The *REG1* Gene Product Is Required for Repression of *INO1* and Other Inositol-Sensitive Upstream Activating Sequence-Containing Genes of Yeast

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ABSTRACT

A search was conducted for suppressors of the inositol auxotrophic phenotype of the *ino4-8* mutant of yeast. The *ino4-8* mutation is a single base pair change that results in substitution of lysine for glutamic acid at position 79 in the bHLH domain of the yeast regulatory protein, Ino4p. Ino4p dimerizes with a second bHLH protein, Ino2p, to form a complex that binds to the promoter of the *INO1* gene, activating transcription. Of 31 recessive suppressors of *ino4-8* isolated, 29 proved to be alleles of a single locus, identified as *REG1*, which encodes a regulatory subunit of a protein phosphatase involved in the glucose response pathway. The suppressor mutation, *sia1-1*, identified as an allele of *REG1*, caused constitutive *INO1* expression and was capable of suppressing the inositol auxotrophy of a second *ino4* missense mutant, *ino4-26*, as well as *ino2-419*, a missense mutation of *INO2*. The suppressors analyzed were unable to suppress *ino2* and *ino4* null mutations, but the *reg1* deletion mutation could suppress *ino4-8*. A deletion mutation in the *OPI1* negative regulator was incapable of suppressing *ino4-8*. The relative roles of the *OPI1* and *REG1* gene products in control of *INO1* expression are discussed.

IN the yeast *Saccharomyces cerevisiae* the products of the *INO2* and *INO4* regulatory genes are responsible for the transcriptional activation of a large number of structural genes encoding phospholipid biosynthetic enzymes. The structural genes subject to this regulation are repressed in response to the phospholipid precursors, inositol and choline. These enzymes are maximally derepressed in the absence of inositol and choline, partially repressed in the presence of inositol alone, and fully repressed when both inositol and choline are added to the growth medium (for review, see Paltauf *et al.* 1992; Henry and Patton-Vogt 1998).

The *INO2* and *INO4* gene products both contain a basic helix-loop-helix (bHLH) domain (Hoshizaki *et al.* 1990; Nikoloff *et al.* 1992), which is characteristic of a family of proteins involved in transcriptional regulation and cell-type determination. The bHLH domain has been shown to be responsible for protein dimerization and DNA binding in a number of transcriptional regulatory proteins in mammalian cells. These include the mammalian oncogene cMyc (Murre *et al.* 1989; Davis *et al.* 1990; Voronova and Baltimore 1990) and the upstream stimulatory factors (USF) that bind to the insulin response sequence of the fatty acid synthase (FAS) promoter (Wang and Sul 1995, 1997). A 10-bp repeated element (consensus sequence: 5′ CATGT GAAAT 3′), first found in the promoter of *INO1* gene (Hirsch 1987), has been identified upstream of all the structural genes that are regulated in response to inositol and choline (Paltauf *et al.* 1992; Bachhawat *et al.* 1995). This repeated element, the inositol-sensitive upstream activating sequence (UAS_{INO}), contains within it the CANNTG motif (*i.e.*, 5′ CATGTG 3′) that has been shown to be the consensus-binding site for bHLH proteins (Lassar *et al.* 1989; Blackwell and Weintraub 1990; Fisher *et al.* 1991). Indeed, the UAS_{INO} core sequence, CATGTG, is identical to the E-box motif reported by Wang and Sul (1997) as the sequence required for USF binding and insulin regulation in the mammalian FAS promoter.

When a DNA fragment from the *INO1* promoter that includes two copies of the 10-bp UAS_{INO} element is incubated with cell extracts prepared from wild-type cells, a protein-DNA complex is formed. This complex is absent when the extracts are derived from *ino2* or *ino4* mutant strains (Lopes and Henry 1991). The *INO2* and *INO4* gene products have been demonstrated to bind directly to the UAS_{INO} site on the *INO1* promoter (Ambroziak and Henry 1994; Nikoloff and Henry 1994; Schwank *et al.* 1995). The *ino2* and *ino4* mutants were originally isolated on the basis of an inositol auxotrophic phenotype (Culbertson and Henry 1975; Donahue and Henry 1981a), which is due to their inability to derepress the *INO1* gene, encoding inositol-1-phosphate synthase (Donahue and Henry 1981b; Hirsch and Henry 1986). Of 12 *ino2* and *ino4* loss-of-function mutants examined by DNA sequencing, 11 have mutations in the basic helix-loop-helix region (Nikoloff 1993; Ambro-

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ziak 1994; Nikoloff and Henry 1994), suggesting the importance of the bHLH domain in the functioning of Ino2p and Ino4p. The *ino4-8* allele contains a single amino acid change from glutamic acid to lysine in the loop region of the bHLH domain at the amino acid in position 79 in Ino4p, while *ino4-26* has a single amino acid change from threonine to isoleucine at amino acid 42 in the basic region of the bHLH domain (Ambroziak 1994). The *ino2-419* mutation contains lysine in place of arginine in the loop region at amino acid 273 (Nikoloff and Henry 1994).

To identify additional factors involved in regulation of phospholipid biosynthesis and to acquire more information about the regulatory network, we conducted a screen for suppressors of inositol auxotrophy in an *ino4-8* strain. We report here the isolation of suppressor mutations which proved to be allelic to the *REG1* locus. The mutations identified in this screen also suppressed *ino4-26* and *ino2-419*. However, these suppressor mutations do not suppress null alleles of either *INO4* or *INO2*, while a *reg1* deletion mutation can suppress the *ino4-8* mutation. *reg1* mutants have been identified in numerous previous genetic screens (Matsumoto *et al.* 1983; Neigeborn and Carlson 1987; Tung *et al.* 1992; Naik *et al.* 1997) designed to identify regulatory loci controlling such diverse functions as glucose repression, RNA processing, and proteinase B expression. *reg1* mutants also exhibit growth and glycogen storage defects (Frederick and Tatchell 1996). Here we demonstrate that the *REG1* gene product is also involved in the control of the coordinately regulated genes of phospholipid biosynthesis.

MATERIALS AND METHODS

Strains, medium, and growth conditions: The genotypes and sources of strains used in this study are listed in Table 1. The following growth media were used: YEPD (1% yeast extract, 2% Bactopeptone, 2% glucose); complete synthetic medium [2% glucose, 0.67% Difco Yeast Nitrogen Base without vitamins, vitamin mix, supplements (amino acids, uracil, and adenine); Greenberg *et al.* 1982a,b], minimal synthetic medium (same as complete synthetic medium but lacking supplements); drop-out medium (same as complete synthetic medium but lacking a single supplement); sporulation medium (0.1% yeast extract, 0.05% glucose, 1% KAc). Solid medium (plates) contained 2.5% agar in addition to the above ingredients. Inositol-free (I^-) plates contained complete synthetic medium with no inositol supplement; inositol-supplemented (I^+) plates contained complete synthetic medium with 75 μm inositol. For liquid growth studies, derepressing medium (D) was defined as complete synthetic medium containing 10 μm inositol. This level of inositol has been shown to allow partial derepression of the *INO1* gene, while still supporting growth of inositol auxotrophs such as *ino4* mutants (Hirsch and Henry 1986). Repressing (R) medium contained 75 μm inositol. Yeast cultures were grown at 30°.

To test for sensitivity to 2-deoxyglucose (2-DG), yeast strains were incubated on plates containing 2% peptone, 1% yeast extract, 2% sucrose, 200 μg of 2-deoxy-d-glucose and 1 μg/ml antimycin A to stimulate anaerobic conditions. Control plates lacked 2-DG.

