MOLECULAR AND CELLULAR BIOLOGY, Oct. 1986, p. 3320–3328
0270-7306/86/103320-09$02.00/0

Vol. 6, No. 10

Expression of the *Saccharomyces cerevisiae* Inositol-1-Phosphate Synthase (*INO1*) Gene Is Regulated by Factors That Affect Phospholipid Synthesis

JEANNE P. HIRSCH AND SUSAN A. HENRY*

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

Received 24 March 1986/Accepted 17 June 1986

The *INO1* gene of *Saccharomyces cerevisiae* encodes the regulated enzyme inositol-1-phosphate synthase, which catalyzes the first committed step in the synthesis of inositol-containing phospholipids. The expression of this gene was analyzed under conditions known to regulate phospholipid synthesis. RNA blot hybridization with a genomic clone for *INO1* detected two RNA species of 1.8 and 0.6 kb. The abundance of the 1.8-kb RNA was greatly decreased when the cells were grown in the presence of the phospholipid precursor inositol, as was the enzyme activity of the synthase. Complementation analysis showed that this transcript encoded the *INO1* gene product. The level of *INO1* RNA was repressed 12-fold when the cells were grown in medium containing inositol, and it was repressed 33-fold when the cells were grown in the presence of inositol and choline together. The *INO1* transcript was present at a very low level in cells containing mutations (*ino2* and *ino4*) in regulatory genes unlinked to *INO1* that result in inositol auxotrophy. The transcript was constitutively overproduced in cells containing a mutation (*opi1*) that causes constitutive expression of inositol-1-phosphate synthase and results in excretion of inositol. The expression of *INO1* RNA was also examined in cells containing a mutation (*cho2*) affecting the synthesis of phosphatidylcholine. In contrast to what was observed in wild-type cells, growth of *cho2* cells in medium containing inositol did not result in a significant decrease in *INO1* RNA abundance. Inositol and choline together were required for repression of the *INO1* transcript in these cells, providing evidence for a regulatory link between the synthesis of inositol- and choline-containing lipids. The level of the 0.6-kb RNA was affected, although to a lesser degree, by many of the same factors that influence *INO1* expression.

The synthesis of membrane phospholipids in the yeast *Saccharomyces cerevisiae* is a coordinately regulated process involving reactions that take place in both the cytoplasmic and membrane compartments of the cell. The enzymes that catalyze these reactions are subject to several control mechanisms. One level of regulation must involve a coordinated response to cell growth, because ongoing membrane synthesis in dividing cells demands a higher rate of phospholipid production than is required in stationary-phase cells.

Another aspect of regulation of phospholipid synthesis that has been more extensively studied is the modulating effect of the concentration of metabolic intermediates on individual enzyme activities. For example, many phospholipid synthetic enzymes are regulated by inositol and choline (for a review, see reference 15), the precursors of the phospholipids phosphatidylinositol (PI) and phosphatidylcholine (PC). These soluble intermediates regulate the synthesis of their respective phospholipids. In addition, the observation that inositol is required for regulation of the enzymes that synthesize PC suggests that inositol and choline may also coordinate the activities of the separate pathways that produce PI and PC. PI and PC are derived from a common precursor, CDP-diacylglycerol (CDP-DG), and communication between the two divergent pathways may be necessary to optimize the proportions of the different phospholipids in the membrane. Further evidence for coordinated control comes from genetic studies showing that regulatory mutations which affect inositol metabolism also affect other aspects of phospholipid synthesis (18, 23, 27). These mutant phenotypes may be due to the disruption of either growth control, metabolite repression functions, or coordination between the separate pathways. Isolation of the structural genes for phospholipid synthetic enzymes provides an opportunity to analyze the regulation of their expression by these various mechanisms.

The synthesis of PI, a major membrane phospholipid in eucaryotes, is initiated in the cytoplasm by the conversion of glucose 6-phosphate to inositol 1-phosphate (I-1-P). I-1-P is then dephosphorylated, and free inositol reacts with CDP-DG in the membrane to form PI. The first step in this reaction series is catalyzed by the cytoplasmic enzyme I-1-P synthase (EC 5.5.1.4), the product of the *INO1* gene (7). Denatured I-1-P synthase has a molecular weight of 62,000. The molecular weight of the native protein is approximately 240,000, indicating that the active form of the enzyme is a tetramer. Growth of cells in the presence of exogenously supplied inositol represses the enzyme activity about 50-fold (6). This repression occurs by regulation of the amount of I-1-P synthase in cells, because the level of the protein is greatly reduced under repressing conditions, as shown by immunoprecipitation (7). The amount of I-1-P synthase is also affected by mutations in genes unlinked to the *INO1* structural gene, resulting in either a very low level (*ino* mutations [7, 27]) or overproduction (*opi* mutations [11–13]) of the enzyme. The *INO1* gene has been cloned by complementation of an *ino1* mutation and was found to be present as a single copy in the genome (22). In cells containing the cloned *INO1* gene on an autonomously replicating plasmid, I-1-P synthase is repressible by inositol, as shown by immunoprecipitation.

* Corresponding author.

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

Strain designation	Relevant genotype	Source or reference
W303-1A (wild type)	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	R. Rothstein
LT13	*MATa leu2-3,112 his3 inol-13*	22
MC2	*MATα lys2 ino2-2*	5
MC21	*MATα lys2 ino2-21*	5
BS1-8D	*MATa ade1 ino4-BS1*	27
MC39	*MATα lys2 ino4-39*	5
OP1	*MATa ade5 opi1-1*	13
C6B	*MATa lys2 cho2-1*	This laboratory

In this report, we demonstrate that modulation of I-1-P synthase activity by inositol and choline, as well as by mutations unlinked to *INO1*, occurs in part by changes in the abundance of *INO1* RNA. Further evidence is presented that regulation of the pathways that lead to the synthesis of PI and PC, the two major membrane phospholipids in *S. cerevisiae*, is not independent but involves an interaction between the two pathways.

(This report was taken in part from a Ph.D. thesis to be submitted by J. P. Hirsch to the Albert Einstein College of Medicine in 1987.)

