Evidence for the Involvement of the Glc7-Reg1 Phosphatase and the Snf1-Snf4 Kinase in the Regulation of *INO1* Transcription in *Saccharomyces cerevisiae*

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ABSTRACT

Binding of the TATA-binding protein (TBP) to the promoter is a pivotal step in RNA polymerase II transcription. To identify factors that regulate TBP, we selected for suppressors of a TBP mutant that exhibits promoter-specific defects in activated transcription *in vivo* and severely reduced affinity for TATA boxes *in vitro*. Dominant mutations in *SNF4* and recessive mutations in *REG1*, *OPI1*, and *RTF2* were isolated that specifically suppress the inositol auxotrophy of the TBP mutant strains. *OPI1* encodes a repressor of *INO1* transcription. *REG1* and *SNF4* encode regulators of the Glc7 phosphatase and Snf1 kinase, respectively, and have well-studied roles in glucose repression. In two-hybrid assays, one *SNF4* mutation enhances the interaction between Snf4 and Snf1. Suppression of the TBP mutant by our *reg1* and *SNF4* mutations appears unrelated to glucose repression, since these mutations do not alleviate repression of *SUC2*, and glucose levels have little effect on *INO1* transcription. Moreover, mutations in *TUP1*, *SSN6*, and *GLC7*, but not *HXK2* and *MIG1*, can cause suppression. Our data suggest that association of TBP with the TATA box may be regulated, directly or indirectly, by a substrate of Snf1. Analysis of *INO1* transcription in various mutant strains suggests that this substrate is distinct from Opi1.

CELL growth and differentiation depend upon accurate gene expression in response to signals from the environment. These signals must be transduced through the cell, and many stimuli ultimately effect activation or repression of transcription. Regulation of transcription requires interactions between sequence-specific activators and repressors, coactivators and corepressors, and the RNA polymerase II general transcription factors. Previous work supports two primary models in explaining assembly of the RNA polymerase II preinitiation complex in response to transcriptional activators. According to both models, promoters are first recognized by the general factor TFIID, which consists of the TATA-binding protein (TBP) and TBP-associated factors (reviewed in Burley and Roeder 1996). One model argues that after TFIID binding to the promoter, the other components of the preinitiation complex assemble in a stepwise manner (Buratowski *et al.* 1989). In the second model, binding of TFIID to the promoter is followed by the recruitment of a protein complex termed the RNA polymerase II holoenzyme (reviewed in Ptashne and Gann 1997).

Using genetic and biochemical approaches, several groups have investigated the regulation of TBP-TATA complex formation. Both *in vitro* and *in vivo*, binding of TBP to the TATA box has been shown to be an important rate-limiting step in transcription (reviewed in Stargell and Struhl 1996). Direct interactions between TBP and certain gene-specific transcriptional activators and repressors have been reported (for examples see Stringer *et al.* 1990; Horikoshi *et al.* 1991, 1995; Emili *et al.* 1994; Melcher and Johnston 1995; Um *et al.* 1995; Zhang *et al.* 1996 and references therein). In addition, genetic studies in *Saccharomyces cerevisiae* have identified several proteins that more generally affect TATA box binding by TBP, including Mot1, Spt3, Rtf1, and the Not proteins (Auble *et al.* 1994; Collart 1996; Madison and Winston 1997; Stolinski *et al.* 1997). However, the mechanisms by which these factors modulate the activity of TBP *in vivo* are not well understood. Therefore, studies of TBP mutants that are impaired in their response to certain activators may provide insights into the significant problem of promoter-specific regulation. We have identified such a class of TBP mutants and determined that these mutants have severely reduced affinity for DNA *in vitro* (Arndt *et al.* 1995). The identification of these and similar TBP mutants (Kim *et al.* 1994; Lee and Struhl 1995) has further established the importance of TATA box binding as a regulatory step in transcription.

While TBP and the other general transcription factors have been extensively studied for their interactions with activator proteins and promoter DNA *in vitro*, the complex regulatory circuitry used by cells to modulate the activity or assembly of the preinitiation complex in response to environmental cues is less well understood. Many of our existing insights into this important problem have come from studies in yeast, where transcrip-

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tional responses to signals such as glucose and inositol availability have been well documented.

