI* gene was excised from pCK1D (16) and cloned into *Bam*HI-*Hin*dIII-treated pUR289 (33) for fusion

TABLE 2. Effects of inositol and choline on the activities of the CDP-choline pathway enzymes in wild-type strain X2180-1B

Supplement[a]	Activity (nmol · min^{-1} · mg^{-1}) of[b]:		
	Choline kinase	Cholinephosphate cytidylyltransferase	Cholinephospho-transferase
None	8.8	4.0	6.8
Inositol	5.6	5.3	6.7
Choline	7.6	3.2	7.4
Inositol + choline	3.8	3.8	5.3

[a] Added to M-i medium at the concentration of 20 μg/ml.
[b] Cells were harvested at the mid-logarithmic phase, and then choline kinase, cholinephosphate cytidylyltransferase, and cholinephosphotransferase activities were determined as described in Materials and Methods.

to the *lacZ* gene. The resulting hybrid gene encoded a 1,453-amino acid protein comprising residues 153 to 582 of yeast choline kinase joined to β-galactosidase at its carboxyl-terminal Gly (originally Lys, but changed to Gly through the above construction). This plasmid, pGK1, was introduced into *E. coli* JM103. The transformant was grown and then treated with isopropyl-D-thiogalactoside to induce the production of the 170-kDa β-galactosidase-choline kinase fusion protein. The protein was extracted from the cells with 4 M urea by the method of Germino et al. (9), purified by polyacrylamide gel electrophoresis, and then used to immunize a rabbit. The immune serum thus obtained neutralized choline kinase in yeast cell extracts (Fig. 2). The

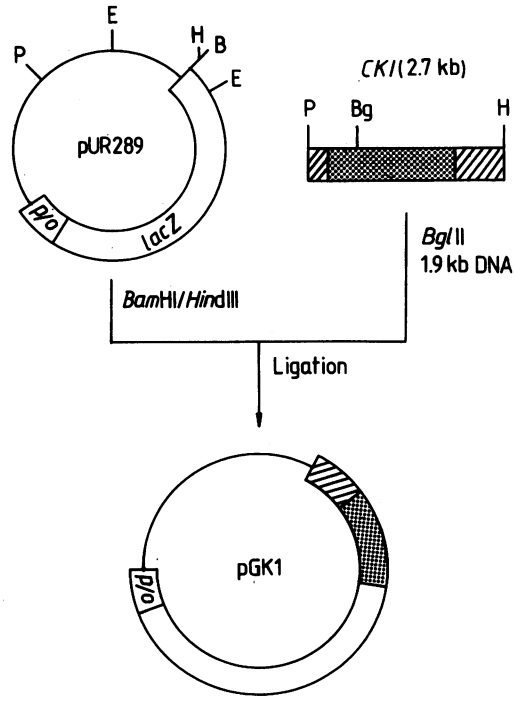

FIG. 1. Construction of a *lacZ-CKI* fusion gene for preparing an anti-choline kinase antibody. The plasmid construction is described in Materials and Methods. The *E. coli lacZ* gene (▭) is indicated; *p* and *o* denote its promoter and operator, respectively. The *CKI* coding region (▩) and its flanking region (▨) are indicated. The sizes of pUR289 and pGK1 are 5.2 and 7.1 kb, respectively. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I.

