Analysis of sequences in the *INO1* promoter that are involved in its regulation by phospholipid precursors

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ABSTRACT

The promoter region of the highly regulated *INO1* structural gene of yeast has been investigated. The major transcription initiation start site (+1) was mapped to a position located five nucleotides upstream of the previously identified initiation codon. The *INO1* TATA is located at −116 to −111. The *INO1* promoter region was used to construct fusions to the *Escherichia coli lacZ* gene. All *INO1* fusion constructs that retained regulation in response to the phospholipid precursors inositol and choline, contained at least one copy of a nine bp repeated element (consensus, 5′-ATGTG-AAAT-3′). The smallest fragment of the *INO1* promoter found to activate and regulate transcription of the fusion gene from a heterologous TATA element was 40 nucleotides in length. This fragment contained one copy of the nine bp repeat and spanned the *INO1* promoter region from −259 to −219. However, when an oligonucleotide containing the nine bp repeated sequence was inserted 5′ to the *CYC1* TATA element, it failed to activate transcription.

INTRODUCTION

In yeast, 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) (1, 2). Enzymes in the PI and PC pathways, as well as the enzyme that synthesizes their common precursor, CDP-DG, are regulated in a coordinate fashion in response to soluble phospholipid precursors (3, 4, 5, 6). The first step in the synthesis of PI is the conversion of glucose-6-phosphate to inositol-1-phosphate (I-1-P). This reaction is catalyzed by the enzyme I-1-P synthase, the product of the *INO1* gene (6). The addition of inositol to the growth medium represses the level of enzyme activity (4, 6) due to a reduction in the level of *INO1* RNA (7). The addition of choline to cells growing in the presence of inositol results in an additional decrease in the level of *INO1* RNA. In the absence of inositol, however, choline has no effect on *INO1* RNA abundance (7). The effects of inositol and choline on *INO1* expression are similar to their effects on the expression of several other phospholipid biosynthetic enzymes. The activities of CDP-DG synthase and phosphatidylserine synthase, enzymes involved in PC synthesis, are unaffected by choline alone, repressed by inositol, and repressed further by the combination of inositol and choline (3, 5).

Two classes of regulatory genes affecting expression of the coordinately regulated enzymes have been identified. The *INO2* and *INO4* genes are believed to encode positive regulators of *INO1* expression because recessive mutations in these genes reduce the level of I-1-P synthesis and *INO1* transcript (7, 8, 9). The *opi1* regulatory mutations cause constitutive derepressed expression of I-1-P synthase (10, 11) and *INO1* transcript (7), suggesting that the wild type *OPI1* gene product is a negative regulator of *INO1* expression. All of these mutations have effects on the PC synthetic enzymes that are similar to their effects on the *INO1* gene product (3, 5, 9). More recently, it has been reported that *INO1* transcription is particularly sensitive to perturbations of the general RNA transcription apparatus. For example, a partial deletion of the carboxy-terminal repeat of the large subunit of RNA polymerase II leads to a pleiotropic phenotype including cold sensitivity, temperature sensitivity, and inositol auxotrophy (Ino^-; 12) due to inability to express the *INO1* transcript (13). Partial deletion of a gene (*SRB2*) encoding a suppressor of the RNA polymerase II phenotype also produced a temperature-sensitive, cold-sensitive, Ino^- phenotype (12). Similar Ino^- phenotypes were also reported in an independent set of mutations defining genes involved in transcription (14).

The sequences of several of the phospholipid biosynthetic genes including *INO1* have been determined. Computer-assisted comparison of these sequences revealed the existence of a nine bp element repeated in the promoters of all of the coregulated genes including *INO1* (1). In the present report, the role of this element and other 5′ sequences has been explored through the fusion of *INO1* promoter sequences to the *Escherichia coli lacZ* gene.

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MATERIALS AND METHODS

Strains, Media and Growth Conditions

The yeast strains used in this study were: W303-1A (*MATa, leu2-3,112, trp1-1, can1-100, ura3-1, ade2-1, his3-11,15*), kindly provided by R.Rothstein; it8 (*MATa, ino2, lys2, ura3*); BRS 1032 (*MATα, ino2, ino4, ura3*); and BRS 1021 (*MATa, opi1, ade5, leu2, trp1, ura3*). Inositol-free and synthetic complete media were prepared as described previously (7). A buffered media containing X-gal (15) was used to score β-galactosidase activity in yeast. In all studies, yeast cells were grown at 30°C.

