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Derepression of *INO1* Transcription Requires Cooperation between the Ino2p-Ino4p Heterodimer and Cbf1p and Recruitment of the ISW2 Chromatin-Remodeling Complex▽†

Ameet Shetty and John M. Lopes*

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts

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The *Saccharomyces cerevisiae INO1* gene encodes the structural enzyme inositol-3-phosphate synthase for the synthesis *de novo* of inositol and inositol-containing phospholipids. The transcription of *INO1* is completely derepressed in the absence of inositol and choline (I^- C^-). Derepression requires the binding of the Ino2p-Ino4p basic helix-loop-helix (bHLH) heterodimer to the UAS_{INO} promoter element. We report here the requirement of a third bHLH protein, centromere-binding factor 1 (Cbf1p), for the complete derepression of *INO1* transcription. We found that Cbf1p regulates *INO1* transcription by binding to sites distal to the *INO1* promoter and encompassing the upstream *SNA3* open reading frame (ORF) and promoter. The binding of Cbf1p requires Ino2p-Ino4p binding to the UAS_{INO} sites in the *INO1* promoter and vice versa, suggesting a cooperative mechanism. Furthermore, Cbf1p binding to the upstream sites was required for the binding of the ISW2 chromatin-remodeling complex to the Ino2p-Ino4p-binding sites on the *INO1* promoter. Consistent with this, ISW2 was also required for the complete derepression of *INO1* transcription.

The *Saccharomyces cerevisiae INO1* (inositol-3-phosphate synthase) gene is required for the *de novo* synthesis of phosphatidylinositol (PI) from glucose-6-phosphate (19, 54). The regulation of *INO1* transcription has been studied for 35 years as a model for understanding the regulation of phospholipid biosynthesis. Its transcription is repressed by inositol and choline (I^+ C^+) and completely derepressed in their absence (I^- C^-) by an intricate cascade of *cis* DNA elements and *trans* factors (11, 15, 32, 39). Most significantly, investigations into its regulation have been driven by the fact that altered *INO1* expression is a hallmark of general defects in transcription (35). This study is significant because it reports novel findings regarding new regulators of *INO1* transcription.

The mechanism for the derepression of *INO1* transcription has been extensively studied. Derepression requires the basic helix-loop-helix (bHLH) transcription factors Ino2p and Ino4p that bind as a heterodimer to two *cis*-regulatory (UAS_{INO}) elements in the *INO1* promoter (Fig. 1) (2, 6, 55, 67, 73). The Ino2p activation domain then recruits the Snf1p histone kinase to the *INO1* promoter, which phosphorylates Ser10 of histone H3 (62). Phosphorylated histone H3 serves as a docking site for the SAGA acetyltransferase, which acetylates Lys14 on histone H3, but also recruits the TATA-binding protein (TBP) (Fig. 1) (61, 62). Ino2p was also shown recently to cause an increase in H3 and H4 acetylation across the *INO1* gene under derepressing conditions (22).

The regulation of *INO1* expression also requires several chromatin remodelers. For example, Ino2p recruits INO80 to the *INO1* promoter, which then recruits SWI/SNF, which leads to chromatin remodeling at the *INO1* promoter (74, 75). Both ISW1 and ISW2 complexes have been reported to play regulatory roles in *INO1* transcription (70). Isw2p has been shown to remodel *INO1* chromatin (53), and the ISW2 complex, containing Isw2p and Itc1p, has been shown to be required for the complete repression of *INO1* through the Ume6p/Sin3p/Rpd3 histone deacetylase (HDAC) (Fig. 1) (24, 31, 53, 82).

INO1 transcription is also affected by the physical location of the gene. Upon derepression, the *INO1* locus is recruited to the nuclear periphery (8, 9). It was shown recently that the localization of *INO1* to the periphery is dependent on sequences, called DNA zip codes, within the *INO1* promoter and upstream region and that these zip codes are required for complete derepression (1).

Under repressing conditions (I^+ C^+), *INO1* gene expression is reduced by the Opi1p repressor protein (9, 36, 44, 47, 48, 50, 63, 65). Opi1p senses phosphatidic acid (PA) levels, which are elevated under derepressing conditions (I^- C^-) (Fig. 1) (63). Opi1p bound to PA is tethered to the endoplasmic reticulum (ER) via Scs2p, an ER integral membrane protein (Fig. 1) (63–65). Under I^+ C^+ conditions, PA levels decrease, releasing Opi1p, which translocates to the nucleus and prevents *INO1* transcription by interacting with Ino2p (Fig. 1) (40, 48, 63). It has been suggested that Opi1p recruits the Sin3p/Rpd3p HDAC complex to the UAS_{INO} elements (87). It is more strongly supported that Sin3p/Rpd3p is recruited by Ume6p to an URS1 element on the *INO1* promoter and leads to the general repression of *INO1* transcription (Fig. 1) (21, 31, 49, 79).

The bHLH transcription factors function by dimerization to regulate transcription (5, 7, 34, 38, 46, 58, 59, 68, 77, 78). Previous studies in our laboratory have shown that multiple yeast bHLH proteins can regulate a single gene (14, 16). This study began with

* Corresponding author. Mailing address: Department of Microbiology, University of Massachusetts, 639 North Pleasant Street, Amherst, MA 01003. Phone: (413) 545-8080. Fax: (413) 545-1578. E-mail: jlopes@microbio.umass.edu.

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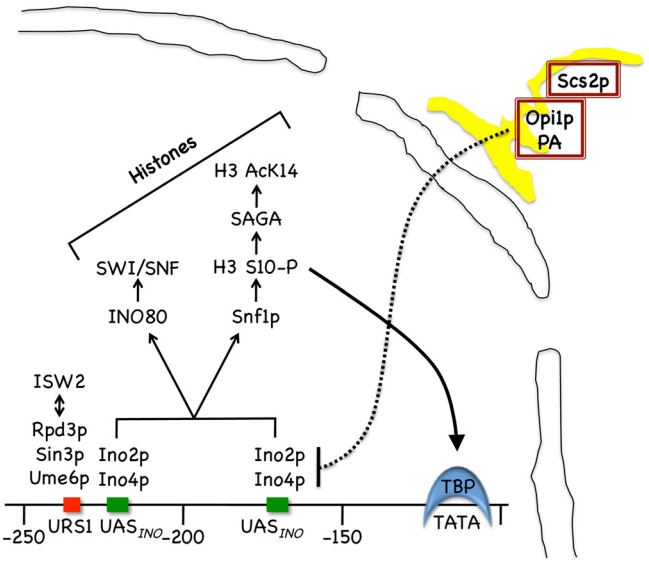

FIG. 1. Model for regulation of *INO1* transcription. Shown is a schematic of the *INO1* promoter containing two UAS$_{INO}$ elements (shown in green) and a repressor site (URS1) (shown in red). Under derepressing conditions (I^- C^-), the Ino2p-Ino4p heterodimer binds to two UAS$_{INO}$ elements and recruits INO80 and Snf1p. INO80 is a chromatin-remodeling complex that recruits another remodeling complex, SWI/SNF. Snf1p is a kinase that phosphorylates serine 10 (S10) on histone H3, which in turn recruits SAGA, which acetylates lysine 14 of histone H3. Histone 3 S10-P promotes the interaction of the TATA-binding protein (TBP) with the *INO1* TATA sequence. Under derepressing conditions, the Opi1p repressor is complexed with phosphatidic acid (PA) and retained in the endoplasmic reticulum (ER) (shown in yellow) by association with Scs2p. Under repressing conditions (I^+ C^+), PA levels drop, and Opi1p is released from the ER, translocates to the nucleus, and associates with Ino2p, repressing transcription. URS1 is a binding site for the general repressor Ume6p, which recruits the Sin3p corepressor and the Rpd3p histone deacetylase (HDAC) complex.

the goal of determining if bHLH proteins other than Ino2p and Ino4p are also involved in the regulation of *INO1*. We found that Cbf1p is also required for the complete derepression of *INO1* transcription. Our results show that Cbf1p and Ino2p-Ino4p bindings are interdependent. Cbf1p plays an important role in chromatin remodeling (25, 52, 70, 71). Consistent with this role, we show that Cbf1p is required for the binding of the ISW2 chromatin-remodeling complex to *INO1* upstream sequences bound by the Ino2p-Ino4p heterodimer.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *S. cerevisiae* strains used in this study were BY4742 (*MAT*α *his3-1 leu2-0 lys2-0 ura3-0*); isogenic strains containing the *ino2*Δ, *ino4*Δ, *pho4*Δ, *cbf1*Δ, *sgc1*Δ, *rtg1*Δ, *rtg3*Δ, *hms1*Δ, *ygr290w*Δ, *isw2*Δ, *itc1*Δ, *isw1*Δ, *ioc2*Δ, *ioc3*Δ, and *ioc4*Δ alleles (30, 88); and a strain harboring an *INO1* promoter deletion (*INO1-100* or *OPI5*) (66, 83). Strains with tandem affinity purification (TAP)-tagged *INO2*, *INO4*, *CBF1*, and *ISW2* were purchased from Open Biosystems (Huntsville, AL) (29).

