Analysis of the Mechanisms Controlling Phospholipid Biosynthetic Gene Expression in the Yeast Saccharomyces Cerevisiae

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ACKNOWLEDGEMENTS

I would first like to recognize the members of my committee for their helpful criticisms and assistance in developing a productive graduate school career: Dr. Sally Amero, Dr. Adam Driks, Dr. Mike Fasullo, Dr. Russ Pieper, and my advisor, Dr. John Lopes. I would also like to acknowledge Dr. Dan Gottschling from the University of Chicago who served on my committee up until the last six months of my graduate career and helped me with several suggestions which saved me a lot of time.

I would like to thank my laboratory colleagues: Melanie Anderson, John Jackson, and Kelly Robinson and wish them the best of luck in the future.

I also would like to acknowledge Dr. Len Erickson and the Molecular Biology Program and Dr. Richard Schultz for all of their support over the past fours.

Finally, I would like to extend my thanks to my wife Kerry for all of her love and support during my graduate career and to my parents for all of their help and encouragement.
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LIST OF ABBREVIATIONS

A adenine
aa amino acid
ATP adenine triphosphate
bp base pair
CAT chloramphenicol acetyltransferase
C cysteine
Ci Curie
CTP cytidine triphosphate
DNA deoxyribonucleic acid
dATP deoxyadenosine triphosphate
dCTP deoxycytosine triphosphate
dGTP deoxyguanosine triphosphate
dNTP deoxynucleotide triphosphate
dTTP deoxythymidine triphosphate
DTT dithiothreitol
EDTA ethylenediamine tetraacetic acid
g gram
GTP guanine triphosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>hr</td>
<td>hour(s)</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactosidase</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LB</td>
<td>Luria broth</td>
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<tr>
<td>L</td>
<td>liter</td>
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<td>M</td>
<td>molar</td>
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<td>mM</td>
<td>millimolar</td>
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<td>minute(s)</td>
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<td>ml</td>
<td>milliliter</td>
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<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDME</td>
<td>phosphatidyl(dimethylethanolamine)</td>
</tr>
<tr>
<td>PMME</td>
<td>phosphatidyl(monomethylethanolamine)</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>sec</td>
<td>second(s)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate acid-EDTA</td>
</tr>
</tbody>
</table>
TE  tris-EDTA

tRNA  transfer RNA

TTP  thymine triphosphate

U  units

v  volts

vol  volumes
ABSTRACT

In the yeast *Saccharomyces cerevisiae*, the phospholipid biosynthetic genes are highly regulated at the transcriptional level in response to the phospholipid precursors inositol and choline. In the absence of inositol and choline, the products of the *INO2* and *INO4* genes form a heteromeric complex which binds to a 10 bp element, UAS\textsubscript{INO}, in the promoters of the phospholipid biosynthetic genes to activate their transcription. In the presence of inositol and choline, the product of the *OPI1* gene represses transcription dictated by the UAS\textsubscript{INO} element. The work described in this dissertation is aimed at understanding how the Ino2 and Ino4 activators and the Opi1 repressor function to regulate phospholipid biosynthetic gene expression.

The first residue of the 10 bp UAS\textsubscript{INO} element is the least well conserved residue. Therefore, the importance of this residue was tested by modifying this position to all four possibilities. These studies indicated that the UAS\textsubscript{INO} element is actually characterized by the sequence: 5'(C/A)ATGTGAAAT3'. The results also indicate that the *OPI1* gene product acts through the 10 bp UAS\textsubscript{INO} element and does not require the presence of an adjacent URS element.

Curiously, a copy of the UAS\textsubscript{INO} element is also present in the promoters of the *INO2* and *INO4* genes, leading to the possibility that their expression is also regulated in response to inositol and choline.
Using a cat reporter gene, INO2-cat expression was found to be regulated 12-fold in response to inositol and choline, but INO4-cat was constitutively expressed. Further, INO2-cat was not expressed in either an ino2 or an ino4 mutant strain and was constitutively over expressed in an opil mutant strain. Expression of the INO4-cat gene was affected only by mutation in the INO4 gene itself. Therefore, INO2-cat transcription is regulated by the products of both the INO2 and INO4 genes whereas INO4 must interact with another protein to activate its own transcription.

The fact that INO2 is regulated in response to inositol and choline suggests that this regulation may be the means by which the inositol/choline response is mediated. To test this, expression of INO2 was uncoupled from the inositol/choline response by placing it under the control of the GAL1 promoter. In this strain, INO2 expression was regulated in response to galactose but was insensitive to inositol. The expression of the INO1 and CHO1 target genes was still regulated in response to inositol even though expression of the INO2 gene was not. 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 OPI1 gene in the GAL1-INO2-containing strain. These data suggest that the OPI1 gene product is the primary target (sensor) of the inositol response and that derepression of INO2 transcription determines the degree of derepression of the target genes.

An analysis of the INO2 promoter was also performed. These results revealed some interesting features of the INO2 promoter.
First, primer extension analysis showed that the *INO2* mRNA includes a 106 bp untranslated leader sequence which includes within it a potential open reading frame of 19 amino acids. This upstream open reading frame overlaps the known translation initiation codon for the *INO2* coding sequences. Deletion analysis of the *INO2* promoter revealed an essential region found between -200 and -250 relative to the translation initiation codon and also showed that the UASINO element in the *INO2* promoter is required for the regulated expression of the *INO2* gene.

Therefore, the regulation of phospholipid biosynthetic gene expression involves two superimposed mechanisms. One mechanism is the regulation of *INO2* activator gene expression which mediates the degree of derepression of its target genes. The second mechanism requires the product of the *OPII* negative regulator which appears to function as a direct regulator of Ino2p:Ino4p activity and is responsible for mediating the inositol/choline response.
CHAPTER I

INTRODUCTION

The membranes of *Saccharomyces cerevisiae* are composed of a typical mixture of phospholipids which serve a general function in cellular compartmentation and may also control the intracellular responses to external stimuli (reviewed in: Greenberg and Lopes, in press). For example, yeast have been shown to synthesize several phosphorylated forms of phosphatidylinositol (PI) (Auger *et al.*, 1989; Lester and Steiner, 1968) that have been proposed to play a role in signal transduction pathways in multicellular eukaryotes (Divecha and Irvine, 1995). Consequently, it is critical to our knowledge of the biology of the eukaryotic cell that we understand how the cell coordinates the synthesis and assembly of the mixture of lipids that comprise the membrane matrix.

In *S. cerevisiae*, the major membrane phospholipids, PI and phosphatidylcholine (PC), are synthesized by two separate pathways that diverge from a common lipid precursor, CDP-diacylglycerol (CDP-DG) (Figure 1) (Kelley *et al.*, 1988; Carman and Henry, 1989). PI can be synthesized directly from exogenous inositol or *de novo* from glucose-6-phosphate (White *et al.*, 1991). The two structural genes, *INO1* and *PIS1*, are required for the *de novo* synthesis of PI from glucose-6-phosphate. The *INO1* gene encodes the enzyme inositol-1-phosphate synthase (IPS) (Klig and Henry, 1984; Dean-Johnson and
Figure 1. Phospholipid biosynthetic pathway
Henry, 1989) which catalyzes the conversion of glucose-6-phosphate to inositol-1-phosphate. This is subsequently dephosphorylated to produce inositol (Culbertson et al., 1976a; Culbertson et al., 1976b). The product of the \textit{PIS1} gene, phosphatidylinositol synthase (PIS), converts inositol and CDP-DG to PI (Fischl and Carman, 1983; Fischl et al., 1986). This pathway is essential since yeast which cannot synthesize inositol die due to a phenomenon called "inositol-less" death, when grown in media lacking inositol (Culbertson and Henry, 1976). Furthermore, disruption of the \textit{PIS1} gene has revealed that this gene is essential for viability (Nikawa \textit{et al.}, 1987). Consequently, PI is an essential component of yeast membranes.

PC is the major phospholipid present in eucaryotic cell membranes (Greenberg and Lopes, in press). In yeast, PC can be synthesized \textit{de novo} from CDP-DG (Figure 1) (White \textit{et al.}, 1991; Paltauf \textit{et al.}, 1992). The first reaction in the PC pathway synthesizes phosphatidylserine (PS) from CDP-DG and free serine using the enzyme PS synthase (PSS). PS is subsequently decarboxylated by PS decarboxylase (PSD) to form phosphatidylethanolamine (PE) which is sequentially methylated to form PC. Formation of PC from PE involves three sequential methylations which are catalyzed by two methyltransferases.

The first reaction in the PC pathway is catalyzed by PSS (Figure 1) and was defined genetically by mutants, \textit{chol}, that failed to grow in the absence of choline (Atkinson \textit{et al.}, 1980a; Atkinson \textit{et al.}, 1980b; Kovac \textit{et al.}, 1980; Nikawa and Yamashita, 1981). The \textit{chol} mutants were found to be defective in the synthesis of PS (Atkinson \textit{et al.}, 1980a; Atkinson \textit{et al.}, 1980b; Kovac \textit{et al.}, 1980) and lacked
PSS activity (Nikawa and Yamashita, 1981). However, chol mutants were able to grow in the presence of either ethanolamine or choline, due to the ability of yeast to synthesize PC via a salvage pathway (Kennedy pathway). The second step in this pathway is the decarboxylation of PS to PE (Figure 1). This step is catalyzed by PSD, the product of the PSD1 gene and PSD2 gene (Clancy et al., 1993; Trotter et al., 1993).

The final three steps in the synthesis of PC de novo involve the sequential methylation of PE (Figure 1) (White et al., 1991; Paltauf et al., 1992). These three reactions are catalyzed by two methyltransferases, the phosphatidylethanolamine methyltransferase (PEMT) and the phospholipid methyltransferase (PMT) (Greenberg and Lopes, in press). PEMT primarily catalyzes the first methylation reaction and consequently PEMT mutant strains (cho2) accumulate elevated levels of PE (Kodaki and Yamashita, 1987; Summers et al., 1988). PMT catalyzes the last two methylation reactions and PMT mutants (opi3) accumulate elevated levels of PMME and PDME (Yamashita et al., 1982; Greenberg et al., 1983; Kodaki and Yamashita, 1987).

Early studies on the product of the INO1 gene, 11P synthase, showed that its activity was regulated in response to the presence of exogenous inositol (Culbertson et al., 1976a; Donahue and Henry, 1981). That is, when cells were grown in the presence of inositol, 11P synthase activity and subunit concentrations were significantly reduced (Culbertson et al., 1976a; Donahue and Henry, 1981). The cloning of the INO1 gene (Klig and Henry, 1984) made it possible to examine if the response to inositol was due to transcriptional
regulation. Quantitative Northern blot hybridization clearly showed that a 1.8 kb \textit{INO1} transcript was most abundant when cells were grown in either the absence of inositol and choline or the presence of choline by itself (Hirsch and Henry, 1986). The addition of inositol to the growth media caused a 90% reduction in the level of the \textit{INO1} mRNA while inositol and choline added in conjunction resulted in a 97% reduction (Hirsch and Henry, 1986). Therefore, the reduction in I1P synthase activity correlated with a decrease in the abundance of the \textit{INO1} transcript.

Deletion analyses of the \textit{INO1} promoter fused to the \textit{lacZ} reporter gene combined with fusions of \textit{INO1} promoter fragments to the \textit{CYC1-lacI'Z} reporter suggested the existence of a repeated element required for the inositol response (Lopes \textit{et al.}, 1991). These studies, coupled with computer-aided analyses of the \textit{INO1} promoter led to the conclusion that there are 9 copies of a repeated 10 bp element (designated UAS\textit{INO}). More recent studies have shown that 2 of the 9 elements are functional when placed upstream of the \textit{CYC1-lacI'Z} reporter (Koipally \textit{et al.}, submitted; Bachhawat \textit{et al.}, in press). Cloning of other genes (\textit{CHO1}, \textit{CHO2}, and \textit{OPI3}) in the pathway also revealed that their expression was regulated at the level of transcription (Bailis \textit{et al.}, 1987; Hudak \textit{et al.}, 1994; Hudak and Henry, unpublished results; Kodaki \textit{et al.}, 1991). Northern blot and quantitative slot blot hybridization analyses revealed that a 1.2 kb \textit{CHO1} transcript was maximally expressed in the absence of inositol and choline and repressed approximately 5 to 6-fold by the addition of inositol and choline to the growth media (Bailis \textit{et al.}, 1987). Examination of the \textit{CHO1}, \textit{CHO2}, and \textit{OPI3} promoters revealed
the presence of UAS\textsubscript{INO} elements in all three promoters (Kodaki \textit{et al.}, 1991a; Kodaki \textit{et al.}, 1991b; Bailis \textit{et al.}, 1992; Paltauf \textit{et al.}, 1992). Therefore, all four of the genes described above, \textit{INO1}, \textit{CHO1}, \textit{CHO2}, and \textit{OPI3}, are coordinately regulated at the level of transcription in response to inositol and choline.

The coordinately regulated expression of the phospholipid biosynthetic genes is dependent on the cis-acting UAS\textsubscript{INO} element which serves as a binding site for a transcriptional activator complex composed of the products of the \textit{INO2} and \textit{INO4} genes (Hirsch and Henry, 1986; Bailis \textit{et al.}, 1987; Klig \textit{et al.}, 1988; Nikoloff \textit{et al.}, 1992). The \textit{INO2} and \textit{INO4} genes were initially identified among the original 10 inositol auxotrophic complementation groups (Culbertson and Henry, 1975; Donahue and Henry, 1981a). The \textit{ino2} and \textit{ino4} mutants fail to express the \textit{INO1} gene product and therefore require inositol for growth (Donahue and Henry, 1981b). Mutations in the \textit{INO2} and \textit{INO4} genes have pleiotropic effects on both the PI and PC branches of the phospholipid biosynthetic pathways (Loewey and Henry, 1984), consistent with their role as transcriptional activators.

Cloning of the \textit{INO2} and \textit{INO4} genes (Klig \textit{et al.}, 1988; Nikoloff \textit{et al.}, 1992) made it possible to speculate about the structure:function relation of these two proteins. For example, the predicted protein sequence of the Ino2p and Ino4p showed a high degree of similarity to members of the \textit{myc} family of basic helix-loop-helix proteins (Hoshizaki \textit{et al.}, 1990; Nikoloff \textit{et al.}, 1992). This observation was striking since the products of the \textit{INO2} and \textit{INO4} genes are known to form a heterodimer (Ambroziak and Henry, 1994) and the HLH domain was recognized as a protein dimerization
motif (Murre et al., 1989). Initial studies using mobility shift assays identified protein:DNA complexes assembled with the INO1 promoter which were dependent on wild type alleles of both the INO2 and INO4 genes (Lopes and Henry, 1991). Experiments using antibodies specific to Ino2p have shown that the INO2 gene product is present in this protein:DNA complex (Nikoloff and Henry, 1994). These same protein:DNA complexes were found to form using in vitro transcribed and translated Ino2p and Ino4p (Ambroziak and Henry, 1994). Finally, use of antibodies generated against an Ino2 fusion protein showed that the Ino4p co-immunoprecipitates with Ino2p in the absence of the UAS_{INO} element, indicating that the Ino2p/Ino4p heterodimer can form independently of the UAS_{INO} element (Ambroziak and Henry, 1994). Therefore, derepression of the phospholipid biosynthetic genes requires wild type alleles of the INO2 and INO4 genes. The Ino2p and Ino4p form a heterodimer which binds specifically to the UAS_{INO} element in the promoters of all of the coregulated phospholipid biosynthetic genes to activate their transcription in the absence of inositol and choline.

In addition to the positively acting products of the INO2 and INO4 genes, phospholipid biosynthetic gene expression is also regulated by a negatively acting protein, the product of the OPI1 gene (Greenberg et al., 1982b). The opil mutants excrete inositol, presumably due to the overproduction of the INO1 gene product (I1P synthase) (Greenberg et al., 1982a; Klig et al., 1985). The phenotype of the opil mutant also shows constitutive overexpression of the product of the CHO1 gene (PSS) (Klig et al., 1985). The constitutive overproduction of the I1P synthase and PSS subunits in the opil
mutant results from a defect in transcriptional regulation of the INO1 and CHOL genes, respectively (Hirsch and Henry, 1986; Bailis et al., 1987). Northern blot analyses revealed that the INO1 and CHOL transcripts are constitutively overexpressed in an opil mutant and more recently, the CH02 and OPI3 transcripts have also been observed to be constitutively overexpressed (Jackson and Lopes, unpublished results).

The exact mechanism by which the Opilp functions to regulate phospholipid biosynthetic gene expression is not yet known. However, it does seem likely that Opilp plays a key role in the response to inositol. One aspect of the function of Opilp that is clear is that it regulates phospholipid biosynthesis through the UASINO element. Strains harboring an opil mutant allele constitutively overexpressed a CYC1-lacI'Z heterologous reporter gene under control of the UASINO element (Koipally et al., submitted; Bachhawat et al., in press). This data provides evidence that Opilp functions through the UASINO element and not some other cis-acting element (such as an upstream repressing sequence element), however it does not provide evidence for a direct interaction between Opilp and the UASINO element.