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 on YEPD plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar). Synthetic complete medium contained 2% glucose, vitamin-free yeast nitrogen base (6.7 gm/liter; Difco Laboratories), biotin (2 μg/liter), calcium pantothenate (400 μg/liter), folic acid (2 μg/liter), niacin (400 μg/liter), *p*-aminobenzoic acid (200 μg/liter), pyridoxine hydrochloride (400 μg/liter), *myo*-inositol (2 mg/liter), lysine (20 mg/liter), arginine (20 mg/liter), methionine (20 mg/liter), threonine (300 mg/liter), tryptophan (20 mg/liter), leucine (60 mg/liter), histidine (10 mg/liter), adenine (20 mg/liter), and uracil (40 mg/liter). Inositol-free medium was identical to synthetic complete medium with the omission of inositol. Choline chloride was added at 1 mM where indicated. Auxotrophic markers were scored on medium lacking a single component of the complete medium. In all studies, cells were grown at 30°C.

Escherichia coli RR1 cultured in LB medium was used to propagate plasmids. Ampicillin was added to 50 μg/ml.

Plasmid construction and isolation. The 5.3-kilobase (kb) yeast genomic insert containing the *INO1* gene in the integrating vector YIp28 (22) was digested with *Ava*I and *Sal*I and recircularized to produce pDK396 (provided by D. Hoshizaki). Subclones pJH305 (fragment A), pJH306 (fragment B1), pJH309 (fragment B2), pJH307 (fragment B3), and pJH308 (fragment C) were constructed by digesting pDK396 with *Pst*I and *Bgl*II and inserting the resulting fragments into the *Pst*I site, the *Bgl*II site, or both sites in the vector pUC9 (35). The B3 fragment from pJH307 was cloned into the *Eco*RI-*Hin*dIII sites of the vectors pGEM1 and pGEM2 (Promega Biotec) to form pJH310 and pJH311, respectively. The B2 fragment from pJH309 was cloned into the *Eco*RI-*Hin*dIII sites of pGEM1 to form pJH316. The D-region probe was made by subcloning the *Eco*RI-*Pvu*II fragment from pJH308 into pGEM1.

The plasmids used for complementation were constructed in the shuttle vector YEp351 (kindly donated by J. Hill). YEp351 contains the yeast *LEU2* gene and 2 μm origin of replication in the bacterial vector pUC18 (J. Hill, A. Myers, T. J. Koerner, and A. Tzagoloff, submitted for publication). The *Sst*I-*Sph*I fragment from pDK396 was cloned into the *Sst*I-*Sph*I sites of YEp351 to give pJH318. The *Bgl*II-*Hin*dIII fragment from pJH318 was cloned into the *Bam*HI-*Hin*dIII sites of YEp351 to give pJH321. The plasmid ptcm3.2, containing the ribosomal protein gene *TCM1* (9), was generously provided by J. Warner.

Plasmid DNA was isolated by the rapid method of Birnboim and Doly (2) for identification of recombinants. Large-scale plasmid preparations were done by the method of Clewell and Helinski (3).

RNA isolation and Northern blot analysis. RNA was isolated from *S. cerevisiae* cells by the glass bead disruption and hot phenol extraction method of Elion and Warner (8). Polyadenylated [poly(A)$^+$] RNA was prepared by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography (1), fractionated on denaturing formaldehyde agarose gels (24) in a modified buffer system (31), and transferred to nitrocellulose (34).

^{32}P-labeled nick-translated probes were synthesized as previously described (30). Hybridization with nick-translated probes was done at 37°C in 50% formamide at salt concentrations of 0.9 M sodium chloride and 0.09 M sodium citrate. ^{32}P-labeled single-stranded RNA probes were synthesized by using SP6 polymerase (NEN Research Products) by the method of Melton et al. (29). Hybridization with RNA probes was done in the hybridization buffer described above at 53°C.

Quantitation of slot-blot hybridization was done by densitometry of autoradiograms with a Quantimet 920 (Cambridge Instruments) image analyzer. The slot-blots used for densitometry underwent multiple exposures, and the linear increase in optical density values with respect to exposure time ensured that the film response was in the linear range.

Transformation of *S. cerevisiae*. Cells were grown overnight to a density of 5×10^7 to 7×10^7 cells/ml. They were transformed with isolated plasmid DNA by the lithium acetate method of Ito et al. (19), with the following modifications. After the first wash of the harvested cells, the pellet was suspended in 20 ml of LTE (0.1 M lithium acetate, 10 mM Tris hydrochloride pH 7.8, 1 mM EDTA) and incubated for 30 min at 30°C with gentle shaking. The culture was spun at 1,000 × *g* and suspended in 3 ml of LTE. Plasmid DNA was added to 0.2-ml portions of this suspension, and the mixtures were incubated for 30 min at 30°C. An equal volume of 60% polyethylene glycol 3350 dissolved in water was added, and the procedure was continued as described previously (19).

Plasmids were retrieved from yeast cells by suspending 1 ml of packed cells in 200 μl of 100 mM NaCl–10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–0.1% sodium dodecyl sulfate and vortexing for 1 min in the presence of glass beads. The sample was extracted twice with phenol and once with chloroform-isoamyl alcohol (24:1). Nucleic acids were ethanol precipitated from 300 mM sodium acetate, and the pellet was washed with 80% ethanol. The sample was suspended in 100 μl of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, and 1 μl of this solution was used to transform *E. coli* cells.