Plasmid Construction and Generation of 5'-end Deletions

Plasmid pAB309 contains a 3.2 Kb *Ava*I-*Bam*HI restriction fragment containing the yeast ribosomal protein gene, *TCM1*, inserted into the *Ava*I-*Bam*HI sites of pGEM1 (3). Plasmid pAB309Δ is a 1.7 Kb *Sal*I deletion of pAB309 that retains the *TCM1* gene. Plasmid pTC101 was constructed by inserting a 750 bp *Eco*RI-*Cla*I restriction fragment from YEp357R (16) into the *Eco*RI-*Acc*I sites of pGEM1. This insert contains the amino-terminal coding sequence of the *Escherichia coli* K-12 *lacZ* gene. Plasmid pLG669Z (17) contains a *CYC1* amino-terminal fusion to *lacI'Z* along with the *CYC1* regulatory sequences. Plasmid pLGΔ312 is a 1.5 Kb *Sma*I deletion of pLG669Z that retains the *CYC1* UAS. Removal of a 430 bp *Xho*I fragment from pLG669Z deletes the *CYC1* UAS and yields pJH304. The *INO1* regulatory sequences that are located 5' to the TATA box were inserted into the unique *Xho*I site of pJH304 using *Xho*I linkers. Plasmid DNA was isolated by the method of Birnboim and Doly (18) for identification of recombinants. Large-scale plasmid

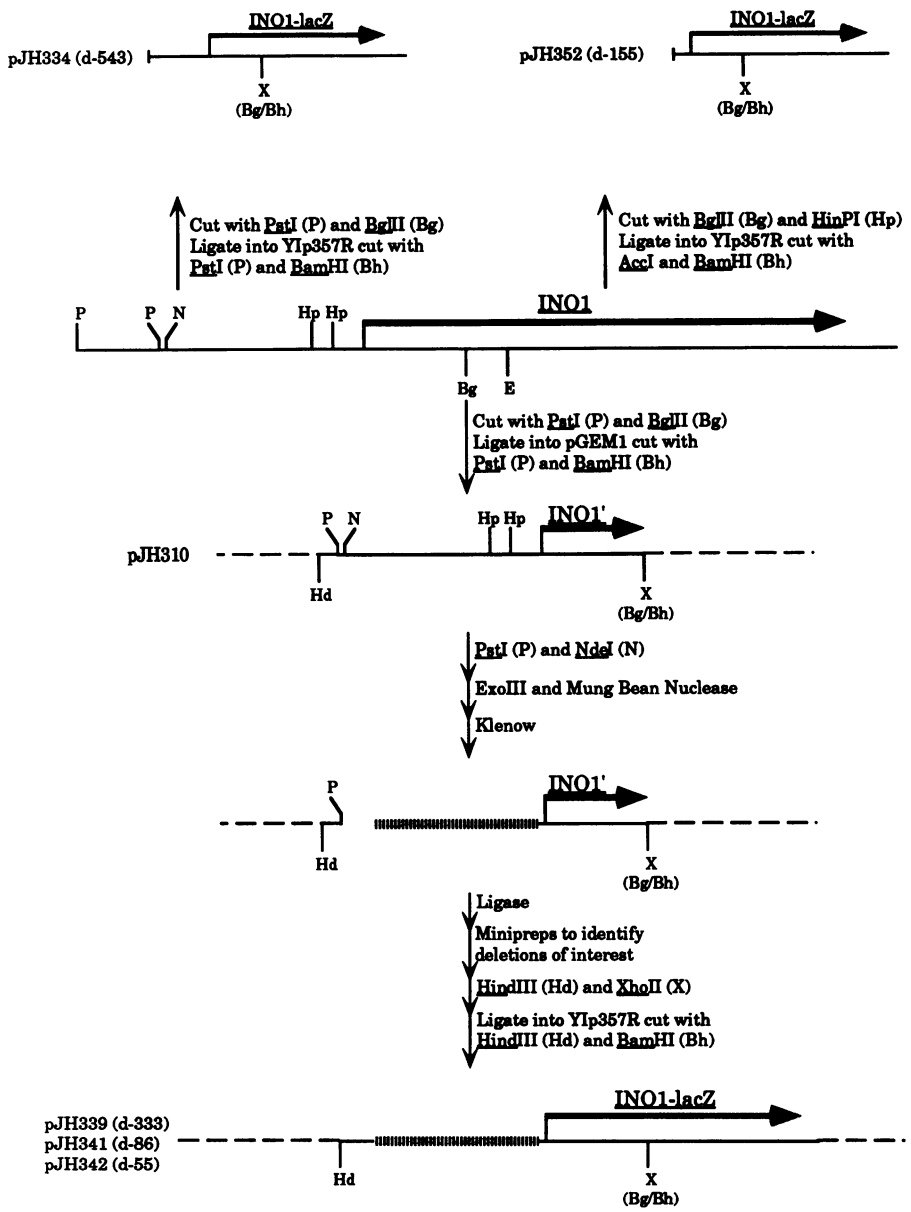


Figure 1. Construction of an *INO1-lacZ* fusion and 5' deletion derivatives. The 1.0 kb *Pst*I-*Bgl*II restriction fragment containing the *INO1* promoter and amino-terminus was cloned into the *Pst*I-*Bam*H1 sites of the vector YIp357R (16), creating an in-frame fusion with the *lacZ* gene (pJH334 and d-543). Likewise, this same restriction fragment was cloned into pGEM1 to yield pJH310 which was subsequently employed to generate deletion derivatives (see also Materials and Methods). Abbreviations: Bg, *Bgl*II; P, *Pst*I; N, *Nde*I; Bh, *Bam*HI; E, *Eco*RV; X, *Xho*II; Hp, *Hin*PI; Hd, *Hind*III.

preparations were done by the method of Clewell and Helinski (19).

