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Autoregulated Expression of the Yeast INO2 and INO4 Helix-Loop-Helix Activator Genes Effects Cooperative Regulation on Their Target Genes

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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 (derepressing), the products of the INO2 and INO4 genes form a heterocomplex which binds to a 10-bp element, upstream activation sequence INO (UASINO), in the promoters of the phospholipid biosynthetic genes to activate their transcription. In the presence of inositol and choline (repressing), the product of the OPII gene represses transcription dictated by the UASINO element. Curiously, we identified a UASINO-like element in the promoters of both the INO2 and INO4 genes. The presence of the UASINO element in these two promoters suggested that the mechanism for the inositol-choline response would involve regulating expression of the two activator genes. Using a cat reporter gene, we find that INO2-cat expression was regulated 12-fold in response to inositol and choline but that INO4-cat was constitutively expressed. We further observed that INO2-cat was not expressed in either an ino2 or an ino4 mutant strain and was constitutively overexpressed in an opi1 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. Our data show that derepression of phospholipid biosynthetic gene expression involves two mechanisms: increasing the levels of the INO2 and INO4 gene products and inactivating the OPII-mediated repression mechanism. We propose a model suggesting that this dual mechanism of regulation accounts for the observed cooperative stimulation of INO1 and CHO1 gene expression (phospholipid biosynthetic genes).

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 positively and negatively acting sequence elements in gene promoters is a primary means of regulating expression (33–35, 41). A further level of control can be imposed by regulation of the steady-state expression of regulatory genes. This can be accomplished by a number of different mechanisms. For example, the genes involved in galactose metabolism in the yeast Saccharomyces cerevisiae are partially controlled by regulation of transcription of the GAL4 regulatory gene (13). In this system, a fourfold transcriptional regulation of the gene encoding the GAL4 activator amplifies to a 170-fold regulation of the GAL1 structural gene. A different mechanism is utilized in control of the genes for amino acid biosynthesis, in which expression of the GCN4 activator protein is regulated at the level of translation (16). A third mechanism for control of expression of activator genes is by regulation of the decay rate of their messages. For example, the message for the MATa1 activator gene, which regulates genes involved in specification of cell type, has a half-life of 5 min (8). The message for the PRP1 regulatory gene, which controls transcription of two genes involved in pyrimidine biosynthesis, has a half-life of 1 min (42). The stabilities of both of these messages are well under the average of 17 min for poly(A)+ mRNA in S. cerevisiae. Regardless of the mechanism, proper control of expression of genes encoding regulatory proteins appears to be important for cells since overexpression of many activators (including GAL4) is deleterious (11) and even small changes in expression of a regulatory gene can be amplified to produce large effects on the expression of target genes (13, 48).

In S. cerevisiae, the major membrane phospholipids, phosphatidylinositol (PI) and phosphatidylcholine (PC), are synthesized by two separate pathways that diverge from a common lipid precursor, CDP-diacylglycerol (CDP-DG) (6, 24). Enzymes in the PI and PC biosynthetic pathways, as well as the enzyme that synthesizes their common precursor, CDP-DG, are regulated in a coordinate fashion in response to the phospholipid precursors inositol and choline (4, 7, 9, 18). The coordinate regulation of the genes that encode these enzymes is accomplished by control of their expression at the level of transcription through a common set of cis-acting regulatory elements and their cognate trans-acting factors (37, 52).

Two classes of regulatory genes affecting expression of the coordinately regulated genes involved in phospholipid biosynthesis have been identified. One class includes the INO2 and INO4 genes, which encode positive regulators of INO1 expression (17) as well as other phospholipid biosynthetic genes (37). Recessive mutations in either of these genes reduce expression of the phospholipid biosynthetic genes to repressed levels (4, 17). Analysis of the sequences of the INO2 and INO4 genes predicts protein products that include a helix-loop-helix (HLH) motif (19, 39) which is common to a number of proteins involved in transcriptional regulation (36). These observations suggest that the products of the INO2 and INO4 genes interact to activate transcription of the phospholipid biosynthetic
genes. Consistent with this hypothesis, a protein-DNA complex that assembles with the INO1 promoter and is dependent on wild-type alleles of the INO2 and INO4 genes has been identified (31). Recently, Ino2p-specific antibodies were used to demonstrate that Ino2p is present in this complex (38). In addition, transcription and translation of both the INO2 and INO4 genes in vitro have been used to show that both Ino2p and Ino4p are present in this complex and that they are capable of forming a heterodimer independently of a DNA template (2).