Mutagenesis and isolation of suppressor mutants: Strains SH405 (*MAT*α *ino4-8*) and SH406 (*MAT***a** *ino4-8*) were mutagenized with ethyl methanesulfonate (EMS) as previously described (Culbertson and Henry 1975) and screened for cells that were able to grow in the absence of inositol (I^- plates). Cells were harvested at stationary phase from 5-ml cultures grown on YEPD medium, washed twice with 10 ml of phosphate-glucose solution (0.2 m Na_2HPO_4, 2% glucose, pH 8.0) and resuspended in 9.7 ml of phosphate-glucose buffer. Cells were mutagenized by adding 0.3 ml of EMS and incubating at 30° for 30 min for strain SH405 (31% survival) and 20 min for strain SH406 (37% survival). The EMS was inactivated as previously described (Culbertson and Henry 1975) and appropriate dilutions were made into liquid YEPD medium and spread onto YEPD plates to achieve a density of about 200 viable cells per plate. Approximately 40,000 colonies were screened by replicating to I^- and I^+ plates.

Yeast genetic manipulations: Genetic techniques such as mating, sporulation, and tetrad dissection were carried out using standard methods (Sherman *et al.* 1978). The potential suppressors were colony-purified and the growth phenotype on I^- plates was reconfirmed. Candidates were then crossed to the *ino4-8* strain of the opposite mating type (*i.e.*, SH405 or SH406), and diploids were scored on I^- plates to determine whether the suppressor mutation was dominant or recessive. Diploids having the parental phenotype of inositol auxotrophy indicated the presence of a recessive suppressor while diploids having the suppressor phenotype growth on I^- medium indicated a dominant suppressor.

The sets of recessive suppressors isolated in strains of opposite mating types were crossed with each other, and the resulting diploids were tested on I^- plates to estimate the number of complementation groups. Representatives of each complementation group were crossed to the *ino4-8* parental strain and the diploids were sporulated and the tetrads, dissected. In many cases, this first cross yielded low sporulation efficiency and/or poor spore viability. A second backcross to the *ino4-8* parental strain, using spore colonies retrieved from the dissection of the first cross of the primary suppressor-bearing strains to an *ino4-8* strain, often resulted in higher sporulation efficiency and better spore viability. Tetrads from the second cross were scored on I^- plates to determine the segregation of the suppressor phenotype.

Test for Opi$^-$ phenotype: The test for the inositol excretion (*o*verproduction of *i*nositol, Opi$^-$) phenotype has been described in detail elsewhere (Greenberg *et al.* 1982b; Swede *et al.* 1992). Briefly, strains to be tested were spotted or replicated onto I^- medium, allowed to grow for 2 days, and then sprayed with an indicator strain, which is an inositol auxotroph. Growth of a halo of the indicator strain around a colony indicated inositol excretion (Opi$^-$ phenotype). An *opi1* mutant strain (SH308) and wild type (W303) were used as positive (Opi$^-$) and negative (Opi$^+$) controls, respectively.

Construction of *reg1*Δ*::URA3*: An ~4.2-kb *Eco*RI-*Xba*I fragment from plasmid pUCsrn1::URA3, kindly provided by A. K. Hopper (Tung *et al.* 1992), was transformed into the diploid strain SH701 and allowed to integrate into the yeast genome by homologous recombination. Southern analysis of four independent Ura$^+$ transformants confirmed the integration of this fragment into the *REG1* locus. Sporulation and subsequent tetrad analysis of one of these transformants confirmed 2:2 segregation for Ura$^+$:Ura$^-$ and cosegregation of *reg1* phenotypes with the Ura$^+$ phenotype. Strains harboring the disruption allele were found to be viable but grew more slowly than their isogenic wild-type counterparts, as previously reported (Tung *et al.* 1992; Frederick and Tatchell 1996). Spore

colonies from this cross were also tested for the Opi$^-$ phenotype, as described above, and all *reg1*Δ segregants were found to be Opi$^-$.

β-Galactosidase assays: A single copy of an *INO1-lacZ* gene fusion stably integrated at the *URA3* locus was introduced into strains of interest (Lopes and Henry 1991). To assay β-galactosidase, cells containing the gene fusion were grown under partially derepressing (with 10 μm inositol) or repressing (with 75 μm inositol) conditions (Hirsch and Henry 1986). The use of completely inositol-free medium for the derepressing condition was not possible because the parental *ino4-8* strains cannot grow in the absence of inositol. Cells were harvested at midlogarithmic phase. Cell extracts were prepared and β-galactosidase assays were performed as described by Lopes and Henry (1991), except that reaction aliquots were removed at 5, 10, and 15 min. β-Galactosidase units are defined as (OD_{420}/min/mg total protein) × 1000.

RNA isolation and analysis: For RNA isolation, yeast cells were grown in repressing (75 μm inositol) and derepressing (10 μm inositol) medium to midlog phase. Cells from a 10-ml culture were harvested and washed once with 5 ml of RE buffer (100 mm LiCl, 100 mm Tris-HCl, pH 7.5, 1 mm EDTA) and suspended in 0.4 ml RE buffer. This solution was transferred to a fresh tube containing ~2/3 volume of ice-cold glass beads and vortexed four times, 1 min each, being placed on ice between pulses. Proteins were removed by sequential extractions with 0.3 ml equilibrated phenol, 0.3 ml phenol/$CHCl_3$/isoamyl alcohol (50:49:1), and 0.3 ml $CHCl_3$. RNA was precipitated at −20° overnight and suspended in 0.1 ml diethyl pyrocarbonate-treated water.

Northern analysis was done by electrophoresis of 20-μg samples of RNA loaded onto 1% agarose-6% formaldehyde/1× MOPS gels and run overnight in 1× MOPS. Gels were electroblotted to Nytran Plus membrane in 1× TAE at 4° for 30 min at 10 V, followed by an additional 1 hr 30 min at 40 V.

Prehybridization and hybridization conditions were as described in Hirsch and Henry (1986). RNA probes for hybridization were enzymatically synthesized from the following plasmids described in Hudak *et al.* (1994): pAB309Δ (*TCM1*); pMH203 (*OPI3*); pJH301 (*INO1*); pAB103 (*CHO1*); pTG109 (*CHO2*). The *TCM1* RNA, whose expression is unaffected by the availability of inositol, was used as a loading control. The results were visualized by autoradiography and quantified by a FUJIX BAS2000 phosphoimager using MacBAS version 2.4 software.

RESULTS

Isolation of mutants that suppress the inositol auxotrophy of the *ino4-8* mutation: From the original screening of 40,000 colonies, 200 suppressor candidates were isolated after two rounds of testing on I$^-$ plates as described in materials and methods. Strains with respiratory deficient phenotypes were eliminated from the collection. Of the 200 original putative suppressor-bearing strains, ~70% appeared to be due to recessive mutations while ~30% appeared to harbor a dominant mutation. The mutations conferring the strongest suppressor phenotypes (showing growth within two days after replicating to I$^-$ plates) were all dominant. Preliminary genetic analysis suggested that most of these strains carried primary reversions of the *ino4-8* allele. The remaining recessive suppressors were subjected to further analysis.

