Regulation of yeast phospholipid biosynthetic gene expression in response to inositol involves two superimposed mechanisms

(activators/basic-helix-loop-helix proteins/repressor/weak promoters/Saccharomyces cerevisiae)

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ABSTRACT Transcription of phospholipid biosynthetic genes in the yeast Saccharomyces cerevisiae is maximally derepressed when cells are grown in the absence of inositol and repressed when the cells are grown in its presence. We have previously suggested that this response to inositol may be dictated by regulating transcription of the cognate gene, INO2. However, it was also known that cells which harbor a mutant opil allele express constitutively derepressed levels of target genes (INO1 and CHO1), implicating the OPII negative regulatory gene in the response to inositol. These observations suggested that the response to inositol may involve both regulation of INO2 transcription as well as OPII-mediated repression. We investigated these possibilities by examining the effect of inositol on target gene expression in a strain containing the INO2 gene under control of the GAL1 promoter. In this strain, transcription of the INO2 gene was regulated in response to galactose but was insensitive to inositol. The expression of the INO1 and CHO1 target genes was still responsive to inositol even though expression of the INO2 gene was unresponsive. However, the level of expression of the INO1 and CHO1 target genes correlated with the level of INO2 transcription. Furthermore, the effect of inositol on target gene expression was eliminated by deleting the OPII gene in the GAL1-INO2-containing strain. These data suggest that the OPII gene product is the primary target (sensor) of the inositol response and that derepression of INO2 transcription determines the degree of expression of the target genes.

Regulation of gene expression in yeast has been extensively documented (reviewed in refs. 1 and 2) and several well-defined systems have emerged as models for how the yeast cell responds to environmental signals by coordinately varying gene transcription (3–5). These model systems have identified specific interactions between cis-acting upstream activation sequences (UASs) (6) and their cognate trans-acting regulatory proteins. Recent investigations have focused on understanding the role(s) of trans-acting regulatory proteins in coordinating gene expression. These roles generally fall into two broad categories. The first category includes regulation of the amount of functional activator—e.g., regulation of GAL4 transcription in response to glucose (7), of GCN4 translation (8) and Gcn4 protein stability (9) in response to amino acid starvation, and of Swi5 and Ace2 transit into the nucleus (10, 11). The second category invokes repressors that specifically interact with activators to inhibit their function—e.g., modulation of the interaction between the Gal80 repressor and the Gal4 activator (12) or between the Pho80 repressor and the Pho4 activator (13). However, it is unusual to find a system that involves both categories in response to a single environmental cue. This report examines the regulation of phospholipid biosynthetic gene expression in response to inositol. We show that the response to inositol involves both transcriptional regulation of the INO2 activator gene and the action of the OPII negative regulatory gene.

Transcription of the phospholipid biosynthetic genes is maximally derepressed in the absence of inositol and repressed in its presence (reviewed in refs. 14 and 15). A highly conserved 10-bp palindrome (5′-CANNTG-3′) found in the galactose regulatory region of the coregulated genes has been shown to be both necessary and sufficient for the inositol response (16, 17). This element (UASINO) includes the canonical binding site for the basic–helix–loop–helix (bHLH) family of proteins (5′-CANNTG-3′) (18, 19). Thus, it was not surprising to find that the UASINO sequence serves as a binding site for a heterodimer composed of two bHLH proteins, Ino2 and Ino4 (20–22). Consistent with their predicted role as transcriptional activators, the INO2 and INO4 genes have been shown to be dispensable for derepression of phospholipid biosynthetic gene expression in response to inositol deprivation (14, 15).

