Copyright © 1988 by the Genetics Society of America

Saccharomyces cerevisiae cho2 Mutants Are Deficient in Phospholipid Methylation and Cross-Pathway Regulation of Inositol Synthesis

Eric F. Summers, Verity A. Letts,[1] Patricia McGraw[2] and Susan A. Henry[2]

Departments of Molecular Biology and Genetics, Albert Einstein College of Medicine, Bronx, New York 10461

Manuscript received June 9, 1988
Revised copy accepted September 6, 1988

ABSTRACT

Five allelic *Saccharomyces cerevisiae* mutants deficient in the methylation of phosphatidylethanolamine (PE) have been isolated, using two different screening techniques. Biochemical analysis suggested that these mutants define a locus, designated *CHO2*, that may encode a methyltransferase. Membranes of *cho2* mutant cells grown in defined medium contain approximately 10% phosphatidylcholine (PC) and 40–50% PE as compared to wild-type levels of 40–45% PC and 15–20% PE. In spite of this greatly altered phospholipid composition, *cho2* mutant cells are viable in defined medium and are not auxotrophic for choline or other phospholipid precursors such as monomethylethanolamine (MME). However, analysis of yeast strains carrying more than one mutation affecting phospholipid biosynthesis indicated that some level of methylated phospholipid is essential for viability. The *cho2* locus was shown by tetrad analysis to be unlinked to other loci affecting phospholipid synthesis. Interestingly, *cho2* mutants and other mutant strains that produce reduced levels of methylated phospholipids are unable to properly repress synthesis of the cytoplasmic enzyme inositol-1-phosphate synthase. This enzyme was previously shown to be regulated at the level of mRNA abundance in response to inositol and choline in the growth medium. We cloned the *CHO2* gene on a 3.6-kb genomic DNA fragment and created a null allele of *cho2* by disrupting the *CHO2* gene *in vivo*. The *cho2* disruptant, like all other *cho2* mutants, is viable, exhibits altered regulation of inositol biosynthesis and is not auxotrophic for choline or MME.

PHOSPHATIDYLCHOLINE (PC) is the major phospholipid component of most eukaryotic cell membranes. The yeast, *Saccharomyces cerevisiae*, like other eukaryotes, synthesizes PC using two different pathways (Figure 1). PC is synthesized from phosphatidylethanolamine (PE) via three sequential methylations of PE (WAECHTER and LESTER 1971). If free choline is present in the growth medium, yeast preferentially utilize it to synthesize PC directly by the CDP-choline pathway first described by KENNEDY and WEISS (1956).

There has been some difficulty in determining the number of phospholipid methyltransferases in mammalian cells (MATO, PAJARES and VARELA 1984; PELECH and VANCE 1984). This difficulty may in part be due to the problems inherent in studying these membrane associated enzymes *in vitro* (AUDUBERT and VANCE 1983). Recently, however, RIDGWAY and VANCE (1987) reported the complete purification from rat liver of an enzyme capable of methylating PE to form PC. Analysis of *Neurospora crassa* mutants auxotrophic for choline suggest that this species has two phospholipid methyltransferases, one that methylates PE to form phosphatidylmonomethylethanolamine (PMME) and another that carries out the next two methylation reactions converting PMME to PC (SCARBOROUGH and NYC 1967). *S. cerevisiae* mutant, *opi3-3*, isolated by GREENBERG, REINER and HENRY (1982) corresponds biochemically to the *Neurospora* mutant defective in the methyltransferase that converts PMME to PC (GREENBERG *et al.* 1983). The *opi3* mutant, however, is not a choline auxotroph. YAMASHITA *et al.* (1982) have reported the isolation of two yeast methyltransferase mutants as choline auxotrophs. Repeated attempts in this laboratory to isolate phospholipid methyltransferase mutants as choline auxotrophs, however, have yielded only mutants at the *CHO1* locus, which encodes phosphatidylserine synthase (LETTS and HENRY 1985). In this report we describe the isolation and characterization of several allelic yeast mutants defective specifically in the methylation reaction that converts PE to PMME. These mutants define a locus, *CHO2*, that appears to encode a PE specific methyltransferase. None of the *cho2* alleles, including a *cho2* null allele constructed by gene disruption, are auxotrophic for any soluble phospholipid precursor.

WAECHTER and LESTER (1971, 1973) have shown that the cellular activities of the yeast phospholipid methyltransferases are repressed by choline in the growth medium. YAMASHITA and OSHIMA (1980)

[1] Present address: Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912.
[2] Present address: Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213.

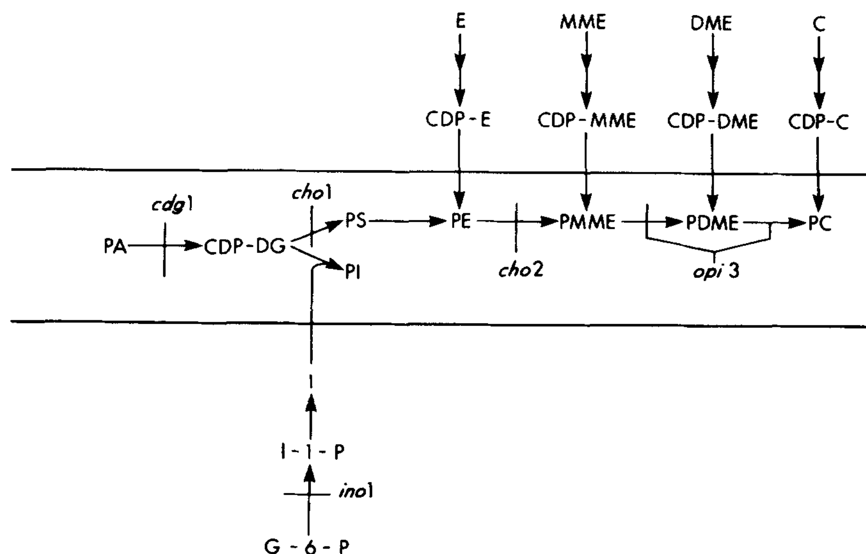

FIGURE 1.—Pathways of phospholipid biosynthesis in *S. cerevisiae*. The membrane associated reactions responsible for *de novo* phospholipid biosynthesis are shown in the space between the two lines (STEINER and LESTER 1972). The three step reaction series converting PE to PC is carried out by the phospholipid *N*-methyltransferases. The reactions shown above the top line represent incorporation of exogenously supplied precursors into their respective phospholipids as described by KENNEDY and WEISS (1956). The positions of genetic lesions affecting specific reactions are indicated by lines intersecting arrows. Abbreviations: E, ethanolamine; MME, monomethylethanolamine; C, choline; PA, phosphatidic acid; CDP-DG, CDP-diacylglycerol; PS, phosphatidyl-serine; PE phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; I, inositol; I-1-P, inositol-1-phosphate; G-6-P, glucose-6-phosphate.

showed that the presence of inositol, the soluble precursor of phosphatidylinositol (PI), is also required for repression of the methyltransferases. Cellular levels of the transcript of the *INO1* gene which encodes the enzyme responsible for *de novo* inositol biosynthesis, inositol-1-phosphate synthase (I-1-PS), are repressed in large part by the presence of inositol in the growth medium but are fully repressed only if choline is also present in the medium (HIRSCH and HENRY 1986). Thus, enzymes involved in the *de novo* biosynthesis of both PC and PI are coordinately regulated (HENRY, KLIG and LOEWY 1984). At least three genes, INO2, INO4 (DONAHUE and HENRY 1981; LOEWY and HENRY 1984) and OPI1 (GREENBERG, GOLDWASSER and HENRY 1982; KLIG *et al.* 1985) are known to be involved in this coordinate regulation. In this report we show that *cho2* methyltransferase mutants excrete inositol if grown on medium lacking inositol and either monomethylethanolamine (MME) or choline and that cellular I-1-PS levels are not repressed at all by inositol unless MME or choline is also present.

MATERIALS AND METHODS

Strains, media and growth conditions: Genotypes and sources of yeast strains used are listed in Table 1. Routine culture and maintenance of yeast strains was carried out in YEPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose) and on YEPD plates (YEPD medium with 2% agar added). All growth and labeling experiments were done using synthetic defined medium containing: 2% glucose, 0.67% Difco Yeast Nitrogen Base (YNB) without amino acids, and supplements (amino acids, adenine and uracil) as described by CULBERTSON and HENRY (1975). Defined medium was brought to 75 μM inositol and supplemented to 1 mM ethanolamine (E), 1 mM MME, or 1 mM choline where indicated. Plasmid bearing strains were always grown in defined medium lacking leucine. Plates contained 2% agar in addition to the above components. Plates used to score specific auxotrophies were made as defined medium lacking a single component. Inositol-free medium was prepared similarly to synthetic defined medium except that Difco Vitamin Free Yeast Nitrogen Base was substituted for YNB without amino acids and vitamins were added as described by GREENBERG, REINER and HENRY (1982). Acetate plates containing 1% potassium acetate, 0.1% yeast extract, 0.1% glucose, 2% agar, and 1 mM choline were used to induce sporulation of diploid yeast for genetic analysis.

Escherichia coli HB101 (*hsdS recA13 supE44 lacZ4 leuB6 proA2 thi1-1* Str^r) was grown in LB medium.

Mutant isolation: Two different screening methods were employed to isolate mutants. *First screening*: The wild-type strain SHID5C was mutagenized with ethylmethane sulfonate (EMS) according to the method of LINDEGREN *et al.* (1965). The mutagenized cells were recovered in rich medium, shifted to defined medium for several hours and then layered onto a continuous Renographin density gradient as described by LETTS and DAWES (1979). The use of a density enrichment for mutants defective in membrane synthesis is based on the observation of HENRY *et al.* (1977) that inositol auxotrophs when deprived of inositol become denser than wild-type cells grown under the same conditions. After centrifugation, denser cell fractions were plated onto medium containing MME. Upon the appearance of colonies these plates were replicated to plates lacking and plates containing MME to screen for MME auxotrophs. *Second screening*: The wild-type strain (*ade5 MAT*α) was mutagenized with EMS as above. Mutagenized cells were recovered and spread onto YEPD plates to give approximately 250 colonies per plate after several days incubation. The colonies were replica plated to inositol-free medium, incubated overnight, and sprayed with the inositol indicator strain (AID-1). Inositol excretion was scored using the cross feeding assay developed by GREENBERG, REINER and HENRY (1982) and modified by GREENBERG *et al.* (1983). Inositol-excreting colonies, detected by the presence of a red halo, were selected for further study. Strains isolated in this manner which proved to excrete inositol only in the absence of MME or choline were considered potential *cho2* mutants and were complementation tested by crossing with *cho2-1* tester strains.