In the presence of high glucose levels, many genes in yeast are repressed, including those encoding proteins needed to metabolize other carbon sources (reviewed in Gancedo 1998; Johnston and Carlson 1992; Trumbly 1992; Ronne 1995). Genetic analyses of glucose repression, using regulation of *SUC2* expression as a paradigm, have identified a complex network of negative and positive regulatory proteins. Current results favor a model in which high glucose levels, which are monitored by the *HXK2* gene product, hexokinase PII, signal the Glc7 phosphatase to negatively regulate the activity of the Snf1 serine-threonine kinase (Johnston and Carlson 1992). Important components of this pathway are the *REG1* and *SNF4* gene products, which bind to and control the Glc7 phosphatase and the Snf1 kinase, respectively, in a positive manner (Tu and Carlson 1995; Jiang and Carlson 1996). During derepression of *SUC2* in low-glucose conditions, the model argues that the Snf1-Snf4 kinase reduces the activity of repressor complexes comprised of Ssn6(Cyc8), Tup1, and the sequence-specific binding proteins Mig1 and Mig2 (Treitel and Carlson 1995; Lutfiyya and Johnston 1996). In this way, the Snf1-Snf4 kinase, in conjunction with the Swi-Snf chromatin-remodeling complex, may counter a repressive chromatin state maintained by Ssn6 and Tup1 (Edmondson *et al.* 1996; Gavin and Simpson 1997). In addition, the kinase may stimulate function of the RNA polymerase II holoenzyme (Carlson 1997).

Another well-studied signaling pathway in yeast affects transcription in response to inositol availability. In the presence of high inositol levels, transcription of the *INO1* gene, which encodes inositol-1-phosphate synthase, is strongly repressed by the *OPI1* and *UME6* gene products (Klig *et al.* 1985; Jackson and Lopes 1996). Opi1 contains a putative leucine zipper adjacent to a basic domain, a motif that has been postulated to bind DNA (White *et al.* 1991); however, direct DNA binding by Opi1 has not been demonstrated (S. A. Henry, personal communication). Ume6 binds to URS1 elements in several promoters, including *INO1*, and recruits a protein complex containing Sin3 and the Rpd3 histone deacetylase (Kadosh and Struhl 1997; Kasten *et al.* 1997). In response to low inositol levels, the Ino2 and Ino4 basic helix-loop-helix proteins activate *INO1* transcription (Ambroziak and Henry 1994). The proteins that transmit the inositol signal to these transcriptional regulatory factors are not completely known, partly because of the complexity of overlapping pathways that impinge on *INO1* expression (Henry and Patton-Vogt 1998). However, *snf1*Δ strains are inositol auxotrophs, suggesting that the Snf1-Snf4 kinase may be involved in *INO1* regulation (Hirschhorn *et al.* 1992). Furthermore, mutations that affect components of the RNA polymerase II holoenzyme, the Swi-Snf complex, and the SAGA histone acetyltransferase complex cause inositol auxotrophy (reviewed in Henry and Patton-Vogt 1998), suggesting that the inositol signal ultimately influences preinitiation complex formation and chromatin structure.

In earlier work, we reported the isolation of TBP mutants that cause inositol auxotrophy as well as a defect in galactose metabolism (Arndt *et al.* 1995). These Ino$^-$ and Gal$^-$ phenotypes correlate strongly with severely impaired induction of the *INO1*, *GAL1*, and *GAL10* genes (Arndt *et al.* 1995). The ability of these TBP mutants to sustain growth of yeast strains indicates that their effects are gene specific. Indeed, transcription of certain other induced and constitutively expressed genes is relatively unaffected in the mutant strains (Arndt *et al.* 1995). *In vitro*, the TBP mutants are severely defective for TATA box binding. By selecting for genetic suppressors of a TBP mutant in this class, TBP-P109A, we have sought to identify factors that regulate the formation or stability of the TBP-TATA complex in a promoter-specific manner. Here, we report the identification of four genes that, when mutated, significantly restore *INO1* transcription to the TBP mutant strain. The finding that two of these genes are *REG1* and *SNF4* implicates proteins previously described for their roles in glucose repression in the control of *INO1* transcription. Our results are consistent with a model in which the Snf1 kinase regulates preinitiation complex assembly at the *INO1* promoter, perhaps by facilitating the TBP-TATA interaction.