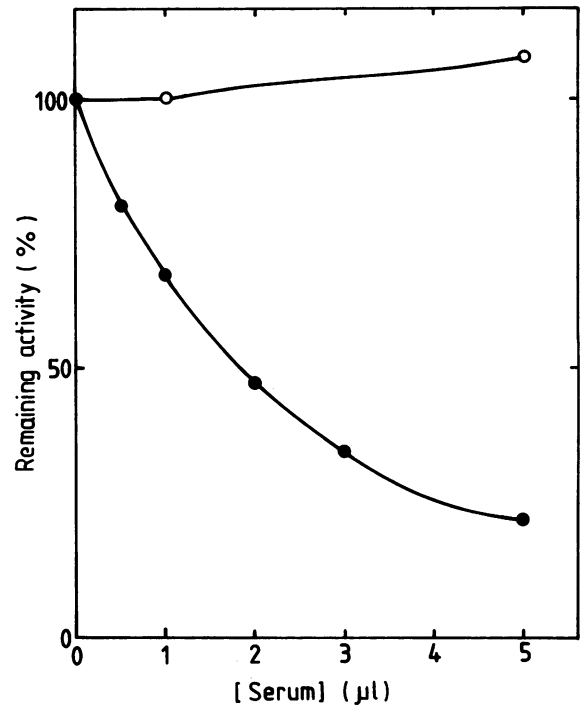

FIG. 2. Immunochemical titration of yeast choline kinase. The 100,000 × *g* supernatant (100 μg of protein; specific activity, 12 nmol · min^{-1} · mg^{-1}) from wild-type strain X2180-1B grown in M-i medium was incubated at 0°C for 60 min with increasing amounts of either immune serum (●) or preimmune serum (○) in 20 mM Tris hydrochloride buffer (pH 7.5) containing 0.15 M NaCl in a final volume of 30 μl. Twenty microliters of cold 10% (wt/vol) Staphylosorb in TBS was then added, and incubation was continued for a further 30 min in an ice bath. The mixture was then centrifuged at 10,000 × *g* for 3 min, and choline kinase activity remaining in the supernatant was determined as described in Materials and Methods.

100,000 × *g* supernatant from wild-type cells grown in the absence of inositol and choline was incubated with the immune serum and then treated with staphylococcal protein A. The mixture was centrifuged, and then the choline kinase remaining in the supernatant was assayed. The enzyme was progressively removed from the solution with increasing amounts of antibody. In contrast, the control serum did not precipitate the enzyme at all. The choline kinase antibody thus obtained was affinity purified by adsorption of the immune serum onto partially purified choline kinase on a nitrocellulose membrane, followed by elution with 1 M propionic acid.

Immunochemical detection of choline kinase in yeast cell extracts. In order to identify the choline kinase protein, we conducted an immunoblot analysis. Three yeast strains (a *cki* mutant, the wild type, and a choline kinase overproducer) were cultured, and then their 4,000 × *g* supernatants were prepared. To minimize protein degradation, cells were disrupted in the presence of protease inhibitors, PMSF and benzamidine, and the extracts were mixed with Laemmli sample buffer (24) and immediately heated at 100°C for 3 min. The samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gel was blotted onto a nitrocellulose membrane. The blot was treated with the affinity-purified anti-choline kinase antibody, and then immunoreactive bands were visualized by using the protein A-peroxidase system. Several immunoreactive

