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INO1-100: an allele of the *Saccharomyces cerevisiae* *INO1* gene that is transcribed without the action of the positive factors encoded by the *INO2*, *INO4*, *SWI1*, *SWI2* and *SWI3* genes

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ABSTRACT

A dominant allele of the *INO1* locus, *INO1-100*, does not require the positive regulators encoded by *INO2* and *INO4* for expression. Sequence analysis showed that *INO1-100* had a 239 bp deletion in the *INO1* promoter. *INO1-100* suppressed the inositol auxotrophy of *ino2*, *ino4*, *swi1*, *swi2* and *swi3* mutants. Transcription of *INO1-100* was constitutive and independent of these regulators. A 20 bp deletion from –247 to –228 caused a similar phenotype. A 38 bp deletion from –245 to –208 suppressed the inositol auxotrophy of an *ino2* mutant, but not an *ino4* mutant, indicating that Ino2p and Ino4p may function alone as well as in a complex. A 40 bp deletion from –287 to –248 that removed a URS1 site caused constitutive transcription that required *INO2* and *INO4*. A deletion from –167 to –128 suppressed the inositol auxotrophy of *swi*, *ino2* and *ino4* mutants, indicating the presence of a previously unidentified URS1. Mutation of the specific negative regulator of phospholipid synthesis encoded by *OPI1* suppressed the inositol auxotrophy of *swi2* mutants. This study indicates that negative regulation of *INO1* is chromatin mediated and provides *in vivo* information on the interaction of both general and specific regulatory factors that function to accomplish negative and positive regulation of the *INO1* promoter in response to inositol.

INTRODUCTION

The *Saccharomyces cerevisiae* enzyme inositol-1-phosphate synthase catalyzes the conversion of glucose-6-phosphate to inositol-1-phosphate and is encoded by the *INO1* gene (1,2). Transcription of *INO1* is highly regulated. *INO1* mRNA is present at derepressed levels when inositol is absent from the growth medium and at repressed levels in the presence of at least 25 μM inositol (3,4). Full repression requires both inositol and choline, although choline by itself has no effect (3). Regulation of the *INO1* response to inositol requires the action of two specific positive regulators, Ino2p and Ino4p (5–7), and one negative regulator, Opi1p (8). These transcriptional regulators also act on other phospholipid biosynthetic genes (5,7–9), the inositol transport gene *ITR1* (11) and fatty acid biosynthetic genes (10). In the absence of inositol, Ino2p and Ino4p act to derepress *INO1* transcription and may function together in a complex (6,11). Elimination of *INO2* or *INO4* results in inositol auxotrophy (1,6,7,12). When inositol is present in the growth medium, Opi1p acts to repress *INO1* transcription; mutations at *OPI1* cause constitutive overexpression of *INO1* and excretion of inositol (8). *INO2* and *INO4* are epistatic to *OPI1*; an *ino2/ino4 opi1* double mutant is an inositol auxotroph (13–15).

In addition to these specific regulators, *INO1* transcription is sensitive to mutations that affect the more general transcriptional apparatus, such as at *SWI1*, *SWI2* and *SWI3*, and these mutant strains are also inositol auxotrophs (16). The *SWI1*, *SWI2* and *SWI3* products are part of a large protein complex that antagonizes chromatin-mediated transcriptional repression (17–20). Mutation of the *SIN3* gene (*CPE1/UME4/RPD1/GAM2/SDI1*) also affects a variety of genes and causes constitutive derepression of *INO1* transcription (21–25). *SIN1* was identified through a mutation that suppressed the effects of *swi1*, *swi2* and *swi3* on the mating type gene *HO* (21). *SIN1* is also a negative regulator of *INO1* (16).

A strain with mutations at *ino2* and *ino4* was used to select a spontaneous inositol prototroph carrying a mutation that suppresses the requirement for functional Ino2p and Ino4p (26,27). The dominant mutation is unlinked to *INO2* or *INO4* and causes constitutive expression of inositol-1-phosphate synthase (27). In the present study we show that the mutation is a 239 bp deletion in the 5′ flanking region of the *INO1* gene. This dominant allele, *INO1-100*, was transcribed constitutively; in the wild-type the level of *INO1* mRNA is repressed 10-fold, whereas *INO1-100* mRNA was only slightly lowered, when inositol was present in the growth medium. Transcription of *INO1-100* did not require *INO2*, *INO4*, *SWI1*, *SWI2* or *SWI3* gene products. Since genetic and biochemical evidence indicates that *INO2* and *INO4* encode products that are required to activate transcription of *INO1*, how could a deletion in the *INO1* promoter region cause constitutive, *INO2/INO4*-independent expression? In order to address this question, *INO1* alleles with a series of smaller deletions spanning

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the region deleted in *INO1-100* were studied. In addition to focusing on functional interactions in the *INO1* promoter region with Ino2p, Ino4p and Opi1p, the effect of *swi* mutations led to the conclusion that relief from chromatin-mediated negative regulation is important to *INO1* function. This study provides *in vivo* information on the regions of the *INO1* promoter that interact with the negative and positive regulatory factors and with each other to achieve a complex mechanism of regulation.

MATERIALS AND METHODS

Media and general molecular methods

Synthetic inositol-free media (I^-) and inositol-containing media (I^+) were described previously, as were the methods used for plasmid DNA isolation and transformation of yeast and *Escherichia coli* (28,29).

Isolation and DNA sequence of the *INO1-100* mutation

The *INO1-100* mutation was isolated from a library of the mutant genome constructed by a *Sau*3A partial digest of DNA from the *INO1-100* strain in the yeast vector pYC1. The *ino2 ino4* yeast strain PMY158 was transformed and a clone conferring inositol prototrophy was isolated. A portion of the insert corresponding to the region from the *Pst*I site at –542 to the *Cla*I site at +372 in the wild-type *INO1* gene was sequenced using the Sanger dideoxy method as adapted by Stratagene.