A second class of regulatory mutation is characterized by the opi1 mutation, which causes a constitutive overexpression of the INO1 transcript, resulting in an overproduction of inositol (51). This suggests that the wild-type OPI1 gene product is a negative regulator of INO1 expression. The OPI1 gene encodes a protein with a leucine zipper domain. While this domain is common to transcription-regulatory proteins (22, 26), it is unlikely that Opi1p binds DNA. In our current model, Opi1p functions by interacting with the Ino2p-Ino4p heterodimer to inhibit its function. This model is based on evidence showing that mutations in both INO2 and INO4 are epistatic to OPI1 mutations (30) and that INO2, INO4, and OPI1 functions are dependent on a common cis-acting element (25). In this respect, OPI1 would function analogously to the product of the GAL80 gene, which interacts with the GAL4 transcriptional activator to inhibit its function (23, 27).

The promoters of the coordinately regulated phospholipid biosynthetic genes contain a common cis-acting regulatory element, upstream activation sequence INO (UASINO), which is both necessary and sufficient to mediate the inositol-choline response (25). This element is defined by the consensus sequence S:\`C/ATGTTGAAT-3\` and serves as the binding site for the heterodimer formed between the products of the INO2 and INO4 genes (2, 38). The UASINO element is similar to that reported for the promoters of two fatty acid synthesis genes (FAS1 and FAS2) which are moderately regulated in response to inositol and choline (45).

A computer-assisted search of the promoters of the INO2 and INO4 genes revealed that they also contain a single copy of the UASINO element (see Fig. 1). This led us to investigate whether expression of these activator genes is also regulated in response to inositol and choline. We propose a model in which the autoregulated transcription of the INO2 and INO4 genes plays a role in cooperative derepression of phospholipid biosynthetic gene expression.

MATERIALS AND METHODS

Strains and growth conditions. The yeast strains used in this study were BR51001 (MAT\(a\) ade2 his3 leu2 can1 trp1 ura3) a1a (MAT\(a\) ade2 his3 leu2 ura3 trp1 ino2::TRPI), NUL 20 (MAT\(a\) ana3 his3 leu2 ino4::LEU2), and BR51021 (MAT\(a\) opil ade5 leu2 trp1 ura3). All cultures were grown at 30°C in synthetic medium (17) containing 2% glucose (vol/vol) and either containing 75 \(\mu\)M inositol and 1 mM choline or lacking inositol and choline.

Plasmid construction and chromosomal integration. All plasmids used in this study were derived from pbBM2015 supplied by Mark Johnston (Washington University, St. Louis, Mo.) (14). 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 and subcloning them into a BamHI site in plasmid pbBM2015 upstream of the GAL4-cat fusion. The 3\' end of each promoter is \(+1\), with +1 being the first nucleotide of the ATG translation start codon. The 5\' ends of the indicated promoters were as follows: INO2 −506; INO4, −493; OPI1, −453; and INO1, −439. The promoter-cat fusions were integrated at the GAL4 chromosomal locus by transformation of yeast with the products of a restriction digestion that releases a 7.6-kb fusion construct from the vector (Fig. 2). Since the ends of DNA fragments are highly recombinogenic (40), UR\(A^4\) transformants arise by recombination between sequences flanking GAL4. Southern blot analysis confirmed proper integration of the fusions in all transformants tested.

Enzyme assays. For CAT assays, 5-mL cultures were grown to 50 to 60 Klett units in synthetic medium containing or lacking inositol and choline. Cells were pelleted, washed with 0.5 ml of 0.25 M Tris-HCl (pH 7.5), and resuspended in 0.2 ml of ice-cold 0.25 M Tris-HCl (pH 7.5). Acid-washed glass beads were added to a level 1 to 2 mm below the meniscus, and the cells were shaken at maximum speed on a Vortex for 4°C for eight 20-s periods, with 20-s pauses between each period. Cellular debris were pelleted in a microcentrifuge at 4°C for 5 min, and cell extracts were stored at −70°C. The protein concentration in each extract was determined with a Bio-Rad protein assay kit (Bio-Rad, Rockville Center, N.Y.).