Growth curves: Precultures in defined medium were inoculated using washed cells from YEPD medium and grown for at least five generations. Precultured cells were washed twice in defined medium and used to inoculate cultures in synthetic medium containing added supplements

TABLE 1

Yeast strains

Designation	Genotype	Source
Wild type	*MAT***a** *ade5*	This laboratory
SHID5C	*MATα ura1 ade6*	This laboratory
VAL572	*MATα cho1-9 cho2-1 ade6 ura1*	This study
VALC3B	*MAT***a** *chol-9*	This study
VALC3D	*MATα cho2-1 lys2 ade6*	This study
VALC6B	*MAT***a** *cho2-1 lys2*	This study
ES1	*MAT***a** *cho2-1 leu2-3,112 lys2*	This study
ES02C	*MAT***a** *cho2-1 ade2 his3 ura3 leu2*	This study
NO37	*MATα cho2-37 ade5*	This study
NO134	*MATα cho2-134 ade5*	This study
NO137	*MATα cho2-137 ade5*	This study
NO143	*MATα cho2-143 ade5*	This study
OP3	*MAT***a** *opi3-3 ade5*	GREENBERG, REINER and HENRY (1982)
ES9	*MAT***a** *cho2-1 opi3-3 leu2-3,112 ade6*	This study
LK1	*MAT***a** *cdg1-1 ade5*	KLIG *et al.* (1988)
ES12	*MAT***a** *cho2-1 cdg1-1 leu2-3,112 ura3 trp1*	This study
JH6d	*MAT***a** *opi1-1 leu2 ura3 ade5*	J. HIRSCH
ESI-1b	*MAT***a** *cho2-1 opi1-1 leu2 ura3 lys2 ade5 ade6*	This study
BL2	*MATα ino2-2 thr1*	B. LOEWY
ESIII-3d	*MATα cho2-1 ino2-2 thr1*	This study
DKH325	*MAT***a** *ino4-39 leu2 ura3 trp1*	D. HOSHIZAKI
ESII-1b	*MATα cho2-1 ino4-39 ura3*	This study
ESXI-1(4d)	*MAT***a** *cho2-Δ::LEU2 leu2 ura3 lys2 ade6*	This study
diploids:		
ES-DC2	*MAT***a***/MATα cho2-1/cho2-137 leu2/+lys2/+ade5/+*	This study
AID-1	*MAT***a***/MATα ino1-13/ino1-13 ade1/ade1 lys2/lys2*	GREENBERG, REINER and HENRY (1982)
ESXI	*MAT***a***/MATα cho2-1/+ leu2/leu2 ade2/+ade6/ +his3/+lys2/+ura3/+*	This study
ESXI-1	*MAT***a***/MATα cho2-1/cho2-Δ::LEU2 leu2/leu2 ade2/ +ade6/+his3/+lys2/+ura3/+*	This study

as indicated. Growth was monitored by measuring the optical density of cultures using a Klett spectrophotometer and cell number was determined using a hemacytometer. Percent viable cells was estimated by staining diluted cultured samples with the vital dye Magdala red (1 mg/ml) and counting red stained (dead) and pale stained (viable) cells on a hemacytometer.

Genetic analysis: Genetic analysis was carried out using standard genetic procedures (SHERMAN, FINK and LAWRENCE 1978).

Linkage relationships of the *cho2* mutation with other mutations affecting PC biosynthesis were tested by performing tetrad analysis. All progeny derived from complete tetrads from all crosses were complementation tested against *cho2-1* by crossing to suitable tester strains and scoring growth on defined medium. *cho2/cho2* diploids grow poorly in the absence of MME and sporulate poorly even in the presence of choline or MME. All segregants were also tested for growth and inositol excretion on inositol-free medium lacking or containing various supplements. It must be noted that the inositol excretion phenotypes of some mutant segregants (especially *cho2* and *opi3*) are difficult to score: excretion halos vary considerably in intensity and are sometimes missing. Thus, while false positives are rare, the absence of an excretion halo is not conclusive evidence that a given mutation is absent. Because of this limitation other tests including *in vivo* labeling of phospholipids were carried out. The specific crosses and the differentiating characteristics of the progeny types of each cross were as follows: (1) *cho2-1 cho1-9* × wild-type (VAL572 × *MATa lys2*): All progeny of this cross were pulse labeled *in vivo* with [*methyl*-^{14}C] methionine in the presence of ethanolamine to identify the presence of the *cho2-1* allele. Absence of growth on plates lacking ethanolamine indicated the presence of the *cho1* mutation. (2) *cho2-1 opi3-3* × wild-type (ES9 × *MATα thr1*):*cho2* and *opi3* mutants are generally distinguishable on the basis of inositol excretion phenotypes. *cho2 opi3* double mutants are MME-choline auxotrophs. Ambiguous tetrads were pulse labeled with [*methyl*-14C]methionine. (3) *cho2-1* × *opi3-3* (ES02C × *MATα opi3-3 lys2*): Scoring of tetrads same as in cross 2 above. (4) *cho2-1* × *opi1-1* (VALC3D × JH6d): This was the most difficult cross to score since none of the progeny have any growth phenotype and pulse labeling does not distinguish the *cho2* strains from the *cho2 opi1* strains. However, the dependability of the inositol excretion phenotype of *opi1* was helpful. All progeny containing *cho2* were detected by complementation testing. Inositol excreters not containing a *cho2* mutation were designated *opi1*. Segregants containing *cho2* which excreted inositol in the presence of choline were designated *cho2 opi1*. The presence of *opi1* in several tetrads had to be determined by crossing to *opi1* tester strains and scoring inositol excretion by the diploids. (5) *cho2* × *ino4* (VALC3D × DKH325): *ino4* containing progeny were scored as inositol auxotrophs on inositol-free medium and *cho2 ino4* progeny were identified as weak MME auxotrophs on defined medium. (6) *cho2* ×

ino2 (VALC6B × BL2): Same as (5) above except that *cho2 ino2* progeny were not auxotrophic for MME.

Immunoprecipitation of inositol-1-phosphate synthase: Relative quantity of the 62,000 D subunit of inositol-1-phosphate synthase was tested by immunoprecipitation of the subunit from crude extracts using rabbit anti-inositol-1-phosphate synthase antisera as described by DONAHUE and HENRY (1981).

***In vivo* labeling conditions:** Cultures were labeled with [*methyl*-^{14}C]-methionine (5.75 mCi/mmole), [^{14}C]ethanolamine (4.0 mCi/mmole), or carrier free [^{32}P]orthophosphate (all isotopes: New England Nuclear). Pulse labelings of 30-min duration with [*methyl*-^{14}C]methionine were carried out as described by LOEWY and HENRY (1984). To pulse label cells with [^{14}C]ethanolamine 10 ml cultures were incubated for 60 min after the addition of 5 μCi of [^{14}C]ethanolamine. The pulse-labeled cultures were washed with defined medium and split into two 5-ml samples: lipid was extracted from one 5-ml sample immediately, and the other 5-ml sample was incubated in defined medium without label for an additional 2-hr chase period. The [^{14}C]ethanolamine- and [^{14}C]methyl-labeled lipids were extracted and separated by one-dimensional paper chromatography.

Steady state labeling with [^{32}P]orthophosphate was performed following the method of ATKINSON, FOGEL and HENRY (1980). Cells were labeled for at least five generations and harvested in late log phase if they were viable in the labeling medium. Several mutant strains lost viability in certain labeling media and therefore could not be labeled for very long periods. Such inviable strains were initially inoculated into media permissive for growth, labeled for 4–6 hours, washed, resuspended in the indicated labeling medium maintaining label at the initial specific activity, and labeled for an additional 12 hr or until approximately 50% viable cells remained—whichever came first. ^{32}P-labeled phospholipids were extracted from labeled cells and separated by two-dimensional chromatography.

Lipid extraction and chromatography: Lipids were extracted as described by ATKINSON, FOGEL and HENRY (1980). Two-dimensional paper chromatography on silica impregnated paper was carried out using the method of STEINER and LESTER (1972). One-dimensional paper chromatograms were carried out using the method of WAECHTER and LESTER (1971). One-dimensional thin layer chromatograms (using silica gel coated aluminum plates from Whatman) were run in a solvent system containing: $CHCl_3$, CH_3OH, NH_4OH, and H_2O at a ratio of 165:67.5:7.5:2. The positions of radioactively labeled lipids on the chromatograms were determined by autoradiography. Labeled spots corresponding to specific lipids were removed and counted by liquid scintillation.

***In vitro* methyltransferase assays:** Phospholipid methyltransferase activities were assayed using crude membrane preparations by a modification of the technique described by WAECHTER and LESTER (1973). Yeast cultures were grown to late log phase in defined medium containing supplementation where indicated. Cells were harvested at 4° and all subsequent steps were carried out at 0–5°. Harvested cells suspended in a sucrose-phosphate buffer (0.2 M sucrose; 50 mM KH_2PO_4-KOH pH 7.2) were broken using a CO_2 cooled Braun homogenizer. Whole cells and glass beads were removed by centrifugation, and a crude membrane fraction was recovered by centrifugation at 40,000 rpm for 40 min. The resulting pellet was suspended in sucrose-phosphate buffer to yield a total protein concentration of 1–2 mg per ml. Phospholipid methyltransferase activities were assayed at 30° in 3-ml reaction mixtures containing: 60 mM sucrose, 15 mM KH_2PO_4-KOH pH 7.2, 100 μg/ml *S*-adenosyl-L-methionine (Sigma No. AA-7007), 20 μCi *S*-[*methyl*-^{3}H]-adenosylmethionine, and 1 ml crude membrane preparation. At specific time points 0.5 ml of each reaction mixture was removed and stopped by adding to 3 ml of a 2:1 mixture of chloroform:methanol and vortexing. The organic phase was utilized to determine the relative incorporation of methyl groups into each methylated phospholipid by separation on one-dimensional paper chromatograms.