Strains

A 1.0 kb *Pvu*II/*Hin*dIII fragment (–654 to +356) from the *INO1-100* clone pO5-1 was isolated and subcloned into YIp351 (30) at the *Sma*I/*Hin*dIII sites to make plasmid pSS1. The 1.0 kb DNA fragment contained the mutation but did not provide a functional fraction of the *INO1* gene. Plasmid pSS1(*LEU2*) was linearized and integrated by homologous recombination (31) into the *INO1* locus of strain PMY158 to form strain SSY10. Therefore, strain SSY10 carries the *INO1-100* mutant allele with *LEU2* linked to it integrated next to a non-functional piece of the original *INO1* gene.

Saccharomyces cerevisiae strains used in the *INO1* promoter deletion allele studies are isogenic to PMY168 (MATα *ura3-52, his3Δ200, trp1Δ1, leu2Δ1*) or PMY169 (isogenic to PMY168, but MAT**a**) (29). The plasmid pBF1 (not shown) was constructed and used for gene replacement (32) of most of *INO1* with *HIS3*. The deletion at *INO2* in strain PMY162 was constructed as described by Nikoloff and Henry (6), using plasmid pMN118 to replace *INO2* with *TRP1* (32). The plasmid pJA25 (33) was used for deletion of *INO4* and replacement with *LEU2* to form strain PMY176. Plasmid pMW20 (8) was used to replace *OPI1* with *LEU2* in strains PMY172 and PMY177.

Other *S.cerevisiae* strains include PMY175 (*ino2::TRP1 opi1::LEU2* isogenic to PMY168), SSY51 (MATα *sin1::TRP1 ino2::TRP1 his3 leu2 lys2 trp1 ura3 ade*), SSY52 (MATα *sin1::TRP1 ino2::TRP1 opi1::LEU2 his3 leu2 lys2 trp1 ura3 ade*), SSY55 (MATα *sin3::TRP1 ino2::TRP1 opi1::LEU2 his3 leu2 lys2 trp1 ura3*), SSY79 (MATα *swi2::HIS3 ade2 his3 leu2 trp1 ura3*) and SSY80 (MAT**a** *swi2::HIS3 opi1::LEU2 ade2 his3 leu2 trp1 ura3*).

Plasmids

Plasmids used in the deletion analysis were based on pRS416 (an ARS/CEN/*URA3* plasmid from Stratagene). Southern analysis (see below) showed that plasmid constructs were present in transformed yeast cells in a single copy. The polymerase chain reaction (PCR) was used in most constructions. Multiple isolates of each construct were tested. The *INO1-100* allele was amplified from genomic DNA (34) using a primer at –422 bp and a primer at +1812 bp relative to the *INO1* initiation codon. The PCR fragment was cut and cloned into the *Bam*HI site of pRS416, giving rise to pINO1-100 (the opposite orientation was pINO1-100A). Except for using PMY168 chromosomal DNA (wild-type *INO1*) for the PCR template, pINO1 was made identical to pINO1-100. These clones were tested for complementation of an *ino1* mutation and were indistinguishable from the analogous clones isolated from genomic libraries. The constructs contained a minimal amount of sequence (66 bp) upstream of the *INO1-100* deletion, so as to minimize potential interference from an opportunistic promoter. No detectable size difference between *INO1* mRNA and any of the plasmid alleles or the genomic *INO1-100* allele was observed.

The other constructs with deletions in the region –366 to –128 relative to the *INO1* transcription initiation site were made by amplifying the region 5′ to the deletion (Fig. 2) and cloning that DNA fragment into pRS416 cut with *Bam*HI and *Eco*RI. The region downstream of each deletion was amplified and then cloned into the 5′ construct using *Eco*RI and *Xho*I. This procedure removed either 40 or 20 bp and replaced it with an *Eco*RI site. The template for these PCR amplifications was pJH318, which carries the *INO1* gene (3). The promoter regions of the deletion alleles were sequenced at the Biopolymer Laboratory (University of Maryland at Baltimore).

Southern analysis

The genomic identification of the *INO1-100* deletion and the copy number of the plasmids used in the deletion analysis was determined by Southern analysis. Genomic DNA was isolated (34), run on a 1% agarose gel and transferred to Nytran electrophoretically. The riboprobe used was made from pO5-8, which contains the *Cla*I–*Sac*I fragment of *INO1-100* cloned into pGEM/7Zf. This probe hybridized to a 1335 bp *Bst*N1 fragment of *INO1* from –554 to +781.

To examine copy number of the plasmids, genomic DNA from PMY173 cells (*ino1::HIS3*) with their respective plasmids was digested with *Bst*N1, blotted and probed with riboprobes *INO1* and *TCM1* (a ribosomal protein gene used as a standard) (35). Determination of copy number was made by PhosphorImager analysis (Molecular Dynamics) with values for the *INO1* bands normalized to their respective *TCM1* bands and compared with PMY168 (wild-type) genomic DNA as the standard for a single copy of *INO1*.

Northern analysis

The procedures for RNA isolation and analysis have been described previously (29).

RESULTS

INO1-100 is an allele of *INO1*

INO1-100 was isolated as a spontaneous mutation that suppresses the requirement of the *ino2 ino4* strain for inositol and thus allows

the *ino2 ino4* strain (A7) to grow on synthetic media lacking inositol (I^- medium) (27). The mutation causes constitutive expression of the protein encoded by the *INO1* gene, *myo*-inositol-1-phosphate synthase, a phenotype that is dominant, as it is observed in both a haploid strain and a heterozygous diploid strain (27). A library was made from A7 DNA (see Materials and Methods) in order to clone the suppressor mutation. A plasmid that complemented the inositol auxotrophy of an *ino2 ino4* strain was identified by transforming the *ino2 ino4* strain PMY158 with the library and selecting transformants that were inositol prototrophs. Further analysis of the primary transformants identified one plasmid, pO5-1, that could complement the inositol auxotrophy of the *ino2 ino4* strain.

Linkage analysis of the cloned gene and the genomic mutation in strain A7 was carried out to confirm that the plasmid that was isolated carried the suppressor mutation present in strain A7, the original isolate. After subcloning to the integrating vector YIp351 (see Materials and Methods), the putative suppressor mutation was integrated into the genome of the *ino2, ino4* strain by homologous recombination to form strain SSY10. The free ends that were generated by cutting the plasmid pSS1 within the suppressor gene sequence were expected to direct the integration to the homologous chromosomal sequence (31). Tetrad analysis of the spores from mating strain SSY10 to strain A7 (*INO1-100, ino2, ino4*) showed 4:0 segregation for the ability to grow without inositol in the medium, indicating that the cloned suppressor mutation was linked to the original mutation in strain A7.

