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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 heteromeric complex which binds to a 10-bp element, upstream activation sequence *INO* (UAS_{INO}), in the promoters of the phospholipid biosynthetic genes to activate their transcription. In the presence of inositol and choline (repressing), the product of the *OPI1* gene represses transcription dictated by the UAS_{INO} element. Curiously, we identified a UAS_{INO}-like element in the promoters of both the *INO2* and *INO4* genes. The presence of the UAS_{INO} 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 *OPI1*-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 *MAT*α*1* activator gene, which regulates genes involved in specification of cell type, has a half-life of 5 min (8). The message for the *PPR1* 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

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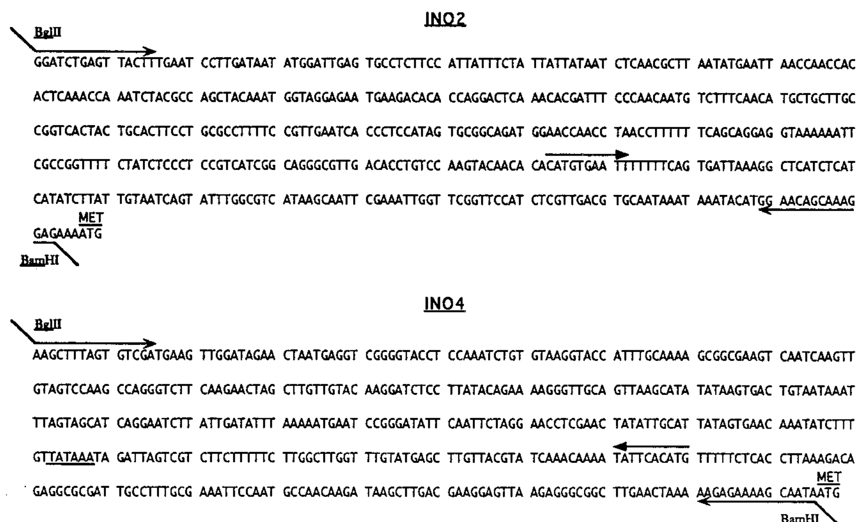

FIG. 1. *INO2* and *INO4* promoter sequences. Noted for reference are the initiation codons (MET), potential UAS_{INO} elements (arrows indicate orientations), a potential TATA box in the *INO4* promoter (underlined), and the oligonucleotide primers (with flanking restriction sites) used for PCR amplification.

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* (UAS_{INO}), which is both necessary and sufficient to mediate the inositol-choline response (25). This element is defined by the consensus sequence 5′-C/ATGTGAAAT-3′ and serves as the binding site for the heterodimer formed between the products of the *INO2* and *INO4* genes (2, 38). The UAS_{INO} 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 UAS_{INO} 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 that 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 BRS1001 (*MAT***α** *ade2 his3 leu2 can1 trp1 ura3*), α1a (*MAT***α** *ade2 his3 leu2 ura3 trp1 ino2*::*TRP1*), NUL 20 (*MAT***α** *ura3 his3 leu2 ino4*::*LEU2*), and BRS1021 (*MAT*α *opi1 ade5 leu2 trp1 ura3*). All cultures were grown at 30°C in synthetic medium (17) containing 2% glucose (vol/vol) and either containing 75 μM inositol and 1 mM choline or lacking inositol and choline.

Plasmid construction and chromosomal integration. All plasmids used in this study were derived from pBM2015 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 *Bam*HI site in plasmid pBM2015 upstream of the *GAL4-cat* fusion. The 3′ end of each promoter is at −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, −495 (Fig. 1); *INO1*, −453; and *OPI1*, −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), URA^+ 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 at 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.), and CAT activities were determined by the phase extraction method (43). Generally, 10 μg of protein was assayed in a 100-μl reaction volume. 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 the time of incubation (in hours).