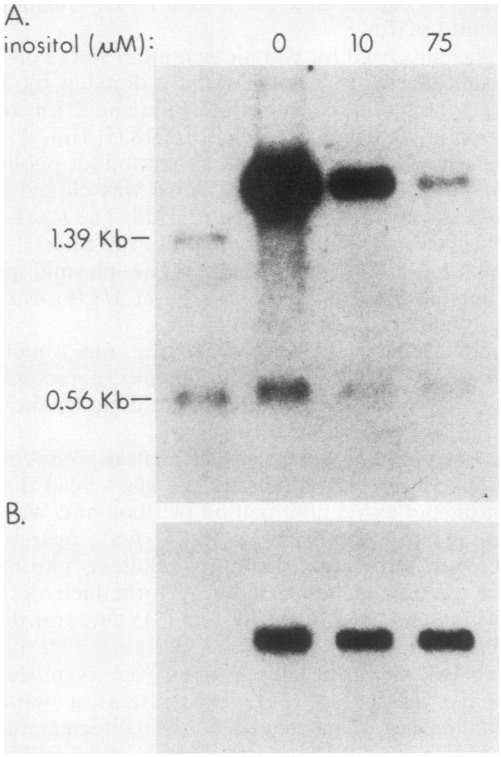

FIG. 1. Northern blot of *INO1* transcripts. Poly(A)$^+$ RNA was isolated from wild-type *S. cerevisiae* cells grown at the indicated concentrations of inositol; 0.8 μg of RNA per lane was fractionated on a 1.2% formaldehyde agarose gel and transferred to nitrocellulose. (A) Blot probed with ^{32}P-labeled nick-translated pDK396, which contains the entire *INO1* insert. The standards in the leftmost lane are ^{32}P-labeled RNAs transcribed by SP6 polymerase from the control template (Promega Biotec). (B) Same blot reprobed with ^{32}P-labeled nick-translated ptcm3.2, which contains the ribosomal protein gene *TCM1*.

RESULTS

Expression of the *INO1* gene in response to inositol. The level of the *INO1* gene product, I-1-P synthase, is known to be regulated by growing cells in the presence of the phospholipid precursor inositol, as shown by measurements of enzyme activity and protein abundance (6, 7). To study the effect of this regulation on the abundance of *INO1* RNA, we used cloned *INO1* DNA to probe a Northern blot of poly(A)$^+$ RNA isolated from cells grown in different concentrations of inositol. RNA from wild-type cells grown in 0, 10, or 75 μM inositol was fractionated on a gel and transferred to nitrocellulose. The blot was probed with ^{32}P-labeled nick-translated DNA from the original 5.3-kb genomic fragment that had been cloned on the basis of complementation of an *ino1* mutation. The resulting autoradiogram is shown in Fig. 1A. Two hybridizing species of RNA were detected, of 1.8 and 0.6 kb. The 1.8-kb RNA was present in high abundance under derepressing conditions (0 μM inositol), was less abundant under intermediate conditions (10 μM inositol), and was greatly reduced under repressing conditions (75 μM inositol). These results correlate qualitatively with the level of I-1-P synthase activity observed in cells grown under similar conditions (6). The 0.6-kb RNA was also present at a reduced level in cells grown in inositol, but the effect on this transcript was much less pronounced. To allow a comparison of the amount of poly(A)$^+$ RNA in each lane, this blot and subsequent Northern blots were reprobed with a ^{32}P-labeled nick-translated plasmid containing a ribosomal protein gene, *TCM1* (Fig. 1B). The level of RNA hybridizing to this gene did not vary under any of the conditions used in this study, as determined by comparison of signals from equal amounts of total RNA quantitated by optical density measurements.

Localization and direction of transcription. The regions within the cloned insert that hybridized to each of the two poly(A)$^+$ RNAs were determined by subcloning adjacent restriction fragments into the vector pUC9 and using the subclones to probe Northern blots. The restriction map of the original insert is shown in Fig. 2. Below it are diagrammed the subclones that were used to probe identical lanes of a Northern blot of RNA from cells grown without inositol (Fig. 3A). Subclones A and B1 did not contain regions complementary to any detectable RNA. Subclone B2 only hybridized to the 0.6-kb RNA, subclone C only hybridized to the 1.8-kb RNA, and subclone B3 detected both transcripts. These results demonstrated that the two RNAs are transcribed from sequences in close proximity, such that the 0.6-kb RNA crosses the middle *Pst*I site at the B2-B3 boundary, and the 1.8-kb RNA crosses the rightmost *Bgl*II site at the B3-C boundary. The possibility that these RNAs are derived from overlapping transcription units cannot be ruled out by this analysis.

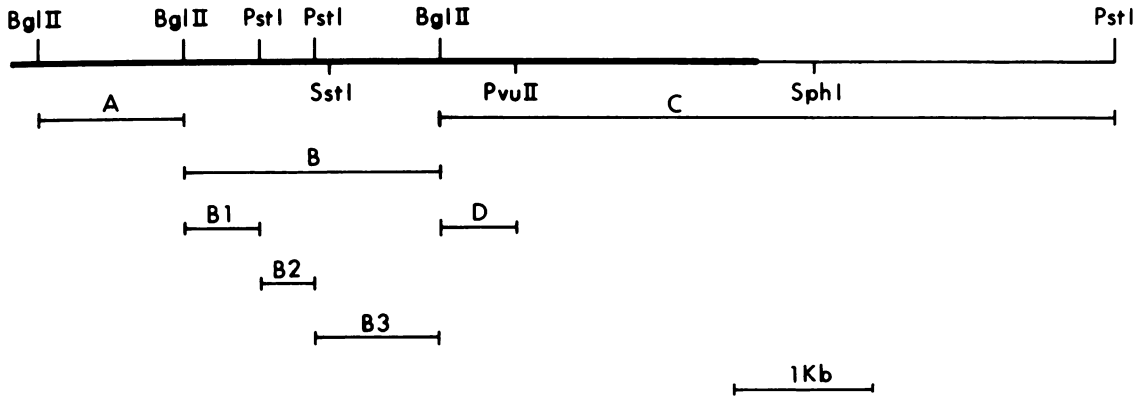

FIG. 2. Restriction map of the cloned *INO1* insert. A partial restriction map of plasmid pDK396 is shown. The heavy line represents the complete *S. cerevisiae* genomic insert containing the *INO1* gene. The light line represents YIp28 vector sequences. This map is a revised version of one that was published previously (22). The fragments shown below the map were subcloned as described in Materials and Methods.

The direction of transcription of the two RNAs was determined by cloning the B3 fragment, which hybridized to both transcripts, into a vector containing the phage SP6 promoter. The B3 fragment was cloned in both orientations with respect to the SP6 promoter, allowing the generation of ^{32}P-labeled single-stranded RNA probes of both polarities. These probes were hybridized with Northern blots of RNA from cells grown in the absence of inositol (Fig. 4). An RNA probe transcribed in direction 1 did not hybridize to either transcript, whereas a probe transcribed in direction 2 detected both transcripts. Therefore, both poly(A)$^+$ RNAs are transcribed in the same direction, from the *Pst*I site toward the *Bgl*II site.

