MOLECULAR AND CELLULAR BIOLOGY, Jan. 1987, p. 167–176
0270-7306/87/010167-10$02.00/0

Vol. 7, No. 1

The Membrane-Associated Enzyme Phosphatidylserine Synthase Is Regulated at the Level of mRNA Abundance

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Received 7 July 1986/Accepted 30 September 1986

To precisely define the functional sequence of the *CHO1* gene from *Saccharomyces cerevisiae*, encoding the regulated membrane-associated enzyme phosphatidylserine synthase (PSS), we subcloned the original 4.5-kilobase (kb) *CHO1* clone. In this report a 2.8-kb subclone was shown to complement the ethanolamine-choline auxotrophy and to repair the defect in the synthesis of phosphatidylserine, both of which are characteristic of *cho1* mutants. When this subclone was used as a hybridization probe of Northern and slot blots of RNA from wild-type cells, the abundance of a 1.2-kb RNA changed in response to alterations in the levels of the soluble phospholipid precursors inositol and choline. The addition of inositol led to a 40% repression of the 1.2-kb RNA level, while the addition of choline and inositol led to an 85% repression. Choline alone had little repressive effect. The level of 1.2-kb RNA closely paralleled the level of PSS activity found in the same cells as determined by enzyme assays. Disruption of the *CHO1* gene resulted in the simultaneous disappearance of 1.2-kb RNA and PSS activity. Cells bearing the *ino2* or *ino4* regulatory mutations, which exhibit constitutively repressed levels of a number of phospholipid biosynthetic enzymes, had constitutively repressed levels of 1.2-kb RNA and PSS activity. Another regulatory mutation, *opi1*, which causes the constitutive derepression of PSS and other phospholipid biosynthetic enzymes, caused the constitutive derepression of the 1.2-kb RNA. When *cho1* mutant cells were transformed with the 2.8-kb subclone on a single-copy plasmid, the 1.2-kb RNA and PSS activity levels were regulated in a wild-type fashion. The presence of the 2.8-kb subclone on a multicopy plasmid, however, led to the constitutive overproduction of 1.2-kb RNA and PSS in *cho1* cells.

The synthesis of phospholipids in the yeast *Saccharomyces cerevisiae* involves both cytoplasmic and membrane-associated enzymes, a number of which are coordinately regulated (19). Within this group are the membrane-associated enzymes CDP diacylglycerol (CDP-DG) synthase (23), phosphatidylserine synthase (PSS) (7, 8, 29), phosphatidylserine decarboxylase (9), and the phospholipid *N*-methyltransferases (N-MTFs) (45–47) and the cytoplasmic enzyme inositol-1-phosphate synthase (I-1PS) (12–14, 21). All the coordinately regulated enzymes respond to alterations in the levels of the soluble phospholipid precursors inositol and choline. Of special interest are the enzymes I-1PS (L-myo-inositol-1-phosphate synthase, EC 5.5.1.4) and PSS (CDP diacylglycerol-L-serine-*O*-phosphatidyl transferase, EC 2.7.8.8) as they catalyze the first steps unique to the synthesis of phosphatidylinositol (PI) and the de novo synthesis of phosphatidylcholine (PC), respectively (Fig. 1). The genes encoding I-1PS (*INO1*) (28) and PSS (*CHO1*) (33) have been cloned.

A number of regulatory mutations which pleiotropically affect the expression of all the coordinately regulated enzymes have been identified. For example, the *ino2* and *ino4* mutations (11, 14), isolated as inositol auxotrophs, have been shown to make constitutively repressed levels of the *INO1* gene product, I-1PS, and the N-MTFs (34). The *opi1* mutant, isolated as an overproducer of inositol (17, 18), constitutively overproduces the *INO1* protein (29) and expresses constitutively derepressed levels of the N-MTFs (29), PSS (29), and CDP-DG synthase (23). The identification of regulatory mutations which pleiotropically affect the regulation of a number of the coordinately regulated enzymes suggests that their expression is controlled by a common regulatory apparatus (19, 29, 34).

The recent cloning of the genes encoding I-1PS (*INO1*) (28) and PSS (*CHO1*) (33) allows the nature of the regulation of phospholipid biosynthesis to be investigated at the molecular level. Recent work has shown that the regulation of I-1PS subunit and activity levels parallels the regulation of *INO1* mRNA abundance (21, 34a). Preliminary experiments with the cloned *CHO1* gene have shown that the levels of a 1.2-kilobase (kb) RNA complementary to *CHO1* closely paralleled the levels of PSS activity in wild-type cells grown under various conditions of inositiol and choline supplementation (18a). In this report we show that the levels of 1.2-kb RNA found in wild type as well as *ino2*, *ino4*, and *opi1* regulatory mutants compare closely to the levels of PSS activity found in the same cells. The 1.2-kb RNA and PSS activity levels also paralleled each other in *cho1* cells transformed with the *CHO1* gene on a multicopy or a single-copy plasmid. Disruption of the *CHO1* gene resulted in the abolition of 1.2-kb RNA and PSS activity. The implications of these findings as they apply to a model for the regulation of *CHO1* gene expression are discussed.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England BioLabs, Inc. (Beverly, Mass.), or P-L Biochemicals, Inc. (Milwaukee, Wis.). DNA polymerase I (large fragment), agarose, and nick translation reagents were purchased from Bethesda Research Laboratories. T4 DNA ligase was from Collaborative Research, Inc. (Waltham, Mass.). SP6 RNA polymerase, $^{32}P_i$, and L-[3-^{3}H]serine were obtained from New England Nuclear Corp. (Boston, Mass.).

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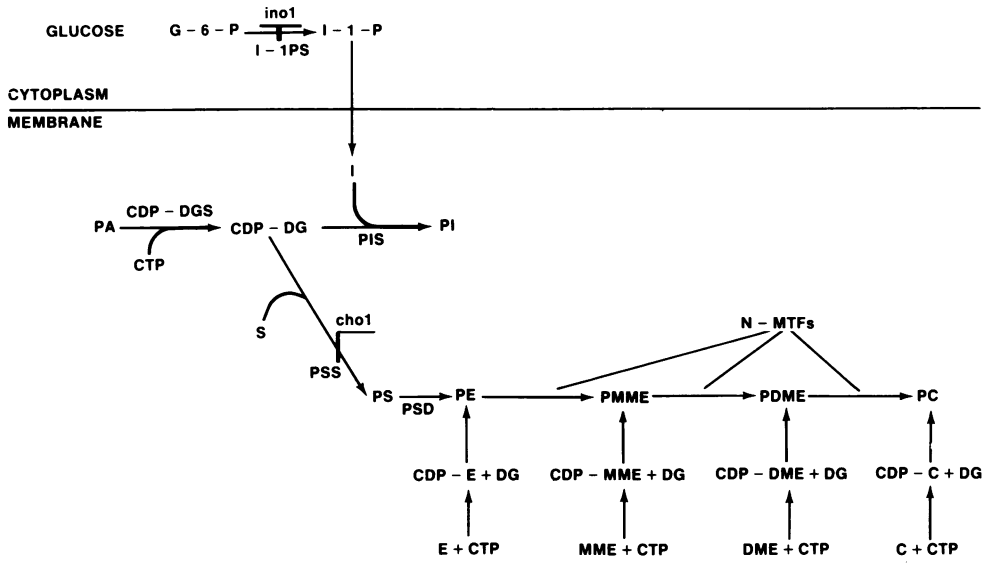