The expression of some yeast genes has been shown to be controlled by repressors that specifically interact with promoter sequences called upstream repressor sequences (URS). Several systems have been shown to be under control of a specific URS element, URS1 with the consensus sequence 5'AGCCGCGCA3' (Sternberg et al., 1987; Buckingham et al., 1990; Luche et al., 1990). Genes under control of the URS1 element include the HO gene
(Sternberg et al., 1987), CAR1 (Luche et al., 1990), and SPO13 (Buckingham et al., 1990). The function of the URS1 element is not likely to be limited to these 3 genes since a substantial number of yeast promoters have sequence elements that resemble the URS1 (Luche et al., 1990), among these is the INO1 promoter (Lopes et al., 1993).

The ability of the URS1NO1 to repress gene transcription is dependent on the products of the SIN3 and UME6 genes (Hudak et al., 1994; Jackson and Lopes, unpublished results). Expression of some genes that have URS1 elements in their promoters are sensitive to mutations in either the SIN3 gene (Vidal et al., 1991; Strich et al., 1994), the UME6 gene (Park et al., 1992), or both (Strich et al., 1994). Consequently, it appears that these repressor proteins are able to interact in different combinations to mediate repression of gene expression.

The role(s) of the SIN3 and UME6 repressor genes on expression of phospholipid biosynthetic genes has recently been examined. Mutations in either gene confer pleiotropical phenotypes on expression of the phospholipid biosynthetic genes by elevating their expression under both repressing and derepressing conditions without affecting the level of regulation (Hudak et al., 1994; Jackson and Lopes, unpublished results). It was surprising that expression of all of the phospholipid biosynthetic genes tested (INO1, CHO1, OPI3, CHO2) were increased since only the INO1 promoter has a recognizable URS1 element (Hudak et al., 1994). These experiments further indicate that Sin3p and Ume6p mediate general repression of phospholipid biosynthetic gene expression, but are not involved in
the inositol/choline response since regulation was unaffected in the
*sin3* and *ume6* mutant strains. Further experiments demonstrated
that the *ume6* and *sin3* mutant alleles affect expression from both
the URS\(^1\)NO\(^1\) element as well as through the UAS\(_{\text{INO}}\) element (Hudak
and Henry, in press; Jackson and Lopes, unpublished results). These
results explain the pleiotropic effect on expression of phospholipid
biosynthetic genes that lack the URS\(_1\) element, but include the
UAS\(_{\text{INO}}\) element. At present it is unclear how these two repressors
function through the UAS\(_{\text{INO}}\) element, however, it seems reasonable
that they would exert the greatest effect on *INO1* expression since
the *INO1* promoter includes both the UAS\(_{\text{INO}}\) elements and the URS\(_1\) element.

Therefore, the regulation of phospholipid biosynthetic gene
expression is a complex regulatory pathway that involves two
activator proteins, the products of the *INO2* and *INO4* genes; a
specific repressor, the product of the *OP11* gene; and two general
repressors, the products of the *SIN3* and *UME6* genes. Understanding
how all of these regulatory proteins function and are regulated is
essential to understanding how the phospholipid biosynthetic
pathway is regulated.
CHAPTER II

REVIEW OF THE RELATED LITERATURE

The proper function of any cell is dependent on the genetically programmed synthesis of a large number of proteins expressed at precise levels. Control of transcription initiation by DNA-binding proteins which recognize sequence elements in gene promoters is a primary means of positively and negatively regulating expression (Morimoto, 1992; Pabo and Sauer, 1992; Buratowski, 1994; Tjian and Maniatis, 1994). The regulation of gene expression in the yeast *Saccharomyces cerevisiae* has been extensively documented (for review see: Guarente, 1992) and several well-defined systems have emerged as models for how the yeast cell responds to environmental signals by coordinately varying gene transcription (Johnston, 1987; Hinnebusch, 1992; Herskowitz, 1992). These model systems have identified specific interactions between *cis*-acting upstream activation sequences (UAS) (Guarente, 1992) 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. That is, how are the regulators themselves regulated? Generally the mechanisms for regulating the regulators fall into two broad categories: 1) Regulation of the amount of functional activator or 2) Regulation of the function of an activator by a repressor
protein, either directly or indirectly. In this section, I will briefly review several metabolic pathways which utilize different means of regulating the cognate activator protein that all fall into these two broad categories.

**Regulation of gene expression by controlling activator protein levels**

There are several means of controlling the amount of a functional activator (category 1). Regulatory mechanisms include those that act at the level of: 1) transcription (the *GAL4* gene in the galactose utilization pathway) (Griggs and Johnston, 1991, 1993); 2) the rate of translation (the *G C N4* activator in the amino acid biosynthetic pathway) (Hinnebusch, 1984; Thereos et al., 1984; reviewed in Hinnebusch, 1994); 3) protein stability (the *G C N4* activator in the amino acid biosynthetic pathway) (Kornitzer et al., 1994); 4) mRNA stability (*MATα1* involved in the specification of cell type and *PPR1* involved in pyrimidine biosynthesis) (Caponigro et al., 1993; Pierrat et al., 1993); and 5) transport into the nucleus (*ACE2* involved in chitinase production and *SW15* involved in mating type switching) (Dohrmann et al., 1992; Nasmyth et al., 1990).

The preferred carbon source for *S. cerevisiae* is glucose. However, a number of alternative carbon sources including galactose, raffinose, maltose, and sucrose can be utilized in the absence of glucose. When yeast are grown on media containing glucose, the genes encoding proteins needed for growth on other carbon sources, such as the *GAL* genes for galactose utilization, are repressed even if those other carbon sources (e.g., galactose) are present in the media. This phenomenon is known as glucose repression and it occurs
primarily at the transcriptional level (Johnston and Carlson, 1992). The extent of glucose repression varies from gene to gene, with some of the \textit{GAL} genes being repressed at least 1000-fold by glucose (Torchia \textit{et al.}, 1984, Yocum and Johnston, 1984).

Glucose repression acts rapidly to reduce gene transcription. For all of the genes which have been tested so far, glucose causes a reduction in the amount of mRNA in the cell (St. John and Davis, 1981; Carlson and Botstein, 1982; Denis \textit{et al.}, 1983; Sedivy and Fraenkel, 1985; Mueller and Getz, 1986), almost certainly due to a reduction in the rate of transcription initiation (Zitomer \textit{et al.}, 1979; Beier and Young, 1982; Federoff \textit{et al.}, 1983; Guarente \textit{et al.}, 1984; Johnston and Davis, 1984; Szekely and Montgomery, 1984; West \textit{et al.}, 1984; Sarokin and Carlson, 1985; Maarse \textit{et al.}, 1988; Flick and Johnston, 1990). Although the specific signal(s) for glucose repression have not yet been identified, three different mechanisms are known to contribute to glucose repression (Johnston and Carlson, 1992). One of these three mechanisms is the inhibition of the \textit{GAL4} transcriptional activator protein.

The Gal4p is a transcriptional activator protein which binds to a specific sequence, \textit{UAS}_{\text{GAL}} , found in the promoters of most of the genes required for galactose utilization (Johnston and Carlson, 1992). The function of Gal4p is almost completely inhibited in cells grown in glucose (Giniger \textit{et al.}, 1985; Flick and Johnston, 1990), and under these growth conditions, Gal4p-binding sites are known to be unoccupied (Giniger \textit{et al.}, 1985; Lohr and Hopper, 1985; Selleck and Majors, 1987). The inhibition of Gal4p function does not require the function of the product of the negative regulatory gene, \textit{GAL80}. 
(Yocum and Johnston, 1984; Torchia et al., 1984) and is due in large part because expression of the \textit{GAL4} gene is repressed about fourfold in the presence of glucose (Griggs and Johnston, 1991; Nehlin et al., 1991). The repression of \textit{GAL4} expression is mediated by the products of the \textit{MIG1}, \textit{SSN6}, and \textit{TUP1} genes through two Mig1p-binding sites in the \textit{GAL4} promoter found between the TATA box and the transcription start site (Griggs and Johnston, 1991; Nehlin et al., 1991). It is thought that the glucose repression of \textit{GAL4} expression by Mig1p, Ssn6p, and Tup1p occurs via the association of a Ssn6p-Tup1p complex with the DNA-binding protein, Mig1 (Keleher et al., 1992). Because the Gal4p is present at very low levels even in galactose-grown cells (Chasman and Kornberg, 1990; Parthun and Jaehning, 1990), and because it binds to DNA cooperatively (Giniger and Ptashne, 1988), the modest reduction in its level in glucose-grown cells results in a significant reduction in its ability to activate transcription (Johnston and Carlson, 1992). Consequently, a 4- to 5-fold reduction in \textit{GAL4} expression leads to at least a 40-fold reduction in \textit{GAL1} expression and in combination with other regulatory mechanisms, leads to a 1000-fold reduction in \textit{GAL1} expression (Griggs and Johnston, 1991).

A second mechanism for regulating the amount of functional activator protein is utilized in the pathway for general amino acid biosynthesis. The \textit{GCN4} gene encodes a positive transcriptional regulatory protein which acts directly to stimulate expression of at least 35 structural genes in 12 different amino acid biosynthetic pathways (Hinnebusch, 1992). The Gcn4p is a DNA-binding protein which interacts specifically with regulatory sequences found
upstream of its target genes (Hope and Struhl, 1985; Arndt and Fink, 1986).

Expression of the Gcn4p is regulated at the translational level through a mechanism involving four short open reading frames (uORF) in the leader of GCN4 mRNA (Hinnebusch, 1992). Translation of the GCN4 coding sequences appears to be regulated by scanning of the 40S ribosomal subunit through the four uORFs in the GCN4 leader. According to this model, 40S ribosomal subunits bind to the 5' end of the mRNA and scan in the 3' direction until the first AUG codon is encountered, where translation usually begins (Hinnebusch, 1992). Thus, when an uORF is present in the mRNA, translation occurs preferentially at that site and precludes initiation at downstream sites because reinitiation is inefficient in eukaryotes (Kozak, 1989).

The uORFs in the GCN4 leader are essential for regulation of its expression since a deletion of all four uORFs (Hinnebusch, 1984; Thireos et al., 1984) or point mutations in their four AUG start codons (Mueller and Hinnebusch, 1986) results in high constitutive expression of GCN4 independent of the factors that normally regulate GCN4 expression. Based on this, a model for the molecular mechanism of GCN4 translational regulation has been proposed. In the absence of amino acid starvation, scanning ribosomes initiate at uORF1 (the most 5' uORF), terminate translation at the uORF1 stop codon, and about 50% of the 40S subunits remain associated with the mRNA and resume scanning downstream (Hinnebusch, 1992). Most of these subunits re-acquire the ternary complex required to reinitiate translation before reaching the start codon of uORF2, 3, or 4
and therefore are able to reinitiate translation at one of these uORFs (Hinnebusch, 1992). Most of the ribosomes dissociate from the mRNA following translation termination at uORF2, 3, or 4, and thus essentially none reach the GCN4 start codon (Hinnebusch, 1992; Dever et al., 1992).

Under amino acid starvation conditions (derepressing conditions), uncharged tRNA accumulates in the cell. This stimulates a protein kinase which phosphorylates several translation initiation factors, resulting in a decrease in the amount of active translation factors available for formation of a translationally competent complex (Hinnebusch, 1992). Because of the diminished amounts of competent complexes present under starvation conditions, about 50% of the 40S subunits scanning downstream after terminating translation at uORF1 fail to rebind the translation factors necessary to initiate translation before reaching uORF4 and thus ignore the start codons at uORF2, 3, and 4 (Abastado et al., 1991). Most of these subunits are able to bind the appropriate translation factors before they reach the GCN4 AUG codon and thus are able to reinitiate translation there (Abastado et al., 1991). Only a few percent of the ribosomes bypass uORFs2, 3, and 4 under nonstarvation conditions, leading to a very low level of Gcn4 expression. Under starvation conditions, 50% of the subunits ignore uORFs2, 3, and 4, and reinitiate translation at GCN4. This would account for the large derepression ratio observed for GCN4 expression in response to amino acid starvation (Dever et al., 1992).

Another mechanism utilized for controlling the amount of the Gcn4 activator protein is through regulation of the stability of the
protein itself. The Gcn4p has been shown to have a short half-life of about 5 min (Kornitzer et al., 1994) under non-amino acid starvation conditions. This instability is due to the presence of 'PEST' sequences adjacent the activation domain in the Gcn4p (Kornitzer et al., 1994). 'PEST' sequences are stretches of a protein rich in the amino acids proline, aspartic acid, glutamic acid, serine, and threonine. These regions are thought to be a general signal for degradation through the ubiquitin degradation pathway based on the fact that they are over-represented in rapidly degraded proteins (Rogers et al., 1986; Rechsteiner, 1988). Under non-starvation conditions pulse-chase experiments show that the Gcn4p disappears within 15 min after the chase (Kornitzer et al., 1994). Under amino acid starvation conditions, nearly 100% of the protein remains 15 min after the chase (Kornitzer et al., 1994).

A forth mechanism for controlling expression of activator proteins is by regulation of the decay rate of their messages. For example, the message for the MATα1 activator gene, which is involved in regulating expression of genes involved in specification of cell type, has a half-life of 5 min (Caponigro et al., 1993). The message for the PPR1 regulatory gene, which controls transcription of two genes involved in pyrimidine biosynthesis, has a half-life of 1 min (Pierrat et al., 1993). The stabilities of both of these messages are well under the average of 17 min for poly(A+) mRNA in S. cerevisiae (Hynes and Philipps, 1976).

Since transcription takes place in the nucleus of the cell, another mechanism for regulating the amount of a functional activator is by regulating its transit into the nucleus. This
mechanism has been described for the Swi5 activator protein (Nasmyth et al., 1990) which regulates expression of the $H O$ endonuclease gene, involved in mating type interconversion. It has also been described for the Ace2 activator protein. This activator protein regulates expression of the $CTS1$ gene (encoding chitinase) (Dohrmann et al., 1992), which is required for cell separation after division. The pattern of expression of the $SWI5$ and $ACE2$ genes are very similar in that they are both transcribed during the G2 phase of the cell cycle but their protein products remain cytoplasmic until late in mitosis (Nasmyth et al., 1990; Dohrmann et al., 1992). Therefore, expression of the target genes ($HO$ for $SWI5$ and $CTS1$ for $ACE2$) is regulated by regulating the transit of the activator proteins into the nucleus.

**Regulation of gene expression by repressor proteins**

The second category involves the regulation of transcriptional activators by transcriptional repressor proteins. Repressor proteins can function in several different ways. For example, a repressor(s) can directly interact with an activator protein to inhibit its function (Gal80 repressor binding to the Gal4 activator and the Pho80/Pho85 complex interacting with the Pho4 activator in the galactose and phosphate utilization pathways, respectively). Repressor proteins play a role in regulating the genes involved in mating type specification by directly binding DNA (reviewed in: Herskowitz et al., 1992). Repressor proteins also can compete for binding DNA with an activator protein (Buf1p, Buf2p, Buf3p and Ume6p binding to the $URS1$ [upstream repressor sequence] element and an activator
protein binding to the adjacent UAS₁ element in the promoter of the \textit{CAR1} gene, in the nitrogen utilization pathway). Repression of transcription can also be mediated by the strategic positioning of nucleosomes over UAS elements to prevent activator proteins from binding their cognate UAS elements (e.g., the \textit{PHO5} promoter) (Almer and Horz, 1986; Almer \textit{et al.}, 1986).

When yeast are grown in media lacking galactose, the genes required for galactose utilization are repressed. This repression is mediated in part by the product of the \textit{GAL80} negative regulatory gene which functions by specifically binding to a region at the carboxyl terminus of the Gal4 activator protein (Johnston and Carlson, 1992), covering the transcriptional activation domain of Gal4p, and preventing it from making contact with the transcriptional apparatus. In galactose-grown cells, all of the Gal4p appears to be associated with Gal80p (Chasman and Kornberg, 1990; Parthun and Jaening, 1990; Leuther and Johnston, 1992; Parthun and Jaening, 1992), suggesting that dissociation of Gal80p is not necessary for Gal4p to activate transcription. Therefore, in the uninduced state (the absence of galactose), Gal4p is present at its DNA binding site with bound Gal80p preventing the function of the activation domain. In the presence of galactose, an as yet unidentified inducer interacts with an inducer-binding domain within the Gal80p (Nogi and Fukasawa, 1989), eliciting a transformation of the Gal4p-Gal80p complex which exposes the Gal4p activation domains (Leuther and Johnston, 1992). The association of Gal80p with Gal4p under inducing conditions suggests that Gal80p may play a slightly repressive role under these conditions. This is
supported by the observation that a deletion of the GAL80 gene leads to higher induced expression of Gal4p target genes (Torchia et al., 1984; Yocum and Johnston, 1984).

A second system which utilizes a direct interaction between activator and repressor proteins is the phosphate utilization pathway. In yeast the preferred source of phosphate is inorganic phosphate (P_i). In the absence of P_i, yeast can obtain phosphate from a variety of organic compounds through the cleavage of phosphoester bonds, catalyzed by phosphatases (Johnston and Carlson, 1992). The presence of P_i causes the genes required for the use of alternative sources of phosphate to be repressed (Johnston and Carlson, 1992). There are five known genes encoding phosphatases which are repressed by the presence of P_i, with the PHO5 gene being more highly expressed and more severely repressed by P_i than the others (Kramer and Andersen, 1980; Thill et al., 1983; Bajwa et al., 1984).