Identification of the *INO1* transcript. Two considerations suggested that the 1.8-kb RNA encodes the *INO1* gene product. First, its abundance varied greatly in response to inositol, as would be expected for the *INO1* mRNA. Second, it was the only transcript complementary to the *INO1* clone that was large enough to code for the 62-kilodalton (kDa) enzyme subunit. Therefore, a subclone was constructed in the shuttle vector YEp351 to test whether a fragment of DNA that could code only for the 1.8-kb RNA was sufficient to complement an *ino1* lesion. The *Sst*I-*Sph*I fragment (see Fig. 2) from the original insert was cloned into YEp351, and the resulting construction was used to transform a strain containing the *ino1-13* allele. This subclone is missing a portion of the 0.6-kb transcription unit (the B2 fragment) and does not include its promoter, which must be to the left of the B3 fragment as it is shown in Fig. 2. Inositol prototrophy was restored to an *ino1-13* mutant strain by transformation with this construction; therefore, the 1.8-kb transcript encodes the *INO1* gene product. The Ino$^+$ phenotype was mitotically unstable and was lost concomitantly with the leucine prototrophy conferred by the *LEU2* gene in the vector. Plasmid DNA was retrieved from the transformed strain and shown to have the same restriction map as the input plasmid DNA. A construction containing the *Bgl*II-*Sph*I fragment from subclone C, which is missing a portion of the 1.8-kb transcription unit, did not complement the *ino1-13* mutation.

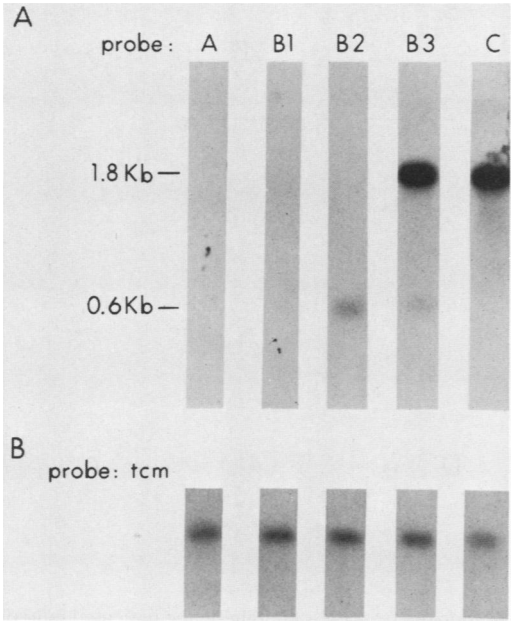

FIG. 3. Northern blot probed with subclones of the *INO1* insert. Poly(A)$^+$ RNA (0.5 μg/lane) was analyzed as described in the legend to Fig. 1. (A) Identical lanes probed with ^{32}P-labeled nick-translated plasmids containing the indicated fragment cloned into the vector pUC9. (B) Same blot reprobed with ptcm3.2.

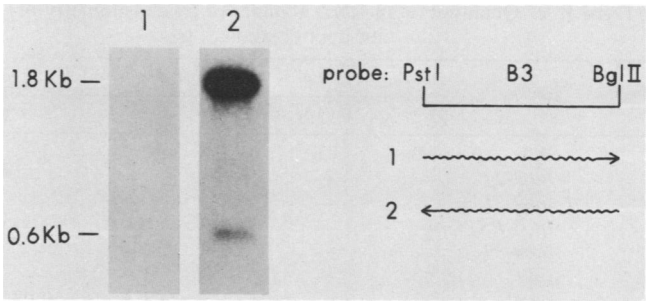

FIG. 4. Direction of transcription. Northern blot analysis of poly(A)$^+$ RNA (0.3 μg/lane) was done as described in the legend to Fig. 1. Lane 1 was probed with ^{32}P-labeled RNA transcribed by SP6 polymerase from fragment B3 cloned into the vector pGEM1 (pJH310). Lane 2 was probed with the SP6 transcription product from B3 cloned into pGEM2 (pJH311).

Quantitation of the regulation by inositol and choline. The relative level of *INO1* RNA in cells grown without inositol compared with that in cells grown with inositol was measured by loading equal amounts of total RNA onto slot-blots and probing with a subclone containing fragment D (see Fig. 2). This fragment is a subclone of fragment C, which hybridized only to the 1.8-kb RNA (Fig. 3). The effect of the addition of choline to cells growing with or without inositol was also determined, because it has been shown that the combination of choline and inositol fully represses many of the enzymes involved in phospholipid synthesis (18, 23, 36). Cells grown without inositol contained a high level of *INO1* RNA whether or not choline was present (Fig. 5, probe D). The addition of 10 μM inositol to the medium slightly reduced the amount of *INO1* RNA, and 75 μM inositol greatly reduced its level. Addition of choline to cells grown in 75 μM inositol further reduced the amount of *INO1* RNA that was present. When the same set of RNAs was probed with a subclone that only detects the 0.6-kb transcript (Fig. 5, probe B2), a small amount of repression by 10 and 75 μM

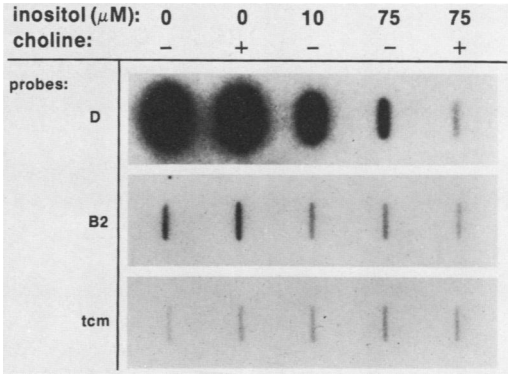

FIG. 5. Quantitation of RNA abundance by slot-blots. Total RNA from wild-type cells grown at the indicated concentration of inositol and either with or without 1 mM choline was loaded directly onto nitrocellulose (4 μg/slot). The strips were probed with ^{32}P-labeled SP6-transcribed RNA from fragment D cloned into pGEM1 (pJH320) or fragment B2 cloned into pGEM1 (pJH316), as indicated. The *TCM1* probe was constructed by cloning the *Ava*I-*Sal*I fragment from ptcm3.2 (24) into pGEM1.