Transformation of *S. cerevisiae* and screening of genomic library: A modification of the alkali cation technique of ITO *et al.* (1983) was used to transform yeast with plasmid DNA. Mitotic instability of plasmids in transformants of interest was tested by spreading a given transformant for single colonies on rich medium and then replicating to selective medium.

A yeast genomic library (a gift from J. HILL) made by ligating *Sau*3a partially digested genomic DNA into the *Bam*HI site of the vector YEp13 was screened for the presence of *cho2* complementing sequences. Approximately 8000 LEU$^+$ transformants of the strain ES12 were screened for MME prototrophy.

DNA preparation and fragment purification: Plasmid DNA was isolated from yeast following the method of STRUHL *et al.* (1979). Plasmid thus purified was used to transform *E. coli* (HB101) by the method of MANDEL and HIGA (1970). The technique of HOLMES and QUIGLEY (1981) was used for small scale preparation of plasmid DNA from *E. coli*. For large scale plasmid purification the CsCl equilibrium gradient method of CLEWELL and HELINSKI (1969) was employed. To isolate a given specific DNA fragment, purified plasmid DNA was digested with restriction enzyme following vendor (Bethesda Research Labs Inc.) specifications and run on a polyacrylamide gel. The ethidium bromide band corresponding to the desired DNA fragment was cut from the gel, crushed, and eluted.

Plasmid constructions: To facilitate restriction analysis 6.6 kb of YEp13 vector sequence was removed from pES21 (the insert of which contains no *Eco*RI sites) by digesting with *Eco*RI, heat inactivating the enzyme, and religating using T4 DNA ligase (Collaborative Research, Inc.). The resulting plasmid, pES21-322, contained the 3.6-kb pES21 insert in the *Bam*HI site of pBR322. The *Eco*RI and *Sal*I sites flanking the *Bam*HI site of pBR322 were utilized to clone the *cho2* complementing insert into the multiple cloning sites of the vectors pGEM1 and pGEM2 (Promega Biotech). A 4-kb insert containing fragment derived from pES21-322 by complete digestion with *Eco*RI and partial digestion with *Sal*I was gel purified and ligated with *Eco*RI-*Sal*I linearized pGEM1 and pGEM2 to generate pES101 and pES102 respectively. To construct a DNA fragment suitable for use in a one-step gene disruption of the *CHO2* gene a 0.95-kb *Hin*dIII-*Eco*RV fragment from the central region of the *cho2* complementing insert was subcloned into the *Hin*dIII-*Hin*cII sites of the pGEM2 multiple cloning site. The resulting plasmid, pES110, was linearized at the central *Sal*I site of the insert to accommodate a 1.2-kb *LEU2* gene containing *Sal*I-*Xho*I fragment derived from YEp13. The chimeric insert of this new plasmid (pES111f) was excised from pGEM2 multiple cloning site by *Hin*dIII-*Xba*I digestion.

Southern blot analysis: Yeast genomic DNA was digested to completion with the restriction enzyme *Eco*RV, separated by agarose gel electrophoresis, transferred to nitrocellulose and analyzed by hybridization (SOUTHERN 1975). Probes were labeled by nick translation using the method of RIGBY *et al.* 1977.

RNA isolation and Northern blot analysis: Yeast were

grown to mid log phase in defined medium, and RNA was isolated using the hot phenol extraction method of ELION and WARNER (1984). The RNA was size fractionated using the system of ROZEK and DAVIDSON (1983) and then transferred to nitrocellulose (THOMAS 1980). The *CHO2* gene containing plasmids pES101 and pES102 were used to generate ^{32}P-labeled single stranded RNA probes using the enzyme SP6 polymerase (NEN Research Products) as described by MELTON *et al.* (1984). The plasmid pAB309 (BAILIS *et al.* 1987) was used to generate single stranded RNA probe complementary to ribosomal protein TCM1 (FRIED and WARNER, 1981) mRNA. The resulting probes were hybridized to Northern blots at 51° overnight in 50% formamide containing 0.9 M sodium chloride and 90 mM sodium citrate.

RESULTS

***cho2-1* mutant isolation and preliminary analysis:** Past efforts to identify mutations affecting PC biosynthesis by screening for choline auxotrophs led, in our hands, only to the isolation of mutations in the *CHO1* gene (LETTS and HENRY 1985). The choline growth requirement of *cho1* mutants, which are defective in the enzyme PS-synthase (ATKINSON, FOGEL and HENRY 1980; KOVAC *et al.* 1980), is equally well satisfied by ethanolamine, the soluble precursor of PE. *cho1* mutants may thus be referred to as choline-ethanolamine auxotrophs. Hypothetically, it would seem possible to isolate phospholipid methyltransferase mutants as choline-MME, choline-DME or strict choline auxotrophs. In an attempt to isolate methyltransferase mutants as auxotrophs we screened colonies derived from an EMS mutagenized culture for the ability to grow in the presence of MME or choline but not in the presence of ethanolamine. In addition, prior to screening, the mutagenized culture was enriched by isolating dense cell fractions from a centrifugal density gradient (described in MATERIALS AND METHODS). Utilizing this selection scheme we isolated one mutant strain, VAL572, which grew better with MME supplementation than with ethanolamine supplementation. However, meiotic segregation of the MME-choline auxotrophy following a cross to wild-type did not show the 2+:2− pattern expected for a single gene defect. While approximately one half of the meiotic segregants were phenotypically wild-type (that is, they were not auxotrophic for MME or choline), the remainder were a mixture of MME-choline and ethanolamine-choline auxotrophs. The ethanolamine-choline auxotrophic segregants failed to complement existing *cho1* alleles. These results suggested that the VAL572 strain contained a *cho1* mutation and a second mutation which acts to restrict the ethanolamine-choline auxotrophy normally caused by lesions in the *CHO1* gene. To test this possibility progeny from five complete tetrads from the VAL572 cross to wild-type were examined for the ability to incorporate labeled methyl groups from [*methyl*-^{14}C]methionine into specific phospholipids when grown in the presence of ethanolamine. The ethanolamine-choline auxotrophic progeny incorporated wild-type levels of *methyl*-^{14}C into methylated phospholipids while the MME-choline auxotrophs and about one half of the non-auxotrophic progeny incorporated reduced levels (data not shown). When the nonauxotrophic but methylation deficient segregants were incubated with [*methyl*-^{14}C]methionine in the presence of MME, normal levels of incorporation into PDME and PC were observed. This result suggested that in addition to a new mutant allele of *cho1* (now designated *cho1-9*) the VAL572 strain carried a second mutation, which we designate *cho2-1*, that caused a specific deficiency in the methylation of PE.

TABLE 2

Plate phenotypes[a]

Relevant genotype	Media supplement[b]: Growth			Inositol excretion			
	None	E	MME	None	E	MME	C
Wild type	+	+	+	−	−	−	−
cho2-1	+	+	+	+	+	−	−
cho2-1 (pES21)	+	+	+	−	−	−	−
cho2-1/cho2-137	−/+	−/+	+	+/−	+/−	−	−
cho2-1/CHO2	+	+	+	−	−	−	−
cho2-Δ::LEU2	+	+	+	+	+	−	−
cho1-9	−/+	+	+	+/−	−	−	−
cho2-1 cho1-9	−	−/+	+	−	+	−	−
opi3-3	+	+	+	+	+	+	−
cho2-1 opi3-3	−/+	−/+	+	+/−	+/−	+	−
cho2-1 opi3-3 (pES21)	+	+	+	+	+	+	−
cdg1-1	+	+	+	++	++	++	++
cho2-1 cdg1-1	−	−	+	+/−	−	++	++
cho2-1 cdg1-1 (pES21)	+	+	+	++	++	++	++
cho2-1 opi1-1	+	+	+	++	++	+	+8
ino2-2	+	+	+	—[c]			
cho2-1 ino2-2	+	+	+	—[c]			
ino4-39	+	+	+	—[c]			
cho2-1 ino4-39	−/+	−/+	+	—[c]			

[a] Relative growth of replicated patches was scored after a 1-day incubation. The same replicated patches were scored for inositol excretion as described in the legend for Figure 4.

[b] E, 1 mM ethanolamine; MME, 1 mM monomethylethanolamine; and C, 1 mM choline.

[c] These four strains are inositol auxotrophs and cannot be tested for inositol excretion.

Growth of the *cho2-1* mutant: In spite of a grossly altered membrane phospholipid composition (see below) *cho2-1* strains showed no deficit compared to wild-type in growth on plates under any condition. In particular, the *cho2-1* mutant was not auxotrophic for choline or MME (Table 2). However, after 5–6 generations of growth in liquid defined medium lacking choline or MME supplementation, *cho2-1* strains did begin to grow more slowly than wild-type (Figure 2). In the absence of supplementation the *cho2-1* strain continued to grow indefinitely at this slowed growth rate without loss of viability. Addition of MME or choline to the growth medium restored growth to a wild-type rate; ethanolamine had no effect.

Phospholipid composition analysis of the *cho2-1*

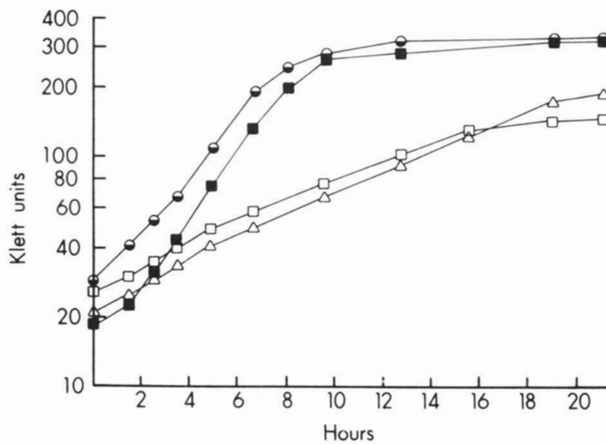

FIGURE 2.—Growth of wild-type and *cho2-1* mutant cells in various media. Cells were precultured in defined medium lacking phospholipid precursors for five to six generations prior to inoculation into indicated growth medium. Wild-type cells grown in defined medium with or without 1 mM MME supplementation (◒). *cho2-1* cells grown in defined medium containing: 1 mM MME (■), 1 mM ethanolamine (□), or no supplement (△).

mutant: Cells were labeled to steady state with [^{32}P]-orthophosphate to determine the effect of the *cho2-1* mutation on membrane phospholipid composition. The autoradiogram of separated phospholipids in Figure 3 illustrates the altered membrane composition of *cho2-1* cells as compared to wild-type. The relative percentages of the cellular phospholipids are shown in Table 3. The percentage of PC, the major phospholipid in wild-type cells, was reduced more than four fold in *cho2-1* cells while the percentage of PE was elevated three-fold over wild-type levels and thus became the major phospholipid in *cho2-1* cells. The methylated intermediates PMME and phosphatidyldimethylethanolamine (PDME), while detectable in wild-type, were undetectable in the *cho2-1* mutant grown in the absence of MME. When MME or choline is present in the growth medium of *cho2* cells, PC levels increased while PE levels were reduced, presumably due to restored regulation of overall phospholipid biosynthesis. PMME produced from exogenous MME via the Kennedy pathway is detected in both wild-type and *cho2* cells grown in medium containing MME. The phospholipid compositions of wild-type and *cho2-1* cells grown in the presence of MME were essentially identical (Table 3).