A plasmid containing a 1.0 Kb *Bgl*II-*Pst*I (*INO1* promoter) restriction fragment, pJH310 (7; Figure 1), was cleaved with *Pst*I and *Nde*I, cutting the plasmid at one end of the insert (upstream relative to *INO1*). Linearized plasmid DNA was employed to create unidirectional deletions (Figure 1) by the method of Henikoff (20). The DNA was precipitated by the addition of ethanol and single-stranded termini were blunt-ended using the Klenow fragment of *E. coli* polymerase I (New England BioLabs) and the conditions described for nick translation (21) in the presence of all four deoxynucleotides. The blunt-ended DNA fragments were ligated and used to transform *E. coli*. Promoter deletions were identified by restriction analysis and sequenced to determine their precise endpoints. The appropriate restriction fragments were then cloned into the *Bam*HI-*Hind*III sites of YIp357R yielding plasmids: pJH339 (d-333); pJH341 (d-86); and pJH342 (d-55).

Plasmids containing deletions were sequenced by the dideoxy chain termination method (22) using denatured supercoiled plasmids as templates (23). Sequencing reactions were carried out using the reagents supplied in a New England BioLabs sequencing kit.

Yeast cells were transformed with plasmid DNA by a modification of the lithium acetate method of Ito *et al.* (24) as described previously (7).

RNA Isolation, Primer Extension and S1 Analysis

DNA primers and probes for S1 analysis were labeled at the 5′ termini after dephosphorylation with calf intestinal phosphatase (Boehringer Mannheim) and labeling with [γ-^{32}P] ATP using T4 polynucleotide kinase (United States Biochemical Corporation) as described by Maniatis *et al.* (25). RNA was isolated from *S. cerevisiae* cells by the glass bead disruption and hot phenol extraction method of Elion and Warner (26). Polyadenylated [poly(A)$^+$] RNA was prepared by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography (27). Primer extension reactions (28) and S1 protective experiments (29) were performed using standard methods previously described. The results were visualized by autoradiography and quantitated by scanning densitometry.

β-galactosidase Assay

To assay β-galactosidase 330 μl of cell extract were added to 1.32 ml β-gal assay buffer and incubated at 28°C for five minutes. The reaction was initiated by adding 660 μl of 4 mg/ml ONPG (in distilled water) and aliquots were removed at 10, 20 and 30 minutes and added to 500 μl 1M Na_2CO_3 (stop solution). The optical density of the final solution was measured at 420 nm. The total protein concentration of each lysate was determined using a BioRad assay kit (30).

RESULTS

Identification of the INO1 Transcript 5′ Terminus

The 5′ terminus of the *INO1* transcript was determined using a 21-nucleotide oligomer complementary to a region 45 nucleotides downstream of the initiation codon (31) to prime reverse transcription from RNA (Figure 2, Lanes 5−7). The extended products revealed a 5′ terminus five nucleotides upstream of the initiation codon, which was designated +1. Minor products mapping to nucleotides −11, −14, and −38 were also observed (Figures 2 and 3). A primer extension reaction using RNA from cells grown in the presence of inositol (Figure 2, Lane 6) showed that the abundance of all the RNA species decreased under repressing conditions, indicating that they are all coordinately regulated.

Within the *INO1* upstream sequence (Figure 3) are seven copies of a nine bp repeated element (consensus, 5′-ATGTGAAAT-3′) that has been detected in the promoter region of a number of genes encoding phospholipid biosynthetic enzymes (1). In addition, the sequence 5′-TATAAA-3′ is observed at nucleotides −116 to −111 (Figure 3). This sequence is identical to the sequence that has been shown to be the most efficient synthetic TATA element, 5′-TATAAA-3′ (32, 33).

Construction of INO1-lacZ Fusions

INO1-lacZ fusions were constructed in the vector YIp357R (16) which lacks a *S. cerevisiae* origin of replication. The initial *INO1-lacZ* fusion construct contained about 1 Kb of *INO1* sequence including 132 amino acids from the I-1-P synthase amino-terminus and 543 nucleotides 5′ to the start of transcription (Figure 1). This construct, d-543 (pJH334), was linearized at a unique restriction site (*Stu*I) within the *URA3* gene and transformed into yeast strain W303-1A (*ura3*). This procedure targets integration of the entire plasmid to the *ura3* locus (34),