A CBF1-TAP-tagged strain harboring an *ino2*Δ mutant allele was generated by transformation with a 2,000-bp fragment amplified from the *ino2*Δ strains using primers KAN*ino2*ΔF and KAN*ino2*ΔR (Table 1) and wild-type (WT) genomic DNA. Genomic DNA was extracted by using a Zymo yeast DNA extraction kit (Zymo Research, Orange, CA). The 2,000-bp fragment contained the KanMX cassette (86) flanked by 200 bp of DNA upstream and 200 bp of DNA downstream of the *INO2* open reading frame (ORF). The *ino4*Δ CBF1-TAP and *cbf1*Δ ISW2-TAP strains were created in a similar manner by using the KAN primer pairs listed in Table 1. INO2-TAP- and INO4-TAP-tagged strains harboring a *cbf1*Δ mutant allele were similarly created by replacing the *CBF1* ORF with the *URA3* gene. This was accomplished by amplifying the *URA3* gene flanked by 200 bp of promoter sequences from plasmid YEp357R (61), using the cbf1orf URA3 primer pair (Table 1). The amplified fragment containing the *URA3* gene flanked by 45 bp of sequence homologous to the *CBF1* ORF was transformed into the INO2-TAP and INO4-TAP strains. Transformants were selected on plates lacking Ura (Ura$^-$), and the *cbf1*Δ::*URA3* allele was confirmed by PCR and sequencing. All integration-based transformations were carried out with a Yeast Maker transformation kit (Clontech, Mountain View, CA).

Yeast cultures were grown at 30°C in a complete synthetic medium lacking inositol, choline, KH_2PO_4 (16, 51, 84), and uracil and/or leucine (in the case of reporter plasmids). Where indicated, 75 μM inositol (I^+) and/or 1 mM choline (C^+) was added. Low-P_i medium contained 0.22 mM KH_2PO_4 and 20 mM KCl, and high-P_i medium contained 11 mM KH_2PO_4. The high-P_i medium was used in all figures where the [P_i] is not indicated. Plasmids containing *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA) were grown at 37°C in Luria-Bertani broth containing 50 μg/ml ampicillin.

Plasmid construction. Plasmid pJH330 was described previously (21) and contains 543 bp upstream of the *INO1* ORF and 132 codons of the *INO1* ORF fused in frame to the *lacZ* reporter in YEp357R (72). The upstream sequences include 439 bp of the *SNA3-INO1* intergenic region and 104 bp of the 3′ end of the *SNA3* ORF. Two E boxes in the *INO1* promoter (positions −173 to −178 and −238 to −243) were mutagenized by using a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). A pGEM-T-INO1 derivative, which contains the *INO1* fragment from pJH330, was used for this mutagenesis. INO1 E-box primers (Table 1) were used to create the single E-box mutants. One of the single mutants was used to create the double mutant. The mutant fragments were digested with EcoRI and KpnI and cloned into YEp357R to yield the *INO1* E1 boxΔ, E2 boxΔ, and E1-E2 boxΔ versions of pJH330. A lithium acetate-based one-step method was used for plasmid transformations (13).

A plasmid to complement the *cbf1*Δ allele was constructed by cloning 500 bp of the *CBF1* promoter and the *CBF1* ORF into pRS315 (80). Plasmid pRS315-*CBF1* was constructed by amplifying a 1,563-bp fragment from BY4742 genomic DNA, using primers Cbf1-XbaI F (position −500) and Cbf1-SalI R (position +1051) (Table 1). The fragment was cloned into pGEM-T, sequenced, excised with XbaI and SalI, and ligated into pRS315.

A strain with an *INO1* promoter mutant lacking both E boxes and a URS1 element (Ume6p-binding site) (239-bp deletion) was previously denoted *INO1-100* (*OPI5*) (66, 83). Two *INO1-lacZ* reporter plasmids that contained either 1,239 bp of *INO1* 5′-flanking sequences from a wild-type strain or 1,000 bp from the *INO1-100* mutant strain were constructed. Wild-type and *INO1-100* genomic sequences were amplified by using primers INO1-100 KpnI F (position −1239) and INO-100 EcoRI R (position −1) (Table 1). The resulting fragments (1,240 bp for the wild type and 1,000 bp for *INO1-100*) were cloned into pGEM-T, sequenced, excised with KpnI and EcoRI, and ligated into YEp357R (72) to yield *lacZ* fusions called *INO1-1200-lacZ* and *INO1-100-lacZ*, respectively.

Enzyme assays. β-Galactosidase assays were performed with microtiter plates as described previously (16). Units of β-galactosidase activity were calculated as the optical density at 420 nm (OD_{420})/min/mg total protein × 1,000.

RNA extraction and quantitative real-time PCR (QRT-PCR) analysis. RNA was extracted by using a hot-acid phenol method (17), subjected to DNase digestion using Qiagen (Valencia, CA) DNase, and purified by using a Qiagen (Valencia, CA) RNeasy RNA extraction kit. RNA (1 μg) was used to synthesize cDNA using either Superscript II or Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). For quantification, cDNA was diluted 1:10, and quantitative PCR (QPCR) was performed as previously described, with either 300 nM or 500 nM primer concentrations (43). *INO1* and *TCM1* transcripts were quantified by using the INO1-ORF2 and TCM1-ORF primer pairs (Table 1).

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (3), with some modifications. Cells were fixed with formaldehyde for 15 min for the INO2-TAP and INO4-TAP strains and 2 h for the CBF1-TAP, *ino2*Δ CBF1-TAP, *ino4*Δ CBF1-TAP, ISW2-TAP, and *cbf1*Δ ISW2-TAP strains (52). Lysis was performed with a Multivortexer using glass beads. The cell extract was sonicated by using a model 100 Sonic Dismembrator with a Branson 250 Microtip sonicator (Fisher Scientific, Pittsburgh, PA) at a 50% duty cycle with a power of 6. Sonication was performed 20 times for 20 s with at least 1 min on ice between pulses to fractionate DNA to ~300 bp. Immunoprecipitations were performed by incubating 800 μl chromatin with 40 μl IgG Sepharose beads for 1 h at room temperature. Beads were washed twice each with FA lysis buffer (3), FA lysis buffer containing 500 mM NaCl, and ChIP wash buffer followed by a wash with Tris-EDTA (TE) buffer. Protein-DNA complexes were eluted from the beads by incubating the beads in ChIP elution buffer for 10 min

