Regulation of the Yeast *INO1* Gene: The Products of the *INO2*, *INO4* and *OPI1* Regulatory Genes Are Not Required for Repression in Response to Inositol

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ABSTRACT

The *ino2*Δ, *ino4*Δ, *opi1*Δ, and *sin3*Δ mutations all affect expression of *INO1*, a structural gene for inositol-1-phosphate synthase. These same mutations affect other genes of phospholipid biosynthesis that, like *INO1*, contain the repeated element UAS_{INO} (consensus 5′ CATGTGAAAT 3′). In this study, we evaluated the effects of these four mutations, singly and in all possible combinations, on growth and expression of *INO1*. All strains carrying an *ino2*Δ or *ino4*Δ mutation, or both, failed to grow in medium lacking inositol. However, when grown in liquid culture in medium containing limiting amounts of inositol, the *opi1*Δ *ino4*Δ strain exhibited a level of *INO1* expression comparable to, or higher than, the wild-type strain growing under the same conditions. Furthermore, *INO1* expression in the *opi1*Δ *ino4*Δ strain was repressed in cells grown in medium fully supplemented with both inositol and choline. Similar results were obtained using the *opi1*Δ *ino2*Δ *ino4*Δ strain. Regulation of *INO1* was also observed in the absence of the *SIN3* gene product. Therefore, while Opi1p, Sin3p, and the Ino2p/Ino4p complex all affect the overall level of *INO1* expression in an antagonistic manner, they do not appear to be responsible for transmitting the signal that leads to repression of *INO1* in response to inositol. Various models for Opi1p function were tested and no evidence for binding of Opi1p to UAS_{INO}, or to Ino2p or Ino4p, was obtained.

THE *INO1* gene of yeast encodes inositol-1-phosphate synthase, the enzyme that catalyzes the rate-limiting step in the synthesis of the eukaryotic phospholipid precursor inositol. *INO1* transcription is a sensitive indicator of defects in the cellular transcription apparatus. Mutations in the large subunit of RNA polymerase II (Scafe *et al.* 1990), the TATA binding protein (Arndt *et al.* 1995; Shirra and Arndt 1999), and in components of the *SWI/SNF* chromatin remodeling complex (Peterson and Herskowitz 1992; Peterson *et al.* 1994) all lead to inositol auxotrophy (Ino^- phenotype) due to an inability to activate the *INO1* gene. Depletion of the general transcription factor, TFIIA, also impairs *INO1* activation (Liu *et al.* 1999). Histone H4 mutations have been characterized that are capable of suppressing the Ino^- phenotype associated with mutations in genes that encode components of the *SWI/SNF* complex (Santisteban *et al.* 1997). Additionally, mutations in the *SIN3* and *UME6* genes lead to high-level *INO1* expression and an associated *o*ver*p*roduction of *i*nositol (Opi^- phenotype; Hudak *et al.* 1994; Jackson and Lopes 1996). The *SIN3* and *UME6* gene products are components of a large complex that contains the *RPD3* gene product, a histone deacetylase (Kasten *et al.* 1997; Kadosh and Struhl 1998; Rundlett *et al.* 1998). Compared to wild type, the *rpd3*Δ mutant displays an increase of 32-fold in *INO1* expression. It also exhibits a 5-fold increase in the acetylation of the lysine 5 residue of histone H4 that is associated with the *INO1* promoter (Rundlett *et al.* 1998).

In wild-type cells, *INO1* is expressed only during the logarithmic phase of growth (Jiranek *et al.* 1998) and only in the absence of inositol (Hirsch and Henry 1986). During logarithmic growth, the *INO1* gene can be repressed in response to inositol only if synthesis of phosphatidylcholine (PC) is ongoing (Griac *et al.* 1996). Excessive turnover of PC via a phospholipase D-mediated route leads to *INO1* derepression, even in the presence of inositol and during stationary phase (Patton-Vogt *et al.* 1997; Sreenivas *et al.* 1998). These observations have led to the hypothesis that one or more metabolites involved in phospholipid biosynthesis and/or turnover are directly involved in generating the signal(s) for derepression/repression of *INO1* (Henry and Patton-Vogt 1998).

The *opi1*, *ino2*, and *ino4* mutants were identified on the basis of specific defects in the regulation and/or expression of *INO1* and other UAS_{INO}-containing genes. The *ino2* and *ino4* mutants were originally isolated as inositol auxotrophs (Culbertson and Henry 1975) and were later shown to be pleiotropic, exhibiting deficiencies in PC biosynthesis (Loewy and Henry 1984), as well as inositol metabolism (Culbertson *et al.* 1976). The *INO2* and *INO4* genes encode basic helix-loop-helix (bHLH) proteins that form a heterodimer and activate

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transcription by binding to a repeated element (UAS_{INO}) (consensus: 5′ CATGTGAAAT 3′) found in the promoter of *INO1* and other coregulated genes of phospholipid biosynthesis (Ambroziak and Henry 1994; Bachhawat *et al.* 1995; Schwank *et al.* 1995). The *opi1* mutants were originally isolated on the basis of an inositol overproduction (Opi$^-$) phenotype (Greenberg *et al.* 1982) and were later shown to express a number of phospholipid biosynthetic enzymes constitutively (Klig *et al.* 1985). The mechanism of action of the *OPI1* gene product, which contains leucine zipper and polyglutamine stretch motifs, is presently unknown (White *et al.* 1991; Graves 1996; Patton-Vogt and Henry 1998; Wagner *et al.* 1999).

A complete understanding of the relative roles of the regulatory genes *INO2*, *INO4*, and *OPI1* is essential to the elucidation of the overall regulatory network controlling *INO1* and other UAS_{INO}-containing, coregulated genes. In particular, it is essential to gain insight into the mechanism by which the signal for repression in response to inositol is transmitted to the regulatory apparatus of the cell. In this article, we examine the phenotypes of strains carrying the *ino4*Δ, *ino2*Δ, *opi1*Δ, and *sin3*Δ mutations relative to growth, *INO1* expression, and ability to form DNA protein complexes with the *INO1* promoter. We present evidence that the *INO1* gene can be repressed in response to inositol in certain genetic backgrounds in which *OPI1*, *INO2*, *INO4*, or *SIN3* has been deleted. We conclude that the Ino2p/Ino4p complex, Opi1p, and Sin3p are not required for the regulatory response to inositol. These findings have important implications for our understanding of the mechanism by which repression of UAS_{INO}-containing genes occurs in response to inositol.

MATERIALS AND METHODS

Yeast strains, media, and growth conditions: In previous studies, null mutations of the *OPI1*, *INO2*, *INO4*, and *SIN3* genes were separately constructed in the W303 *MAT***a** or α genetic background, producing the strains OP-Δ2, Disr 1D, NUL2, and SH296, respectively (Table 1). These four strains were used in crosses and tetrads were dissected according to the methods of Sherman *et al.* (1978) to generate a set of genetically related strains containing all possible combinations of the four regulatory mutations (Table 1; JAG strains). The genotypes of these strains were verified in part by replica plating on various drop-out media. Regulatory mutations were identified by plate assays for Opi$^-$ and Ino$^-$ phenotypes and confirmed by analysis of PCR reactions of genomic DNA using primers designed to determine whether the individual deletions were present (data not shown).