Complementation and segregation analysis: The strains carrying recessive suppressors were classified according to growth on I$^-$ plates. Approximately 31 putative suppressor-bearing strains with stronger growth phenotypes (showing definite growth within 3–4 days after replicating to I$^-$ plates) were selected for further analysis. Because the mutants had been isolated in two strains of opposite mating type, it was possible to conduct an initial complementation analysis crossing the mutant collections of opposite mating types against each other. The recessive mutants were found to fall into three complementation groups with one complementation group (*sia1*) containing 29 members exhibiting the stronger growth phenotypes. The other two groups had only a single representative each and only one of these (*sia2*) was subjected to further characterization. The suppressor mutations representing the *sia1* and *sia2* (for suppressor of *i*nositol *a*uxotrophy) complementation groups were subjected to further genetic analysis.

Two suppressor-bearing strains from the larger (*sia1*) complementation group and the single *sia2* strain were backcrossed to the *ino4-8* parent strain of the opposite mating type (*i.e.*, SH405 or SH406; Table 1). Twenty-three tetrads with four surviving spores were recovered from the crosses of the two representatives from the *sia1* complementation group and all exhibited 2:2 segregation of the growth phenotype on I$^-$ plates. Nineteen tetrads with four surviving spores were tested from crosses involving suppressors of the single *sia2* representative, and all showed 2:2 segregation for growth on I$^-$ plates, indicative of a mutation in a single gene.

***sia1* and *sia2* are not linked to the *INO4* locus or to each other:** Strains carrying the *sia1-1* or the *sia2-1* mutation (*i.e.*, *ino4-8 sia1-1* or *ino4-8 sia2-1*) were crossed to wild-type strains SH155 or SH224 (see Table 1 for full genotypes). In 34 tetrads with four surviving spores recovered from crosses of an *ino4-8 sia1-1* strain to wild type, 22 showed 3$^+$:1$^-$ segregation for inositol auxotrophy (*i.e.*, Ino$^+$:Ino$^-$), 10 exhibited 2$^+$:2$^-$ segregation and 2 segregated 4$^+$:0$^-$ (Table 2A). In the 17 tetrads with four surviving spores from crosses involving *ino4-8 sia2-1* strains to wild-type strains, 14 tetrads segregated 3$^+$:1$^-$ for inositol auxotrophy, 3 segregated 2$^+$:2$^-$, and no 4$^+$:0$^-$ tetrads were recovered. In these crosses, the 4$^+$:0$^-$ segregation represents the parental ditype category, 3$^+$:1$^-$ reflects a tetratype ascus, and 2$^+$:2$^-$ segregation is expected for nonparental ditype asci. However, the proportion of 4$^+$:0$^-$ asci was lower than the expected ratio of 1 in 6. Explanations include the possibility that the suppressor phenotype might not be fully penetrant (*i.e.*, some *ino4-8 sia1-1* or *ino4-8 sia2* segregants may score as Ino$^-$), or spores of the *ino4-8 sia1-1* and *ino4 sia2-1* genotypes may not germinate as well as other genotypes. Since only tetrads with four surviving spores were analyzed, a lower viability for the *ino4-8 sia1-1* (or *sia2-1*) genotype would lead to a reduced percentage of 4$^+$:0$^-$ tetrads among the tetrads analyzed. Consistent with either of these explanations, an excess of 2$^+$:2$^-$

TABLE 1

Yeast strains

Strain	Genotype	Source
SH155	*MATα his3 leu2 trp1 trp5 ura3::INO1-lacZ*	Lopes and Henry (1991)
SH224	*MAT***a** *his3 leu2 trp1 ura3::INO1-lacZ*	Lopes and Henry (1991)
SH405	*MATα his3 lys2 trp1 ino4-8 ura3::INO1-lacZ*	This study
SH406	*MAT***a** *ade2 his3 trp1 ino4-8 ura3::INO1-lacZ*	This study
SH702	*MATα ade3 his3 leu2 trp1 ura3 ino4-8*	This study
SH407	*MATα his3 lys2 trp5 ino4-26 ura3::INO1-lacZ*	Graves (1996)
SH408	*MAT***a** *ade3 his3 leu2 lys2 ino4-26 ura3::INO1-lacZ*	This study
SH352	*MAT***a** *his3 lys2 trp1 ino4-8 sia1-1 ura3::INO1-lacZ*	This study
SH355	*MAT***a** *ade2 his3 trp1 ino4-8 sia1-1 ura3::INO1-lacZ*	This study
SH351	*MAT***a** *ade2 his3 trp1 ino4-8 sia1-2 ura3::INO1-lacZ*	This study
SH361	*MATα his3 lys2 trp1 ino4-8 sia1-2 ura3::INO1-lacZ*	This study
SH357	*MATα his3 lys2 trp1 ino4-8 sia2-1 ura3::INO1-lacZ*	This study
SH358	*MAT***a** *ade2 his3 trp1 ino4-8 sia2-1 ura3::INO1-lacZ*	This study
SH366	*MATα his3 leu2 lys2 trp1 trp5 sia1-1 ura3::INO1-lacZ*	This study
SH367	*MATα his leu2 lys2 trp1 or trp5 sia1-1 ura3::INO1-lacZ*	This study
SH368	*MAT***a** *his3 leu2 trp1 sia1-1 ura3::INO1-lacZ*	This study
SH369	*MAT***a** *his3 lys2 trp1 or trp5 sia1-1 ura3::INO1-lacZ*	This study
SH371	*MAT***a** *ade2 his3 trp1 or trp5 sia1-1 ura3::INO1-lacZ*	This study
SH374	*MATα his3 leu2 trp1 sia2-1 ura3::INO1-lacZ*	This study
SH376	*MAT***a** *his3 trp1 or trp5 sia2-1 ura3::INO1-lacZ*	This study
SH309	*MATα his3 leu2 trp1 ura3 ino4Δ::LEU2*	This laboratory
SH295	*MAT***a** *his3 leu2 trp1 ura3 ino2Δ::TRP1*	This laboratory
SH313	*MAT***a** *ade5 ura3 ino2-419*	This laboratory
SH308	*MAT***a** *his3 leu2 trp1 ura3 opi1Δ::LEU2*	Graves (1996)
SH150	*MATα his3 leu2 trp1 trp5 sin3-102 ura3::INO1-lacZ*	This laboratory
DID	*MATα ade2 his3 ura3 dep1Δ::HIS3 leu2::INO1-lacZ*	S. Kohlwein
SH700	*MATα his3 leu2 lys2 trp1 ino4-8 sia1-1 ura3::INO1-lacZ*	This study
SH701	**a**/α *ura3-1/ura3-1 leu2-3, 112/leu2::INO1-lacZ ade201/ade2-1, his3-11/his3/11, 15 trp1-1/trp1-1*	This laboratory
W303 (SH14)	*MAT***a** *ura3-1 leu2-3, 112 trp1-1 ade2-1 his3-11, 15 can1-100*	R. Rothstein
SH703	*MATα ade2 his3 leu2 trp1 or 5 ino4-8 ura3::INO1-lacZ*	This study
SH704	*MAT***a** *ade2 his3 trp1 ura3 leu2::INO1-lacZ reg1Δ::URA3*	This study
SH706	*MATα ade2 his3 leu2 trp1 reg1Δ::URA3*	This study
SH707	*MATα ade2 his3 leu2 trp1 reg1Δ::URA3 ino4-8*	This study

tetrads was observed, compared to $4^+:0^-$ tetrads, in reciprocal crosses (*i.e.*, crosses of strains of the *ino4-8 SIA1* or *ino4-8 SIA2* genotypes to strains of the *INO4 sia1-1* or *INO4 sia2-1* genotypes, respectively; data not shown). However, the excess of $2^+:2^-$ *vs.* $4^+:0^-$ tetrads and the high proportion of $3^+:1^-$ and $2^+:2^-$ asci shown in Table 2A indicate that it is unlikely that either *sia1* or the *sia2* is closely linked to the *INO4* gene.