DNA sequence analysis of the suppressor mutation (see Materials and Methods) demonstrated that the mutation was an allele of *INO1*. As can be seen in Figure 1A, the suppressor allele *INO1-100* was identical to wild-type *INO1* except for a deletion of 239 bp in the region of *INO1-100* that is 5′ to the *INO1* open reading frame. The missing sequence was from –366 to –128 inclusive, relative to the transcription initiation site of *INO1* (+1) at –5 from the *INO1* AUG (36). All sequence location designations in this paper are relative to the transcription initiation site (+1). The fusion junction of the deletion occurs within a string of three T residues and is shown in Figure 1B. Figure 1C shows that Southern analysis of genomic DNA from a strain carrying the original *INO1-100* mutation (A7) confirmed a size difference of about 250 bp (Fig. 1B). The *INO1-100* deletion removes two previously identified UAS_{INO} elements at –239 to –229 (UAS_{-239}) and –177 to –167 (UAS_{-177}) (36) and a URS1 (37) site at –255 to –247 (URS_{-255}) (38; Fig. 1). Ino2p and Ino4p are thought to act at UAS_{INO} sites (6,11). We identified an additional candidate URS1 sequence at –143 to –135 (URS_{-143}). Seven of the nine consensus bases (37) are present at this location and evidence presented in this paper indicated a site of negative regulation is located in the vicinity of this URS1.

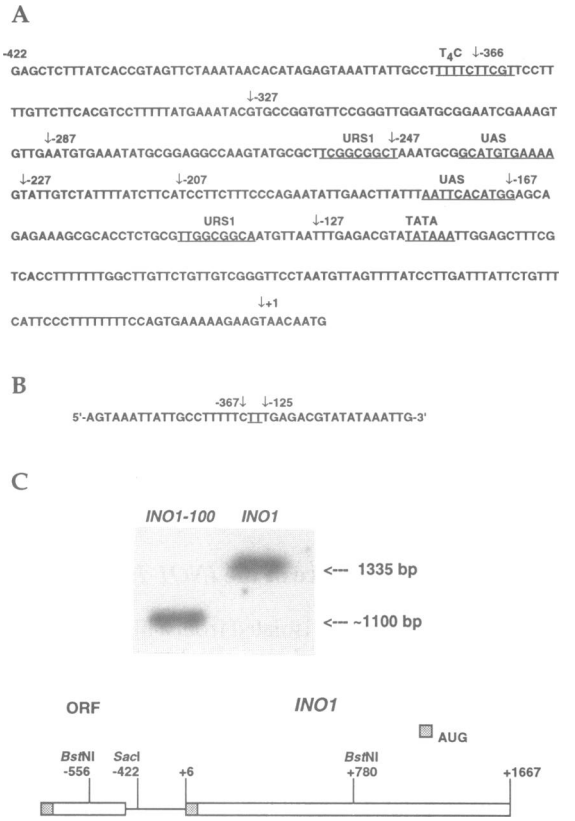


Figure 1. Sequence of the *INO1-100* allele. (**A**) Sequence of the 5′ region of *INO1*. The underlined sequences represent $URS1_{-255}$, UAS_{INO} (UAS_{-239} and UAS_{-177}), $URS1_{-143}$ and the TATA box. The arrows indicate the boundaries of various deletions. The sequence from –366 to –128 inclusive relative to transcription initiation (+1) was deleted in the *INO1-100* allele. The boundaries of the deletions are: A1, –366 to –328; B2, –327 to –288; C3, –287 to –248; D4, –247 to –208; E5, –207 to –168; F6, –167 to –128; D41, –247 to –228; D42, –245 to –208; D43, –227 to –208. (**B**) Sequence of the region resulting from the *INO1-100* deletion. A region with three T residues fused within a region with two T residues to form a new joint containing three T residues. (**C**) The original *INO1-100* isolate contains a genomic deletion at *INO1*. Southern analysis of the 5′ regions of *INO1* (strain PMY168) and *INO1-100* (strain A7) was done using genomic DNA digested with *Bst*NI (see Materials and Methods).

Identification of two 40 bp regions that each suppress the requirement for *INO2* and *INO4*

In order to narrow down the region responsible for suppression of the inositol auxotrophy of *ino2* and *ino4* strains, *INO1* alleles with deletions internal to the larger deleted region in the *INO1-100* allele were constructed. Six 40 bp deletions were made corresponding to the plasmids shown in Figure 2 (pA1, pB2, pC3, pD4, pE5 and pF6). These and other *INO1* alleles used in the studies in this paper were constructed in single copy CEN/ARS vectors; copy number was determined as described in Materials and Methods and ranged between 0.8 and 1.4 copies/cell for the series. In addition, the promoter region of each construct was sequenced to establish that no new mutations occurred during the process of constructing the series of deletion alleles (see Materials and Methods). Each allele complemented a strain (PMY173) with a growth requirement for inositol caused by deletion of the *INO1* coding region; all deletion alleles supported normal growth on medium without inositol (Fig. 2, column A). Thus none of the deletions removed a region essential for transcription or translation of *INO1* and all of them produced a fully functional inositol-1-phosphate synthase. The *INO1-100* allele was cloned into the vector pRS416 that was used in all other constructions in both orientations to show that expression of the alleles was independent of plasmid sequences.