RNA analysis. RNA was isolated from yeast by a glass bead disruption and hot phenol extraction procedure (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: pAB309Δ, *Eco*RI, SP6 (*TCM1*);

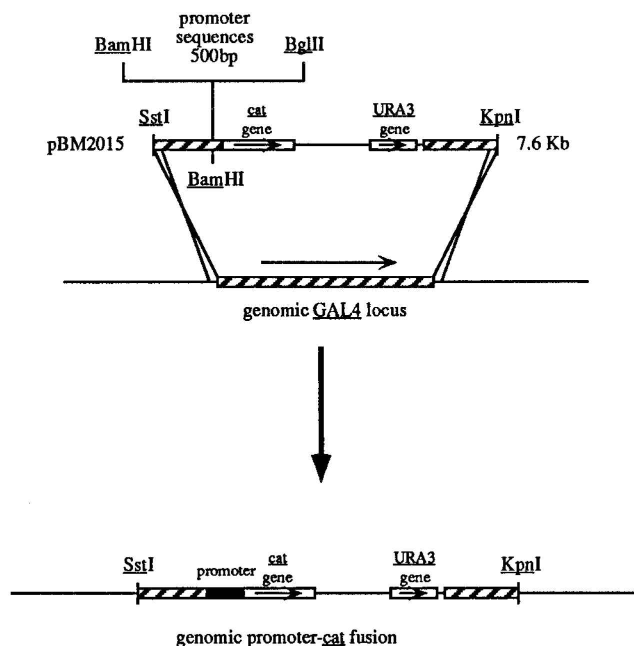

FIG. 2. Integration of *INO2*- and *INO4-cat* fusions in a single copy at the *GAL4* locus of *S. cerevisiae*. DNA fragments (approximately 500 bp) containing the *INO2* or the *INO4* promoter flanked by *Bam*HI-*Bgl*II sites were inserted into the *Bam*HI site of pBM2015 (14). A 7.6-kb *Sst*I-*Kpn*I 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.

pJH310, *Hin*dIII, T7 (*INO1*); pAS103, *Hin*dIII, T7 (*CHO1*) (20). Northern hybridizations were performed as previously described (17), and results were visualized by autoradiography and quantitated by densitometry.

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 to 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 (see the text) and assayed for CAT activity as described earlier.

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 by recombination at the *GAL4* locus (Fig. 2). We chose this assay because we suspected that expression of *INO2* and *INO4* would be low since genes encoding activators generally tend to be weakly expressed (14, 42). The data in Table 1 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. As a control, a promoterless *cat* construct was also analyzed and the results show that there was no *cat* expression from this vector (Table 1). Therefore, any expression observed in this system must originate 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*.

TABLE 1. Regulation of *INO2-cat* gene expression

Reporter gene[a]	CAT activity (U)[b] in: I−C−[d]	CAT activity (U)[b] in: I+C+[e]	Fold difference[c]
INO2-cat	0.75	0.08	12.3 (2.8)
INO4-cat	4.41	4.37	1.0 (0.1)
OPI1-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] Each reporter gene was integrated in a single copy at the *GAL4* locus of BRS1001 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.

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

TABLE 2. Autoregulation of *INO2-cat* and *INO4-cat* gene expression

Reporter gene[a]	Relevant strain genotype[b]	CAT activity (U)[c] in medium with: 10 μM inositol, 1 mM choline	CAT activity (U)[c] in medium with: 75 μM inositol, 1 mM choline
INO2-cat	Wild type	0.43	0.08
INO2-cat	*ino2*	0.02	0.02
INO2-cat	*ino4*	0.01	0.02
INO4-cat	Wild type	ND[d]	4.37
INO4-cat	*ino2*	3.35	4.20
INO4-cat	*ino4*	0.01	0.02
Promoterless-*cat*	*ino2*	0.02	0.02
Promoterless-*cat*	*ino4*	0.02	0.02

[a] Each reporter gene was integrated in single copy at the *GAL4* locus as described in Materials and Methods.
[b] The strains used in these experiments were BRS1001 (wild type), α1A (*ino2*), and NUL20 (*ino4*).
[c] 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 three experiments.
[d] ND, not determined.

TABLE 3. Regulation of *INO2-cat* expression by the *OPI1* gene

Reporter gene[a]	Relevant strain genotype[b]	CAT activity (U)[c] in:	
		I−C−[d]	I+C+[e]
INO2-cat	Wild type	0.75	0.08
INO2-cat	*opi1 INO2 INO4*	4.00	5.23
INO4-cat	Wild type	4.41	4.37
INO4-cat	*opi1 INO2 INO4*	4.36	5.85
Promoterless-*cat*	Wild type	0.01	0.01
Promoterless-*cat*	*opi1 INO2 INO4*	0.02	0.02

[a] Each reporter gene was integrated in a single copy at the *GAL4* locus as described in Materials and Methods.
[b] The strains used in these experiments were BRS1001 (wild type) and BRS1021 (*opi1 INO2 INO4*).
[c] 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 three experiments.
[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.