FIG. 1. Phospholipid biosynthetic pathways in yeasts. The first step unique to the synthesis of PI is the conversion of glucose-6-phosphate (G-6-P) into inositol-1-phosphate (I-1-P) by the cytoplasmic enzyme (I-1PS) (11), the product of the *INO1* gene (13). Inositol-1-phosphate is subsequently dephosphorylated to form free inositol. The syntheses of PI and PS require the precursor CDP-DG which is synthesized in the membrane from phosphatidic acid (PA) and CTP by the action of CDP-DG synthase (CDP-DGS). The membrane-bound enzyme PI synthase (PIS) catalyzes the synthesis of PI from free inositol and CDP-DG. The *CHO1* gene product, PSS (1, 2, 33), an integral membrane protein (7), catalyzes the first step unique to the de novo synthesis of PC by forming PS from CDP-DG and free serine. PS is subsequently decarboxylated to form PE by the membrane-bound enzyme PS decarboxylase (PSD). PE is then methylated in three sequential steps to form PC by the membrane-bound N-MTFs (41, 44). The intermediate methylated phospholipids phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) as well as PE and PC can alternatively be formed from the soluble precursors ethanolamine (E), choline (C), etc., by the action of a pathway originally described by Kennedy and Weiss (27).

[α^{32}P]GTP and [α-^{32}P]dATP were from Amersham Corp. (Arlington Heights, Ill.). SG81 silica gel-impregnated chromatography paper was purchased from Whatman, Inc. (Clifton, N.J.). Nitrocellulose paper was from Schleicher & Schuell, Inc. (Keene, N.H.). Antibiotics, solvents, and other reagents were from Fisher Scientific Co. (Pittsburg, Pa.). CDP-DG (6) and [5-^{3}H]CDP-DG (4) were prepared as previously described.

Yeast strains. The *cho1-3* mutant strain clu18 (*MATa cho1-3 leu2-3,112 his2 his3 ura3-52*) was used as the recipient of subcloned fragments of the *CHO1* gene in complementation experiments. The diploid strain ctW1 (*MATa/MAT*α *cho1-3/CHO1 leu2-3,112/LEU2 trp1-1/trp1-1 his3/his3 ade2/ADE2 ade6/ADE6 ura3-1/URA3 can1-100/CAN1*) was used as the parent strain in the gene disruption experiments. The diploid was constructed by mating ct3-1 (*MAT*α *cho1-3 trp1-1 his3 ade6*) to W3031A (*MATa leu2-3,112 can1-100 ura3-1 ade2 his3*), a gift from R. Rothstein. The strains DKT307 (*MATa ino2-21 leu2-3,112 ade2-1 trp1-1 ura3-1 his3-11,15*), DKT308 (*MAT*α *ino4-39 leu2-3,112 ura3-1*), and DKT313 (*MAT*α *opi1-1 ura3-1 trp1-1 ade5 lys*) were the gift of D. Hoshizaki. A *MAT*α *ade5* laboratory strain (12) was used as the wild type in all experiments. The conditions and media utilized for growth, maintenance, and genetic analysis of wild type as well as *cho1*, *ino2*, *ino4*, and *opi1* mutant yeast strains have been described previously (1, 2, 12, 14, 17, 18).

Bacterial strains and plasmids. *Escherichia coli* HB101 (*hsdS recA13 supE44 lacZ4 leuB6 proA2 thi1-1* Str^r) was maintained on LB medium (36). When appropriate, ampicillin was added to a final concentration of 100 μg/ml. All plasmids used had an *E. coli* replicon and an ampicillin resistance gene for selection in *E. coli*.

Plasmid DNA preparation. *E. coli* cells bearing plasmids with the ampicillin resistance gene were grown to the stationary phase in ampicillin-containing medium. Plasmid DNA was prepared from these cells by the centrifugation of cell lysates to equilibrium in cesium chloride-ethidium bromide gradients (10). Plasmids were digested with restriction endonucleases under conditions specified by the manufacturer. When appropriate the cohesive ends left by endonuclease digestion were made flush with DNA polymerase I (large fragment) and the four deoxyribonucleotides under conditions specified by the manufacturer. Mixtures of appropriate fragments were ligated with T4 DNA ligase. The products of ligation were used to transform *E. coli* HB101 to ampicillin resistance. Miniplasmid DNA preparations were made from individual ampicillin-resistant colonies by the rapid-boiling technique of Holmes and Quigley (22). The structures of the plasmids were analyzed by electrophoresis of restriction endonuclease-generated fragments on 0.8% agarose gels. Plasmids of the desired structure were amplified.

Yeast transformation and mitotic stability analysis. Subclones of the 4.5-kb *CHO1* clone from the primary clone bank isolate YEp*CHO1* (33) were placed into the multiple cloning site of YEp351 (19a), a yeast shuttle plasmid bearing the 2μm replicon and the *LEU2* selective marker. The *CHO1* subclone from pAB206 (Fig. 2), including more than 20 base pairs of flanking polylinker sequences on both sides, was placed into the yeast centromere plasmid YCp50 (courtesy of Ron Davis), yielding the plasmid pAB501. In addition to bearing the centromere from *S. cerevisiae* chromosome IV, the plasmid has the 2μm replicon and *URA3* selective marker. These plasmids were transformed into an appropriately marked recipient strain by the divalent cation technique of Ito et al. (25), selecting for complementation of leucine or uracil auxotrophy. If a secondary screening for choline prototrophy proved positive, the transformants were

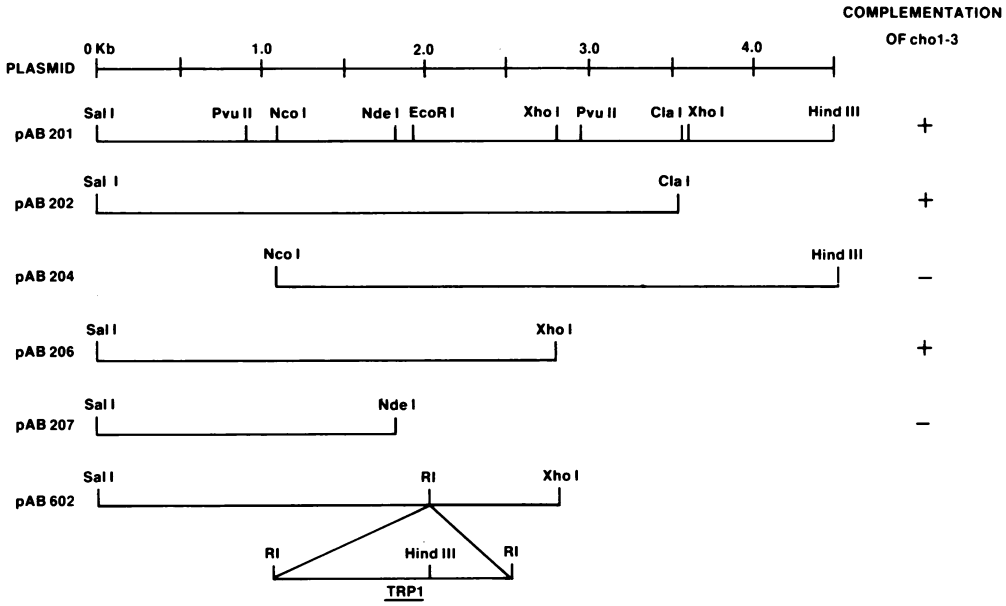

FIG. 2. Restriction map of *CHO1* subclones and their complementation activities in *cho1-3* cells. The *CHO1* subclones were obtained from the 4.5-kb primary clone bank isolate of *CHO1* present in the plasmid YEp*CHO1* (33) by cutting with the appropriate restriction endonucleases and inserting the fragments into the multiple cloning site of appropriately cut YEp351 (19a) as discussed in the text. A haploid yeast strain (clu18) carrying the *cho1-3* allele was transformed with the subclone-bearing plasmids, and the transformants were tested for their choline phenotype as related in the text. Plasmids that gave choline prototrophic transformants are marked + on the chart. Plasmids that failed to complement the choline auxotrophy are marked − on the chart. Plasmid pAB602 is derived from pAB206 and bears the *TRP1* gene in the *Eco*RI site of the *CHO1* subclone (see text).