Each of the six deletion alleles was tested in *ino2* and *ino4* strains to determine whether they would suppress inositol

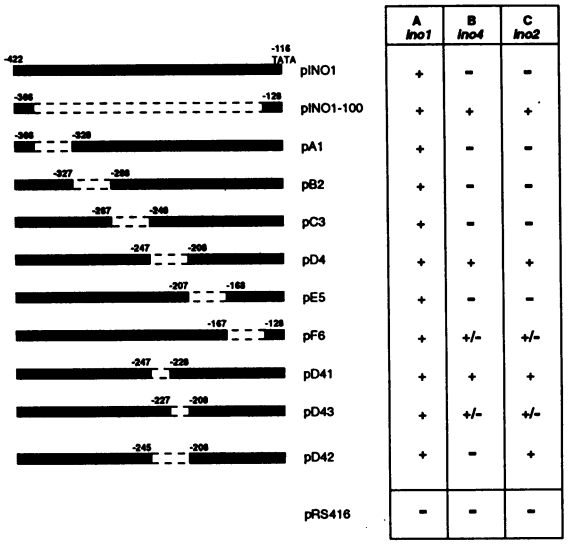


Figure 2. Complementation of *ino1*, *ino2* and *ino4* mutations by *INO1* deletion alleles. The assay was for growth on synthetic medium without inositol (I^-). Choline was added to achieve the growth indicated for the strains that carried pD41, pD42 and pD43. Choline had no effect on the growth of the other strains. Construction of the plasmids carrying the deletion alleles is described in Materials and Methods. pRS416 was the parent vector. +, normal growth; +/–, less than normal growth; –, no growth.

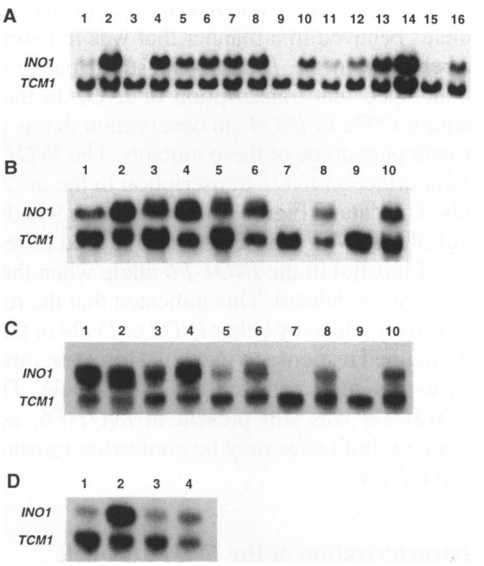


Figure 3. *INO1* mRNA expressed from the *INO1* deletion alleles. (**A**) Alleles expressed in an *ino1* null strain. Lanes 1 and 2, *INO1-A1*; 3 and 4, *INO1-B2*; 5 and 6, *INO1-C3*; 7 and 8, *INO1-D4*; 9 and 10, *INO1-E5*; 11 and 12, *INO1-F6*; 13 and 14, *INO1-100*; 15 and 16, *INO1*. (**B**) Alleles expressed in an *ino4* strain. (**C**) Alleles expressed in an *ino2* strain. For (B) and (C) lanes 1 and 2, *INO1-100*; 3 and 4, *INO1-D4*; 5 and 6, *INO1-D41*; 7 and 8, *INO1-D43*; 9 and 10, *INO1-F6*. (**D**) Northern analysis of *INO1-D42*. Lanes 1 and 2 are *INO1-D42* in an *ino1* null strain and lanes 3 and 4 are *INO1-D42* in an *ino2* strain. RNA was extracted from cells grown to early log stage in the presence of 65 μM inositol (odd lanes) or in its absence (even lanes). The riboprobe for the *INO1* mRNA was generated from plasmid pJH319 (3). The riboprobe for the *TCM1* mRNA was generated from plasmid pAB309 (55). Riboprobes were labeled by incorporation of [α-^{32}P]CTP. *TCM1* mRNA was used as an internal control that does not respond to inositol and the relative quantities were determined by PhosphorImager analysis (see Materials and Methods).

auxotrophy of these mutants (Fig. 2, columns B and C). When the *ino2* and *ino4* strains carried the *INO1-D4* allele (deletion –208 to –247) they did not require inositol and were able to grow as well as both the *INO1-100* and wild-type strains. *INO1-F6* (deletion –128 to –167) also suppressed inositol auxotrophy of the *ino2* and *ino4* strains, although to a lesser extent than the *INO1-D4* allele. This data suggested that the deletions in *INO1-D4* and *INO1-F6* removed functional negative elements. The UAS_{-239} element and one base pair (T) of $URS1_{-255}$ were removed in the *INO1-D4* allele and the putative $URS1_{-143}$ was removed in the *INO1-F6* allele. The *INO1-A1*, *INO1-B2*, *INO1-C3* and *INO1-E5* alleles required the *INO2* and *INO4* genes for growth on medium without inositol (Fig. 2). Therefore, none of these deletion alleles were able to suppress the *ino2* or *ino4* mutations, indicating that they do not remove the critical region of the *INO1* promoter that is missing in the *INO1-100* deletion allele.

Transcription of *INO1* is independent of *INO2* and *INO4* in the *INO1-100*, *INO1-D4* and *INO1-F6* strains

Northern analysis of *INO1-100*, *INO1-D4* and *INO1-F6* mRNA levels in the PMY173 background (*ino1::HIS3*, *INO2*, *INO4*) showed that in the presence of wild-type alleles of *INO2* and *INO4*, *INO1* mRNA was observed in both the presence and absence of inositol in the growth medium (Fig. 3A). Wild-type *INO1* mRNA (Fig. 3A, lanes 15 and 16) was repressed ~10-fold in the presence of inositol, as previously reported for the genomic allele (3). This result indicated that this wild-type plasmid-borne *INO1* allele was regulated normally in response to inositol. mRNA levels from the *INO1-100* strain (Fig. 3A, lanes 13 and 14) were essentially constitutive, but did respond to the presence of inositol in the growth medium with an ~2-fold reduction. *INO1-F6* (Fig. 3A, lanes 11 and 12) was repressed somewhat in the presence of inositol; the repressed level of mRNA was higher than in the wild-type.