Unless noted otherwise, strains were grown at 30° in either YEPD (1% yeast extract, 2% bacto-peptone, 2% glucose) or vitamin-defined synthetic complete media, with or without supplements of 10 μm inositol or 75 μm inositol and 1 μm choline, containing 3% glucose, 0.17% yeast nitrogen base salts, 0.5% ammonium sulfate, 0.0002% trace components, and 1% vitamin mix as previously described (Greenberg *et al.* 1982). Additionally, the following amino acids and supplements were added (milligrams per liter): adenine (20), arginine (20), histidine (20), leucine (60), lysine (230), methionine (20), threonine (300), tryptophan (230), and uracil (20).

Medium supplemented with 75 μm inositol and 1 mm choline is designated I(75)C. The combined presence of inositol and choline at these concentrations has been shown to result in the full repression of transcription of the phospholipid biosynthetic structural genes (Hirsch and Henry 1986; Bailis *et al.* 1987; Henry and Patton-Vogt 1998). Medium supplemented with 10 μm inositol is designated I(10). This concentration of inositol permits an intermediate level of expression of the structural genes, while allowing the inositol auxotrophs to grow (Hirsch and Henry 1986). Medium completely lacking inositol and choline (designated I$^-$) was also utilized. This growth condition has been demonstrated to allow complete derepression of the transcription of the phospholipid structural genes, but it does not permit growth of inositol auxotrophs.

To score markers or to select diploids or transformants based on nutritional prototrophies, strains were grown in synthetic medium lacking the appropriate amino acid or other nutrients. Strains were maintained on the appropriate plates containing 2% agar. Potassium acetate plates (0.1% yeast extract, 0.05% glucose, 1% potassium acetate, 2% agar) were used to induce diploids to sporulate.

RNA isolation and analysis: Total RNA was purified from yeast grown to the middle of the logarithmic growth phase using glass bead disruption and hot phenol extractions (Elion and Warner 1984). RNA was analyzed by the Northern and slot blot assay methods of Hirsch and Henry (1986). The ribosomal gene *TCM1* was used as a control for total RNA levels. As previously described (Hirsch and Henry 1986), *TCM1* expression is not influenced by the presence or absence of phospholipid precursors. Quantitation of the blots was performed using the AMBIS (San Diego) imaging system.

To generate the riboprobes used in this study, the appropriate plasmids (Table 2) were linearized (*INO1*, pJH310 → *Hin*dIII; *TCM1*, pAB309Δ → *Eco*RI; Table 2) and purified by phenol extraction and ethanol precipitation. The DNA was resuspended in 5 μl H_2O for use in the Gemini *in vitro* transcription system (Promega Corp., Madison, WI). The probes were transcribed with the appropriate polymerase (*INO1* → T7, *TCM1* → SP6) in the presence of [α-^{32}P]CTP, which was substituted for unlabeled CTP. The probes were run over a NucTrap column (Stratagene, La Jolla, CA) to remove the unincorporated nucleotides.

Electrophoretic mobility shift assays: Yeast strains were grown to midlogarithmic phase, and whole cell extracts were prepared as described by Lopes and Henry (1991). The protein concentration of extracts was determined using the microassay of the Bio-Rad (Richmond, CA) protein assay kit. Electrophoretic mobility shift assays (EMSAs) were performed according to the methods of Lopes and Henry (1991). A fragment of the *INO1* promoter (−259 to −154), termed template B, containing two copies of UAS_{INO}, was prepared as described by Lopes and Henry (1991).

A cocktail was made for the entire series of reactions [per reaction: 4 mm Tris/HCl pH 8.0, 4 mm $MgCl_2$, 4% glycerol, 1 mm dithiothreitol (DTT), 20,000 cpm DNA probe, and 1 μg double-stranded poly(dI-dC) nonspecific competitor]. The experiments employed a final concentration range of 25 mm to 250 mm KCl, with most assays being performed at 50 mm. Once the cocktail had been aliquotted, an aliquot of whole cell extract, containing 50–100 μg of protein, was added. The reactions were incubated at 30° for 15 min and stopped by the addition of 2 μl of 10× dye (0.4% bromophenol blue, 0.4% xylene cyanol, 50% glycerol). Reactions were fractionated on a 4% nondenaturing TBE-polyacrylamide gel, with 1× TBE used as a running buffer.

TABLE 1

Strains

Strain	Genotype	Source
Disr 1D	*his3-11 leu2-3,-112 trp-1 ino2*Δ*::TRP1 MAT***a**	Nikoloff and Henry (1994)
NUL2	*ade2-1 his3-11,-15 trp1-1 ura3-5 ino4*Δ*::TRP1 MAT*α	Ambroziak and Henry (1994)
SH296	*his3-11 leu2-3,-112 ura3-5 trp1-1 sin3*Δ*::TRP1 MAT***a**	Hudak *et al.* (1994)
OP-Δ2	*leu2-3,-112 his3-11,-15 opi1*-Δ*::LEU2 MAT***a**	White *et al.* (1991)
JAGWT	*his3 leu2 trp1 ura3 MAT*α	This study
JAG1	*his3 leu2 trp1 ura3 opi1*Δ*::LEU2 MAT***a**	This study
JAG2	*his3 leu2 trp1 ura3 ino2*Δ*::TRP1 MAT***a**	This study
JAG3	*his3 leu2 trp1 ura3 sin3*Δ*::TRP1 MAT***a**	This study
JAG4	*his3 leu2 trp1 ura3 ino4*Δ*::LEU2 MAT***a**	This study
JAG1/2	*his3 leu2 trp1 ura3 opi1*Δ*::LEU2 ino2*Δ*::TRP1 MAT***a**	This study
JAG1/3	*his3 leu2 trp1 ura3 opi1*Δ*::LEU2 sin3*Δ*::TRP1 MAT***a**	This study
JAG1/4	*his3 leu2 trp1 ura3 opi1*Δ*::LEU2 ino4*Δ*::LEU2 MAT*α	This study
JAG2/3	*his3 leu2 trp1 ura3 ino2*Δ*::TRP1 sin3*Δ*::TRP1 MAT***a**	This study
JAG2/4	*his3 leu2 trp1 ura3 ino2*Δ*::TRP1 ino4*Δ*::LEU2 MAT*α	This study
JAG3/4	*his3 leu2 trp1 ura3 sin3*Δ*::TRP1 ino4*Δ*::LEU2 MAT***a**	This study
JAG1/2/3	*his3 leu2 trp1 ura3 opi1*Δ*::LEU2 ino2*Δ*::TRP1 sin3*Δ*::TRP1 MAT*α	This study
JAG1/2/4	*his3 leu2 trp1 ura3 opi1*Δ*::LEU2 ino2*Δ*::TRP1 ino4*Δ*::LEU2 MAT*α	This study
JAG1/3/4	*his3 leu2 trp1 ura3 opi1*Δ*::LEU2 sin3*Δ*::TRP1 ino4*Δ*::LEU2 MAT***a**	This study
JAG2/3/4	*his3 leu2 trp1 ura3 ino2*Δ*::TRP1 sin3*Δ*::TRP1 ino4*Δ*::LEU2 MAT***a**	This study
JAG1/2/3/4	*his3 leu2 trp1 ura3 opi1*Δ*::LEU2 ino2*Δ*::TRP1 sin3*Δ*::TRP1 ino4*Δ*::LEU2 MAT*α	This study

***In vitro* transcription/translation:** To perform *in vitro* transcription (Butler and Chamberlin 1982; Nielsen and Chapiro 1986), 4 μg of the respective plasmids carrying the genes of interest were linearized: *OPI1* (pJAG15 → *Not*I), *INO2* (pMN103 → *Sal*I), and *INO4* (pJA755 → *Eco*RI) (Table 2). These complete open reading frames were transcribed with the Promega Gemini transcription kit. The transcripts were phenol extracted and resuspended in sterile water containing pyrocarbonic acid diethyl ester (DEPC). Approximately 4 μg of each *in vitro*-transcribed RNA was used in *in vitro* translation reactions using rabbit reticulocyte lysates (Promega; Pelham and Jackson 1976). The reactions were performed in the presence of ^{3}H-leucine.