MATERIALS AND METHODS

Genetic methods and media: Rich (YPD), minimal (SD), synthetic complete (SC), 5-fluoroorotic acid (5-FOA), as well as presporulation and sporulation media were prepared as described (Rose *et al.* 1990). Auxotrophic requirements were scored on SD media supplemented with the appropriate nutrients or SC media lacking the appropriate nutrients. Canavanine tests were performed using SC media lacking arginine and containing 60 mg/liter canavanine sulfate (SC-Arg+CAN, Schild *et al.* 1981). 2-Deoxyglucose media contained YEP (1% yeast extract, 2% Bacto-peptone), 2% sucrose, 200 μg/ml 2-deoxyglucose (Sigma, St. Louis), and 1 μg/ml antimycin A (Sigma), which was added to simulate anaerobic conditions. Media lacking inositol (−Ino) were prepared as described (Sherman *et al.* 1981); control media (+Ino) contained 200 μm inositol. Solid −Ino media contained adenine, uracil, and the amino acids histidine, lysine, leucine, and tryptophan (SD-Ino). Because we noticed that adenine caused a small induction of *INO1* transcription (M. K. Shirra and K. M. Arndt, unpublished observations), liquid −Ino media did not contain adenine. For growth of *spt15-328 hxk2*Δ and *spt15-328 reg1*Δ double mutants, solid −Ino media contained adenine, uracil, and all 20 amino acids (SC-Ino). Transformation of yeast cells was performed using the lithium acetate procedure (Ito *et al.* 1983). Plasmids were recovered from yeast as described (Arndt *et al.* 1995) and transformed into *Escherichia coli* strain MH1 (Hall *et al.* 1984) for propagation. Plasmids were transformed into *E. coli* strain DH5α for sequencing.

Yeast strains: With the exception of MCY2616 (Tu and Carlson 1994), Y153 (Durfee *et al.* 1993), and KA20, all strains

TABLE 1

***Saccharomyces cerevisiae* strains**

Strain	Genotype
KY87	*MAT***a** *his4-917*δ *lys2-173R2 ade8*
FY630	*MAT*α *his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY108	*MAT***a** *his4-917*δ *lys2-173R2 ura3-52 ade8*
KY214	*MAT*α *spt15-328 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY484	*MAT***a** *spt15-328 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 ade8*
KY485	*MAT*α *spt15-328 SNF4-204 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY486	*MAT*α *spt15-328 reg1-230 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY487	*MAT*α *spt15-328 opi1-312 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY488	*MAT***a** *spt15-328 rtf2-315 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 ade8*
KY489	*MAT***a** *spt15-328 opi1-319 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 ade8*
KY490	*MAT***a** *reg1-230 his4-917*δ *lys2-173R2 ura3-52 trp1*Δ*63*
KY491	*MAT*α *SNF4-204 his4-917*δ *lys2-173R2 leu2*Δ*1 trp1*Δ*63*
KY492	*MAT*α *opi1-319 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY493	*MAT*α *rtf2-315 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52*
KY494	*MAT***a**/*MAT*α *spt15-328/SPT15 his4-917*δ/*his4-917*δ *lys2-173R2/lys2-173R2 leu2*Δ*1/leu2*Δ*1 ura3-52/ura3-52 TRP1/trp1*Δ*63 ade8/ADE8*
KY495	*MAT***a** *SNF4-204 his4-917*δ *lys2-173R2 ade8*
KY496	*MAT***a**/*MAT*α *spt15-328/SPT15 his3*Δ*200/his3*Δ*200 lys2-173R2/lys2-173R2 ura3-52/ura3-52 TRP1/trp1*Δ*1 ade8/ADE8*
KY497	*MAT***a** *reg1-230 his4-917*δ *lys2-173R2 ura3-52*
KY498	*MAT*a *reg1*Δ*::URA3 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52*
KY499	*MAT*α *spt15-328 mig1*Δ*::URA3 his3*Δ*200 lys2-173R2 leu2*Δ*1 ura3-52*
KY500	*MAT***a** *spt15-328 tup1*Δ*::HIS3 his3*Δ*200 lys2-173R2 ura3-52*
KY501	*MAT***a** *spt15-328 reg1*Δ*::URA3 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 ade8*
KY502	*MAT*α *spt15-328 hxk2*Δ*::LEU2 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 ade8*
KY503	*MAT***a** *SNF4::URA3::SNF4 his4-917*δ *lys2-173R2 ura3-52 ade8*
KY504	*MAT***a** *spt15-328 SNF4-313 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 ade8*
KY529	*MAT***a** *spt15-328 ssn6*Δ*::HIS3 his3*Δ*200 lys2-173R2 ura3-52 trp1*Δ*63*
KY530	*MAT*α *spt15-328 ssn6*Δ*::HIS3 his3*Δ*200 lys2-173R2 ura3-52 ade8*
KY531	*MAT***a** *opi1-319 his4-917*δ *lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63 ade8*
KY532	*MAT***a** *spt15-328 reg1-230 his3*Δ*200 lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY533	*MAT*α *spt15-328 SNF4-204 his3*Δ*200 lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY534	*MAT***a** *opi1*Δ*::HIS3 his3*Δ*200 lys2-173R2 ura3-52 ade8*
KY535	*MAT*α *spt15-328 opi1*Δ*::HIS3 his3*Δ*200 lys2-173R2 ura3-52*
KY536	*MAT*α *spt15-328 opi1*Δ*::HIS3 reg1-230 his3*Δ*200 lys2-173R2 leu2*Δ*1 ura3-52 trp1*Δ*63*
KY537	*MAT***a** *spt15-328 opi1*Δ*::HIS3 SNF4-204 his3*Δ*200 lys2-173R2 ura3-52 trp1*Δ*63*
FY1179	*MAT***a** *mig1*Δ*::URA3 his3*Δ*200 lys2-*Δ*202 leu2*Δ*1 ura3-52*
FY1193	*MAT*α *snf1*Δ*10 his3*Δ*200 leu2*Δ*1 ura3-52 trp1*Δ*63*
MCY2616	*MAT***a** *glc7-T152K his3*Δ*200 lys2-801 ura3-52 trp1*Δ*1*
KA20	*MAT***a** *spt15-328 glc7-T152K his4-917*δ *lys2-173R2 ura3-52 trp1*Δ*1*
Y153	*MAT***a** *URA3::GAL-lacZ LYS2::GAL-HIS3 gal4*Δ *gal80*Δ *his3*Δ*200 leu2-3,112 ura3-52 trp1*Δ*1 ade2-101*

