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# The Promoter of the Yeast *INO4* Regulatory Gene: a Model of the Simplest Yeast Promoter

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In *Saccharomyces cerevisiae*, the phospholipid biosynthetic genes are transcriptionally regulated in response to inositol and choline. This regulation requires the transcriptional activator proteins Ino4p and Ino2p, which form a heterodimer that binds to the UAS$_{INO}$ element. We have previously shown that the promoters of the *INO4* and *INO2* genes are among the weakest promoters characterized in yeast. Because little is known about the promoters of weakly expressed yeast genes, we report here the analysis of the constitutive *INO4* promoter. Promoter deletion constructs scanning 1,000 bp upstream of the *INO4* gene identified a small region ($-58$ to $-46$) that is absolutely required for expression. S1 nuclease mapping shows that this region contains the transcription start sites for the *INO4* gene. An additional element ($-114$ to $-86$) modestly enhances *INO4* promoter activity (fivefold). Thus, the region required for *INO4* transcription is limited to 68 bp. These studies also found that *INO4* gene expression is not autoregulated by Ino2p and Ino4p, despite the presence of a putative UAS$_{INO}$ element in the *INO4* promoter. We further report that the *INO4* steady-state transcript levels and Ino4p levels are regulated twofold in response to inositol and choline, suggesting a posttranscriptional mechanism of regulation.

Transcriptional regulation is a mechanism, in many organisms, by which to coordinate the expression of multiple genes in response to various cellular conditions. Specific *cis* and *trans* elements mediate the control of transcription. In *Saccharomyces cerevisiae*, an upstream activating sequence (UAS) located within 1,400 bp of the translational start site dictates the binding of activator proteins (11). Once bound to the UAS element, activator proteins interact with the general transcriptional machinery located at the TATA box ($-60$ to $-120$ bp). Therefore, it is the interactions between promoter elements and transcription factors that direct the timing and strength of gene expression. However, much of what is known about yeast promoters comes from studies of genes expressed in easily detectable quantities. With the complete genome sequence available and the advent of genome-wide approaches to the study of gene expression, it is becoming clear that there are a large number of weakly expressed genes that play an important role in the regulation of various processes in yeast. However, little is known about the *cis* and *trans* elements necessary for weakly expressed genes, although it is clear from studies on the *GAL4* promoter that novel *cis*-acting elements are required for expression. For example, studies on the *GAL4* promoter have identified an upstream essential sequence element that is required for basal transcription but is not interchangeable with a TATA element (10). Here we present an analysis of the *INO4* promoter to further our understanding of the *cis*-acting elements necessary for weak promoter expression.

In *S. cerevisiae*, transcriptional regulation of the phospholipid biosynthetic genes is mediated by the *INO4* and *INO2* genes (9). Low levels of chloramphenicol acetyltransferase (CAT) activity from *INO4-cat* and *INO2-cat* promoter fusions places these genes into a small group of yeast promoters which drive weak gene expression (5). Sequence analysis of these two genes suggests that they belong to the basic helix-loop-helix (bHLH) family of transcriptional activators (15, 20). Consistent with this observation, the products of the *INO4* and *INO2* genes form a heterodimer that binds to a canonical bHLH binding site termed the UAS$_{INO}$ element (C/AATGTGAAAT) (3, 27). The first six base pairs of this element contain the canonical bHLH binding site (5′ CANNTG 3′) (1). The UAS$_{INO}$ element is found in the promoters of the coordinately regulated phospholipid biosynthetic genes *INO1*, *CHO1*, *CHO2*, and *OPI3* (9). Transcription of these genes is derepressed when inositol and choline are absent from the growth medium and repressed in the presence of inositol and choline. The UAS$_{INO}$ element is both necessary and sufficient to confer inositol-and-choline-mediated regulation (6, 17, 25).

Ino2p contains a transcriptional activation domain, which can activate a reporter gene when artificially tethered to DNA (27). On the other hand, it has been suggested that Ino4p cannot activate transcription on its own (27). This suggests that Ino4p is responsible for recruiting Ino2p to the UAS$_{INO}$ element that enables the transcriptional activation domain of Ino2p to activate transcription of the phospholipid biosynthetic genes. Strains containing mutant alleles of *INO2* or *INO4* are unable to derepress *INO1* expression, resulting in inositol auxotrophy (9, 13). Repression of the phospholipid genes in response to inositol-and-choline supplementation is mediated by the *OPI1* gene, which encodes a leucine zipper protein. Strains containing a mutant allele of the *OPI1* gene overexpress *INO1*, resulting in an overproduction-and-excretion-of-inositol phenotype (Opi$^+$) (14).

A previous study showed that an *INO2-cat* reporter gene is regulated by inositol and choline (5). Addition of inositol and choline repressed *INO2-cat* gene expression 12-fold. This regulation required both the *INO2* and *INO4* genes, showing that *INO2* is regulated in the same manner as the phospholipid biosynthetic genes. However, an *INO4-cat* gene was expressed constitutively with respect to inositol and choline and did not require the *INO2* gene (5). This suggested that the mechanism