Recently, we showed that expression of an INO2 promoter–chloramphenicol acetyltransferase (CAT) fusion gene was regulated in response to inositol in a pattern that was indistinguishable from that of its target genes (23). This observation suggested that regulation of the phospholipid biosynthetic genes in response to inositol may involve regulation of transcription of the INO2 activator gene. However, regulation of phospholipid biosynthetic gene expression is also dependent on a negative-acting regulatory gene, OPII (24). Strains that harbor null alleles of OPII constitutively overexpress the phospholipid biosynthetic structural genes (24) as well as the INO2 gene (23). Therefore, the response to inositol may involve both categories of mechanisms—i.e., regulation of expression of the INO2 activator gene and repression by the OPII gene product (Fig. 1 Upper). However, we could not preclude the possibility that the role of OPII might be to regulate INO2 gene expression (Fig. 1 Lower). To distinguish between these two models we uncoupled INO2 expression from the inositol response by placing it under the control of the GAL1 promoter. In a strain that contains the GAL1-INO2 fusion, expression of the target genes (INO1 and CHO1) was found to be regulated in response to both inositol and galactose concentrations. However, the inositol response was eliminated when the OPII gene was deleted in this same strain.

MATERIALS AND METHODS

Strains and Growth Conditions. Yeast strains used in this study were BRS1001 (MATa, ade2-1, his3-11,15, leu2-3,112, can1-100, ura3-1, trpl-1), BRS2002 (MATa, ade2-1, his3-11,15, leu2-3,112, can1-100, ura3-1, trpl-1, ino12::TRP1), and BRS2005 (MATa, ade2-1, his3-11,15, leu2-3,112, can1-100, ura3-1, trpl-1, opilΔ::LEU2), BRS2011 (MATa, ade2-1, his3-

Abbreviations: CAT, chloramphenicol acetyltransferase; UAS, upstream activation sequence.

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were synthesized were procedures. The construction of strains containing the INO2-cat reporter gene integrated in single copy at the GAL4 locus has been previously described (23). All cultures were grown at 30°C in synthetic medium (25) either supplemented with 75 μM inositol and 1 mM choline or lacking inositol and choline. Media containing different carbon sources are described in the text.

**Plasmid Construction and Chromosomal Integration.** Plasmid pBM2289 (26) was used to fuse the INO2 coding sequence to the GAL1 promoter. This plasmid contains the wild-type GAL1 promoter, upstream of an SpI restriction site, and the URA3 selectable marker. The INO2 gene was amplified by PCR using a 5’ primer (5’-GATCATTGCAACAAACAGC-3’) which included the translational initiator codon for the INO2 gene flanked by an SpI restriction site for subcloning purposes. The 3’ PCR primer (5’-GATCTTNGACCGTTT-3’) was targeted to sequences downstream of the translational stop codon for the INO2 gene. This was done to ensure that sequences important for mRNA 3’-end maturation were included. The INO2 PCR product was cloned into the pGEM-T vector (Promega) to create pGEM-INO2. An SpI restriction fragment containing the INO2 coding sequence was cloned into an SpI restriction site in pBM2289, creating pGAL1-INO2. The pGAL1-INO2 construct places the INO2 coding DNA immediately downstream of the wild-type GAL1 promoter. The pGAL1-INO2 plasmid was linearized within the URA3 gene (SulI) and used to transform an ino2 deletion mutant strain (BRS2002) to create BRS2011. Single-copy integrants at the URA3 locus were confirmed by Southern blot analysis. A derivative of BRS2001 that contained a null allele of the OPI1 gene (opi1Δ::LEU2) was constructed by transformation with a restriction fragment carrying the opi1Δ null allele (24) and was designated BRS2012.

**RNA Analyses.** RNA was isolated from yeast by a glass-bead disruption/hot phenol extraction procedure (27). RNA probes for Northern and quantitative slot blot hybridizations (23, 25) were synthesized with the Gemini II core system (Promega) from plasmids linearized with a restriction enzyme as follows (shown as plasmid, restriction enzyme, RNA polymerase) for the indicated (parenthesized) probe: pGEM-INO2, Sal I, T7 (INO2); pFLG, BamH1, SP6 (ACT1). Probes for INO1, CHO1, and TCM1 have been described (23). The results of Northern and slot blot hybridizations were visualized by autoradiography and quantitated by densitometry.