submitted to mitotic stability analysis. The transformants were grown in nonselective medium for more than 10 generations before plating onto nonselective agar and replica plating to selective agar. Loss of leucine or uracil prototrophy in some percentage of the colonies suggested mitotic loss of an extrachromosomal element bearing the complementing gene. Concomitant loss of choline prototrophy mapped the complementing activity to the plasmid.

Phospholipid analysis. Cells were grown in minimal medium containing inositol (75 μm) and choline (1 mM) at 30°C to a density of 1×10^7 to 2×10^7 cells per ml. The cells were washed twice with medium lacking choline and suspended in inositol- and choline-free medium. After 2 h of choline-free growth at 30°C, $^{32}P_i$ was added to the medium (final specific activity, 50 μCi/ml) and incubated with the cells for 30 min. After the pulse, the cells were washed, and the lipids were extracted by the method of Atkinson et al. (1). A portion of the extract was counted by liquid scintillation to determine total lipid-extractable counts. The remaining lipids were applied to Whatman SG81 chromatography paper treated as described by Steiner and Lester (40) and separated by two-dimensional chromatography (40, 41). The positions of the labeled lipids on the chromatograms were determined by autoradiography. The identity of each species was determined by comparison of its mobility relative to the mobilities of pure phospholipid standards (1, 2). The amount of each labeled phospholipid was determined by liquid scintillation counting of the corresponding spots on the chromatogram.

RNA isolation and Northern and slot blots. Total cellular RNA was prepared by glass bead disruption of cells grown to a density of 1.0×10^7 to 3.0×10^7 cells per ml in the appropriate media (15). The RNA was size fractionated on 1.2% agarose–3% formaldehyde–20 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.4)–1 mM EDTA and blotted to nitrocellulose by the method of Thomas (44). Slot blots were prepared by application of RNA directly to nitrocellulose under high-salt conditions (3 M sodium chloride, 0.3 M sodium citrate) with a Hybrislot slot-blot manifold from Bethesda Research Laboratories.

Riboprobe generation and hybridization. The 2.8-kb *CHO1* subclone was removed from pAB206 by restriction endonuclease digestion and ligated into the SP6 vectors pGEM1 and pGEM2 (Promega Biotech, Madison, Wis.) to form pAB306 and pAB307, respectively. A 3.2-kb restriction fragment bearing the gene for the *S. cerevisiae* ribosomal protein L3 (*TCM1*) (courtesy of J. Warner) was ligated to pGEM1 to form pAB309. The plasmids were linearized before their use in the synthesis of the probe. ^{32}P-labeled RNAs complementary to one strand of the cloned sequence were synthesized by the method published by Melton et al. (35). The resulting probes were hybridized to blots at 53°C in 50% formamide–0.6 M sodium chloride–60 mM sodium citrate–0.1% sodium dodecyl sulfate–0.05% bovine serum albumin–0.05% Ficoll–0.05% polyvinylpyrrolidone. Slot-blot hybridization signals were quantitated by densitometric analysis of autoradiograms with a Quantimet 520 image analyzer (Cambridge Instruments, Inc.). All measurements were obtained with samples that were within the linear limits of the film and densitometer.

Preparation of cell extracts. Cells were disrupted with glass beads with a Mini-Bead-Beater (Biospec Products) in 50 mM Tris hydrochloride buffer (pH 7.5) containing 1 mM disodium EDTA, 0.3 M sucrose, and 10 mM 2-mercaptoethanol (29, 38). Glass beads and unbroken cells were removed by centrifugation at 1,500 × *g* for 5 min. The supernatant (cell extract) was used for enzyme activity assays.

PSS enzyme assays. PSS activity was measured at 30°C by following the incorporation of L-[3-^{3}H]serine (10,000 cpm/nmol) into chloroform-soluble material or the release of

TABLE 1. Quantitation of incorporation of $^{32}P_i$ into the phospholipids of yeasts[a]

Strain	% Phospholipids[b]						
	PA	PL	PI	PS	PE	PC	Other
Wild type	13.3	14.4	12.9	27.1	23.2	7.9	1.2
cho1-3	12.3	29.0	33.2	ND[c]	9.8	8.4	7.3
cho1-3(pAB206)	9.9	9.5	16.4	30.4	23.2	8.4	2.2
cho1-3(pAB501)	8.5	13.7	11.3	28.7	24.2	10.4	3.3
TVc-1a (spore colony[d])	10.4	11.2	12.2	28.9	23.8	10.5	3.0
TVc-1b (spore colony)	10.9	17.9	42.9	ND	10.8	10.5	7.0
TVc-1c (spore colony)	15.9	18.1	39.4	ND	10.5	8.7	7.4
TVc-1d (spore colony)	10.9	9.1	14.0	28.4	20.4	13.7	3.5

[a] The figures rendered here are given as percentages of total lipid extractable counts. Total incorporation into lipid was 2.5 to 8.4 cpm/10^4 cells and did not vary significantly with respect to strain or growth condition.

[b] Phospholipid designations: PA, phosphatidic acid; PL, polar lipid (CDP-DG); Other, phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine, lysophospholipids, and cardiolipin.

[c] ND, Not detectable.

[d] Spore colonies derived from gene disruption experiments (see text).

water-soluble [5-^{3}H]CMP from radioactive CDP-DG (400 cpm/nmol) as previously described (7). The assay mixture contained 50 mM Tris hydrochloride (pH 8.0), 0.60 mM $MnCl_2$, 0.5 mM L-serine, 0.25 mM CDP-DG, 4 mM Triton X-100, and cell extract in a total volume of 0.1 ml. Activity was linear with respect to time and protein concentration under the specified assay conditions when measured with either labeled substrate. A unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product per min under the conditions described. Specific activity was defined as the number of units per milligram of protein.

Protein determination. Protein concentration was determined by the method of Bradford (5) as previously described (3), with bovine serum albumin as the standard.

Gene disruption. The method used here was the single-step gene disruption technique of Rothstein (39). A 1.5-kb *Eco*RI DNA fragment bearing the *TRP1* gene from *S. cerevisiae* was inserted into the *Eco*RI site of the 2.8-kb *CHO1* subclone resulting in the plasmid pAB602 (Fig. 2). The recombinant *CHO1*::*TRP1* subclone was separated from vector sequences by *Bam*HI and *Sal*I endonuclease digestion. The resulting 4.3-kb DNA fragment was used to transform a diploid strain, ctW1 (*trp1-1/trp1-1 cho1-3/CHO1*). Tryptophan prototrophic transformants were subsequently screened for their choline phenotype. Choline auxotrophic and prototrophic transformants were identified and submitted to the mitotic stability analysis as discussed above. Those transformants that had stable tryptophan and choline phenotypes were submitted to Southern blot and genetic analyses.