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TABLE 1. Oligonucleotides used in this study

| INO4-BamHI (0) | 5′-GGGGGGATCCTATTGCTTTTCTCTT-3′ |
|---|---|
| INO4-1000 | 5′-AGATCTGATGCATCGCCGACC-3′ |
| INO4-750 | 5′-AGATCTTGGATTATAGTTGTT-3′ |
| INO4-A (−496) | 5′-GGGGAGATCTAAGCTTTAGTGTCGA-3′ |
| INO4-B (−396) | 5′-AGATCTTGTAGTCCAAGCCAG-3′ |
| INO4-C (296) | 5′-AGATCTTTTAGTAGCATCAGG-3′ |
| INO4-D (−186) | 5′-AGATCTGATTAGTCGTCTTCT-3′ |
| INO4-UAS (−125) | 5′-AGATCTTATTCACATGTTTTTC-3′ |
| INO4-E (−114) | 5′-AGATCTTTTTTTCTCACCTTAA-3′ |
| INO4-E1 (−86) | 5′-AGATCTTTGCCTTTGCGAAAT-3′ |
| INO4-E2 (−58) | 5′-AGATCTAGATAAGCTTGACGA-3′ |
| INO4-E3 (−30) | 5′-AGATCTGCGGCTTGAACTAAA-3′ |
| INO4-F (−46) | 5′-GGATCCGTCAAGCTTATCTTG-3′ |
| INO4-G (−186) | 5′-GGATCCTATTTATAACAAAGA-3′ |
| INO4-H (−201) | 5′-GGATCCTATTTGTTCACTATA-3′ |
| INO4-I (−346) | 5′-GGATCCGGAGATCCTTGTACA-3′ |
| INO4-3′ | 5′-CCCGGGTTAATTGTT-3′ |
| INO4-HA 5′ | 5′-ATGACGAAAGATCTTAAGGAGATACAA-3′ |
| INO4-HA 3′ | 5′-TATCTCCTTAAGATCTTTCGTCATTAT-3′ |
| INO4-PE | 5′-GCCAGTTCACCCTTTATCTCC-3′ |

controlling *INO4* expression might be different from that of the phospholipid biosynthetic genes and the *INO2* gene.

In the present study, we set out to define the *cis* elements necessary for *INO4* gene expression. We identified two elements of the *INO4* promoter that are necessary for full expression. One of these elements maps to the same region as the transcriptional start site. We also found that expression of the native *INO4* gene, and its protein product, is modestly regulated by inositol and choline. Our *INO4-cat* reporter gene fusions suggest that this regulation does not occur at the level of transcription initiation.

## MATERIALS AND METHODS

**Strains and growth conditions.** The yeast strains used in this study were BRS1001 (*MAT***a** *ade2-1 his3-11,15 leu2-3, 112 can1-100 ura3-1 trp1-1*), BRS2001 (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 ino2Δ::TRP1*), BRS2004 (*MAT*α *ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 ino4Δ::LEU2*), BRS2005 (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 opi1Δ::LEU2*), Null20 (*MAT***a** *his3-11,15 leu2-3,112 ura3-1 ino4Δ::LEU2*), SH307 (*MAT*α *his3-11,15 leu2Δ1 ura3-52 ino4Δ::LEU2*), and YB588 (*MAT***a** *ade2 ade3 his3 leu2 ura3 trp1 nmt1-451D ino4Δ::HIS3*). All cultures were grown at 30°C in synthetic medium either supplemented with 75 μM inositol and 1 mM choline or lacking inositol and choline (16).

**Plasmid constructions.** A nested set of *INO4* promoter deletions to be fused to the *cat* reporter gene was created by PCR using appropriate primers (Table 1). The 5′-terminal deletion PCRs used the 3′ primer INO4-BamHI along with the 5′ primers INO4-1000 through INO4-E3 (Table 1). The 3′-terminal promoter deletions used the 5′ primer INO4-A along with the 3′ primers INO4-F through INO4-I. The individual PCR products were inserted into pGEM-T (Promega, Madison, Wis.). Each deletion fragment was excised from the pGEM-T derivative by digestion with *Bgl*II and *Bam*HI and inserted into pBM2015 (10). The pBM2015 derivatives were transformed into yeast as previously described (5).

Plasmids derived from YCp50 were used to test the various promoter deletions for the ability to direct *INO4* expression. PCR was performed using the various 5′ deletion primers with the 3′ primer INO4-3′ to create the nested set of promoter deletions upstream of the entire *INO4* open reading frame (ORF). These PCR products were cloned into the pGEM-T vector. *Sal*I-*Sph*I fragments were isolated from the pGEM-T derivatives and cloned into YCp50.

A YCp50-*INO4*-HA construct was created by mutational PCR. PCR was used to introduce a *Bgl*II site three codons into the *INO4* ORF. To do this, a PCR using the primers INO4-3′ and INO4-HA 5′ created an 851-bp product containing the *INO4* ORF and a 5′ *Bgl*II site. A second PCR with the primers INO4-A and INO4-HA 3′ created a 500-bp product containing the *INO4* promoter and a 3′ *Bgl*II site. The PCR products were digested with *Bgl*II and ligated to create a product which contained the *INO4* promoter and ORF with a *Bgl*II site immediately downstream of the start codon. The ligated fragment was used as a template for another round of PCR containing the primers INO4-A and INO4-3′, resulting in a 1,350-bp product. This PCR fragment was cloned into pGEM-T. The pGEM-T derivative was partially digested with *Bgl*II, and a 120-bp *Bgl*II fragment containing three tandem copies of the hemagglutinin (HA) epitope

(provided by Susan Henry, Carnegie Mellon University) was inserted. A *Sal*I-*Sph*I fragment was isolated from the pGEM-T derivative and cloned into YCp50. All promoter constructs and the YCp50-*INO4*HA construct was confirmed by DNA sequencing. The plasmid pTA-*INO4* contains the entire *INO4* ORF, starting at the translational initiator codon and extending past the translational stop codon.

**CAT assays.** Five-milliliter cultures were grown to 50 to 60 Klett units in appropriate media. Assays were conducted as previously described (5). Units of CAT activity were defined as counts per minute measured in the organic phase and expressed as a percentage of the total counts per minute (percent conversion) divided by the amount of protein assayed (in micrograms) and the time of incubation (in hours).