Incorporation of methyl groups and ethanolamine into phospholipids in *cho2-1* cells: To examine more closely the metabolic defect in the *cho2-1* mutant, cells were pulse labeled with [*methyl*-^{14}C]methionine or [^{14}C]ethanolamine, and the incorporation of label into various phospholipids was quantitated. Table 4 shows the incorporation of methyl groups derived from [*methyl*-^{14}C]methionine into the methylated phospholipids in a 30-min pulse labeling, and Figure 4 shows the corresponding one-dimensional chromatograms. *cho2-1* cells grown in defined medium or in defined medium containing ethanolamine incorporated very few labeled methyl groups into PMME, PDME or PC in contrast to wild-type cells grown in the same media. Addition of MME to the growth medium resulted in greatly increased incorporation of methyl groups into PDME and PC but not into PMME in *cho2-1* cells.

Most of the labeled ethanolamine incorporated into PE in a one-hour pulse labeling of wild-type cells was chased into PC during a 2-hr chase period (Figure 5). Incorporation into PMME and PDME was very low indicating that these phospholipids are only transient intermediates in the synthesis of PC. In *cho2-1* cells, labeled ethanolamine was incorporated into PE at an even higher rate than in wild-type cells but much less was chased into PC. Virtually no label was found in the two methylated intermediates in *cho2-1* cells. It is important to note, however, that PC was synthesized from PE at a reduced but detectable level in *cho2-1* cells.

***In vitro* analysis of phospholipid methylation:** To observe directly the enzymatic defect in *cho2* cells, we measured the incorporation of labeled methyl groups from *S*-[*methyl*-^{3}H]-adenosyl-methionine into the three methylated phospholipids (PMME, PDME and PC) *in vitro* as described in MATERIALS AND METHODS. Wild-type membranes incorporated tritiated methyl groups into all three of the methylated phospholipids. Label accumulated in PDME and PC throughout the 20-min labeling period while incorporation into PMME leveled off within 5 min (Figure 6). This suggests that

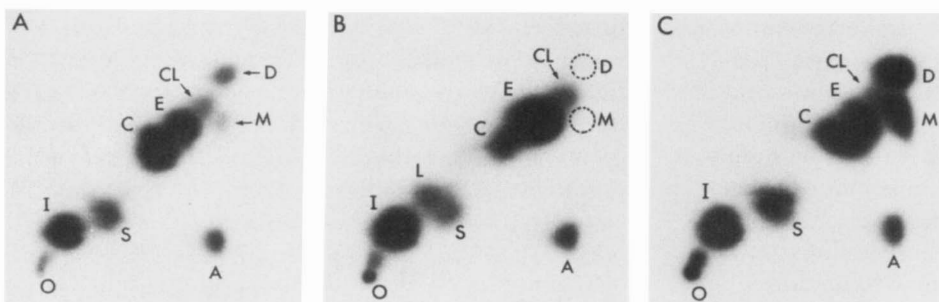

FIGURE 3.—Lipids of wild-type and *cho2-1* cells. Autoradiograms of ^{32}P steady-state labeled phospholipids separated by two-dimensional paper chromatography. A, wild-type cells grown in defined medium; B, *cho2-1* cells grown in defined medium; C, *cho2-1* cells grown in defined medium supplemented with 1 mM MME. Labels (see Figure 1 legend): O, orgin; I, PI; S, PS; L, lyso-PE; A, PA; C, PC; E, PE; CL, cardiolipin; M, PMME; and D, PDME.

TABLE 3

Phospholipid compositions of mutant and wild-type strains

Relevant genotype	Media supplement[b]	Phospholipids (%)[a]							
		PA	PI	PS	PE	PMME	PDME	PC	Other
Wild type		3.3	21.8	6.6	17.7	0.8	2.9	42.2	4.7
Wild type	MME	2.9	20.7	6.9	13.5	11.9	17.9	19.0	7.2
cho2-1		3.5	24.8	4.1	51.0	ND[c]	ND	9.1	7.5
cho2-1	MME	3.8	22.6	8.2	13.0	11.2	13.1	20.1	8.0
cho2-1 (pES21)		4.8	17.9	7.2	26.5	3.4	0.9	30.9	8.4
cho2-134		3.2	28.6	5.5	42.7	ND	ND	10.4	9.6
cho2-137		3.8	32.9	5.4	39.7	ND	ND	9.9	8.3
cho2-Δ::LEU2		4.1	27.3	5.2	49.6	ND	ND	6.8	7.0
cho2-1/cho2-137[d]		3.3	27.8	4.7	48.2	ND	ND	11.3	4.7
opi3-3		3.4	17.3	1.9	9.2	44.3	15.5	3.7	4.7
cho2-1 opi3-3[d]		3.4	26.3	2.8	53.9	ND	ND	5.1	8.5
ino2-2		2.9	30.1	7.0	28.3	6.2	8.7	11.5	5.3
cho2-1 ino2-2		3.1	33.8	6.3	44.0	ND	ND	4.2	8.6
ino4-39		2.6	30.6	3.7	34.0	2.3	4.4	12.9	9.5
cho2-1 ino4-39[d]		3.0	34.2	7.3	44.2	ND	ND	2.7	8.6
cho1-9[d]		5.1	37.9	ND	5.6	ND	0.6	44.9	5.9
cho2-1 cho1-9[d]		2.3	61.0	ND	9.2	ND	ND	20.4	7.1
cdg1-1		5.0	12.4	8.0	14.7	ND	0.9	53.7	5.3
cho2-1 cdg1-1[d]		6.8	15.8	9.2	41.2	ND	ND	16.3	10.7

[a] Relative steady state percentages of each phospholipid were determined after labeling with (^{32}P)orthophosphate as described in the MATERIALS AND METHODS. Phospholipid abbreviations are as indicated for Figure 1. The designation "other" includes polar lipids migrating at the origin, phosphatidylglycerol, cardiolipin and lysophospholipids.
[b] MME, 1 mM monomethylethanolamine.
[c] ND, not detectable.
[d] These strains lost viability; therefore, they were not labeled to absolute steady state.

PMME is a transient intermediate in the *de novo* synthesis of PC. *cho2-1* membrane fractions also incorporated tritiated methyl groups into PC, but at a reduced rate. There was no detectable accumulation of label in the intermediates PMME and PDME. The reduced rate of phospholipid methylation in *cho2-1* membranes cannot be due to a lowered substrate concentration because PE comprises 50% of the phospholipid in *cho2-1* cells. Addition of MME to the growth medium of *cho2-1* cells increased the *in vitro* incorporation rate of labeled methyl groups into PDME and PC to essentially wild-type levels. However, there was no observed incorporation into PMME in the same membranes.

Inositol excretion phenotype of *cho2* mutants: The *cho2-1* mutant excretes inositol into the growth medium if grown in the absence of MME or choline (Figure 7 and Table 2). Immunoprecipitation of the major enzyme involved in inositol biosynthesis, I-1-PS, from cells grown in different media showed that I-1-PS enzyme levels were repressed by inositol in *cho2* cells only if MME or choline were present (Figure 8). The presence of ethanolamine failed to restore regulation of I-1-PS in response to inositol.

Isolation of additional *cho2* alleles as inositol excreters: Utilizing the unique inositol excretion phenotype of *cho2* mutant cells we isolated four new alleles of *cho2*: *cho2-37*, *cho2-134*, *cho2-137*, and *cho2-143*. Each of the new alleles closely resembled the original *cho2-1* allele both phenotypically and biochemically. All four mutants grew well and excreted inositol on solid media lacking MME or choline. Pulse labeling with [*methyl*-^{14}C]methionine demonstrated that each of these new *cho2* mutants was equally deficient in PE methylation (Table 4; Figure 4B). The membrane phospholipid compositions of *cho2-134* and *cho2-137* were also very similar to that of the *cho2-1* mutant (Table 3).

Interestingly, when these new isolates were complementation tested against *cho2-1* tester strains, a new *cho2* mutant phenotype was uncovered: *cho2/cho2* diploid strains grew poorly on plates in the absence of MME or choline supplementation. Figure 7B shows photographs of two plates illustrating this *cho2/cho2* diploid growth requirement for several *cho2* allele combinations. Also, unlike *cho2* haploid cells, homozygous *cho2/cho2* diploid cells grew very slowly and gradually lost viability in unsupplemented liquid defined medium (not shown). However, the membrane phospholipid composition of a *cho2-1/cho2-137* diploid strain was not substantially different from that of the haploid *cho2* mutant strains (Table 3). This homozygous diploid phenotype proved very useful in detecting the presence of a *cho2* mutation in any given strain.

The *cho2* mutation is not closely linked to other mutations affecting PC biosynthesis: A number of

TABLE 4

[*methyl*-^{14}C]methionine pulse labelings

Relevant genotype	Media supplement[b]	cpm/10^6 cells incorporated into each phospholipid[a]			
		PMME	PDME	PC	NL[c]
Wild type		42	179	386	163
Wild type	E	57	201	356	176
Wild type	MME	40	251	244	165
cho2-1		3	11	16	220
cho2-1	E	3	11	15	242
cho2-1	MME	5	142	132	221
cho2-37		2	6	43	218
cho2-37	MME	3	214	246	206
cho2-134		3	6	57	235
cho2-134	MME	2	135	180	183
cho2-137		2	5	43	210
cho2-137	MME	2	117	160	151
cho2-143		1	4	40	244
cho2-143	MME	3	182	190	164
cho2-1 (YEp13)		2	7	96	143
cho2-1 (pES19)		4	18	289	233
cho2-1 (pES20)		50	196	733	526
cho2-1 (pES21)		34	122	308	250
cho2-1 (pES22)		36	120	327	268
opi3-3		207	150	50	240
cho2-1 opi3-3		3	13	2	171
cho2-1 opi3-3 (pES21)		171	137	53	234

[a] cpm incorporated into each phospholipid per 10^6 cells in a 30 min pulse were determined as described in MATERIALS AND METHODS.