DNA from the stable transformants was prepared by the method of Struhl et al. (43). DNA (5 μg) from each transformant was digested with *Pvu*II endonuclease before size fractionation on 0.8% agarose-TBE (89 mM Tris base, 89 mM boric acid, 20 mM EDTA) gels. DNA fragments were transferred to nitrocellulose after in situ denaturation by the method of Southern (39). These blots were hybridized to a 0.9-kb *Pvu*II-*Eco*RI fragment of the 2.8-kb *CHO1* subclone labeled with ^{32}P by nick translation. The hybridizations were performed at 37°C in 50% formamide–1.0 M sodium chloride–100 mM sodium citrate–0.1% sodium dodecyl sulfate –0.05% bovine serum albumin–0.05% Ficoll–0.05% polyvinylpyrrolidone–10 mg of denatured salmon sperm DNA per ml.

Stable transformants were also placed onto sporulation medium. Ten tetrads of the transformant ctW1-602.1 were dissected, and the individual spores were replica plated onto dropout media to determine their phenotypes. The spores (TVc-1a, b, c, and d) from one of these tetrads were analyzed in more detail. Each spore was submitted to Southern blot, Northern blot, and PSS activity analyses in the manner discussed above.

RESULTS

Subcloning and complementation. Subclones were constructed by placing restriction fragments of the original 4.5-kb genomic clone of the *CHO1* gene (33) into the multiple cloning site of YEp351 (Fig. 2). These plasmids were then transformed into a *cho1-3 leu2-3,112* strain. Transformants were selected on the basis of leucine prototrophy and subsequently screened for choline prototrophy. Subclones pAB201, pAB202, and pAB206 complemented the choline auxotrophy, while pAB204 and pAB207 did not (Fig. 2). These results showed that the region of the original *CHO1* clone which complements *cho1-3* has one border between the *Sal*I and *Nco*I sites and the other between the *Nde*I site and the *Xho*I site nearer the *Nde*I site.

One feature of *cho1* mutant strains is a marked decrease in the ability to synthesize phosphatidylserine (PS) (1, 32). To determine whether the smallest subclone which repaired the choline auxotrophy would also repair the phospholipid defect, we subjected the pAB206 transformant to phospholipid analysis as described in the Materials and Methods. The *cho1-3* strain transformed with pAB206, a multicopy plasmid, exhibited a level of PS synthesis that was comparable to that of wild-type cells, while the untransformed *cho1-3* cells exhibited the low level of PS synthesis characteristic of *cho1* mutants (Table 1). Phospholipid analysis of *cho1-3* cells transformed with the plasmid pAB501, a single-copy centromere plasmid bearing the 2.8-kb *CHO1* subclone, revealed the same level of PS synthesis found in the cells transformed with the multicopy plasmid (Table 1). The 2.8-kb subclone, therefore, repairs the phospholipid defect in *cho1-3* cells whether it is present as a single copy or in multiple copies.

Determination of steady-state levels of *CHO1* RNA and PSS activity in wild-type cells. The level of *CHO1* RNA in wild-type cells was assessed. Labeled RNA probes were used to probe Northern and slot blots of total RNA isolated from wild-type cells grown under various conditions of inositol and choline supplementation. The 2.8-kb *CHO1* subclone from pAB206 was cloned into the SP6 vectors pGEM1 and pGEM2 to produce the plasmids pAB306 and pAB307. These plasmids served as templates for the synthe-

sis of ^{32}P-labeled RNAs homologous to opposite strands of the subclone. Another plasmid, pAB309, bears the gene encoding the ribosomal protein L3 (*TCM1*) from *S. cerevisiae* (16) and yielded antisense *TCM1* RNA. The *TCM1* hybridization signal was used as a constitutive standard to which the *CHO1* signals were compared. To draw parallels between changes in the steady-state level of *CHO1* RNA and the regulation of PSS activity, we performed PSS enzyme assays in tandem with the RNA analysis.

Radiolabeled RNA prepared from pAB307 was used to detect RNAs transcribed starting from the *Sal*I end of the 2.8-kb *CHO1* subclone, while probe from pAB306 was used to detect RNAs of the opposite polarity. The pAB307 probe hybridized to a low-abundance, poly(A)$^-$ RNA of 120 nucleotides (Fig. 3). The abundance of this RNA did not change with respect to the abundance of 28S and 18S rRNAs used as standards (data not shown) when inositol or choline or both were added to the growth medium. The pAB306 probe hybridized weakly to an unregulated 1.6-kb RNA and strongly to a regulated 1.2-kb RNA (Fig. 3). Both RNAs were poly(A)$^+$ (data not shown). The profile of 1.2-kb RNA abundance paralleled the profile of PSS activity determined by enzyme assays: both RNA abundance and PSS activity were highest in the supplement-free and choline-grown cells (lanes C and D), lower in the inositol-grown cells (lane B), and at their lowest in the cells supplemented with both inositol and choline (Fig. 3, lane A; Table 2).

Quantitation of the level of *CHO1* RNA in wild-type cells was performed by probing slot blots with ^{32}P-labeled RNA prepared from pAB306. Because the unregulated 1.6-kb species detected by hybridization with pAB306 RNA was barely discernible on Northern blots, the levels of *CHO1* cRNA determined in these experiments were taken to be an accurate indication of the 1.2-kb RNA level. This analysis showed that the addition of inositol and choline to the growth medium led to an 85% reduction in the 1.2-kb RNA level, while the addition of inositol alone led to an approximately 40% reduction. The cells grown in the presence of choline alone had approximately 30% less 1.2-kb RNA than cells grown without supplements (Fig. 3). The PSS activity levels corresponded closely to the 1.2-kb RNA levels found in the same cells. The one exception is the enzyme activity level found in choline-grown cells which somewhat exceeded that found in cells grown without supplements (Table 2). The correlation between the levels of 1.2-kb RNA and PSS expression suggest that the 1.2-kb RNA is the *CHO1* mRNA and that the regulation of PSS is at the level of *CHO1* mRNA abundance.

Disruption of *CHO1* gene. To obtain more definitive evidence for the relationships proposed above, we disrupted the *CHO1* gene by the single-step gene disruption technique of Rothstein (38). A 1.5-kb *Eco*RI restriction fragment bearing the *TRP1* gene was inserted into the unique *Eco*RI site within the 2.8-kb *CHO1* subclone (Fig. 2). A *Bam*HI and *Sal*I fragment containing *CHO1*::*TRP1* was transformed into the diploid ctW1 (*trp1/trp1 cho1/CHO1*), selecting for tryptophan prototrophs. In the subsequent screening of choline phenotype both choline auxotrophs and prototrophs were identified as would be expected if *CHO1*::*TRP1* replaced the wild-type or mutant *CHO1* alleles, respectively. Since *CHO1*::*TRP1* bears homology to *TRP1* as well as *CHO1* the recombinant gene could also have integrated at the *TRP1* locus. Southern blot analysis of DNA from several transformants revealed that the *CHO1*::*TRP1* construction had integrated as a single copy at the *CHO1* locus, disrupting one, the other, or both *CHO1* alleles (data not shown). As double disruptants were choline auxotrophs, the insertion of the *TRP1* gene must inactivate the *CHO1* gene. The existence of viable double disruptants demonstrated that the disruption of the *CHO1* gene was not lethal.