INO1 mRNA levels for the four alleles that required *INO2/INO4* for growth were also investigated. *INO1-A1* (Fig. 3A, lanes 1 and 2), *INO1-B2* (Fig. 3A, lanes 3 and 4) and *INO1-E5* (Fig. 3A, lanes 9 and 10) responded to the presence and absence of inositol in the growth medium normally. Repressed and derepressed levels of *INO1* mRNA from *INO1-E5* were identical to those observed with wild-type *INO1* (Fig. 3A, lanes 15 and 16). The derepressed levels of *INO1* mRNA from *INO1-A1* and *INO1-B2* were slightly elevated over those observed in the wild-type *INO1* strain. The *INO1-C3* allele (deletion –248 to –287) is missing all but one base of the URS1 located at –247 to –255. *INO1* mRNA levels in the presence and absence of inositol in the growth medium (Fig. 3A, lanes 5 and 6) showed a response in the *INO1-C3* strain that was similar to the response observed in the *INO1-100*, *INO1-D4* and *INO1-F6* strains; essentially constitutive expression, with an ~2-fold decrease in *INO1* mRNA observed when inositol was present in the growth medium. Expression of *INO1* mRNA in the *INO1-C3* strain, however, required *INO2* and *INO4*, whereas expression of *INO1* mRNA in the *INO1-100*, *INO1-D4* and *INO1-F6* strains was independent of *INO2* and *INO4*.

Northern analysis of the *INO1-100*, *INO1-D4* and *INO1-F6* mRNA levels in the PMY176 (*ino4::LEU2*) and PMY162 (*ino2::TRP1*) backgrounds is shown in Figure 3. The *INO1-100*

(Fig. 3B and C, lanes 1 and 2) and *INO1-D4* (Fig. 3B and C, lanes 3 and 4) alleles behaved in a manner that was indistinguishable from their behavior in the *INO2/INO4* strain background. We therefore concluded that transcription of *INO1* in these alleles does not require *INO2* or *INO4*, an observation that is consistent with the growth phenotype of these mutants. The *INO1-F6* allele had a different effect on *INO1* transcription in the *ino2* and *ino4* backgrounds. The data in Figure 3B and C (lanes 9 and 10) show that the level of the *INO1* transcript was reduced in response to the presence of inositol in the *INO1-F6* allele when the *INO2* or the *INO4* genes were deleted. This indicated that the response to inositol can be maintained by either *INO2* or *INO4* or that another regulator maintained response to inositol. However, this response to inositol was not seen with the *INO1-D4* allele. The region deleted in *INO1-D4* was still present in *INO1-F6*, as was the UAS_{-239} element; both sites may be conferring inositol responsiveness to *INO1-F6*.

Further characterization of the *INO1-D4* allele

The region of the *INO1* promoter that when deleted produced a phenotype similar to the original *INO1-100* deletion was from –208 to –247, as demonstrated with the *INO1-D4* allele. In order to further define the specific region conferring the *INO2/INO4*-independent constitutive phenotype, three additional *INO1* deletion alleles were characterized. Three deletions were made corresponding to the plasmids shown in Figure 2 (pD41, pD42 and pD43). Strains carrying the deletion alleles *INO1-D41* (deletion –228 to –247), *INO1-D42* (deletion –208 to –245) and *INO1-D43* (–208 to –227) all complemented inositol auxotrophy of the PMY173 strain (*ino1::HIS3*, *INO2*, *INO4*) (Fig. 2, column A).

The deletion alleles were tested in *ino2* and *ino4* strains to see if they could suppress inositol auxotrophy of these mutants (Fig. 2, columns B and C). The *INO1-D41* deletion removed the UAS_{-239} element and one base from the $URS1_{-255}$ site. When *ino2* and *ino4* strains carried *INO1-D41*, suppression of inositol auxotrophy was observed at a level just slightly less than with *INO1-100* or *INO1-D4*. *INO2* and *INO4* are also required for wild-type level *de novo* synthesis of phosphatidylcholine (PC) (26). Supplementing with choline allows synthesis of PC by the CDP–choline pathway (39) and compensates for the deficiency. If *ino2/ino4* are supplemented with adequate inositol, a choline supplement is not required (26). If inositol availability is lower than normal, phosphatidylinositol levels are affected and phosphatidylcholine deficiency limits growth. When *INO1-D41* was supplemented with choline, suppression of inositol auxotrophy was equivalent to that observed with *INO1-D4*. This indicates, however, that *INO1* mRNA is slightly lower in *INO1-D41* than in *INO1-D4*. The requirement for *INO2/INO4* was weakly suppressed in strains carrying *INO1-D43* (Fig. 2, columns B and C). We concluded that the region missing in *INO1-D41* from –228 to –247 must contain most of the critical sequence. In order to find out whether the 1 bp of the $URS1_{-255}$ that was missing in the *INO1-D4* allele was involved in suppression of the requirement for *INO2* or *INO4*, the *INO1-D42* allele was tested. The *INO1-D42* allele was almost identical to the *INO1-D4* allele except that it contained a complete URS1 site along with one more 3′ flanking base pair. The *ino4* strain carrying *INO1-D42* was auxotrophic for inositol (Fig. 2, column B), although *INO1-D42* did suppress inositol auxotrophy of the *ino2* strain (Fig. 2, column C). We concluded from these results that inactivation or removal of both $URS1_{-255}$ and UAS_{-239} combine to cause the *INO1-100* phenotype, allowing essentially constitutive expression of *INO1* in the absence of its known activators *INO2* and *INO4*.

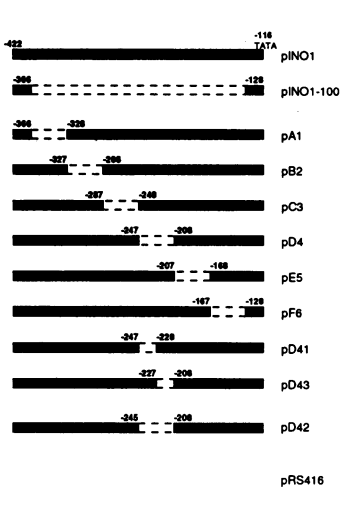
An analysis of *INO1* mRNA levels in the *INO1-D41* and *INO1-D43* strains in the *ino2* and *ino4* genetic backgrounds is shown in Figure 3B and C. The expression observed from *INO1-D41* (Fig. 3B and C, lanes 5 and 6) was less than that from *INO1-D4* (Fig. 3B and C, lanes 3 and 4) and *INO1-100* (Fig. 3B and C, lanes 1 and 2) and expression was lower in the *ino2* mutant than in *ino4*. *INO1-D41* was also expressed at higher levels in the absence of inositol than in its presence. Expression of *INO1* mRNA was also observed in the absence of inositol from *INO1-D43* (Fig. 3B and C, lane 8), although compared with *INO1-D41*, mRNA levels were reduced (Fig. 3B and C, lanes 6 and 8). The phenotype of the *INO1-D43* strain was similar to the *INO1-F6* strain. An analysis of *INO1* mRNA levels in the *INO1-D42* strains in the *ino1* and *ino2* genetic backgrounds is shown in Figure 3D. In the *ino1*, *INO2*, *INO4* background, transcription from the *INO1-D42* allele was repressed in the presence of inositol and derepressed in the absence of inositol. In the *ino2* background, *INO1-D42* was derepressed to a lower level than in the *ino1* background. This suggested that some derepression of *INO1-D42* in the *ino1* background was dependent on *INO2*. In comparison with *INO1-D4*, the levels of mRNA from *INO1-D42* were lower overall, indicating that loss of URS1 function was responsible for some of the constitutive transcription from *INO1-D4*.