TABLE 2. Quantitation of RNA abundance by densitometry of slot-blot hybridization[a]

Probe	Strain	Relative amt of RNA at inositol concn (μM)[b]:				
		0 (−)	0 (+)	10 (−)	75 (−)	75 (+)
D	WT[c]	1.0	1.6	0.59	0.08	0.03
	ino2-2	—[d]	—	0.07	—	—
	ino2-21	—	—	0.07	—	—
	ino4-BS1	—	—	0.10	—	—
	ino4-39	—	—	0.02	—	—
	opi1	2.5	—	—	1.9	—
	cho2	2.1	2.2	—	1.3	0.02
B2	WT	1.0	1.3	—	0.81	0.71

[a] Cells were grown at the indicated concentrations of inositol. Total RNA from the strains shown was analyzed by slot-blot hybridization as described in the legend to Fig. 5. The numbers are normalized to the signal obtained with the *TCM1* probe and are expressed as the fraction of the amount of RNA present in cells grown with no supplement.

[b] Cells were grown with (+) or without (−) 1 mM choline.

[c] WT, Wild type.

[d] —, Not done.

inositol was observed. Exposures of these slot-blots that were in the linear response range of the film were quantitated by densitometry, and the results of several measurements are summarized in Table 2. The numbers were normalized to the values obtained with the ribosomal protein gene clone to correct for differences in RNA loading, and they are expressed as the fraction of the RNA level present in cells grown with no supplements. The addition of choline alone caused a slight increase in *INO1* RNA abundance (Table 2). When cells were grown in 75 μM inositol, the steady-state level of *INO1* RNA decreased about 12-fold compared with the level in cells grown without inositol. Addition of both choline and inositol resulted in a further reduction of about 3-fold, giving a fully repressed level 33-fold lower than the derepressed level. The 0.6-kb RNA was 1.5 times as abundant in cells grown with no additions as in cells grown in 75 μM inositol (Table 2, probe B2). The regulation of abundance of the 0.6-kb RNA by inositol suggested that it may encode a function involved in phospholipid synthesis. Its accumulation was examined in subsequent experiments to determine whether it varied as a result of mutations in regulatory genes that affect many aspects of phospholipid metabolism.

Effect of unlinked mutations on *INO1* expression. A number of regulatory mutations have been isolated that affect the amount of I-1-P synthase detectable in cells (13, 23, 27). These mutations act in *trans* because they do not map to the *INO1* locus (5, 13, 27). Two categories of mutants have been isolated: *ino* mutants, which are inositol auxotrophs but carry mutations unlinked to *INO1*, and *opi* mutants, which overproduce inositol and were isolated by using a bioassay which detects excreted inositol (13). The level of *INO1* RNA present in cells containing these mutations was determined by Northern blot analysis. In each case, the blot was probed with ^{32}P-labeled RNAs transcribed by SP6 polymerase which were homologous to both the 1.8- and 0.6-kb RNAs.

Figure 6A shows a Northern blot of total RNA isolated from cells containing mutations in two different regulatory genes, *INO2* and *INO4*, and from cells containing a mutation in the *INO1* structural gene. Two *ino2* alleles and two *ino4* alleles were studied, all of which confer inositol auxotrophy on the cells that carry them. The *ino1-13* lesion, a putative missense mutation, results in the production of a protein that is immunologically cross-reactive with I-1-P synthase. These strains were grown in 10 μM inositol, an intermediate condition which allows growth of inositol auxotrophs but results in partial derepression of the *INO1* gene in wild-type cells (Fig. 1). The response of wild-type cells grown under these conditions is shown for comparison. No 1.8-kb *INO1* RNA was detectable in the *ino2* and *ino4* strains. A wild-type

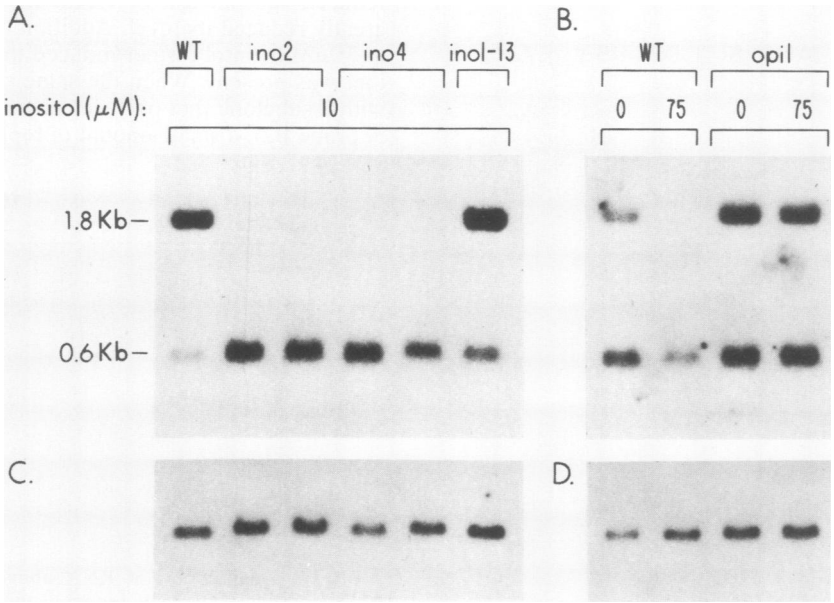

FIG. 6. Northern blot of *INO1* transcripts in *ino* and *opi* mutants. Total RNA (5 μg/lane) from cells containing the indicated mutations was analyzed as described in the legend to Fig. 1. (A) RNA from wild-type (WT), *ino2*, *ino4*, and *ino1* strains grown in 10 μM inositol. The mutant alleles (from left to right) are *ino2-2*, *ino2-21*, *ino4-BS1*, *ino4-39*, and *ino1-13*. (B) RNA from a wild-type strain and a strain carrying the *opi1-1* mutation grown at the indicated concentration of inositol. The lanes in both panels A and B were probed with ^{32}P-labeled SP6-transcribed RNA from plasmids containing sequences homologous to both the 1.8-kb RNA (pJH320) and the 0.6-kb RNA (pJH316). (C and D) Blots shown in panels A and B, respectively, reprobed with nick-translated ptcm3.2.

level of transcript was present in the *ino1-13* mutant strain, as expected. The 0.6-kb transcript was more abundant in the *ino2*, *ino4*, and *ino1-13* strains than in a wild-type strain grown under the same conditions.