[b] E, 1 mM ethanolamine; MME, 1 mM monomethylethanolamine.

[c] NL, neutral lipids.

mutations that have effects on PC biosynthesis have been characterized (for review see HENRY, KLIG and LOEWY 1984). Crosses were performed in which the segregation of the *cho2-1* mutation could be scored with respect to the segregation of these other mutations (Table 5). Spore viability was poor in these crosses, thus the number of tetrads scored was somewhat low. However, in each case only complete tetrads were used, and all segregants were examined exhaustively to confirm their genotype (see MATERIALS AND METHODS). The table shows that the majority of tetrads resulting from each cross are of the recombinant type; therefore, the *CHO2* locus is not closely linked to any of the other loci defined by the mutations present in these crosses.

Double mutants containing *cho2-1*: In the course of carrying out crosses between *cho2* mutant strains and other phospholipid mutants, double mutant strains were generated that had novel phospholipid compositions (Table 3) and exhibited new phenotypes (Table 2). Some double mutant combinations were auxotrophic or partially auxotrophic for choline whereas neither of the single mutants alone had such phenotypes. For example, while neither haploid *cho2* mutants or *opi3* mutants were auxotrophic for choline or showed a substantial loss of viability within 24 hr following a shift to medium lacking choline, the *cho2 opi3* double mutant grew markedly better if supplemented with choline. Furthermore, strains of this genotype exhibited a decrease in viable cells to 60% after a 24-hr incubation in medium lacking choline. *cho2 opi3* mutant cells grown in unsupplemented medium contained high levels of PE (53%) and very low levels of methylated phospholipid (5%) at the time when growth ceased. (As explained in MATERIALS AND METHODS the labeling procedure used for strains which were being starved for an essential growth factor may not yield a true steady-state labeling of the phospholipids). The *cho2 ino4* double mutant was clearly auxotrophic for MME or choline as well as inositol and lost viability when shifted to synthetic medium lacking both of these precursors (40% viable cells after 24 hr). The viability loss of *cho2 ino4* cells coincided with a major decrease in the level of PC to about 3%. However, the *cho2 ino2* double mutant which contained only about 4% PC was not auxotrophic for MME or choline. The *cho2 cdg1* double mutant strain has a strict requirement for MME or choline as described previously by KLIG *et al.* (1988). This strain had a less severe drop in PC levels than

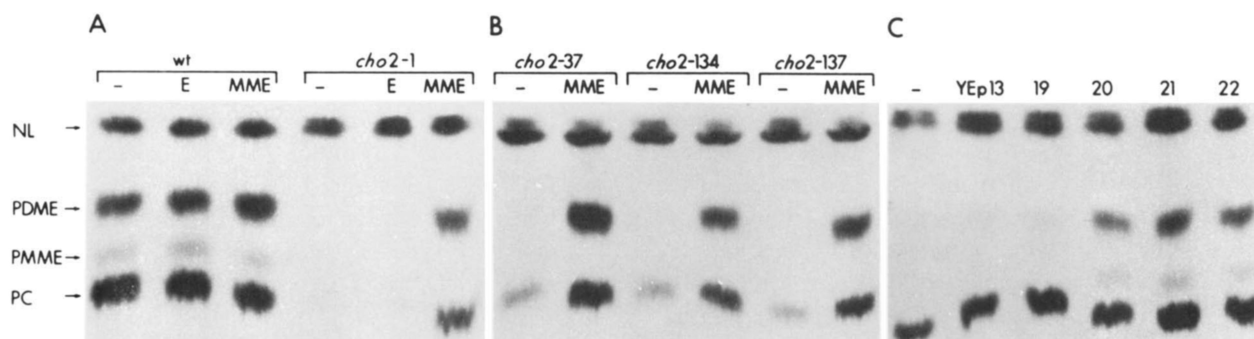

FIGURE 4.—Synthesis of methylated phospholipids. Autoradiograms of *methyl*-^{14}C-labeled phospholipids isolated from cell cultures pulse labeled with [*methyl*-^{14}C]methionine and separated by one-dimensional paper chromatography. Abbreviations: NL, neutal lipid; for others see Figure 1. A and B show labeling patterns of wild-type and different *cho2* mutants grown in synthetic medium containing various supplements as indicated. C, Labeling pattern of a *cho2-1* strain grown in unsupplemented synthetic medium and transformed with, from left to right, no plasmid, Yep13 vector, pES19, pES20, pES21 and pES22.

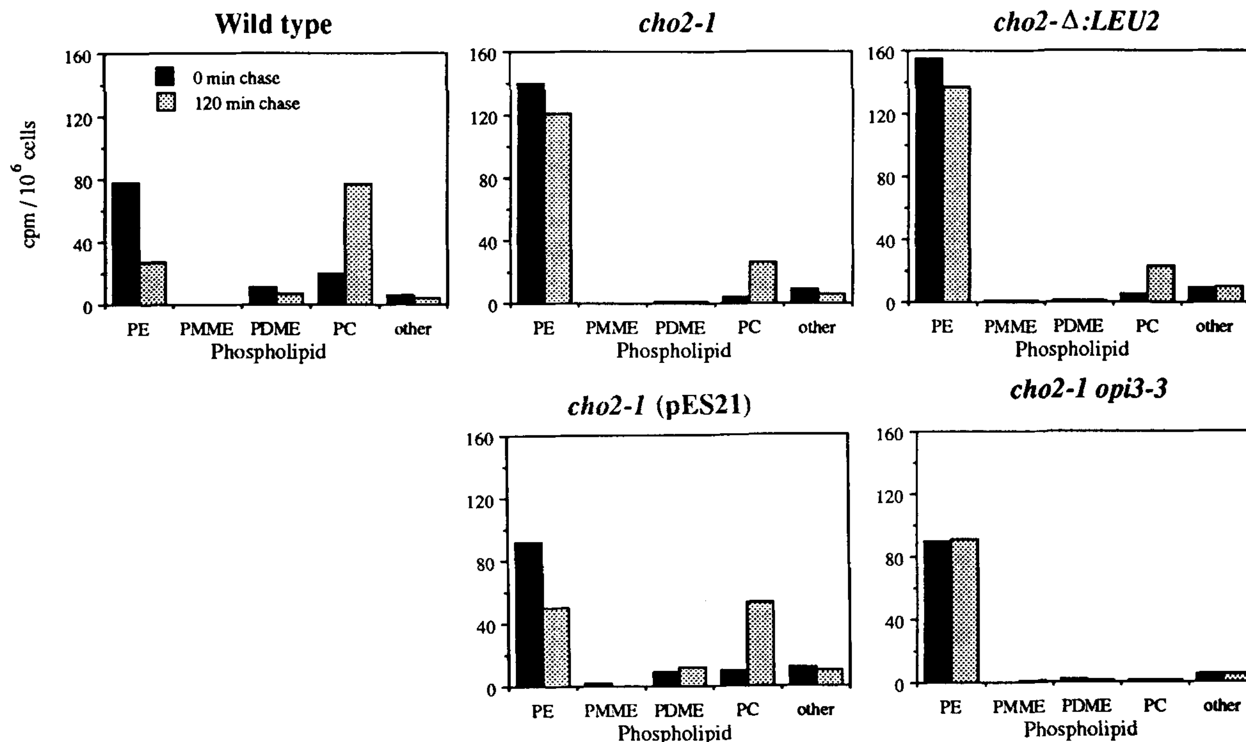

FIGURE 5.—Incorporation of [^{14}C]ethanolamine into phospholipids. Incorporation displayed in cpm incorporated per million cells following a 60-min pulse (black columns) and after a subsequent 120-min chase (shaded columns). All labelings were performed in unsupplemented synthetic medium. Relevant genotypes are indicated; phospholipid abbreviations are as in Figure 1.

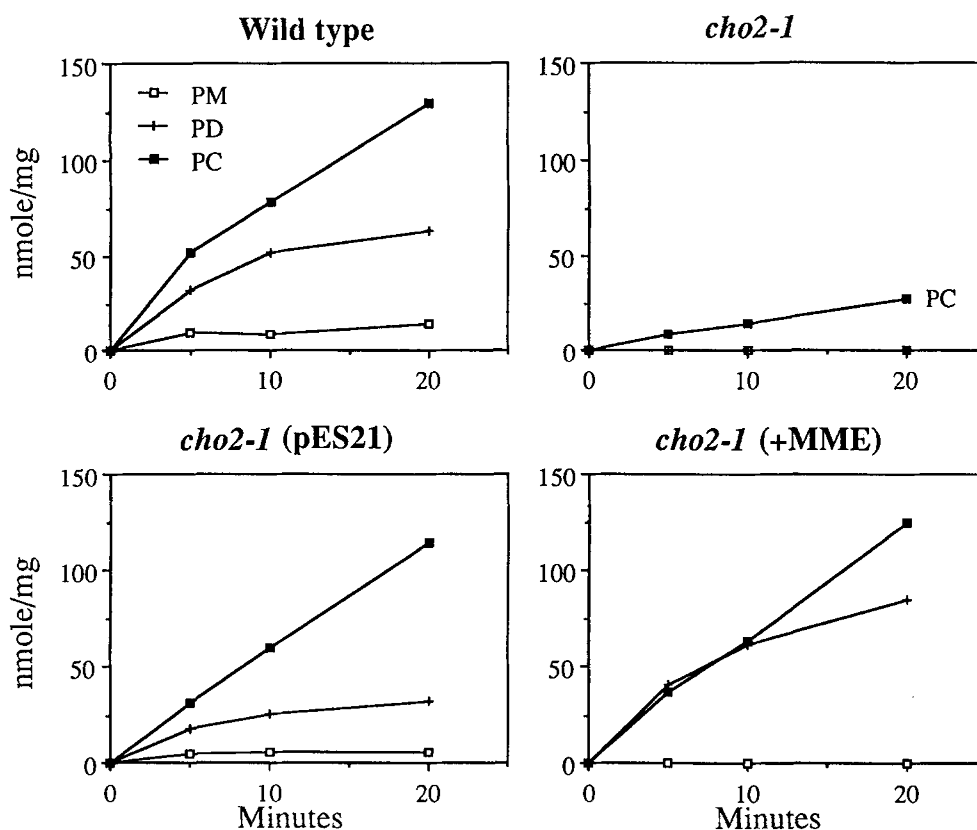

FIGURE 6.—Phospholipid methyltransferase activities assayed *in vitro*. Displayed as nanomoles of methyl group incorporated from *S*-adenosyl-L-methionine into specific phospholipids per milligram total protein in the crude membrane fractions used in the assays (see MATERIALS AND METHODS). Graph symbols are indicated in the wild-type graph (upper left); PM = PMME and PD = PDME as in Figure 1. (pES21), indicates transformation by plasmid; (+MME), indicates growth medium supplemented to 1 mM MME.

