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The *INO2* and *INO4* Loci of *Saccharomyces cerevisiae* Are Pleiotropic Regulatory Genes

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We isolated a mutant of *Saccharomyces cerevisiae* defective in the formation of phosphatidylcholine via methylation of phosphatidylethanolamine. The mutant synthesized phosphatidylcholine at a reduced rate and accumulated increased amounts of methylated phospholipid intermediates. It was also found to be auxotrophic for inositol and allelic to an existing series of *ino4* mutants. The *ino2* and *ino4* mutants, originally isolated on the basis of an inositol requirement, are unable to derepress the cytoplasmic enzyme inositol-1-phosphate synthase (*myo*-inositol-1-phosphate synthase; EC 5.5.1.4). The *INO4* and *INO2* genes were, thus, previously identified as regulatory genes whose wild-type product is required for expression of the *INO1* gene product inositol-1-phosphate synthase (T. Donahue and S. Henry, J. Biol. Chem. 256:7077–7085, 1981). In addition to the identification of a new *ino4* allele, further characterization of the existing series of *ino4* and *ino2* mutants, reported here, demonstrated that they all have a reduced capacity to convert phosphatidylethanolamine to phosphatidylcholine. The pleiotropic phenotype of the *ino2* and *ino4* mutants described in this paper suggests that the *INO2* and *INO4* loci are involved in the regulation of phospholipid methylation in the membrane as well as inositol biosynthesis in the cytoplasm.

The *ino2* and *ino4* mutants of *Saccharomyces cerevisiae* require inositol and are unable to derepress inositol-1-phosphate synthase (*myo*-inositol-1-phosphate synthase; EC 5.5.1.4; I-1-P S), the cytoplasmic enzyme responsible for the conversion of glucose 6-phosphate to inositol 1-phosphate (3) (see Fig. 1). The structural gene for I-1-P S is the *INO1* gene (6), which is not linked to the *INO4* or *INO2* gene (3). The *INO4* and *INO2* genes have been identified as regulatory genes whose wild-type product is required for expression of the *INO1* gene product I-1-P S (6). In the present study, we isolated a new mutant which proved to be allelic to existing *ino4* mutants. However, the new *ino4* mutant was not isolated on the basis of inositol auxotrophy. Rather, it was isolated on the basis of a defect in the synthesis of phosphatidylcholine (PC) and was subsequently found to be auxotrophic for inositol and allelic to existing *ino4* mutants.

Neurospora crassa mutants with a defect in PC biosynthesis exhibit an absolute auxotrophic requirement for choline (19). In contrast, the *opi3* mutant of *S. cerevisiae*, which has a defect in PC biosynthesis similar to that of the *Neurospora* mutants, is not auxotrophic for choline, despite the fact that its phospholipids contain as little as 2 to 5% PC, as compared with 40 to 50% in wild-type cells (8). The *cho1* mutant of *S. cerevisiae*, which is auxotrophic for choline, has a defect in the synthesis of phosphatidylserine (PS) (see Fig. 1 and references 1, 2, 14, 16, and 17; V. Letts, Ph.D. thesis, University of Edinburgh, Edinburgh, Scotland, 1980). The auxotrophic requirement of the *cho1* mutant is fulfilled by either ethanolamine or choline, which are incorporated into lipids by the pathway described by Kennedy and Weiss (13) (see Fig. 1), bypassing PS as an intermediate in the synthesis of phosphatidylethanolamine (PE) and PC. The phospholipid compositions of the *cho1* and *opi3* mutants of *S. cerevisiae* are aberrant under all growth conditions. Studies of the *cho1* and *opi3* mutants have shown that it is possible for *S. cerevisiae* cells to grow vegetatively with major alterations in the proportions of their phospholipids (1, 2, 8). This flexibility in the phospholipid composition of the yeast membrane may explain why stringent choline auxotrophs of *S. cerevisiae* have been difficult to isolate (10).

As the *cho1* mutant requires either ethanolamine or choline, we reasoned that selection in the *cho1* background for mutants having a requirement satisfied only by choline might result in the isolation of a mutant defective in the conversion of PE to PC (see Fig. 1). Such a mutant was isolated and indeed exhibited reduced conversion of PE to PC, but it was also found to be auxotrophic for inositol and allelic to the existing series of *ino4* mutants (3). Further characterization of the existing series of *ino4* and *ino2* mutants demonstrated that they all have a defect in the formation of PC via methylation of PE. The consistent reduction in PC biosynthesis in these mutants suggests that the *INO2* and *INO4* genes are involved in the regulation of PC biosynthesis in the membrane as well as in the regulation of I-1-P S, a cytoplasmic enzyme. The pleiotropic phenotype of the *ino2* and *ino4* mutants is discussed in relation to the coordinate regulation of phospholipid synthesis in *S. cerevisiae*.

(This report was taken in part from a Ph.D. thesis to be submitted by B. S. Loewy to the Albert Einstein College of Medicine, Bronx, N.Y., 1984.)