The *sia1*-bearing strains were also crossed to *sia2* strains (Table 2A). Although a relatively small number of tetrads with four spores surviving were recovered (13 in two crosses), the high proportion of $3^+:1^-$ and $2^+:2^-$ tetrads indicated that *sia1* and *sia2* mutations are not closely linked. No unusual growth phenotype was observed for *sia1 sia2* strains, which were viable and resembled the *sia1* and *sia2* single mutants in ability to suppress *ino4-8*. In contrast, a cross involving two *sia1* alleles (*i.e.*, *ino4-8 sia1-1* with *ino4-8 sia1-2*) produced 21 $4^+:0^-$ tetrads and only 1 tetrad exhibiting a $3^+:1^-$ segregation pattern (which could be due to reversion of *ino4-8* or a gene conversion; Table 2A).

Both the *sia1-1* and the *sia2-1* mutations can suppress a second *ino4* missense allele, but neither can suppress *ino4*Δ: Strains SH407 and SH408 carrying the *ino4-26* allele, a missense mutation in the basic region of the bHLH domain at amino acid position 42 (Ambroziak 1994), were crossed to strains SH368 and SH372 carrying the *sia1-1* or the *sia2-1* mutation, respectively, in the *INO4* genetic background (Table 2B). The high proportions of $3^+:1^-$ and $4^+:0^-$ tetrads recovered from these crosses indicate that the *sia1-1* and the *sia2-2* mutations can both suppress the inositol auxotrophy of the *ino4-26* strain. In contrast, crosses of *sia1-1*- or *sia2-1*-bearing strains SH369 and SH376 to strain SH309 carrying an *ino4* deletion mutation (*ino4*Δ) produced only tetrads exhibiting a $2^+:2^-$ segregation for inositol auxotrophy (Table 2B), indicating that neither *sia1-1* nor *sia2-1* can suppress the *ino4*Δ allele.

The *sia1* and *sia2* mutations can suppress an *ino2* missense mutation, but not an *ino2*Δ allele: Strains harboring *sia1-1* or *sia2-1* were crossed to a strain carrying the *ino2-419* allele (a missense mutation in the loop

TABLE 2

Crosses of suppressor-bearing strains

Cross	$4^+:0^-$	$2^+:2^-$	$3^+:1^-$
A. Crosses involving *sia1*- and *sia2*-bearing strains with each other and with wild type			
SH352 (*ino4-8 sia1-1*) × SH155 (*INO4 SIA1*)	1	8	20
SH355 (*ino4-8 sia1-1*) × SH155 (*INO4 SIA1*)	1	2	2
SH357 (*ino4-8 sia2-1*) × SH224 (*INO4 SIA2*)	0	2	7
SH358 (*ino4-8 sia2-1*) × SH224 (*INO4 SIA2*)	0	1	7
SH355 (*ino4-8 sia1-1*) × SH357 (*ino4-8 sia2-1*)	2	2	3
SH351 (*ino4-8 sia1-2*) × SH357 (*ino4-8 sia2-1*)	0	1	5
SH359 (*ino4-8 sia1-1*) × SH351 (*ino4-8 sia1-2*)	21	0	1
B. Crosses of *sia1-1*- and *sia2-2*-bearing strains with *ino4-26* and *ino4*Δ strains			
SH368 (*INO4 sia1-1*) × SH407 (*ino4-26 SIA1*)	6	7	16
SH374 (*INO4 sia2-1*) × SH408 (*ino4-26 SIA2*)	2	0	8
SH369 (*INO4 sia1-1*) × SH309 (*ino4*Δ *SIA1*)	0	9	0
SH376 (*INO4 sia2-1*) × SH309 (*ino4*Δ *SIA2*)	0	12	0
C. Crosses of *sia1-2*- and *sia2-2*-bearing strains with *ino2-419* and *ino2*Δ strains			
SH366 (*INO2 sia1-1*) × SH313 (*ino2-419 SIA1*)	3	4	13
SH367 (*INO2 sia1-1*) × SH313 (*ino2-419 SIA1*)	4	5	13
SH374 (*INO2 sia2-1*) × SH313 (*ino2-419 SIA2*)	1	5	8
SH367 (*INO2 sia1-1*) × SH295 (*ino2*Δ *SIA1*)	0	7	0
SH374 (*INO2 sia2-1*) × SH295 (*ino2*Δ *SIA2*)	0	8	0
D. Crosses of *reg1*Δ and *opi1*Δ to *ino4-8*			
SH368 (*sia1-1*) × SH707 (*reg1*Δ *ino4-8*)	23	0	0
SH703 (*ino4-8*) × SH704 (*reg1*Δ)	7	4	17
SH703 (*ino4-8*) × SH308 (*opi1*Δ)	0	38	0

Tetrad ratios are given for growth on inositol-free media (Ino^+/Ino^-) phenotypes.

region of the bHLH domain at amino acid 273; Nikoloff and Henry 1994). A high proportion of tetrads exhibiting $3^+:1^-$ and $4^+:0^-$ ratios was observed, suggesting that neither *sia1-1* nor *sia2-1* is linked to *INO2*, but both are capable of suppressing the *ino2-419* allele. Strains bearing the *sia1-1* or the *sia2-2* mutation were also crossed to a strain carrying an *ino2*Δ null allele. The observed $2^+:2^-$ segregation for inositol auxotrophy (Table 2C) indicates that neither *sia1-1* nor *sia2-1* can suppress the *ino2*Δ mutation.

The growth characteristics of suppressor strains: Growth of strains carrying the *sia1-1* (Figure 1) and *sia2-1* (data not shown) mutations was compared to growth of *ino4-8* and wild-type strains in medium containing 10 μm inositol (derepressing, D) and 75 μm inositol (repressing, R). As expected, the suppressor-bearing strain SH352 (*ino4-8 sia1-1*) grew more rapidly and reached a higher optical density in D medium containing 10 μm inositol than did SH406 (*ino4-8 SIA1 SIA2*; Figure 1). However, the *ino4-8 sia1-1* strain did not grow as rapidly as wild type or *sia1-1 INO4* (SH368) in D medium (Figure 1). Similarly, the *sia2-1 ino4-8* strain grew more rapidly than the *ino4-8* (SH406) strain, but not as rapidly as wild type or SH374 (*INO4 sia2-1*) in D medium containing 10 μm inositol (data not shown). Strain SH374 (*INO4 sia2-1*) exhibited a growth rate comparable to wild type in either D or R medium (data not shown). However, SH368 (*sia1-1 INO4*) grew slightly more slowly and reached a lower optical density than wild type (SH155; Figure 1) in medium containing either 10 μm or 75 μm inositol.