the *cho2 opi3* mutant or the *cho2 ino4* mutant and exhibited a simultaneous drop in PI levels (Table 3). The *cho2 cdg1* strain also lost viability with a shift to medium lacking MME or choline and tended to form large visible clumps in liquid medium. A *cho2 cho1* double mutant was a MME-choline auxotroph,

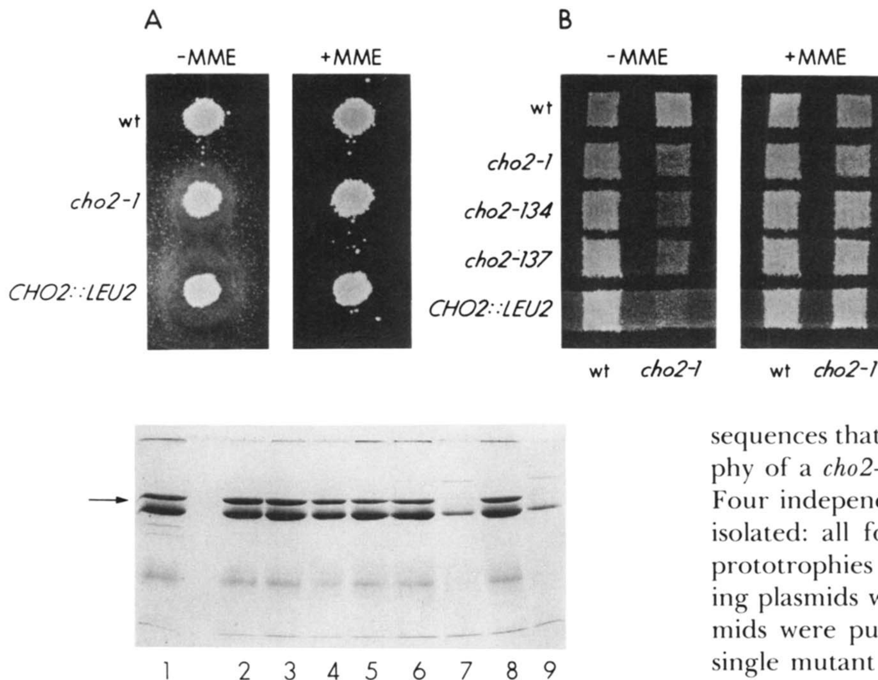

FIGURE 7.—Plate phenotypes of *cho2* mutants. A, Inositol excretion assay. Cell patches were replicated to defined medium either lacking (−MME) or containing (+MME) monomethylethanolamine and sprayed with an inositol indicator stain (AID-1). Presence of a red halo (here seen as white) indicates inositol excretion by a given cell patch. B, Complementation tests between different *cho2* alleles. A wild-type strain and several *cho2* alleles were cross replica plated against both a wild-type and a *cho2-1* tester strain and subsequently replicated to medium lacking and medium containing MME.

1 2 3 4 5 6 7 8 9

FIGURE 8.—Immunoprecipitation of inositol-1-phosphate synthase from *cho2-1* cells. Polyacrylamide gel electrophoresis of immunoprecipitates of inositol-1-phosphate synthase (I-1-PS) from crude extracts of *cho2-1* cells grown in various media. Gel is stained with coomassie blue. An immunoprecipitate of partially purified I-1-PS is shown in lane 1 as a standard. The arrow indicates the position of the band corresponding to the 62 kD I-1-PS subunit, and the dark band underneath it is the immunoglobulin heavy chain. The even numbered lanes (2, 4, 6 and 8) derived from extracts of cells grown in inositol-free medium and the odd numbered lanes (3, 5, 7 and 9) represent extracts from cultures grown in medium containing 75 μM inositol. In addition, cultures used for lanes 4 and 5 were supplemented with 1 mM ethanolamine, cultures used for lanes 6 and 7 were supplemented with 1 mM MME, and cultures used for lanes 8 and 9 were supplemented with 1 mM choline.

TABLE 5

Segregation analysis

Cross[a]	Tetrad type[b] PD	T	NP	Total 4-spored asci
cho2 cho1 × wt	1	4	0	5
cho2 opi3 × wt	0	8	2	10
cho2 × *opi3*	2	2	3	7
cho2 × *opi1*	2	13	2	17
cho2 × *ino4*	1	8	4	13
cho2 × *ino2*	1	6	0	7

[a] Strain genotypes and methods of scoring are described in the MATERIALS AND METHODS.
[b] Abbreviations: PD, parental ditype; T, tetratype; NP, nonparental ditype.

showed a striking increase in PI levels (61%) relative to all other phospholipids and lost viability rapidly to less than 10% viable cells within 12 hr following a shift to medium lacking MME or choline.

Cloning of the *CHO2* gene: A YEp13 based yeast genomic library was screened for the presence of sequences that would complement the MME auxotrophy of a *cho2-1 cdg1-1* double mutant strain (ES12). Four independent LEU$^+$ MME$^+$ transformants were isolated: all four showed simultaneous loss of both prototrophies when mitotic stability of the transforming plasmids was tested. The four transforming plasmids were purified and used to transform a *cho2-1* single mutant strain (ES1). Three of the four transforming plasmids (pES20, pES21, and pES22) clearly complemented the methylation deficiency of a *cho2* strain (Figure 6C, Table 4) while one of the transforming plasmids (pES19) only partially complemented the deficiency. The pES19 clone proved to be identical in restriction map to a clone containing the *OPI3* gene which appears to encode the methyltransferase catalyzing the final two methylation reactions leading to PC (S. KOHLWEIN and P. MCGRAW, personal communication). Transformation with the vector alone had no effect. Preliminary restriction analysis indicated that pES20, 21 and 22 have overlapping restriction maps and that pES19 is unrelated. The shortest *cho2* complementing clone, pES21, was selected for further analysis. A restriction map of the 3.6-kb insert of pES21 is shown in Figure 9A. The restriction map of pES21 corresponds closely to the restriction map of the *PEM1* clone published by KODAKI and YAMASHITA (1987) with two differences: the single *Sal*I site and the *Ssp*I site situated between the *Nhe*I and *Bgl*II sites of the pES21 insert are not predicted by the published sequence of *PEM1*.

Transformation by the plasmid pES21 complemented the *cho2* lesion at all levels. A *cho2-1*(pES21) transformed strain grew at a wild-type rate in unsupplemented defined medium lacking leucine (not shown) and did not excrete inositol under any growth condition (Table 2). Also, a *cho2-1 opi3-3*(pES21) double mutant transformed strain resembled the *opi3-3* single mutant strain phenotypically, and a *cho2-1 cdg1-1*(pES21) transformant resembled the *cdg1-1* single mutant (Table 2). *In vivo* and *in vitro* phospholipid labeling experiments demonstrated that the biochemical lesion in *cho2* cells was corrected by transformation with pES21 (Table 3, Figures 4 and 6).

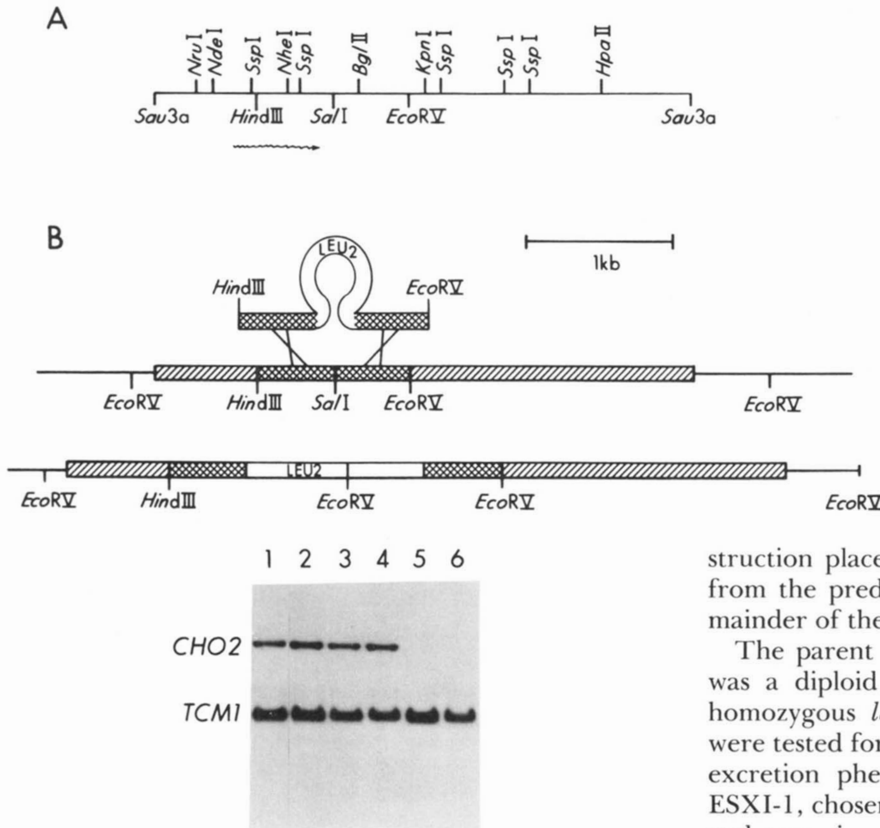

FIGURE 9.—Restriction map and *in vivo* disruption of the *CHO2* gene. A, Restriction map of the 3.6-kb pES21 *cho2*-complementing insert. The underlying wavy arrow indicates the direction of transcription of the *CHO2* mRNA. B, Illustrates the method used to disrupt the *CHO2* gene *in vivo*. The top fragment was used to transform the yeast strain, ESXI. Hatched areas in B correspond to cloned sequences within the pES21 insert (A and B maps are drawn to the same scale). The open bar corresponds to an introduced *LEU2* gene containing fragment.