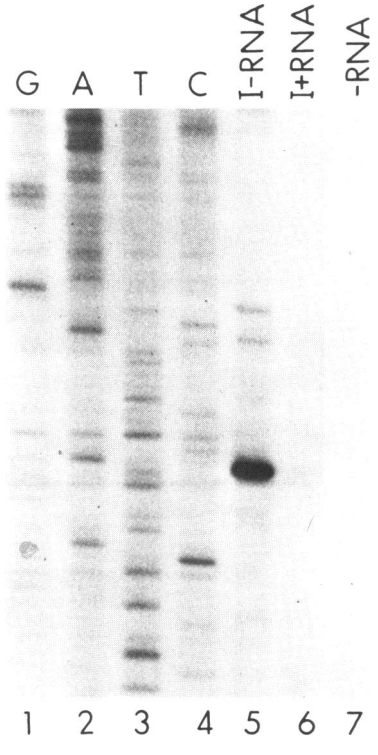


Figure 2. Primer extension of an oligonucleotide complementary to the *INO1* gene. Poly(A)$^+$ RNA (0.5 μg/reaction) from cells grown in the absence (Lane 5) or presence (Lane 6) of inositol was annealed with 2 ng of 5′ end-labeled primer for one hour at 60°C, and complementary DNA was synthesized by the addition of 25 units of reverse transcriptase. The products of the reaction were fractionated on a 10% polyacrylamide/6M urea gel. The procedure was carried out without the addition of RNA (Lane 7) as a control. Lanes 1−4 contain G, A, T, and C dideoxy sequencing reactions, respectively, using the same oligonucleotide as a primer.

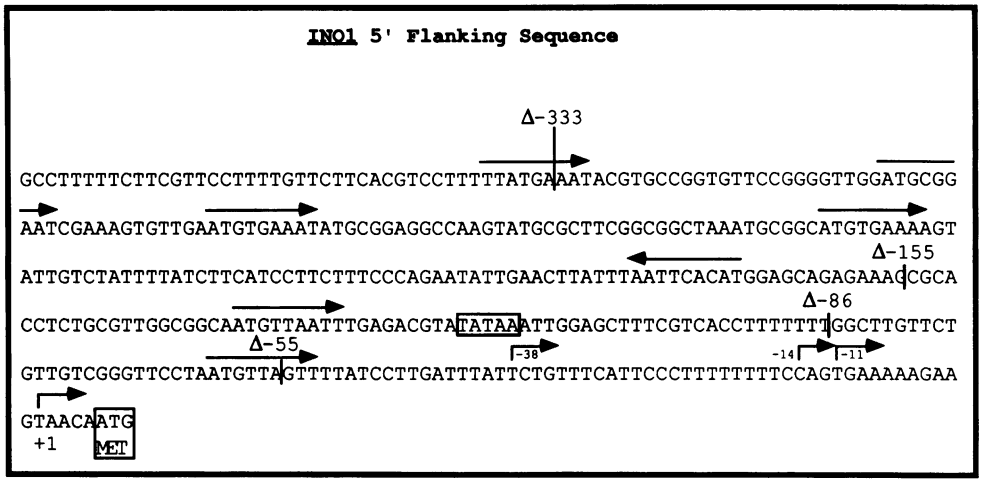
leaving the genomic *INO1* locus intact. Single copy integrants were confirmed by Southern blot analysis.

An S1 protection assay was carried out using RNA extracted from the d-543 construct. The probe used to detect *INO1-lacZ* fusion RNA is shown in Figure 4, Panel B. The labeled site (*Mst*II) was within the *lacZ* gene, resulting in a probe that detects only the fusion RNA. The probe used to assay the genomic *INO1* transcript was labeled at a site (*Eco*RV) downstream of the *Bgl*II site (Figures 1 and 4A). This probe detects only RNA transcribed from the intact genomic *INO1* locus. The size of the protected fragment from the fusion probe was identical or nearly identical to the size of the protected fragment from the probe that detects *INO1* genomic RNA (Figure 4). The 5′ terminus of the fusion construct was confirmed on a high resolution polyacrylamide gel and was found to be identical to the 5′ terminus of the genomic *INO1* gene (data not shown). Furthermore, the *INO1-lacZ* RNA produced by the pJH334 construct was comparable in abundance to the RNA transcribed from the native *INO1* locus (Figure 4) and was reduced in equivalent fashion when the cells were grown in the presence of inositol or inositol plus choline (Figure 4, Panels A and B). Moreover, wild type cells transformed with this construct yielded 89.9 units of β-galactosidase activity when cells were grown in the absence of inositol and choline, and 1.1 units when inositol and choline were present.

Deletion constructs were made by removing DNA sequentially from the 5′ end of the *INO1* upstream region. Transformants containing the deletion constructs integrated in single copy at the *URA3* locus were grown under derepressing (no inositol or choline) or partially repressing (only inositol) conditions. Total cellular RNA was purified and the relative levels of *INO1-lacZ* fusion mRNA was quantitated by S1 analysis. Constructs d-543 (Figure 4) and d-333 (data not shown) exhibit virtually identical repression ratios of approximately ten-fold or greater in response to inositol. Construct d-155, retaining only one copy of the nine bp repeat 5′ to TATA (Figure 1, 3) was expressed at an abundance comparable to the d-543 and -333 constructs under derepressing conditions. However, in the presence of inositol the d-155 construct, exhibited only four-fold repression. The d-86 and d-55 constructs eliminate the apparent TATA element at −111 to −116 and these constructs were not expressed under any condition (data not shown).