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
KAN*ino2*ΔF	5′-TTTTCTATCTCCCTCCGTCAT-3′
KAN*ino2*ΔR	5′-ATGAAGATACTGGTAATTCTT-3′
KAN*ino4*ΔF	5′-TCTTTGTTATAAATAGATTAG-3′
KAN*ino4*ΔR	5′-TATAGTAAGTTGAACACTAAA-3′
KAN*cbf1*ΔF	5′-ACGAGAAAAGTATTGGGCAAA-3′
KAN*cbf1*ΔR	5′-TAACGTACAAAGACATATTTG-3′
cbf1orf URA3 F	5′-TCAAGTGCTTAAAATATAATACGGTTTTCTACACTTTTATTAACGTAGCTTTTCAATTCAATTCA-3′
cbf1orf URA3 R	5′-TACATAGGGAGACTCGAAATACATTTAGCTATCTATTTTTAACTCGTTTTGCTGGCCGCATCTT-3′
ISW2 URA3 F	5′-ATGACGACCCAGCAAGAGGAGCAACGAAGTGATACCAAGAATAGCTAGCTTTTCAATTCAATTCA-3′
ISW2 URA3 R	5′-TCATGCTTCTTGATCAATTTTGGTTCTTTTATCAACATGATCGTTGTTTTGCTGGCCGCATCTTC-3′
INO1-Ebox1F	5′-CCCAGAATATTGAACTTATTTAATTGAGCTCGAGCAGAGAAAGCGCACCTCTGCGTTGG-3′
INO1-Ebox1R	5′-CCAACGCAGAGGTGCGCTTTCTCTGCTCGAGCTCAATTAAATAAGTTCAATATTCTGGG-3′
INO1-Ebox2F	5′-CCAAGTATGCGCTTCGGCGGCTAAATGCGGTCTAGAAAAAGTATTGTCTATTTTATCTTCATCC-3′
INO1-Ebox2R	5′-GGATGAAGATAAAATAGACAATACTTTTTCTAGACCGCATTTAGCCGCCGAAGCGCATACTTGG-3′
Cbf1-XbaI F	5′-TCTAGAACATGTCATCCGTGAGCG-3′
Cbf1-Sal1R	5′-GTCGACGCAGATACATAGGGAGACT-3′
INO1-100 KpnI F	5′-GGTACCAAAACAAGTAGAGGAAAAG-3′
INO1-100 EcoRI R	5′-GAATTCATTGTTACTTCTTTTTCACTG-3′
SNA3-500F (F)	5′-TCCTCTTTGTGTGGGACGAT-3′
SNA3-500R (F)	5′-TCAATGCAACGCTTTACTGC-3′
SNA3-200F (E)	5′-ACGTGATGAAGGCTCGTTTT-3′
SNA3-200R (E)	5′-TGGTTGTTTGCTTTCTGCTG-3′
INO1-849F (D)	5′-TAATTTAGAAATGGACAGAGACCA-3′
INO1-849R (D)	5′-GTATCCCTGTTGAACATACCCTTA-3′
INO1-549F (C)	5′-CCCTGCAGAGGAATCTCAAG-3′
INO1-549R (C)	5′-CACTAAGTACGGCCGGAAGA-3′
INO1-383F (B)	5′-ATTGCCTTTTTCTTCGTTCC-3′
INO1-383R (B)	5′-CATTCAACACTTTCGATTCC-3′
INO1E1F (A)	5′-CTTCATCCTTCTTTCCCAGAATATTGAAC-3′
INO1E1R (A)	5′-GACGAAAGCTCCAATTTATATACGTCTC-3′
INO1-ORF1 F	5′-CAAACTACTTCGGCTCCATGAC-3′
INO1-ORF1 R	5′-CTTGACTTCTCTGCATAGCTTCG-3′
INO1-ORF2 F	5′-GTATTAAACCGGTCTCCATTGC-3′
INO1-ORF2 R	5′-CCGACGGGCTTCATATATTTG-3′
TCM1-ORF F	5′-CCAGAGCTGGTCAAAGAGGT-3′
TCM1-ORF R	5′-ACCGTAGTGGACGAAACCAC-3′

at 65°C followed by TE buffer. The supernatants from the two steps were combined, treated with 25 μg RNase A (Invitrogen, Carlsbad, CA), and incubated for 15 min at 37°C. DNA was eluted by incubating the supernatant at 65°C overnight with 5 μg proteinase K (Invitrogen, Carlsbad, CA) and 0.1% SDS. DNA was purified by using ChIP DNA Clean and Concentrator kits (Zymo Research, Orange, CA). For QPCR analysis, ChIP DNA and input DNA were diluted 1:10 and 1:100, respectively. QPCR analysis was performed as previously described (43). Primers used for QPCR analysis are listed in Table 1.

RESULTS

Transcription of *INO1* is regulated by Cbf1p. The Ino2p and Ino4p bHLH proteins are known regulators of *INO1* transcription (2, 67, 73). We recently reported that the expression of two well-studied bHLH target genes, *CIT2* and *ENO1*, is regulated by multiple bHLH factors (14, 16). Thus, we tested whether other bHLH proteins, besides Ino2p and Ino4p, also regulate *INO1* transcription. To this end, we transformed WT and isogenic bHLH knockout strains with an *INO1-lacZ* reporter (pJH330) that contains 543 bp of the *INO1* promoter. Inositol (I) and choline (C) regulate Ino2p and Ino4p function (11, 15, 32, 39), and phosphate (P_i) concentrations regulate Pho4p function (45, 81); hence, transformants were grown in four different media that varied these components (I^- C^- high P_i, I^- C^- low P_i, I^+ C^+ high P_i, and I^+ C^+ low P_i). In the case of *ino2*Δ and *ino4*Δ mutant strains, the I^- C^- media contained 10 μM inositol, which allows growth but still derepresses the expression of Ino2p-Ino4p target genes (4, 20).

As expected, Ino2p and Ino4p were required for *INO1-lacZ* expression under both derepressing conditions (I^- C^-) (Fig. 2A). In addition, the data showed that mutations in all bHLH proteins affected transcription from the *INO1* promoter, although in most cases the effect was modest (see *rtg1*Δ, *rtg3*Δ, and *pho4*Δ strains in Fig. 2A). The most dramatic defect, besides those seen with the *ino2*Δ and *ino4*Δ strains, was observed with the *cbf1*Δ strain. Activity was reduced to 21% of that seen for the WT under both derepressing conditions (I^- C^-) (Fig. 2A). For the remainder of this study, we focused on Cbf1p because it was found to exhibit the most dramatic effect on *INO1* derepression. While we conducted most experiments under the four growth conditions described above, for the reminder of this report we show only data for the high-P_i conditions to facilitate presentation and because the low-P_i results recapitulated the high-P_i results.

Two different experiments were carried out to confirm the effect of the *cbf1*Δ allele on *INO1* expression. A complementation test was performed to confirm that the phenotype of the *cbf1*Δ strain was due to the knockout allele (Fig. 2B). The *cbf1*Δ mutant strain was transformed with either a pRS315 plasmid or a pRS315 derivative carrying the *CBF1* gene driven