The regulation of PHO gene expression by P_i is accomplished by several positive and negative regulatory proteins. The product of the PHO4 gene encodes the major transcriptional activator protein in the pathway and is required for PHO gene expression (Vogel et al., 1989; Ogawa and Oshima, 1990). The PHO80 and PHO85 genes encode negative regulators of the PHO genes since mutations in either of these genes cause constitutive expression of the PHO5 gene (Ueda et al., 1975; Kaneko et al., 1985). Evidence indicates that the Pho80p and Pho85p form a complex which interacts with Pho4p to repress its function (Kaffman et al., 1994). The actual mechanism by which Pho80p/Pho85p repress Pho4p function is not yet known. A direct
interaction between the Pho80p and two regions of the Pho4p has been demonstrated (Jayaraman et al., 1994), suggesting the mechanism may be a masking of the activator through direct interactions with the repressor complex. However, a more specific role for the Pho80p/Pho85p complex has been proposed based on the predicted sequence these two proteins. Sequence analysis of the predicted Pho85p showed that it has greater than 50% identity with the CDC28 cyclin-dependent protein kinase (Uesono et al., 1987; Toh-e et al., 1988). Furthermore, the predicted Pho80p showed homology to two yeast cyclins, Hsc26 (Ogas et al., 1991) and OrfD (Frohlich et al., 1991). Therefore, it is possible that Pho80p/Pho85p might inactivate Pho4p by phosphorylation. Indeed, it has been shown that phosphorylation of Pho4p by the Pho80p/Pho85p complex in the presence of P_i is sufficient to inhibit the Pho4p transcriptional activation function (Kaffman et al., 1994) resulting in repression of \textit{PHO5} expression. In the absence of P_i, Pho4p is under phosphorylated due to the inhibition of the Pho80p/Pho85p complex by the product of the \textit{PHO81} gene, thus allowing Pho4p to activate transcription (Schneider et al., 1994).

Haploid yeast may be of two mating types, \( a \) or \( \alpha \), depending on the gene present at the MAT locus. In \( \alpha \) cells two transcriptional regulatory proteins are expressed from the \textit{MAT} \( \alpha \) gene, Mat\( \alpha 1p \) and Mat\( \alpha 2p \). In \( a \) cells, the \textit{MAT} \( a \) gene produces a single protein, Mat\( a1p \). These three proteins are responsible for regulating the expression of \( \alpha \)-specific genes, \( a \)-specific genes, haploid-specific genes, and sporulation-specific genes. The Mat\( \alpha 1p \) is required for activating transcription of \( \alpha \)-specific genes, Mat\( \alpha 2p \) is a repressor of
transcription of α-specific genes, and in diploid cells, Mata1p-Mata2p is a repressor of haploid-specific genes (reviewed in: Herskowitz et al., 1992).

The Mata1p functions in conjunction with the non-cell-type-specific Mcmlp to activate transcription of α-specific genes (Bender and Sprague, 1987; Tan et al., 1988; Grayhack, 1992). These proteins bind to adjacent sites in the promoters of α-specific genes, and shown to mutually enhance each others binding to their specific sites (Bender and Sprague, 1987; Tan et al., 1988; Grayhack, 1992). Further, the Mcmlp is the true activator while Mata1p primarily assists in the binding of Mcmlp to DNA and directs the binding of Mcmlp to the promoters of α-specific genes (Bender and Sprague, 1987; Jarvis et al., 1988).

In contrast, Mata2p represses expression of α-specific genes in α cells in conjunction with the Mcmlp (Jarvis et al., 1989; Keleher et al., 1989). Mata2p is a DNA binding protein and the promoters of α-specific genes contain an Mcmlp-binding site flanked on both sides by Mata2p-binding sites (Keleher et al., 1989). Therefore, the proposed function for Mata2p-mediated repression is by binding to DNA adjacent to Mcmlp and masking the activation domain of Mcmlp.

In diploid cells, the Mata2p and Mata1p associate to repress expression of haploid-specific genes by binding to a specific element in the promoters of these genes (Miller et al., 1985; Siliciano and Tatchell, 1986; Goutte and Johnson, 1988). This repression may occur by two different mechanisms. The first is by preventing transcriptional activators from binding to DNA, thereby preventing
activation. The Ssn6 general repressor protein is required for repression by Mata1p-Mata2p (Mukai et al., 1991). Therefore, in the second mechanism, Mata1p-Mata2p may actively repress transcription by recruiting Ssn6p (and likely Tup1p) to the promoters of haploid-specific genes (Herskowitz et al., 1992).

The pathway regulating the utilization of nitrogen uses a complex of repressor proteins consisting of Ume6p, Buf1p, Buf2p, and Buf3p to regulated gene expression (Sumrada and Cooper, 1987; Luche et al., 1990). These proteins bind directly to a repressor element found in the promoter of genes in the nitrogen utilization pathway to repress their expression (Kovari et al., 1990; Luche et al., 1990; Luche et al., 1992; Luche et al., 1993). When the repressor proteins are bound to their cognate site, they compete with an activator protein for binding to its cognate site, and therefore inhibits transcriptional activation (Viljoen et al., 1992).

Under conditions of nitrogen starvation (derepressing conditions), a catabolic pathway is induced which allows yeast cells to degrade and utilize arginine as a source of nitrogen (Magasanik, 1993). The catabolism of arginine requires the CAR1 and CAR2 genes, encoding arginase and ornithine transaminase, respectively (Magasanik, 1993). When cells are grown in the presence of efficiently utilized nitrogen sources, expression of the CAR1 gene is repressed (Bossinger and Cooper, 1977). In the presence of arginine (and absence of other nitrogen sources), both the CAR1 and CAR2 genes are strongly derepressed (Messenguy et al., 1991; Messenguy and Dubois, 1993).
Control of expression of the \textit{CAR1} gene is dictated by the presence of three different UAS elements in its promoter designated UAS\textsubscript{C1}, UAS\textsubscript{C2}, and UAS\textsubscript{I} (Kovari \textit{et al.}, 1990) and one upstream repression sequence, URS\textsubscript{1} (Sumrada and Cooper, 1987; Luche \textit{et al.}, 1990). UAS\textsubscript{C1} and UAS\textsubscript{C2} mediate inducer-independent transcriptional activation (Kovari and Cooper, 1991; Kovari \textit{et al.}, 1992) and consist of multiple binding sites for the products of the \textit{ABF1} and \textit{RAP1} general transcription factor genes (Dorsman \textit{et al.}, 1988; Buchman and Kornberg, 1990; Kurtz and Shore, 1991). Under repressing conditions, the proteins which bind to the negatively acting URS\textsubscript{1} site overcome the inducer-independent transcription supported by the Abf1p and Rap1p bound at UAS\textsubscript{C1} and UAS\textsubscript{C2} (Kovari \textit{et al.}, 1990; Luche \textit{et al.}, 1990; Luche \textit{et al.}, 1992; Luche \textit{et al.}, 1993). When arginine is present, the third UAS, UAS\textsubscript{I}, whose operation is inducer dependent, is bound by its cognate activator (Viljoen \textit{et al.}, 1992). Under these conditions, the combined activation capability of the three UASs is able to overcome the negative regulation by the proteins bound at URS\textsubscript{1} (Viljoen \textit{et al.}, 1992), allowing for derepression of \textit{CAR1} expression. Therefore, in this system, it appears that there is a competition going on between the repressor complex and the activator. Under repressing conditions, the repressor complex is bound to DNA, preventing transcriptional activation, presumably by preventing binding of the transcriptional activator. Under derepressing conditions, the inducer, arginine, causes the repressor complex to be removed, allowing the activator to bind DNA and transcription to be induced.
Another means of repressing transcription that does not involve specific repressor proteins is by the strategic positioning of nucleosomes in the promoter, preventing binding of either the basal transcriptional machinery or activator proteins, and thereby preventing transcriptional activation. An example of a gene where this mechanism is used is in regulation of \textit{PHO5} expression (Almer and Horz, 1986; Almer \textit{et al.}, 1986). The chromatin structure at the promoter of the \textit{PHO5} gene undergoes a dramatic transition upon \textit{PHO5} activation (Almer and Horz, 1986; Almer \textit{et al.}, 1986). In the repressed state, the \textit{PHO5} promoter is organized in an array of positioned nucleosomes that is only interrupted by a short restriction enzyme hypersensitive site containing a major UAS (Almer and Horz, 1986). Upon activating the gene by starving the cells for phosphate, two nucleosomes upstream and two downstream of the hypersensitive site disappear, and the entire promoter becomes accessible for the transcriptional machinery (Almer \textit{et al.}, 1986). This nucleosome disruption has been shown to be independent of DNA replication (Schmid \textit{et al.}, 1992) and therefore must involve some factor(s) which are capable of disrupting the nucleosome structure to allow transcription factor access.

Further evidence for a role of nucleosomes in regulating gene expression come from experiments in which a yeast strain was constructed where the only histone H4 gene is controlled by a repressible promoter (Han \textit{et al.}, 1988; Durrin \textit{et al.}, 1992). Repression of histone H4 synthesis (which inhibits nucleosome formation) results in the activation of several yeast promoters, including the \textit{PHO5}, \textit{CUP1}, and \textit{HIS3} promoters (Han \textit{et al.}, 1988;
Durrin et al., 1992), under conditions which would otherwise be non-inducing. Furthermore, the activation observed when nucleosome assembly is inhibited does not require the UAS elements in the promoters of these genes (Han and Grunstein, 1988; Durrin et al., 1992). Therefore, it is apparent that nucleosomes play a role in repressing expression of a number of genes and this repression must somehow be overcome to allow activation of transcription.

All of the metabolic pathways described above utilize different mechanism for regulating their respective transcriptional activators and all fall into one of the two broad categories of regulation. However, it is unusual to find a system that invokes a mechanism from both categories in response to a single environmental cue. For example, in the galactose utilization pathway, both GAL4 gene expression and Gal4p function (by the Gal80 repressor protein) are regulated, but they are in response to different signals. That is, expression of the GAL4 gene is repressed when cells are grown in the presence of glucose, while Gal80p repression of Gal4p is in response to the presence or absence of galactose. In this report, the regulation of phospholipid biosynthetic gene expression in response to inositol is described. This metabolic pathway is unique compared to those described above because it superimposes both transcriptional regulation of the INO2 activator gene and the action of the OPI1 negative regulatory gene in response to a single environmental cue, inositol.
CHAPTER III
MATERIALS AND METHODS

Bacteria Strains and Growth Conditions

*Escherichia coli* DH5α cells [F−; endA1; hsdR17(rK−, mK+); supE44; thi-1; recA1; gyrA96; relA1; Δ(argF-lacZYA)U169; φ80lacZ; ΔM15 λ−] were cultured in LB medium (10% (w/v) Bacto-tryptone, 5% (w/v) yeast extract, 10% (w/v) NaCl) supplemented with 50 µg/ml Ampicillin for the propagation of plasmids. Indicator plates containing 50 µl 2% (w/v) X-gal (5-bromo-4-chloro-3-indoly1-β-D-galactoside) in dimethylformamide were used to detect recombinant colonies. All bacterial strains were grown at 37°C and stored at 4°C or frozen at -80°C. Bacterial transformations were performed by the CaCl2 method (Sambrook *et al.* 1989) using transformation competent DH5α cells (Gibco-BRL).

Yeast Strains and Growth Conditions

The genotypes and sources of *S. cerevisiae* strains used in this study are listed in Table 1. Strain BRS1001 (lab wild type strain) was originally called W303-1a (R. Rothstein). All yeast strains were maintained at 30°C on YEPD plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and stored at 4°C.
Table 1. *Saccharomyces cerevisiae* strains used in this study.

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<th>Strain designation</th>
<th>Genotype</th>
<th>Source</th>
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</tr>
<tr>
<td>BPA202</td>
<td>BRS1001 with gal4::INO2-2-cat::URA3</td>
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<tr>
<td>BPA203</td>
<td>BRS1001 with gal4::INO2-3-cat::URA3</td>
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<td>Source</td>
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<tr>
<td>BPA205</td>
<td>BRS1001 with gal4::INO2-5-cat::URA3</td>
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<td>BPA206</td>
<td>BRS1001 with gal4::INO2-6-cat::URA3</td>
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<td>BPA207</td>
<td>BRS1001 with gal4::INO2-7-cat::URA3</td>
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<tr>
<td>BPA211</td>
<td>BRS1001 with gal4::INO2-7/3-cat::URA3</td>
<td>this study</td>
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<tr>
<td>BPA212</td>
<td>BRS1001 with gal4::INO2-8/2-cat::URA3</td>
<td>this study</td>
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<td>BRS2001</td>
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<td>BRS2011</td>
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<td>this study</td>
</tr>
</tbody>
</table>
**Yeast Transformations**

Yeast transformations were performed using the one step transformation of yeast protocol (Chen *et al.*, 1992). The strain to be transformed was grown overnight at 30°C in YEPD. One ml of cells (about 2.5 X 10^8 cells) was transferred to a sterile microfuge tube and pelleted by centrifugation for 5 min at 7500 rpm. The pellet was resuspended in 100 µl of freshly made one step buffer (0.2 N lithium acetate, 40% PEG 3350, 100 mM DTT) and 50 ng-1mg of DNA and 5 µl of 10 mg/ml salmon sperm DNA was added. The tubes were mixed by vortexing and incubated at 45°C for 1 hr. Cells were then spread on selective plates and incubated at 30°C for 3-4 days.

**Plasmid Isolation**

Large scale isolation of plasmid DNA from *E. coli* was performed by the alkaline lysis method. A 40 ml overnight culture grown in LB media was pelleted at 5000 rpm. The cell pellet was washed once in 10 ml of saline solution (100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 8.0), resuspended in 2.4 ml of freshly prepared lysozyme buffer (25 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM sucrose, 2 mg/ml lysozyme) and incubated on ice for 20 min. A solution of 0.2 M NaOH, 1% SDS (4.8 ml) was then added. The tubes were mixed gently by inverting and incubated on ice for 10 min. Three ml of NaOAc, pH 4.6 was added and the tubes were mixed by inverting and incubated on ice for 20 min. Cellular debris was pelleted by centrifugation at 15,000 rpm for 15 min. The supernatant was collected and treated with 5 µl of 10 mg/ml RNase A for 15 min at 37°C. The samples
were extracted once with 10 ml of phenol:chloroform (1:1) and the plasmid DNA was precipitated by adding 2 volumes of ethanol and centrifuging at 10,000 rpm for 15 min. The pellet was resuspended in 525 µl of sdH2O and transferred to a microfuge tube. The plasmid DNA was reprecipitated by adding 100 µl of 5 M NaCl and 625 µl of 13% PEG (6000-8000). The samples were incubated overnight at -20 C and plasmid DNA was pelleted by centrifugation at 15,000 rpm for 15 min. The pellet was resuspended in 150 µl of sdH2O. DNA concentration was determined by measuring the optical density of the solution at 260 nm (1 O.D. = 50 µg/ml).

Plasmid Construction

A. Plasmids used to assay the first position of UASINO

Plasmids used to assay the first position were all derived from pJH304 (Lopes et al., 1991). This is an autonomously replicating plasmid which contains the CYC1 basal promoter and the CYC1-lacI'Z reporter gene but lacks the UASCYC elements. The constructs were generated by first annealing complementary synthetic oligonucleotides to each other (SH1/SH2; SH3/SH4; SH5/SH6; JML9/JML10; Table 2). Each oligonucleotide contained the 9 bp core sequence, 5'ATGTGAAAT3' (Hirsch, 1987, Lopes et al., 1991). The tenth base at the 5' end (i.e., first position of the 10 bp UASINO) was varied to produce all four possible configurations (i.e., G, A, T, or C). The annealed oligonucleotides were then inserted into a XhoI site in pJH304.
Table 2. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Designation</th>
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<tr>
<td>SH1</td>
<td>5'-TCG AGA ATG TGA AAT C-3'</td>
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<tr>
<td>SH2</td>
<td>5'-TCG AGA TTT CAC ATG C-3'</td>
</tr>
<tr>
<td>SH3</td>
<td>5'-TCA AGG ATG TGA AAT C-3'</td>
</tr>
<tr>
<td>SH4</td>
<td>5'-TCG AGA TTT CAC ATC C-3'</td>
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<td>SH5</td>
<td>5'-TCG ACT ATG TGA AAT C-3'</td>
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<td>JML10</td>
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<td>IN02-Bgl</td>
<td>5'-GGG GAG ATC TGG ATC TGA GTT ACT T-3'</td>
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<tr>
<td>IN02-Bam</td>
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<td>IN04-Bgl</td>
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<tr>
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<td>5'-GGG GGG ATC CTA TTG CTT TTC TCT T-3'</td>
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<tr>
<td>IN02-3'</td>
<td>5'-GAT CAT TGC ACC GTI-3'</td>
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<tr>
<td>IN02-Sph</td>
<td>5'-GCA TGC ATG CAA CAA GCA ACT-3'</td>
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<td>5'-CCC GGG GTA ATT GTI-3'</td>
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<td>IN02(586-600)</td>
<td>5'-ACG AGT CGT CAT TGA-3'</td>
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<td>IN02(537-552)</td>
<td>5'-CCA TAG GAT CTA GAC C-3'</td>
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Table 3. Plasmids used in this study.