The *in vitro*-translated lysate of interest was diluted ~10-fold in immunoprecipitation buffer (150 mm NaCl, 0.1% NP-40, 50 mm Tris-HCl, pH 8.0). Serum containing polyclonal antibodies directed against Ino2p was added, and the tubes were incubated on ice for 1 hr. The Ino2p antibody was prepared as described in Nikoloff and Henry (1994). Approximately 4 mg of Protein A-Sepharose bead slurry (Pharmacia, Piscataway, NJ) was added, and the reaction mixture was allowed to incubate at 4° on a rocking bed for at least 1 hr. Samples were centrifuged at 12,000 rpm for 1 min to pellet the immunocomplex. Pellets were washed four times with 100 μl immunoprecipitation buffer and analyzed by gel electrophoresis.

RESULTS

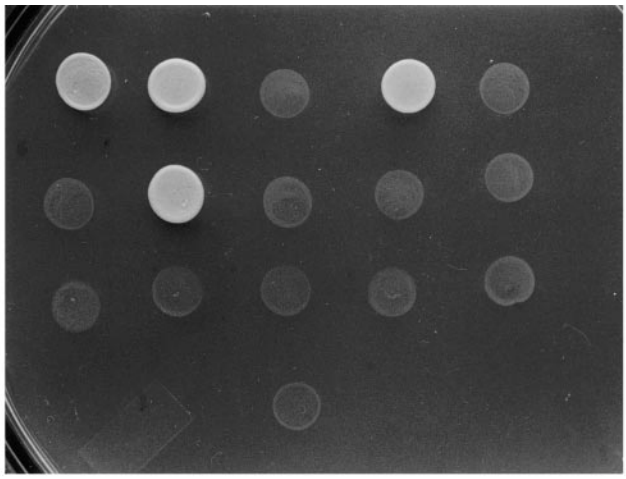
Growth of yeast strains carrying the *ino2*Δ, *ino4*Δ, *opi1*Δ, and *sin3*Δ mutations, singly and in combination: Strains carrying all possible combinations of *ino2*Δ, *ino4*Δ, *opi1*Δ, and/or *sin3*Δ mutations (Table 1) were grown to the midlogarithmic phase of growth in YEPD medium, washed, and spotted onto inositol-free (I^-) plates. All strains containing an *ino2*Δ or an *ino4*Δ mutation, including double and triple mutants containing *ino2*Δ and/or *ino4*Δ in any combination with *opi1*Δ and/or *sin3*Δ, exhibited no growth after 2 days of incubation at 30° on I^- plates (Figure 1).

The same strains were then grown in liquid culture under two conditions capable of supporting growth of inositol auxotrophs. The first growth condition consisted of supplementation with 10 μm inositol but no choline. This medium, designated I(10), permits wild-type cells to express *INO1* and other UAS_{INO}-containing genes. However, the level of *INO1* expression under this growth condition is lower (*i.e.*, partially depressed) compared to the level of expression observed in wild-

TABLE 2

Plasmids used in this study

Plasmid	Description	Source
pJH310	*INO1* riboprobe	Hirsch and Henry (1986)
pAB309Δ	*TCM1* riboprobe	Hudak *et al.* (1994)
pJL105	95-bp *Hin*dIII-*Eco*RI fragment of the *INO1* promoter (template B)	Lopes and Henry (1991)



WT	1Δ	4Δ	3Δ	2Δ
1/2Δ	1/3Δ	1/4Δ	2/3Δ	2/4Δ
3/4Δ	1/2/3Δ	1/2/4Δ	1/3/4Δ	2/3/4Δ
		1/2/3/4Δ		

Figure 1.—Growth of phospholipid regulatory mutants on inositol-free (I^-) plates. Cells were grown in liquid YEPD medium to midlogarithmic phase, harvested by centrifugation, washed, and spotted at equivalent cell densities onto I^- plates. (Top) Growth of strains spotted on an I^- plate after 2 days of incubation at 30°. (Bottom) Identification of the strains (full genotypes given in Table 3) as follows: 1Δ, *opi1*Δ; 2Δ, *ino2*Δ; 4Δ, *ino4*Δ; 3Δ, *sin3*Δ. Strains with multiple mutations are identified as follows: 1/4Δ, *opi1*Δ *ino4*Δ; 1/2/3/4Δ, *opi1*Δ *ino2*Δ *sin3*Δ *ino4*Δ, etc.

type cells growing in medium that is completely inositol free (I^-) (Figure 2, A–C; and Hirsch and Henry 1986). Partially derepressing, I(10) medium also supports growth of inositol auxotrophs, such as *ino2* and *ino4* (Hirsch and Henry 1986). However, the *ino2*Δ and *ino4*Δ strains both grew more slowly and reached a lower final culture density in I(10) medium than the wild-type strains (Table 3). The *ino4*Δ mutant exhibited a doubling time of 4.9 hr in I(10) medium, whereas the *ino2*Δ strain essentially failed to grow in I(10) medium. The growth pattern of the *ino2*Δ *ino4*Δ double mutant in I(10) medium more closely resembled that of the *ino4*Δ mutant than that of the *ino2*Δ mutant (Table 3).

The second growth condition consisted of supplementation of synthetic complete medium with 75 μm inositol and 1 μm choline (I(75)C medium). Wild-type cells grown in I(75)C medium exhibit full repression of *INO1* and other UAS_{INO}-containing genes (Hirsch and Henry 1986; Bailis *et al.* 1987; Griac *et al.* 1996). Under these conditions, wild-type cells derive essentially all their inositol from exogenous sources. They also synthesize a substantial proportion of PC via the cytidine diphosphate (CDP)-choline pathway, utilizing exogenous choline (Hirsch and Henry 1986; Griac *et al.* 1996). This fully supplemented growth condition resulted in optimal growth of *ino2*Δ and *ino4*Δ mutants, permitting them to grow with no apparent deficiency compared to wild type (Table 3). In fact, in I(75)C medium, the *ino4*Δ culture achieved a higher final optical density than wild type, whereas the *ino2*Δ culture reached a final optical density indistinguishable from the wild-type strain. The *ino2*Δ and *ino4*Δ strains also exhibited doubling times slightly faster than wild type in I(75)C medium (Table 3). Even wild-type strains exhibit growth stimulation in response to supplementation with inositol and choline (Griac *et al.* 1996). In our study, we observed that the wild-type strain doubled every 2.8 hr in I(75)C compared to a doubling time of 3.8 hr in I(10) medium. The wild-type strain also reached a higher final culture density in I(75)C than in I(10) medium (Table 3).

In contrast to the *ino2*Δ, the *ino4*Δ, or the wild-type strain, growth of the *opi1*Δ strain was not stimulated in I(75)C medium as compared to I(10) medium. In both media, the *opi1*Δ strain grew somewhat faster than the wild-type strain did under the most favorable growth condition [*i.e.*, in I(75)C medium]. The *opi1*Δ strain also achieved a higher final optical density than wild type in both I(10) and I(75)C medium (Table 3). The fact that the *opi1*Δ defect renders the yeast cell constitutive for a high level of expression of enzymes of phospholipid biosynthesis (Klig *et al.* 1985; White *et al.* 1991; Bachhawat *et al.* 1995) may account for the relatively rapid growth of the *opi1*Δ strain under inositol-limiting conditions [*i.e.*, I(10) medium, Table 3].