The *opi1* mutation is representative of a class of overproducer mutations that result in the constitutive production of I-1-P synthase, whether or not inositol is present (13). Total RNA was isolated from a strain carrying the *opi1* mutation that had been grown in 75 μM inositol or in medium lacking inositol. Northern blot analysis of this RNA showed that there was a high level of the *INO1* transcript present in the *opi1* strain grown either in the presence or absence of inositol (Fig. 6B). The 0.6-kb transcript was also expressed constitutively in this strain.

The expression of the *INO1* gene was also studied in the context of the *cho2* mutation, another mutation which affects phospholipid synthesis. Cells with this mutation have an unusual pattern of I-1-P synthase regulation. Immunoprecipitation studies have shown that I-1-P synthase is present when these cells are grown in a high concentration of inositol; that is, the enzyme is constitutively expressed. However, the addition of choline or its methylated precursors to medium containing inositol represses I-1-P synthase expression (15; V. A. Letts and E. Summers, personal communication). Strains containing the *cho2* mutation also excrete inositol. The *cho2* mutation has an effect on PC synthesis in addition to its effect on inositol excretion. A Northern blot of RNA isolated from cells with the *cho2* mutation grown with and without inositol and choline is shown in Fig. 7. In the absence of inositol, choline did not repress the level of *INO1* RNA. When these cells were grown in medium containing 75 μM inositol, there was no reduction of *INO1* RNA abundance, in contrast to what was seen in wild-type cells (Fig. 1). The combination of inositol and choline, however, resulted in a large decrease in the level of *INO1* RNA. Inositol and choline also reduced the amount of the 0.6-kb transcript to the level seen in wild-type cells under these conditions.

The amount of *INO1* RNA present in cells carrying *ino2*, *ino4*, *opi1*, and *cho2* mutations was quantitated by using slot-blots, and the results are summarized in Table 2. The *ino* mutants grown in 10 μM inositol contained a level of *INO1* RNA that was comparable to the level in wild-type cells grown in 75 μM inositol. Cells carrying the *opi1* mutation had 2.5 times as much *INO1* RNA as fully derepressed wild-type cells in depressing conditions, and this level was only slightly reduced when these cells were grown in 75 μM inositol. The *cho2* mutant strain had twice as much *INO1* RNA as wild-type cells when grown under derepressing conditions, and there was only a 38% decrease in this level when the cells were grown in a high concentration of inositol. Growth of *cho2* cells in inositol plus choline reduced the amount of *INO1* RNA to the level detected in fully repressed wild-type cells.

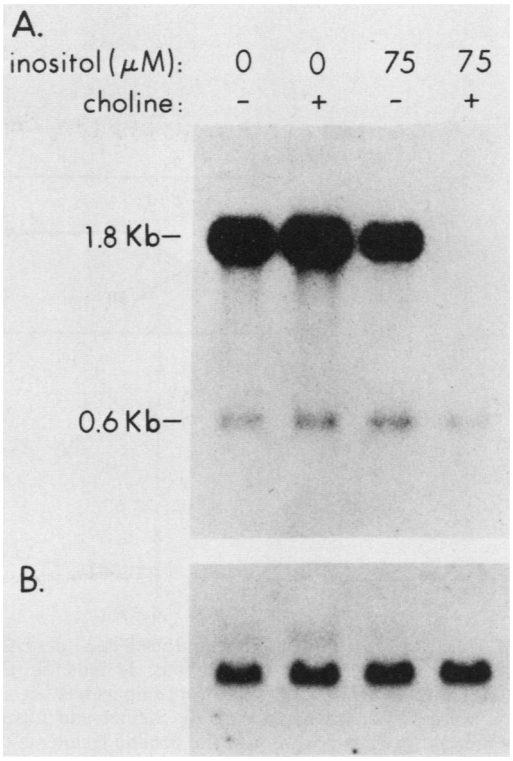

FIG. 7. Northern blot of *INO1* transcripts in a *cho2* strain. Total RNA (5 μg/lane) from cells containing a *cho2* mutant allele was analyzed as described in the legend to Fig. 1. Cells were grown at the indicated inositol concentrations and either with or without 1 mM choline. (A) Blot probed with ^{32}P-labeled SP6-transcribed RNA from plasmids pJH320 (fragment D) and pJH316 (fragment B2). (B) Same blot reprobed with nick-translated ptcm3.2.

DISCUSSION

Modulation of the amount of *INO1* gene product by the phospholipid precursors inositol and choline, as well as by mutations defining unlinked genes, occurs in part by changes in the steady-state level of *INO1* mRNA. By analogy with other regulatory networks found in *S. cerevisiae* (for example, the galactose-inducible genes [14]), it seems likely that this regulation occurs by changes in the rate of transcription of the gene.

The level of *INO1* RNA was repressed 12-fold when cells were grown in inositol, in contrast to the 50-fold repression of enzyme activity reported for cells grown under conditions identical to those used here (6). This discrepancy may reflect the involvement of an additional, posttranscriptional mechanism that affects the amount of active enzyme. For example, a critical concentration of the 62-kDa subunit might be necessary before assembly into the active tetramer occurs. The addition of choline to cells growing in inositol further represses the level of *INO1* RNA. The effect of choline had not been observed by immunoprecipitation of I-1-P synthase, because inositol alone reduces the amount of enzyme to a virtually undetectable level (23). The finding that choline influences the expression of *INO1* RNA in cells grown in the presence of inositol demonstrates that I-1-P synthase is regulated according to the pattern seen for other enzymes involved in phospholipid synthesis, such as CDP-DG synthase (18) and phosphatidylserine synthase (23).