**RNA analysis.** RNA was isolated from yeast by a glass bead disruption and hot phenol extraction procedure (8). RNA probes (cRNA) for Northern (RNA) hybridizations were synthesized with the Gemini II Core System (Promega, Madison, Wis.) from plasmids linearized with a restriction enzyme and transcribed with an RNA polymerase as follows (shown as plasmid, restriction enzyme, RNA polymerase) for the probe indicated in parentheses: pTA-*INO4*, *Sal*I, T7 (*INO4*); pJH310, *Hin*DIII, T7 (*INO1*); and pAB309Δ, *Eco*RI, SP6 (*TCM1*). Northern hybridizations were performed as previously described (14), results were visualized by autoradiography, and gene-specific counts per minute were quantitated using a Betascope 603 Blot Analyzer (Beta-gen, Waltham, Mass.).

For the S1 nuclease digestion assay, BRS1001 transformed with pJA201 (contains the entire *INO4* gene) and BRS2004 were grown in the appropriate media. RNA was isolated as described above. A single-stranded probe was created using asymmetric PCR (12). A standard 100-μl PCR mixture contained 1 μg of the INO4-PE oligonucleotide and 0.3 ng of the INO4-A oligonucleotide. The single-stranded PCR product was purified by gel electrophoresis and labeled at the 5′ terminus with [γ-$^{32}$P]ATP using T4 polynucleotide kinase (Gibco BRL, Grand Island, N.Y.) (22). Approximately 10 ng of labeled probe was coprecipitated with 20 μg of total yeast RNA. The pellet was resuspended in 10 μl of S1 hybridization buffer [80% formamide, 0.4 M NaCl, 0.04 M piperazine-*N*,*N*′-bis(2-ethane-sulfonic acid) (PIPES; pH 6.6), 0.1 mM EDTA]. The hybridization reaction was overlaid with mineral oil, denatured at 95°C for 3 min, and hybridized overnight at 46°C. Next, 300 μl of S1 digestion buffer (0.28 M NaCl, 0.03 M sodium acetate [pH 4.5], 4.5 mM ZnCl₂, salmon sperm DNA at 20 μg/ml, S1 nuclease at 1,200 U/ml (Boehringer Mannheim, Ind.) was added and the reaction mixture was incubated at 15°C for 1 h. The digestion reaction was terminated by addition of 50 μl of 2.5 M ammonium acetate–50 mM EDTA. The reaction mixture was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and the digestion products were precipitated. The pellet was resuspended in 10 μl of gel loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95°C for 5 min, and fractionated through a 4% denaturing polyacrylamide gel. A sequencing ladder generated using the INO4-PE primer was used as the standard.

**Western blots.** Preparation of whole-cell extracts of *S. cerevisiae* was performed as previously described (18), and 50 μg of yeast extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose by electroblotting at 150 mA for 2 h. The blot was stained with Ponceau to confirm equal loading and transfer of proteins. The blot was incubated in BLOTTO (100 mM Tris, 1.5 M NaCl, 0.5% Tween 40, 5% powder milk) overnight at 4°C with constant shaking. Anti-HA antibody (Boehringer Mannheim) was added to a final dilution of 1/250 and incubated for 1 h at room temperature with shaking. The blot was then washed in BLOTTO five times for 5 min each time. An alkaline phosphatase-conjugated anti-immunoglobulin G antibody (Zymed Laboratory Inc., San Francisco, Calif.) was added to BLOTTO at a final dilution of 1/1,000 and incubated for 1 h at room temperature. The blot was washed in BLOTTO five times for 5 min each time. The blot was suspended in alkaline phosphate buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl₂). Nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (BCIP) (Promega) was added in a 2:1 ratio to the buffer and allowed to react for 1 h. The reaction was stopped by the addition of stop buffer (200 mM Tris [pH 8.0], 5 mM EDTA).

## RESULTS

Previous results obtained by using an *INO4* promoter fusion to the *cat* reporter gene suggested that *INO4* expression is unresponsive to inositol and choline (5). Until this report, we had been unable to detect the *INO4* transcript using Northern blot hybridization. In part, this is due to the low abundance of the *INO4* transcript, which was overcome by generating a high-specific-activity cRNA probe. In addition, the *INO4* sequences included in the probe itself are also important since the ORF and 3′ untranslated region are required. Probes containing the *INO4* ORF or parts of the ORF are not sufficient to detect the *INO4* transcript (J. M. Lopes, unpublished data). To determine if the *INO4-cat* reporter accurately reflected expression

of the native *INO4* gene, steady-state transcript levels were quantitated by Northern blot hybridization. RNA was isolated from strains grown in medium that normally represses (with 75 μM inositol and 1 mM choline), partially derepresses (with 10 μM inositol and 1 mM choline), or completely derepresses (lacking inositol and choline) most of the phospholipid biosynthetic structural genes (9, 13). An *INO4*-specific cRNA probe recognized a single major RNA of approximately 600 nucleotides (Fig. 1A). Quantitation of this transcript revealed that steady-state *INO4* levels are reduced by 60% in a wild-type strain in response to inositol-and-choline supplementation (Fig. 1B). Repression of the phospholipid biosynthetic genes in response to inositol and choline is known to require the negative regulatory protein encoded by *OPI1*. Like the phospholipid biosynthetic genes, the 60% reduction of *INO4* expression by inositol and choline is dependent on a wild-type allele of *OPI1*. However, unlike the phospholipid biosynthetic genes, regulation of *INO4* by inositol and choline does not require a functional copy of *INO2* (9) (Fig. 1A and B). To ensure that the *ino2*Δ strain lacked the *INO2* gene function, we quantified expression of the *INO1* transcript. As expected, *INO1* expression was eliminated in the *ino2*Δ mutant strain (Fig. 1C).