MATERIALS AND METHODS

Strains. The genotypes and sources of the strains used in this study are shown in Table 1.

Media and growth conditions. Strains were maintained in YEPD plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar). Synthetic complete medium contained 2% glucose; 0.67% yeast nitrogen base (Difco Laboratories) without amino acids; and the amino acids (in milligrams per liter) lysine (20), arginine (10), leucine (10), methionine (10), threonine (60), tryptophan (10), histidine (10), adenine (10), uracil (10), and *myo*-inositol (3, brought to a total of 5 with the inositol contained in yeast nitrogen base). The supplement 1 mM choline or 1 mM ethanolamine (Sigma Chemical Co.) was added as required. Auxotrophic markers were scored on media lacking a single component of the complete

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TABLE 1. *S. cerevisiae* strains used in this study

Laboratory strain designation	Genotype	Source or reference
Wild type	*MATa ade5*	3
Wild type	*MATα ade5*	3
S5	*MATa ade1*	3
MC13	*MATα ino1-13 lys2 can1*	3
MC13	*MATa ino1-13 ade1*	3
KA101	*MATα can1 cho1 ino1-13 lys2*	1
MC40	*MATα ino4-40 lys2*	3
MC38	*MATα ino4-38 lys2*	3
MC39	*MATα ino4-39 lys2*	3
MC26	*MATa ino4-26 ade5*	3
MC8	*MATα ino4-8 lys2*	3
MC2	*MATα ino2-2 lys2*	3
MC21	*MATα ino2-21 lys2*	3
BSI	*MATα cho1 ino1-13 ino4-BSI lys2*	This study
BSI-8D	*MATa ino4-BSI ade1*	This study

medium. Medium lacking inositol was prepared with vitamin-free yeast base (Difco) as described by Culbertson and Henry (3).

Mutagenesis and genetic analysis. The parental strain KA101 (*cho1 ino1-13 lys2 MATα*) was mutagenized with ethyl methanesulfonate by the method of Lindegren et al. (18). Mutagenized cells were diluted, plated on YEPD medium (ca. 100 colonies per plate), and incubated at 30°C. The colonies were replica plated to synthetic complete medium supplemented with 1 mM choline or 1 mM ethanolamine or not supplemented. Colonies that grew in the presence of exogenous choline but did not grow or did not grow well on ethanolamine-supplemented plates were identified.

Genetic analysis was carried out by standard procedures. Diploids were induced to sporulate on acetate medium. Asci were digested with 10% Glusulase and dissected by micromanipulation on a YEPD plate. Spore colonies were replica plated to the various media to determine the segregation of auxotrophic requirements.

Labeling conditions. Cultures were labeled with [*methyl*-^{14}C]methionine (5.75 mCi/mmol) or $^{32}P_i$ (carrier free). All isotopes were obtained from New England Nuclear Corp. Cells were grown overnight in YEPD medium or in synthetic medium with inositol. Similar results were obtained in both cases. The cells were washed twice with the synthetic medium in which they were to be labeled. All labeling experiments were done with logarithmic-growth-phase cultures at 30°C in synthetic medium. For *cho1* cells, ethanolamine or choline supplement was added. For most [^{14}C]methionine labeling studies, the cultures were incubated at 30°C for 1.5 to 2 h in inositol-free medium. These conditions were used because inositol and choline affect the regulation of the phospholipid methyltransferases (10, 21, 22, 25). This brief period of incubation in inositol-free medium has no effect upon the growth or viability of inositol-requiring strains (9). Subsequent studies showed that similar results were obtained when *ino4* cells were labeled with [*methyl*-^{14}C]methionine in inositol-containing medium. Cold L-methionine (15 μg/ml) and [*methyl*-^{14}C]methionine (0.5 μCi/ml; specific activity, 57.5 mCi/mmol) were then added, and the cultures were incubated at 30°C for an additional 30 min. A 0.5-ml sample of each labeled culture was mixed with 0.5 ml of 10% trichloroacetic acid (TCA) and incubated for a minimum of 20 min. The samples were transferred to filters (Millipore Corp.) and washed with 5% TCA. The filters were counted in a scintillation counter. The remainder of the cells were harvested by centrifugation and washed twice with distilled water, and the lipids were extracted as described below.

The phospholipid composition was determined as described by Atkinson et al. (2) by labeling cells with $^{32}P_i$ for five to six generations to obtain steady-state labeling. The final specific activity of the ^{32}P in the cultures used in the steady-state labeling experiments was 0.544 mCi/mmol. Lipids were extracted from 5 ml of culture and analyzed as described below.