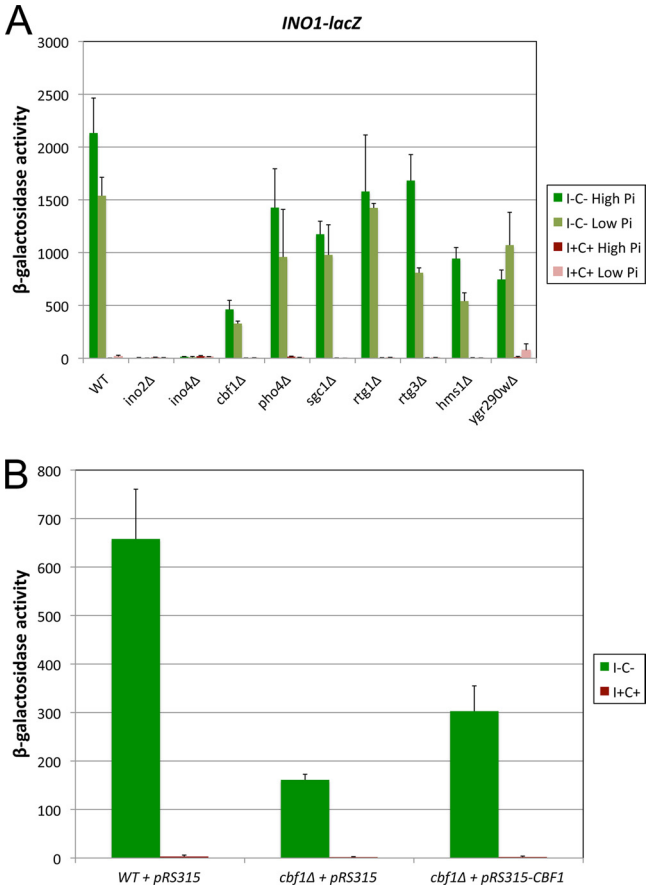

FIG. 2. *CBF1* regulates *INO1-lacZ* expression. (A) WT and isogenic bHLH knockout strains were transformed with an *INO1-lacZ* plasmid (pJH330). Transformants were grown in four different media: I^- C^- high P_i, I^- C^- low P_i, I^+ C^+ high P_i, and I^+ C^+ low P_i. Green and red bars indicate derepressing and repressing conditions, whereas dark and light indicate high and low P_i, respectively. In the case of *ino2*Δ and *ino4*Δ, the I^- C^- media had 10 μM inositol to allow the growth of these auxotrophic strains. Cells were harvested in mid-log phase and assayed for β-galactosidase activity. (B) Complementation test of the *cbf1*Δ *INO1-lacZ* phenotype. The *cbf1*Δ strain was cotransformed with the *INO1-lacZ* plasmid and either a pRS315-CBF1 plasmid or pRS315, and an isogenic WT strain was cotransformed with *INO1-lacZ* and plasmid pRS315. These transformants were assayed for β-galactosidase activity. The data represent means and standard errors of the means from at least three different experiments.

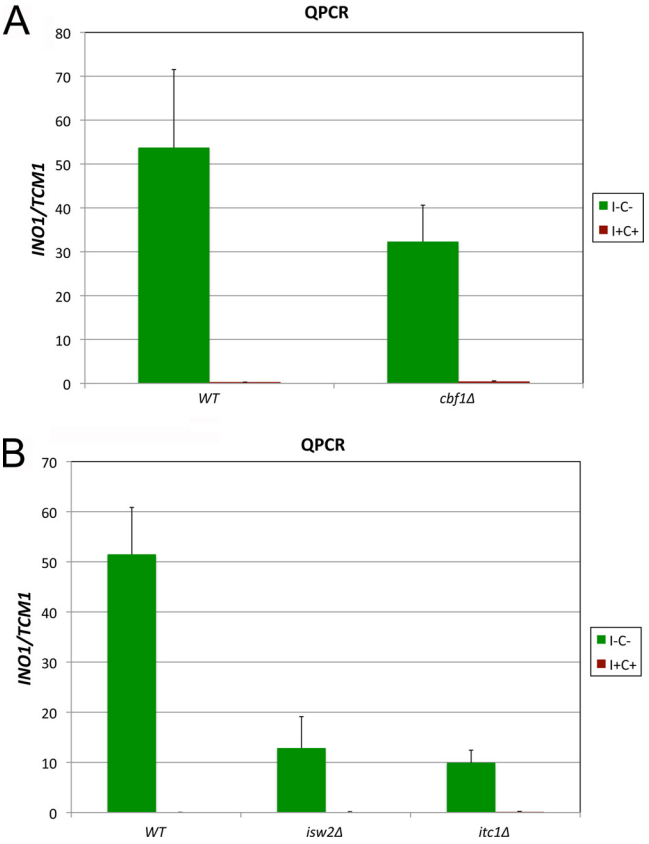

FIG. 3. Quantification of *INO1* transcript levels in WT and *cbf1*Δ strains. (A) Isogenic WT and *cbf1*Δ strains were grown to mid-log phase under derepressing (I^- C^-) and repressing (I^+ C^+) conditions, and *INO1* transcript levels were quantified by QRT-PCR. (B) Similarly, isogenic WT, *isw2*Δ, and *itc1*Δ strains were grown as described above. *INO1* transcript levels were normalized to *TCM1* transcript levels. The data represent means and standard errors of the means from at least three different experiments.

by its own promoter, and an isogenic wild-type strain was transformed with the empty plasmid pRS315. The *cbf1*Δ strain carrying plasmid pRS315-*CBF1* yielded *INO1-lacZ* expression that was 2-fold higher than that of the pRS315 transformant, confirming that the phenotype was due to the *cbf1*Δ mutation. However, plasmid pRS315-*CBF1* did not completely restore activity to wild-type levels (Fig. 2B). This is not an unusual observation, since the expression of the plasmid-based *CBF1* may not completely recapitulate the native state. We also directly tested the effect of *cbf1*Δ on the transcription of the *INO1* gene by QRT-PCR analysis. The data showed that *INO1* transcript levels were reduced by ~2-fold in a *cbf1*Δ strain compared to a WT strain under derepressing conditions (I^- C^-) (Fig. 3A). Thus, both the QRT-PCR analysis and the *lacZ* reporter assay showed that Cbf1p is required for the complete derepression of *INO1* transcription and that this occurs through the *INO1* promoter.

Cbf1p binds distal sites in the *INO1* promoter. bHLH transcription factors regulate transcription by binding DNA (42, 77). As stated above, Ino2p and Ino4p interact with the *INO1* promoter at two E boxes (UAS_{INO} elements) (2, 67, 73), and Cbf1p activates the expression of *MET* genes by binding their promoters (52). Since Cbf1p regulates *INO1* transcription, ChIP was used to determine if Cbf1p binds to the *INO1* promoter.

We probed for the binding of Ino2p, Ino4p, and Cbf1p on the *INO1* promoter and regions further upstream (Fig. 4A) under derepressing and repressing conditions. The experiments with Ino2p and Ino4p served as controls, since they are known to bind the two E boxes in the *INO1* promoter, but in addition, we also tested binding to regions much further upstream (60, 76). We used C-terminal TAP-tagged bHLH strains and quantitative PCR with primers to six different regions spanning ~1.6 kb upstream of the *INO1* ORF (Fig. 4A). As expected, Ino2p-TAP and Ino4p-TAP were enriched on two regions of the *INO1* promoter that contain E boxes (E1 and E2) under derepressing conditions (Fig. 4B and C). The B set of primers (Fig. 4A) did not overlap the E2 element but