It is interesting that the modest regulation of steady-state *INO4* levels in response to inositol and choline was not observed with the *INO4-cat* reporter gene (5). One possible explanation for this difference is that promoter sequences absent from the *INO4-cat* reporter gene are required for the response to inositol and choline. The original *INO4-cat* gene contained 500 bp of the *INO4* promoter (5). To ensure that all of the potential promoter elements were included, two additional *INO4-cat* constructs were created that contained 750 and 1,000 bp upstream of the *INO4* translational start site. However, these constructs also failed to exhibit a response to inositol-and-choline supplementation (Fig. 2). It seems unlikely that the *INO4* promoter would encompass sequences farther upstream than 1,000 bp, since this would include most of a divergent ORF, YOL107W. The lack of regulation of the *INO4-cat* gene suggests that *INO4* expression may be regulated at the level of mRNA stability by inositol and choline. This possibility was explored in greater detail as described below.

The possibility that inositol and choline may also exert regulation at the level of mRNA stability prompted us to examine Ino4p levels under repressing and derepressing conditions. Toward this end, we tagged Ino4p at the N terminus with three tandem copies of the HA epitope. This fusion was inserted into the YCp50 (YCp50-*INO4*HA) vector and subsequently transformed into an *ino4*Δ mutant strain (BRS2004). Yeast extracts were prepared from strains transformed with either YCp50-*INO4* or YCp50-*INO4*HA grown in medium that normally represses and derepresses the phospholipid biosynthetic genes. Western blotting using an anti-HA antibody revealed that Ino4p protein levels are decreased in the presence of inositol and choline (Fig. 3). This result is consistent with the steady-state *INO4* mRNA levels.

Transcriptional regulation of the phospholipid biosynthetic genes and the *INO2* regulatory gene is dependent on both the *INO2* and *INO4* genes. However, *INO4* transcription does not require *INO2* (Fig. 1A and B). This is consistent with a previous report showing that expression of an *INO4-cat* promoter fusion did not require the *INO2* gene (5). Genes that are responsive to inositol and choline are typically dependent on both the *INO2* and *INO4* genes for derepression. However, some genes have been found which require *INO4* but not *INO2* for their expression (9). In fact, this laboratory and others have reported that *INO4-cat* and *INO4-lacZ* reporter gene expression does not require *INO2* but does require *INO4* (5, 26).
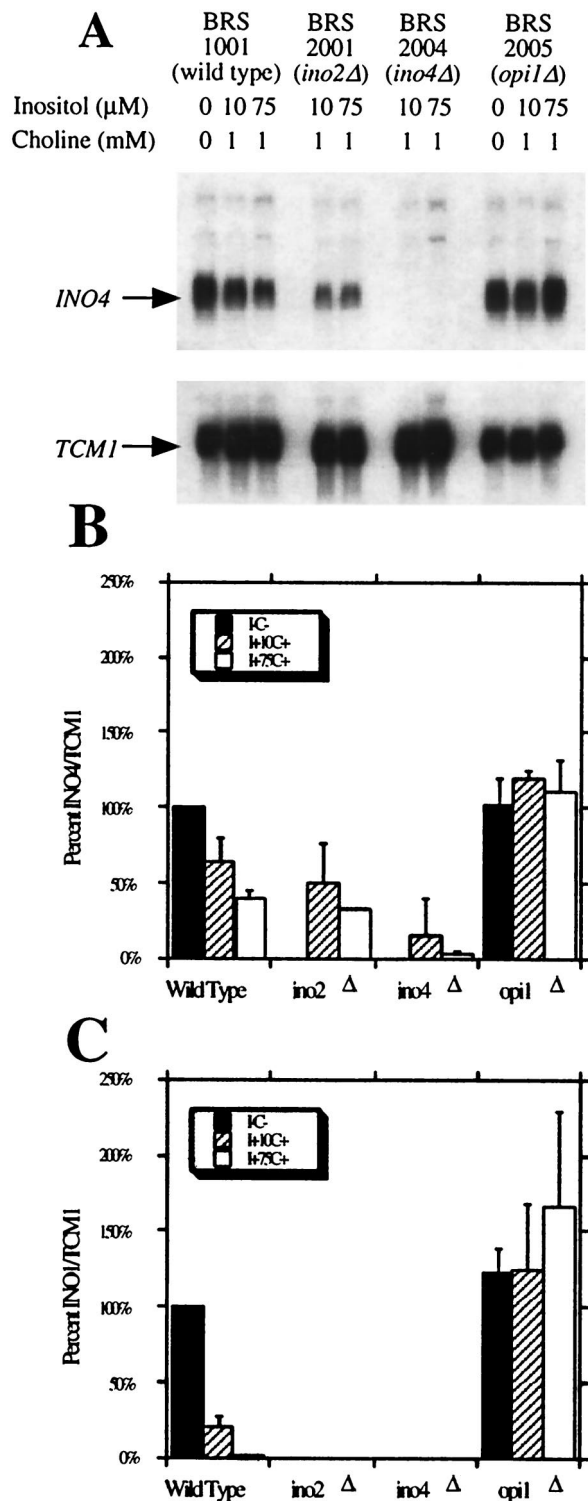