Preparation of spheroplasts and extraction of lipids. Lipids were extracted after spheroplasting of the cells as described by Atkinson et al. (1, 2). Washed, labeled cells were digested for 15 min at room temperature in a mixture consisting of 1.2 M glycerol, 100 mM sodium thioglycolate, 50 mM Tris sulfate (pH 7.5), and 0.5 mg of Zymolyase 5000 (Kirin Breweries) per ml. Spheroplasts were pelleted, and the supernatant was drawn off with a Pasteur pipette. Lipids were extracted by a modification of the method described by Folch et al. (7). Spheroplasts were suspended in 5 ml of chloroform-methanol (2:1), vortexed, and extracted for 1 h at room temperature. Distilled water (1 ml) was then added,

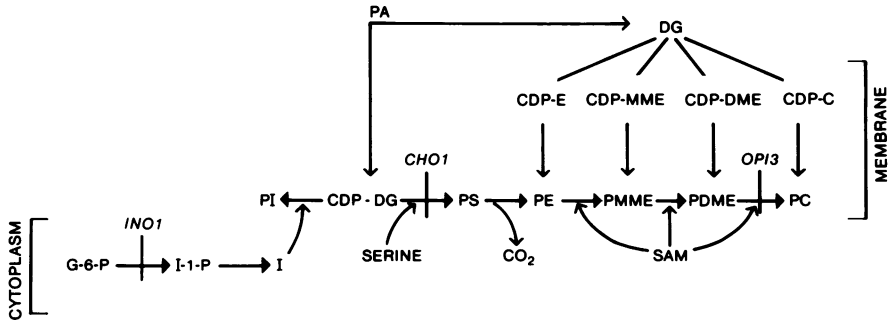

FIG. 1. Phospholipid biosynthesis in *S. cerevisiae*. Shown are reactions in the cytoplasm and the membrane which are involved in the synthesis of the major phospholipids and their precursors in *S. cerevisiae*. Steiner and Lester (20) detected most of these reactions in vitro in isolated *S. cerevisiae* membranes. Waechter and Lester (21, 22) reported the synthesis of PC via methylation of PE in *S. cerevisiae* membranes. Kennedy and Weiss (13) described the formation of PE, PC, PMME, and PDME from exogenous precursors. The cytoplasmic synthesis of inositol 1-phosphate (I-1-P) from glucose 6-phosphate (G-6-P) in *S. cerevisiae* was described by Culbertson et al. (4). PA, Phosphatidic acid; CDP-DG, cytidine diphosphate diglyceride; PI, phosphatidylinositol; SAM, *S*-adenosylmethionine; DG, diglyceride; CDP-E, cytidine diphosphate ethanolamine; CDP-MME, cytidine diphosphate monomethylethanolamine; CDP-DME, cytidine diphosphate dimethylethanolamine; CDP-C, cytidine diphosphate choline; I, inositol. The assignments of genes known with certainty to be structural genes (*INO1 CHO1*) or identified as possible structural genes (*OPI3*) are shown by a gene designation above a given reaction.

TABLE 2. Tetrad analysis of a cross of the original BSI mutant (*MATα chol inol-13 ino4-BSI lys2*) with strain S5 (*MATa adel*)

Tetrad	Spore	Growth on[a]:				Synthesis[b] of PC	Genotype[c]			
		Com	Com+ E	Com + C	I⁻ + C		*ino4/BSI*	*inol-13*	*chol*	*MAT*
1	A	−	+/−	+	−	−	*ino4*	+	*chol*	**a**
	B	−	+	+	−	+	+	*inol*	*chol*	**a**
	C	+	+	+	−	+	+	*inol*	+	α
	D	+	+	+	−	−	*ino4*	+	+	α
2	A	−	+/−	+	−	−	*ino4*	+	*chol*	**a**
	B	+	+	+	+	+	+	+	+	α
	C	−	+/−	+	−	−	*ino4*	*inol*	*chol*	**a**
	D	+	+	+	−	+	+	*inol*	+	α
3	A	−	+/−	+	−	−	*ino4*	*inol*	*chol*	α
	B	−	+	+	+	+	+	+	*chol*	**a**
	C	+	+	+	−	−	*ino4*	+	+	α
	D	+	+	+	−	+	+	*inol*	+	**a**
4	A	−	+	+	+	+	+	+	*chol*	α
	B	+	+	+	−	−	*ino4*	*inol*	+	**a**
	C	+	+	+	+	+	+	+	+	α
	D	−	+/−	+	−	−	*ino4*	*inol*	*chol*	**a**
Parent strain										
S5		+	+	+	+	+	+	+	+	**a**
KA101		−	+	+	−	+	+	*inol*	*chol*	α
BSI		−	+/−	+	−	−	*ino4*	*inol*	*chol*	α

[a] Medium abbreviations: Com, synthetic complete medium; Com + C, Com with choline; Com + E, Com with ethanolamine; I⁻ + C, Com without inositol but with choline. Plates were scored for relative growth after 48 h at 30°C. +, Growth; +/−, leaky growth, −, lack of growth.

[b] Measured as the incorporation of [*methyl*-^{14}C]methionine. −, BSI pattern (see Fig. 3 and Table 3); +, wild-type pattern.