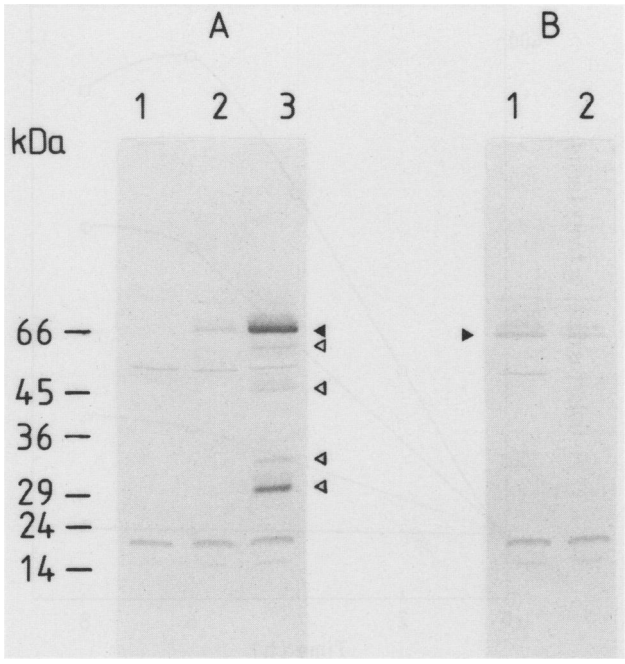

FIG. 3. Immunoblot analysis of choline kinase in a gene disruptant, the wild type, and an overproducer (A) and the effect of inositol and choline on the amount of choline kinase (B). (A) Gene disruptant 2014 (lane 1), wild-type X2180-1B (lane 2), and overproducer D302-3C(pCK1D) (lane 3) were grown in M-i medium. (B) The wild type was grown in the absence (lane 1) and presence (lane 2) of 20 μg each of inositol and choline per ml. The cells were harvested at an A_{550} of 0.5 to 0.6, washed with distilled water, and then disrupted in GTV-PMSF buffer containing 10 mM benzamidine hydrochloride by shaking with glass beads and centrifuged at 4,000 × *g* for 10 min. The supernatant was mixed with Laemmli sample buffer (24) and then immediately heated in a boiling water bath for 3 min. Fifty-three micrograms of each sample was subjected to immunoblot analysis as described in Materials and Methods. The choline kinase activities in the yeast cells in panel A were <0.1 (lane 1), 8.9 (lane 2), and 185 nmol · min^{-1} · mg^{-1} (lane 3), respectively. Those in panel B were 8.9 (lane 1) and 2.3 nmol · min^{-1} · mg^{-1} (lane 2), respectively. The numbers to the left of the gel in panel A denote the positions of the following protein standards: bovine serum albumin, ovalbumin, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, bovine erythrocyte carbonic anhydrase, trypsinogen, and α-lactoalbumin. Symbols: ▲, location of the enzyme; △, bands thought to be the hydrolytic products.

bands were detected (Fig. 3A). The following observations led us to conclude that the 66-kDa band was choline kinase. First, this band was not observed for the extract from the *cki* null mutant (lane 1) but was detectable for the wild-type extract (lane 2). Second, this protein was present in a large amount in the choline kinase overproducer, which carried multiple copies of the *CKI* gene (lane 3). Its molecular weight was very close to that predicted from the nucleotide sequence of *CKI* (66,316) (16) as well as to that reported by Brostrom and Browning (4) for their partially purified enzyme preparation (67 kDa). Several minor bands detected on the blot (Fig. 3, △) were thought to be due to proteolytic products of the enzyme.

Decrease in enzyme amount in response to inositol and choline. To examine the effect of inositol and choline on the enzyme amount, we performed similar immunoblot analyses on extracts obtained from cells grown in the presence or absence of inositol and choline. The unsupplemented cells

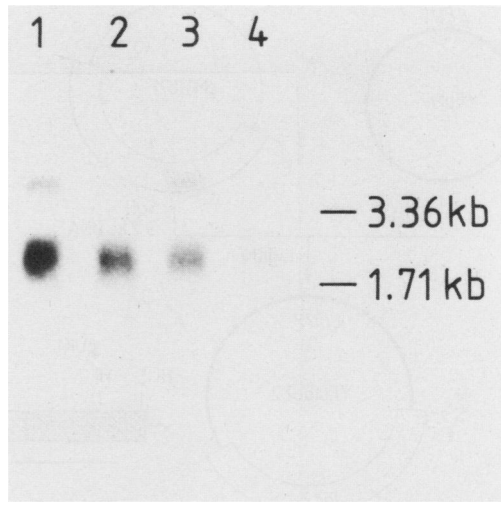

FIG. 4. Northern blot analysis of the *CKI* transcript in wild-type cells cultured in the presence of 20 μg each of choline and inositol per ml. Two micrograms of poly(A)$^+$ RNA from wild-type cells (X2180-1B) grown in M-i medium with no supplement (lane 1), choline (lane 2), inositol (lane 3), or both choline and inositol (lane 4) was electrophoresed on a formaldehyde-containing 1% agarose gel and subsequently transferred to a Biodyne A membrane. The blot was probed with the ^{32}P-labeled 0.8-kb *Mva*I-*Bam*HI fragment of *CKI*.

(lane 1) contained severalfold more 66-kDa protein than the supplemented cells (Fig. 3B, lane 2). Thus, the above-mentioned reduction in choline kinase activity caused by inositol and choline was correlated with a decrease in the enzyme amount.

Effect of inositol and choline on choline kinase mRNA abundance. The abundance of choline kinase mRNA was analyzed by Northern blot analysis with a DNA probe prepared from the *CKI* coding region. The results of the analysis of poly(A)$^+$ RNA isolated from wild-type cells grown with different supplementation are shown in Fig. 4. An intense 1.9-kilobase band was observed for the sample from cells grown in the absence of inositol and choline (lane 1). We concluded that this RNA band represented the *CKI* transcript for the following reasons. First, the size was fairly consistent with the molecular weight of choline kinase as determined above. Second, this 1.9-kilobase band was not observed for the *CKI* disruptant (strain 2014) obtained by partial substitution of its coding region with *LEU2*. Instead, a very faint 1.2-kilobase band, presumably a transcript from the *LEU2*-disrupted *CKI* gene, was detected (data not shown).