FIGURE 10.—Northern blot analysis of *CHO2* transcripts from different strains. Lanes: 1, wild-type; 2, cho2-1/CHO2 (ESXI); 3, cho2-1/cho2-Δ::LEU2 (ESXI-1); 4, cho2-1; 5, cho2-Δ::LEU2 (ESXI-1(4d)); 6, cho2-Δ::LEU2 (7a). The blot also shows ribosomal protein *TCM1* mRNA as a control.

pES21 derived sequences hybridize to a single RNA: Two ^{32}P-labeled single stranded RNA probes generated from the two opposite strands of the pES21 insert (see MATERIALS AND METHODS) were used to hybridize a Northern blot of total yeast RNA. One of the probes did not hybridize to any RNA that we could detect (not shown); the other probe hybridized to a single RNA species just under 3 kb in length (Figure 10). The directionality of transcription of the detected RNA (shown in Figure 9) agrees with that predicted by the sequence analysis of KODAKI and YAMASHITA (1987).

Disruption of the *CHO2* gene *in vivo*: To generate a null allele of the *CHO2* locus we disrupted the *CHO2* gene *in vivo* as described in MATERIALS AND METHODS using the single-step gene disruption technique of ROTHSTEIN (1983). The constructed DNA fragment used to transform the parent strain is diagramed in Figure 9B. The *Sal*I site used for the insertion of the *LEU2* gene is one of the two sites present in the cloned *CHO2* gene which is not predicted in the *PEM1* sequence published by KODAKI and YAMASHITA (1987). This makes it difficult to determine the precise location of the disruption relative to the putative open reading frame. However, we estimate that the construction places the insert at about amino acid 180 from the predicted N terminus, eliminating the remainder of the 869 amino acid open reading frame.

The parent strain (ESXI) used for the disruption was a diploid *cho2-1/CHO2* heterozygote that was homozygous *leu2/leu2*. Stable LEU$^+$ transformants were tested for the characteristic *cho2* mutant inositol excretion phenotype and sporulated. One isolate, ESXI-1, chosen for further analysis, excreted inositol and gave rise to tetrads (4 out of 4 tested) that were 4:0 inositol excreter positive and 2:2 leu$^+$/leu$^-$. Presumably, the wild-type *cho2* allele of the parent strain had been disrupted and the haploid disruptant was viable. Sporulation and spore viability of the choline/MME requiring diploid transformants was poor. Therefore, from the segregants, several *cho2* leucine prototrophic spores were selected for further analysis. Mating of one presumed *cho2Δ::LEU2* haploid segregant (ESXI-1(4d) - see Table 1 for full genotype) to *LEU2* and *leu2* tester strains indicated that the integration event was not at the *LEU2* locus. The leucine prototrophy cosegregated with the *cho2* inositol excretion phenotype in all twenty of the four spored tetrads analyzed in detail. Complementation analysis of all *cho2* segregants from these crosses confirmed the presence of a *cho2* allele. Southern blot analysis (not shown) confirmed that the disruption had occurred at the *CHO2* locus. Northern blot analysis (Figure 10) showed that no detectable *CHO2* mRNA was present in haploid cells containing the *cho2-Δ* allele establishing that it was indeed a null allele. *In vivo* labeling experiments indicated that the *cho2-Δ* disruptant was not biochemically distinct from the *cho2* mutant strains originally isolated (Table 3 and Figure 5). The disruptant also had the same growth and inositol excretion phenotype as the original *cho2* mutants (Figure 7A).

DISCUSSION

The *cho2* mutants exhibit reduced synthesis of PC via methylation of PE. Five independently isolated

cho2 mutants and one null mutant constructed by disrupting the *CHO2* gene *in vivo* all methylate PE to form PC at a similar but reduced rate as compared to wild-type. When free MME is added to the growth medium of *cho2* mutant cells, it is incorporated directly into PMME and normal levels of *de novo* methylated PDME and PC are synthesized. This result and the absence of detectable incorporation of methyl label into PMME in *cho2* mutant cells under any condition indicate that the metabolic deficiency lies specifically in the first phospholipid methylation reaction converting PE into PMME. Pulse chase labelings and *in vitro* assays of phospholipid methyltransferase activities show, however, that *cho2* cells are able to synthesize PC via methylation of PE at a reduced rate. This residual PC synthesis cannot be ascribed to a partial activity of an altered enzyme in a leaky mutant because the *cho2-Δ::LEU2* null mutant has the same phenotype. A *cho2 opi3* double mutant synthesizes no methylated phospholipid *de novo* suggesting that the *OPI3* gene product is responsible for the residual methylation activity. An *OPI3* clone was found to complement the *cho2 cdg1* MME/choline auxotrophy, and it partially restored phospholipid methylation in a *cho2* strain (Table 4), again suggesting that the *OPI3* gene product is able to repair the metabolic defect in *cho2* mutants to some degree.

KODAKI and YAMASHITA (1987) concluded that there are two genes in yeast that encode phospholipid methyltransferases. In fact, the *cho2* complementing clone (pES21) that we have characterized is virtually identical in restriction map to the *PEM1* clone sequenced by KODAKI and YAMASHITA (1987). KODAKI and YAMASHITA (1987) believe that the cloned DNA fragment contains the structural gene for PE methyltransferase. While we agree that the evidence is consistent with this interpretation, phospholipid methyltransferases of yeast have not been characterized or purified and the evidence is, as yet, insufficient to permit unambiguous identification of the structural genes. KODAKI and YAMASHITA (1987) reported that they isolated the *PEM1* clone by complementation of a mutant that they have named *pem1*. This mutant has been described as a choline auxotroph (YAMASHITA *et al.* 1982; KODAKI and YAMASHITA 1987). No detailed genetic analysis of the *pem1* strain was published in the original report of its isolation (YAMASHITA *et al.* 1982) and in the subsequent cloning paper, no gene disruption or other integrative transformation was performed. It seems likely, on the basis of our analysis that the original *pem1* strain is a composite of two or more unidentified mutations, one of which may be allelic to *cho2*.

It is clear, however, that even total disruption of the *CHO2* gene does not lead to choline auxotrophy. We believe that the ability of a *cho2* null mutant to grow without choline is due to two factors: (1) *cho2* strains retain limited ability to methylate PE, presumably due to the presence of a second phospholipid methyltransferase as described above. (2) *S. cerevisiae* cells appear to be very flexible with respect to what they will tolerate in variations of phospholipid composition. For instance, *cho1* mutant cells grow well in defined medium supplied with exogenous choline yet contain no detectable phosphatidylserine and quite diminished phosphatidylethanolamine content (ATKINSON, FOGEL and HENRY 1980). Also, *opi3* mutant cells do not require choline for growth and are viable with very low levels of PC (2–3%) and very high levels of PMME (>40%) (GREENBERG *et al.* 1983).

Analysis of several double mutant strains, however, indicated that there are limitations to the permissible variation in the levels of certain phospholipids. In particular, it appears that yeast cells require some level of methylated phospholipid (*i.e.*, total of PMME, PDME and PC). Strains such as *cho2 opi3* and *cho2 ino4* are auxotrophs and lose viability in medium lacking supplementation. The *cho2 opi3* double mutant combination had an apparent PC content of 5% at the point when it began to lose viability after growth in the absence of MME or choline. However, the *cho2 ino2* strains grew with no apparent viability loss with a PC content of only 4%. It is not known why *cho2 ino2* strains should be able to grow with a PC content below that which is tolerated in *cho2 opi3* strains but it may be that other differences in the phospholipid composition modulate the cellular requirement for PC. Overall, the data support the conclusion that a level of 4–5% methylated phospholipid is required for cell growth and viability in yeast. The finding that *cho2/cho2* homozygous strains, which have essentially the same phospholipid composition as *cho2* haploid strains, behave as weak MME-choline auxotrophs suggests that diploid cells may have more stringent requirements for levels of certain lipids such as PC.

A number of enzymes involved in phospholipid biosynthesis are coordinately regulated. The cellular activities of the enzymes CDP-DG synthase (HOMANN, HENRY and CARMAN 1985), PS synthase (KLIG *et al.* 1985) and the phospholipid *N*-methyltransferases (WAECHTER and LESTER, 1973; YAMASHITA *et al.* 1982) are all fully repressed by the combination of both choline and inositol in the growth medium. Cellular levels of the enzyme I-1-PS are repressed in wild-type cells by the presence of inositol in the growth medium (DONAHUE and HENRY 1981). In the case of the enzymes PS synthase (BAILIS *et al.* 1987) and I-1-PS (HIRSCH and HENRY 1986), repression occurs at the level of mRNA abundance. At least three loci, *INO2*, *INO4* and *OPI1*, are involved in the coordinated regulation of these enzymes (HENRY, KLIG and LOEWY 1984). *ino2* and *ino4* mutants are inositol auxotrophs

due to their failure to derepress I-1-PS and are likewise unable to derepress enzymes involved in PC biosynthesis via the methylation pathway. *opi1* mutants are constitutive overproducers of inositol and fail to repress the enzymes PC biosynthesis under any condition.

In addition, several classes of mutations that appear to represent lesions in structural genes involved in PC biosynthesis have secondary and conditional effects upon cross-pathway regulation of I-1-PS. *cho1* mutants which are defective in the structural gene for PS synthase excrete inositol and fail to repress I-1-PS in response to inositol unless the medium in which they are grown contains ethanolamine or choline (LETTS and HENRY 1985). Likewise, in *cho2* cells, *INO1* RNA is expressed at derepressed levels unless choline is added to inositol (HIRSCH and HENRY 1986). In this report, we have examined inositol excretion and the expression of the *INO1* gene product, I-1-PS, in *cho2* cells grown in the presence of ethanolamine, MME or choline. The addition of MME or choline to the growth medium of *cho2* cells restores PC biosynthesis and at the same time eliminates inositol excretion and permits regulation of I-1-PS by inositol (Table 2, Figure 8). The addition of ethanolamine, in contrast, does not result in PC biosynthesis in *cho2* cells and, under these conditions, the cells excrete inositol and I-1-PS fails to be repressed in response to inositol. This result should be compared to the situation in *cho1* mutants where the addition of ethanolamine permits PC biosynthesis and simultaneously restores regulation of I-1-PS in response to inositol (Table 2 and LETTS and HENRY 1985). Thus, regulation is restored in both *cho1* and *cho2* mutants when PC is synthesized directly from choline via the CDP-choline pathway. Regulation is also restored in both mutants when an intermediate (such as ethanolamine for *cho1* mutants or MME for *cho2* mutants) enters the pathway at a point which bypasses the respective metabolic lesion and restores PC biosynthesis via the methylation pathway. It is clear that PC biosynthesis itself is important in restoration of the regulatory signal and not the *CHO2* gene product because the *cho2* null mutant regulates I-1-PS normally in response to inositol if PC biosynthesis is occurring. Thus, it would appear that PC biosynthesis itself is a component of the regulatory circuitry or generates a signal essential for the transcriptional regulation of I-1-PS and presumably other coordinately regulated phospholipid biosynthetic enzymes.

