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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 UASINO, 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 UASINO, 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 UASINO, element (C/AATGTGAAAT) (3, 27). The first six base pairs of this element contain the canonical bHLH binding site (5′ CANNTG 3′) (1). The UASINO, 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 UASINO, 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 UASINO, 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...
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 inositol and 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 (MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1), BRS2001 (MATa ade2-1 his3-11,15 leu2-112 can1-100 ura3-1 trp1-1), BRS2004 (MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 I-LEU2), BRS2005 (MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 I-LEU2), Null20 (MATa his3-11,15 leu2-3,112 can1-100 ura3-1 I-LEU2), NH170 (MATa his3-11,15 leu2-3,112 can1-100 ura3-1 I-LEU2), and YBS88 (MATa ade2-1 ade3-1 his3-1 ura3-1 trp1-1 ade2-1 his3-1 ade3-1 null1-2 I-Sph1 I-LEU2). 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).

Phenol/phenol constructs. 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’-terminus of the deletion PCR product was used as the 3’-primers INO4-A through the 3’-primers INO4-F and parts of the ORF were isolated. Three sets of primers were created and used the 5’-primers INO4-A through the 3’-primers INO4-F, respectively. The individual PCR products were inserted into pGEM-T (Promega, Madison, Wis.). Each deletion PCR product was excised from the pGEM-T derivative by digestion with BglII and BamHI and inserted into pBMB2015 (10). The pBMB2015 derivatives were transformed into yeast as previously described (5).

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 nativeINO4gene, steady-state transcript levels were quantitated by Northern blot hybridization. RNA was isolated from strains grown in medium that normally represses (with 75 
\mu \text{M} \text{inositol and 1 mM choline}), partially derepresses (with 10 
\mu \text{M} \text{inositol and 1 mM choline}), or completely derepresses (lacking inositol and choline) most of the phospholipid biosynthetic structural genes (9, 13). AnINO4-specific cRNA probe recognized a single major RNA of approximately 600 nucleo-
tides (Fig. 1A). Quantitation of this transcript revealed that steady-state transcript 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 neg-
ative regulatory protein encoded by OP11. Like the phospho-
lipid biosynthetic genes, the 60\% reduction ofINO4expression by inositol and choline is dependent on a wild-type allele of OP11. However, unlike the phospholipid biosynthetic genes, regulation ofINO4by inositol and choline does not require a functional copy ofINO2(9) (Fig. 1A and B). To ensure that theino2Astrain lacked theINO2gene function, we quantified expression of theINO1transcript. As expected,INO1expression was eliminated in theino2Δmutant strain (Fig. 1C).

It is interesting that the modest regulation of steady-stateINO4levels in response to inositol and choline was not ob-
erved with theINO4-catreporter gene (5). One possible expla-
nation for this difference is that promoter sequences absent from theINO4-catreporter gene are required for the response to inositol and choline. The originalINO4-catgene contained 500 bp of theINO4promoter (5). To ensure that all of the potential promoter elements were included, two additionalINO4-catconstructs were created that contained 750 and 1,000 bp upstream of theINO4translational start site. However, these constructs also failed to exhibit a response to inositol-
and-choline supplementation (Fig. 2). It seems unlikely that theINO4promoter would encompass sequences farther up-
stream than 1,000 bp, since this would include most of a di-
vergent ORF, YOL107W. The lack of regulation of theINO4-
catgene suggests thatINO4expression 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 reg-
ulation at the level of mRNA stability prompted us to examineINO4levels under repressing and derepressing conditions. Toward this end, we taggedINO4at the N terminus with three tandem copies of the HA epitope. This fusion was inserted into theYCP50(YCP50-IN04HA)vector and subsequently trans-
formed into anino2Δmutant strain (BRS2004). Yeast extracts
were prepared from strains transformed with either YCP50-
INO4or YCP50-IN04HA grown in medium that normally represses and derepresses the phospholipid biosynthetic genes. Western blotting using an anti-HA antibody revealed thatINO4protein levels are decreased in the presence of inositol and choline (Fig. 3). This result is consistent with the steady-
stateINO4mRNA levels.

Transcriptional regulation of the phospholipid biosynthetic
genes and theINO2regulatory gene is dependent on both the 
INO2andINO4genes. However,INO4transcription does not requireINO2(Fig. 1A and B). This is consistent with a previ-
uous report showing that expression of anINO4-catpromoter fusion did not require theINO2gene (5). Genes that are responsive to inositol and choline are typically dependent on both theINO2andINO4genes for derepression. However, some genes have been found which requireINO4but notINO2for their expression (9). In fact, this laboratory and others have reported thatINO4-catandINO4-lacZreporter gene expression does not requireINO2but does requireINO4(5, 26).

![FIG. 1. Quantitative analysis ofINO4andINO1mRNAs. (A) Representative Northern blot hybridization showingINO4transcript levels. TCM1, a consti-
tutively 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 ofINO4toTCM1counts per minute quantified using a Betascope 603 Blot Analyzer (Beta-gen). (C) Bar values represent ratios ofINO1toTCM1counts per minute. Cells were grown in complete synthetic medium supplemented with 75 
\mu \text{M} \text{inositol and 1 mM choline (I } \text{−} 75 \text{C} \text{−} \text{+)} or 10 
\mu \text{M} \text{inositol and 1 mM choline (I } \text{+10C} \text{−} \text{+)} 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%).}
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 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 UASINO element. The INO4 promoter contains two putative bHLH binding sites, with the proximal bHLH binding site resembling a UASINO 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 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
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. 4. Complementation of ino4Δ (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 ino4Δ 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.

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 ino4Δ 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.

To aid in the interpretation of the promoter deletion studies,
ment, when fused to a CYC1-lacZ reporter system (6, 25) but not functional in a CYC1-lacZ construct, proved 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 thiolutilin and by the use of a yeast temperature-sensitive RNA polymerase (rpb1-1) mutant. Incubation with thiolutilin 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 thiolutilin 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 opI1A strain (Fig. 1A and B). This suggests that OP11 may regulate both tran-
scription initiation and mRNA stability in response to inositol and choline.

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