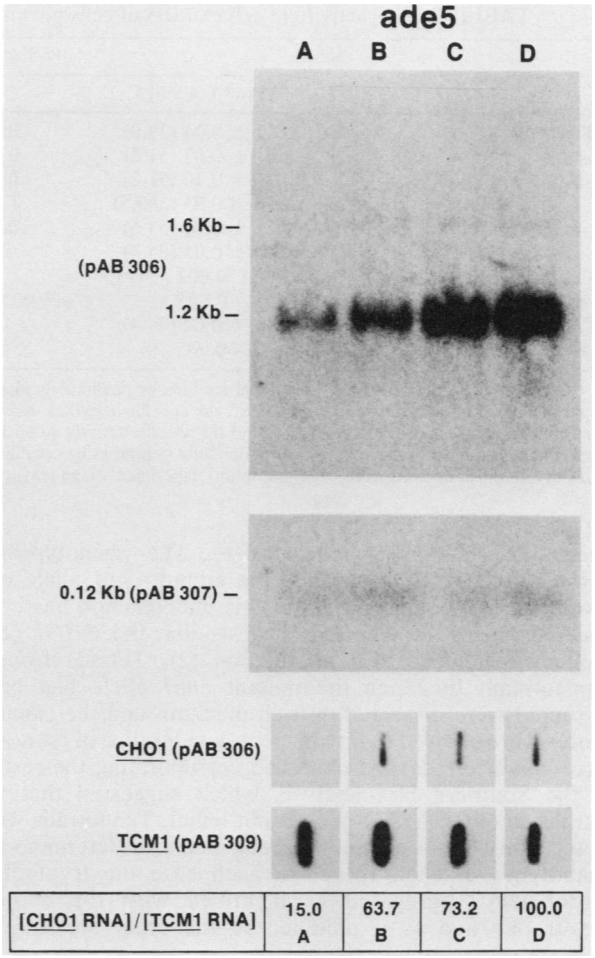

FIG. 3. Northern and slot-blot analyses of steady-state levels of *CHO1* mRNA from wild-type cells (*ade5*) grown under various conditions of inositol and choline supplementation. Wild-type (*ade5*) cells were grown in vitamin-free minimal medium or medium supplemented with inositol (75 μM) or choline (1 mM) or both as related in the text. Northern blots of total RNA (10 μg per lane) were prepared as related in the text. Identical blots were hybridized to ^{32}P-labeled RNA probes generated from pAB306 or pAB307 which are complementary to opposite strands of the 2.8-kb *CHO1* subclone from pAB206. The identical RNA preparations were used to make a slot blot which was sequentially probed with ^{32}P-labeled RNA from pAB306 and ^{32}P-labeled RNA complementary to mRNA encoding the ribosomal protein L3 (*TCM1*) generated from pAB309. The resulting autoradiographs were scanned with a Quantimet 520 image analyzer to determine the amounts of *CHO1* and *TCM1* RNA that were present. Only films with signals that were within the linear range of the film and densitometer were used in our determinations. Using the amounts of *TCM1* RNA as a constitutive standard, the amounts of *CHO1* RNA found in each growth condition are expressed as the quotient of the [*CHO1* RNA]/[*TCM1* RNA] value found in each growth condition divided by the [*CHO1* RNA]/[*TCM1* RNA] value determined for wild-type cells grown in fully derepressing conditions (supplement-free) and then multiplied by 100. Lanes: A, 75 μM inositol, 1 mM choline; B, 75 μM inositol; C, 1 mM choline; D, no supplement.

To further define the effect of *CHO1* gene disruption, we initiated genetic analysis of the transformed diploids. Interestingly, when transformants disrupted at both alleles or at the wild-type *CHO1* allele were put onto sporulation me-

TABLE 2. PSS activity in cell extracts of cells grown under various conditions of inositol and choline supplementation

Strain	PSS activity (U/mg ± SD) with the following growth conditions[a]			
	75 μM I, 1 mM C	75 μM I	1 mM C	Unsupplemented
WT (*ade5*)	0.12 ± 0.04 (29.0)	0.25 ± 0.01 (62.0)	0.45 ± 0.07 (109.0)	0.41 ± 0.02 (100.0)
ino2-21	0.21 ± 0.07 (51.2)	0.18 ± 0.07 (43.9)		
ino4-39	0.21 ± 0.10 (51.2)	0.18 ± 0.06 (43.9)		
cho1-3 (pAB206)	1.15 ± 0.03 (280.5)	1.15 ± 0.01 (280.5)	1.00 ± 0.11 (243.9)	1.01 ± 0.15 (246.3)
cho1-3(pAB501)	0.22 ± 0.04 (53.6)	0.32 ± 0.04 (78.0)	0.59 ± 0.02 (143.9)	0.64 ± 0.11 (156.3)
TVc-1a (spore colony)[b]	0.10 ± 0.01 (24.4)			
TVc-1b (spore colony)	0.002			
TVc-1c (spore colony)	0.005			
TVc-1d (spore colony)	0.10 ± 0.02 (24.4)			
cho1-3	0.005			

[a] Cells were grown in vitamin-free minimal medium or medium supplemented with inositol (I), choline (C), or both as related in the text. Cell extracts were prepared and assayed as described in the text. the specific activities of PSS were calculated from 12 determinations from two independent growth studies. The values in parentheses represent the quotient of the specific activity value determined for the particular strain and growth condition divided by the specific activity value obtained for wild-type cells grown under fully derepressing conditions (no supplements) and multiplied by 100.

[b] Spore colonies derived from gene disruption experiments (see text).

dium, the cells failed to sporulate. The phenotypically CHO^+ transformants in which the mutant *cho1* allele was disrupted sporulated normally. This phenomenon has been documented previously and suggests that the *CHO1* gene product is required for sporulation (2). Tetrads from a transformant in which the mutant *cho1* allele had been disrupted were dissected on rich medium, and the isolated spores were allowed to divide. A preponderance of four- and three-spore tetrads was observed corroborating the results of the Southern blot analysis which suggested that the disruption of the *CHO1* gene is not lethal. Ten tetrads were plated onto various single dropout media to determine the phenotypes of each spore. In each case the tryptophan prototrophy segregated as if linked with the choline auxotrophy and as if unlinked to the other auxotrophic markers segregating in the cross.

One of these tetrads was chosen for further analysis. The haploid spores were submitted to Southern blot analysis as described in the Materials and Methods (Fig. 4). The choline prototrophic (tryptophan auxotrophic) spores possessed the 2.0-kb *Pvu*II fragment predicted for cells carrying an undisrupted *CHO1* gene. The choline auxotrophic (tryptophan prototrophic) spores exhibited a 3.5-kb *Pvu*II fragment, the size predicted for cells carrying *CHO1*::*TRP1*. The parent diploid ctW1-602.1, heterozygous for the disruption, had both 2.0- and 3.5-kb bands, while the *cho1-3* haploid strain used to construct ctW1 had only the 2.0-kb band (data not shown).