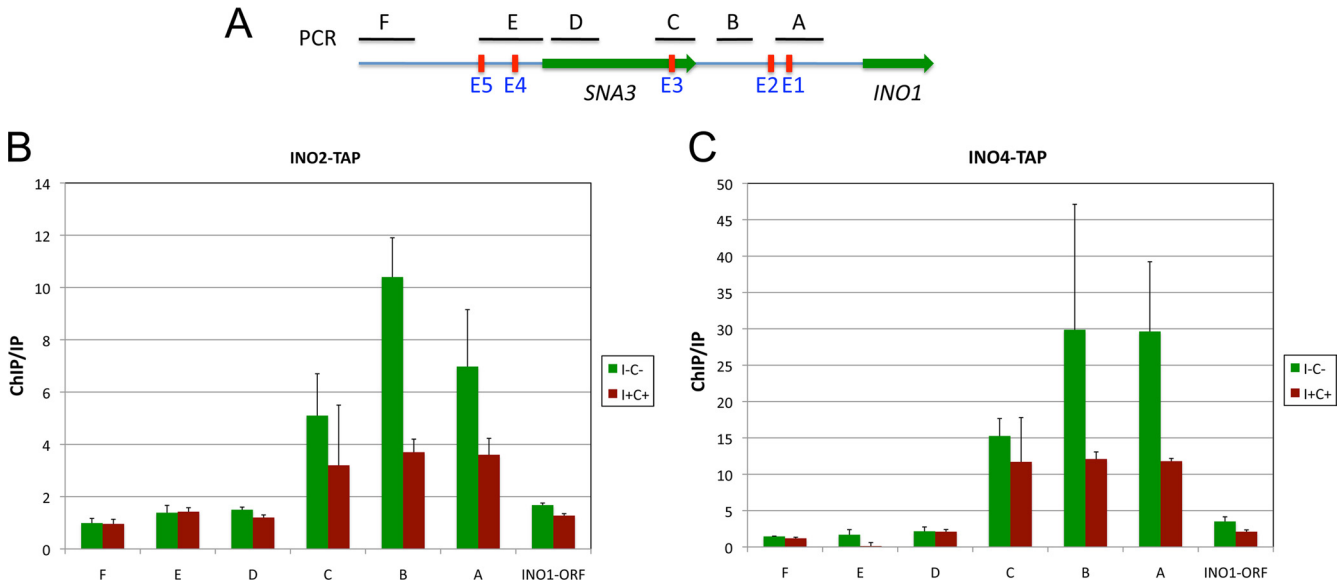

FIG. 4. ChIP analysis of Ino2p-Ino4p binding to the *INO1* promoter and upstream regions. (A) Schematic showing primer positions (A to E) and E boxes (E1 to E5) relative to the *INO1* and *SNA3* ORFs. (B and C) Ino2p-TAP and Ino4p-TAP bind to the *INO1* promoter. ChIP analysis was performed by using TAP-tagged strains grown under derepressing (I^- C^-) and repressing (I^+ C^+) conditions. Enrichment on the *INO1* promoter and upstream regions was quantified by using QRT-PCR. ChIP/immunoprecipitation (IP) ratios were normalized by using *TCM1*. The INO1 ORF primers cover a region within the *INO1* coding sequence and serve as a negative control. The data represent means and standard errors of the means from at least three different experiments.

were close enough (46 bp) to identify binding at this site. We did make repeated attempts to identify a primer set that overlapped the E2 element, but every combination tested yielded multiple PCR products or other artifacts. The enrichment of Ino2p-TAP was 7- to 10-fold higher at the *INO1* promoter than at *TCM1* (normalizer) or the *INO1* ORF (Fig. 4B) under derepressing conditions. Ino4p-TAP was enriched ~30-fold at the *INO1* promoter. There was also enrichment at a third region that includes the 3′ end of the adjacent *SNA3* ORF (primer set C in Fig. 4). These results are in good agreement with data from previous studies, which showed that the Ino2p-Ino4p heterodimer binds the *INO1* promoter and activates *INO1* transcription (2, 9, 67, 73).

Surprisingly, we did not observe any significant enrichment of Cbf1p-TAP in the same region bound by Ino2p-TAP and Ino4p-TAP in the *INO1* promoter (compare Fig. 4 and 5). Instead, Cbf1p was enriched in the region from −439 bp upstream to −1,019 bp upstream of *INO1* covered by three primer pairs (Fig. 4A and 5). Notably, two of the three PCR primer sets that identified Cbf1p binding include three potential E boxes. Binding was enhanced 2-fold under derepressing conditions, suggesting that it might depend on Ino2p-Ino4p binding. Thus, Cbf1p regulates *INO1* transcription by binding regions upstream of the canonical promoter that include the upstream *SNA3* ORF and its promoter.

Cbf1p regulation of *INO1* expression is dependent on an Ino2p-Ino4p-binding site. The results described above raised the possibility that Cbf1p regulation of *INO1* might depend on Ino2p-Ino4p binding to the *INO1* promoter. We tested this possibility in two ways. First, we used a construct carrying a deletion of the *INO1* promoter (previously called *INO1-100* or *OPI5*) fused to *lacZ*. The *INO1-100* mutation has been described to be a dominant allele of *INO1* lacking 239 bp of the *INO1* promoter, including the two UAS_{INO} elements (E1 and E2 in Fig. 4); however, it demonstrates incomplete dominance with respect to *INO1* transcription (83). The *INO1-100* allele exhibits a nearly constitutive expression of *INO1*, which is independent of Ino2p and Ino4p (83). The repressive region required for the *INO1-100* phenotype was mapped to a 20-bp region adjacent to the URS1 element and overlapping the distal UAS_{INO} element (E2 box) (Fig. 1) (83). It is not definitively known why the *INO1*

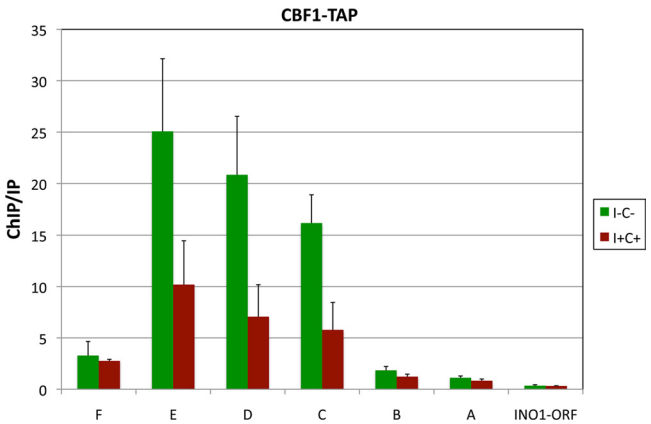

FIG. 5. ChIP analysis of Cbf1p binding to the *INO1* promoter and upstream regions. Cbf1p-TAP binds to regions upstream of the *INO1* promoter within and upstream of the *SNA3* ORF. ChIP analysis was performed by using a CBF1-TAP-tagged strain grown under derepressing (I^- C^-) and repressing (I^+ C^+) conditions. Enrichment on the *INO1* promoter and upstream regions was quantified by using QRT-PCR. ChIP/IP ratios were normalized by using *TCM1*. The data represent means and standard errors of the means from at least three different experiments.