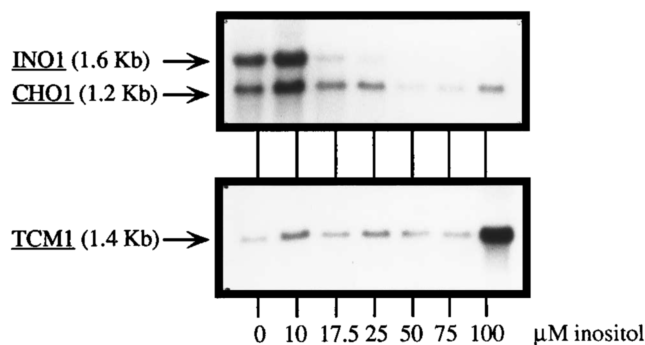

FIG. 3. Northern blot hybridization of phospholipid biosynthetic genes. A representative Northern blot hybridization showing *INO1* and *CHO1* transcript levels in cells grown in complete synthetic media supplemented with various inositol concentrations (0 to 100 μM) is displayed. The constitutively expressed ribosomal protein gene *TCM1* (32) was used to normalize for loading variations.

cat construct. Further, the repressed level of expression of *INO2-cat* in the *ino2* and *ino4* mutant strains was about four-fold 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 *opi1* 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 UAS_{INO} element. 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 *opi1* mutant strain. The data show that in the *opi1* 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* was used to normalize for loading variations. 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 UAS_{INO} element (3). 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 (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

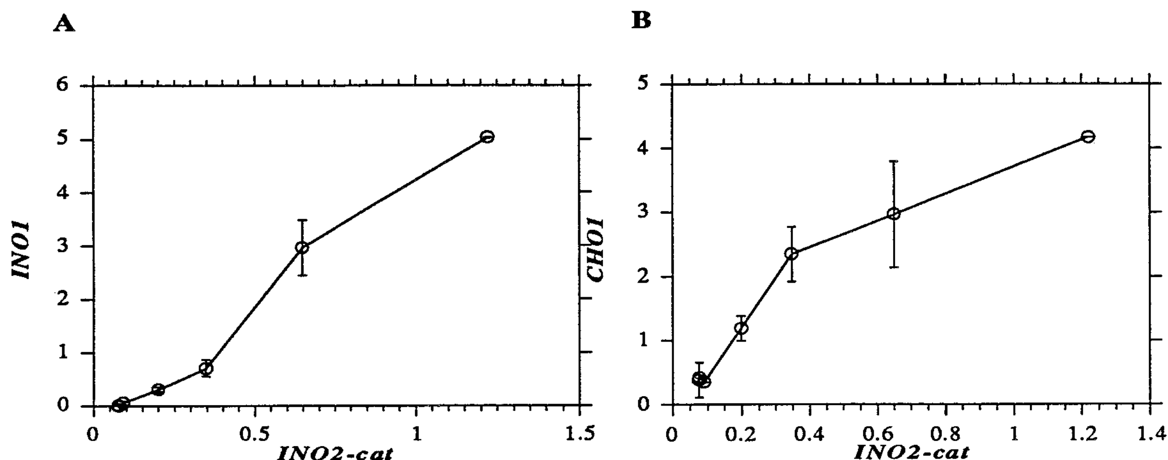

FIG. 4. Effects of variation in levels of *INO2* expression on *INO1* (A) and *CHO1* (B) expression. *INO2* expression was determined by assay of *INO2-cat* activity. *INO1* and *CHO1* expression was determined by Northern blot hybridization. The extent of hybridization was quantitated by densitometry (arbitrary units), normalized for loading variations with the constitutively expressed ribosomal protein gene *TCM1* (32). All assays were performed with cultures growing exponentially in complete synthetic medium (17) containing 1 mM choline and either lacking inositol or containing 10, 17.5, 25, 50, 75, or 100 μM inositol.