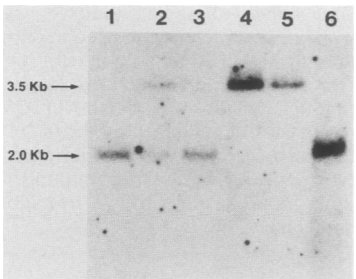

FIG. 4. Southern blot analysis of a gene disruption diploid and haploid progeny. A Southern blot of DNA (5 μg per lane) from the following strains was prepared and analyzed as described in the text: lane 1, ctW1 (untransformed diploid); lane 2, ctW1 pAB602.1 heterozygous for disruption; lane 3, TVc-1a (choline prototrophic spore); lane 4, TVc-1b (choline auxotrophic spore); lane 5, TVc-1c (choline auxotrophic spore); lane 6, TVc-1d (choline prototrophic spore).

Phospholipid analysis was also performed on this tetrad. The results for the choline prototrophic spores were essentially identical to those found for wild-type cells (Table 1). The spores carrying *CHO1*::*TRP1*, however, had the low level of PS synthesis characteristic of *cho1* mutants and, in addition, had a somewhat more elevated level of PI synthesis than that found in *cho1-3* cells (Table 1).

Northern blot analysis of RNA profiles in these spores revealed that the spores carrying the wild-type *CHO1* allele had the 1.2-kb *CHO1* RNA as well as the 1.6-kb species (Fig. 5). The 1.2-kb RNA was absent from the spores carrying *CHO1*::*TRP1*, while the 1.6-kb RNA was present (Fig. 5). No other RNA species complementary to the 2.8 kb *CHO1* subclone was identified. A normal level of 1.2-kb RNA was found in the *cho1-3* haploid strain (ct3-1) used in the making of the ctW1 diploid (Fig. 5).

PSS enzyme activity levels in the spores were assessed and found to be consistent with the RNA results. The spores lacking the 1.2-kb RNA had negligible PSS activity levels, while the spores with wild-type levels of 1.2-kb RNA exhibited wild-type PSS activity levels. These results confirm that the 1.2-kb RNA encodes the *CHO1* gene product.

Determination of steady-state levels of *CHO1* RNA and PSS activity in regulatory mutants. The expression of the 1.2-kb RNA and PSS in mutants which exhibit altered regulation of multiple phospholipid biosynthetic enzymes was also investigated. The *ino2* and *ino4* mutations have been shown to have pleiotropic effects, as they result in the constitutive

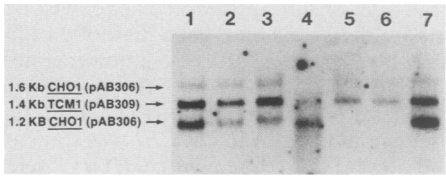

FIG. 5. Northern blot analysis of RNA from gene disruption diploid and haploid progeny. Total RNA (10 μg) from cells grown in minimal medium supplemented with inositol (75 μg) and choline (1 mM) were prepared as related in the text. These blots were probed with ^{32}P-labeled *TCM1* (pAB309) and *CHO1* (pAB306) riboprobes and autoradiographed. An autoradiogram exposed five times longer than the one pictured revealed no 1.2-kb *CHO1* signal for lanes 5 and 6. Lanes: 1, ctW1 (untransformed diploid); 2, ctW1 (pAB602.1) (transformed diploid); 3, ct3-1 (haploid *cho1-3* parent of ctW1); 4, TVc-1a (choline prototrophic spore); 5, TVc-1b (choline auxotrophic spore); 6, TVc-1c (choline auxotrophic spore); 7, TVc-1d (choline prototrophic spore).

repression of I-1PS and the N-MTFs (34). The extremely low level of I-1PS expression in these mutants results in an inositol auxotrophy which necessitates the addition of inositol to all growth media (12, 34). The *opi1* mutation has also been shown to be pleiotropic as it causes the constitutive derepression of PSS as well as of I-1PS and the N-MTFs (29).

Northern blots of RNA prepared from *ino2* and *ino4* cells revealed that the level of 1.2-kb RNA is constitutive since it does not vary with the addition of choline to inositol-containing growth medium (Fig. 6). Regardless of the growth conditions, the quantity of 1.2-kb RNA in these cells was determined by slot-blot analysis to be approximately equal to the fully repressed level in wild-type cells (Fig. 6). The level of PSS activity in *ino2* and *ino4* cells also did not respond to the addition of choline to inositol-containing medium, being between the intermediate and fully repressed levels in wild-type cells regardless of the growth conditions (Table 2).

Northern blots of RNA from *opi1* cells revealed that the 1.2-kb RNA level did not vary with the addition of inositol, choline, or both to the growth medium (Fig. 7). Through slot-blot analysis it was determined that the constitutive level of 1.2-kb RNA in *opi1* cells was approximately equal to the fully derepressed level in wild-type cells (Fig. 7). The PSS activity level in *opi1* cells was invariant and was three times the wild-type fully derepressed level (29). The above results suggest that the *ino2* and *ino4* mutants are incapable

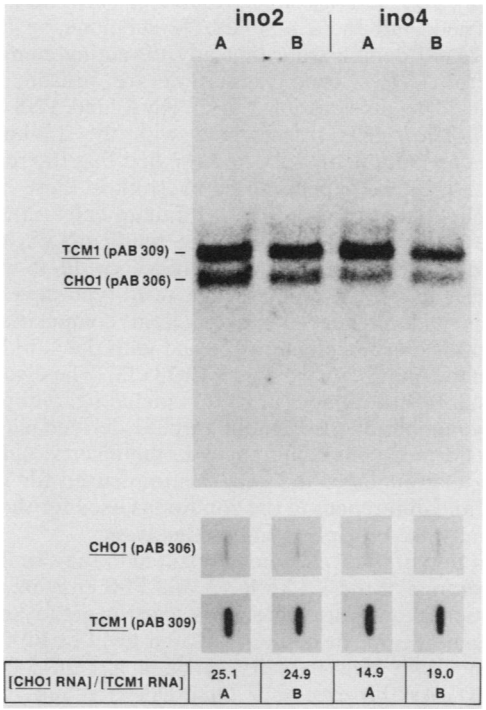

FIG. 6. Northern and slot-blot analyses of steady-state levels of *CHO1* RNA from *ino2* and *ino4* cells grown under various conditions of choline supplementation. Cells bearing the regulatory mutations *ino2* (DKT307) or *ino4* (DKT308) were grown in inositol-containing medium (75 μM) with or without supplemental choline (1 mM) as related in the text. Total RNA was applied to a Northern blot (20 μg per lane) and a slot blot (10 μg per slot), probed, and analyzed as described in the legend to Fig. 5. A probe complementary to the mRNA of the ribosomal protein L3 (pAB309) was included in the Northern blot hybridization milieu, while pAB307 probe was omitted. Lanes: A, 75 μM inositol, 1 mM choline; B, 75 μM inositol.