The *sin3*Δ strain, like the *opi1*Δ strain, exhibited no growth stimulation in I(75)C medium compared to I(10) medium. However, in contrast to the *opi1*Δ mutant, the *sin3*Δ strain grew significantly slower and achieved a lower optical density than either wild type or *opi1*Δ under both growth conditions (Table 3). The fact that the growth of the *sin3*Δ strain was not stimulated in I(75)C medium suggests that its growth deficiency is not primarily due to defects in phospholipid metabolism. However, the *opi1*Δ *sin3*Δ double mutant exhibited a growth rate in both media resembling *opi1*Δ, suggesting that the elimination of the *OPI1* gene product suppresses the relative *sin3*Δ growth deficiency. This is somewhat surprising since Sin3p participates in a large protein complex involved in histone deacetylation (Kasten *et al.* 1997; Rundlett *et al.* 1998). This complex has global effects on cellular metabolism and regulation (Kadosh and Struhl 1998; Sun and Hampsey 1999). In contrast, to date, Opi1p has only been demonstrated to affect lipid metabolism (Henry and Patton-Vogt 1998).

The *opi1*Δ mutation, however, did not restore inositol prototrophy (Figure 1) or alleviate the slow growth phe-

notype of *ino2*Δ and *ino4*Δ mutants in I(10) medium. The *opi1*Δ *ino2*Δ, the *opi1*Δ *ino4*Δ, and the *opi1*Δ *ino2*Δ *ino4*Δ strains all failed to grow on I^- medium (Figure 1) and grew more slowly and reached lower optical densities in I(10) medium than the *opi1*Δ strain (Table 3). Under fully supplemented conditions [I(75)C medium], the *opi1*Δ *ino4*Δ double-mutant strain grew somewhat more slowly than either of the single mutants (*i.e.*, *opi1*Δ or *ino4*Δ) but ultimately reached an optical density as high as the *opi1*Δ single mutant. The *opi1*Δ *ino2*Δ strain exhibited a doubling time faster than *opi1*Δ *ino4*Δ in I(75)C medium and reached a culture density similar to *opi1*Δ (Table 3).

The *sin3*Δ *ino2*Δ double-mutant strain grew faster than the *sin3*Δ strain under fully supplemented conditions [I(75)C medium], and it grew as well as the *sin3*Δ strain in inositol-limited medium [I(10)]. Thus, the presence of the *sin3*Δ mutation, unlike the *opi1*Δ mutation, appeared to alleviate the severe *ino2*Δ growth deficiency under inositol-limiting conditions [I(10) medium, Table 3]. The *sin3*Δ *ino4*Δ strain, in contrast, exhibited a growth rate in I(10) medium that was slower than either the *ino4*Δ or the *sin3*Δ single-mutant strain. The strains containing the triple and quadruple combinations of the four mutations, for the most part, grew more poorly, especially in I(10) medium, than any of the single or double mutant combinations (Table 3).

Expression of the *INO1* transcript in strains carrying *ino2*Δ, *ino4*Δ, *opi1*Δ, and *sin3*Δ mutations: Expression of the *INO1* transcript was assayed in each strain in the media used for the growth studies described above. The wild-type strain exhibited the expected pattern (Hirsch and Henry 1986) of *INO1* expression and regulation: full derepression in I^- medium and a somewhat lower level of expression (~50% of the level observed in I^- medium) in inositol-limiting medium [I(10)]. Under fully supplemented conditions [I(75)C medium], the level of *INO1* transcript in the wild-type strain was ~10% of the level observed in I^- medium (Figure 2, A–C). The expression of *INO1* transcript was also examined in the *opi1*Δ mutant strain under all three growth conditions [*i.e.*, I^-, I(10), and I(75)C media; Figure 2B]. As previously reported (Hirsch and Henry 1986; White *et al.* 1991), the *INO1* transcript was overexpressed about twofold in the *opi1*Δ strain compared to wild type under fully derepressing conditions (I^- medium) and showed