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).

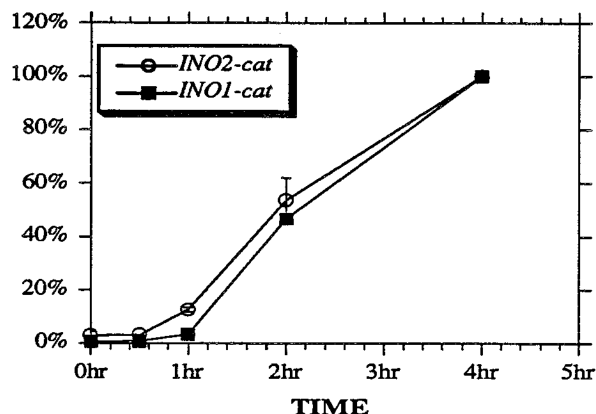

FIG. 5. Comparison of derepression kinetics of *INO1-cat* and *INO2-cat* expression. Strains harboring an *INO2-cat* fusion or an *INO1-cat* fusion were grown to mid-log phase in repressing medium (containing inositol and choline) and switched to derepressing medium (containing neither inositol nor choline). Samples were taken at various time points and assayed for CAT activity.

DISCUSSION

Regulation of phospholipid biosynthesis is dictated by the UAS_{INO} 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_{GAL4} and UES_{GAL4} (UES stands for upstream essential sequence), which can only function together (14). That is, UAS_{GAL4} is not able to function in concert with a canonical TATA element and UES_{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_{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_{INO} element in the *INO4* promoter. Conceivably, single-base changes from the UAS_{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_{INO} element.

The data showed that cooperativity plays a role in control of expression of *INO1* and *CHO1* (Fig. 4). This is not unprecendented in *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_{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). We therefore 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 decrease expression of the *INO2*, *INO1*, and *CHO1* genes and reduce the amount of Ino2-Ino4 heterodimer available to bind the UAS_{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

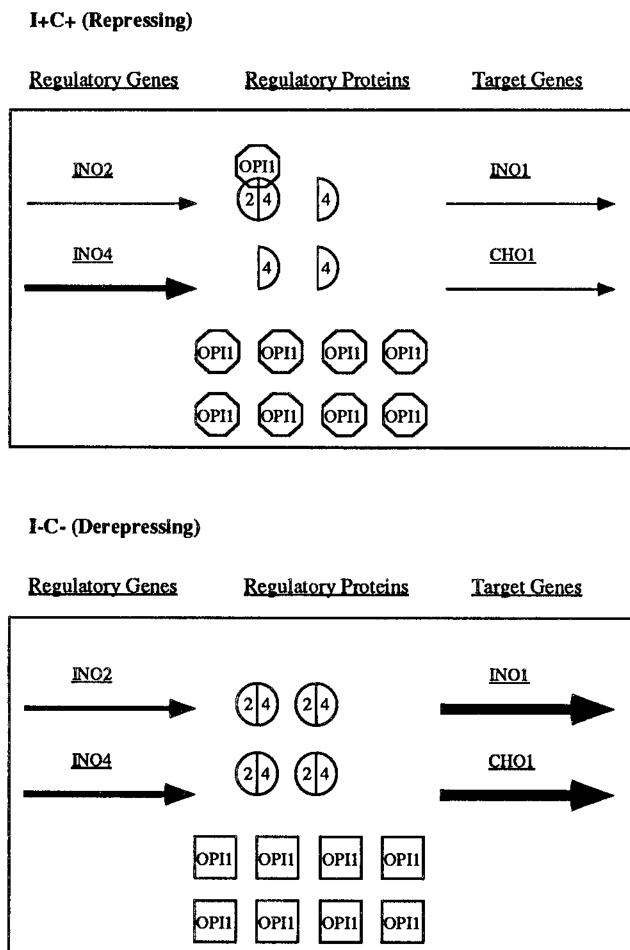

FIG. 6. Model for cooperative derepression of phospholipid biosynthetic gene expression in *S. cerevisiae*. See Discussion for a detailed explanation. I+C+, containing inositol and choline; I−C−, containing neither inositol nor choline.

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_{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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