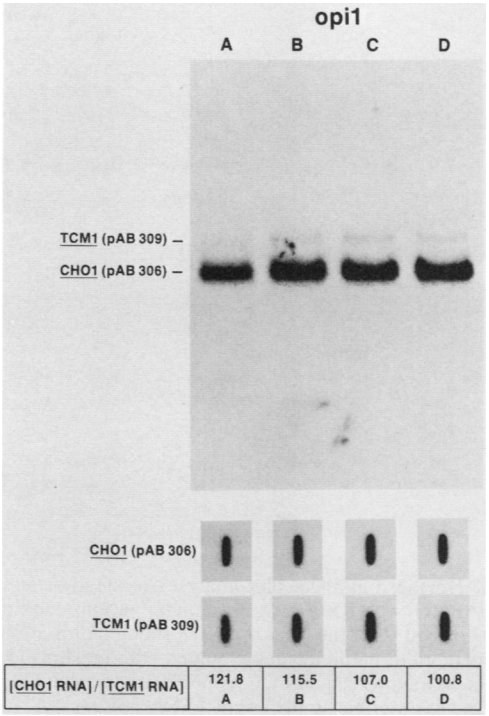

FIG. 7. Northern and slot-blot analyses of steady-state levels of *CHO1* mRNA in *opi1* cells grown under various conditions of inositiol and choline supplementation. Cells bearing the regulatory mutation *opi1* (DKT313) were grown in vitamin-free minimal medium or medium supplemented with inositol (75 μm) or choline (1 mM) or both as described in the text. Total RNA was prepared and applied to a Northern blot (20 μg per lane) and a slot blot (10 μg per slot), probed with pAB306 (*CHO1*) and pAB309 (*TCM1*) riboprobes, and analyzed as described in the legend to Fig. 5. Lanes: A, 75 μM inositol, 1 mM choline; B, 75 μM inositol; C, 1 mM choline; D, no supplements.

of derepressing *CHO1* gene expression, while the *opi1* mutant is defective in its repression.

Gene expression from plasmid-borne *CHO1* gene. In earlier experiments we established that *cho1* cells bearing the original *CHO1* clone in a multicopy plasmid (YEpCHO1) made more PS and had more PSS activity than wild-type cells grown under the same conditions (33). Further analysis revealed that the PSS activity level in YEpCHO1-transformed *cho1-3* cells was constitutive and was six to seven times the wild-type derepressed level regardless of the growth conditions (V. Letts and G. Carman, personal communication). The possibility that the original *CHO1* clone might have been missing elements required for the normal regulation of *CHO1* gene expression prompted the investigation of *CHO1* gene expression in *cho1-3* strains transformed with the 2.8-kb *CHO1* subclone in either a multicopy (pAB206) or a single-copy (pAB501) plasmid.

Northern blots of RNA from pAB206-transformed *cho1-3* cells revealed that the 1.2-kb RNA levels were high and abnormally regulated since the addition of supplemental inositol, choline, or both had little effect (Fig. 8). Quantitation of 1.2-kb RNA and PSS activity levels showed that both were two to three times the wild-type fully derepressed levels (Fig. 8; Table 2). The pattern of 1.2-kb RNA and PSS regulation in *cho1-3* cells transformed with the single-copy plasmid pAB501 was wild type, however. Northern blots of RNA from these cells showed that the 1.2-kb RNA was least abundant in inositol- and choline-supplemented cells, more

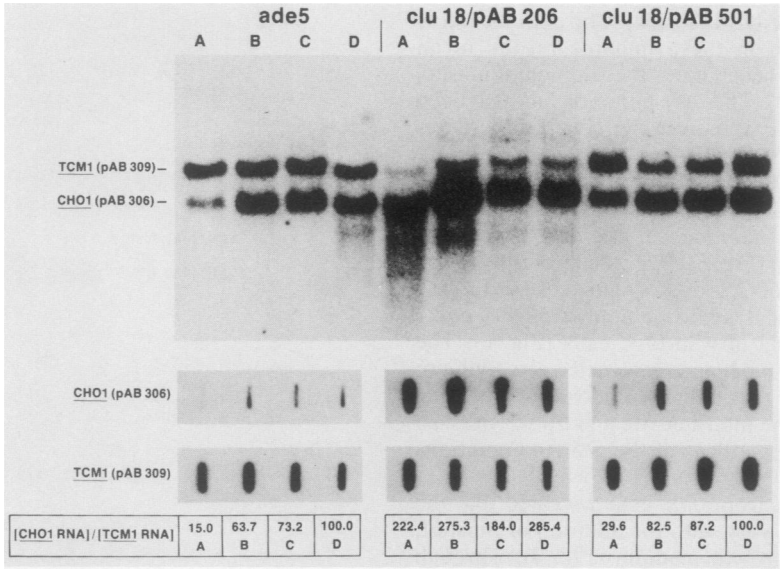

FIG. 8. Northern and slot-blot analyses of steady-state levels of *CHO1* mRNA in wild-type and *cho1-3* mutant cells transformed with plasmid pAB206 or pAB501 and grown under various conditions of inositol and choline supplementation. Wild-type and *cho1-3* (clu18) mutant cells transformed with the 2.8-kb *CHO1* subclone on a multicopy (pAB206) or single-copy (pAB501) plasmid were grown in vitamin-free minimal medium or medium supplemented with inositol (75 μM), choline (1 mM), or both as described in the text. Total RNA was prepared and applied to a Northern blot (20 μg per lane) and a slot blot (10 μg per slot), probed with pAB306 (*CHO1*) and pAB309 (*TCM1*) riboprobes, and analyzed as described in the legend to Fig. 5. Lanes: A, 75 μM inositol, 1 mM choline; B, 75 μM inositol; C, 1 mM choline; D, no supplement.

abundant when inositol alone was present, and most abundant when either choline alone or no supplement was present (Fig. 8). Quantitation of the 1.2-kb RNA levels in pAB501-transformed cells revealed amounts that were wild type with the exception of an increase in the level found in the inositol-supplemented condition to approximately that seen in choline-supplemented cells (Fig. 8). The PSS activity levels were approximately 1.5 times the levels observed for wild-type cells grown under the same conditions (Table 2).

DISCUSSION

In this report we showed that a 2.8-kb subclone of the original 4.5-kb clone of the *S. cerevisiae CHO1* gene complements the ethanolamine-choline auxotrophy and repairs the defect in PS synthesis characteristic of *cho1* mutants. When this smallest functional sequence was used as a probe of wild-type RNA on Northern blots three species were identified, only one of which was regulated in response to changing levels of the phospholipid precursors inositol and choline. Quantitation of the steady-state levels of this 1.2-kb regulated RNA showed that they corresponded closely to the PSS activity levels in the same cells. Disruption of the *CHO1* gene resulted in the simultaneous disappearance of 1.2-kb RNA and PSS activity. On the basis of these results we conclude that the 1.2-kb RNA is the *CHO1* mRNA. As all *cho1* mutants isolated thus far have defects in PSS activity (1, 2, 30, 31), including a mutant which possesses PSS with an increased k_m for serine (30), it has been suggested that *CHO1* is the structural gene for PSS. The data presented here are consistent with the hypothesis that the 1.2-kb *CHO1* mRNA encodes PSS. More substantial proof of this assignment awaits the establishment of a correspondence between the nucleotide sequence of the *CHO1* gene and the amino acid sequence of PSS.

Furthermore, we demonstrated that PSS is regulated at the level of mRNA abundance. Analysis of 1.2-kb RNA and PSS activity levels in cells carrying regulatory mutations revealed that *CHO1* gene regulation is linked to the regulation of other phospholipid biosynthetic genes. The 1.2-kb RNA and PSS activity levels found in *cho1* mutant cells transformed with the 2.8-kb *CHO1* subclone on a single-copy plasmid demonstrate that any *cis*-acting elements required for *CHO1* gene regulation are present on the subclone. The analysis of 1.2-kb RNA and PSS activity levels in *cho1* cells transformed with the 2.8-kb *CHO1* subclone on a multicopy plasmid showed that the regulation of the *CHO1* gene appears to be aberrant in these cells.