***INO1* gene expression in *sia1* and *sia2* strains:** Strains containing a single copy of an *INO1-lacZ* gene fusion at the *URA3* locus were used to assay *INO1* gene expression (Table 3). As in the growth experiments described above, medium containing a low amount of inositol (10 μm), which allows partial derepression of *INO1*, was used as the D growth condition and 75 μm inositol was used as the fully R growth condition (Donahue and Henry 1981b; Hirsch and Henry 1986). The wild-type strain (SH155) expressed ~155 units of activity in derepressing D medium (Table 3). In fully repressing R medium, the wild-type strain expressed <20 units of β-galactosidase. Such repression of the *INO1* reporter construct in response to high levels of inositol is consistent with previous reports (Lopes and Henry 1991). Also consistent with previous reports (Hirsch and Henry 1986; Hoshizaki *et al.* 1990), the *ino4-8* parental strain (SH406) exhibited no detectable β-galactosidase activity under either D or R growth condition. In contrast to the *ino4-8* parental strain, strains SH352 and SH357 (carrying the *sia1-1* or the *sia2-1* suppressor, respectively, in an *ino4-8* genetic background) expressed measurable β-galactosidase from the *INO1 lacZ* fusion in D medium

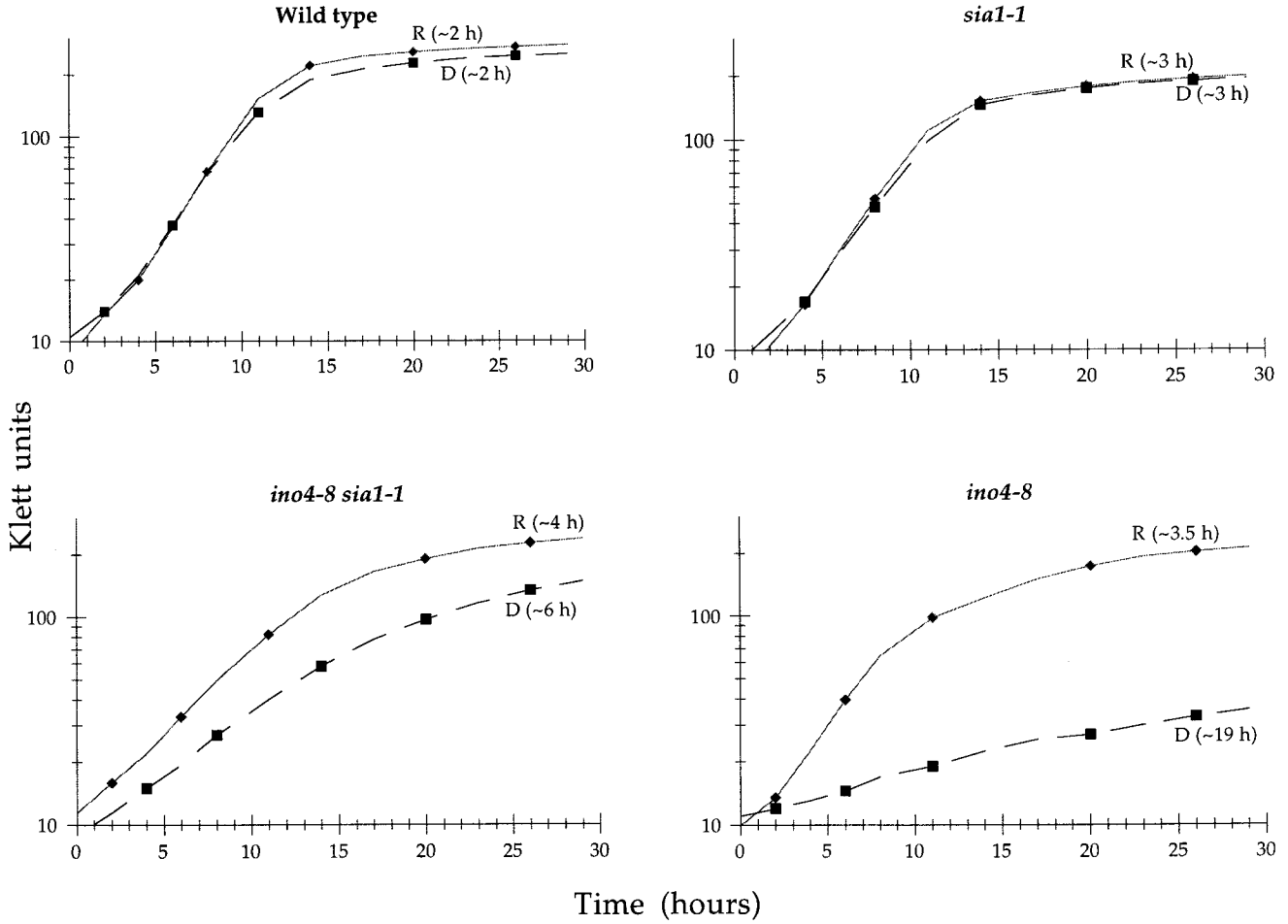


Figure 1.—Growth of *sia1-1* (SH368) and *sia1-1 ino4-8* (SH352) strains compared to wild-type (SH155) and *ino4-8* (SH406) strains in the presence and absence of inositol. Strains were inoculated from overnight cultures into synthetic complete medium with 75 μm inositol (R; ◆) and 10 μm inositol (D; ■), as described in the materials and methods. Growth at 30° was monitored by optical density using a Klett-Summerson spectrophotometer. The doubling times of each strain in each type of medium are shown in parentheses on the figure next to the medium designation (R or D).

(Table 3). The level of activity in D medium [~29 units in the case of SH352 (*ino4-8 sia1-1*) and 35 units in the case of SH357 (*ino4-8 sia2-1*)] was lower than the level observed in wild type (SH155), but higher than in the parental strain (SH406, *ino4-8 SIA1 SIA2*).

Expression of derepressed levels of β-galactosidase from the *INO1-lacZ* reporter construct was observed in R medium in the *sia1-1 INO4* strain (SH368). Constitutive expression, but at a lower level, was observed in the *sia1-1 ino4-8* genetic background (strain SH352). Thus, the *sia1-1* mutation results in constitutive expression of the *INO1-lacZ* reporter construct in both genetic backgrounds (*i.e.*, *ino4-8* or *INO4*). The *sia1-1* mutant strains were tested for the Opi$^-$ phenotype, but none of these strains was Opi$^-$.

In contrast to the constitutive pattern of *INO1* expression observed in *sia1-1*-bearing strains, expression of the *INO1-lacZ* gene fusion was repressed in R medium in *sia2-1*-bearing strains (SH357 and SH374). Levels of β-galactosidase activity observed in both D and R medium in the SH357 strain (*ino4-8 sia2-1*) were lower than the levels observed in SH374 (*INO4 sia2-1*; Table 3).