Construction of INO1-CYC1-lacI′Z Fusions

A second set of constructs was created in the vector pLG669Z (17), a multi-copy autonomously replicating vector that contains elements of the yeast *CYC1* gene fused in-frame to the *E. coli lacI′Z* gene. The *CYC1* UAS was removed, leaving the *CYC1* TATA sequence intact, thus creating vector pJH304 (Figure 5). Fragments from the *INO1* promoter (flanked by *Xho*I linkers) were inserted 5′ to the *CYC1* TATA in pJH304, creating a tri-hybrid *INO1-CYC1-lacI′Z* fusion. Initially, a *Mae*II restriction fragment from *INO1* (−329 to −119) was cloned into pJH304,



Occurence of a 9 base pair conserved sequence in the regulatory regions of other phospholipid biosynthetic structural genes

Summary of substitutions:	A	16	—	3	2	1	21	21	16	8
	T	5	23	—	19	4	—	—	4	14
	C	1	—	—	1	—	—	1	2	—
	G	1	—	20	1	18	2	1	1	1
Consensus:		A	T	G	T	G	A	A	A	T

Figure 3. Sequence of the *INO1* 5′ flanking region. The sequence of the *INO1* 5′ flanking region up to the initiation codon is shown. The TATA element and the initiation codon are boxed. The major site of transcription initiation is designated by an arrow at (+1) as are three minor sites (−11, −14 and −38). The positions of the deletion endpoints are indicated by vertical lines. Small arrows show the positions of the nine bp repeated element. Also noted is a summary of the substitutions for each nucleotide of the conserved nine base pair element found 23 times total upstream of the *INO1* (seven copies), *CHO1* (one copy), *CHO2*, *OPI3*, and *PIS* genes of *S. cerevisiae* (1). The re-evaluation of the sequence of the *INO1* 5′ prime region also revealed a minor error in the previously-published *INO1* sequence (31), namely: there are four G residues instead of three at positions −314 to −311; and two T residues at position −83 and −82 instead of a single one.

5′ to the *CYC1* TATA box, creating pJH359 (Figure 5). The pJH359 construct omits the *INO1* TATA element and contains potential *INO1* UAS sequences 5′ to the *CYC1* TATA. Cells of strain W303-1A transformed with either pJH359 (−359 to −119 *INO1-CYC1-lacI′Z*) or pKS102 contains a (*Eco*RI-*Hind*III) fragment spanning nucleotides −259 to −154 of the *INO1* 5′ region expressed approximately 135 units and 136 units of β-galactosidase activity (Figure 5) when grown in the absence of inositol and choline (derepressing condition), respectively.

The 105 nucleotide *INO1* fragment in pKS102 is inverted relative to its orientation in the intact *INO1* promoter (i.e., 5′ −154 to −259 3′). A 40 bp sub-fragment of the pKS102 *INO1* fragment spanning nucleotides −259 to −219 was inserted into pJH304 to make pKS101 (Figure 5). This construct expressed only 19.3 units of β-galactosidase under derepressing conditions. None of the three *INO1-CYC1-lacI′Z* transformants described above expressed detectable levels of β-galactosidase activity when grown in media supplemented with 75 μM inositol and 1 mM choline (repressed; Figure 5).

The 40 nucleotide (*Eco*RI-*Fok*I) fragment of DNA employed in constructing pKS101 contains one copy of the nine bp repeated element that occurs seven times throughout the intact *INO1* promoter (Figure 3). The 105 bp nucleotide fragment employed in creating pKS102 contains two copies of the repeated element and pJH359 contains five copies of the repeat. In order to ascertain whether the nine bp repeated element by itself would support the observed expression and/or regulation, a 21 bp oligonucleotide was constructed that contained within it the nine bp consensus sequence, 5′-ATGTGAAAT-3′. The sequence in the oligonucleotide flanking the nine bp consensus is unrelated