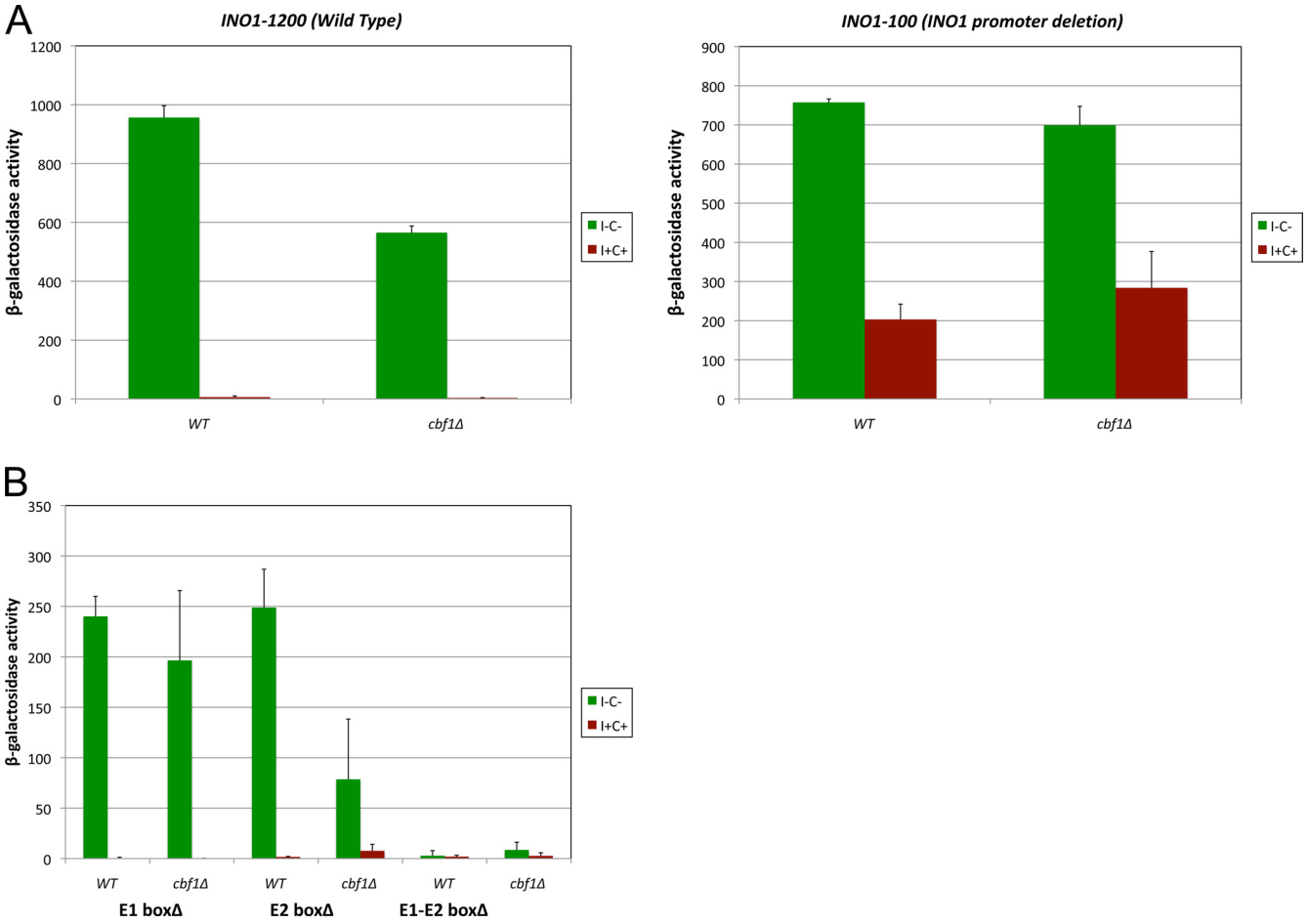

FIG. 6. Cbf1p regulation of *INO1* expression depends on Ino2p-Ino4p-binding sites in the *INO1* promoter. (A) WT and isogenic *cbf1*Δ knockout strains were transformed with an *INO1-1200-lacZ* plasmid (wild-type *INO1* promoter) or an *INO1-100-lacZ* plasmid (UAS_{INO}-deleted *INO1* promoter). (B) WT and isogenic *cbf1*Δ knockout strains were transformed with pJH330 containing mutations of the E1 box, the E2 box, or both the E1 and E2 boxes. These transformants were assayed for β-galactosidase activity as described above. The data represent means and standard errors of the means from at least three different experiments.

gene is expressed in the absence of the two UAS_{INO} elements. For the purposes of our studies, the *INO1-100* allele was used simply to determine if Cbf1p regulation of *INO1* expression required the region, including Ino2p-Ino4p-binding sites. As a control, we transformed a construct containing 1,200 bp of DNA upstream of the *INO1* ORF fused to the *lacZ* reporter into WT and *cbf1*Δ strains and assayed for β-galactosidase activity. This full-length construct recapitulated the results observed with the *cbf1*Δ allele in the *INO1-lacZ* reporter described above (pJH330, which contained only 543 bp upstream of *INO1*), namely, decreased expression in the *cbf1*Δ strain (compare Fig. 6A and 2). However, expression from the *INO1-100* promoter was unaffected by the *cbf1*Δ mutation (Fig. 6A). Also, in accordance with previously reported results (83), the *INO1-100* construct yielded only 50% repression in inositol and choline (Fig. 6A) compared to the wild-type *INO1* promoter construct (Fig. 6A). These results support the model whereby the Cbf1p-mediated regulation of *INO1* requires the Ino2p-Ino4p heterodimer bound to the *INO1* regulatory region.

The results described above did not preclude that Cbf1p regulation of *INO1* might be due to other factors that bind the 239-bp region deleted in *INO1-100*. To more precisely define if Ino2p-Ino4p binding to the *INO1* promoter is required for Cbf1p binding, we created specific E-box point mutations in the pJH330 construct. The E1-boxΔ, E2-boxΔ, and E1-E2-boxΔ mutants were transformed into WT and *cbf1*Δ mutant strains and assayed for *lacZ* expression. Deleting the two E boxes eliminated the expression of the reporter (Fig. 6B). However, the data also show that deleting the E1 box effectively eliminated the *cbf1*Δ phenotype (Fig. 6B). This epistatic effect suggests that the *cbf1*Δ phenotype is dependent on Ino2p-Ino4p binding to the E1 box. The results also show that eliminating the E2 box did not affect the *cbf1*Δ phenotype. It is curious that the E box that is more distal to the Cbf1p-binding sites appears to be required for Cbf1p activity. A model to explain this result is discussed below.

Ino2p-Ino4p and Cbf1p bindings are interdependent. The results described above led us to determine the sequence of events of Cbf1p and Ino2p-Ino4p binding to the *INO1* promoter and upstream sequences. Given that the effect of Cbf1p on the derepression of *INO1* depended on the Ino2p-

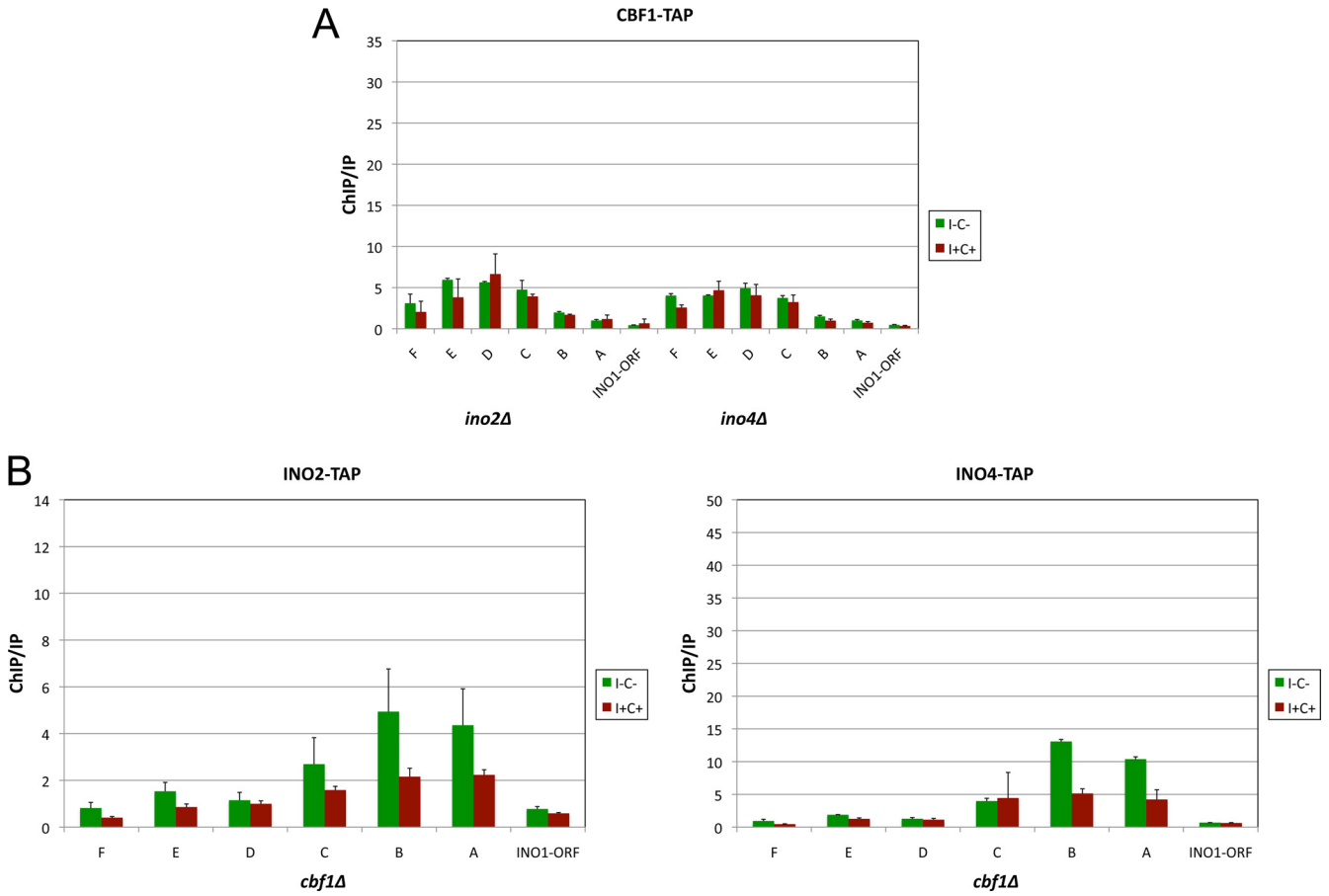

FIG. 7. Cbf1p and Ino2p-Ino4p bindings to the *INO1* promoter are interdependent. (A) Cbf1p binding requires the Ino2p-Ino4p heterodimer. ChIP analysis was performed by using CBF1-TAP-tagged *ino2*Δ and *ino4*Δ strains under derepressing ($I^- C^-$) and repressing ($I^+ C^+$) conditions. (B) Ino2p-Ino4p binding requires Cbf1p. ChIP analysis was performed by using INO2- and INO4-TAP-tagged *cbf1*Δ strains as described above. Enrichment on the *INO1* promoter and upstream regions was quantified by using QRT-PCR and the primer pairs described in the legend of Fig. 4A. ChIP/IP ratios were normalized by using *TCM1*. The data represent means and standard errors of the means from at least three different experiments.