[c] The genotype was determined by examination of growth and the phenotype pattern of phospholipid synthesis and by genetic complementation (to distinguish *inol* and *ino4*). Not shown is the segregation of *lys2* and *adel*, both of which segregated 2:2 in all cases. +, Wild type.

and the tubes were vortexed again and centrifuged to separate the aqueous and organic phases. The bottom phase was removed and dried under N_2.

Separation of lipids. Lipid residues were resuspended in a small volume of chloroform-methanol (2:1) and spotted on Whatman SG81 silica-impregnated paper which had been dipped in 2% EDTA (pH 7) and heated for 20 min (20). The ^{32}P-labeled lipids were separated in two dimensions by ascending chromatography with the solvent systems described by Steiner and Lester (20). The first-dimension separation was done with chloroform-methanol-ammonium hydroxide (30%)-H_2O (66:27:3:0.8), and the second-dimension separation was done with chloroform-methanol-acetic acid-H_2O (32:4:5:1). The lipids labeled with [*methyl*-^{14}C]methionine were separated in one dimension in the solvent chloroform-methanol-ammonium hydroxide (66:17:3). The radioactive lipids were located by autoradiography and counted by liquid scintillation in toluene–2,5-diphenyloxazole–1,4-bis-(5-phenyloxazolyl)benzene or Betafluor (National Diagnostics).

Immunoprecipitation. Strains were tested by immunoprecipitation for the presence of the 62,000-dalton subunit of I-1-P S. The technique, which has been described previously in more detail (6), involved preparing a crude extract (by Braun homogenization of pelleted cells) of mutant or wild-type *S. cerevisiae* cells suspended in a buffer consisting of 20 mM Tris-hydrochloride (pH 7.2) containing 2 mM phenylmethylsulfonyl fluoride (to inhibit proteolysis) in 1% dimethyl sulfoxide; 10 mM NH_4Cl; and 10 mM 2-mercaptoethanol. The lysate was centrifuged at 4°C for 5 min at 7,000 × *g* (to remove intact cells) and then at 27,000 × *g* for 20 min. Immunoprecipitations of the extracts were carried out with rabbit anti-I-1-P S serum prepared as described by Donahue and Henry (6). Volumes of crude extracts corresponding to 1.0 to 2.0 mg of protein were incubated with 10 μl of rabbit antisynthase serum for 3 h at 4°C in 1.5-ml Eppendorf centrifuge tubes and then indirectly precipitated with *Staphylococcus aureus* cells (10% of the volume of the incubation mixture) for 1 h at 4°C. Samples were centrifuged, and the precipitates were washed twice with phosphate-buffered saline containing 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.1% deoxycholate, and 0.5 M NaCl. The washed precipitates were then electrophoresed through 10% sodium dodecyl sulfate-polyacrylamide gels under fully dissociating conditions (15) as described in detail by Donahue and Henry (6). On such gels the 62,000-dalton subunit of I-1-P S, when present in crude extracts at wild-type derepressed levels, is readily visualized by Coomassie blue staining of the gels. The band is absent in extracts prepared from wild-type cells grown under repressing conditions (6).

RESULTS

Mutant isolation and preliminary analysis. As discussed above, the goal of this study was to obtain mutants defective in the conversion of PE to PC (Fig. 1). We reasoned that the failure to isolate mutants defective in the conversion of PE to PC by direct selection for choline auxotrophs was probably due to the fact that yeast cells tolerate considerable alterations in the proportions of the various phospholipids present in the membrane (10). Starting with the *chol* mutant, which already has major alterations in phospholipid composition, we selected for a limitation of its auxotrophic requirement (i.e., absolute choline auxotrophy, not satisfied by ethanolamine). In so doing, we hoped to directly select mutants defective in PC biosynthesis via methylation of PE.

The *chol* mutant totally lacks PS but has no defect in the conversion of PE to PC (1, 2). The parental strain, KA101, is auxotrophic for inositol as well as choline or ethanolamine

TABLE 3. Incorporation of [*methyl*-^{14}C]methionine into phospholipids in mutant strains[a]

Strain[b]	Genotype[b]	Relative incorporation (%) of [*methyl*-^{14}C]methionine into[c]:					Incorporation (cpm) of [*methyl*-^{14}C]methionine into[d]:	
		PMME	PDME	PC	NL	Other	TCA	PC
KA101	*cho1* +	4 (2.3)	11 (6.7)	27 (15.9)	36 (21.6)	22	9	1.5
BSI	*cho1 ino4-BSI*	8 (2.8)	9 (3.4)	6 (2.3)	53 (20.2)	24	8	0.2
Wild type (*ade5*)	+ +	1 (0.6)	9 (5.1)	69 (33.2)	20 (11.2)	1	17	5.6
MC40	+ *ino4-40*	14 (4.5)	26 (8.6)	19 (6.4)	36 (11.9)	5	15	1.0
MC39	+ *ino4-39*	13 (3.6)	20 (5.3)	15 (3.7)	46 (12.3)	6	10	0.4
MC38	+ *ino4-38*	13 (3.4)	19 (5.0)	12 (3.2)	52 (13.6)	4	13	0.4
MC8	+ *ino4-8*	11 (3.4)	20 (6.2)	16 (4.9)	48 (13.7)	5	14	0.7
BSI-8D	+ *ino4-BSI*	11 (2.3)	24 (5.2)	22 (5.0)	37 (7.5)	6	16	0.8
MC2	+ *ino2-2*	10 (2.1)	17 (3.3)	13 (2.6)	52 (9.8)	8	8	0.2
MC21	+ *ino2-21*	8 (1.9)	14 (3.5)	11 (2.7)	61 (15.4)	6	14	0.4