We thank JOHN HILL for donating the genomic library used in this study and LISA KLIG for bringing the *cdg1 cho2* mutant phenotype to our attention. We also thank ADAM BAILIS, JEANNE HIRSCH, DEBORAH HOSHIZAKI, MARGARET JOHNSON and SEPP KOHLWEIN for their helpful advice and discussion.

This work was supported by U.S. Public Health Service grant GM-19629 to S.A.H. E.F.S. was supported on National Institutes of Health (NIH) training grant T32GM7288. This work is taken in part from a thesis to be submitted by E.F.S. in partial fulfillment of the requirements of the Ph.D. degree at the Albert Einstein College of Medicine. P. McG. was supported on an NIH postdoctoral fellowship.

LITERATURE CITED

ATKINSON, K. D., S. FOGEL and S. A. HENRY, 1980 Yeast mutant defective in phosphatidylserine synthesis. J. Biol. Chem. **255:** 6653–6661.

AUDUBERT, F., and D. E. VANCE, 1983 Pitfalls and problems in studies on the methylation of phosphatidylethanolamine. J. Biol. Chem. **258:** 10695–10701.

BAILIS, A. M., A. P. POOLE, G. M. CARMAN and S. A. HENRY, 1987 The membrane-associated enzyme phosphatidylserine synthase is regulated at the level of mRNA abundance. Mol. Cell. Biol. **7:** 167–176.

CLEWEL, D. B., and D. R. HELINSKY, 1969 Supercoiled circular DNA-protein complex in Escherichia coli: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA **62:** 1159–1166.

CULBERTSON, M., and S. A. HENRY, 1975 Inositol requiring mutants of *Saccharomyces cerevisiae.* Genetics **80:** 23–40.

DONAHUE, T. F., and S. A. HENRY, 1981 *myo*-Inositol-1-phosphate Synthase: Characteristics of the enzyme and identification of its structural gene in yeast. J. Biol. Chem. **256:** 7077–7085.

DONAHUE, T. F., and S. A. HENRY, 1982 Inositol mutants of *Saccharomyces cerevisiae*: mapping the *INO1* locus and characterizing alleles of the *INO1 INO2* and *INO4* loci. Genetics **98:** 491–503.

ELION, E. A., and J. R. WARNER, 1984 The major promoter element of rRNA transcription in yeast lies 2 kb upstream. Cell **39:** 663–673.

FRIED, H. M., and J. R. WARNER, 1981 Cloning of the yeast gene for triclodermin resistance and ribosomal protein L3. Proc. Natl. Acad. Sci. USA **78:** 238–242.

GREENBERG, M. L., P. GOLDWASSER and S. A. HENRY, 1982 Characterization of a yeast regulatory mutant constitutive for the synthesis of inositol-1-phosphate synthase. Mol. Gen. Genet. **186:** 157–163.

GREENBERG, M. L., B. REINER and S. A. HENRY, 1982 Regulatory mutations of inositol biosynthesis in yeast: isolation of inositol-excreting mutants. Genetics **100:** 19–33.

GREENBERG, M. L., L. S. KLIG, V. A. LETTS, B. S. LOEWY and S. A. HENRY, 1983 Yeast mutant defective in phosphatidylcholine synthesis. J. Bacteriol. **153:** 791–799.

HENRY, S. A., L. S. KLIG and B. S. LOEWY, 1984 The genetic regulation and coordination of biosynthetic pathways in yeast: amino acid and phospholipid synthesis. Annu. Rev. Genet. **18:** 207–231.

HENRY, S. A., K. D. ATKINSON, A. I. KOLAT and M. R. CULBERTSON, 1977 Growth and metabolism of inositol starved cells of Saccharomyces cerevisiae. J. Bacteriol. **130:** 472–484.

HIRSCH, J. P., and S. A. HENRY, 1986 Expression of the *Saccharomyces cerevisiae* inositol-1-phosphate synthase (*INO1*) gene is regulated by factors that affect phospholipid synthesis. Mol. Cell Biol. **6:** 3320–3328.

HOLMES, D. S., and M. QUIGLEY, 1981 A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:** 193–197.

HOMANN, M. J., S. A. HENRY and G. CARMAN, 1985 Regulation of CDP-diacylglycerol synthase activity in *Saccharomyces cerevisiae.* J. Bacteriol. **163:** 1265–1266.

ITO, H., F. YASUKI, K. MURATA and A. KIMURA, 1983 Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:** 163–168.

KENNEDY, E. P., and S. B. WEISS, 1956 The function of cytidine

coenzymes in the biosynthesis of phospholipids. J. Biol. Chem. **222:** 193–214.

KLIG, L. S., M. J. HOMMAN, G. M. CARMAN and S. A. HENRY, 1985 Coordinate regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae*: pleiotropically constitutive *opi1* mutant. J. Bacteriol. **162:** 1135–1141.

KLIG, L. S., M. J. HOMMAN, S. D. KOHLWEIN, M. J. KELLY, S. A. HENRY and G. M. CARMAN, 1988 Saccharomyces cerevisiae mutant with a partial defect in the synthesis of CDP-diacylglycerol and altered regulation of phospholipid biosynthesis. J. Bacteriol. **170:** 1878–1886.

KODAKI, T., and S. YAMASHITA, 1987 Yeast phosphatidylethanolamine methylation pathway. J. Biol. Chem. **262:** 15428–15435.

KOVAC, L., I. GBELSKA, V. POLIACHOVA, J. SUBIK and V. KUVACOVA, 1980 Membrane mutants: a mutant with a lesion in phosphatidylserine biosynthesis. Eur. J. Biochem. **111:** 491–501.

LETTS, V., and I. DAWES, 1979 Mutations affecting lipid biosynthesis of *Saccharomyces cerevisiae*: isolation of ethanolamine auxotrophs. Biochem. Soc. Trans. **7:** 976–977.

LETTS, V. A., and S. A. HENRY, 1985 Regulation of phospholipid synthesis in phosphatidylserine synthase-deficient (*cho1*) mutants of *Saccharomyces cerevisiae.* J. Bacteriol. **163:** 560–567.

LINDEGREN, G., W. L. HWANG, W. OSHIMA and C. C. LINDEGREN, 1965 Genetical mutants induced by ethylmethane sulfonate in *Saccharomyces.* Can. J. Genet. Cytol. **7:** 491–499.

LOEWY, B. S., and S. A. HENRY, 1984 The *INO2* and *INO4* loci of *Saccharomyces cerevisiae* are pleiotropic regulatory genes. Mol. Cell Biol. **4:** 2479–2485.

MANDEL, M., and A. HIGA, 1970 Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53:** 154.

MATO, J. M., M. A. PAJARES and I. VARELA, 1984 How many phospholipid methyltransferases are there in mammalian cells? Trends Biochem. Sci. **9:** 471–472.

MELTON, D. A., P. A. KRIEG, M. R. REBAGLIATI, T. MANIATIS, K. KINN and M. R. GREEN, 1984 Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **18:** 7035–7056.

PELECH, S. L., and D. E. VANCE, 1984 Regulation of phosphatidylcholine biosynthesis. Biochim. Biophys. Acta **779:** 217–251.

RIDGWAY, N. D., and D. E. VANCE, 1987 Purification of phosphatidyl-ethanolamine N-methyltransferase from rat liver. J. Biol. Chem. **262:** 17231–17239.

RIGBY, P. W., J. M. DIECKMANN, C. RHODES and P. BERG, 1977 Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113:** 237–251.

ROTHSTEIN, R. J., 1983 One step gene disruption in yeast. Method Enzymol. **101C:** 202–211.

ROZEK, C. E., and N. DAVIDSON, 1983 Drosophila has one myosin heavy-chain gene with three developmentally regulated transcripts. Cell **32:** 23–34.

SCARBOROUGH, G. A., and J. F. NYC, 1967 Methylation of ethanolamine phosphatides by microsomes from normal and mutant strains of *Neurospora crassa.* J. Biol. Chem. **242:** 238–242.

SHERMAN, F., G. R. FINK and C. W. LAWRENCE, 1978 *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SOUTHERN, E. M., 1975 Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:** 503–517.

STEINER, S., and R. L. LESTER, 1972 In vitro studies of phospholipid synthesis in *Saccharomyces cerevisiae.* Biochim. Biophys. Acta **260:** 222–243.

STRUHL, K., D. T. STINCHCOMB, S. SCHERER and R. W. DAVIS, 1979 High frequency transformations of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76:** 1035–1039.

THOMAS, P. S., 1980 Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77:** 5201–5205.

WAECHTER, C. J., and R. L. LESTER, 1971 Regulation of phosphatidylcholine synthesis in *Saccharomyces cerevisiae.* J. Bacteriol. **105:** 837–843.

WAECHTER, C. J., and R. L. LESTER, 1973 Differential regulation of the N-methyltransferases responsible for phosphatidylcholine synthesis in *Saccharomyces cerevisiae.* Arch. Biochem. Biophys. **158:** 401–410.

YAMASHITA, S., and A. OSHIMA, 1980 Regulation of phosphatidylethanolamine methyltransferase by *myo*-inositol in *Saccharomyces cerevisiae.* Eur. J. Biochem. **104:** 611–616.

YAMASHITA, S., A. OSHIMA, J. NIKAWA and K. HOSAKA, 1982 Regulation of the phosphatidyl-ethanolamine methylation pathway in *Saccharomyces cerevisiae.* Eur. J.Biochem. **128:** 589–595.

Communicating editor: M. CARLSON