**CAT Enzyme Assays.** CAT activity was determined with a phase-extraction procedure (7, 23). Units of CAT activity were defined as counts per minute measured in the organic phase and expressed as a percentage of the total counts per minute (percent conversion) divided by the amount of protein assayed (in micrograms) and the time of incubation (in hours).

**RESULTS**

**Uncoupling INO2 Transcription from the Inositol Response.** Expression of the cat reporter gene driven by the INO2 promoter (integrated in single copy at the GAL4 locus in BRS1001) (23) was sensitive to different inositol concentrations in the growth medium (Fig. 2). Specifically, we observed increased levels of CAT activity with decreasing concentrations of inositol. The effect of the different inositol concentrations on expression of the INO2-cat gene was similar to the effect on expression of the INO2-target genes INO1 and CHO1 (23, 25, 28). This suggested that regulation of INO2 expression may be the primary mechanism for the coordinated response to inositol. To directly determine the role of INO2 expression in the regulation and/or expression of the target genes, we uncoupled INO2 expression from the inositol response by placing it under the control of the galactose-inducible GAL1 promoter. To do this, we constructed a plasmid (pGAL1-INO2) that placed the INO2 coding sequence downstream of the GAL1 promoter in plasmid pBM2289 (26). Plasmid pGAL1-INO2 (containing the URA3 selectable marker) was stably integrated in single copy at the ura3 locus of strain BRS2002 (ino2Δ) to yield BRS2011 (pGAL1-INO2::URA3, ino2Δ). We chose to use BRS2002 because it contained a deletion allele of the INO2 gene and therefore ensured that INO2 expression originated exclusively from the GAL1-INO2 hybrid gene.

Expression of the INO2 gene in BRS2011 (pGAL1-INO2::URA3, ino2Δ) was expected to be sensitive to carbon source (GAL1 promoter-driven) but insensitive to inositol. We tested this prediction by using two assays for INO2 expression. First, we compared the growth phenotype of BRS2011 (pGAL1-INO2::URA3, ino2Δ) on media containing different carbon sources and either lacking or containing inositol (Table 11). We also assayed CAT activity from extracts of mutants (BRS1001) containing a single copy of the reporter gene integrated in single copy at the GAL4 locus (23). Cells were grown in media containing various concentrations of inositol. All values are presented as a percentage of completely derepressed levels and are the average of at least three independent assays. Standard deviations were less than 15% in all cases.

**Fig. 1.** Models depicting potential regulatory cascades for phospholipid biosynthetic gene expression. Genes are designated in italics and proteins by the "p" suffix. Refer to the text for a complete description.

**Fig. 2.** Expression of the INO2-promoter driven CAT reporter gene is sensitive to inositol concentration. CAT activity was assayed from extracts of wild-type cells (BRS1001) containing a single copy of the reporter gene integrated in single copy at the GAL4 locus (23). Cells were grown in media containing various concentrations of inositol. All values are presented as a percentage of completely derepressed levels and are the average of at least three independent assays. Standard deviations were less than 15% in all cases.
Table 1. Growth phenotype of GAL1–INO2-containing ino2Δ strain

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<tr>
<th>Strain (genotype)</th>
<th>Inositol</th>
<th>No inositol</th>
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<tbody>
<tr>
<td>BRS1001 (INO2)</td>
<td>+ + + +</td>
<td>+ + + + +</td>
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<tr>
<td>BRS2002 (ino2Δ)</td>
<td>+ + + +</td>
<td>- - - - -</td>
</tr>
<tr>
<td>BRS2011 (pGAL1-IN02::URA3, ino2Δ)</td>
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Strains were tested by spotting ~10⁶ cells on complete synthetic medium (21) that was either supplemented with 75 μM inositol or lacked inositol and that contained either 2% galactose (Gal), 2% raffinose (Raf), or 2% glucose (Glc) by weight. Growth was scored after 48 hr (30°C) as wild type (+), no growth (-), or slow growth (±).