Phospholipid analysis of *cho1-3* mutant cells transformed with the 2.8-kb *CHO1* subclone on a multicopy plasmid or a single-copy plasmid restored the synthesis of PS to wild-type levels (Table 1). A greater than wild-type level of PS synthesis and an altered phospholipid composition were reported for *cho1-3* cells transformed with the 4.5-kb *CHO1* clone on the multicopy plasmid YEp13 (33). The discrepancy may result from a number of factors including: differences in the copy number of YEp13- and YEp351-derived plasmids in the transformed cells, differences in the inserts, differences in the backgrounds of the *cho1-3* strains used for transformation, and differences in the conditions used for the growth of the cells used for phospholipid analysis.

Disruption of the *CHO1* gene resulted in the simultaneous disappearance of the 1.2-kb RNA and PSS enzyme activity. Phospholipid analysis of spores carrying the disrupted *CHO1* gene demonstrated an elevated level of PI synthesis compared with that in cells carrying a *cho1-3* mutation (Table 1). As the precursor CDP-DG is required for the syntheses of both PI and PS, a possible explanation for this increase may be that by completely abolishing PS synthesis in the disruptant, CDP-DG formerly used in the synthesis of PS is used for the synthesis of PI instead. Cells carrying the *cho1-3* allele, however, may make a weakly functional enzyme which utilizes a small amount of CDP-DG, reducing the amount available for PI synthesis. Since cells carrying the *cho1-3* allele make normal amounts of *CHO1* mRNA (Fig. 5) they could, in fact, make a defective PSS.

While the disruption of the *CHO1* gene is not a lethal event, when the wild-type *CHO1* allele or both alleles of a

cho1-3/CHO1 diploid are disrupted the diploids are unable to sporulate, suggesting that PSS is required for sporulation. This phenomenon has been previously documented for *cho1/cho1* diploids and has been attributed to mitochondrial deficiencies in these cells (2).

In *S. cerevisiae*, the regulation of gene expression at the RNA level often requires the activity of *cis*-acting regulatory elements located 5′ to the start of transcription and *trans*-acting elements which interact with these *cis*-acting elements (42). The initial step taken toward identifying *cis*-acting elements required for *CHO1* gene regulation was to analyze the level of *CHO1* gene expression obtained from the 2.8-kb *CHO1* subclone. When the 2.8-kb *CHO1* subclone was placed into the multicopy plasmid YEp351 and transformed into *cho1-3* cells, the regulation of the 1.2-kb RNA and PSS activity was aberrant. The unregulated overproduction of PSS was previously reported in *cho1* cells transformed with YEp*CHO1*, a multicopy plasmid in which the 4.5-kb *CHO1* clone was inserted into YEp13 (33). YEp13 and YEp351 differ substantially in their construction, and the *CHO1* clone in YEp*CHO1* contains almost 2 kb more 5′-flanking sequence than is present on the 2.8-kb subclone. Therefore the unregulated overproduction of PSS and the 1.2-kb mRNA documented here probably is not due to the specific vector or flanking sequences.

Normal regulation of PSS and 1.2-kb mRNA was observed when the copy number of the *CHO1* subclone was reduced to approximately one by placing it into a centromere-containing plasmid. The *cho1-3* cells into which this plasmid was introduced contain a genomic copy of the gene which is capable of producing the 1.2-kb mRNA but are bereft of PSS activity (Fig. 5; Table 2). Because the total pool of 1.2-kb mRNA responds in a wild-type fashion to the addition of inositol, choline, or both to the medium it appears that the abundance of 1.2-kb mRNA transcribed from the genomic and plasmid-borne *CHO1* genes is regulated normally. The normal regulation of PSS in these cells suggests that the 2.8-kb subclone possesses all the *cis*-acting sequences necessary for *CHO1* gene regulation since PSS activity in the transformants can only be attributed to the presence of the plasmid-borne *CHO1* gene.

The evidence discussed thus far pertaining to the coregulation of phospholipid biosynthetic enzyme activities in response to inositol and choline does not demonstrate that the regulation of any of the enzymes involves a common genetic apparatus. The pleiotropic effects of the *ino2*, *ino4*, and *opi1* regulatory mutations in influencing the activities of all the coregulated enzymes establishes that these enzymes and regulatory elements form a regulon. The reduced and constitutive levels of the 1.2-kb RNA and PSS activity in cells bearing the *ino2* or *ino4* mutations suggest that the wild-type alleles of these genes encode positive regulators of *CHO1* gene expression. Conversely, high constitutive levels of the 1.2-kb RNA and PSS in *opi1* cells suggest that the wild-type *OPI1* gene encodes a negative regulator of *CHO1* gene expression. The results presented here for *CHO1* and elsewhere for *INO1* (21, 34a) suggest that these regulators exert their effects primarily at the level of structural gene mRNA abundance.

The minor discrepancies between enzyme activity and RNA data observed in the analysis of *ino2*, *ino4*, and *opi1* cells may have been at least partially due to inaccuracies in the assessment of 1.2-kb RNA concentration, since the *TCM1* mRNA concentrations which were used for normalization may vary between strains. Alternatively, *ino2*, *ino4*, and *opi1* cells may degrade PSS more slowly or synthesize it more rapidly than wild-type cells. Yet another possible explanation may be that an altered phospholipid composition alters the activity of PSS. Carman and colleagues have shown that varying the relative concentrations of PI, PS, phosphatidylethanolamine (PE), and PC in vesicles constructed in vitro affects the activity of PSS (J. Hromy and G. Carman, personal communication). Therefore, the altered phospholipid compositions found in *ino2*, *ino4*, and *opi1* cells may account for some fraction of the discrepancy between the RNA and enzyme activity data (29, 34).

Since the *OPI1*, *INO2*, and *INO4* gene products act across pathways to regulate *INO1* and *CHO1* gene expression, the regulation of phospholipid biosynthesis resembles the general control of amino acid biosynthesis in yeasts (19, 26). In general amino acid control, the activities of a number of enzymes involved in the synthesis of a group of amino acids respond coordinately to changes in the concentration of any one or more of the amino acids in that group. The *INO2*, *INO4*, and *OPI1* regulatory genes are analogous to the *GCN* and *GCD* genes since the *GCN* and *GCD* genes encode factors which mediate general control by altering the levels of transcription of the genes encoding the enzymes under general control (20, 37). Recent experiments have determined the molecular role of one of the general control regulatory factors (*GCN4*) (24). The molecular mechanisms of *INO2*, *INO4*, and *OPI1* control of phospholipid biosynthetic gene expression are currently under investigation.

ACKNOWLEDGMENTS

We thank Deborah Hoshizaki, John Hill, David Botstein, Jon Warner, and Rodney Rothstein for contributing yeast strains and plasmids used in this study. We thank John Hill, Jeanne Hirsch, Deborah Hoshizaki, Margaret Johnson, Kathryn Jones, Sepp Kohlwein, Pat McGraw, and Eric Summers for fruitful discussions during the preparation of this manuscript.

This work was supported by Public Health Service grants GM-19629 and GM-11301 to S.A.H. and GM-28140 to G.M.C. from the National Institutes of Health. A.M.B. was supported by Public Health Service training grant GM-07491 from the National Institutes of Health.

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