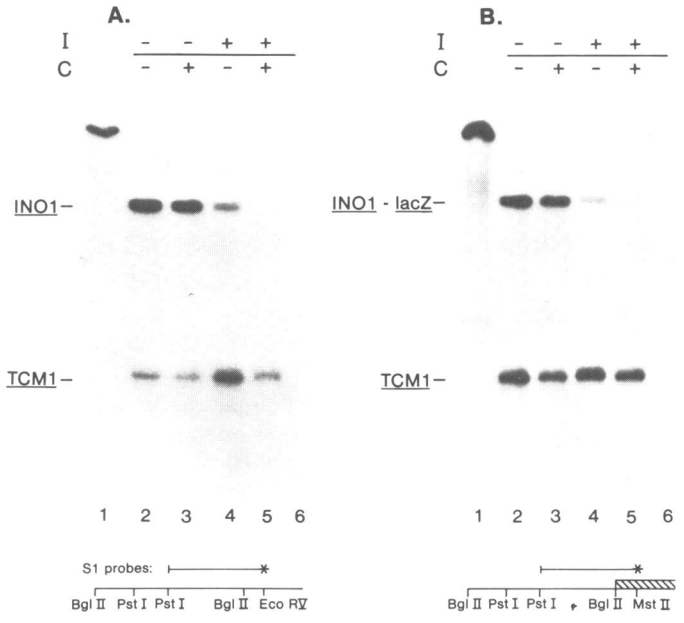
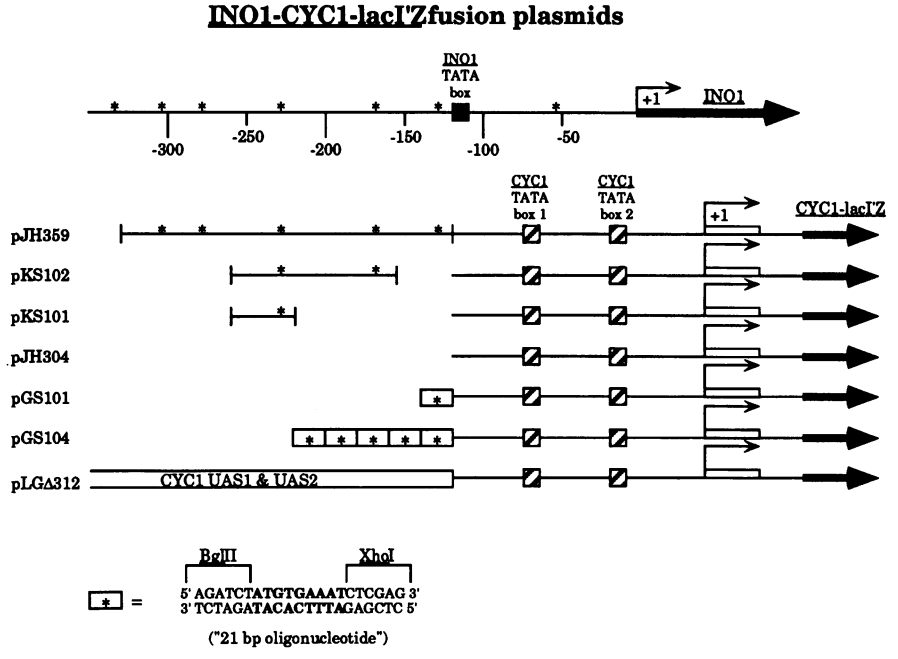


Figure 4. S1 analysis of *INO1* and *INO1-lacZ* RNA. Total RNA (5 μg/reaction) from a strain containing an integrated copy of an *INO1-lacZ* fusion (pJH334) was assayed by an S1 protection experiment, and the products of the reaction were fractionated on a denaturing 5% acrylamide gel. The S1 probe was labeled at the *Eco*RV site in the *INO1* gene (Panel A) or at the *Mst*II site in the *INO1-lacZ* fusion (Panel B). RNA was isolated from cells grown with or without 75 μM inositol and with or without 1 mM choline, as indicated. A probe complementary to the 5′ end of *TCM1* RNA was included in the reactions shown in Lanes 2−5 in each panel. Lanes 1 and 6 in each panel shows the results of control reactions carried out in the absence of S1 and RNA, respectively.



β-galactosidase activity

MEDIA	Wild type	ino2	opi1
I-C-	135.2	NP	546.5
I+C+	<1.0	<1.0	694.9
I-C-	136.4	NP	591.5
I+C+	<1.0	<1.0	459.3
I-C-	19.3	NP	77.8
I+C+	<1.0	<1.0	76.4
I-C-	NP	NP	NP
I+C+	<1.0	<1.0	<1.0
I-C-	<1.0	NP	<1.0
I+C+	<1.0	<1.0	<1.0
I-C-	<1.0	NP	<1.0
I+C+	<1.0	<1.0	<1.0
I-C-	159.3	NP	NP
I+C+	228.4	NP	NP

Figure 5. Schematic representation of *INO1* regulatory region fusions to a *CYC1-lacI′Z* chimaera. Noted are the relative positions of the TATA boxes and starts of transcription (+1). Plasmid pJH359 contains *INO1* sequences −329 to −119 generated by insertion of a *Mae*II restriction fragment with *Xho*I linkers into a *Xho*I site in pJH304 that contains the *CYC1* TATA elements. Likewise, pKS102 and pK101 contain *INO1* sequences −259 to −154 and −259 to −219, respectively. Plasmid pJH304 is the parental vector and contains no *INO1* DNA. Plasmids pGS101 and pGS104 were constructed by inserting one and five copies of the 21 bp oligonucleotide, respectively, into the *Xho*I site of pJH304. The 21 bp oligonucleotide includes the conserved nine bp element discussed in the text. Plasmid pLGΔ312 contains the intact *CYC1* promoter fused to *lacI′Z*. Units of β-galactosidase are equal to X1000 (optical density at 420 nm/min/mg of total protein). Abbreviations: I-C-, minimal media; I+C+, minimal media supplemented with 75 μM inositol and 1 mM choline; NP, not performed.

to *INO1* sequence and consists of an *Xho*I restriction site 3′ and a *Bgl*II site 5′ of the nine bp element (Figure 5). Construct pGS101 contains a single copy of the 21 bp oligonucleotide while pGS104 contains five copies of the oligonucleotide (Figure 5). Neither construct supported any expression of β-galactosidase under any growth condition when transformed into a wild type yeast strain (Figure 5).