1). BRS2011 (pGAL1-IN02::URA3, ino2Δ) grew normally on galactose-containing medium regardless of the presence or absence of inositol. That is, the level of GAL1 promoter-driven INO2 gene expression in medium containing galactose rescued the inositol auxotrophy associated with the ino2Δ mutant allele. However, this same strain grew slowly on a raffinose medium, and failed to grow on a glucose medium when inositol was omitted. The inability of BRS2011 (pGAL1-IN02::URA3, ino2Δ) to grow on glucose and grow slowly on raffinose is due to expression from the GAL1 promoter, which is severely repressed when cells are grown on glucose-containing medium and reduced on raffinose-containing medium (3). Consequently, INO2 expression may be limiting under these two growth conditions, which would affect the ability of the ino2Δ strain to grow in the absence of inositol. As controls, we also examined the growth of an isogenic INO2 strain (BRS1001) and the isogenic parental strain carrying the ino2Δ allele (BRS2002). As expected, the INO2 wild-type strain grew under all conditions whereas the ino2Δ strain required inositol for growth regardless of the carbon source (Table 1).

The second assay involved direct quantitation of INO2 transcription in BRS2011 (pGAL1-IN02::URA3, ino2Δ) by Northern and slot blot hybridizations. For this, we grew cells in media that contained different concentrations of galactose and either lacked or contained inositol. INO2 expression from the GAL1 promoter was not sensitive to the presence of inositol in the growth medium (Fig. 3 A and B) but was sensitive to the concentration of galactose in the medium (Fig. 3B). The presence of different concentrations of galactose had previously been shown to result in different levels of expression from the GAL1 promoter (29). Consequently, we uncoupled INO2 expression from the inositol response and made it sensitive to galactose concentration.

**Transcription of the INO2 Gene Correlates with Transcription of Its Target Genes.** The BRS2011 strain (pGAL1-IN02::URA3, ino2Δ) allowed us to determine whether regulation of INO2 expression is a component of the coordinated response to inositol. That is, does yeast coordinately derepress expression of the phospholipid biosynthetic genes in response to inositol by simply derepressing expression of the INO2 gene? To address this question we directly quantitated transcription of two INO2-target genes, INO1 (25) and CHO1 (28), in BRS2011 (pGAL1-IN02::URA3, ino2Δ) grown in media containing varying concentrations of galactose in both the presence and the absence of inositol. Transcription of the INO1 gene in BRS2011 (pGAL1-IN02::URA3, ino2Δ) was sensitive to both galactose and inositol in the growth medium (Fig. 4). That is, in the absence of inositol, transcription of the INO1 gene correlated with the concentration of galactose in the growth medium. However, in the presence of inositol, INO1 transcription was repressed regardless of the galactose concentration. Similarly, transcription of the CHO1 target gene was also

**Fig. 3.** Uncoupling INO2 expression from the inositol response. (A) Expression of INO2 transcript from strain BRS2011 (pGAL1-IN02::URA3, ino2Δ) grown in media containing 0.5% galactose and various concentrations of inositol (from left to right: 0, 5, 10, 17.5, 25, 50, 75, and 100 μM). The same blot was rehybridized with the ACT1-specific probe to normalize for loading variations. (B) Relative levels of INO2 transcription (arbitrary densitometry units) from BRS2011 (pGAL1-IN02::URA3, ino2Δ) grown in media containing various concentrations of galactose either lacking (hatched bars) or containing (solid bars) 75 μM inositol and 1 mM choline. The amount of INO2 transcript was determined by densitometric scanning of quantitative slot blots and normalized for loading variations by using the ACT1 transcript. Values represent the average of three independent assays. Standard deviations were less than 15% in all cases.