INO2/INO4-independent transcription is also independent of *SWI1*, *SWI2* and *SWI3*

Strains with mutations at *swi1*, *swi2* and *swi3* make reduced amounts of *INO1* mRNA and are inositol auxotrophs (16). These *swi* strains were tested to see whether the requirement for inositol could be suppressed by any of the *INO1* deletion alleles and the results are shown in Figure 4. It was first established that the plasmid carrying the wild-type *INO1* gene, pINO1, did not suppress inositol auxotrophy of the *swi* strains, in agreement with the results of Peterson and colleagues (16) that wild-type *INO1* is expressed at very reduced levels in these *swi* mutant backgrounds. pINO1-100, carrying the *INO1-100* allele, however, complemented inositol auxotrophy of all three *swi* mutants. As can be seen in Figure 4, *INO1-D4* also suppressed the need for the *SWI1/2/3* gene products, suggesting a link between *SWI1/2/3* function and *INO2/INO4* function in regulating *INO1* transcription. Suppression of *swi1* inositol auxotrophy was also observed with *INO1-F6*, which also suppressed inositol auxotrophy of the *swi2* and *swi3* strains to a lesser degree. That the function of the *SWI* gene products extends beyond interaction with either *INO2* or *INO4* was suggested by the data in Figure 4, which shows that some degree of suppression of the requirement for the *SWI1/2/3* gene products was also observed with *INO1-C3*, an allele that required both *INO2* and *INO4* for expression. *INO1-D42* weakly suppressed *swi2* inositol auxotrophy and *INO1-D43* weakly suppressed inositol auxotrophy of all three *swi* strains.

Interactions of *trans*-acting factors that affect *INO1*

In order to better understand various *trans*-acting mutations that affect *INO1*, several double and triple mutant strains were constructed. It is known that *ino2* mutants are inositol auxotrophs and so are *ino2 opi1* double mutants (9). *INO2* is required for positive regulation and *OPI1* for negative regulation. Would



swi1	swi2	swi3
-	-	-
+	+	+
-	-	-
-	-	-
+	+/-	+/-
+	+	+
-	-	-
+	+/-	+/-
+	+	+
+/-	+/-	+/-
nd	+/-	nd
-	-	-

Figure 4. Complementation of *swi1*, *swi2* and *swi3* inositol auxotrophy by *INO1* deletion alleles. The assay was for growth on synthetic medium without inositol (I^-). +, normal growth; +/–, less than normal growth; +/– –, poor but visible growth; –, no growth; nd, not done. The plasmids carrying the deletion alleles are the same as those in Figure 2.

eliminating another negative regulator overcome the auxotrophy? The mutation *sin1* suppresses inositol auxotrophy of *swi2* (16); when combined into a triple mutant with the genotype *sin1 ino2 opi1*, the *sin1* mutation did not alleviate inositol auxotrophy of *ino2 opi1* (Table 1). Mutation of another negative regulator encoded by *SIN3* is not sufficient to overcome inositol auxotrophy due to *ino2* mutation (22). A strain that carried the combination *ino2 opi1 sin3* remained an inositol auxotroph (Table 1).

Table 1. Genetic analysis of negative regulatory mutations in *swi2* and *ino2* strains

Strains	Relevant genotype	Growth on I^-
PMY162	*ino2*	–
PMY172	*opi1*	+
PMY233	*sin3*	+
PMY72	*sin1*	+
PMY175	*ino2 opi1*	–
SSY51	*ino2 sin1*	–
SSY52	*ino2 sin1 opi1*	–
SSY55	*ino2 sin3 opi1*	–
SSY79	*swi2*	–
SSY80	*swi2 opi1*	+

Although neither *opi1* mutations nor *opi1* mutations in combination with *sin* mutations were able to suppress inositol auxotrophy of *ino2* strains, we wanted to know whether *opi1* mutation would suppress inositol auxotrophy of a *swi* strain. Previous work (16) showed that a *sin1* mutation suppressed inositol auxotrophy of *swi1*, *swi2* and *swi3*. We found that the double mutant *swi2 opi1* was an inositol prototroph (Table 1). Therefore, either mutation at *sin1* (16) or mutation at *opi1* suppressed inositol auxotrophy caused by *swi2*, suggesting that the negative regulators encoded by *SIN1* and *OPI1* interact at some level with the *SWI2* positive factor, and perhaps with each other as well.

DISCUSSION

The failure of any of our deletions to 'kill' *INO1* transcription is curious. If molecular and genetic evidence indicates that *INO2* and *INO4* are positive regulators of *INO1* transcription and they bind to the UAS_{INO}, absence of the target should cause a similar phenotype. Instead, a spontaneous genomic deletion that removed both of the binding sites for the activation complex was found to rescue mutants lacking *INO2/INO4*. Although there are additional copies of the UAS_{INO} that are potentially functional, the mutant phenotype did not depend on these copies and they were not included in the construction of the deletion alleles. Removal of the binding site for the negative regulator *OPI1* might be expected to lead to an *INO1-100* phenotype, except that *ino2/ino4* mutations are epistatic to *opi1* mutations. An *opi1* mutation does not rescue an *ino2/ino4* double mutant; *ino2/ino4 opi1* mutants are inositol auxotrophs (13–15). Therefore, *INO1-100* was transcribed without Ino2p and Ino4p or the products of *SWI1*, *SWI2* or *SWI3*, genes that have been implicated in antagonism of chromatin-mediated repression and in activation of transcription (18,20,40–43). The *INO1-100* mutation relieved negative regulation and in the process achieved constitutive transcription independent of the known positive regulators.