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</tr>
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<td>pBMINO4</td>
<td>INO4 promoter-cat fusion</td>
<td>this study</td>
</tr>
<tr>
<td>pBMINO2-1</td>
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<td>INO2 promoter deleted from -300 to -500-cat fusion</td>
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<td>pBM2289</td>
<td>GAL1 promoter-cat fusion</td>
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<td>pBMGAL1-INO2</td>
<td>GAL1 promoter fused to INO2 coding sequences. Derived from pBM2289</td>
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<td>pAS103</td>
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<td>pGEM-INO4</td>
<td>pGEM1 derivative with INO4 gene</td>
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</table>
A. Construction of CAT reporter fusion vectors.

All CAT reporter fusion plasmids were derived from pBM2015 supplied by M. Johnston (Washington University, St. Louis, Mo.)(Griggs and Johnston, 1993). This plasmid contained the chloramphenicol acetyltransferase (CAT) reporter gene (*cat*) fused to the first eleven codons of the *GAL4* gene, the *URA3* yeast selectable marker, and 1.5 to 2.0 kb of DNA from the region surrounding the *GAL4* chromosomal locus. Fusions of the *INO2, INO4, OPI1*, and *INO1* promoters to the *cat* reporter were constructed by amplifying the respective promoters by PCR. PCR primers were designed such that the 5' primer had a *BgIII* site at its 5' end and the 3' primer had a *BamHI* site at its 5' end. The primers used to amplify the promoters are as follows: *INO2*-Bgl and *INO2*-Bam (*INO2*); *INO4*-Bgl and *INO4*-Bam (*INO4*); *OPI1*-Bgl and *OPI1*-Bam (*OPI1*); and *INO1*-Bgl and *INO1*-Bam (*INO1*)(Table 2). PCR products were cloned into the pGEM®-T vector (Promega). A *BgIII*/*BamHI* restriction fragment containing the promoter of interest was then cloned into a *BamHI* site in pBM2015 to create: pBMINO2 (*INO2* promoter-*cat* fusion), pBMINO4 (*INO4* promoter-*cat* fusion), pMOPI1 (*OPI1* promoter-*cat* fusion), and pBMINO1 (*INO1* promoter-*cat* fusion)(Table 3). Proper orientation of the inserted promoter was determined by digesting the plasmid with *BamHI* and *SstII*.

B. Fusion of *INO2* coding sequences to the *GAL1* promoter.

*GAL1*-INO2 fusions plasmids were derived from pBM2289 provided by M. Johnston (Washington University, St. Louis, Mo.)(Griggs and Johnston, 1993). This plasmid contains the wild type *GAL1* promoter, upstream of an *SphI* restriction site, and the *URA3*
selectable marker. The INO2 gene was amplified by PCR using a 5' primer (INO2-Sph; Table 2) which included the translational initiator codon for the INO2 gene flanked by an SphI restriction site for subcloning purposes. The 3' PCR primer (INO2-3'; Table 2) was targeted to sequences downstream of the translational stop codon for the INO2 gene. This was done to insure that sequences important for RNA 3' end maturation were included. The INO2 PCR product was cloned into the pGEM®-T vector (Promega) to create pGEM-IN02. An SphI restriction fragment containing the IN02 coding sequence was cloned into an SphI restriction site in pBM2289 creating pGAL1-IN02 (Table 3). Proper orientation of the inserted fragment was confirmed by digesting pGAL1-IN02 with BamHI and BglIII. The pGAL1-IN02 construct places the IN02 coding DNA immediately downstream of the wild type GAL1 promoter.

Generation of Yeast Strains

A. Yeast strains used for CAT assays.

Yeast strains used for CAT assays were generated by transforming yeast strains (BRS1001, α1a, Nul 20, or BRS1021) with a 7.6 kb SstI/KpnI restriction fragment. These fragments contained the promoter-cat fusion gene and the URA3 selectable marker flanked by 1-2 kb of sequences from the GAL4 chromosomal region (Figure 2). Since the ends of DNA fragments are highly recombinogenic (Orr-Weaver et al., 1981), Ura+ transformants arise by recombination between sequences flanking GAL4. Southern blot
Figure 2. Integration of INO2- and INO4-cat fusions in single copy at the GAL4 locus of *S. cerevisiae*. DNA fragments (approximately 500 bp) containing the INO2 or the INO4 promoter flanked by BamHI-BglII sites were inserted into the BamHI site of pBM2015 (Griggs and Johnston, 1993). A 7.6-kb SstI-KpnI restriction fragment was used to transform a uracil auxotrophic yeast strain, yielding stable single-copy integrants. Shown for reference are sequences that include the GAL4 locus (hatched box), the direction of transcription (arrows), and the locations of the URA3 and cat genes.
Figure 3. Integration of GAL1-INO2 fusion at the URA3 locus. The plasmid pBMGAL1-INO2 was linearized with StuI and used to transform a uracil auxotrophic strain (α1a) to yield stable integrants.
analysis confirmed proper integration of the fusions in all transformants tested.

B. Strains harboring GAL1-INO2 fusion gene.

Yeast strains harboring the GAL1 promoter-INO2 fusion genes were constructed by transforming an ino2 deletion mutant strain (α1a) with the plasmid pBMGAL1-INO2 linearized within the URA3 selectable marker gene using StuI. The linearized plasmid integrates at the ura3 locus, giving rise to Ura+ transformants (Figure 3). Proper integration was confirmed by Southern blot analysis.

Southern Blot Analysis

A. Preparation of yeast genomic DNA

Isolation of yeast genomic DNA was performed as described by Hoffman and Winston (1987). Cells from a 10 ml saturated culture were collected by centrifugation. The cell pellet was rinsed with 0.5 ml of sdH2O, transferred to a microfuge tube and pelleted. The cell pellet was then resuspended in 0.2 ml of lysis buffer (2% Triton, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA) and 0.2 ml of phenol/chloroform/isoamyl alcohol (25:24:1). Glass beads (0.3 gm) were added to each tube and the tubes were vortexed for 4 min. 0.2 ml of 1X TE-8.0 was added to the mixture and the tubes were then centrifuged for 5 min. The aqueous supernatant was transferred to a new tube and precipitated by adding 2 volumes of ethanol and centrifuging for 5 min. The DNA pellet was resuspended in 0.4 ml 1X TE-8.0, treated with 30 µg of RNase A for 10 min at 37°C, and
precipitated as above. The pellet was washed with 100 µl of 70% ethanol, dried under vacuum, and resuspended in 50 µl of 1X TE-8.0.

B. Southern blot hybridization analysis

Ten µl of genomic DNA were digested overnight at 37°C with the appropriate restriction endonucleases and fractionated through a 0.8% (w/v) agarose gel in 1X TBE (89 mM Tris base, 89 mM boric acid, and 2mM EDTA (pH 8.0)). DNA transfer to Nytran Modified Nylon Membrane (0.45 µm) (Micron Separations Inc.) was performed by capillary action after subjecting the gel to three washes in 0.2 N HCl for 15 min each (depurination), three 15 min denaturation washes (1.5 M NaCl, 0.5 N NaOH), and three 15 min neutralization washes (1.5 M NaCl, 0.5 M Tris, pH 7.4). DNA transfer proceeded overnight in 10X SSC buffer (1X SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.0). After transfer, the blot was vacuum dried for two hours.

Nick-translated hybridization probes were synthesized from purified fragments containing relevant DNA sequences to a specific activity of greater than 1 X 10⁸ cpm/µg DNA with [α-³²P]-dCTP (Ci/µl) according to instructions specified in the Gibco-BRL Nick-Translation Kit. Prehybridization of filters was done at 42°C for 2-4 hrs in solution containing 50% formamide, 20% NaHPO₄ solution (5 M NaCl, 50 mM Na₂PO₄, 0.5% pyrophosphate), 5X Denhardt's solution (50X Denhardt's: 5 g Ficoll 400, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin in 500 ml sdH₂O), 0.5% SDS (v/v), and 0.1 mg/ml denatured salmon sperm DNA. Labelled probe that had been denatured at 100°C for 5 min was added directly to the prehybridization solution and filters were incubated at 42°C
overnight. The filters were then washed three times for 15 min each in 2X SSC, 0.1% SDS at 65°C. Filters were blotted dry and exposed to film overnight.

RNA Analysis by Northern and Slot Blot Hybridizations

A. Isolation of total cellular RNA

Total RNA was isolated from yeast strains by the glass bead disruption and hot phenol extraction method of Elion and Warner (1984). Twenty-five ml cultures were harvested at mid-log phase (between 60-80 Klett units). Cells were pelleted by centrifugation and washed once with ice cold sdH2O, repelleted and resuspended in 0.5 ml of LET-1% SDS (0.1 M LiCl, 10 mM EDTA, 0.01 M Tris-HCL pH 7.4, 1% SDS). This suspension was transferred to a sterile 15 ml disposable glass screw cap tube and placed at -80°C overnight. The next day, cell pellets were thawed on ice. The cellular contents were released by vortexing each sample with 0.5 ml volume of glass beads for 25 seconds. One hundred µl of phenol/chlorform were added to each sample. Each sample was then vortexed 4 x 25 sec, placing them on ice between each vortexing pulse. Two ml of LET containing 0.2% SDS were added to each sample. Two successive hot phenol extractions were then performed for each sample by transferring the mixture into a tube containing 2.5 ml of hot phenol placed in a water bath at 65°C. Each sample was vortexed for 5 sec, placed on ice for 4 min, and centrifuged to recover the aqueous layer each time. The last fraction was transferred to a 15 ml Corex tube. LiCl was added to a final concentration of 0.3 M and the RNA was precipitated by adding 2.5 volumes of ethanol and placing the samples at -20°C
overnight. The RNA was pelleted at 10,000 rpm for 30 min at 4°C and each pellet was dissolved in 0.4 ml of sdH2O. The RNA was then reprecipitated by adding 0.1 ml of 0.5 M NaCl and 1 ml of ethanol. The RNA was pelleted by centrifugation and resuspended in 200 µl of sdH2O. The concentration of RNA was determined by measuring the optical density at 260 nm (1 O.D. = 40 µg/ml).

B. Northern blot hybridization analysis

Ten µg of total RNA were dissolved in a 3X volume of sample buffer (50% formamide, 20 mM MOPS [3-(N-morpholino)propanesulfonic acid], 1 mM EDTA, 5 mM NaOAc, 2.2 M formaldehyde), heated to 65°C for 5 min to reduce secondary structure and fractionated on a 1.2% (w/v) agarose, 3% (v/v) formaldehyde, 20 mM MOPS, 1mM EDTA gel. The running buffer was 20 mM MOPS pH 7.4, 1 mM EDTA. RNA was transferred to Nytran modified nylon membrane (0.45 µm)(Micron Separations Inc.) by capillary blotting overnight in 20X SSC and the membrane was baked at 80°C under vacuum for 2 hrs.

Single-stranded RNA probes (riboprobes) were synthesized with the Riboprobe® Gemini II Core System (Promega) according to manufacturers suggestions with plasmids linearized with a restriction enzyme and transcribed with a RNA polymerase as follows (shown as plasmid, restriction enzyme, RNA polymerase for the indicated (parenthesized) probe: pAB309Δ, EcoRI, SP6 (TCM1)(Hudak et al., 1994); pJH310, HindIII, T7 (INO1)(Hudak et al., 1994); pAS103, HindIII, T7 (CHO1)(Hudak et al., 1994); pGEM-INO2, SalI, T7 (INO2)(Ashburner and Lopes, 1995b); pPLg, BamHI, SP6, (ACT1)(Ashburner and Lopes, 1995b); pGEM-INO4, SalI, T7, (INO4).
Synthesis of riboprobes was performed in a 30 µl total volume with 6 µl of 5X buffer (200 mM Tris-HCl [pH 7.9], 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl), 2 µl of 100 mM DTT, 20 units Rnasin® ribonuclease inhibitor, 6 µl of 2.5 mM NTPs (CTP, GTP, and ATP), 3.6 µl of 100 µM UTP, 0.5 µg of linearized template DNA, 5 µl of [α-32P]-UTP (10 mCi/ml), and 20 units of RNA polymerase (SP6 or T7). Reactions were carried out for 1 hr at 37°C. Reactions were stopped by adding 1.0 µl of 0.5 M EDTA. The riboprobes were precipitated by adding 1.0 µl of 0.5 M NaCl, 1.0 µl of 10 mg/ml yeast tRNA, and 80 µl of ethanol. The pellets were resuspended in 100 µl of sdH₂O. Filters (Northern or Slot blots) were prehybridized in solution containing 50% formamide, 0.25 M NaHPO₄, pH 7.4, 5X Denhardt's solution, 0.5% SDS. 0.1 mg/ml denatured salmon sperm DNA for 2-4 hrs at 55°C. Labelled riboprobes (50 µl) were added directly to the prehybridization solution and the filters were hybridized overnight at 55°C. The next day, filters were washed three times for 15 min each in 2X SSC, 0.1% SDS at 65°C. Filters were blotted dry and exposed to X-ray film.

C. Quantitative slot blot hybridization analysis

Slot blot analysis was performed using a slot blot apparatus from Gibco-BRL. Typically, 3 µg of total RNA was loaded into each well with 0.4 ml of 20X SSC. RNA was immobilized onto Nytran modified nylon membrane (0.45 mm)(Micron Separations Inc.) by applying vacuum to the slot blot apparatus to remove liquid from the wells. Each well was washed once with 0.4 ml of 20X SSC by applying vacuum. Filters were baked under vacuum for 2-4 hrs at 80°C. Prehybridization and hybridization were performed as
described for Northern blot filters. Quantitation was done using a densitometer. The constitutively expressed \textit{TCM1} or \textit{ACT1} gene transcripts were used to normalize for loading variations.

\textbf{CAT Assays}

\textbf{A. Preparation of yeast extracts}

Whole cell yeast extracts to be assayed for CAT activity were prepared by growing 5 ml cultures of cells to 50-60 Klett units. Cells were pelleted by centrifugation and washed in 0.5 ml of cold 0.25 M TRIS, pH 7.5. Cells were transferred to microfuge tubes and centrifuged for 5 min at full speed. The cell pellets were resuspended in 0.2 ml of cold 0.25 M TRIS, pH 7.5. Cells were broken open by adding 200 µl of 0.5 mm glass beads and shaking on a vortexer in the cold room for 8 20-sec rounds of shaking with 20-sec pauses between each round to allow the cells to cool. The extracts were then centrifuged for 5 min at 4°C to pellet the glass beads and cell debris. The supernatants were removed to new tubes and protein concentrations of each extract were determined using the Bio-Rad protein assay kit according to manufacturers specifications. Extracts were assayed immediately or stored at -80°C for up to one week.

\textbf{B. Phase-extraction assay for CAT activity}

CAT assays were performed using the phase-extraction method (Seed and Sheen, 1988). Typically, 10 µg of protein was assayed in a 100 µl reaction volume containing 1 µl of 25 mM n-butyryl coenzyme A (Sigma) and 1 µl of 14C-chloramphenicol (0.025 µCi/µl) (Amersham). The reactions were incubated at 37°C for 1 hr.
Reactions were stopped by adding 200 µl of a 2:1 mixture of tetramethylpentadecane:xylenes and vortexing. Samples were centrifuged for 5 min at room temperature. After centrifugation, 160 µl of the upper (organic) phase was mixed with 4 ml of liquid scintillation fluid (Bio-Safe II, Research Products International). Eighty µl of the lower phase from a few samples was dried on glass fiber filters (Fisher) and the filter was placed in 4 ml of liquid scintillation fluid. Samples were then counted using a Beckman LS 6500 scintillation counter and CAT activity in each sample was determined. Units of CAT activity are defined as counts per minute measured in the organic phase and expressed as a percentage of total counts per minute (percent conversion) divided by the amount of protein assayed (in micrograms) and time of incubation (in hours).

β-galactosidase Assays

β-galactosidase assays were performed essentially as described in Lopes et al. (1991). Five ml yeast cultures were grown overnight at 30°C in complete synthetic media that contained the appropriate supplements for each experiment (I-C- or I+C+), and lacked the uracil for plasmid maintenance. Part of the 5 ml overnight culture was used to inoculate 25 ml of fresh media to a Klett reading of 15-20. Cultures were allowed to grow at 30°C, and cells were harvested at a density range of 60-80 Klett units (mid-logarithmic stage). The cells were pelleted and suspended in 1 ml β-galactosidase buffer and frozen overnight at -80°C. To prepare cell extracts, frozen cells were thawed on ice and broken open by vortexing 6 X for 15 sec with 1 ml acid washed glass beads. The supernatant was transferred to a 1.5
ml microfuge tube, and cell debris was pelleted at 8,000 rpm for 15 min. The supernatant was collected, and used in β-galactosidase activity assays.

To assay β-galactosidase, 330 µl of cell extract was added to 1.32 ml β-gal assay buffer and incubated at 28°C for 5 min. The reaction was initiated by adding 660 µl of 4 mg/ml ONPG. Aliquots of 700 µl were removed after 5, 10, and 15 min and added to 500 µl 1 M Na₂CO₃ to stop the reaction. Optical densities of the final solutions were measured at 420 nm. The total protein concentration of each extract was determined using a Bio-Rad assay kit (Bradford, 1965). β-galactosidase units are defined as (O.D.₄₂₀/min/mg total protein) x 1000.