**Fig. 4.** Transcription of the INO1 gene is sensitive to both galactose concentration and inositol in the GAL1–INO2-containing strain. Data were generated as described in the legend to Fig. 3B.
sensitive to both galactose and inositol (Fig. 5). Thus, in BRS2011 (pGAL1-IN02::URA3, ino2Δ), transcription of the INO1 and CHO1 target genes was still repressed in response to inositol supplementation even though INO2 transcription was no longer sensitive to inositol (Fig. 3). Because the GAL4 promoter is significantly stronger than the INO2 promoter, we conducted a set of experiments parallel to those shown in Fig. 4, by expressing the INO2 gene under control of the weak GAL4 promoter. The GAL4 promoter is about twice as strong as the INO2 promoter (unpublished observations) and is repressed when cells are grown in glucose-containing medium (7). We obtained the same results with the GAL4–INO2-containing strain as we report here with the GAL1–INO2 strain. That is, INO1 gene expression was still subject to regulation by inositol even though INO2 expression was now under control of a glucose-repressible promoter (7). However, it was not possible to confirm that INO2 expression driven by the GAL4 promoter was uncoupled from the inositol response, because of the weakness of the GAL4 promoter (ref. 7; unpublished observations).

Thus, the coordinated response to inositol does not appear to be exclusively dictated by controlling INO2 expression. However, the degree of derepression of the INO1 and CHO1 genes did correlate with the level of INO2 transcription in BRS2011 (pGAL1-IN02::URA3, ino2Δ). That is, there was a correlation between the level of expression of the INO2 activator gene (Fig. 3B) and the target genes at galactose concentrations between 0 and 0.5% (Figs. 4 and 5). However, while INO2 transcription continued to increase at galactose concentrations greater than 0.5% (Fig. 3B), INO1 and CHO1 transcription did not increase under these same growth conditions (Figs. 4 and 5).

The OPII Gene Is Required for the Inositol Response in a GAL1–INO2 Strain. Since regulation of INO2 gene transcription was not the primary target of the inositol response, we reasoned that the OPII negative regulatory gene might be the primary target. This line of reasoning was supported by the phenotype of strains carrying opi1 mutant alleles. In an opi1 mutant strain, expression of the INO1 (24, 25) and CHO1 (28) target genes is insensitive to the presence of inositol in the growth medium. This suggests that the product of the OPII gene either regulates INO2 expression (Fig. 1 Upper) or directly regulates the function of the Ino2 protein (Fig. 1 Lower). To distinguish between these two models, we examined the effect of deleting the OPII gene in BRS2011 (pGAL1-IN02::URA3, ino2Δ) on regulation of INO1 gene expression. If the response to inositol was mediated by Opi1 regulating the function of Ino2 (Fig. 1 Lower), then deletion of OPI1 gene in BRS2011 (pGAL1-IN02::URA3, ino2Δ) should yield constitutive expression of the INO1 target gene.

To directly examine the role of the OPII gene in the response to inositol we deleted the OPI1 gene in strain BRS2011 (pGAL1-IN02::URA3, ino2Δ) to yield BRS2012 (pGAL1-IN02::URA3, opi1Δ, ino2Δ). We then examined the effect of the opi1Δ allele on regulation of INO1 expression in BRS2012 (pGAL1-IN02::URA3, opi1Δ, ino2Δ) by Northern blot hybridization. Total RNA was purified from strains grown in media that contained 0.5% galactose and either lacked or included inositol. The level of INO1 transcripts was quantitated by densitometry and normalized for loading by use of the ACT1 gene probe. As expected, BRS2012 (pGAL1-IN02::URA3, opi1Δ, ino2Δ) expressed constitutively elevated levels of INO1 relative to the isogenic BRS2011 strain (pGAL1-IN02::URA3, ino2Δ) (Fig. 6 Right). In fact, the pattern of regulation in the strains containing pGAL1-IN02 (Fig. 6 Right) was virtually indistinguishable from that in the strains containing the native INO2 gene (Fig. 6 Left).