INO1-100 is missing all known regulatory sites in the promoter and yet constitutive expression is observed at a higher than normal level. It is unlikely that the TATA box by itself supports this level of transcription in the *INO1-100* mutant strain. Instead, there may be another positive element that drives the high level of transcription. The location of this element must be downstream of the *INO1-100* deletion (–128), since almost no sequence upstream of the deletion was included in the constructions. Expression driven by this positive downstream element (DSE) must require something in the region missing in *INO1-100* for repression to occur in response to external inositol. Since transcription of *INO1-D4* closely resembles that of *INO1-100*, the region primarily responsible for repression of the DSE can be localized to –247 to –208. The region deleted in *INO1-F6* may also contribute to repression, since this allele was capable of some transcription in the absence of *INO2* and *INO4*. Why have a downstream element if Ino2p/Ino4p are satisfactory activators? *INO1* is also regulated by growth stage (44) and may be regulated during sporulation and other cellular events. This regulation could be independent of *INO2/INO4* and *OPI1* and require other elements, such as the DSE. The DSE could act synergistically with the upstream elements to achieve maximal transcription of *INO1* in the absence of inositol, or the region upstream of the TATA box and the battery of regulators may confer regulated expression in response to exogenous inositol and choline; in their absence the default setting for the gene is constitutive transcription.

From our observations we propose the following model of genetic interaction:

SWI1, SWI2, SWI3 → *SIN3, OPI1, SIN1 (UME6)* → *INO2, INO4* → R → DSE

where R represents the negative element removed by *INO1-100* and DSE represents a downstream element that stimulates transcription in the absence of inhibition. The *SWI* complex antagonizes the negative regulators encoded by *SIN1*, *SIN3*, *OPI1* and *UME6*. The action of these negative regulators would be to promote a transcriptionally repressive chromatin structure; targets could include the UAS_{INO} sites, the TATA box and the DSE. The *INO2* and *INO4* gene products would then act to alleviate the repressive chromatin structure, to antagonize the R

element(s) and to directly stimulate transcription. The R element acts to repress the DSE.

Several regions were identified that affected negative regulation. The deletion in *INO1-C3* effectively removed URS1$_{-255}$ and constitutive transcription was observed. This URS1 site has been shown previously to be a functional negative regulatory element (38). We observed that transcription of *INO1-C3* depended on Ino2p and Ino4p. Like *INO1-C3*, *opi1* mutations also confer constitutive transcription of *INO1* that is dependent on Ino2p and Ino4p. Loss of negative regulation of *INO1-C3* could result from elimination of the binding site for Opi1p. This model is unlikely. First of all, Opi1p is a specific negative regulator involved in phospholipid regulation and URS1 sites are found in many unrelated genes (23). Second, *opi1* mutants overexpress many phospholipid biosynthetic genes, including *ITR1* (29), which encodes the major inositol transporter. *ITR1* does not have a URS1 site, so Opi1p must be able to act independent of URS1. Furthermore, there is evidence that Opi1p acts through UAS$_{INO}$ (38). It is more likely that removal of the URS1 site eliminates repression mediated by the *UME6* protein and the *UME4/SIN3/CPE1* protein. Ume6p requires the URS1 site in the promoter of the arginase gene *CAR1* in order to repress transcription (45). It has been reported that *UME6* is also required for repression of the *INO1* gene (38). The *UME4/SIN3/CPE1* protein is also a negative regulator of *INO1* (22) that may act at this site (23,46). Expression of *INO1* in the presence of a *sin3* mutation is dependent on *INO2* and *INO4* (22), consistent with Sin3p acting at URS1 through interactions with another protein. Therefore the negative regulation lost in the *INO1-C3* allele is that conferred by *UME6* and *SIN3*.

The region deleted in *INO1-F6* contains a potential URS1 site 5′-TTGGCGGCA-3′ at –143 with substitutions at positions 2 and 9 of the consensus sequence 5′-TCGGCGGCT-3′ that have been shown to retain URS1 function (37). *INO1-F6* was expressed in the absence of Ino2p/Ino4p, indicating that a negative regulatory region was removed. Both *INO1* mRNA and growth on media without inositol were less than the wild-type and expression was not constitutive. The F6 deletion moved UAS$_{-177}$ from 50 bp upstream of the TATA box with URS1$_{-143}$ in between to 16 bp from the TATA box with URS1$_{-143}$ deleted. Evidence indicates that UAS$_{-177}$ is functional (38), suggesting that URS1$_{-143}$ is functional, but it cannot be completely ruled out that shifting UAS$_{-177}$ closer to the TATA box contributed to expression from *INO1-F6*.

Like *INO1-100*, *INO1-D4* was transcribed constitutively in the absence of *INO2* and *INO4*. Forty base pairs between –247 and –208 were deleted, including UAS$_{-239}$ and a critical base at the last position of URS1$_{-255}$. The resulting substitution in the last position eliminates function of the URS1 element (37). Since the C3 deletion removed all but one base of URS1$_{-255}$ and *INO2* and *INO4* were still required for expression, the phenotype of *INO1-D4* is not due to loss of URS1$_{-255}$, although that loss may contribute to the level of transcription observed. Thirty eight base pairs between –245 and –208 were deleted in *INO1-D42*, removing UAS$_{-239}$, but leaving URS1$_{-255}$ intact. The level of *INO1* mRNA was lower in *INO1-D42* strains than in *INO1-D4* strains, confirming that loss of URS1$_{-255}$ contributes to the mRNA levels observed for *INO1-D4*. Like *INO1-100*, *INO1-D4* did not require *SWI1*, *SWI2* or *SWI3*. Deletions D41 (–247 to –228) and D43 (–227 to –208) split the region missing in *INO1-D4* in half. Expression of *INO1-D41* was nearly equal to that of *INO1-D4*, indicating that deletion of UAS$_{-239}$ and one base of URS1$_{-255}$ is the major reason for the *INO1-100* phenotype. Surprisingly, removal of the region between –227 and –208 allowed weak expression of *INO1-D43* in the absence of *INO2* and *INO4* with a phenotype similar to *INO1-F6*; transcription was not constitutive and growth was less than wild-type on media without inositol. The region deleted in *INO1-D43* may contain yet another site of negative regulation or the deletion may cause a spatial shift of interacting elements that effectively removes the requirement for Ino2p and Ino4p while retaining responsiveness to inositol.