Primer Extension Analysis

The precise site of transcription initiation of the IN02 transcript was determined by primer extension analysis (Elion and Warner, 1984). An oligonucleotide (INO2(586-600);Table 2) was end labeled at its 5' end with [γ-³²P]-ATP. The reaction was carried out in a 20 µl volume containing 50 mM TRIS, pH 7.8, 10 mM MgCl₂, 5 mM DTT, 50 µCi [γ-³²P]-ATP (Amersham), 0.2 µg of DNA and 30 units of T4 polynucleotide kinase (Gibco-BRL). The reaction was incubated at 37°C for 30 min and was stopped by adding 2 ml of 0.5 M EDTA. The reaction was extracted once with an equal volume of phenol/chloroform (1:1) and precipitated by adding 5 µl of 3 M sodium acetate, 1 µl of 10 mg/ml yeast tRNA, and 2 volumes of ethanol. The labeled oligo was resuspended in 10 µl of 1X TE-pH 8.0. Primer extensions were carried out by ethanol precipitating RNA (10
µg/reaction), washing with 70% ethanol and resuspending in 7 µl of 1X TE-pH 8.0. 2 µl of primer extension buffer (10 mM TRIS pH 7.8, 1 mM EDTA, 0.25 M KCl) and 2 ng of labeled oligonucleotide were added to the reaction. Reactions were heated to 90°C for 5 min then incubated at 55°C for 6 hrs to anneal the oligo with the RNA. Following the annealing step, 25 µl of reverse transcription buffer (20 mM TRIS pH 8.0, 10 mM MgCl₂, 5 mM DTT, 0.4 mM dATP, 0.4 mM dGTP, 0.4 mM dTTP, 0.4 mM dCTP, 10 µg/µl actinomycin D) and 25 units of reverse transcriptase (Gibco-BRL) were added. The reaction was incubated at 42°C for 1 hr and then precipitated by adding 7.4 µl of 0.5 M NaCl and 2 volumes of ethanol. The primer extension products were resuspended in 4 µl of sterile distilled water and 4 µl of sequencing dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Products were fractionated on a sequencing gel along side a DNA sequencing ladder to determine the size of the products.

DNA Sequencing

Double-stranded DNA templates were sequenced by the dideoxy chain termination method of Sanger et al. (1977) using the Promega fmol™ sequencing system. One µg of purified plasmid DNA (pTAIN04) was mixed with 1.0 ng of primer (1NO4-Bam; Table 2). Five µl of fmol™ sequencing buffer and 1 µl of [α-35S]-dATP (Amersham) were added. Sterile distilled water was added to bring the final volume to 16 µl. One µl of sequencing grade Taq DNA polymerase (5 units/µl) was added to the primer/template mix. Four µl of the enzyme/primer/template mix was then added to each of
four tubes containing 2.5 µl of the appropriate d/ddNTP mix (d/ddGTP, d/ddATP, d/ddTTP, d/ddCTP). Twenty µl of mineral oil was added to each reaction tube. The reactions were then subjected to PCR amplification (1 cycle at 95°C for 2 min, followed by 30 cycles at 95°C for 30 sec (denaturation), 55°C for 30 sec (annealing), 72°C for 1 min (extension)). After the thermocycling program was completed, 3 µl of fmol™ Sequencing stop solution was added to each tube. Reactions were heated to 70°C for 2 min prior to loading on a sequencing gel. The reactions were fractionated on a 6% (w/v) polyacrylamide, 7 M urea wedge gel (0.25 mm- 0.75 mm) at a constant 90 watts. Sequencing gels were dried directly onto Whatmann 3M filter paper and exposed to X-ray film for 24-48 hours.

Derepression Assay

To determine the kinetics of derepression of the INO2 and INO1 genes, wild-type strains harboring either an INO2-cat reporter fusion or an INO1-cat reporter fusion were inoculated into 20 ml of medium supplemented with inositol and choline (repressing). Cultures were grown to mid-log phase (50-60 Klett units), and cells were collected by filtration onto a nitrocellulose filter with a Millipore filter apparatus. Cells were washed twice with prewarmed medium lacking inositol and choline and then resuspended in 25 ml of medium lacking inositol and choline (derepressing). Samples (5 ml) were taken at various times and assayed for CAT activity as described earlier.
CHAPTER IV
RESULTS

A 5' Cytosine or Adenosine residue is essential for maximal UAS\textsubscript{INO}-
driven gene expression

In order to test the effectiveness of the 10 bp consensus
sequence of the UAS\textsubscript{INO} element as a UAS, a set of oligonucleotides
was constructed (Table 2) and inserted into pJH304 (Table 3). This
plasmid contains the CYC1 basal promoter and the reporter gene
fusion CYC1-lacl'Z, but it lacks a functional UAS (Lopes \textit{et al.}, 1991).
Each oligonucleotide contained the 9 bp core sequence, 5'
ATGTGAAAT3' (Hirsch, 1987; Lopes \textit{et al.}, 1991). The tenth base at
the 5' end (\textit{i.e.}, the first position of the 10 bp UAS\textsubscript{INO}) was varied to
produce all four possible configurations. Previously, it had been
shown that the 9 bp core sequence was completely incapable of
serving as a UAS (Lopes \textit{et al.}, 1991). When a T or a G residue was
placed at the first position of the 10 bp UAS\textsubscript{INO}, to produce the
sequences, 5'TATGTGAAAT3' or 5'GATGTGAAAT3', the constructs
were found to be incapable of serving as a UAS in a wild type strain
(Table 4). However, positioning an adenosine in the first position
(5'ATGTGAAAT3') produced an element that was capable of serving
as a UAS. The construct with this element supported expression of
45 units of \textbeta-galactosidase activity under derepressing conditions
(\textit{i.e.}, in the absence of lipid precursors; I-C-; Table 4). Expression
Table 4. Effect of 5' nucleotide substitutions on UASINO activity

<table>
<thead>
<tr>
<th>Nucleotide 5' to the 9-mer b</th>
<th>Wild Type</th>
<th>opil</th>
</tr>
</thead>
<tbody>
<tr>
<td>I+C+ c</td>
<td>I-C- d</td>
<td>I+C+</td>
</tr>
<tr>
<td>9 mer c</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>C</td>
<td>8.4 ± 2.4</td>
<td>122.0 ± 21.8</td>
</tr>
<tr>
<td>A</td>
<td>&lt;1.0</td>
<td>45.0 ± 4.5</td>
</tr>
<tr>
<td>T</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>G</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

a unit = 1000 x (optical density @ 420nm/min/mg of total protein)
b The 9-mer has the sequence: 5'- ATGTGAAAT - 3'. The sequences referred to above were constructed with XhoI flanking sequences (ctcgag) and ligated into the XhoI site of the UAS-less vector, pJH304.
c I+C+, complete synthetic media supplemented with 75 µM inositol and 1 mM choline.
d I-C-, unsupplemented complete synthetic media.
e In this construct there is no nucleotide intervening between the XhoI flanking sequences and the 9-mer. The XhoI nucleotide 5' of the 9-mer is a G.
from the construct containing this synthetic element was completely repressed in the presence of inositol and choline (I+C+, repressing; Table 4). Positioning of a cytosine residue at the first position (5'CATGTGAAAT3') produced an element capable of supporting 122 units of β-galactosidase activity from the reporter gene under derepressing conditions (Table 4). This construct, unlike the construct containing an adenosine at the 5' end, was not completely repressed in the presence of the lipid precursors inositol and choline. The ratio of repressed to derepressed levels of expression was approximately 15-fold for the construct containing a C at the first position. Based on these results, the functional UASINO element is proposed to be a 10 bp element, 5' (C/A)ATGTGAAAT 3'.

These four variations of the UASINO element were also tested in a repressor-deficient (opiI) strain. In the opiI genetic background, those elements with a C or A at the first position supported a high constitutive level of β-galactosidase activity, consistent with the phenotype of an opiI mutant strain (Hirsh and Henry, 1986; Bailis et al., 1987). The T- and G-containing UASINO elements were inactive in the opiI background.

INO2-cat, but not INO4-cat, expression is regulated by inositol and choline

Computer-assisted searches of the INO2 and INO4 promoters revealed that they each contain a copy of the UASINO element (Figure 4). This led to speculation that expression of the INO2 and/or INO4 genes may be transcriptionally regulated in response to inositol and choline. Therefore, to determine if INO2 and/or INO4 expression
were regulated, plasmids were constructed that fused sequences upstream of the AUG translation start codons of the INO2 and INO4 genes to a GAL4-cat fusion reporter gene. A single copy of these fusions was integrated into the yeast genome without any associated vector sequences by homologous recombination at the GAL4 locus (see Figure 2) to create BPA101 and BPA102 (Table 1).

The data in Table 5 show that expression of the INO2-cat reporter gene was approximately 12-fold higher in the absence of inositol and choline than in their presence. In contrast, there was no difference in the level of expression of the INO4-cat fusion in the presence or absence of inositol and choline (Table 5). The level of expression of the INO4-cat construct was approximately six-fold higher than was the INO2-cat construct under derepressing conditions, suggesting that INO2 expression is limiting relative to that of INO4. As a control, a strain (BPA103) containing a promoterless cat construct was also analyzed and the results show that there was no cat expression from this vector (Table 5). Therefore, any expression observed in this system must originate from the inserted promoters.

Interestingly, the pattern of INO2-cat expression is reminiscent of the pattern of expression of one of its target genes, INO1 (Hirsch and Henry, 1986). This point was illustrated by analysis of INO1-cat expression (Table 5). The results agree with published data describing INO1 regulation (Hirsch and Henry, 1986) and show that although the degree of regulation differs for INO2-cat and INO1-cat (12-fold vs. 27-fold, respectively), the pattern of regulation is the same. In addition, the INO2-cat fusion was
Figure 4. \textit{INO2} and \textit{INO4} promoter sequences. Noted for reference are the initiation codons (MET), potential UAS\textit{INO} elements (arrows indicate orientations), a potential TATA box in the \textit{INO4} promoter (underlined), and the oligonucleotide primers (with flanking restriction sites) used for PCR amplification.
| Reporter gene | CAT activitya | | | |
|---------------|---------------|---------------|-------------|
| | I-C-c | I+C+d | Fold regulation |
| INO2-cat | 0.75 | 0.08 | 12.3 (2.8) |
| INO4-cat | 4.41 | 4.37 | 1.0 (0.1) |
| OPII-cat | 18.50 | 11.00 | 1.7 (0.2) |
| INO1-cat | 33.90 | 1.30 | 27.0 (9.6) |
| promoterless-cat | 0.01 | 0.01 | 1.1 (0.6) |

a Assays were carried out employing extracts from yeast transformants harboring each of the reporter genes. Each value represents the average of data from at least 4 experiments.
b Each reporter gene was integrated in single copy at the GALA locus of BRS1001 as described in the Materials and Methods.
c I-C-, complete synthetic media (17) lacking inositol and choline.
d I+C+, complete synthetic media (17) supplemented with 75 µM inositol and 1 mM choline.
e Average of the fold-regulation (I-C-/I+C+) for each experiment with standard deviation in parentheses.
expressed at a level approximately 45-fold less than was *INO1-cat*.

**INO2**: but not *INO4*, mRNA level is regulated in response to inositol

The levels of the native *INO2* and *INO4* transcripts were also examined by quantitative slot blot analysis. Previous attempts to identify the *INO2* transcript had been unsuccessful, presumably due to the weak level of expression from the *INO2* promoter. However, the use of high specific activity riboprobes allowed for detection of both the *INO2* and *INO4* transcripts by quantitative slot blot analysis. This data showed that the *INO2* transcript is regulated approximately 5-fold in response to inositol, while the *INO4* transcript was expressed constitutively (Table 6). These results are in agreement with those described above for *INO2-cat* and *INO4-cat* regulation.

**INO2-cat** expression requires wild-type alleles of the *INO2* and *INO4* genes

Because Ino2p may be able to bind to its own promoter through the UAS\(_{INO}\) element and since the *INO2-cat* fusion is regulated in response to inositol and choline, it is possible that *INO2* expression may be autoregulated. In order to determine if *INO2* expression is autoregulated, the 7.6 kb *KpnI/SstI* fragment containing the *INO2-cat* fusion was integrated at the *GAL4* locus in an *ino2* null strain (α1a, Table 1). The *ino2* mutant strain harboring the *INO2-cat* reporter fusion (BPA104) was then assayed for CAT activity under repressing conditions (75 μM inositol and 1mM...
TABLE 6. Comparison of native levels of *INO2* and *INO4* transcripts in a wild type strain.

<table>
<thead>
<tr>
<th></th>
<th>I+C+$^1$</th>
<th>I-C−$^1$</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>INO2/ACT1</em>$^2$</td>
<td>0.077</td>
<td>0.344</td>
<td>4.5</td>
</tr>
<tr>
<td><em>INO4/ACT1</em>$^2$</td>
<td>0.935</td>
<td>1.085</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^1$, I+C+, 75 µM inositol, 1 mM choline; I-C-, no inositol or choline

$^2$, levels of *INO2* and *INO4* transcripts normalized to *ACT1*
The data in Table 7 show that the \textit{INO2-cat} fusion was not expressed in the \textit{ino2} mutant strain. It should be noted that the expression of the \textit{INO2-cat} reporter at this concentration of inositol was reduced to a level equal to that of a promoterless \textit{cat} construct. Further, the repressed level of expression of \textit{INO2-cat} in the \textit{ino2} mutant strain was about four-fold lower than the repressed level in the wild-type strain. The \textit{ino2} mutant strain containing the \textit{INO2-cat} fusion was also grown in medium containing 10 \text{µM} inositol to test the effect of the \textit{ino2} mutation under derepressing conditions. This concentration of inositol is the minimal amount required for growth of the \textit{ino2} strain while simultaneously allowing partial derepression of phospholipid biosynthetic gene expression in a wild-type strain. While in the wild-type strain an intermediate level of expression was observed (Table 7), \textit{INO2-cat} was not expressed in the \textit{ino2} mutant strain at this concentration of inositol. These results suggest that a wild-type allele of the \textit{INO2} gene is required for \textit{INO2} expression, thus, the Ino2p does autoregulate its own expression. Since wild-type alleles of both \textit{INO2} and \textit{INO4} are required for expression of the genes encoding the phospholipid biosynthetic enzymes, then \textit{INO4} may also be required for \textit{INO2} expression. To determine this, the \textit{INO2-cat} reporter fusion was integrated at the \textit{GAL4} locus in an \textit{ino4} null genetic background (Nul 20, Table 1) to create BPA107. This strain was then assayed for CAT activity under repressing conditions (75 \text{µM} inositol and 1 \text{mM} choline) and in medium containing 10 \text{µM} inositol. The \textit{INO2-cat} reporter fusion was not expressed in this strain when grown under either condition (Table 7). Therefore, in addition to being
### TABLE 7. Autoregulation of *INO2* and *INO4* gene expression

<table>
<thead>
<tr>
<th>Reporter gene(^b)</th>
<th>Relevant strain</th>
<th>genotype(^c)</th>
<th>CAT activity(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{INO2-cat})</td>
<td>Wild type</td>
<td></td>
<td><strong>10 µM inositol</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1mM choline</td>
</tr>
<tr>
<td>(\text{INO2-cat})</td>
<td></td>
<td>(\text{ino2})</td>
<td>0.43</td>
</tr>
<tr>
<td>(\text{INO2-cat})</td>
<td></td>
<td>(\text{ino4})</td>
<td>0.02</td>
</tr>
<tr>
<td>(\text{INO4-cast})</td>
<td>Wild type</td>
<td></td>
<td>N.D.</td>
</tr>
<tr>
<td>(\text{INO4-cat})</td>
<td>(\text{ino2})</td>
<td></td>
<td>3.35</td>
</tr>
<tr>
<td>(\text{INO4-cat})</td>
<td>(\text{ino4})</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>(\text{promoterless-cat})</td>
<td>(\text{ino2})</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>(\text{promoterless-cat})</td>
<td>(\text{ino4})</td>
<td></td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^a\) Assays were carried out employing extracts from yeast transformants harboring each of the reporter genes. Each value represents the average of data from at least 3 experiments.

\(^b\) Each reporter gene was integrated in single copy at the *GALA* locus as described in the Materials and Methods.

\(^c\) The strains used in these experiments were BRS1001 (wild type), \(\alpha 1\)A (*ino2*), and NUL20 (*ino4*).

N.D., not determined
autoregulated, INO2 expression also requires a wild-type allele of the
INO4 gene.

INO4-cat expression requires a wild-type allele of INO4, but does not require INO2

Expression of the INO4-cat reporter fusion was found to be
constitutively expressed (Table 5). Therefore, although INO4 expression is not sensitive to regulation in response to inositol and choline, its promoter does contain a copy of the UASINO element, so its expression may still require wild-type copies of the INO2 and INO4 genes. In order to test this, the INO4-cat reporter fusion was integrated at the GAL4 locus in both an ino2 null (α1a) and ino4 null (Nul 20) genetic background to create BPA105 and BPA106 and assayed for CAT activity under repressing (75 µM inositol and 1 mM choline) conditions and in 10 µM inositol. In contrast to INO2-cat expression, INO4-cat was expressed at wild-type levels in the ino2 mutant strain both at 10 µM inositol and at 75 µM inositol (Table 7). However, INO4-cat expression was abolished in the ino4 mutant strain (Table 7). This suggests that the constitutive expression of INO4 requires a wild-type copy of the INO4 gene but does not require the INO2 gene.