[a] The data represent the percentage of label from [*methyl*-^{14}C]methionine incorporated in a 30-min pulse into each phospholipid, normalized against the incorporation into total lipid and total TCA-precipitable material. The data represent an average of at least five separate experiments.

[b] +, Wild type.

[c] Represented as the incorporation into each lipid as a percentage of total lipid-soluble label. The numbers in parentheses represent the incorporation into each lipid as a percentage of total incorporation into TCA-precipitable material. NL, Neutral lipids. The category "other" includes the polar lipids remaining near the origin and other unresolved lipids. Labeling, extraction, and separation of the lipids were carried out as described in the text.

[d] Represented as counts per minute incorporated by 10^4 cells into TCA-precipitable material or PC in a 30-min pulse, as described in the text.

due to the presence of the *ino1-13* mutation (see Table 1 for complete genotypes and Table 2 for a complete description of growth requirements). The *ino1-13* mutation is in the structural gene (*INO1*) for I-1-P S. It is a stable missense mutation which produces an inactive subunit of I-1-P S which can be detected with antibody to wild-type I-1-P S (6). Strain KA101 was mutagenized as described above. Out of ca. 1,000 mutagenized colonies plated, 1 colony, designated BSI, which grew poorly on ethanolamine-supplemented plates but grew well in the presence of choline, was identified and subjected to further analysis. The choline auxotrophic requirement was "leaky" and somewhat difficult to score on plates after replica plating. However, optimum growth was detected only when the mutant strain was supplied with choline (see Table 2 for a complete description of phenotypes and growth patterns of the mutant and parental strains).

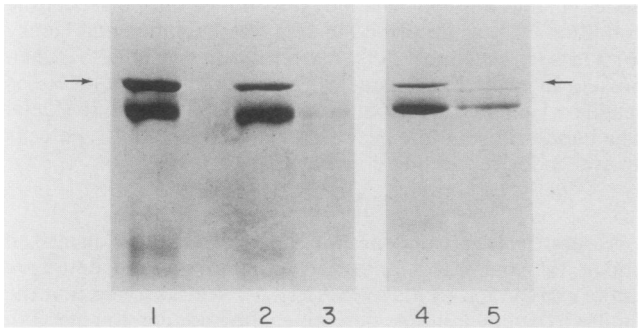

FIG. 2. Polyacrylamide gel electrophoresis of immunoprecipitates of I-1-P S from crude extracts (see text) of various strains, all grown under derepressing conditions (10 μM inositol in the growth medium). Immunoprecipitated partially purified wild-type I-1-P S is included as a reference (lane 1). The arrows indicate the position of the 62,000-dalton subunit of I-1-P S; the heavy band immediately below the arrows is the immunoglobulin heavy chain. Lanes 2 through 5 contain immunoprecipitates of extracts from the wild type (*ade5*) (lane 2), a BSI-8D spore colony (*ino4-BSI*) derived from the cross of the wild type (*ade5*) with strain BSI (lane 3), the parental strain (KA101) (*ino1-13 cho1*) (lane 4), and the original mutant strain BSI (*ino4-BSI cho1 ino1-13*) (lane 5). (See Tables 1 and 2 for complete genotypes and phenotypes.)

To determine whether the conversion of PE to PC was defective in the mutant BSI, we labeled the parental and mutant strains with [*methyl*-^{14}C]methionine as described above (Table 3). The relative incorporation of *methyl*-^{14}C label into individual phospholipids was normalized against the incorporation of label into total TCA-precipitable material (Table 3), serving as a measure of total metabolism of the label into macromolecules. In addition, the incorporation was calculated on a per-cell basis. By either criterion, the rate of incorporation of [*methyl*-^{14}C]methionine into PC in the mutant was 80% lower than in the parental strain (KA101). However, strain KA101 exhibits altered rates of synthesis of virtually every phospholipid (2). Its pattern of labeling of lipids with [*methyl*-^{14}C]methionine is somewhat different from that of the wild type (Table 3). Thus, to fully assess the nature of the biochemical defect, it was desirable to study the BSI mutant without the *cho1* lesion.