INO1-D42 suppressed inositol auxotrophy of an *ino2* strain but not an *ino4* strain. In a study of the promoter of another gene regulated by *INO2* and *INO4*, the choline permease gene *CTR*, Li and Brendel (47) found that a deletion down to –435 relative to the *CTR* AUG permitted approximately equivalent, although lower than normal, expression in *ino2* and *ino4* strains. A deletion down to –381 permitted expression in the *ino2* strain but not in the *ino4* strain. The UAS$_{INO}$ in this promoter is located at –271, so the region between the two deletions does not contain a UAS$_{INO}$. Ashburner and Lopes (48), using *INO2*– and *INO4–CAT* fusions, showed that regulation of *INO2–CAT* transcription requires *INO2* and *INO4*, whereas they suggest regulation of *INO4* transcription requires *INO4* and some other protein, again suggesting that the Ino2p and Ino4p functions can be separated. If Ino2p cannot function without Ino4p, or the reverse, then behavior should not be different in *ino2* versus *ino4* mutants. Our results indicate that Ino4p can act separately from Ino2p in the context of the *INO1-D42* promoter. The separate functions of Ino2p and Ino4p are in addition to their previously characterized binding as a heterodimer (6,11).

INO2 and *INO4* were required for derepression when URS1$_{-255}$ was eliminated. If Sin3p and Ume6p act at this URS1 site, constitutive transcription due to the loss of either of these negative regulators should also be dependent on *INO2/INO4*. This is true for *sin3* mutants (22). *opi1 ino2/ino4* mutants are also inositol auxotrophs, as are *sin1 ino2* mutants. Thus elimination of any of the four known negative regulators of *INO1* transcription is insufficient to overcome inositol auxotrophy caused by an *ino2* mutation. In fact, we found that *sin3 opi1 ino2* and *sin1 opi1 ino2* triple mutants were also inositol auxotrophs. The hypothesis that Ino2p/Ino4p are needed for nucleosome displacement is compatible with these results, since such a function would make it unlikely that elimination of any combination of negative regulators could suppress inositol auxotrophy of an *ino2/ino4* strain.

Transcription of *INO1* requires the action of a complex (the *SWI* complex) that incorporates the gene products from *SWI1*, *SWI2/SNF2*, *SWI3*, *SNF5* and *SNF6* (16,17,19). The *SWI* complex has been shown to antagonize chromatin structure and aid in activator binding (21,23,40–43). Inositol auxotrophy due to *swi1*, *swi2* or *swi3* mutations was suppressed by *INO1-100*, *INO1-C3*, *INO1-D4*, *INO1-D41*, *INO1-D42*, *INO1-D43* and *INO1-F6* to varying degrees. The deletions may promote a more open chromatin structure that alleviates the need for the *SWI* complex. All of the above deletions either remove a known URS site and/or permit transcription without *INO2*. The sites themselves and/or their spatial arrangement may affect nucleosome positioning. When the transcriptional activator encoded by *PHO4* (49) binds to its UAS in the promoter of the *PHO5* gene, nucleosomes are displaced (50). If binding of an activator can displace or shift nucleosomes, then binding of a repressor protein

may also affect chromatin structure, potentially positioning a nucleosome over the TATA region.

The *opi1* mutation suppressed inositol auxotrophy caused by the *swi2* mutation, indicating that the *SWI* complex aids in antagonizing *OPI1*-mediated repression of *INO1*. Therefore Opi1p may promote a chromatin structure that involves positioning of nucleosomes to prevent transcription. This may be the primary function of Opi1p or a secondary function in addition to a more direct antagonism of Ino2p/Ino4p involving the UAS_{INO}. The *SWI* complex in conjunction with Ino2p/Ino4p may also antagonize *OPI1*-mediated repression and facilitate binding of Ino2p and/or Ino4p to the *INO1* promoter. Once bound, these activators cause the displacement of nucleosomes in the TATA region. Previously, Peterson and colleagues (16) showed that *sin1* suppresses inositol auxotrophy due to *swi1*, *swi2* and *swi3* mutations. *SIN1* has been shown to bind to DNA non-specifically (51). *SIN1* may function through interactions with other proteins. Wang and colleagues (52) showed that inositol auxotrophy due to *swi1* mutation is suppressed by *sin3* mutation, indicating that *SIN3* has a role in maintaining chromatin-mediated repression of transcription. Sin3p is a regulatory protein that does not bind to DNA, but rather exerts its effects by interacting with other proteins (21,52–54). Therefore, Sin1p, Sin3p and Opi1p may interact to stabilize a repressive chromatin structure at the *INO1* promoter when sufficient exogenous inositol is present. When inositol drops below a certain level, the *SWI* complex, in concert with Ino2p/Ino4p, antagonizes these repressors to promote a more open chromatin structure. Support for this model is found in the work of Cote and colleagues (40). Using purified *SWI* complex components, they demonstrated that the *SWI* complex aided activators in binding to nucleosomal DNA. Once bound or through the act of binding, Ino2p/Ino4p would then antagonize the negative regulatory region removed in *INO1-D4*. This would facilitate activation from the DSE, as well as any potential additional activation functions of Ino2p/Ino4p, and lead to normal derepression.

With the multitude of regulatory proteins and elements involved, regulation of *INO1* transcription is clearly very complex. The model developed in this study forms the basis for future research that should establish the function of the DSE and determine how the repertoire of regulatory proteins interact with each other and with chromatin to regulate transcription of *INO1*.

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