INO2-cat expression is sensitive to regulation by OP11

In addition to the positive activator, INO2 and INO4 genes, the phospholipid biosynthetic genes are also regulated by the negative regulatory protein encoded by the OP11 gene (White et al., 1991b),
and its action is also dependent upon the UASINO element. Strains harboring mutant alleles of the OPII gene constitutively overexpress the phospholipid biosynthetic genes (Hirsh and Henry, 1986; Bailis et al., 1987). To determine if INO2 and INO4 expression is also negatively regulated by OPII, the INO2-cat and INO4-cat reporter fusions were integrated at the GAL4 locus of an opil mutant strain (BRS1021) to create BPA110 and BPA111 and assayed for CAT activity under repressing (75 µM inositol and 1 mM choline) and derepressing (no inositol and choline) conditions. The data show that in the opil strain INO2-cat was overexpressed constitutively at a level higher than the fully derepressed level in the wild-type strain (Table 8). Curiously, the level of INO2-cat expression in the opil strain was equivalent to that of INO4-cat in the wild-type strain (Table 8 and Table 5). In contrast, INO4-cat expression was unaffected by this mutation (Table 8). Therefore, INO2, but not INO4, expression is negatively regulated by the product of the OPII gene.

**OPII-cat is modestly regulated and is nonlimiting**

Since the OPII negative regulatory gene was required for the inositol-choline response, it was possible that its expression may also be regulated in response to inositol and choline. This type of mechanism has already been reported for the GAL80 negative regulatory gene of *S. cerevisiae* (Igarashi et al., 1987; Shimada and Fukasawa, 1985). Therefore, the level of cat expression driven by the OPII promoter was determined in a strain harboring an OPII-cat
TABLE 8. Regulation of \textit{INO2} expression by the \textit{OPI1} gene

<table>
<thead>
<tr>
<th>Reporter gene(^b)</th>
<th>genotype(^c)</th>
<th>I-C(^d)</th>
<th>I+C(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{INO2-cat}</td>
<td>Wild type</td>
<td>0.75</td>
<td>0.08</td>
</tr>
<tr>
<td>\textit{INO2-cat}</td>
<td>\textit{opil, INO2, INO4}</td>
<td>4.00</td>
<td>5.23</td>
</tr>
<tr>
<td>\textit{INO4-cat}</td>
<td>Wild type</td>
<td>4.41</td>
<td>4.37</td>
</tr>
<tr>
<td>\textit{INO4-cat}</td>
<td>\textit{opil, INO2, INO4}</td>
<td>4.36</td>
<td>5.85</td>
</tr>
<tr>
<td>promoterless-\textit{cat}</td>
<td>Wild type</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>promoterless-\textit{cat}</td>
<td>\textit{opil, INO2, INO4}</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^a\) Assays were carried out employing extracts from yeast transformants harboring each of the reporter genes. Each value represents the average of data from at least 3 experiments.

\(^b\) Each reporter gene was integrated in single copy at the \textit{GAL4} locus as described in the Materials and Methods.

\(^c\) The strains used in these experiments were BRS1001 (wild type), and BRS1021 (\textit{opil, INO2, INO4}).

\(^d\) I-C\(^-\), complete synthetic media (17) lacking inositol and choline.

\(^e\) I+C\(^+\), complete synthetic media (17) supplemented with 75 µM inositol and 1 mM choline.
fusion (BPA113). The data show that there was a modest regulation of \( OP11 \)-\( cat \) expression (1.7-fold; Table 5). Based on this data, it is not clear that this modest regulation of \( OP11 \) expression is important for the inositol-choline response. However, the more significant observation was that \( OP11 \)-\( cat \) expression was always in large excess relative to \( INO2 \)-\( cat \) and \( INO4 \)-\( cat \) expression. This suggests that the inositol-choline response may be initiated by the product of the \( OP11 \) gene.

Kinetics of \( INO2 \) and \( INO1 \) derepression

The regulation of \( INO2 \)-\( cat \) expression is reminiscent of that of its target genes. That is, the expression of both \( INO2 \)-\( cat \) and \( INO1 \)-\( cat \) was regulated (Table 5), sensitive to \( ino2 \) and \( ino4 \) mutant alleles (Table 7) (Hirsch and Henry, 1986), and regulated by the \( OP11 \) repressor (Table 8) (Hirsch and Henry, 1986). Since \( INO2 \) is required for its own expression as well as expression of the \( INO1 \) gene, the kinetics of \( INO2 \) and \( INO1 \) derepression might be different.

Strains harboring either an \( INO1 \)-\( cat \) reporter fusion (BPA114) or an \( INO2 \)-\( cat \) reporter fusion (BPA101) were used to determine the kinetics of derepression. Cultures were grown in medium supplemented with inositol and choline (repressing) and were then switched to medium lacking inositol and choline (derepressing). Samples were taken at various time points after the switch to derepressing medium and assayed for CAT activity. The data show that the pattern of \( INO2 \)-\( cat \) derepression was similar to that of \( INO1 \)-\( cat \); however (Figure 5). Both genes were fully derepressed at 2.5 hrs.
Cooperative regulation of the phospholipid biosynthetic genes by INO2

One very sensitive mechanism for coordinating the expression of several target genes is to regulate expression of the cognate activator proteins. For example, a 4-fold regulation of GAL4 expression contributes to a 1000-fold regulation of the GAL1 gene (Griggs and Johnston, 1991). A major part of this regulation is the result of cooperative binding of the GAL4 activator to multiple sites in the GAL1 promoter. Since INO2-cat expression was regulated and several of its target genes have multiple INO2-binding sites, we examined whether there is cooperativity in regulation of phospholipid biosynthetic gene expression. To do this, expression of two INO2 target genes was quantitated under growth conditions that establish different levels of INO2 expression. The two target genes were INO1 and CHO1, which have two and one binding site(s), respectively.

To establish different levels of INO2 expression, cells were grown in media supplemented with different concentrations of inositol (range, 0 to 100 µM). The amount of INO2 expression was determined by assay of CAT activity in a wild-type strain harboring the INO2-cat reporter fusion, whereas INO1, CHO1, and TCM1 expression was determined by Northern blot hybridization. A representative Northern blot hybridization showing the patterns of expression and the specificity of the probes is shown (Figure 6). The constitutively expressed ribosomal protein gene TCM1 was used to normalize for loading variations. The patterns of expression of INO1
Figure 5. Comparison of derepression kinetics of \textit{INO1-cat} and \textit{INO2-cat} expression. Strains harboring an \textit{INO2-cat} fusion or an \textit{INO1-cat} fusion were grown to mid-log phase in repressing medium (containing inositol and choline) and switched to derepressing medium (containing neither inositol or choline). Samples were taken at various time points and assayed for CAT activity.
and $CHO1$ relative to $INO2-cat$ were best fit by a sigmoidal curve (Figure 7). In support of this observation, the Hill coefficients were determined to be 3.04 and 1.42 for the $INO1$ and $CHO1$ curves, respectively (EnzFit version 1.05; Elsevier-Biosoft). This sigmoidal relation was characteristic of a cooperative mechanism (Giniger and Ptashne, 1988; Griggs and Johnston, 1991), which was surprising since the $CHO1$ promoter only has a single UAS$_{INO}$ element (Bailis et al., 1992). Even the $INO1$ cooperativity cannot be explained on the basis of multiple UAS$_{INO}$ elements since there was no synergism between the two $INO1$ UAS$_{INO}$ elements (Koipally et al., submitted; Lopes and Henry, 1991). A model to describe these results will be proposed in the discussion.

Uncoupling $INO2$ expression from the inositol/choline response

Expression of a reporter gene ($cat$) driven by the $INO2$ promoter (integrated in single copy at the $GAL4$ locus in a wild type strain) was found to be sensitive to different inositol concentrations in the growth medium (Figure 8). Specifically, increased levels of CAT activity were observed 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 $CHO1$ (see Figure 7) (Hirsch and Henry, 1986; Bailis et al., 1987). This raised the possibility 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
Figure 6. Northern blot hybridization of phospholipid biosynthetic genes. A representative Northern blot hybridization showing INO1 and CHO1 transcript levels grown in complete synthetic media supplemented with various inositol concentrations (0 to 100 µM) is displayed. The constitutively expressed ribosomal protein gene TCM1 (Lopes et al., 1991) was used to normalize for loading variations.
Figure 7. Effects of variation in levels of \textit{INO2} expression on \textit{INO1} (A) and \textit{CHO1} (B) expression. \textit{INO2} expression was determined by assay of \textit{INO2-cat} activity. \textit{INO1} and \textit{CHO1} expression was determined by Northern blot hybridization. The extent of hybridization was quantitated by densitometry (arbitrary units), normalized for loading variations with the constitutively expressed ribosomal protein gene \textit{TCM1} (Lopes \textit{et al.}, 1991). All assays were performed with cultures growing exponentially in complete synthetic medium (Hirsh and Henry, 1986) containing 1 mM choline and either lacking inositol or containing 10, 17.5, 25, 50, 75, or 100 µM inositol.
expression of the target genes, INO2 expression was uncoupled from the inositol response by placing it under the control of the galactose inducible GAL1 promoter. To do this, a plasmid was constructed (pGAL1-INO2) that placed the INO2 coding sequence downstream of the GAL1 promoter in plasmid pBM2289 (Griggs and Johnston, 1993). Plasmid pGAL1-INO2 (containing the URA3 selectable marker) was stably integrated in single copy at the ura3 locus of strain BRS2001 (ino2Δ) to yield BRS2011 (pGAL1-INO2::URA3, ino2Δ). This strain also lacked a functional INO2 gene to insure that INO2 expression originated exclusively from the GAL1-INO2 hybrid gene.

Expression of INO2 in BRS2011 (pGAL1-INO2::URA3, ino2Δ) was expected to be sensitive to carbon source (GAL1 promoter-driven) but insensitive to inositol. To test this, two assays were used to examine INO2 expression in BRS2011 (pGAL1-INO2::URA3, ino2Δ). First, the growth phenotype of BRS2011 (pGAL1-INO2::URA3, ino2Δ) was assayed on media containing different carbon sources and either lacking or containing inositol (Table 9). BRS2011 (pGAL1-INO2::URA3, ino2Δ) grew normally on galactose media regardless of the presence or absence of inositol. That is, the level of GAL1 promoter-driven INO2 gene expression in media containing galactose rescued the inositol auxotrophy associated with the ino2Δ mutant allele. However, this strain grew slowly in the presence of raffinose, and failed to grow on glucose when inositol was omitted. The inability of BRS2011 (pGAL1-INO2::URA3, ino2Δ) to grow on glucose and grow slowly on raffinose is presumably due to the low level of expression from the GAL1 promoter which is repressed when cells are grown on glucose and reduced on raffinose (Johnston, 1987).
Figure 8. *INO2-cat* expression is sensitive to different inositol concentrations. CAT activity was assayed from extracts of wild type cells (BRS1001) containing a single copy of an *INO2-cat* reporter gene integrated in single copy at the *GAL4* locus. Cells were grown in media containing different concentrations of inositol. All values are presented as a percentage of completely derepressed levels and are the average of at least 3 independent assays. Standard deviations were less than 15% in all cases.
Consequently, *INO2* expression may be repressed in these strains under these two growth conditions thereby preventing the *ino2Δ* strain from growing in the absence of inositol. As controls, growth of an isogenic wild-type strain (BRS1001) and the isogenic parent strain carrying the *ino2Δ* allele (BRS2001) were also assayed under the same growth conditions. As expected, the wild-type strain grew under all conditions whereas the *ino2Δ* strain required inositol for growth regardless of the carbon source (Table 9).

The second assay involved direct quantitation of *INO2* transcription in BRS2011 (pGAL1-*INO2::URA3*, *ino2Δ*) by Northern and slot blot hybridizations. To do this, cells were grown in media that contained different concentrations of galactose and either lacked or contained inositol. The presence of different concentrations of galactose had previously been shown to result in different levels of expression from the *GAL1* promoter (Aparicio and Gottschling, 1994). *INO2* expression from the *GAL1* promoter was found to be sensitive to the concentration of galactose in the growth medium (Figure 9, A) but was insensitive to inositol (Figure 9, B). Consequently, expression of the *INO2* gene has been uncoupled from the inositol response and made sensitive to galactose concentration.

Transcription of the *INO2* gene correlates with transcription of its target genes

The BRS2011 strain (pGAL1-*INO2::URA3*, *ino2Δ*) was used to determine if 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
## TABLE 9. Growth phenotype of *GAL1*-INO2-containing *ino2Δ* strain

<table>
<thead>
<tr>
<th>Strain (Genotype)</th>
<th><strong>Gal</strong></th>
<th><strong>Raf</strong></th>
<th><strong>Glu</strong></th>
<th><strong>Gal</strong></th>
<th><strong>Raf</strong></th>
<th><strong>Glu</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>BRS1001 (<em>INO2</em>)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BRS2001 (<em>ino2Δ</em>)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BRS2011 (*pGAL1-INO2::URA3, <em>ino2Δ</em>)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Strains were tested by spotting approximately 1x10^6 cells on the appropriate media.
  
  Growth was scored after 48 hrs (30°C) as wild type (+), no growth (-), or slow growth (+/-).

* Complete synthetic media (21) was either supplemented with 75 µm inositol (+) or lacked inositol (-).

* Media contained either 2% galactose (Gal), 2% raffinose (Raf), or 2% glucose (Glu)
Figure 9. Uncoupling \textit{INO2} expression from the inositol response. (A) Expression of \textit{INO2} transcript from strain BRS2011 (pGAL1-
\textit{INO2::URA3, ino2Δ}) grown in media containing different concentrations of inositol and 0.5% galactose. The inositol concentrations used were (from left to right): 0; 5; 10; 17.5; 50; 75; 100 µM. The same blot was re-hybridized with the \textit{ACT1}-specific probe to normalize for loading variations. (B) Relative levels of \textit{INO2} transcription (arbitrary densitometry units from BRS2011 grown in media containing different concentrations of galactose either lacking (I-C-) or containing 75 µM inositol and 1 mM choline (I+C+). The amount of \textit{INO2} transcript was determined by densitometric scanning of quantitative slot blots and normalized for loading variations using the \textit{ACT1}-specific probe. Values represent the average of 3 independent assays. Standard deviations were less than 15% in all cases.
response to inositol exclusively by derepressing expression of the \textit{INO2} gene? To address this question, expression of two Ino2p target genes, \textit{INO1} (Hirsch and Henry, 1986) and \textit{CHO1} (Bailis et al., 1987) was directly quantitated in BRS2011 (pGAL1-INO2::URA3, \textit{ino2Δ}) in media containing varying concentrations of galactose both in the presence and absence of inositol. Transcription of the \textit{INO1} gene in BRS2011 (pGAL1-INO2::URA3,\textit{ino2Δ}) was sensitive to both galactose and inositol in the growth medium (Figure 10, top). That is, in the absence of inositol, transcription of the \textit{INO1} gene correlated with the concentration of galactose in the growth medium. However, in the presence of inositol, \textit{INO1} transcription was repressed regardless of the galactose concentration. Similarly, transcription of the \textit{CHO1} gene was also sensitive to both galactose and inositol (Figure 10, bottom). Thus, in BRS2011 (pGAL1-INO2::URA3,\textit{ino2Δ}), transcription of the \textit{INO1} and \textit{CHO1} target genes was still repressed in response to inositol supplementation even though \textit{INO2} transcription was no longer sensitive to inositol.

\textit{OPI1} is required for the inositol/choline response in the \textit{GAL1-INO2} strain

Since regulating transcription of the \textit{INO2} gene was not the primary target of the inositol response, it is possible that the \textit{OPI1} negative regulatory gene might be the primary target. This line of reasoning was supported by the phenotype of strains carrying \textit{opi1} mutant alleles. In an \textit{opi1} mutant strain, expression of the \textit{INO1}
Figure 10. Transcription of the \textit{INO1} gene (top) and the \textit{CHO1} gene (bottom) is sensitive to both galactose concentration and inositol in the \textit{GAL1-INO2}-containing strain. Data was generated as described in the legend to Figure B.
(Hirsh and Henry, 1986; White et al., 1991) and CHO1 (Bailis et al., 1987) target genes is insensitive to the presence of inositol in the growth medium. This suggests that the product of the OP11 gene either regulates INO2 expression or directly regulates the function of the Ino2p. To distinguish between these two models, the effect of deleting the OP11 gene in BRS2011 (pGAL1-INO2::URA3, ino2Δ) on regulation of INO1 gene expression was examined. If the response to inositol was mediated by Op1p regulating the function of the Ino2p, then deleting the OP11 gene in BRS2011 (pGAL1-INO2::URA3, ino2Δ) should yield constitutive expression of the INO1 target gene, since INO2 expression is no longer sensitive to inositol in this strain.