RNA analysis. RNA was isolated from yeast by a glass bead disruption and hot phenol extraction method (10). RNA probes (cRNA) for Northern (RNA) hybridizations were synthesized with the Gemini II Core System (Promega) from plasmids linearized with a restriction enzyme and transcribed with an RNA polymerase as follows (shown as plasmid, restriction enzyme, RNA polymerase) for the indicated (parenthesized) probe: pAB309\(\Delta\), EcoRI, SP6 (T7CI);
RESULTS

INO2-cat, but not INO4-cat, expression is regulated by inositol and choline. To determine if INO2 and/or INO4 are regulated in response to inositol and choline, we constructed plasmids that fused sequences upstream of the ATG 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. Strains harboring the fusion constructs were assayed for CAT activity under derepressing conditions (75 μM inositol and 1 mM choline). The data show that INO2-cat was not expressed in either the ino2 or ino4 mutant strain (Table 2). It should be noted that the expression of the INO2-cat construct at this concentration of inositol was reduced to a level equal to that of a promoterless

TABLE 1. Regulation of INO2-cat gene expression

<table>
<thead>
<tr>
<th>Reporter gene</th>
<th>CAT activity (U) in:</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I−C−</td>
<td>I+C+</td>
</tr>
<tr>
<td>INO2-cat</td>
<td>0.75</td>
<td>0.08</td>
</tr>
<tr>
<td>INO4-cat</td>
<td>4.41</td>
<td>4.37</td>
</tr>
<tr>
<td>OP11-cat</td>
<td>18.50</td>
<td>11.00</td>
</tr>
<tr>
<td>INO1-cat</td>
<td>33.90</td>
<td>1.30</td>
</tr>
<tr>
<td>Promoterless-cat</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

a Each reporter gene was integrated in a single copy at the GAL4 locus of BR51001 as described in Materials and Methods.

b Assays were carried out with extracts from yeast transformants harboring each of the reporter genes. Each value is the average of data from at least four experiments.

c Average of the fold differences (I−C−/I+C+) for each experiment, with standard deviation in parentheses.

d I−C−, complete synthetic medium (17) lacking inositol and choline.

e I+C+, complete synthetic medium (17) supplemented with 75 μM inositol and 1 mM choline.

originates from the inserted promoters. In addition, the INO4-cat construct was expressed at a level sixfold higher than was the INO2-cat construct under derepressing conditions, which suggests that INO2 expression is limiting relative to that of INO4.

Interestingly, the pattern of INO2-cat expression is reminiscent of the pattern of expression of one of its target genes, INO1 (17). This point was illustrated by analysis of INO1-cat expression (Table 1). The results agree with published data describing INO1 regulation (17) and show that INO2-cat was expressed at a level roughly 45-fold less than was INO1-cat.

INO2-cat and INO4-cat expression is autoregulated. To determine if INO2 and INO4 expression is autoregulated, the INO2-cat and INO4-cat reporter constructs were transformed into both ino2 and ino4 null mutant strains. Strains harboring the fusion constructs were assayed for CAT activity under repressing conditions (75 μM inositol and 1 mM choline). The data show that INO2-cat was not expressed in either the ino2 or ino4 mutant strain (Table 2). It should be noted that the expression of the INO2-cat construct at this concentration of inositol was reduced to a level equal to that of a promoterless
cat construct. Further, the repressed level of expression of INO2-cat in the ino2 and ino4 mutant strains was about fourfold lower than the repressed level in the wild-type strain (Table 2). The INO2-cat strains were also grown in medium containing 10 μM inositol to test the effects of the ino2 and ino4 mutations under derepressing conditions. This concentration of inositol is the minimal amount required for growth of the ino2 and ino4 strains while simultaneously allowing partial derepression of gene expression. While in the wild-type strain an intermediate level of expression of INO2-cat was observed (Table 2), INO2-cat was not expressed in either the ino2 or ino4 mutant strains at this concentration (Table 2). These results suggest that wild-type alleles of both the INO2 and INO4 genes are required for expression of the INO2 gene.

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 2). However, INO4-cat expression was abolished in the ino4 mutant strain (Table 2). This suggests that the constitutive expression of INO4 requires a wild-type copy of the INO4 gene but does not require the INO2 gene.