Many of the same factors that regulate *INO1* abundance affect the level of a 0.6-kb poly(A)$^+$ RNA encoded by a closely linked region. The clustering of genes involved in the same metabolic pathway has been observed previously in *S. cerevisiae* (for example, the *GAL* [33] and DAL [4] clusters), and it is possible that this transcript encodes a protein that is involved in some aspect of phospholipid synthesis. Proof of this hypothesis awaits disruption of the gene to determine whether the resulting strain has an altered phospholipid composition.

The results presented here suggest that the *INO1* gene product, like other enzymes involved in phospholipid synthesis, is subject to coordinated regulation involving inositol

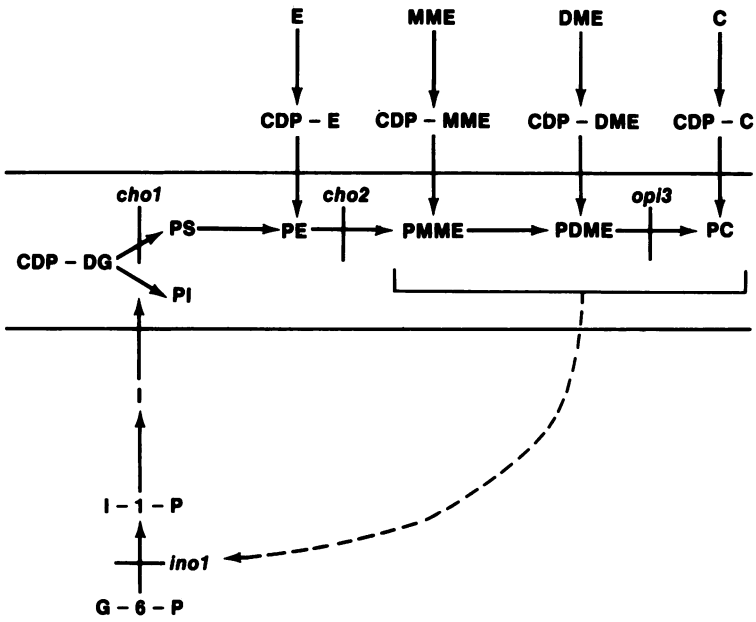

FIG. 8. Pathways for phospholipid synthesis in *S. cerevisiae*. Reactions that take place in the membrane are shown between the rules. Abbreviations: G-6-P, glucose 6-phosphate; I, inositol; E, ethanolamine; C, choline; PS, phosphatidylserine. The incorporation of ethanolamine, MME, DME, and choline into lipids has been described by Kennedy and Weiss (21). The pathways for de novo synthesis of PI and PC were demonstrated in vitro by Steiner and Lester (32). The synthesis of inositol from glucose 6-phosphate was described by Culbertson et al. (6). The positions of the genetic lesions in the *cho1* and *ino1* mutants are shown, as are the probable positions of the *cho2* and *opi3* (12) defects.

and choline. However, this regulation must allow for flexibility in the responses of the various structural genes. For the other enzymes studied, the degree of repression by the combination of inositol and choline is twice the degree of repression by inositol alone (18, 23). For *INO1*, the effect of inositol (12-fold repression) is much greater than the additional effect of choline in combination with inositol (3-fold). The effectors of this regulation are the precursors of two major membrane phospholipids, PI and PC. Figure 8 shows the series of reactions that result in the formation of these phospholipids. The role of the soluble precursors may be to allow communication between the pathways leading to the synthesis of PI and PC so that optimal proportions of different phospholipids in the membrane are maintained.

Further evidence that these enzymes are under coordinated control comes from studies of regulatory mutations that affect *INO1*. The *ino2* and *ino4* mutants were isolated on the basis of their inositol auxotrophy but were subsequently found to have alterations in other steps of phospholipid synthesis (27). The effect of *ino2* and *ino4* mutations on the level of *INO1* RNA suggests that they encode positive regulators that are required for the expression of structural genes, analogous to the functions of *GAL4* and *GCN4*, the positive regulators required for the expression of galactose-inducible genes (20), and genes in the general amino acid control system (17), respectively. Unlike the *GAL4* and *GCN4* proteins (10, 16), however, neither *INO2* nor *INO4* is likely to encode the ultimate positive regulator of *INO1* because a dominant mutation (*OPI5*$^-$) has been identified that allows *INO1* expression in an *ino2 ino4* background (28; B. S. Loewy, Ph.D. thesis, Albert Einstein College of Medicine, 1985).

The *opi1* mutation was isolated on the basis of its inositol excretion phenotype but was also found to affect the regulation of the pathway leading to PC (see Fig. 8). A mutation in the *OPI1* locus causes constitutive expression of many enzymes in the phospholipid pathway (18, 23). An *opi1* mutant strain makes twice as much *INO1* RNA as a fully derepressed wild-type strain, and this overproduction occurs in both the absence and presence of inositol. It seems likely that the wild-type product of this gene is an overall negative regulator of the expression of phospholipid synthetic enzymes.

Further evidence suggesting that the regulation of PI and PC synthesis is coupled comes from the study of the *cho2* mutation. This mutation may define a structural gene for a phospholipid methyltransferase that synthesizes PC (12, 15). In addition, it has effects on inositol metabolism. Cells carrying the *cho2* mutation do not require choline for growth, although they have an aberrant phospholipid composition in its absence. *cho2* cells contain very little PC and a large proportion of phosphatidylethanolamine (PE) (15; Letts and Summers, personal communication). PE is an intermediate in the pathway that culminates in PC, so it appears that the *cho2* lesion results in a block in the methylation of PE that normally occurs to produce PMME (see Fig. 8). The addition of monomethylethanolamine (MME), the soluble precursor of PMME, to *cho2* cells allows them to make PC, indicating that they specifically lack the ability to methylate PE. This result is consistent with the hypothesis that *CHO2* is the structural gene for a phospholipid methyltransferase (15). Cells containing the *cho2* mutation excrete inositol under derepressing conditions, consistent with the observation that they contain a twofold-higher level of *INO1* RNA under these conditions than wild-type cells. The results presented here demonstrate that *cho2* mutants have an unusual pattern of *INO1* expression. Addition of inositol to these cells results in a slight decrease in the level of *INO1* RNA compared with the large decrease seen under these conditions in wild-type cells. Inositol and choline together cause full repression, as they do with wild-type cells. This result can be reconciled with

the hypothesis that *cho2* cells are blocked in the pathway leading to PC if it is postulated that an effector that detects the level of PC synthesis must be present to allow regulation by inositol. This molecule could be a breakdown product of PC or one of its methylated phospholipid precursors (PMME or PDME) which transmits a signal that affects *INO1* expression (dotted line in Fig. 8). PC is capable of providing the regulatory link because *cho2* cells grown in the presence of choline use it to synthesize PC directly, and they still exhibit normal regulation of *INO1* by inositol. The addition of either of the methylated precursors of PC (MME or dimethylethanolamine [DME]) to *cho2* cells results in the synthesis of PC. These additions also allow repression of I-1-P synthase by inositol, as detected by immunoprecipitation (Letts and Summers, personal communication). Thus, restoration of the pathway leading to PC results in proper I-1-P synthase regulation.