The *CKI* transcript thus identified was most abundant in cells cultured in the absence of inositol and choline (lane 1) but was least abundant in cells cultured in the presence of both (lane 4). Cells grown in the presence of either inositol or choline contained this RNA species at an intermediate level (lanes 2 and 3). However, in agreement with the data for the activity and amount of the enzyme, inositol had a greater effect than choline on the mRNA level. Thus, qualitatively, the change in the enzyme activity was correlated with a change in the mRNA abundance. The additional 3.6-kilobase band (lanes 1 and 3), which hybridized very weakly with the *CKI* probe used here, may be a transcript from a different but related gene. The abundance of this RNA appeared to vary in response to choline supplementation. However, further analysis was not performed.

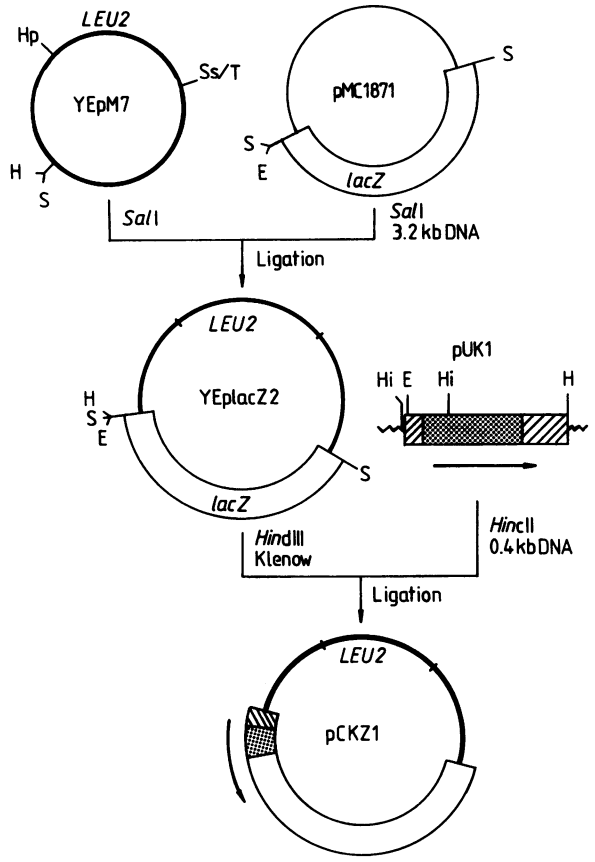

FIG. 5. Construction of a *CKI-lacZ* fusion gene by replacing the coding region of *CKI* with that of *lacZ*. The details of the procedure are given in Materials and Methods. The *E. coli lacZ* gene (□), *CKI* coding region (▒), and *CKI* flanking region (▨) are indicated. The thick line represents the YEpM7 sequence, the wavy line represents the pUC19 sequence, and the arrow shows the direction of transcription. The sizes of YEpM7, pMC1871, YEplacZ2, and pCKZ1 are 5.7, 7.5, 8.9, and 9.3 kb, respectively. Restriction enzyme sites: E, *Eco*RI; H, *Hin*dIII; Hi, *Hin*cII; Hp, *Hpa*I; S, *Sal*I; Ss, *Ssp*I; T, *Tth*111I.

Analysis of the expression of *CKI* with the *lacZ* reporter gene. We examined the expression of the choline kinase gene using *lacZ* as a reporter gene. A fusion gene was constructed which contained the 5′-flanking region (308 base pairs) and N-terminal sequence (amino acids 1 to 30) of *CKI* followed by the in-frame *lacZ* coding region (Fig. 5). We transformed a *leu2* strain, D451-3, with the pCKZ1 plasmid carrying this fusion gene as well as *LEU2*. The resultant transformant, D451-3(pCKZ1), was grown in M-i medium with or without inositol and choline, and then β-galactosidase activity was assayed. The specific activities in the supplemented and unsupplemented cells were 72 and 285 U (for definition of the β-galactosidase unit, see Materials and Methods), respectively, proving that the fusion gene was fully responsive to inositol and choline. Hence, this fusion gene contained the entire regulatory sequence of *CKI*.