To directly examine the role of the OP11 gene in the response to inositol, the OP11 gene was deleted in strain BRS2011 (pGAL1-INO2::URA3, ino2Δ) to yield BRS2012 (pGAL1-INO2::URA3, op1Δ, ino2Δ) (Table 1). The effect of the op1Δ allele on regulation of INO1 expression in BRS2012 (pGAL1-INO2::URA3, op1Δ, ino2Δ) was then examined by Northern blot hybridization. Total RNA was purified from strains grown in media that contained 0.5% galactose which either lacked or included inositol. The level of INO1 transcription was quantitated by densitometry and normalized for loading using the ACT1 gene probe. As expected, BRS2012 (pGAL1-INO2::URA3, op1Δ, ino2Δ) expressed constitutively elevated levels of INO1 relative to the isogenic BRS2011 strain (pGAL1-INO2::URA3, ino2Δ) (Figure 11). Moreover, the pattern of regulation of INO1 expression in the pGAL1-INO2-containing strains (Figure 11, right panel) was virtually indistinguishable from that of the strains containing the native INO2 gene (Figure 11, left panel).
Characterization of the \textit{INO2} promoter

A. Mapping of the \textit{INO2} transcription initiation site

The 5' end of the \textit{INO2} transcript was mapped using primer extension analysis (Elion and Warner, 1984). Initial attempts to map the 5' end of the \textit{INO2} transcript were unsuccessful because of the low level of \textit{INO2} transcript present in wild type yeast cells, even under derepressing conditions. Therefore, to increase the level of \textit{INO2} transcript, a wild type strain (BRS1001), was transformed with a multicopy plasmid (YES.B) which contains a 14.2 kb insert including the entire \textit{INO2} promoter and coding sequences. This strain was grown in both repressing (I+C+) and derepressing (I-C-) conditions and total cellular RNA was isolated. Total cellular RNA was then used to map the 5' end by primer extension analysis. A major transcription initiation site was found 106 bp upstream (-106) from the AUG (A= +1) translation start codon (Figure 12). Several minor transcription initiation sites were also observed between -90 to -146 relative to the AUG translation start codon (Figure 12). The major transcription initiation start site at -106 is 28 bp downstream from the potential \textit{UAS\textsubscript{INO}} element and 94 bp downstream from a region found to be essential for \textit{INO2} expression (see next section for details). Curiously, this long leader includes within it a second potential AUG translational start codon found 17 bp upstream from the known translational start codon. If translation occurred from this upstream AUG, it would encode a potential open reading frame (ORF) of 57 nt which would overlap the \textit{INO2} ORF.
Figure 11. Effect of an opilΔ allele on INO1 expression in the GAL1-INO2-containing strain. Representative Northern blot hybridization of INO1 transcription in strains containing: INO2 and OPI1 (BRS1001); INO2 and opilΔ (BRS2005); pGAL1-INO2::URA3 and OPI1 (BRS2011); and pGAL1-INO2::URA3 and opilΔ (BRS2012). Each strain was grown in media containing 0.5% galactose which either lacked (-) or was supplemented with 75 µM inositol and 1mM choline (+). The values below each lane represent relative levels of INO1 transcript (normalized for loading using the ACT1 transcript) determined by densitometry. Shorter exposures were used for densitometric scanning of RNA from the opilΔ strains. B.D.; below detection.
Figure 12. Primer extension analysis of INO2. Primer extension products were separated along side a DNA sequencing ladder in order to size the fragments. I-C-, absence of inositol and choline; I+C+, presence of 75 µM inositol and 1 mM choline; control, RNA was left out of the primer extension reaction.
B. Identification of cis-acting elements in the *INO2* promoter

In order to identify cis-acting promoter sequences that are required for the regulated expression of *INO2-cat*, a series of promoter deletions was constructed by PCR. These promoter deletions were fused to the *cat* reporter gene in pBM2015, and integrated in single copy at the *GAL4* locus (see Figure 2). Southern blot analysis was performed to confirm the proper size and integration of the promoter deletion constructs. A representative Southern blot analysis is shown in Figure 13. Transformants harboring the proper integrated promoter deletion mutants were grown in repressing (I+C+) and derepressing (I-C-) media and assayed for CAT activity. The data from this analysis are summarized in Figure 14. Deletion from -400 to -500 (relative to the AUG translation start codon) and from -300 to -500 had no affect on *INO2-cat* expression compared to the full length promoter (Figure 14, deletion numbers 1 and 2). A further deletion of 100 bp, from -200 to -500 resulted in a complete loss of *INO2-cat* expression (Figure 14, deletion number 3) as did deletion from -100 to -500 (deletion number 4). A deletion from the 3' end of the *INO2* promoter, from -1 to -50 gave a constitutively high level of CAT activity (Figure 14, deletion number 5). It should be noted that this deletion retains the normal *INO2* translation start codon, but deletes the upstream AUG codon. A further deletion of 100 bp, from -1 to -150 results in a very low level of *INO2-cat* expression (Figure 14, deletion number 6). This deletion removes both the upstream AUG codon as well as the potential UAS\_*NO* element. Deletion from -1 to -250 results in a complete loss of *INO2-cat* expression (Figure 14, deletion
Figure 13. Representative Southern blot hybridization of strains harboring INO2 promoter deletion-cat fusion genes.
number 7), as does deletion from -1 to -350 (deletion number 8) and from -1 to -450 (deletion number 9). An internal deletion from -100 to -150, which removes the potential UAS\textsubscript{INO} element and flanking sequences, results in a constitutively high level of \textit{INO2-cat} expression (Figure 14, deletion number 6/4). Deletion from -200 to -250 results in a complete loss of \textit{INO2-cat} expression (Figure 14, deletion number 7/3), suggesting that this region is essential for \textit{INO2-cat} expression. Deletion from -300 to -350, as expected, has no affect on \textit{INO2-cat} expression (Figure 14, deletion number 8/2).
Figure 14. Summary of INO2 promoter deletion analysis. Promoter fragments were fused to the *cat* reporter gene and integrated in single copy at the *GAL4* locus in a wild type (BRS1001) strain. CAT activity values are the average from at least three independent experiments. I-C-, complete synthetic medium (Hirsh and Henry, 1986) lacking inositol and choline. I+C+, complete synthetic medium (Hirsh and Henry, 1986) supplemented with 75 µM inositol and 1 mM choline.
CHAPTER V
DISCUSSION

Regulation of phospholipid biosynthesis is dictated by the UASINO element, which serves as a binding site for the Ino2p/Ino4p heterodimer (Ambroziak and Henry, 1994; Koipally et al., submitted). However, the exact mechanism(s) of regulation has not yet been determined. Examination of the molecular and genetic properties of the trans-acting factors suggests that protein-protein interactions may play an important role in repressing transcription of the phospholipid biosynthetic genes. For example, the inositol/choline response may involve a direct interaction between the Opi1 negative regulatory protein and either the Ino2 or Ino4 activator proteins. This mechanism is reminiscent of the regulation of GAL gene expression in response to galactose (Johnston and Carlson, 1992; Leuther and Johnston, 1992) and regulation of the PHO genes in response to phosphate starvation (Jayaraman et al., 1994).

Alternatively, regulation of phospholipid gene expression may be regulated by modulating activator protein levels. A mechanism such as this is utilized in the general control of amino acid biosynthesis by regulating the rate of translation of the Gcn4 activator and by regulating the stability of the Gcn4p (Hinnebusch, 1984; Kornitzer et al., 1994). Furthermore, repression of the structural genes for galactose utilization in response to glucose
(glucose repression) is also partly established by repressing expression of the *GAL4* activator gene (Griggs and Johnston, 1991; Lamphier and Ptashne, 1992). Therefore, it is possible that another mechanism for regulating expression of phospholipid biosynthetic gene expression involves controlling the levels of the Ino2 and/or Ino4 activator proteins or the Opi1 repressor protein.

The work described in this dissertation is aimed at understanding the mechanism(s) by which phospholipid biosynthetic gene expression is regulated. The results indicate a dual mechanism which superimposes regulation of expression of the gene encoding the Ino2 transcriptional activator protein and repression of function of the Ino2p/Ino4p heterodimer by the Opi1 negative regulatory protein. This is significant because it is unusual for a metabolic pathway, such as phospholipid biosynthesis, to utilize two mechanisms for regulating expression of its structural genes in response to a single signal.

**Identification of the minimal UAS_{INO} element**

The UAS_{INO} element was originally identified as a 9 bp element with the consensus 5'ATGTGAAAT3' (Lopes *et al.*, 1991), from the promoter of the *INO1* gene. However, when this element was tested in the heterologous *CYCl-lacI'Z* reporter gene, it was unable to support transcription (Table 4). Based on this, it was suggested that the consensus element actually consists of 10 bp, having the consensus 5'CATGTGAAAT3' (Paltauf *et al.*, 1992). Of the nine copies of the repeated element located in the *INO1* promoter, four have a cytosine residue at the 5' end preceeding the 9 bp, three have an
adenine, and there is one each with a 5' thymine or guanine (Koipally et al., submitted). When all of the known UAS_{INO} elements are aligned, the first (most 5') residue is the least well conserved residue of the 10 bp element, with a cytosine being represented in about 40% of the copies. Therefore, it appears that this first residue may be the most important residue for determining the functionality of the UAS_{INO} element.

The effectiveness of the 10 bp consensus sequence as a UAS was tested using a set of oligonucleotides containing the 9 bp core sequence and differing only at the first (most 5' residue). These results support the conclusion that the active core sequence is 5'C/AAATGTGAAAT3' (Table 4). The construct containing a cytosine residue at the 5' end was a stronger UAS element than the construct with an adenine at the 5' end, although both constructs were regulated in response to inositol and choline. Thus, the consensus element 5'C/AAATGTGAAAT3', is capable of serving as a UAS_{INO} and confers regulated expression in response to inositol and choline. Furthermore, the UAS_{INO} sequence is responsible for mediating the response to the OPII gene product. In the opil mutant background, expression from both the adenine- and cytosine-containing constructs was constitutively overexpressed compared to expression in the wild type strain. Therefore, it is apparent that this consensus sequence alone is capable of conferring the OPII-mediated response and does not require the presence of an adjacent URS element.

The Ino2 and Ino4 activator proteins are proposed to be members of the basic helix-loop-helix family of transcriptional activator proteins (Hoshizaki et al., 1990; Nikoloff et al., 1992).
Members of this family of proteins form dimers which bind to a canonical DNA sequence 5'CANNTG3' (with NN being any nucleotide) (Blackwell et al., 1990; Blackwood and Eisenman, 1991; Berberich et al., 1992). The 10 bp consensus UAS_INO contains within it this canonical sequence, 5'CATGTGAAAT3', consistent with the Ino2p and Ino4p being members of the bHLH family. Although in multicellular eukaryotes a cytosine is required at the 5' end, the fact that a UAS_INO element with an adenine at this site functions probably indicates a less stringent requirement for the canonical bHLH binding site in yeast.

Regulation of INO2 expression

Computer-assisted searches of the INO2 and INO4 promoter regions revealed that they both contain a copy of the UAS_INO element. This raised the possibility that phospholipid biosynthetic gene expression might be regulated by regulating the expression of either the INO2 and/or INO4 genes. In order to test this, the INO2 and INO4 promoters were fused the the cat reporter gene and the constructs were integrated into the yeast genome at the GAL4 locus. These strains were then assayed for CAT activity under repressing (I+C+) and derepressing (I-C-) conditions.

Expression of the INO2-cat gene was regulated 12-fold in response to inositol and choline, whereas the INO4-cat gene was constitutively expressed (see Table 5). These results were confirmed by quantitative slot blot analysis which showed that the INO2 transcript was regulated about 5-fold in response to inositol (see Table 6), while the INO4 transcript was constitutively expressed.
Furthermore, under derepressing conditions the *INO2-cat* gene was expressed at a level less than the *INO4-cat* gene, suggesting that *INO2* expression may be limiting relative to *INO4* expression. Also, in the *opil* mutant background, *INO2-cat* was constitutively overexpressed but its level of expression was equal to that of the *INO4-cat* construct in a wild-type strain (see Table 8), indicating that *INO4* expression may become limiting relative to *INO2* expression in this background. Finally, the overexpression of the *INO2-cat* gene in the *opil* strain is a likely explanation as to why the phospholipid biosynthetic genes are constitutively overexpressed in this mutant background (Hirsh and Henry, 1986; Bailis et al., 1987).

Since both the *INO2* and *INO4* promoters contain a potential copy of the UASINO element and since this element serves as a binding site for the Ino2p/Ino4p heterodimer, it is possible that expression of these genes may be regulated by the Ino2 and Ino4 proteins. In order to test this possibility, the *INO2-cat* and *INO4-cat* constructs were each integrated in single copy into *ino2* and *ino4* mutant strains and assayed for CAT activity. These results showed that *INO2-cat* requires a wild type allele of both the *INO2* and *INO4* genes to be expressed whereas *INO4-cat* expression requires a wild type copy of the *INO4* gene, but does not require the *INO2* gene (see Table 7). Therefore, both *INO2* and *INO4* are under autogenous control.

Expression of *INO2-cat* requires wild type Ino2p and Ino4p and its expression is sensitive to regulation by the Opi1 repressor protein. This indicates that the inositol/choline response is mediated somehow through either the *INO2* gene or the Ino2p. Since expression of
the $INO4\text{-}cat$ gene is not sensitive to inositol and choline nor is it regulated by the Opi1 repressor protein, it in turn is not likely to mediate the inositol/choline response. A likely scenario is that the Opi1p interacts directly with the Ino2p to inhibit its function. This would then mediate two simultaneous events: 1) inhibiting the function of the Ino2p/Ino4p heterodimer, preventing activation of the phospholipid biosynthetic structural genes; and 2) repress expression of the $INO2$ gene itself. In support of this model, an $OPI1\text{-}cat$ gene is expressed at a level approximately 25-fold higher than the $INO2\text{-}cat$ gene (see Table 5), thus assuring that there is enough Opi1p in the cell to repress all of the available Ino2p. Further evidence supporting this model will be described in detail later.

$INO4$ is expressed constitutively

As stated above, the $INO4\text{-}cat$ gene was expressed constitutively. This data disagrees with preexisting data (Schuller et al., 1992) which showed that $INO4\text{-}lacZ$ expression was repressed three- to four-fold in response to inositol and choline. However, the level of $INO4\text{-}lacZ$ expression was actually below the limits of sensitivity for the assay and was compared with that of an entirely different control vector. This was apparent since the derepressed level of $INO4\text{-}lacZ$ expression (20 units) is barely above the levels from a UAS-less reporter (14 units) (Schuller et al., 1992), while the repressed levels of $INO4\text{-}lacZ$ expression (6 units) are actually below the control levels (14 units) (Schuller et al., 1992). In the data reported here, the level of $INO4\text{-}cat$ expression was substantially
above the lower limits of sensitivity for this system. Moreover, the experiments described here examined INO4 promoter activity in a native context (chromosomal) and in a single copy, thus avoiding any potential multicopy or plasmid-related artifacts.

Expression of the INO4-cat gene was dependent on a wild type allele of the INO4 gene but not the INO2 gene (see Table 7). Since the Ino4 protein does not appear to have any transcriptional activation domain (Schwank et al., 1995) and does not homodimerize (Ambroziak and Henry, 1994), it must form a heterodimer with some other protein that has an activation domain to support its own transcription. In support, it is not unprecedented for HLH proteins to form dimers with multiple partners. For example, the mammalian protein Max can form homodimers or heterodimers with Myc, Mad, and Mxi (Amati and Land, 1994). There are three other known yeast transcriptional activator HLH proteins, Pho4 (Ogawa and Oshima, 1990), Cbf1 (Cai and Davis, 1990), and Rtg1 (Liao and Butow, 1993). All three of these were tested for defects in phospholipid biosynthesis and were found not to be required for phospholipid biosynthetic gene expression, indicating that they are not likely to be required for INO4 expression. Consequently, there must be another as-yet-unidentified partner for the INO4 gene.

Another issue concerning expression of the INO4 gene is the presence of the UAS\textsubscript{INO} element in its promoter. Since the INO4-cat gene is constitutively expressed, what then is the function of the UAS\textsubscript{INO} element in the INO4 promoter? Conceivably, single-base changes from the UAS\textsubscript{INO} element consensus may dictate specificity for binding of different sets of partners. This has been shown to be
the case for mammalian HLH proteins in which base changes in a Myc/Max-binding site will create a Max/Max-binding site (Solomon et al., 1993). Interestingly, the yeast CTR1 gene, encoding the choline transporter protein, also requires INO4 but not INO2 for its expression (Li and Brendel, 1993). A comparison of the CTR1 and INO4 promoters identified a consensus HLH binding site (CAA/TTG) that deviates from the UASINO element. This additional HLH binding site is found in the upstream region of the INO4 promoter, and a deletion of this upstream region results in loss of INO4 expression (Ambroziak). Ongoing studies in the Lopes laboratory are aimed at identifying the function of the UASINO as well as the second potential HLH binding site in the INO4 promoter and in identifying potential partners for the Ino4p.