FIG. 1. Quantitative analysis of *INO4* and *INO1* mRNAs. (A) Representative Northern blot hybridization showing *INO4* transcript levels. *TCM1*, a constitutively expressed ribosomal protein gene, was used to normalize for loading variations. The relevant genotypes of the strains used are shown in parentheses. (B) Bar values represent ratios of *INO4* to *TCM1* counts per minute quantified using a Betascope 603 Blot Analyzer (Beta-gen). (C) Bar values represent ratios of *INO1* to *TCM1* counts per minute. Cells were grown in complete synthetic medium supplemented with 75 μM inositol and 1 mM choline (I+75C+) or 10 μM inositol and 1 mM choline (I+10C+) or lacking inositol and choline (I−C−). The values shown are averages of at least three separate trials and are normalized to that of the wild type without inositol and choline (100%).
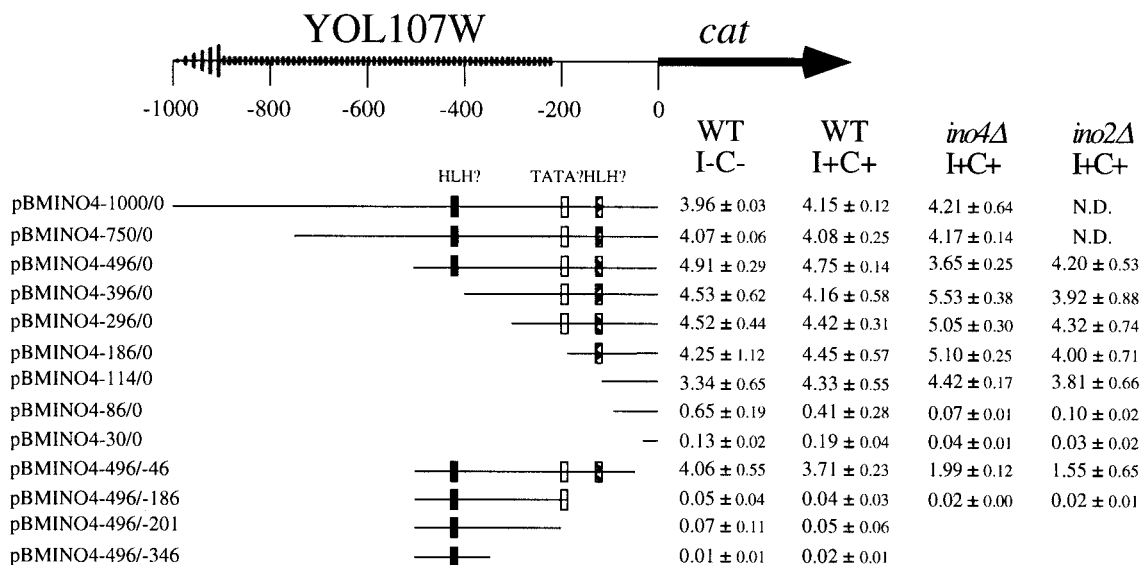
| | WT I−C− | WT I+C+ | ino4Δ I+C+ | ino2Δ I+C+ |
|---|---|---|---|---|
| pBMINO4-1000/0 | 3.96 ± 0.03 | 4.15 ± 0.12 | 4.21 ± 0.64 | N.D. |
| pBMINO4-750/0 | 4.07 ± 0.06 | 4.08 ± 0.25 | 4.17 ± 0.14 | N.D. |
| pBMINO4-496/0 | 4.91 ± 0.29 | 4.75 ± 0.14 | 3.65 ± 0.25 | 4.20 ± 0.53 |
| pBMINO4-396/0 | 4.53 ± 0.62 | 4.16 ± 0.58 | 5.53 ± 0.38 | 3.92 ± 0.88 |
| pBMINO4-296/0 | 4.52 ± 0.44 | 4.42 ± 0.31 | 5.05 ± 0.30 | 4.32 ± 0.74 |
| pBMINO4-186/0 | 4.25 ± 1.12 | 4.45 ± 0.57 | 5.10 ± 0.25 | 4.00 ± 0.71 |
| pBMINO4-114/0 | 3.34 ± 0.65 | 4.33 ± 0.55 | 4.42 ± 0.17 | 3.81 ± 0.66 |
| pBMINO4-86/0 | 0.65 ± 0.19 | 0.41 ± 0.28 | 0.07 ± 0.01 | 0.10 ± 0.02 |
| pBMINO4-30/0 | 0.13 ± 0.02 | 0.19 ± 0.04 | 0.04 ± 0.01 | 0.03 ± 0.02 |
| pBMINO4-496/-46 | 4.06 ± 0.55 | 3.71 ± 0.23 | 1.99 ± 0.12 | 1.55 ± 0.65 |
| pBMINO4-496/-186 | 0.05 ± 0.04 | 0.04 ± 0.03 | 0.02 ± 0.00 | 0.02 ± 0.01 |
| pBMINO4-496/-201 | 0.07 ± 0.11 | 0.05 ± 0.06 | | |
| pBMINO4-496/-346 | 0.01 ± 0.01 | 0.02 ± 0.01 | | |

FIG. 2. Expression of INO4-cat fusions in the wild type (WT) (BRS1001), ino4Δ (BRS2004), and ino2Δ (BRS2001) strains. Transformants containing the various promoter deletions were grown in uracil-lacking synthetic medium either containing 75 μM inositol and 1 mM choline (I+C+) or lacking inositol and choline (I−C−). Extracts from the yeast transformants were prepared and assayed for CAT activity. The values shown are averages of at least three separate trials. YOL107W is a divergent ORF. The locations of two putative bHLH binding sites and a potential TATA box are shown. N.D., not determined.

Surprisingly, in the present study, we observed that the level of CAT activity in an ino4Δ strain (BRS2004) was equivalent to that in the wild-type strain, suggesting that INO4 is not required for INO4-cat expression (Fig. 2).