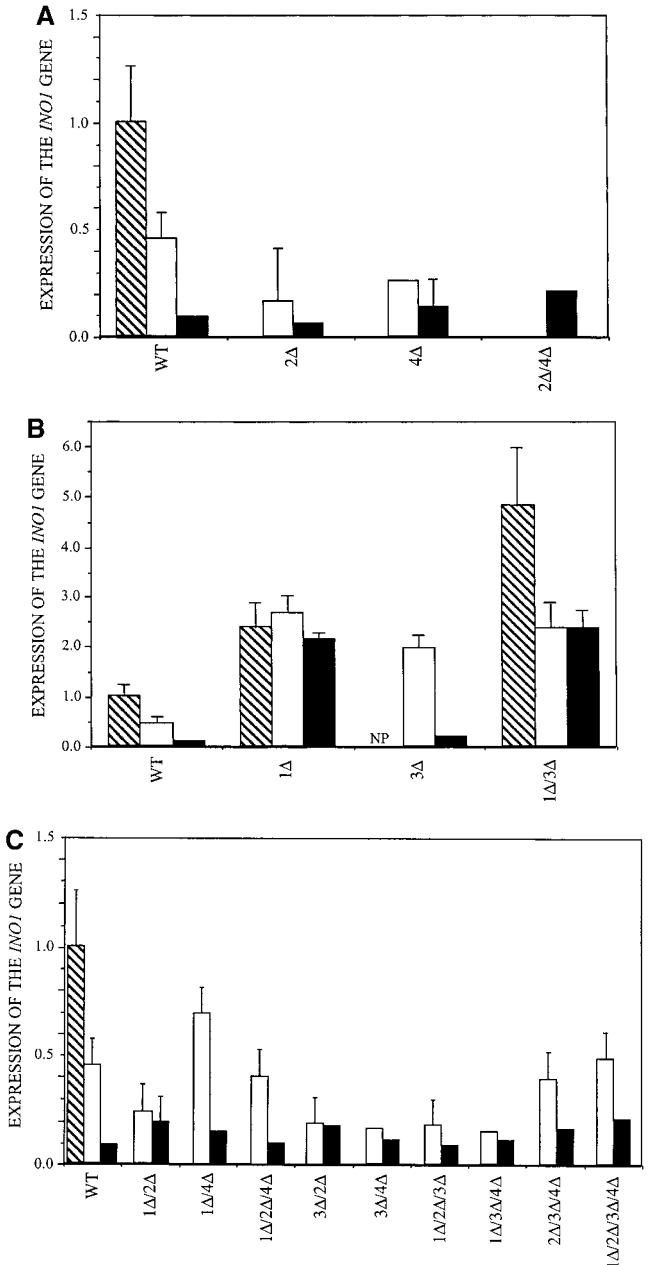


Figure 2.—*INO1* expression in strains carrying mutations in phospholipid biosynthetic regulatory mutants. Total RNA was harvested from the strains grown to the midlogarithmic phase of growth. The full genotypes of the strains are given in Table 1. The mutations are abbreviated as in Figure 1 as follows: WT, wild type (no mutation); 1Δ, *opi1*Δ; 2Δ, *ino2*Δ; 4Δ, *ino4*Δ; and 3Δ, *sin3*Δ. The media in which the strains were grown are represented by the following bars: hatched, inositol-free (I^-, medium); open, 10 μm inositol [I(10) medium]; and solid, 75 μm inositol and 1 mm choline [I(75)C medium]. The specificity of the riboprobes was verified by Northern blot analysis (data not shown). Subsequently, expression was quantified by slot blot analysis and is presented as a ratio of the counts per minute of the *INO1* hybridization to the counts per minute of *TCM1* as measured by the AMBIS visualization system. All samples are normalized to the *INO1*/*TMC1* ratio calculated for wild-type cells grown in the absence of inositol. Each data point is representative of three to five experimental repeats. Wild-type *INO1* expression is repeated in A–C for purposes of comparison. Note that the scale of B differs from A and C in order to represent the two- to fivefold overexpression of *INO1* by *opi1*Δ- and/or *sin3*Δ-bearing strains in comparison to wild type. (A) Expression of *INO1* in strains containing *ino2*Δ and/or *ino4*Δ compared to wild type. (B) Strains carrying *opi1*Δ and/or *sin3*Δ mutations. (C) Strains carrying *ino2*Δ and/or *ino4*Δ mutations in combination with *opi1*Δ and/or *sin3*Δ.

TABLE 3

Growth of mutant strains

Strain	Genotype	Doubling time medium (hr.)		Culture density medium (%)	
		I(75)C	I(10)	I(75)C	I(10)
JAGWT	Wild type	2.8	3.8	100	68
JAG1	*opi1*Δ	2.1	2.3	121	111
JAG3	*sin3*Δ	4.4	5.0	65	64
JAG2	*ino2*Δ	1.9	10.1	101	5
JAG4	*ino4*Δ	2.0	4.9	126	45
JAG2/4	*ino2*Δ *ino4*Δ	2.0	5.0	127	20
JAG1/2	*opi1*Δ *ino2*Δ	2.7	8.5	127	8
JAG1/4	*opi1*Δ *ino4*Δ	3.6	5.8	128	26
JAG1/3	*opi1*Δ *sin3*Δ	2.2	2.3	108	104
JAG3/2	*sin3*Δ *ino2*Δ	2.2	4.6	69	47
JAG3/4	*sin3*Δ *ino4*Δ	4.8	7.7	124	35
JAG1/2/3	*opi1*Δ *sin3*Δ *ino2*Δ	4.1	7.4	115	50
JAG1/2/4	*opi1*Δ *ino2*Δ *ino4*Δ	3.4	7.8	144	9
JAG1/3/4	*opi1*Δ *sin3*Δ *ino4*Δ	6.0	6.5	115	46
JAG2/3/4	*sin3*Δ *ino2*Δ *ino4*Δ	3.5	7.3	117	5
JAG1/2/3/4	*opi1*Δ *sin3*Δ *ino2*Δ *ino4*Δ	3.2	9.0	131	16

Culture density was determined using a Klett-Summerson colorimeter. Doubling times are expressed in hours. Culture density for each strain is expressed as a percentage of the stationary density achieved by the wild-type strain grown in I(75)C; the value for wild-type strain growing in I(75)C is set at 100. See Table 1 for full genotypes.

essentially no repression in response to the presence of inositol and choline in the growth medium.

The *sin3*Δ mutant also overexpressed *INO1* transcript severalfold compared to the wild-type strain in I(10) medium. In contrast to the *opi1*Δ mutant, however, *INO1* expression was repressed in the *sin3*Δ strain in response to high levels of inositol and choline [I(75)C medium; Figure 2B], as previously reported (Hudak *et al.* 1994). The level of *INO1* transcript in the *opi1*Δ *sin3*Δ double mutant strain in I^- medium (Figure 2B) exceeded the levels observed in all other strains studied here, reaching a level fourfold higher than wild type and about twofold higher than in the *opi1*Δ strain growing in I^- medium. However, in I(10) and I(75)C medium, *INO1* expression in the *opi1*Δ *sin3*Δ mutant was reduced to a level approximating that observed in the *opi1*Δ mutant (*i.e.*, about twofold higher than wild-type derepressed level in I^- medium).

The *ino2*Δ and *ino4*Δ single-mutant strains both expressed low levels of *INO1* transcript in both I(10) and I(75)C medium (Figure 2A), consistent with previous reports (Hirsch and Henry 1986). In I(75)C medium, *INO1* transcript was detected in the *ino2*Δ*ino4*Δ strains at a level slightly higher than that seen in the *ino4*Δ strain. However, *INO1* transcript was not detected above background when the *ino2*Δ*ino4*Δ strain was grown on I(10) medium (Figure 2A). The *opi1*Δ*ino2*Δ double-mutant strain expressed *INO1* transcript at a level only slightly higher than the *ino2*Δ single mutant in both I(10) and I(75)C medium, as did the *sin3*Δ *ino2*Δ strain. The *sin3*Δ *ino4*Δ strain also expressed very low levels of *INO1* transcript under both growth conditions (Figure 2C).

Surprisingly, given its lack of growth on I^- plates (Figure 1), the *opi1*Δ *ino4*Δ strain, grown under partially derepressing conditions [*i.e.*, I(10) medium], expressed a level of *INO1* transcript slightly higher than that seen in the wild-type strain under the same growth conditions. The effects of the *opi1*Δ and *ino4*Δ mutations on *INO1* expression appeared to be additive under partially derepressing conditions [*i.e.*, I(10) medium], since the double mutant exhibited a level of *INO1* expression intermediate between the level observed in the two single mutants under these growth conditions (Figure 3, A–C). Moreover, regulation in response to inositol and choline, which is absent in the *opi1*Δ mutant, was restored in the *opi1*Δ *ino4*Δ double mutant, resulting in *INO1* repression in I(75)C medium. The *opi1*Δ *ino2*Δ *ino4*Δ triple-mutant strain also expressed a relatively high level of *INO1* transcript in I(10) medium, despite its very poor growth in this medium (Table 3), and this expression was repressed in I(75)C medium (Figure 3). The *sin3*Δ *ino2*Δ *ino4*Δ triple mutant also exhibited residual expression in I(10) medium, as did the quadruple mutant *opi1*Δ *sin3*Δ *ino2*Δ *ino4*Δ, and in both cases *INO1* expression was repressed in I(75)C medium. Thus, the elimination of all of these regulatory factors did not completely eliminate *INO1* expression or its repression in response to inositol.

The *OPI1* gene product does not appear to bind di-

rectly to UAS_{INO}: EMSAs were performed using whole-cell extracts of the strains in this study as described in materials and methods. For these studies, we employed a fragment of the *INO1* promoter containing nucleotides −259 to −154 (template B), prepared as described in the materials and methods. This fragment of DNA contains two copies of UAS_{INO} (Lopes and Henry 1991) and has been shown to support the formation of a complex with the Ino2p/Ino4p dimer (Lopes and Henry 1991; Ambroziak and Henry 1994; Nikoloff and Henry 1994), and it is also capable of driving regulated transcription of a heterologous reporter gene (Lopes and Henry 1991).

When wild-type extracts were incubated with template B, two major complexes were detected (Figure 3A), as previously described (Lopes and Henry 1991). Using an oligonucleotide competition assay (data not shown), the complex indicated by the lower arrow (Figure 3A) was identified as the nonamer binding factor (NBF) described by Lopes and Henry (1991). The complex migrating directly above the NBF complex (top arrow, Figure 3A; middle arrow, Figure 3B) was absent when *ino2*Δ (Figure 3B, lane 4) or *ino4*Δ (Figure 3B, lane 5) extracts were used and is therefore identified as the Ino2p/Ino4p complex, as previously described (Lopes and Henry 1991; Ambroziak and Henry 1994; Nikoloff and Henry 1994).