Ino4p-binding sites in the *INO1* promoter (Fig. 6), we reasoned that the Ino2p-Ino4p heterodimer might recruit Cbf1p to the upstream regions. To test this, we performed a ChIP assay using *ino2*Δ CBF1-TAP and *ino4*Δ CBF1-TAP strains, where the *INO2* and *INO4* genes have been replaced with a KanMX cassette. The data show that deleting *INO2* and *INO4* severely decreased (~80% decrease) Cbf1p-TAP binding to regions upstream of *INO1* (compare Fig. 7A and 5). These results indicate that Cbf1p recruitment to the region from positions −439 to −1019 upstream from the *INO1* gene is dependent on Ino2p-Ino4p binding to downstream regions. Likewise, we performed ChIP using *cbf1*Δ INO2-TAP and INO4-TAP strains (Fig. 7B). The data show that Cbf1p is required for the complete binding of the Ino2p-Ino4p heterodimer. There was a ~60% drop in Ino2p and Ino4p binding in the *cbf1*Δ strain relative to the wild-type strain (compare Fig. 4B and C and 7B). Note that the ChIP experiments were normalized by using binding to *TCM1*, which allows comparisons between data sets.

The ISW2 chromatin-remodeling complex binds to the *INO1* promoter and is required for complete derepression of *INO1* expression. Cbf1p has been shown to regulate transcription by recruiting chromatin remodelers of the imitation switch (ISWI) class family, composed of ISW1a, ISW1b, and ISW2 (53, 70, 71). ISWI family proteins were shown previously to be involved in the repression of *INO1* expression (70, 82). We reasoned that Cbf1p might recruit ISWI complexes to regulate *INO1* transcription under derepressing conditions. We first tested the effect of ISWI complex mutants on *INO1-lacZ* expression (pJH330). We found that Isw2p and Itc1p were required for the complete derepression of the *INO1* promoter (see Fig. S1 in the supplemental material). These two proteins are members of the ISW2 complex (70). We also tested the effect of ISW2 mutants on the transcription of *INO1* by QRT-PCR. Both Isw2p and Itc1p were required for the complete derepression of *INO1* transcription (Fig. 3B). *INO1* transcript levels were reduced more than 60% in *isw2*Δ and *itc1*Δ mutant strains compared to WT levels.

The results described above led us to determine if ISW2 components are associated with the *INO1* promoter. We performed a ChIP analysis using TAP-tagged Isw2p under activating and repressing conditions using the same primer sets described above in order to compare ISW2 binding to Ino2p-Ino4p and Cbf1p binding patterns. We found that Isw2p-TAP

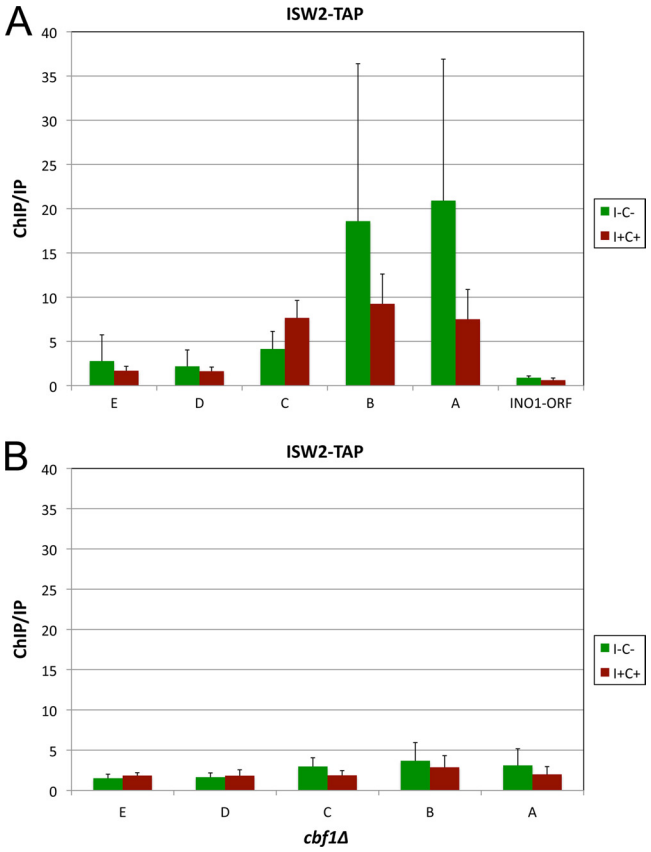

FIG. 8. Isw2p recruitment to the *INO1* promoter requires Cbf1p. ChIP analysis was performed by using ISW2-TAP (A) and ISW2-TAP *cbf1*Δ strains (B) under derepressing (I^- C^-) and repressing (I^+ C^+) conditions. Enrichment on the *INO1* promoter was quantified by using QRT-PCR and the primer pairs described in the legend of Fig. 4A. ChIP/IP ratios were normalized by using *TCM1*. The data represent means and standard errors of the means from at least three different experiments.

binding was enriched in the *INO1* promoter (Fig. 8A). The pattern of ISW2 binding was similar to that seen for Ino2p-TAP and Ino4p-TAP (compare Fig. 6B to 4B and C). Note that the large variability in binding for Isw2p was previously documented by others in the field. However, our results are consistent with genomic ChIP analysis using a catalytically inactive Isw2p that was found to bind the *INO1* promoter (28).

To further elucidate the role of Cbf1p in *INO1* transcription, we explored the possibility that Cbf1p plays a role in Isw2p binding to the *INO1* promoter. We performed a ChIP assay using a *cbf1*Δ ISW2-TAP strain. The data showed that the binding of Isw2p-TAP was substantially decreased in the *cbf1*Δ strain (Fig. 8B).

DISCUSSION

The expression of the *S. cerevisiae INO1* gene is regulated by a variety of environmental cues such as inositol, nitrogen starvation, and the unfolded-protein response (9, 11, 12, 15, 33, 39). These responses require intricate regulatory cascades involving the concerted action of at least 16 activators, repressors, general transcription factors, histone modifiers, and chromatin remodelers (15, 26, 27). For more than 3 decades, the *INO1* gene has been a model for studies of transcription regulation (15). In spite of the wealth of information available, novel mechanisms of transcription regulation are still being uncovered from studies of *INO1*. For instance, just this year it was reported that the *INO1* promoter and regions further upstream harbor sequences called DNA zip codes (1). These sequences are important for the recruitment of *INO1* to the nuclear periphery and are required for optimal transcription. The current study underscores the fact that there is still more to be learned from analyzing the regulation of *INO1* expression.

In recent years we have reported that multiple bHLH proteins regulate the transcription of single genes in yeast (e.g., *CIT2* and *ENO1*) (14, 16). We can now add *INO1* to this target list, since we showed here that Cbf1p acted in concert with the two known activators of *INO1* transcription, Ino2p and Ino4p (Fig. 2 and 3). Cbf1p plays an important role in chromosome segregation and the regulation of methionine biosynthesis (10, 57). Strains bearing *cbf1*Δ alleles display several phenotypes, such as increased chromosome loss, sensitivity to microtubule-disrupting drugs (thiabendazole and benomyl), and methionine auxotrophy (69, 77). In addition, genomic studies have unearthed a myriad of new physical (protein-protein) and genetic interactions, suggesting that Cbf1p has other important biological functions in the cell (18, 56, 85). Collectively, these observations suggest that PI or, more generally, phospholipid synthesis may be coordinated with all of these processes.