The INO2-cat gene is overexpressed in an op1 mutant strain. Regulation of expression of the phospholipid biosynthetic genes is also controlled by a negative regulator, encoded by the OPI1 gene (51), and its action is dependent upon the UASINO gene. Strains harboring mutant alleles of the OPI1 gene constitutively overexpress the phospholipid biosynthetic genes (4, 17). To determine if INO2 and INO4 expression is also negatively regulated by OPI1, the INO2-cat and INO4-cat fusion constructs were used to transform an op1 mutant strain. The data show that in the op1 strain INO2-cat was overexpressed constitutively at a level higher than the fully derepressed level in the wild-type strain (Table 3). Curiously, INO2-cat expression in this strain was equivalent to that of INO4-cat in the wild-type strain. In contrast, INO4-cat expression was unaffected by this mutation. Therefore, INO2, but not INO4, expression is negatively regulated by the product of the OPI1 gene.

OPI1-cat gene expression is modestly regulated and nonlimiting. Since the OPI1 negative regulatory gene was required for the inositol-choline response, we reasoned that its expression may also be regulated. This type of mechanism has already been reported for the GAL80 gene of S. cerevisiae (21, 46).

Therefore, we determined the level of expression from the OPI1 promoter using the cat reporter system. The data show that there was a modest regulation of OPI1-cat expression (1.7-fold; Table 1). However, the more significant observation was that OPI1-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 OPI1 gene.

Cooperative regulation of the phospholipid biosynthetic genes by INO2. Since INO2-cat expression was regulated and the target genes have multiple INO2-binding sites, we examined whether there is cooperativity in regulation of phospholipid biosynthetic gene expression. To do this, we quantitated expression of two INO2 target genes 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 (Fig. 3). The constitutively expressed ribosomal protein gene TCM1 (32) was used to normalize for loading variations.

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The patterns of expression of INO1 and CHO1 relative to INO2-cat were best fit by a sigmoidal curve (Fig. 4). 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 (12, 13), which was surprising since the CHO1 promoter only has a single UASINO element (3). Even the INO1 cooperativity cannot be explained on the basis of multiple UASINO elements since there was no synergism between the two INO1 UASINO elements (25, 31). A model to explain this cooperativity will be discussed in detail later.

Kinetics of INO2 and INO1 derepression. Regulation of INO2-cat expression is reminiscent of that of its target genes. For example, the expression of both INO2-cat and INO1-cat was regulated (Table 1), sensitive to ino2 and ino4 mutant alleles (Table 2) (17), and regulated by the OPI1 repressor (Table 3) (17). 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 for these two genes. Strains harboring either an INO1-cat fusion or an INO2-cat fusion 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, INO2-cat expression is moderately higher than INO1-cat expression at an earlier time point (Fig. 5). Both genes were fully derepressed at 2.5 h.

Relative promoter strengths. Our analysis of INO2-cat and INO4-cat expression suggests that the promoters of these two genes are extremely weak. To examine this, we compared fully derepressed expression from several yeast promoters using cat fusions (data not shown). The experiments revealed that the GAL4 promoter (previously the weakest known promoter) was about 2.1-fold stronger than the INO2 promoter while the INO4 promoter was slightly stronger than the GAL4 promoter (2.8-fold). The OPI1 and the INO1 promoters were substantially stronger than the INO2 promoter (24.7- and 45.2-fold, respectively).

DISCUSSION

Regulation of phospholipid biosynthesis is dictated by the UASINO element, which serves as a binding site for Ino2-Ino4 heterodimer (2, 25, 34). Here, we report that expression of an INO2-cat reporter gene was found to be controlled by this same regulatory scheme (autoregulation) while an INO4-cat gene was expressed constitutively (Table 1). The regulated expression of the INO2-cat gene suggests that regulation of INO2 transcript abundance may be an important mechanism for control of phospholipid biosynthetic gene expression. However, a caveat that should be noted is that we have not determined if the levels of Ino2 protein reflect the regulation observed from the INO2-cat reporter gene.