We next used this fusion gene for studying the derepression of *CKI*. The transformant carrying pCKZ1 was precultured in the presence of inositol and choline and then shifted to a new medium with different supplementation for further culture. The change in β-galactosidase activity was monitored by removing cells from the culture at different times and permeabilizing them and then assaying the enzyme

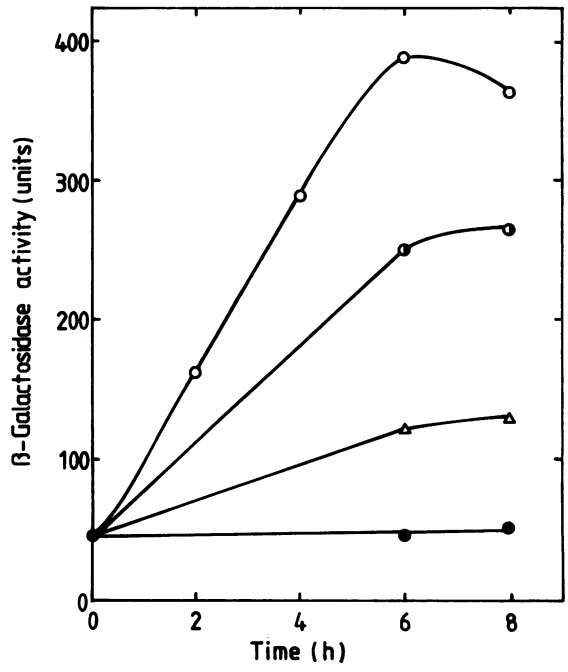

FIG. 6. Derepression of *CKI* examined by using *lacZ* as a reporter. Strain D451-3 harboring pCKZ1 was precultured in 150 ml of M-i medium supplemented with inositol, choline, and uracil (20 μg of each per ml) for 12 h. The cells were washed three times with M-i medium, suspended in 10 ml of M-i medium supplemented with uracil and the components indicated below, and then cultured. The initial A_{550} was 0.37. At the times indicated, 0.25 to 1.0 ml was withdrawn from the culture and β-galactosidase activity was assayed as described in Materials and Methods. Additions to the culture are symbolized as follows: ○, none; ◑, choline; △, inositol; ●, inositol and choline.

activity. The specific activity increased with time after the removal of inositol and choline, reaching a maximum at 6 h (Fig. 6). However, when choline was added to the culture medium, the highest level was somewhat lower. Inositol greatly inhibited restoration of the activity. When both inositol and choline were present, the activity did not increase at all. These results are quite consistent with those obtained above and strongly suggest that the transcription of the yeast choline kinase gene is regulated by inositol and choline.

DISCUSSION

The results presented here show that yeast choline kinase, the initial enzyme in the CDP-choline pathway, is regulated at the level of mRNA abundance by inositol and choline. As in the case of other regulated enzymes in yeast phospholipid synthesis, such as phosphatidylethanolamine methyltransferase (37), phospholipid methyltransferase (38), CDP-diacylglycerol synthase (14), phosphatidylserine synthase (22), and inositol-1-phosphate synthase (12), choline kinase is maximally repressed when both inositol and choline are added to the culture medium, but when added separately, inositol has a greater effect than choline. Thus, the control of choline kinase and that of the other key enzymes in phospholipid synthesis have common features. This suggests that the expression of choline kinase is controlled by a genetic mechanism similar to that of the above enzymes. In this context, it would be interesting to examine the control of

choline kinase in mutants with pleiotropic defects in the regulation of phospholipid synthesis (22, 25).

The present results show that the concept of the coordinate regulation of phospholipid synthesis can be extended to the CDP-choline pathway. On the basis of disruption of the choline kinase gene as well as expression of the gene in *E. coli* cells, we have previously shown that choline kinase accounts for most of the ethanolamine kinase activity in yeast cells (16). Therefore, the current data indicate that the CDP-ethanolamine pathway is subject to repression by inositol and choline as well.

At present, it is not known whether repression is the sole mechanism for the regulation of choline kinase. Because our previous survey revealed the occurrence of putative phosphorylation sites (Arg-Arg-X-Ser) in the primary sequence of choline kinase predicted from the nucleotide sequence of the *CKI* gene, the possibility may be considered that yeast choline kinase is subject to regulation through covalent modification of the enzyme, such as phosphorylation, as in the case of phosphatidylserine synthase (21).

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