**DISCUSSION**

We have determined that the response to inositol requires two superimposed mechanisms. One mechanism is the regulation of INO2 activator gene expression, which is subject to autoregulation by the INO2 gene product (23). The second mechanism requires the product of the OPII negative regulator, which may function as a direct regulator of Ino2/ino4 activity (Fig. 1 Upper). Our data favor a model wherein the OPII gene product (Opi1) is the primary target of the inositol response. We had previously shown that Opi1 is required to regulate expression of the INO2 activator gene (23). However, here we show that it is also required to directly regulate expression of the INO1 target gene (Fig. 6). These observations are paradoxical because both mechanisms are in operation in a wild-type yeast. Thus, it is difficult to determine if the primary role of the OPII gene product is to regulate target gene expression directly or indirectly through regulation of INO2 regulatory gene expression. In part, the resolution of this paradox is
dependent on determining how much Ino2 protein is present in the cell under various growth conditions.

Nevertheless, it is clear that OPI1 is absolutely required for the inositol response, whereas regulation of INO2 expression can be eliminated without affecting regulation of the target genes in response to inositol (Figs. 4 and 5). Furthermore, OPI1 seems a likely target for the inositol response, since it appears to be expressed at a level higher than either INO2 or INO4 (23). We have previously shown that the OPI1 promoter is capable of driving constitutive expression (i.e., unresponsive to inositol) of a car reporter gene at a level that is substantially higher than either the INO2 or INO4 promoters (23). Curiously, the relative levels of expression of the OPI1 and INO2 regulatory genes (23) are reminiscent of the relative levels of GAL80 and GAL4 expression (26, 30). Consistent with this line of reasoning, it has been proposed that the GAL80 gene product is the sensor for the intracellular inducer of the GAL system (31).

We observed a strong correlation between INO2 expression driven by the GAL1 promoter and expression of two target genes, INO1 and CHO1. This suggests that regulation of INO2 expression does play a role in the response to inositol. For example, if Ino2 levels are extremely low under repressing conditions, then the cell would have to express INO2 prior to activating transcription of the target genes. Alternatively, the "pump may be primed" by a small amount of Ino2 and derepression of INO2 expression may serve to establish the degree of derepression of the target genes. We favor the latter model, since it has been shown that extracts prepared from cells grown under repressing conditions form the Ino2/Ino4/UASINO complex (32). Furthermore, the kinetics of derepression of an INO2-cat gene and an INO1-cat gene were essentially identical (23), suggesting that derepression of INO2 expression does not precede that of its target genes.

The role of derepressing INO2 expression may be to establish the degree of derepression of the target genes. Consistent with this hypothesis, we have observed a correlation between INO2 expression and target gene expression at different concentrations of inositol (23). Thus, depending on the inositol concentration, INO2 may be expressed at different levels which will determine the level of target gene expression. Moreover, since the number and sequence of potential Ino2/Ino4 target sequences vary among the promoters of the coregulated genes (15), it seems likely that different levels of INO2 expression may be required to activate expression of different target genes.

The experiments presented here provide further evidence that INO2 expression is limiting relative to INO4 (23) and that target gene expression is most likely limited by the amount of INO4 expression. This latter point is evidenced by the fact that INO2 expression from the GAL1 promoter increased linearly as a function of galactose concentration up to 2% galactose (Fig. 3B), whereas INO1 and CHO1 expression reached a plateau at galactose concentrations between 0.25% and 0.5%. This result was not entirely surprising, since we previously observed that the INO2 promoter was substantially weaker than the INO4 promoter (23). Furthermore, overexpression of INO2 (but not INO4) from a multicopy plasmid yielded an elevated level of the Ino2/Ino4/UASINO complex in mobility-shift assays (20). Thus, the role of the INO4 gene product may be to establish an upper limit to the level of derepression of the target genes.