An explanation for this difference presented itself when we noticed that in the earlier study we had employed a different ino4Δ strain, Null20 (5). Curiously, Null20 and BRS2004 both originated as ino4Δ mutant spores isolated from the same tetrad and are predicted to be isogenic (J. Ambroziak, personal communication). To determine if the two ino4Δ strains behaved differently, we retransformed the INO4-cat construct into the Null20 strain and assayed for CAT activity. As seen in the earlier report, no CAT activity was observed in the Null20 transformants. Therefore, the discrepancy lay within the ino4Δ strains. One of the strains may have obtained a second mutation that alters expression of the INO4-cat reporter. To determine the correct INO4-cat phenotype, we assayed for CAT activity in two additional, independently isolated ino4Δ mutant

strains with entirely different genetic histories: SH307 (kindly provided by Miriam Greenberg, Wayne State University) and YB588 (kindly provided by Steven Cok, Washington University) The CAT expression levels in these two strains were comparable to the levels in strain BRS2004. We measured INO4-cat expression in multiple ino4Δ strains and found the following mean levels of CAT activity (± the standard deviations): BRS2004, 4.75 ± 0.14 U; SH307, 2.97 ± 0.53 U; YB588, 3.15 ± 0.84 U; Null20, 0.02 ± 0.01 U. These results support the conclusion that INO4 is not autoregulated.

Transcriptional regulation of the phospholipid biosynthetic genes, and the INO2 regulatory gene, in response to inositol and choline is mediated by a UAS$_{INO}$ element. The INO4 promoter contains two putative bHLH binding sites, with the proximal bHLH binding site resembling a UAS$_{INO}$ element. This prompted us to delineate the region(s) of the INO4 promoter that is required for gene expression. To do this, we created a nested set of INO4 promoter deletions fused to the cat reporter gene. The constructs were integrated at the GAL4 locus in single copy, and all integrations were confirmed by Southern blotting. Cultures were grown in media with or without both inositol and choline and assayed for CAT activity. This assay demonstrated that the −86 to −46 region of the INO4 promoter is required to drive expression of the cat gene in an inositol-and-choline-independent manner (Fig. 2). As shown above, expression from the complete INO4 promoter does not require INO2 or INO4 (Fig. 2). However, the possibility remained that elements within the promoter require INO2 and INO4 to maintain appropriate INO4 expression levels. Therefore, the promoter deletions were assayed in ino2Δ and ino4Δ mutant strains under completely repressing conditions. The results confirmed that the −86 to −46 region of the INO4 promoter is necessary and sufficient for INO4 expression (Fig. 2). This also demonstrated that the two putative bHLH binding sites within the INO4 promoter are not functional and not dependent on the INO2 and INO4 genes.

Because it is possible that integration of the INO4-cat constructs at the GAL4 locus could artificially alter expression of
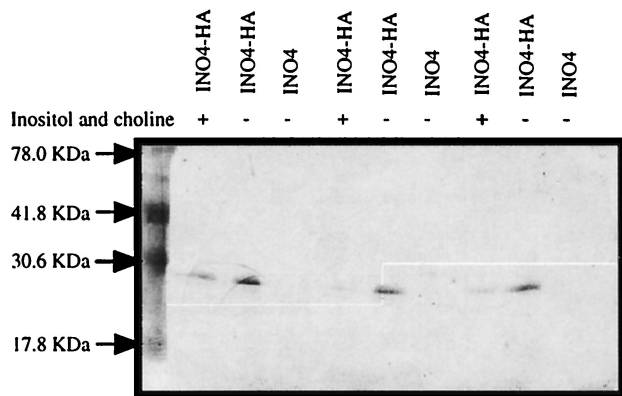


FIG. 3. Western blot analysis of HA-tagged Ino4p. This representative Western blot shows Ino4p levels in cells grown in complete synthetic medium either lacking (−) or supplemented with (+) 75 μM inositol and 1 mM choline.

FIG. 4. Complementation of *ino4*Δ (BRS2004) inositol auxotrophy by various YCp50-*INO4* promoter deletions. Respective transformants were plated on uracil-lacking synthetic medium containing inositol and choline and replica plated to uracil-lacking synthetic medium also lacking inositol and choline. Growth is indicated by a plus sign, and absence of growth is indicated by a minus sign. Quantitation of steady-state *INO1* mRNA transcript levels by Northern blot hybridization in the wild-type strain (BRS1001) and an *ino4*Δ mutant strain (BRS2004) transformed with the YCp50-*INO4* promoter deletion constructs is shown. Strains were grown in uracil-lacking synthetic medium either containing 75 μM inositol and 1 mM choline (I+C+) or lacking inositol and choline (I−C−). *TCM1* was used to normalize for loading variations. The values shown represent ratios of *INO1* to *TCM1* expression levels. The locations of two putative bHLH binding sites and a potential TATA box are shown as in Fig. 2.

the *INO4-cat* gene, we assayed for the ability of the *INO4* promoter deletions to drive expression of the *INO4* gene. PCR products that contained the various promoter deletions upstream of the entire *INO4* ORF were created. The PCR products were ligated into the YCp50 vector and transformed into *ino4*Δ mutant strain BRS2004. Expression of the *INO4* gene from the various promoter deletions resulted in complementation of the inositol auxotrophy of BRS2004. This assay provided further proof that the −58 to −46 region of the *INO4* promoter is absolutely required to express *INO4* at levels sufficient to complement the inositol auxotrophy of an *ino4*Δ mutant strain (Fig. 4).

Placement of the deletion constructs on a centromeric plasmid may cause overexpression of the *INO4* gene. This may alter the regulation of the *INO1* gene, potentially leading to the abnormal expression of *INO1*. To address this issue, we measured the steady-state *INO1* transcript levels in these strains by Northern blot hybridization. The regulation of *INO1* transcript levels in the transformants was comparable to the regulation observed in a wild-type strain (compare Fig. 4 to Fig. 1C).