Genetic analysis. Mutant strain BSI was crossed with strain S5 (*ade1 CHO1 INO1 MAT***a**; see Tables 1 and 2), and tetrad analysis was carried out. The phenotypes and genotypes of four extensively studied tetrads are shown in Table 2. The defect in PC synthesis, measured as the incorporation of [*methyl*-^{14}C]methionine into PC, was found to segregate independently of the *cho1* and *ino1-13* mutations (Table 2). When the new mutation in PC biosynthesis segregated out of the *cho1* genetic background, however, the mutant no longer required choline (or ethanolamine) for growth (see Table 2, spores 1D, 3C, and 4B). Tetrad analysis, surprisingly, also revealed a two-gene segregation for inositol auxotrophy (Table 2), whereas the segregation of only the *ino1-13* mutation (present in the KA101 background; Table 1) was expected. Allele testing (3) confirmed that the *ino1-13* allele was segregating in a normal 2:2 fashion (Table 2). The new inositol auxotrophy segregated independently of the *ino1* mutation but cosegregated with the defect in PC biosynthesis (Table 2). The new inositol auxotrophy present in strain BSI was tested for complementation with various known *ino* mutants (3). It was found to be an allele of the *INO4* locus. The new *ino4* mutant (BSI) was examined for expression of I-1-P S (the *INO1* gene product) by immunoprecipitation and was found, like other *ino4* mutants (6), to lack the I-1-P S subunit (Fig. 2; lane 5), whereas the parental strain (KA101) produced the inactive subunit produced by the *ino1-13*

TABLE 4. Phospholipid compositions of *ino2*, *ino4*, *ino1*, and *cho1* mutants and of a wild-type strain

Strain	Genotype	Phospholipid composition[a]						
		PI	PS	PE	PMME	PDME	PC	Other
Wild type	*ade5*	28.0	5.5	12.1	0.9	1.8	39.7	12.0
MC13	*ino1-13*	28.6	4.9	13.2	1.1	1.9	37.8	12.5
KA101	*ino1-13 cho1*	32.8	—[b]	15.4	1.0	1.1	42.4	7.3
BSI-8D	*ino4-BSI*	34.5	4.3	19.7	5.4	13.5	13.8	8.8
MC8	*ino4-8*	29.2	5.1	22.8	2.1	12.3	13.4	15.1
MC26	*ino4-26*	36.4	4.6	18.5	2.5	7.8	17.8	12.4
MC38	*ino4-38*	36.3	4.1	23.8	2.0	7.8	11.1	14.9
MC39	*ino4-39*	37.0	3.8	19.9	1.5	9.6	15.3	12.9
MC40	*ino4-40*	32.3	5.2	21.1	2.2	10.8	15.7	12.7
MC2	*ino2-2*	36.5	4.6	28.3	1.3	9.5	11.9	7.9
MC21	*ino2-21*	38.3	4.9	27.9	1.3	8.2	11.3	8.1

[a] Phospholipid compositions were determined as described in the text. The cells of all strains were grown in synthetic complete medium with 75 μM inositol. KA101 cells were supplied with 1 mM ethanolamine as well. Data represent percentages of total lipid phosphorus content determined for each lipid by chromatographic analysis. The total lipid phosphorus content was 11.5 to 12.9 nmol/10^7 cells and was not found to be significantly different in any of the strains tested. PI, Phosphatidylinositol. The category "other" includes the pooled percentages of phosphatidic acid, cytidine diphosphate diglyceride, cardiolipin, phosphatidylglycerol, and other minor lipid species.

[b] —, No lipid detected.

mutation (Fig. 2, lane 4). Likewise, immunoprecipitation of strain BSI-8D (*ino4-BSI INO1 CHO1*) showed that the 62,000-dalton subunit was not produced by the mutant in a wild-type (i.e., *INO1 CHO1*) background (Fig. 2, lane 3).

Biochemical analysis. The phospholipid compositions of the new *ino4* allele (*ino4-BSI*) and several other *ino4* mutants were consequently examined as described above and compared with those of the *ino1-13*, KA101 and wild-type strains (Table 4). No significant difference in the total amount of phospholipid per cell was found among any of the strains (Table 4). However, *ino4* mutants were found to have phospholipid compositions which contained significantly decreased proportions of PC. Whereas the phospholipid compositions of wild-type, *ino1*, and *cho1* strains contained ca. 40% PC, all of the *ino4* mutants had phospholipid compositions which consisted of <20% PC.

Phospholipid synthesis in the *ino4* mutants was also examined by labeling with [*methyl*-^{14}C]methionine as described above. A one-dimensional separation of *methyl*-^{14}C-labeled phospholipids from three different *ino4* mutants is shown in Fig. 3. In comparison with wild-type cells, all the *ino4* mutants exhibited a decrease in the labeling of PC with [*methyl*-^{14}C]methionine and accumulated a higher proportion of label in the two methylated intermediates phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) (Table 3 and Fig. 3). Whereas the wild-type strain incorporated more than 60% of the lipid-soluble label from [*methyl*-^{14}C]methionine into PC in 30 min, the *ino4* mutants incorporated only 12 to 22%. The mutants, however, incorporated 19 to 26% of the lipid-soluble label into PDME and 11 to 14% into PMME, as compared with 9 and 1%, respectively, for the wild type. The rate of incorporation of label from [*methyl*-^{14}C]methionine into PC in the mutants was found to be at least fivefold lower than that in the wild type (1.0 to 0.2 cpm per 10^4 cells in the mutants and 5.6 cpm per 10^4 cells in the wild type [Table 3]). In contrast, the incorporation of label into total TCA-precipitable material was comparable or only slightly lower in the mutants than in the wild-type strains (Table 3).