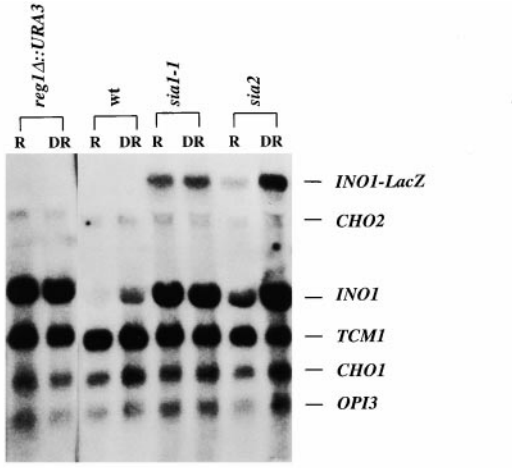
Expression of phospholipid biosynthetic structural genes in *sia1-1* and *sia2-1* strains: The *INO1*, *CHO1*, *CHO2*, and *OPI3* genes are coordinately regulated in response to inositol (Paltauf *et al.* 1992). An autoradiogram of a Northern blot showing the expression of these transcripts in the wild type, *sia1-1 INO4*, and *sia2-1 INO4* strains is shown in Figure 2. Under the derepressing growth condition employed in this study (10 μm inositol; D medium), the wild-type strain exhibited 4-fold derepression of *INO1* compared to levels observed in cells grown under repressing conditions (R). This level of derepression is comparable to previous reports of *INO1* expression in cells grown in the presence of 10 μm inositol, whereas 10-fold or greater derepression is typically seen in cells grown in completely inositol-free medium (Hirsch and Henry 1986). In D medium containing 10 μm inositol, the *sia2-1* cells exhibited approximately 9-fold derepression of *INO1*, a much greater de-

TABLE 3

Expression of the *INO1-lacZ* reporter gene in various strains

Strain	Genotype	β-Galactosidase activity D	β-Galactosidase activity R
SH155	*INO4 SIA1 SIA2*	155.1 ± 15.6	17.4 ± 9.1
SH406	*ino4-8 SIA1 SIA2*	≤1	≤1
SH352	*ino4-8 sia1-1 SIA2*	28.8 ± 3.2	22.8 ± 4.7
SH368	*INO4 sia1-1 SIA2*	199.3 ± 34.6	208.5 ± 24.1
SH357	*ino4-8 SIA1 sia2-2*	35.5 ± 6.6	3.3 ± 0.3
SH374	*INO4 SIA1 sia2-2*	154.8 ± 28.1	28.8 ± 4.4

β-Galactosidase levels were measured in cells grown in the presence of 10 μm inositol, derepressing condition (D), and in 75 μm inositol, repressing condition (R).



Strain	INO1 D	INO1 R	INO1 D/R	CHO1 D	CHO1 R	CHO1 D/R	OPI3 D	OPI3 R	OPI3 D/R	CHO2 D	CHO2 R	CHO2 D/R
W303 (WT)	1.0	0.27	3.7	1.0	0.65	1.5	1.0	1.0	1.0	1.0	0.8	0.8
SH706 (*reg1Δ*)	7.2	6.6	1.1	0.96	1.0	0.9	1.7	2.1	0.8	1.6	2.0	0.8
SH368 (*sia1*)	6.2	5.4	1.1	1.0	0.8	1.2	1.6	1.8	0.9	1.5	1.5	1.0
SH376 (*sia2*)	18	2.1	8.5	1.73	0.7	2.5	3.0	0.7	4.3	2.9	0.9	3.2

Figure 2.—Northern analysis of transcripts of genes, *INO1*, *OPI3*, *CHO2*, and *CHO1*, encoding enzymes subject to repression by inositol in wild-type, *sia1-1 INO4*, *sia2 INO4*, and *reg1*Δ *INO4* strains. DR denotes partially derepressing growth condition (10 μm inositol), as described in materials and methods. R denotes fully repressing growth condition (75 μm inositol). Ribosomal protein gene *TCM1* is included as a control for RNA loading. Lanes from left to right: Lane 1, SH706 (*reg1*Δ), 75 μm inositol; lane 2, SH706 (*reg1*Δ), 10 μm inositol; lane 3, W303 (wild type), 75 μm inositol; lane 4, W303 (wild type), 10 μm inositol; lane 5, SH368 (*sia1-1 INO4*), 75 μm inositol; lane 6, SH368 (*sia1-1 INO4*), 10 μm inositol; lane 7, SH376 (*sia2-1 INO4*), 75 μm inositol; lane 8, SH376 (*sia2-1 INO4*), 10 μm inositol. Quantification of the Northern blot is depicted below the blot. The numbers were obtained by normalizing to the signal obtained with the *TCM1* probe and are expressed as a proportion of the amount of the specific RNA present in wild-type cells grown under derepressing (D) growth conditions (*i.e.*, the amount of each RNA in wild type normalized to *TCM1* is set at 1.0 for each of the four probes). The repression ratio (D/R) was obtained by dividing the level of each transcript expressed in D medium by the level observed in R medium.

gree of derepression than observed in the wild-type strain (Figure 2). The *sia2-1* strain also had a copy of the *INO1-lacZ* reporter construct integrated at the *URA3* locus, and the transcript from this construct was regulated in a pattern comparable to the native *INO1* transcript (Figure 2).

The *OPI3*, *CHO1*, and *CHO2* transcripts, in general, exhibit much less dramatic repression ratios than *INO1* and are not fully repressed in medium containing inositol unless choline is present in addition to inositol (Bailis *et al.* 1987; Gaynor *et al.* 1991). In the present study, no choline was added to the R growth medium and, consistent with previous reports, we observed that the *OPI3*, *CHO1*, and *CHO2* transcripts showed much less repression than the *INO1* transcript under these conditions in both wild-type and *sia2-1* cells. Compared to the wild-type strain, the *sia2-1* strain showed elevated derepression of all of these transcripts in D medium. The *sia1-1* strain exhibited no significant repression of any of the transcripts tested under these conditions (Figure 2).

Analysis of *INO1-lacZ* expression in diploid strains: The coregulated structural genes of phospholipid biosynthesis are also constitutively expressed in *opi1* (Hirsch and Henry 1986), *sin3* (Hudak *et al.* 1994; Slekar and Henry 1995), and *dep1* (Lamping *et al.* 1995) mutant strains. *sia1-1*-bearing strains were mated to strains carrying *opi1*, *sin3*, and *dep1* mutations, and β-galactosidase activity was assayed in the resulting diploids under both derepressing and repressing conditions (Table 4). The diploids all exhibited wild-type regulation in response to growth in R medium. Thus, the *sia1-1* mutation complements the *opi1*, *dep1*, and *sin3* mutations and is unlikely to be an allele of any of these loci. A plasmid containing the *OPI1* gene was also transformed into *sia1-1* strain and failed to complement the *sia1* suppressor phenotype (data not shown).

Cloning of an *sia1* complementing clone: Strain SH700 (*sia1-1 ino4-8*) was transformed with a CEN-based genomic library and transformants were tested for their ability to grow in the absence of inositol. Because the *sia1-1* mutation is recessive, we reasoned that the presence of the wild-type copy of this gene in a *ino4-8 sia1-1* strain would render it auxotrophic for inositol. Therefore, we looked for those transformants that had lost their ability to grow in the absence of inositol upon transformation. Among 3600 transformants tested, only one showed the expected phenotype. Sequencing analysis of a portion of the genomic fragment containing the complementing activity (pMR1034), followed by a search of the Saccharomyces Genome Database, showed that this fragment maps to coordinates 494,689–504,734 on the right arm of chromosome IV (inserts numbered as in Saccharomyces Genome Database). This fragment contains five

TABLE 4

Expression of the *INO1-lacZ* reporter gene in diploid strains

Diploid	Medium	
	D	R
SH352 (*sia1-1 ino4-8*) × SH155 (wild type)	107.7 ± 27.0	16.8 ± 3.6
SH367 (*sia1-1*) × SH371 (*sia1-1*)	103.7 ± 11.8	90.4 ± 19.8
SH368 (*sia1-1*) × SH308 (*opi1*Δ)	88.8 ± 10.9	16.6 ± 4.3
SH369 (*sia101*) × SH150 (*sin3-102*)	97.6 ± 22.4	11.9 ± 0.8
SH368 (*sia1-1*) × DID (*dep1*Δ)	211.1 ± 20.7	32.9 ± 4.9
SH376 (*sia2-1*) × DID (*dep1*Δ)	199.7 ± 14.5	32.8 ± 1.8
SH352 (*ino4-8 sia1-1*) × SH707 (*ino4-8 reg1*Δ)	14 ± 4	9.8 ± 2.6
SH368 (*sia1-1*) × SH706 (*reg1*Δ)	59.6 ± 8	55.4 ± 6
SH224 (wild type) × DID (*dep1*Δ)	190.7 ± 5.5	34.9 ± 2.1