To aid in the interpretation of the promoter deletion studies,

we mapped the *INO4* transcriptional start site using an S1 nuclease assay. However, native *INO4* expression levels were too low to be detected by this assay. Therefore, wild-type strain BRS1001 was transformed with pJA201, a multicopy plasmid containing the entire *INO4* gene driven by its own promoter (2). Overexpression of the *INO4* gene in this transformed strain allowed detection of the *INO4* initiation sites. The assay revealed one major start site at −48 and two additional minor transcript start sites at −54 and −47 (Fig. 5). Therefore, the transcriptional start sites of *INO4* fall within the minimal region of the promoter required for expression as defined by the *INO4-cat* deletion studies and the YCp50-*INO4* derivatives.

The *INO4-cat* promoter deletion studies also identified a potential regulatory element. Deletion of the region from
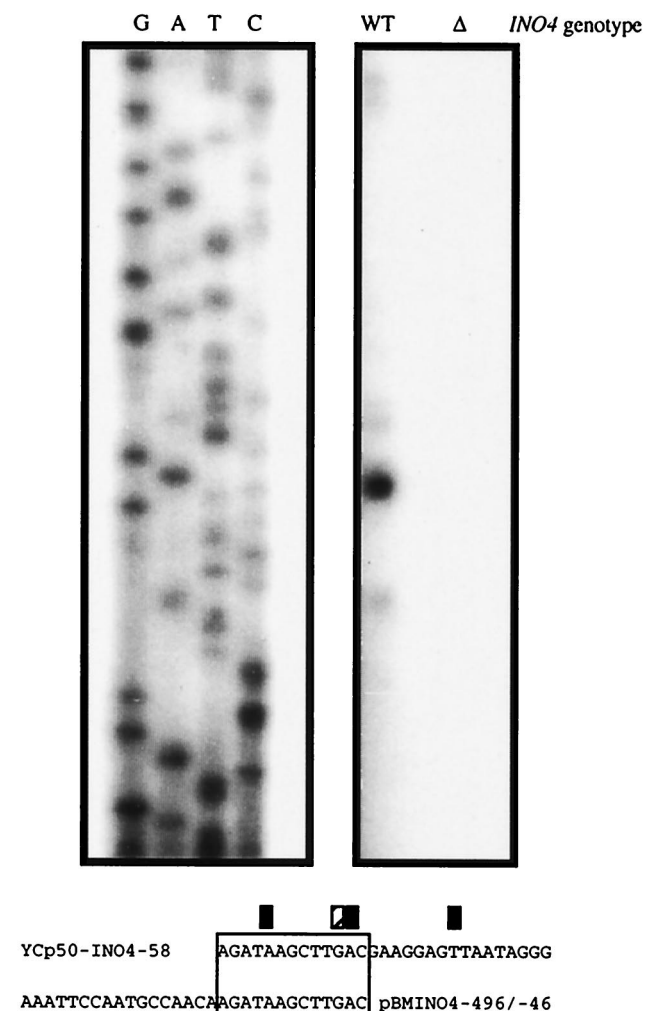


FIG. 5. S1 nuclease digestion assay of *INO4* mRNA. A single-stranded PCR probe was hybridized to total cellular mRNA from the wild type (WT) strain (BRS1001) transformed with pJA201, and an *ino4*Δ mutant strain (BRS2004). BRS1001 was grown in leucine-lacking synthetic medium containing 75 μM inositol and 1 mM choline, while BRS2004 was grown in complete synthetic medium containing 75 μM inositol and 1 mM choline. S1 digestion fragments were run on a denaturing polyacrylamide gel next to the *INO4* sequence that was generated with the same primer used to synthesize the probe. Different exposures of the sequencing ladder (1 day) and S1 digestion products (4 days) are shown. Nucleotide assignment of *INO4* mRNA start sites. The stippled box represents the major start site. The solid boxes represent minor start sites. Sequences refer to the exact nucleotides present in the respective promoter deletions.

−114 to −86 resulted in a 5- to 10-fold decrease in *cat* expression from the *INO4* promoter (Fig. 2). Yet, the YCp50 derivatives containing *INO4* promoter sequences to −86 and −58 were still able to complement the inositol auxotrophy of BRS2004 (Fig. 4). This suggested that in spite of the 5- to 10-fold decrease in *INO4*, as seen in the CAT assay, there is sufficient Ino4p present to regulate its target genes.

## DISCUSSION

Because little is known about elements necessary to direct expression from a weak promoter in yeast, we determined the element(s) necessary for *INO4* expression. We created various promoter deletions within the *INO4* promoter and fused them upstream of the *cat* reporter gene. These constructs did reveal that the region from −86 to −46 bp upstream of the *INO4* translational start site is absolutely necessary for *INO4* expression (Fig. 2). To eliminate the possibility of artifacts from the *INO4-cat* constructs, we inserted the *INO4* promoter deletions fused upstream of the entire *INO4* ORF into YCp50. We assayed the deletions for the ability to complement the inositol auxotrophy of an *ino4Δ* strain. These experiments supported the *INO4-cat* construct data demonstrating that the −58 to −46 region of the *INO4* promoter is required for expression. An S1 nuclease digestion assay demonstrated that the major *INO4* transcriptional start site, along with two minor start sites, lies within the −58 to −46 region (Fig. 5). Therefore, various *INO4* promoter deletions and the S1 nuclease assay have shown that *INO4* contains an initiator element and no TATA box. To our knowledge, *INO4* is the first yeast gene that requires an initiator element but contains no TATA box. However, this phenomenon is very prevalent in the housekeeping genes of higher eukaryotes. Housekeeping genes are constitutively expressed genes with promoters that usually do not contain TATA boxes or other regulatory elements (7).