The *ino2* mutants are another class of inositol auxotrophs which, like the *ino4* mutants, fail to express I-1-P S (6). The *INO2* locus is not linked to the *INO4* and *INO1* loci (3). Phospholipid synthesis was examined in the two existing *ino2* mutants (*ino2-2* and *ino2-21*) (3). The *ino2* mutants (Fig. 3 and Table 3) were found to have a defect in PC biosynthesis resembling the defect in *ino4* mutants. They were also found to have phospholipid compositions resembling those of the *ino4* mutants (Table 4). Furthermore, like *ino4* mutants, they incorporated a reduced proportion of [*methyl*-^{14}C]methionine into PC (Table 3).

To compare PC biosynthesis in *ino2* and *ino4* mutants with PC synthesis in fully repressed wild-type cells, we grew wild-type cells in the presence and absence of inositol and choline. (The phospholipid *N*-methyltransferases of yeasts are repressed only by the simultaneous addition of inositol and choline to the growth medium [10, 21, 22, 25].) The addition of choline to cells grown in medium lacking inositol had little or no effect on the labeling of the three methylated lipids with [*methyl*-^{14}C]methionine (Table 5). However, when cells were grown in medium supplemented with inositol, additional supplementation with choline caused about a threefold reduction in the amount of label recovered in PC in a 30-min pulse (Table 5). Furthermore, in cells grown in the presence of inositol and choline, only 32% of the lipid-soluble label was recovered in PC, as compared with 59 to 69% under the other three conditions (Table 5). There also appeared to be a slightly higher accumulation of label in the intermediates PDME and PMME when cells were grown in the presence of inositol and choline. Thus, the patterns of labeling of wild-type cells most resembled those of the *ino2*

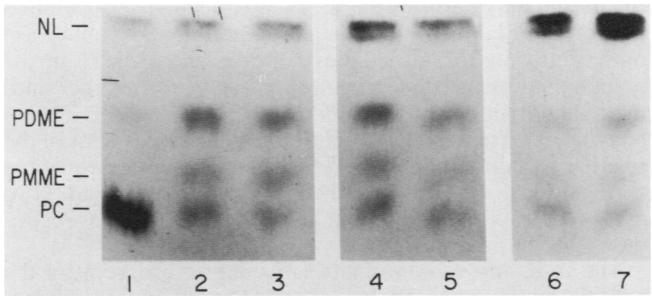

FIG. 3. Synthesis of methylated phospholipids in *ino4* and *ino2* mutants. Autoradiogram of a one-dimensional chromatographic separation of *methyl*-^{14}C-labeled lipids from various *ino4* and *ino2* strains (labeled, extracted, and separated as described in the text). Lanes: 1, wild type (*ade5*); 2, MC40 (*ino4-40*); 3, MC38 (*ino4-38*); 4, MC8 (*ino4-8*); 5, BSI-8D (*ino4-BSI*); 6, MC2 (*ino2-2*); 7, MC21 (*ino2-21*). NL, Neutral lipids. (See Table 1 for complete genotypes.)

TABLE 5. Incorporation of [*methyl*-^{14}C]methionine into phospholipids in a wild-type strain (*ade5*)[a]

Supplement	Relative incorporation (%) of [*methyl*-^{14}C]methionine into[b]:					Incorporation (cpm) of [methyl-^{14}C]methionine into[c]:	
	PMME	PDME	PC	NL	Other	TCA	PC
None	1.2 (0.6)	9.0 (5.1)	68.7 (33.2)	19.9 (11.2)	1.2	19	6.3
Choline	1.6 (0.7)	8.5 (3.8)	64.7 (28.5)	23.1 (10.1)	2.1	17	4.8
Inositol	2.0 (0.9)	14.4 (6.4)	59.5 (26.5)	21.4 (9.2)	2.7	25	6.5
Inositol and choline	5.3 (1.9)	19.5 (7.1)	32.3 (11.7)	37.7 (13.6)	5.2	17	2.0

[a] See Table 3, footnote *a*.
[b] See Table 3, footnote *c*.
[c] See Table 3, footnote *d*.

and *ino4* mutants when the wild-type cells were supplemented with inositol and choline. However, even under these conditions, which correspond to full repression of the phospholipid methyltransferases (10, 22, 25), the wild-type cells incorporated more label into PC (Table 5) than did the *ino2* and *ino4* mutants (Table 3).