D, 10 μm inositol, derepressing condition; R, 75 μm inositol, repressing condition.

different open reading frames (YDR027–YDR031) of which only one corresponded to a previously characterized gene, *REG1*/*HEX2*/*SRN1* (Neigeborn and Carlson 1987; Tung *et al.* 1992; Naik *et al.* 1997). Subsequent subcloning of this ~10-kb fragment showed that the only complementing subclones were those that carried a full-length copy of the *REG1* gene. Additional *REG1* clones, kindly provided by M. Johnston and K. M. Arndt, further confirmed this observation. Thus, it appeared likely that the *SIA1* gene was, in fact, *REG1*.

To explore this possibility further, we constructed a *reg1*Δ mutant as described in materials and methods and examined its growth and other characteristics. The *reg1*Δ mutant exhibited a longer lag period than wild type or *sia1-1* when inoculated into fresh medium. However, once it reached logarithmic phase, its growth was no more impaired than the growth of *sia1-1* (which is shown in Figure 1). As previously described (Frederick and Tatchell 1996), and similar to the *sia1-1* mutant, the *reg1*Δ mutant grew slightly more slowly than wild type under all growth conditions employed in this study. The doubling time of the *reg1*Δ mutant in R medium containing 75 μm inositol was ~2.5 hr compared to approximately 3 hr for *sia1-1* and ~2 hr for wild type. Unlike the *sia1-1 INO4* mutant strain, however, the *reg1*Δ *INO4* strain had a weak inositol excretion (Opi$^-$) phenotype. The *reg1*Δ strain also exhibited elevated constitutive expression of *INO1*, comparable to the expression pattern seen in *sia1-1* cells (Figure 2).

Among 28 full four-spore tetrads recovered from a cross of SH703 (*ino4-8*) to SH703 (*reg1*Δ), 17 exhibited 3^+:1^- segregation of inositol auxotrophy, 4 showed 2^+:2^- segregation, and 7 tetrads exhibited 4^+:0^- segregation (Table 2D). Furthermore, all *reg1*Δ *ino4-8* segregants grew in the absence of inositol. Thus, the null allele, *reg1*Δ*::URA3*, has the same ability to suppress *ino4-8* as does *sia1-1* (Table 2A).

A *reg1*Δ *ino4-8* strain (SH707) was crossed with a *sia1-1 ino4-8* strain (SH352) and the diploid was found to be inositol prototrophic, indicating that the *sia1-1* and *reg1*Δ mutations do not complement. A second diploid was constructed by crossing SH368 (*sia1-1 INO4*) with SH706 (*reg1*Δ *INO4*). Both diploids were analyzed for β-galactosidase expression from the *INO1-lacZ* reporter gene (Table 4). In both cases, consistent with failure of *sia1-1* and *reg1*Δ to complement, β-galactosidase expression was constitutive (Table 4). However, the level of expression of the reporter construct was lower in the strain that was homozygous for *ino4-8* (*i.e.*, SH352 × SH707). This diploid sporulated poorly. Therefore, a diploid heterozygous for *ino4-8* was produced by crossing strains SH368 (*sia1-1*) and SH707 (*reg1*Δ*::URA3 ino4-8*). This strain was sporulated and dissected. Among 23 tetrads recovered from this cross, all showed 4^+:0^- segregation for growth on inositol-free plates (Table 2D). In addition, all spore colonies were tested for resistance to 2-DG (Lobo and Maitra 1977), a phenotype associated with *reg1* mutants (Tu and Carlson 1995). The parental strains, *sia1-1* and *reg1*Δ, and all progeny from the cross exhibited 2-DG resistance. In all 23 tetrads, 2-DG resistance segregated 4^R:0^S resistant:sensitive. Thus, *reg1*Δ and *sia1-1* are allelic and we have renamed *sia1-1* as *reg1-600*.

The *opi1*Δ mutation cannot suppress *ino4-8*: The observation that a *REG1* null allele is able to suppress the *ino4-8* growth phenotype indicates that the mechanism of suppression does not involve specific contact between the *REG1* gene product and the mutant *ino4-8* gene product. This caused us to question whether the absence of a negative regulator in the *ino4-8* genetic background was sufficient to confer suppression. To determine whether mutations in the negative regulator encoded by the *OPI1* gene would have a similar effect, a strain carrying the *opi1*Δ mutation (SH308) was crossed to strain SH703 (*ino4-8*). In all 38 tetrads (Table 2D), from which four surviving spores were recovered, 2^+:2^- segregation for inositol auxotrophy was observed, indicating that *opi1*Δ cannot suppress *ino4-8*.

DISCUSSION

INO1 expression is regulated not only in response to the availability of inositol in the growth medium, but also in response to growth phase (Lamping *et al.* 1995; Griac *et al.* 1996; Jiranek *et al.* 1998), ongoing phosphatidylcholine biosynthesis (Griac *et al.* 1996), and turnover (Patton-Vogt *et al.* 1997; Sreenivas *et al.* 1998). Specific regulatory genes shown to affect *INO1* expression and regulation include *INO2, INO4*, and *OPI1* (Paltauf *et al.* 1992). Under derepressing conditions (absence of inositol), *opi1* mutants excrete inositol (Opi$^-$ phenotype) due to overexpression of the *INO1* gene product, inositol-1-phosphate synthase (Greenberg *et al.* 1982b). *opi1* mutants also fail to repress the *INO1* gene in response to the presence of inositol or stationary growth phase signals (Hirsch and Henry 1986; White *et al.* 1991; Jiranek *et al.* 1998). The *INO2* and *INO4* loci encode transcriptional activators of the bHLH class which form a heterodimer that binds to the repeated element UAS_{INO} found in the *INO1* promoter (Ambroziak and Henry 1994; Nikoloff and Henry 1994; Bachhawat *et al.* 1995). Deletion of either the *INO2* or the *INO4* locus causes inositol auxotrophy (Ino$^-$ phenotype) which is not alleviated by mutations at the *OPI1* locus (Graves 1996). However, the precise function of the *OPI1* gene product (Opi1p) is not yet established and Opi1p does not appear to bind to DNA directly (Graves 1996).

Mutations at a large number of other loci produce Ino$^-$ and Opi$^-$ phenotypes, indicating defects in *INO1* expression and/or regulation (reviewed in Henry and Patton-Vogt 1998). For example, mutations in RNA polymerase II (Scafe *et al.* 1990a,b,c), the TATA binding protein (Arndt *et al.* 1995), and the global regulatory factors *SWI1*/*ADR6*, *SWI2*/*SNF2*, and *SWI3* (Peterson and Herskowitz 1992; Peterson *et al.* 1994; Peterson and Tamkun 1995) all cause inositol auxotrophy. In contrast, mutations in the *SIN3* (Hudak *et al.* 1994; Slekar and Henry 1995) and *UME6* (Jackson and Lopes 1996) regulatory factors result in Opi$^-$ phenotypes and/or constitutive expression of *INO1*. The Ino$^-$ phenotypes of the *SWI*/*SNF* genes are caused by the dependence of *INO1* transcription on the *SWI*/*SNF* complex (Peterson *et al.* 1994; Peterson and Tamkun 1995), which is involved in chromatin remodeling. Furthermore, mutations in histone H4 can bypass the requirement for the *SWI*/*SNF* complex, thus permitting *INO1* expression (Santisteban *et al.* 1997) in Swi$^-$ or Snf$^-$ strains. The Ino$^-$ and Opi$^-$ phenotypes described above are all associated with mutations that affect the general transcription apparatus and/or chromatin structure, suggesting that expression of the *INO1* gene is highly sensitive to perturbation in the general state of cellular transcription.