Effects of Regulatory Mutations on Expression of lacZ Fusions

The *INO1-CYC1-lacI′Z* fusion constructs were transformed into strains bearing mutations at regulatory loci that are known to affect *INO1* expression. The pJH359 (−329 to −119) and pKS102 (−154 to −259) *INO1-CYC1-lacI′Z* constructs expressed high constitutive levels of β-galactosidase (Figure 5) in an *opi1* background. The *opi1* transformants carrying either of these constructs expressed 450 to 700 units of β-galactosidase constitutively (as compared to 135 units of activity under derepressing conditions in a wild type strain). The pKS101 (−259 to −219) construct expressed 76 to 78 units of β-galactosidase constitutively when transformed into the *opi1* background (as compared to 19.3 units at the derepressed condition in a wild type strain; Figure 5). The parent vector pJH304 and the two constructs, pGS101 and pGS104, that contained copies of the 21 bp oligonucleotide containing the nine bp repeat, failed to express any detectable activity in any strain background. None of the *INO1-CYC1-lacI′Z* transformants expressed any detectable activity in an *ino2* genetic background when grown in the presence of inositol plus choline.

DISCUSSION

Examination of the DNA sequence of the *INO1* 5′ flanking region (Figure 3) revealed an element that corresponds to the TATA consensus, spanning nucleotides −116 to −111. The location of this element is typical of *S. cerevisiae* genes, which usually contain functional TATA sequences in a region from 60 to 120 nucleotides upstream of the start site (35). The sequence present in the *INO1* promoter, 5′-TATAAA-3′, is identical to the sequence that has been shown to be the most efficient synthetic TATA element, 5′-TATAAA-3′ (32, 33). The d-86 and d-55 *INO1-lacZ* fusion constructs (Figures 1, 3) eliminate the putative TATA sequence at −116 and neither construct supports transcription, consistent with the hypothesis that the sequence at −116 to −111 is a functional TATA element.

The expression of β-galactosidase from plasmid pJH359 containing the *CYC1* TATA and *INO1* sequences spanning nucleotides −329 to −119, confirms that sequences 5′ to the *INO1* TATA are capable of functioning as a UAS and, furthermore, that they are capable of conferring regulation in response to inositol and inositol plus choline. A 105 bp sub-fragment spanning nucleotides −154 to −259 (construct pKS102, Figure 5) was also capable of supporting the same degree of expression and regulation from the *CYC1* TATA as the 210 bp fragment contained in pJH359. The 105 nucleotide fragment contained in pKS102 was inverted compared to its orientation in the intact *INO1* promoter. This lends further support to the idea that pKS102 contains within it an *INO1* UAS sequence since these elements function regardless of orientation (36). A sub-fragment from pKS102 spanning the 40 nucleotides from −259 to −219 (pKS101, Figure 5) also supported regulated expression, but at a reduced level. Furthermore, the *INO1* fragment in pKS102 and the smaller sub-fragment contained in pKS101 both contain a site(s) responsible for *INO1* response to the *OPI1* gene product since both constructs are expressed constitutively at elevated levels in the *opi1* mutant background. The pKS102 and pKS101 constructs also appear to be dependent on the *INO2* gene product since they are not expressed in an *ino2* genetic background. However, since the *ino2* mutants are inositol auxotrophs, they must always be grown in the presence of inositol. A rigorous test of *INO2* dependence will require an assessment of derepression during a transient growth period following removal of inositol from the medium.

Thus, all the information necessary for regulation of the *INO1* gene is contained within the 105 nucleotides spanning −259 to −154 and a subset of that information is contained between −259 and −219. The only sequence detected in the *INO1* promoter that is found in promoters of other co-regulated genes, is the nine bp consensus, 5′-ATGTGAAAT-3′. The 105 bp fragment in pKS102 contains two copies of the nine bp repeated element, and the smaller 40 bp sub-fragment in pKS101 contains one copy of the repeat. The d-333 *INO1-lacZ* deletion construct maintained full repression in response to inositol. This construct removes half of the most upstream nine base pair repeated element, leaving six intact copies (Figure 3). The d-155 construct that retains only two copies of the nine bp repeat; one 5′ to the TATA sequence and one 3′ to TATA was still partially repressed in response to inositol. Comparison of the 105 nucleotides (spanning −259 to −154) that conferred regulation on pKS102 to the sequence 5′ to TATA remaining in the d-155 construct (Figure 3), revealed only one sequence in common; namely, the nine bp element.

However, the completely artificial constructs, pGS101 and pGS104, containing one or five copies, respectively (Figure 5), of the nine bp repeat contained within a 21 bp oligonucleotide of sequence otherwise unrelated to *INO1*, did not activate transcription from the heterologous TATA in the *CYC1-lacI′Z* fusion. Thus, the nine bp repeat is not sufficient in and of itself to activate transcription and therefore, cannot by itself define the *INO1* UAS. This result does not, however, eliminate the possibility that the nine bp element could function as an essential element in *INO1* expression in conjunction with other as yet unidentified adjacent sequences. This type of element has recently been observed in the *DAL7* gene (37) and termed a UIS (upstream induction sequence). In that system, the UIS by itself does not activate transcription of a heterologous gene, but increases the level of activation driven by the *DAL7* UAS (37). Alternatively, the nine bp element could be involved in repression (37).