Our results showed that Cbf1p was required for the full derepression of *INO1* transcription under derepressing (I^- C^-) conditions (Fig. 2 and 3). We also found that the Cbf1p-mediated regulation of *INO1* required one of the two Ino2p-Ino4p-binding sites (Fig. 6B). Surprisingly, we found that Cbf1p did not bind the *INO1* promoter, or at least not the *SNA3-INO1* intergenic region (bound by the Ino2p-Ino4p heterodimer) (Fig. 5). Instead, it bound to sites distal to the *INO1* promoter within the *SNA3* ORF and promoter sequences. Genome-wide transcription factor binding analyses done previously did not identify a binding of Cbf1p to these regions (37). This could be due to a number of reasons such as growth conditions or cross-linking conditions. It is important that while data from the ChIP studies suggest that Cbf1p may bind multiple locations, they do not conclusively prove that it does bind multiple locations. In fact, the pJH330 construct that was used for most experiments in this study contains 439 bp of the *SNA3-INO1* intergenic region and 104 bp of the 3′ end of the *SNA3* ORF. The latter 104 bp includes the E3 box (Fig. 4). Thus, maybe this is the only E box required for regulation by Cbf1p. Alternatively, multiple sites may be involved *in vivo*, but the E3 box is enough to yield a phenotype on a reporter plasmid.

Our results also showed that Cbf1p was required for maximal Ino2p-Ino4p binding to the *INO1* promoter (Fig. 4 and 7). This finding suggests that the bindings of Cbf1p and the Ino2p-Ino4p heterodimer are cooperative. Our results showed that Cbf1p was more dependent on the Ino2p-Ino4p heterodimer than vice versa. This finding is consistent with the effect of mutations in each of the factors on the expression of *INO1*. An interesting observation was that the E1 box was required for a *cbf1*Δ phenotype (Fig. 6B). One possible explanation for this

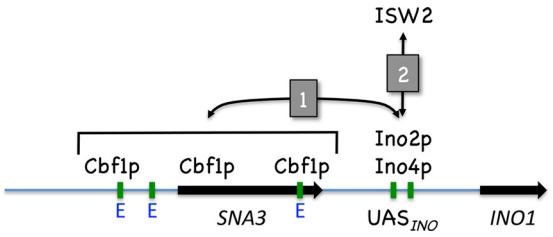

FIG. 9. Model for regulation of *INO1* transcription by Ino2p-Ino4p, Cbf1p, and ISW2. Black arrows indicate the positions of genes, and green bars indicate the positions of UAS$_{INO}$ elements and other potential E boxes. Numbered arrows indicate the sequence of events. Refer to Discussion for a complete description of the model.

observation may lie in the fact that the E1 and E2 boxes lie in different orientations (2, 6, 55, 67, 73). Thus, Cbf1p may interact with either Ino4p or Ino2p but not both. Another possible explanation may have to do with the phasing of the binding sites.

Our results also showed that the ISW2 complex was required for complete *INO1* derepression (Fig. 3). Isw2p appeared to regulate *INO1* transcription through the same pathway as that of Cbf1p since *INO1* transcript levels were nearly identical in the *isw2*Δ and *isw2*Δ *cbf1*Δ strains (see Fig. S2 in the supplemental material). Furthermore, Cbf1p was required for the recruitment of Isw2p onto the *INO1* promoter (Fig. 8). This is consistent with the current model for ISW2 activity, which includes a requirement for a DNA-binding factor to recruit the ISW2 complex to promoters (23, 28). It seemed likely Cbf1p was the target-specific DNA-binding factor required for the ISW2 chromatin-remodeling activity on *INO1*. However, our experiments suggest that ISW2 binding is likely through the Ino2p-Ino4p heterodimer (or something recruited by these bHLH proteins) and that the requirement for Cbf1p is indirect, since Cbf1p is required for maximal Ino2p-Ino4p binding.

Based on our results, we propose the following model for Cbf1p-mediated *INO1* derepression (Fig. 9). Under activating conditions (I^- C^-), the Ino2p-Ino4p heterodimer bound to the *INO1* promoter enhances the binding of Cbf1p to more-distal sites. Likewise, Cbf1p binding enhances Ino2p-Ino4p binding. Cbf1p binds across a region, which includes the upstream *SNA3* ORF and its promoter. Cbf1p is required for the interaction of the ISW2 complex with the Ino2p-Ino4p heterodimer. ISW2 remodels chromatin in the *INO1* promoter, facilitating transcription under derepressing conditions. It is important that ISW2 has been associated with the repression of *INO1* (I^+ C^+ conditions) (70, 82). This phenotype was not as obvious in the bar graphs presented here, because they included derepressed levels of expression. However, it is evident that the *isw2*Δ and *itc1*Δ mutants yielded elevated expression levels of *INO1-lacZ* under derepressing conditions (see Fig. S1 in the supplemental material).

Our results raise several questions. Where does Cbf1p bind specifically, and how does the Ino2p-Ino4p heterodimer mediate the recruitment of Cbf1p to distal sites? Genomic regulator localization studies showed that ~83% of yeast intergenic regions that contain the palindromic E-box sequence CACGTG are likely to bind Cbf1p (52, 60). It is therefore plausible that Cbf1p binds to E box 5 (CACGTG) upstream of the *SNA3* ORF (Fig. 4A). However, our ChIP results indicated that Cbf1p is likely to interact at multiple sites in the *SNA3* promoter and ORF (Fig. 5). This region contains three potential E boxes that could serve as binding sites for Cbf1p. The fact that Cbf1p enrichment at the *INO1* flanking sites is lost in the absence of Ino2p and Ino4p (Fig. 7) shows that Cbf1p binding is consequential for *INO1* transcription and not merely an artifact of the ChIP assay. One possible explanation for our results is that one or more of these E boxes are weak binding sites for Cbf1p and that the proximal binding of the Ino2p-Ino4p heterodimer cooperatively enhances the binding of Cbf1p to the distal sites.

Another interesting question is whether Cbf1p regulates *SNA3* gene expression. It has been known for some time that *SNA3* transcription is repressed by inositol and choline (similarly to *INO1*), but in contrast to *INO1*, *SNA3* expression is upregulated in the absence of *INO2* and *INO4* (41). Since the absence of the Ino2p-Ino4p heterodimer results in the loss of Cbf1p binding at distal sites (Fig. 7), it is possible that the binding of Cbf1p at the *SNA3* promoter regulates the transcription of both *INO1* and *SNA3*.

Yet another question is whether ISW2-mediated chromatin remodeling at the *INO1* locus occurs under depressing conditions. ISW1 and ISW2 complexes have both been reported to be required for *INO1* repression (28, 70). So how is ISW2 involved in derepression? *INO1* transcript levels were reported previously to be higher in an *isw2*Δ background than in a wild-type background, suggesting that ISW2 is a repressor of *INO1* transcription (53, 82). However, those studies were done only under repressing conditions (I^+ C^+). Our data clearly showed that under activating (I^- C^-) conditions, *isw2*Δ strains show reduced *INO1* transcript levels (Fig. 3), indicating that ISW2p is also required for complete derepression. Previous reports showed that nucleosome profiles on the *INO1* promoter are similar in *isw2* and *ume6* strains, suggesting that they act in the same pathway (53). However, the removal of the Ume6p-binding site has no effect on Isw2p enrichment on the *INO1* promoter (28), suggesting that ISW2 is recruited independent of Ume6p. These results, combined with our data, suggest that the Ino2p-Ino4p heterodimer may be the target-specific DNA-binding factor that interacts with ISW2 on the *INO1* promoter under derepressing conditions but that this interaction is driven by Cbf1p.

Finally, it will also be necessary to determine how Cbf1p regulation is coordinated with other cascades that regulate *INO1* expression. For example, how is it coordinated with the INO80 and Snf1p pathways that are also driven by Ino2p-Ino4p binding to the UAS$_{INO}$ elements (Fig. 1)? Moreover, how are all of these regulatory cascades coordinated with the recruitment of the *INO1* gene to the nuclear periphery by DNA zip codes present within the *INO1* regulatory region and distal sites within *SNA3* (1)?

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