In this study, we have demonstrated that mutations at the *REG1* locus can also result in constitutive expression of the *INO1* gene and that the *reg1*Δ mutation confers an Opi$^-$ phenotype. The *REG1* gene product has been shown to be necessary for repression of genes such as *SUC2* which are under catabolite repression by the glucose response signal transduction pathway. The *REG1* gene product is a regulatory subunit of Glc7p, a type 1 protein phosphatase that regulates the *SNF1* kinase (Tu and Carlson 1994, 1995). Another component of the glucose response pathway is *SNF4*, a regulatory subunit of the *SNF1* kinase (Celenza and Carlson 1986; Jiang and Carlson 1996). The *SNF1* gene encodes a serine-threonine protein kinase required to activate those genes under catabolite repression (Celenza and Carlson 1986).

In addition to their effects upon glucose-repressible genes, *snf1* mutations have Ino$^-$ phenotypes (Hirschhorn *et al.* 1992). The *INO1* gene and the other coregulated genes, *CHO1*, *OPI3*, and *CHO2*, are all constitutively expressed in the *sia1-1* (*reg1-600*) mutant (Table 3; Figure 2). These data and the observation that *snf1* mutants are Ino$^-$ suggest that UAS_{INO}-containing genes require the action of the *SNF1* kinase for their expression and the action of the *REG1* gene product for their repression. Thus, *INO1* expression and regulation appear to require a fully functioning glucose response pathway. It is curious that insulin regulation of FAS in mammalian cells acts through an E-box element (Wang and Sul 1997) homologous to UAS_{INO}. The yeast FAS (Schüller *et al.* 1995) and the acetyl Co-A carboxylase (*ACC1*) promoters (Hasslacher *et al.* 1993) also contain UAS_{INO} sequences. Thus, it is tempting to speculate that the regulatory mechanisms controlling fat metabolism in response to glucose availability in animals and fungi might have had a common ancestry. However, *INO1* and other coregulated genes of phospholipid biosynthesis are expressed and regulated by inositol in the presence of glucose, as shown by the experiments reported here, all of which were conducted in glucose-containing medium. Furthermore, in a related report, Shirra and Arndt (1998) report that glucose has little effect on *INO1* expression. Thus, it appears that the glucose response signal transduction cascade is required for *INO1* expression and regulation. However, the *REG1* gene product must function via a mechanism that is distinct from that by which it governs classical catabolite repressible genes such as *SUC2*, which is not measurably expressed in wild-type cells grown in the presence of glucose.

Furthermore, the expression of *INO1* is influenced by at least one other signal transduction cascade. The *ire1* and *hac1* mutations (Nikawa and Yamashita 1992; Cox and Walter 1996; Nikawa *et al.* 1996; Cox *et al.* 1997) have also been reported to cause inositol auxotrophy. The *IRE1* locus encodes a protein kinase involved in the unfolded protein response pathway and *HAC1* encodes a positive regulator of *IRE1* (Cox and Walter

1996). Regulation of *INO1* expression must, therefore, integrate inputs from several transduction pathways.

In an independent study, Shirra and Arndt (1998) report the isolation of suppressors of the inositol auxotrophy of the *spt15-328* mutation in the TATA binding protein (TBP). Consistent with our findings that *reg1* mutants can suppress the inositol auxotrophy of certain *ino4* and *ino2* missense alleles, Shirra and Arndt isolated a recessive suppressor of the inositol auxotrophy of *spt15-328* that proved to be an allele of *REG1*. They also identified a dominant suppressor that is an allele of *SNF4*. Shirra and Arndt also reported that one of their suppressors is an allele of *OPI1*. However, we found that the *opi1*Δ allele does not suppress the inositol auxotrophy of *ino4-8* (Table 2D). One explanation for the different effects of *opi1* mutants on the Ino$^-$ phenotypes of the *ino4-8* and *spt15-328* mutations could be that Opi1p represses transcription of *INO1* (and other genes whose transcription is dependent on the binding of the Ino2p/Ino4p complex) via an interaction with the TBP. Curiously, however, Shirra and Arndt report that the expression of *INO1* transcript is regulated by inositol in the *opi1 spt15-328* double mutant. Yet, in the *opi1 SPT15* strain, *INO1* expression is constitutive (Shirra and Arndt 1998). This result suggests that Opi1p attenuates the level of transcription of *INO1* via TBP but does not actually control the regulatory response to inositol.

If this hypothesis is correct, then the regulation in response to inositol by *INO1* is mediated by regulatory factors working at a point in the regulatory cascade that precedes the steps mediated by both Opi1p and the TBP. The Ino2p/Ino4p complex is a possible target for such regulation. The *ino4-8* mutation is a point mutation, glutamic acid to lysine at residue 79 in the loop region of the bHLH motif (Ambroziak 1994). The recessive suppressors isolated in this study, *sia1-1* (*reg600*) and *sia2-1*, proved to have the unusual property that they suppressed not only the inositol auxotrophy of *ino4-8* strains and *ino4-26* (threonine to isoleucine in the basic region of the bHLH motif; Ambroziak 1994), but also *ino2-419* (arginine to lysine in the loop region of the bHLH motif; Nikoloff and Henry 1994). However, neither the *sia1-1* nor the *sia2-1* mutations were able to suppress either the *ino4*Δ or the *ino2*Δ mutation (Table 2). Thus, the suppression mechanism appears to depend on some residual function of the mutated Ino2p/Ino4p complex.

Cell extracts prepared from strains carrying *ino2-419*, *ino4-8*, or *ino4-26* point mutations have very low or undetectable ability to heterodimerize and bind DNA as a complex (Ambroziak 1994; Nikoloff and Henry 1994). However, when wild-type Ino2p is cotranslated *in vitro* with the *ino4-8* or *ino4-26* mutant gene products, the heterodimers formed have some residual ability to bind UAS_{INO} *in vitro* (Ambroziak 1994). The mutation in *REG1*, which deregulates the protein kinase activity of the *SNF1* kinase, may lead to strengthening of the mutated residual Ino2p/Ino4p complex permitting it to function, at least partially, *in vivo*. We propose that Reg1p affects *INO1* transcription, via its role in regulating the *SNF1*/*SNF4* complex, by influencing the interaction of Ino2p/Ino4p with each other and, thus, with UAS_{INO}. This, in turn, affects the interaction of the *INO1* promoter with the *SPT15* gene product, TBP. *opi1* mutations, including *opi1*Δ, do not suppress *ino4-8* and, thus, we believe that the mechanism of Opi1p action does not influence the activity of the Ino2p/Ino4p complex or its binding to UAS_{INO}. Because *opi1* mutations suppress *spt15-328*, Opi1p could function as a mediator between TBP and its recruitment to UAS_{INO} by the active Ino2p/Ino4p complex.

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