To determine the optimal binding conditions for the two types of complexes (*i.e.*, Ino2p/Ino4p and NBF), the concentration of potassium chloride in the binding mixture was varied systematically. As the concentration of KCl was increased from 25 mm to 250 mm in binding reactions with wild-type extracts, the intensity of the Ino2p/Ino4p complex was reduced and the intensity of the NBF complex increased (Figure 3A). However, both complexes can be observed at intermediate concentrations such as 50 mm KCl (Figure 3A, lane 3). This intermediate concentration was, therefore, used for subsequent analyses of the mutant strains (Figure 3B). A complex of lower mobility was seen in binding reactions performed with wild-type extracts when a higher concentration of protein was employed (top arrow, Figure 3B, lane 10; compare to lane 2). We believe that this complex is due to separate binding of the Ino2p/Ino4p complex at each of the two UAS_{INO} sequences in template B, since oligonucleotides containing only one copy of the UAS_{INO} failed to form the slow migrating complex, no matter how much extract was added (data not shown). Reactions conducted with *sin3*Δ extracts contained all of the same bands observed in wild-type reactions (Hudak *et al.* 1994), as previously reported. As expected, the *ino2*Δ (Figure 3B, lane 4) and *ino4*Δ (Figure 3B, lane 5) mutants lacked the Ino2p/Ino4p complex and displayed only the NBF complex.

The EMSAs performed with extracts produced from the *opi1*Δ strain (Figure 3B, lane 3) produced results

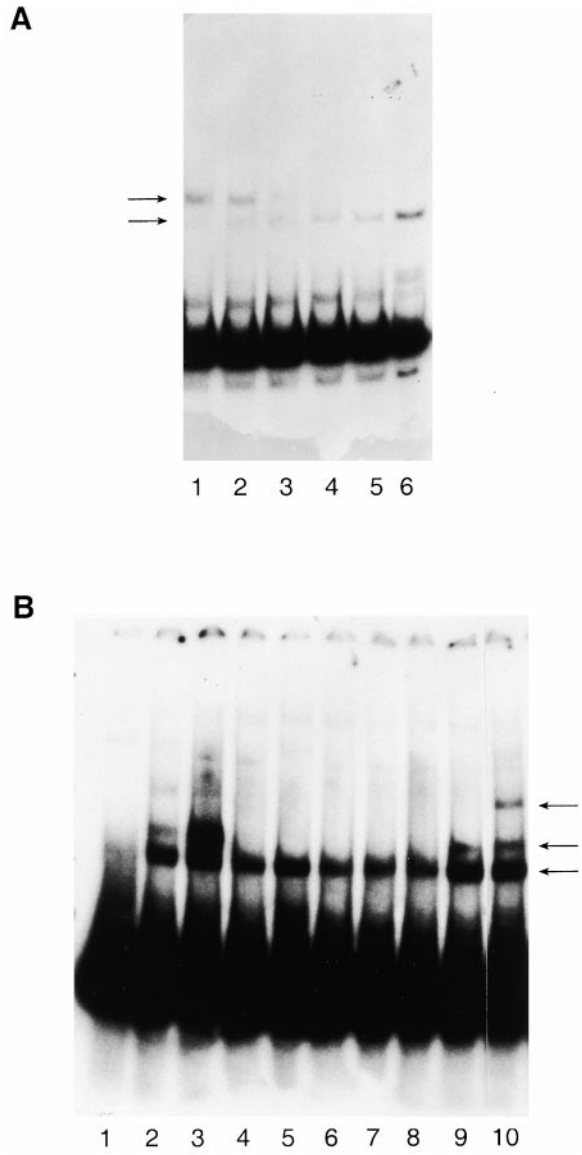


Figure 3.—Electrophoretic mobility shift assays performed with extracts of strains bearing mutations in phospholipid regulatory genes. EMSA reactions were fractionated on 4% nondenaturing gels as described in materials and methods. Extracts were prepared from cells grown to midlogarithmic phase in YEPD medium. (A) Reactions with aliquots of wild-type extracts containing 50 μg total protein were performed at varying concentrations of KCl: lane 1, 10 mm; lane 2, 25 mm; lane 3, 50 mm; lane 4, 75 mm; lane 5, 100 mm; lane 6, 200 mm. The top arrow indicates the Ino2p/Ino4p complex, and the bottom arrow marks the NBF complex. (B) EMSA reactions were performed in the presence of 50 mm KCl, using cell extracts containing 50 μg of total protein. The extracts correspond to the following lanes: 1, none; 2, wild type; 3, *opi1*Δ; 4, *ino2*Δ; 5, *ino4*Δ; 6, *opi1*Δ *ino2*Δ; 7, *opi1*Δ *ino4*Δ; 8, *opi*Δ *sin3*Δ *ino2*Δ *ino4*Δ; 9, *sin3*Δ; and 10, 2× wild-type extract (100 μg protein). The middle arrow indicates the Ino2p/Ino4p complex, and the bottom arrow marks the NBF complex. The top arrow indicates a complex that is observed in wild-type (lane 10) and *sin3*Δ (not shown) extracts when a higher proportion of protein is used in the reaction, as discussed in results.

similar to those obtained with wild-type extract (Figure 3B, lane 2), except that the levels of both the Ino2/Ino4p complex and NBF appeared to be elevated relative to wild type. However, no complex was observed that was absent in reactions performed with *opi1*Δ extracts that was present in reactions carried out with wild-type extract or vice versa. Extracts from all of the double mutants harboring either an *ino2*Δ or an *ino4*Δ mutation formed the NBF complex, but none of these extracts supported formation of an Ino2p/Ino4p complex (Figure 3B, lanes 6–8, and data not shown). EMSA reactions performed with extracts derived from the *opi1*Δ *sin3*Δ strain produced results identical to the *sin3*Δ mutant extracts (data not shown).

In vitro-cotranslated Ino2p and Ino4p also formed complexes with template B (data not shown), as previously described (Ambroziak and Henry 1994). To further assess the possibility that Opi1p might bind UAS_{INO} directly, *in vitro*-translated Opi1p was incubated with a fragment of the *INO1* promoter (template B) as described in materials and methods. The *in vitro*-translated Opi1p failed to form any complex with template B, either alone or in concert with cotranslated Ino2p or Ino4p, and when Opi1p was cotranslated with both Ino2p and Ino4p, only Ino2p/Ino4p complexes were formed (data not shown). Opi1p, Ino2p, and Ino4p cotranslated *in vitro* were also immunoprecipitated with anti-Ino2p antibody. This analysis reconfirmed the coimmunoprecipitation of Ino4p with Ino2p previously described (Ambroziak and Henry 1994). However, Opi1p did not immunoprecipitate with anti-Ino2p when cotranslated with Ino2p, or when Opi1p was cotranslated with Ino2p and Ino4p (data not shown).