Cooperative regulation of the phospholipid biosynthetic genes by INO2

The data showed that cooperativity plays a role in control of expression of INO1 and CHOL (see Figure 7). This is not unprecedented in S. cerevisiae, since cooperativity was also observed in GAL4 activation of GAL1 expression (Giniger and Ptashne, 1988; Griggs and Johnston, 1991). In the GAL system the observed cooperativity is due to the cooperative binding of the Gal4 activator protein to four binding sites in the GAL1 promoter. A similar mechanism could exist for activation of the INO1 gene, since there are two functional UASINO elements in this promoter (Koipally et al., unpublished results). However, there is no evidence of synergism between these elements or cooperativity of binding to these two sites.
(Lopes and Henry, 1991; Koipally et al., submitted). Furthermore, cooperativity was observed in activation of the \textit{CHO1} gene, which only has a single copy of the UAS\textsubscript{INO} element. Therefore the observed cooperativity must be due to a mechanism other than activator binding. An alternative model to explain the cooperative activation of \textit{INO1} and \textit{CHO1} is shown in Figure 15. When cells are grown in medium containing inositol and choline (repressing), two mechanisms exist for repression of phospholipid biosynthetic gene expression. The Opi1 repressor interacts with the Ino2/Ino4 heterodimer to decrease expression of the \textit{INO2}, \textit{INO1}, and \textit{CHO1} genes and therefore reduce the amount of Ino2/Ino4 heterodimer available to bind the UAS\textsubscript{INO} element. The mechanism for derepression requires that the repressing action of the Opi1p be inactivated, allowing the phospholipid biosynthetic genes as well as the \textit{INO2} activator gene to be derepressed, resulting in complete derepression of these genes. Therefore, the cooperativity in this system results from the concomitant derepression of the \textit{INO2} activator gene and inactivation of the Opi1 repressor protein. This model predicts that Opi1p provides the initial response; however, it cannot predict the nature of the interaction between the repressor and the two activators. Several additional observations support this model. As stated earlier, the \textit{OPI1-cat} gene is overexpressed relative to both \textit{INO2-cat} and \textit{INO4-cat}, and its expression is essentially unaffected by inositol and choline (see Table 5). Furthermore, it has already been reported that the amount of Ino2p/Ino4p-UAS\textsubscript{INO} complex is affected by inositol and choline (Lopes and Henry, 1991). Therefore, the model predicts that \textit{OPI1} is required for the initial
Figure 15. Model for cooperative derepression of phospholipid biosynthetic gene expression in *S. cerevisiae*. See text for a detailed explanation. I+C+, containing 75 µM inositol and 1 mM choline; I-C-, containing neither inositol or choline.
response to inositol and choline but that regulation of \(INO2\) expression establishes the magnitude and cooperativity of the response.

**Uncoupling of \(INO2\) transcription from the inositol response**

Data described earlier showed that expression of a reporter gene (\(cat\)) driven by the \(INO2\) promoter was sensitive to different concentrations of inositol in the growth media (see Figure 8). 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 \(CHO1\) (Hirsh and Henry, 1986; Bailis et al., 1987; Ashburner and Lopes, 1995a). This raised the possibility that regulation of \(INO2\) expression may be the primary mechanism for the coordinated response to inositol. To directly examine the role of \(INO2\) expression in the regulation and/or expression of the target genes, \(INO2\) expression was uncoupled from the inositol response by placing it under the control of the galactose inducible \(GAL1\) promoter.

Transcription of the \(INO2\) gene in the strain harboring the \(GAL1\)-\(INO2\) fusion gene (BRS2011) was uncoupled from the inositol response since its expression was completely insensitive to inositol and was regulated in response to galactose, with increasing levels of expression observed with increasing concentrations of galactose in the growth media (see Figure 9). However, when this strain was used to determine regulation of the \(INO2\) target genes, \(INO1\) and \(CHO1\), their expression was still regulated in response to inositol, response (see Figure 10). This implied that there is some other
mechanism regulating the inositol response. A likely candidate for this other mechanism is the Opi1 negative regulatory protein. To examine this possibility, the effect of deleting the \textit{OPI1} gene in strain BRS2011 (\textit{GALI-IN02}) on \textit{INO1} expression was tested. These results showed that a wild type allele of the \textit{OPI1} gene is required for the inositol response since even in the strain with \textit{INO2} expression uncoupled from the inositol response, the \textit{INO1} gene was constitutively overexpressed in the \textit{opil} mutant background (see Figure 11).

Therefore, based on the above results, it appears that the response to inositol requires two superimposed mechanisms. One mechanism is the regulation of \textit{INO2} activator gene expression which is subject to autoregulation by the \textit{INO2} gene product. The second mechanism requires the product of the \textit{OPI1} negative regulator which may function as a direct regulator of Ino2p/Ino4p activity. This data favors a model wherein the \textit{OPI1} gene product is the primary target of the inositol response. Data described earlier showed that the \textit{OPI1} gene product is required to regulate expression of the \textit{INO2} activator gene (see Table 7). This data suggested that the inositol response is mediated by Opi1p regulation of \textit{INO2} expression. However, in the \textit{GALI-IN02} strain, Opi1p has no control over \textit{INO2} expression, but it still regulates \textit{INO1} expression (see Figure 11). This implies that the mechanism by which Opi1p functions is by directly regulating the Ino2p/Ino4p heterodimer.

Regardless of the exact mechanism of Opi1p function, it is clear that \textit{OPI1} is absolutely required for the inositol response whereas regulation of \textit{INO2} expression can be eliminated without affecting
regulation of the target genes in response to inositol (see Figure 10). Furthermore, OPI1 seems a likely target for the inositol response since it appears to be expressed at a level higher than either the INO2 or INO4 genes (see Table 5). Data described in Table 5 show that an OPI1-cat reporter gene is expressed constitutively (i.e., unresponsive to inositol) at a level that is substantially higher than either the INO2 or INO4 promoters. Curiously, the relative levels of expression of the OPI1 to INO2 regulatory genes are reminiscent of the relative levels of GAL80 to GAL4 expression (Shimada and Fukusawa, 1985; Griggs and Johnston, 1993). 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 (Nogi and Fukasawa, 1989).

A strong correlation between the level of INO2 expression driven by the GAL1 promoter and the level of expression of two target genes, INO1 and CHO1, was observed (see Figures 10 and 11). This suggests that regulation of INO2 expression does play a role in the response to inositol. For example, if Ino2p levels are extremely low under repressing conditions, the cell would have to express INO2 prior to activating transcription of the target genes. Alternatively, a small amount of Ino2p may be enough to initiate the response and derepression of INO2 expression may serve to establish the degree of derepression of the target genes, depending on the concentration of exogenous inositol. The model seems more likely since it has been previously shown that extracts prepared from cells grown under repressing conditions form the Ino2p:Ino4p:UASINO complex (Lopes and Henry, 1991). Furthermore, the timing of derepression of an
$INO2$-cat gene and an $INO1$-cat gene were essentially identical (see Figure 5) 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, a correlation has been observed between $INO2$ expression and target gene expression at different concentrations of inositol. 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 Ino2p/Ino4p target sequences vary among the promoters of the coregulated genes (Greenberg and Lopes, in press), it seems likely that different levels of $INO2$ expression may be required to activate expression of different target genes.

Characterization of the $INO2$ promoter

An analysis of the $INO2$ promoter region was conducted to identify the cis-acting elements responsible for directing $INO2$ expression. These results revealed some unique features of the $INO2$ promoter (summarized in Figure 16). Primer extension analysis to determine the precise transcription initiation site of the $INO2$ gene showed that there is a leader of approximately 106 bp relative to the AUG translation initiation codon (see Figure 12). Several minor transcription initiation sites were also observed between -90 and -146 relative to the AUG translation start codon. The major transcription initiation start site at -106 is 28 bp downstream from the potential UAS$_{INO}$ element and 94 bp downstream from a region
found to be essential for \textit{INO2} expression. Curiously, this leader includes within it a second potential AUG translational start codon found 17 bp upstream from the known translational start codon. If translation occurred from this upstream AUG, it would encode a potential ORF of 57 nt (19 amino acids) which would overlap the \textit{INO2} ORF (Figure 16). At this point, it is not known if this short ORF is translated and what affect it might have on \textit{INO2} expression. Since the uORF overlaps the \textit{INO2} translation initiation codon, the only way in which \textit{INO2} could be translated is through "leaky" scanning of the 40S ribosomal subunit. One possible cause of leaky scanning could be due to the formation of stem-loop structures which are predicted to form in the \textit{INO2} leader (J.M. Lopes, unpublished observation), that would allow the 40S subunit to bypass the upstream AUG.

Deletion analysis of the \textit{INO2} promoter revealed several potential regions which contribute to the expression of \textit{INO2}. First, a region between -200 and -250 (relative to the translation start codon) was found to be essential for transcription. Although no canonical TATA element is present in the \textit{INO2} promoter based on computer assisted searches, the region between -200 and -250 is adenine and thymine rich and therefore could potentially serve as a TATA element. It is more likely that this region is a very weak TATA element as opposed to an upstream essential sequence (UES) element described in the \textit{GAL4} promoter (Griggs and Johnston, 1993). The reason for this is that the UES\textsubscript{GAL4} cannot function with a heterologous UAS element, such as the UAS\textsubscript{GAL} found in the
Figure 16. Diagram of the INO2 promoter region. Shown are the essential region from -200 to -250, the UAS\textsubscript{INO} element, at -136, the major transcription initiation region at -106, and the potential upstream ORF overlapping the INO2 ORF.
promoters of the \textit{GAL4} target genes. That is, it is only able to function with the UAS element found in the \textit{GAL4} promoter (Griggs and Johnston, 1993). Since a UAS\textsubscript{INO} element is present in the \textit{INO2} promoter and since it is known to function with a canonical TATA element (e.g., in the promoters of the \textit{INO2} target genes), it lends support to the notion that this essential region in the \textit{INO2} promoter is a weak or divergent TATA element.

A 3' deletion which removed the first 50 bp of the promoter (from -1 to -50) resulted in a constitutively high level of expression from the \textit{INO2-cat} gene. Curiously, this deletion retains the normal \textit{INO2} translation start codon, but deletes the upstream AUG codon, indicating that this codon may play a role in regulating the translation of the \textit{INO2} mRNA. Translational control of expression is not unprecedented in yeast. This mechanism of control has already been described for the \textit{GCN4} activator gene (Hinnebusch, 1984). The \textit{CPA1} gene, encoding a subunit of the arginine pathway carbamoylphosphate synthetase, also has a single uORF of 25 amino acids (Werner \textit{et al.}, 1987). This uORF begins about 134 nt upstream of the normal AUG start codon for the \textit{CPA1} gene and ends about 60 nt upstream of the AUG (Werner \textit{et al.}, 1987) and has been shown to confer repression by arginine on expression of the \textit{CPA1} gene (Delbecq \textit{et al.}, 1994). Another gene which contains uORFs is the \textit{UME6} transcriptional repressor gene. The leader of the \textit{UME6} mRNA contains 5 short uORFs (from 5 to 23 amino acids) (Strich \textit{et al.}, 1994). The function of these uORFs in the \textit{UME6} leader are not yet known, however, it is known that the \textit{UME6} mRNA is expressed constitutively (Strich \textit{et al.}, 1994), so it is likely that these uORFs play
a role in translational regulation of UME6 expression. Therefore, it is
not surprising that INO2 expression may also be under translational
control. What is unusual is that this would add another level of
regulation to INO2 expression in addition to the transcriptional
regulation of INO2 and regulation by Opi1p. Further experiments
into the role of the upstream AUG, including direct mutagenization to
eliminate it, will need to be performed to determine its role in
regulating INO2 expression.

The INO2 promoter contains a potential UASINO element
between -134 and -144 relative to the AUG translation start codon.
Deletion from -1 to -150, which includes the UASINO element, results
in a loss of INO2-cat expression (see Figure 14, deletion no. 6) under
both repressing and derepressing conditions, indicating that the
UASINO element does function in regulating INO2 expression. In
contrast, an internal deletion which removes from -100 to -150,
including the UASINO element, but retains from -1 to -100, results in
a constitutively high level of INO2-cat expression. This data implies
that there is some other positively-acting element between -1 and
-100 which communicates with the UASINO element to regulate INO2
expression.

The data described in this section provides some preliminary
insights into the cis-acting elements responsible for controlling INO2
expression. However, further experiments will be required to
narrow down the exact location of certain elements such as a
potential TATA element in region -200 to -250 and the potential
positively acting element in region -1 to -100. Also, site-directed
mutagenesis of the UAS_{INO} element and the upstream AUG codon will need to be performed to determine their function.

*INO2* and *INO4* as yeast analogues of mammalian Myc and Max oncogenes

There are several features which suggest that the *INO2*/*INO4* genes are analogous to the mammalian Myc/Max oncogenes. For example, the basic organization of the two sets of proteins is similar with Ino2p providing the activation domain to the Ino2p/Ino4p heterodimer (Schwank *et al.*, 1995) but requiring Ino4p to bind DNA (Ambroziak and Henry, 1994; Nikoloff and Henry, 1994) just as Myc provides the activation domain but requires Max to bind DNA. Both Ino2 and Myc are phosphoproteins and the phosphorylation site (Ser-62 in Myc and Ser-69 in Ino2p) is located in the transcriptional activation domain (Hammond *et al.*, 1993; Henriksson *et al.*, 1993; Pulverer *et al.*, 1994). Also, the pattern of expression of the two genes is conserved since *INO2* and Myc expression is regulated and limiting relative to *INO4* and Max (see Table 5; Ayer and Eisenman, 1993; Larsson *et al.*, 1994; Ashburner and Lopes, 1995a). Conversely, *INO4* and Max expression is constitutive and non-limiting (see Table 5; Berberich *et al.*, 1992; Ayer and Eisenman, 1993; Ashburner and Lopes, 1995a). The observation that the human Sin3 repressor protein interacts with Max via the Mad and Mxi proteins has recently been described (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995). Previously it had been shown that the yeast Sin3 protein is required for proper regulation of the phospholipid biosynthetic genes (Hudak *et al.*, 1994). Finally, it is also clear that these two sets of
proteins have common targets such as genes required for polyamine synthesis (Bello-Fernandez et al., 1993) and cell cycle progression (Hammond et al., 1993; Amati and Land, 1994). As more target genes of Myc/Max regulation are discovered, it will be interesting to see how many of these genes overlap with those known to be regulated by the Ino2p/Ino4p complex. The striking similarities between these two sets of proteins may help in further understanding the function of Myc/Max in mammalian cells by utilizing the knowledge of INO2/INO4 function in yeast.

**Future directions**

There are several avenues to pursue in order to further understand how the INO2, INO4, and OPI1 gene products contribute to regulation of phospholipid biosynthetic gene regulation. Among these are determining the exact interaction between Opi1p and Ino2p. One possibility is to look for mutations in INO2 which prevent an interaction between the Ino2p and Opi1p. A mutation such as this would presumably display the Opi+ phenotype, and would be indicative of a direct interaction between Ino2p and Opi1p. A second possibility is to look for OPI1 superrepressor mutations. That is, mutations in OPI1 which do not allow the phospholipid biosynthetic genes to be derepressed in the absence of inositol. The phenotype of these mutations would likely be due to the inability of Opi1p to dissociate from the Ino2p/Ino4p complex.

A second avenue to pursue is to identify other partners for the Ino4p and to identify other genes which require the INO4 gene for their expression, but do not require INO2. A genetic screen is
currently underway in the Lopes laboratory to identify other partners for Ino4p. It will be interesting to determine if these other partners have any homologies to the mammalian Mad and Mxi proteins, two other proteins known to heterodimerize with Myc. There are currently two known genes, \textit{INO4} and \textit{CTR1}, which require Ino4p but not Ino2p for their expression. Given the pleiotropic phenotype of \textit{ino4} mutant strains, it is likely that there are many other genes which require Ino4p but not Ino2p.

Finally, a more detailed analysis of the \textit{INO2} promoter is needed to better understand its regulation. This includes identifying the TATA element, determining the function of the UAS\textsubscript{INO} element, identifying any other positively acting elements, and determining the function of the upstream AUG translation start codon.

Concluding remarks

In this work, I have shown that phospholipid biosynthetic gene expression is regulated by two superimposed mechanisms, the regulated expression of the \textit{INO2} activator gene and the \textit{OPT1} repressor gene. This is significant since it is the only known pathway in yeast which utilizes two mechanisms to regulate gene expression in response to a single signal (inositol). Furthermore, it has become apparent through this work and other ongoing work in this laboratory that the \textit{INO2} and \textit{INO4} genes are analogous to the mammalian Myc and Max oncogenes. Therefore, the study of \textit{INO2} and \textit{INO4} function and regulation could be an important paradigm for better understanding the function of these oncogenes.


VITA

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Mr. Ashburner received a Bachelor of Arts in Biology from St. Anselm College in Manchester, New Hampshire in May of 1989. He then went on to work in the laboratory of Glenn J. Bubley, M.D. at Beth Israel Hospital/Harvard Medical School from June of 1989 to July of 1991, studying the mechanism of cis-diaminedichloroplatinum-mediated mutagenesis. In 1991, he enrolled in the Program in Molecular Biology at Loyola University Chicago, Maywood, Illinois. He joined the laboratory of John M. Lopes, Ph.D., in 1992, where he studied the mechanisms controlling phospholipid biosynthetic gene expression in the yeast Saccharomyces cerevisiae. In 1994, Mr. Ashburner was awarded a fellowship by the Arthur J. Schmitt Foundation.

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