DISCUSSION

We isolated mutants of *S. cerevisiae* defective in the formation of PC via methylation of PE. Our selection scheme resulted in the isolation of a mutant which synthesized PC at a reduced rate and at the same time accumulated increased amounts of the methylated phospholipid intermediates (Tables 3 and 4 and Fig. 3). Unlike *Neurospora* mutants (19) defective in PC biosynthesis, the new *S. cerevisiae* mutant was not a strict choline auxotroph. The fact that the mutant grew despite a quite aberrant phospholipid composition reinforces the idea that yeast cells tolerate substantial alterations in the relative proportions of the major phospholipids.

However, in addition to its defect in PC synthesis, the mutant also failed to express the cytoplasmic enzyme I-1-P S and was thus an inositol auxotroph. The mutant was found to be allelic to existing *ino4* mutants previously selected on the basis of inositol auxotrophy. In addition to their effect on inositol metabolism, all the *ino4* and *ino2* mutants also simultaneously affected PC biosynthesis, as shown by biochemical analyses. The *ino2* and *ino4* mutants exhibited a reduced proportion of PC in their phospholipid compositions, ranging from 11 to 18% in the mutants and 38 to 42% in the wild-type strains examined in this study (Table 4). The reduction in the proportion of PC in the phospholipid compositions of the *ino4* and *ino2* mutants while the intermediate PDME accumulated (Table 4) suggests that the activity of the third methylation step (Fig. 1) is reduced. The accumulation of label in the pulse-labeling experiments (Table 3) in both methylated intermediates (PMME and PDME) in the mutants suggests that the second as well as the third methylation step may be affected. However, these in vivo labeling experiments do not permit a clear assessment of the relative reduction in activity of each of the three sequential methylations. Such an assessment will require in vitro assays of each of the enzymatic activities. These experiments are in progress.

The *ino2* and *ino4* mutants are unable to derepress the cytoplasmic enzyme I-1-P S (5, 6). The pleiotropic phenotypes of the *ino2* and *ino4* mutations described here suggests that the *INO2* and *INO4* loci are also involved in the regulation of phospholipid methylation. The level of incorporation of [*methyl*-^{14}C]methionine into PC in the mutants was lower than that in wild-type cells grown in the presence of inositol and choline (Tables 3 and 5), the growth condition which corresponds to full repression of the phospholipid *N*-methyltransferases (10, 21, 22, 25). The *ino2* and *ino4* mutants incorporated label into PC at a rate ca. 50% or less of the rate in fully repressed wild-type cells (grown in the presence of inositol and choline). Thus, the synthesis of PC in the mutants most resembles the synthesis of PC in wild-type cells grown under conditions of full repression of the phospholipid *N*-methyltransferases.

As the conversion of PE to PC was reduced in the *ino2* and *ino4* mutants, it appears that the wild-type (*INO4 INO2*) gene products may be required for maximum expression (derepression) of the phospholipid *N*-methyltransferases, just as they are required for I-1-P S derepression. In the case of I-1-P S, the gene product of the *INO1* gene, it is known that the *ino4* mutation leads to a reduced level of total *INO1* transcription (L. S. Klig, Ph.D. thesis, Albert Einstein College of Medicine, Bronx, N.Y., 1983; L. S. Klig and S. A. Henry, J. Biol. Chem., in press). The level at which the *ino4* and *ino2* mutations affect the regulation of the phospholipid *N*-methyltransferases, however, cannot be determined until the structural genes for those enzymes are identified and isolated. The finding that these genes affect the expression of activities in both pathways (i.e., inositol biosynthesis in the cytoplasm and the conversion of PE to PC in the membrane) suggests that these functions are under coordinate control. Other mutants having pleiotropic phenotypes which may be related to this coordinate regulation have been reported. For example, Yamashita and Oshima (24) isolated a class of mutants which had very low levels of the phospholipid *N*-methyltransferases, but only in the presence of inositol. In addition, the *S. cerevisiae opi3* mutant, which was isolated on the basis of an inositol overproduction and excretion phenotype, was found to be defective in the third methylation reaction in PC biosynthesis (i.e., PDME → PC) (8).

The pleiotropic phenotypes of these mutants and the observation that both choline and inositol are required for the repression of the phospholipid *N*-methyltransferases (10, 21, 22, 25) suggest that there exists a complex network of regulations coordinating the synthesis of choline- and inositol-containing phospholipids and their precursors. There are other precedents in *S. cerevisiae* for the coordinate control of related biosynthetic pathways. For example, a large number of enzymes involved in the biosynthesis of a variety of amino acids are coordinately regulated. Not only are the amino acid biosynthetic enzymes coordinately derepressed upon starvation for any one amino acid, but all the enzymes also respond to the same series of positive and negative regulatory genes (11, 12, 23). The finding that the *INO2* and *INO4* genes regulate several phospholipid biosynthetic activities suggests that phospholipid biosynthesis in *S. cerevisiae* may also be under coordinate regulation.

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