DISCUSSION

INO1 is one of the most highly regulated genes in yeast. Not only is its expression repressed some 10- to 30-fold in response to the exogenous phospholipid precursors inositol and choline (Hirsch and Henry 1986), but it is also regulated in response to growth phase (Griac *et al.* 1996; Jiranek *et al.* 1998). *INO1* is also one of a group of apparently unrelated, but highly regulated, genes, the expression of which is commonly found to be affected in mutants having global transcriptional defects (Henry and Patton-Vogt 1998; Shirra and Arndt 1999). Thus, it has become common to check for Ino^- phenotypes in mutants defective in the cellular transcription apparatus (Scafe *et al.* 1990; Santisteban *et al.* 1997; Liu *et al.* 1999). The suppression of Ino^- phenotypes has also been used in screens for suppressors of mutants with global transcription defects (Shirra and Arndt 1999).

Recently, mutations in several major signal transduction pathways, the unfolded protein response pathway (UPR; Cox *et al.* 1997) and the glucose response pathway (Ouyang *et al.* 1999; Shirra and Arndt 1999), have also been reported to confer Ino^- or Opi^- phenotypes. The UPR and the glucose response pathways both involve protein phosphorylation and both are required for *INO1* expression in a wild-type genetic background. In both cases, the *opi1*Δ mutation relieves inositol auxotrophy conferred by mutations (*snf1* and *ire1*) that inactivate protein kinases (Snf1p and Ire1p; Cox and Walter 1996; Shirra and Arndt 1999). These kinases are required for activation of the glucose response and UPR pathways, respectively.

In contrast to the mutants discussed above, which have defects in the general transcription machinery and/or major cellular signal transduction pathways, the *ino2*, *ino4*, and *opi1* mutants are believed to be defective primarily in regulation of lipid metabolism (Henry and Patton-Vogt 1998). However, until this study, no comprehensive assessment of the epistasis of the *ino2*Δ, *ino4*Δ, and *opi1*Δ mutants had been conducted. An earlier, preliminary study of epistasis involving *ino2*, *ino4*, and *opi1* point mutants isolated after chemical mutagenesis was limited to a qualitative assessment of Ino^- and Opi^- plate phenotypes (Loewy *et al.* 1986). The *opi1*Δ *ino2*Δ and *opi1*Δ *ino4*Δ strains studied here failed to grow on I^- plates (Figure 1), as previously reported for point mutants (Loewy *et al.* 1986). This observation is also consistent with the failure of *opi1* mutants to be isolated as suppressors of *ino4-8* (Ouyang *et al.* 1999) and with the relatively slow growth of the *opi1*Δ *ino4*Δ, *opi1*Δ *ino2*Δ, and *opi1*Δ *ino2*Δ *ino4*Δ strains in inositol-limiting I(10) medium (Table 3).

Whereas Ino2p and Ino4p are known to bind as a heterodimer to UAS_{INO} (Ambroziak and Henry 1994; Schwank *et al.* 1995), at present neither the precise role nor the mechanism of Opi1p function is known (Graves 1996; Wagner *et al.* 1999). One possible explanation for the constitutive overproduction of inositol (Opi^-) phenotype of the *opi1*Δ mutant could be that Opi1p interacts with either one or both of the positive regulators, Ino2p or Ino4p, preventing them from binding to each other and/or to UAS_{INO}. Alternatively, Opi1p might bind to UAS_{INO} directly, blocking access to the element by the Ino2p/Ino4p complex. However, the data presented in this report do not support either of these models. We obtained no evidence for direct interaction of Opi1p with Ino2p or Ino4p. Both Ino2p and Ino4p have been shown to be present in a DNA protein complex that binds to UAS_{INO}-containing DNA fragments (Ambroziak and Henry 1994; Nikoloff and Henry 1994; Bachhawat *et al.* 1995; Schwank *et al.* 1995) and this complex is clearly identifiable in the experiments reported here (Figure 3B). However, no Opi1p-dependent DNA-protein complex was observed when extracts of wild-type cells were incubated with an *INO1* promoter fragment (Figure 3B). Furthermore, no complex present in the wild-type strain was observed to be absent in the *opi1*Δ mutant (Figure 3B). These results are consistent with those of Wagner *et al.* (1999), who

detected no evidence for Opi1p binding to DNA or to Ino2p or Ino4p. They did observe that overproduction of Opi1p from a *GAL* promoter caused yeast strains to become auxotrophic for inositol, consistent with the role of Opi1p as a negative regulator. However, the mechanism of Opi1p function remains elusive.

Consistent with the growth phenotypes observed here (Figure 1, Table 3), there was very little *INO1* expression in *ino2*Δ and *ino4*Δ strains (Figure 2A) in either I(10) or I(75)C medium. Deletion of Opi1p had only a modest effect on residual *INO1* expression in the *ino2*Δ genetic background (Figure 2C). However, *INO1* expression in the *opi1*Δ *ino4*Δ strain, grown in I(10) medium, was quite high relative to wild type. The expression of *INO1* transcript in the *opi1*Δ *ino4*Δ strain did not seem to be dependent on Ino2p, since *INO1* expression was only slightly reduced in the *opi1*Δ *ino2*Δ *ino4*Δ strain compared to the *opi1*Δ *ino4*Δ strain (Figure 2C), even though this strain grew even more poorly in I(10) medium than did the *opi1*Δ *ino4*Δ strain (Table 3).

The results reported here reveal subtle differential effects of the *ino2*Δ and *ino4*Δ mutations on growth and *INO1* expression, especially in the *opi1*Δ genetic background, suggesting that Ino2p and Ino4p may have some functions distinct from their common role as pairing partners in the Ino2p/Ino4p heterodimer. It has already been demonstrated that these two regulatory genes are differentially regulated. Both the *INO2* and *OPI1* genes are regulated by the presence of inositol and choline in a manner analogous to *INO1* (Ashburner and Lopes 1995a,b; Jiranek *et al.* 1998), whereas *INO4* is constitutively expressed (Schüller *et al.* 1992; Ashburner and Lopes 1995a). Moreover, the levels of Ino2p appear to be limiting for the formation of the Ino2p/Ino4p heterodimer (Nikoloff and Henry 1994), which binds to UAS_{INO}, whereas the level of Ino4p does not appear to be limiting for *INO1* expression (Ashburner and Lopes 1995a,b). In addition, Ino2p contains an activation domain, while Ino4p does not (Schwank *et al.* 1995). A previous study of the effects of *ino2* and *ino4* point mutants showed greater residual expression of *CHO1* (the UAS_{INO}-containing structural gene for phosphatidylserine synthase) in *ino4* mutants than in *ino2*Δ mutants (Bailis *et al.* 1992). Furthermore, *ino4*Δ strains can utilize glycerolphosphoryl inositol as a source of inositol, whereas *ino2*Δ strains cannot (Patton-Vogt and Henry 1998). In all of the above-cited instances, *ino2*Δ mutants are observed to have more severe defects than *ino4*Δ mutants. If Ino2p retained any residual ability to bind to UAS_{INO} elements in the absence of Ino4p, this could explain the more severe phenotypes of the *ino2*Δ mutant. However, no evidence of Ino2p binding in the absence of Ino4p was detected *in vitro* (Ambroziak and Henry 1994; and data not shown). In addition, this hypothesis is not consistent with the observed residual *INO1* expression in the *opi1*Δ *ino2*Δ *ino4*Δ strain reported here (Figure 2C). Thus, the residual *INO1* expression in the *opi1*Δ *ino4*Δ genetic background does not seem to be due simply to the presence of the Ino2p activator.