The *INO4* promoter study also identified a region that is required for full *INO4-cat* expression. Deletion of nucleotides −114 to −86 resulted in an 80 to 90% decrease in *cat* expression (Fig. 2). However, YCp50-*INO4* constructs lacking these sequences still complemented the inositol auxotrophy and maintained proper *INO1* regulation (Fig. 4). This suggested that *INO4* expression is in excess of what is needed to regulate the phospholipid biosynthetic genes. This idea is also supported by the fact that overexpression of Ino4p, but not Ino2p, does not result in elevated levels of the Ino2p/Ino4p/UAS$_{INO}$ complex in mobility shift assays (19).

Sequence analysis illuminated two potential bHLH binding sites and a TATA-like element. However, the promoter deletions demonstrated that none of these elements were necessary for *INO4-cat* expression. Studies with in vitro-transcribed and -translated Ino2p and Ino4p have shown that they can bind to the proximal putative *INO4* bHLH binding site which most closely resembles the UAS$_{INO}$ element (28). Also, this element, when fused to a *CYC1-lacZ* reporter, was sufficient to confer regulation by inositol and choline. However, it has been observed that certain UAS$_{INO}$ elements are functional in the *CYC1-lacZ* reporter system (6, 25) but not functional in a native context (4). We have demonstrated that neither of the *INO4* bHLH promoter elements is required for expression or regulation (Fig. 2 and 4).

The bHLH region of Ino4p shares homology with the bHLH region of the mammalian protein Max (20). Max forms heterodimers with Myc, Mad, and Mxi-1 to regulate genes required for cell proliferation and differentiation. Myc levels increase in cells undergoing proliferation, while Mad levels increase in cells as they differentiate (1). Max is constitutively expressed (1). Therefore, it is Myc and Mad concentrations that are rate limiting for heterodimer formation and ultimately responsible for determining which cell program is expressed. The similarity between the sequence and expression of Ino4p and Max suggests that Ino4p, like Max, may bind other bHLH proteins in yeast. Results of a two-hybrid study suggest that this is, in fact, true (K. A. Robinson and J. M. Lopes, unpublished data). By binding to bHLH proteins other than Ino2p, it is possible that Ino4p is responsible for regulating genes other than the phospholipid biosynthetic genes. Results obtained by using a whole-genome array suggest that there are genes independent of the phospholipid biosynthetic genes, which are each regulated positively and negatively by *INO4* (Robinson and Lopes, unpublished data). Therefore, the *INO4* pattern of expression suggests that it is a housekeeping gene in yeast.

A previous report from this laboratory (5) and data presented here (Fig. 2) showed that the *INO4-cat* construct is expressed constitutively. Another report using *INO4-lacZ* suggested that *INO4* is autoregulated in response to inositol and choline (26). Because of this conflict, we decided to look at the in vivo steady-state levels of the *INO4* mRNA transcript. Northern analysis demonstrated that the *INO4* transcript is regulated two- to threefold in response to inositol and choline (Fig. 1A and B). Consistent with the steady-state *INO4* mRNA levels, we found that the Ino4p levels are also regulated in response to inositol and choline. To eliminate the possibility that the constitutive expression of the *INO4-cat* construct was the result of omitted upstream regulatory sequences, we created additional *INO4-cat* constructs. Constructs containing sequences 750 and 1,000 bp upstream of the *INO4* translational start site still elicited constitutive CAT activity (Fig. 2). One possible cause of the discrepancy is that *INO4* is regulated at the level of mRNA stability. *INO4* would not be the first gene in the phospholipid biosynthetic pathway to be regulated at the level of mRNA stability in response to inositol and choline. *INO1* and *CHO2* transcript stability is regulated 50 to 60% by inositol and choline (J. Yates and J. M. Lopes, unpublished data). In fact, the threefold regulation seen with the *INO4-lacZ* gene may result from mRNA stability, since the *INO4-lacZ* construct is a translational fusion (26).

There are currently four methods for measuring mRNA half-life (21). One method, labeling of cells to steady state (or by pulse-chase) in vivo, has not been successfully used with low-abundance transcripts. We attempted to determine the half-life of the *INO4* transcript using thiolutin and by the use of a yeast temperature-sensitive RNA polymerase (*rpb1-1*) mutant. Incubation with thiolutin resulted in rapid degradation of the *INO4* transcript, which made it impossible to detect the transcript by Northern blot hybridization. Experiments using the *rpb1-1* mutant strain yielded the same result as the thiolutin assay. Moreover, the *rpb1-1* mutant is an inositol auxotroph (23, 24), which would make it difficult to exclude the possibility that artifacts resulted from the auxotrophy. A final method for determining mRNA half-life requires placing the gene under the control of the tightly regulated strong *GAL1* promoter (21). Therefore, the increase in transcript numbers may facilitate detection by Northern blot hybridization. However, in the case of *INO4*, this would create gross overexpression of the *INO4* transcript, well beyond physiological levels.

Transcriptional regulation of the phospholipid biosynthetic genes in response to inositol and choline is mediated by *INO2* and *INO4* (9). However, *INO4* expression does not require *INO2* or *INO4* (Fig. 1 and 2). Nevertheless, as is the case with the coordinately regulated phospholipid biosynthetic genes, *INO4* is constitutively overexpressed in an *opi1Δ* strain (Fig. 1A and B). This suggests that *OPI1* may regulate both tran-

scription initiation and mRNA stability in response to inositol and choline.

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