This model is supported by results obtained with cells carrying the *cho1* mutation. *CHO1* is the structural gene for phosphatidylserine synthase (26), but a mutation in this gene also causes loss of regulation of the *INO1* gene product by inositol (25). The *cho1* mutant strain has an ethanolamine auxotrophy, and when these cells are starved for ethanolamine they derepress I-1-P synthase even in the presence of a high concentration of inositol. Therefore, a block in the reaction sequence leading to PC disrupts the regulation of *INO1* by inositol, supporting an interactive system of regulation between these metabolic pathways.

ACKNOWLEDGMENTS

We thank John Hill, Deborah Hoshizaki, and Jonathan Warner for kindly providing plasmids for use in this study.

This work was supported by Public Health Service research grants GM-19629 and GM-11301 from the National Institutes of Health. J.P.H. was supported by Public Health Service training grant GM-07128 from the National Institutes of Health.

LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Clewell, D. B., and D. R. Helinski.** 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.
4. **Cooper, T. G.** 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*, p. 39–99. *In* J. N. Strathern, E. W. Jones, and J. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Culbertson, M., and S. Henry.** 1975. Inositol requiring mutants of *Saccharomyces cerevisiae*. Genetics **80**:23–40.
6. **Culbertson, M. R., T. F. Donahue, and S. A. Henry.** 1976. Control of inositol biosynthesis in *Saccharomyces cerevisiae*: properties of a repressible enzyme system in extracts of wild-type (Ino^+) cells. J. Bacteriol. **126**:232–242.
7. **Donahue, T., and S. Henry.** 1981. Myoinositol-1-phosphate synthase: characteristics of the enzyme and identification of its structural gene in yeast. J. Biol. Chem. **256**:7077–7085.
8. **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.
9. **Fried, H. M., and J. R. Warner.** 1981. Cloning of yeast gene for trichodermin resistance and ribosomal protein L3. Proc. Natl. Acad. Sci. USA **78**:238–242.
10. **Giniger, E., S. M. Varnum, and M. Ptashne.** 1985. Specific DNA binding of *GAL4*, a positive regulatory protein of yeast. Cell **40**:767–774.
11. **Greenberg, M. L., P. Goldwasser, and S. A. Henry.** 1982. Characterization of a yeast regulatory mutant constitutive for synthesis of inositol-1-phosphate synthase. Mol. Gen. Genet. **186**:157–163.
12. **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.
13. **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.
14. **Guarente, L., R. Yocum, and P. Gifford.** 1982. A *GAL10-CYC1* hybrid yeast promoter identifies the *GAL4* regulator as an upstream site. Proc. Natl. Acad. Sci. USA **79**:7410–7414.
15. **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.
16. **Hinnebusch, A. G.** 1985. A hierarchy of *trans*-acting factors modulates translation of an activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **5**:2349–2360.
17. **Hinnebusch, A., and G. R. Fink.** 1983. Positive regulation in the general amino acid control of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **80**:5374–5378.
18. **Homann, M. J., S. A. Henry, and G. M. Carman.** 1985. Regulation of CDP-diacylglycerol synthase activity in *Saccharomyces cerevisiae*. J. Bacteriol. **163**:1265–1266.
19. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
20. **Johnston, S. A., and J. E. Hopper.** 1982. Isolation of the yeast regulatory gene *GAL4* and analysis of its dosage effects on the galactose/melibiose regulon. Proc. Natl. Acad. Sci. USA **79**:6971–6975.
21. **Kennedy, E. P., and S. G. Weiss.** 1956. The function of cytidine coenzymes in the biosynthesis of phospholipids. J. Biol. Chem. **222**:193–214.
22. **Klig, L. S., and S. A. Henry.** 1984. Isolation of the yeast *INO1* gene: located on an autonomously replicating plasmid, the gene is fully regulated. Proc. Natl. Acad. Sci. USA **81**:3816–3820.
23. **Klig, L. S., M. J. Homann, 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.
24. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
25. **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.
26. **Letts, V. A., L. S. Klig, M. Bae-Lee, G. M. Carman, and S. Henry.** 1983. Isolation of the yeast structural gene for the membrane-associated enzyme phosphatidylserine synthase. Proc. Natl. Acad. Sci. USA **80**:7279–7283.
27. **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.
28. **Loewy, B., J. Hirsch, M. Johnson, and S. Henry.** 1986. Coordinate regulation of phospholipid synthesis in yeast, p. 551–565. *In* J. Hicks (ed.), Yeast cell biology (UCLA Symposium vol. 33). Alan R. Liss, Inc., New York.
29. **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.
30. **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.
31. **Rozek, C. E., and N. Davidson.** 1983. *Drosophila* has one myosin heavy-chain gene with three developmentally regulated transcripts. Cell **32**:23–34.
32. **Steiner, M., and R. L. Lester.** 1972. In vitro studies of phospholipid synthesis in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **260**:222–243.
33. **St. John, T. P., and R. W. Davis.** 1981. The organization and transcription of the galactose gene cluster of *Saccharomyces*. J. Mol. Biol. **152**:285–315.
34. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
35. **Vieira, J., and J. Messing.** 1982. The pUC9 plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
36. **Yamashita, S., A. Oshima, J. Nikawa, and K. Hosaka.** 1982. Regulation of the phosphatidylethanolamine methylation pathway in *Saccharomyces cerevisiae*. Eur. J. Biochem. **128**:589–595.