Expression of the INO2-cat gene was regulated 12-fold in response to inositol and choline, but even under derepressing conditions its expression was limiting relative to expression of the INO4-cat genes. Interestingly, in the opi1 mutant strain INO2-cat was constitutively overexpressed and its expression was equal to that of the INO4-cat gene in a wild-type strain (Table 3). This suggests that INO4 expression may act as a backstop to ensure that INO2 expression does not exceed that of INO4. Furthermore, the overexpression of the INO2-cat gene explains why phospholipid biosynthetic genes are constitutively overexpressed in an opi1 mutant strain (4, 17).

Our finding that the INO4-cat gene was expressed constitutively disagrees with preexisting data (45) which showed that INO4-lacZ expression was repressed twofold in response to inositol and choline. However, the level of INO4-lacZ expression was actually below the limits of sensitivity for the assay and was compared with that of an entirely different control vector. In the present report, the level of INO4-cat expression was substantially above the lower limits of sensitivity for this system. Moreover, our experiments examined INO4 promoter activity in a native context (chromosomal) and in a single copy, thus avoiding any potential multicopy or plasmid-related artifacts. We have also determined that the native INO4 mRNA is
constitutively expressed (2a), using a reverse transcription-PCR protocol (50).

Expression of the INO2-cat gene was weak relative to the expression of several other yeast genes, including GAL4. Previously, the GAL4 regulatory gene was recognized as the weakest promoter in yeast (13). The low level of GAL4 transcription is established by two elements, UAS\textsubscript{GAL4} and UES\textsubscript{GAL4} (UES stands for upstream essential sequence), which can only function together (14). That is, UAS\textsubscript{GAL4} is not able to function in concert with a canonical TATA element and UES\textsubscript{GAL4} is not able to function with a heterologous UAS element (14). However, the mechanism that establishes weak expression of INO2 must be different, since the INO2 promoter does contain a UAS\textsubscript{INO} element which is sufficient to confer inositol- and choline-dependent expression from a heterologous TATA element (25). A computer-assisted search of the INO2 promoter failed to identify an element resembling a TATA box. Therefore, it is likely that a poor TATA element and/or a weak transcription initiation site (15, 49) may be responsible for the weak expression of the INO2 gene.

Expression of the INO4-cat gene was dependent on a wild-type allele of the INO4 gene but not the INO2 gene (Table 2). Since the Ino4 protein does not appear to have any transcriptional activation domain (2a) and does not homodimerize (2), it must form a heterodimer with another protein that has a transcriptional activation domain. 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 (1). We have already determined that the other three known yeast transcriptional activator HLH proteins, Pho4 (44), Cbf1 (5), and Rtg1 (29), are not required for INO4 expression. Consequently, there must be another as-yet-unidentified partner for the INO4 gene. Another issue that must be addressed is that of 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 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 (47). Interestingly, the yeast CTR1 gene also requires INO4 but not INO2 for its expression (28). A comparison of the CTR1 and INO4 promoters identified a consensus HLH binding site (CAA/TTG) that deviates from the UAS\textsubscript{INO} element.

The data showed that cooperativity plays a role in control of expression of INO1 and CHO1 (Fig. 4). This is not unprecedented in \textit{S. cerevisiae}, since cooperativity was also invoked in GAL4 activation of GAL1 expression (12, 13). A similar mechanism could exist for activation of the INO1 gene, since there are two UAS\textsubscript{INO} elements in this promoter (25). However, there is no evidence of synergism between these elements or cooperativity of binding to these two sites (25, 31). Therefore, we propose an alternative model to explain the cooperative derepression of INO1 and CHO1 expression (Fig. 6). 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 derepress expression of the INO2, INO1, and CHO1 genes and 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 Opi1 protein be inactivated, allowing the phospholipid biosynthetic genes as well as the INO2 activator gene to be derepressed and resulting in complete derepression of these genes. Therefore, cooperativity in this system results from the concomitant derepression of the INO2 activator gene and inactivation of the Opi1 repressor protein. This model predicts that Opi1 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. The OPI1-cat gene is overexpressed relative to both INO2-cat and INO4-cat, and its expression is essentially unaffected by inositol and choline (Table 1). Furthermore, it has already been reported that the amount of Ino2-Ino4-UAS\textsubscript{INO} complex is affected by inositol and choline (31). Therefore, the model predicts that OPI1 is required for the initial response to inositol and choline but that regulation of INO2 expression establishes the magnitude and cooperativity of the response.

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