It is possible that transcription factors, other than Ino2p and Ino4p, could be involved in the expression of UAS_{INO}-containing genes. In a study that may have relevance to this discussion, Cok *et al.* (1998) studied *INO2* and *INO4* expression in a mutant, *nmt1-451D*, which has a temperature-sensitive defect in protein myristoylation. The *INO2* transcript was found to be elevated, while the *INO4* transcript was lower, in *nmt1-451D* cells in both the presence and absence of inositol. Cok *et al.* (1998) found that expression of the UAS_{INO}-containing *FAS1* gene (fatty acid synthase) is Ino2p dependent in *nmt1-451D* cells growing at their permissive temperature. However, *FAS1* expression is Ino2p independent at the *nmt1-451D* restrictive temperature where protein myristoylation becomes limiting for growth. Cok *et al.* (1998) suggest that another as-yet-unidentified transcription factor might replace Ino2p function, at least with respect to *FAS1* expression, under the circumstances they analyzed.

The residual *INO1* transcript expressed in the *opi1*Δ *ino4*Δ and *opi1*Δ *ino2*Δ *ino4*Δ strains under inositol-limiting conditions was also observed to be regulated in response to inositol and choline (Figure 2C). Thus, at the level of *INO1* expression, *ino4*Δ is not epistatic to *opi1*Δ. Rather, the double mutant appears to have a pattern of gene expression closer to wild type than to either single mutant. The *opi1*Δ *ino2*Δ *ino4*Δ strain, and even the *opi1*Δ *sin3*Δ *ino2*Δ *ino4*Δ quadruple-mutant strain, exhibited patterns of *INO1* expression and regulation similar to the *opi1*Δ *ino4*Δ strain. Indeed, in all of the strains, other than the *opi1*Δ single mutant in which *INO1* was expressed at significant levels in I(10) medium, repression was observed in I(75)C medium. The fact that significant regulated *INO1* expression was observed in the *opi1*Δ *ino4*Δ, *opi1*Δ *ino2*Δ *ino4*Δ, and other *ino2*Δ and *ino4*Δ-bearing strains leads to the question as to why these strains fail to grow on I^- plates and also why they grow quite poorly in I(10) medium. Such slow growth under inositol-limiting conditions is not due simply to failure to express *INO1*, since the *opi1*Δ *ino4*Δ strain expressed substantial levels of *INO1* transcript (Figure 3C). It also grew more slowly than either single mutant (*i.e.*, *opi1*Δ or *ino4*Δ) under fully supplemented conditions, *i.e.*, in I(75)C medium, suggesting that the *opi1*Δ and *ino4*Δ mutations may have synergistic negative effects upon processes outside lipid metabolism.

The substantial residual, regulated *INO1* expression, which is observed in *opi1*Δ *ino4*Δ and *opi1*Δ *ino2*Δ *ino4*Δ strains, is surprising and has major implications for any model that purports to explain the regulation of *INO1* and other UAS_{INO}-containing genes in response to inositol and choline. *INO1* expression is constitutive when *OPI1* is deleted in an otherwise wild-type genetic back-

ground (Hirsch and Henry 1986; White *et al.* 1991). Nevertheless, the results obtained in our study show that Opi1p is not required, at least in an *ino4*Δ or an *ino2*Δ *ino4*Δ genetic background, for the transmission of the signal that leads to repression of *INO1* in response to inositol and choline. In addition to observing repression of *INO1* in response to inositol and choline in *opi1*Δ *ino4*Δ and *opi1*Δ *ino4*Δ *ino2*Δ strains, we observed severalfold repression of *INO1* expression in the *opi1*Δ *sin3*Δ and *opi1*Δ *sin3*Δ *ino2*Δ *ino4*Δ strains (Figure 2C). Thus, Sin3p is also not required for the transmission of the inositol-responsive signal, but it does affect overall levels of *INO1* expression in both *OPI1* and *opi1*Δ genetic backgrounds. Furthermore, since substantial regulated expression of the *INO1* gene is observed in the *opi1*Δ *ino4*Δ and the *opi1*Δ *ino2*Δ *ino4*Δ strains, we are forced to conclude that the Ino2p/Ino4p complex is not essential for repression of *INO1* in response to inositol. The above conclusions are entirely unexpected since the Ino2p/Ino4p heterodimer clearly binds directly to UAS_{INO} (Ambroziak and Henry 1994; Schwank *et al.* 1995) and both Ino2p and Ino4p are required for significant expression of *INO1* in a wild-type (*i.e.*, *OPI1*) genetic background (Figure 2) (Hirsch and Henry 1986). We speculate that Opi1p could be responsible for maintaining a cellular condition conducive to selective binding of Ino2p/Ino4p at UAS_{INO}, but that neither Opi1p nor the Ino2p/Ino4p complex is responsible for transmitting the signal that leads to repression of UAS_{INO}-containing genes in response to inositol.

We propose that when Opi1p is eliminated but Ino2p and Ino4p are both present, the Ino2p/Ino4p heterodimer gains enhanced access to UAS_{INO} elements. Since Ino2p is a highly potent activator (Schwank *et al.* 1995), the level of transcription that is sustained under these conditions could "swamp" an underlying regulatory mechanism that is required for repression in response to inositol. Under this scenario, when Ino2p and Ino4p are eliminated along with Opi1p, access to UAS_{INO}-containing promoters could still be enhanced and would allow access by other less efficient activators. *INO1* expression in the strains lacking Opi1p, Ino4p, and/or Ino2p in this case would be due to the effects of transcription factors other than the Ino2p/Ino4p complex having access to the *INO1* promoter. The first six nucleotides of the core sequence of UAS_{INO} (*i.e.*, 5′ CATGTG 3′) is a fairly generic binding site for proteins of the bHLH family. There are a number of transcription factors in yeast in the bHLH class, in addition to Ino2p/Ino4p, which could potentially recognize such a binding site (Bachhawat *et al.* 1995). Alternatively, the as-yet-unidentified NBF (Figure 3) could play a role in basal *INO1* expression. NBF is the only complex observed when binding reactions are performed with *ino2*Δ or *ino4*Δ extracts, whether Opi1p is present or not (Figure 3B).

Whatever activators are responsible for *INO1* transcription in the absence of the Ino2p/Ino4p complex in the *opi1*Δ genetic background (*i.e.*, in the *opi1*Δ *ino4*Δ and the *opi1*Δ *ino2*Δ *ino4*Δ strains), it seems likely that the *INO1* promoter becomes much more accessible to the basal transcription machinery in the absence of Opi1p. This hypothesis is consistent with the results of Shirra and Arndt (1999), who demonstrated that deletion of *OPI1* results in substantially enhanced *INO1* transcription in the *spt15-328* mutant, which has a partially defective TATA binding protein. Shirra and Arndt (1999) also observed repression of *INO1* in response to inositol in *opi1*Δ *spt15-328* strains. This result is consistent with our conclusion that Opi1p is not required for the regulatory response to inositol. Whatever the explanation for the inositol/choline-regulated *INO1* expression in *opi1*Δ *spt15-328*, *opi1*Δ *ino2*Δ *ino4*Δ, and *opi1*Δ *ino4*Δ strains, we conclude that neither Opi1p nor the Ino2p/Ino4p complex is primarily responsible for transmitting the signal from lipid biosynthesis to the transcription machinery controlling UAS_{INO}-containing genes. Rather, it appears that Opi1p and the Ino2p/Ino4p complex work antagonistically to attenuate the overall level of expression of UAS_{INO}-containing genes. Thus, some other as-yet-unidentified factor or condition associated with the transcription of UAS_{INO}-containing promoters must be responsible for regulating the relative level of transcription in response to the signal produced by the presence of inositol and choline.

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