It is possible that the nine bp repeated element in the *INO1* upstream region does not define a positive or a negative regulatory site and that its existence in the promoter is completely unrelated to the observed regulation. However, it has recently been demonstrated that the nine bp consensus sequence defines a binding site for a specific protein. Band mobility shift assays (J. Lopes, unpublished) have revealed that the protein is capable of binding to each of the multiple sites within the *INO1* promoter and that the addition of the 21 bp oligomer containing the nine bp repeat is sufficient to compete away the binding activity. The protein that binds specifically to the nine bp repeat, however, is present in extracts of all yeast strains thus far examined including *ino2*, *ino4* and *opi1* strains. Therefore, this protein cannot be the gene product of any locus thus far defined in genetic terms as a regulator of *INO1*. A biochemical characterization of this protein is in progress. Experiments are presently in progress to test, directly, the ability of the nine bp element to serve as

a repressor in conjunction with heterologous UAS elements and to further define the precise minimum sequence and characteristics of the *INO1* UAS.

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REFERENCES

1. Carman, G. M., and Henry, S. A. (1989) *Annual Review of Biochemistry*, **58**, 635–669.
2. Kelley, M. J., Bailis, A. M., Henry, S. A., and Carman, G. M. (1988) *J. Biol. Chem.*, **263**, 18078–18084.
3. Bailis, A. M., Poole, M. A., Carman, G. M., and Henry, S. A. (1987) *Mol. Cell. Biol.*, **7**, 167–176.
4. Culbertson, M. R., Donahue, T. F., and Henry, S. A. (1976) *J. Bacteriol.*, **126**, 232–242.
5. Homann, M. J., Henry, S. A., and Carman, G. M. (1985) *J. Bacteriol.*, **163**, 1265–1266.
6. Donahue, T., and Henry, S. (1981) *J. Biol. Chem.*, **256**, 7077–7085.
7. Hirsch, J. P., and Henry, S. A. (1986) *Mol. Cell. Biol.*, **6**, 3320–3328.
8. Culbertson, M., and Henry, S. (1975) *Genetics*, **80**, 23–40.
9. Loewy, B. S., and Henry, S. A. (1984) *Mol. Cell. Biol.*, **4**, 2479–2485.
10. Greenberg, M. L., Reiner, B., and Henry, S. A. (1982) *Genetics*, **100**, 19–33.
11. Greenberg, M. L., Goldwasser, P., and Henry, S. A. (1982) *Mol. Gen. Genet.*, **186**, 157–163.
12. Nonet, M. L., and Young, R. A. (1989) *Genetics*, **123**, 715–724.
13. Scafe, C., Chao, D., Lopes, J., Hirsch, J. P., Henry, S., and Young, R. A. (1990) *Nature*, (In press).
14. Arndt, K. T., Styles, C. A., and Fink, G. R. (1989) *Cell*, **56**, 527–537.
15. Ruby, S. W., Szostak, J. W., and Murray, A. W. (1983) *Methods in Enzymol.*, **101**, 253–267.
16. Myers, A. M., Tzagoloff, A., Kinney, D. M., and Lusty, C. J. (1986) *Gene*, **45**, 299–310.
17. Guarente, L., and Ptashne, M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2199–2203.
18. Birnboim, H. C., and Doly, J. (1979) *Nucleic Acids Res.*, **7**, 1513–1523.
19. Clewell, D. B., and Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. USA*, **62**, 1159–1166.
20. Henikoff, S. (1984) *Gene*, **28**, 351–359.
21. Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P. (1977) *J. Mol. Biol.*, **113**, 237–251.
22. Sanger, F., Nicklen, S., and Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
23. Chen, E. J., and Seeburg, P. H. (1985) *DNA*, **4**, 165–170.
24. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.*, **153**, 163–168.
25. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
26. Elion, E. A., and Warner, J. R. (1984) *Cell*, **39**, 663–673.
27. Aviv, H., and Leder, P.. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 1408–1412.
28. Reddy, V. B., Ghosh, P. K., Lebowitz, P., Piatak, M., and Weissman, S. M. (1979) *J. Virol.*, **30**, 279–296.
29. Berk, A. J., and Sharp, P. A. (1977) *Cell*, **12**, 721–732.
30. Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248–254.
31. Dean-Johnson, M., and Henry, S. A. (1989) *J. Biol. Chem.*, **264**, 1274–1283.
32. Chen, W., and Struhl, K. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2691–2695.
33. Nagawa, F., and Fink, G. R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8557–8561.
34. Orr-Weaver, T. L., Szostak, J. W. and Rothstein, R. J. (1983) *Methods in Enzymol.*, **101**, 228–245.
35. Hahn, S., Hoar, E. T., and Guarente, L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8562–8566.
36. Guarente, L. (1984) *Cell*, **36**, 799–800.
37. Yoo, H. S. and Cooper, T. G. (1989) *Mol. Cell. Biol.*, **9**, 3231–3243.