are congenic with FY2, a *GAL2*$^+$ *ura3-52* derivative of S288C (Winston *et al.* 1995; Table 1). Strains were constructed by standard methods for tetrad analysis (Rose *et al.* 1990) or one-step gene replacement (Rothstein 1983). Strains containing the suppressor mutations in an *SPT15*$^+$ background were identified from the nonparental ditypes of crosses performed to determine if the suppressor mutations were linked to *SPT15*$^+$ (see below) and were confirmed by reconstruction of the suppression. *reg1*Δ strains and *hxk2*Δ strains were constructed by transforming KY494 with the 4.2-kb *Xba*I-*Eco*RI fragment from pUCsrn1::URA3 (Tung *et al.* 1992) or with the 3.0-kb *Hin*dIII-*Pvu*II fragment from pMR226 (gift from K.-D. Entian), respectively, and sporulating the resulting transformants. *tup1*Δ strains were constructed by transforming KY496 with the 3.6-kb *Sac*I-*Spe*I fragment from pMC134 (gift from H. Ronne) and sporulating the resulting transformants. KA20 and KY499 were obtained as spores from matings between KY214 and MCY2616 or FY1179, respectively. *ssn6*Δ strains and *opi1*Δ strains were constructed by PCR-mediated, one-step gene disruptions (Ausubel *et al.* 1998) in KY494 followed by sporulation and dissection. Oligonucleotides used to amplify *ssn6*Δ*::HIS3* were as follows: 5′-GCTATAAGCCTTTAGACT AGTACTACAACTACAACAGCAACTGTGCGGTATTTCACA CCG-3′ and 5′-TGATTATAAATTAGTAGATTAATTTTTTGA ATGCAAACTTAGATTGTACTGAGAGTGCAC-3′. Oligonucleotides used to amplify *opi1*Δ*::HIS3* were as follows: 5′-TGT TTACAGTGCTGATTAAAGCGTGTGTATCAGGACAGTGCT GTGCGGTATTTCACACCG-3′ and 5′-TTACTGGTGGTAATG CATGAAAGACCTCAATCTGTCTCGGAGATTGTACTGAGAG TGCAC-3′. Disruptions of *REG1*, *TUP1*, *SSN6*, and *OPI1* were confirmed by Southern blotting (Ausubel *et al.* 1998). Disruption of *HXK2* or the presence of *glc7-T152K* in strains was confirmed by resistance to 2-deoxyglucose (Lobo and Maitra 1977; Neigeborn and Carlson 1987). Because *ssn6*Δ strains are also temperature sensitive, the *SPT15* genotype of KY529 and KY530 was determined by backcrossing to strains con-

taining *SPT15*$^+$ *SSN6*$^+$. The *trp1*Δ genotype of KA20 was determined by PCR analysis. KY503 was created by transforming KY108 with pPS52 (see below), which had been linearized with *Eco*RI. Insertion of an additional copy of *SNF4*, marked by *URA3*, was confirmed by Southern blotting (Ausubel *et al.* 1998).

Isolation of extragenic suppressors of *spt15-328*: To reduce the likelihood of recovering true revertants of *spt15-328* (see results), we created derivatives of KY214 and KY484 that carried pPS5, a 2μ plasmid containing *spt15-328* and *URA3*. Sixteen individual colonies from each strain were patched onto SC-Ura media and replica plated to media lacking inositol and uracil. Patches were mutagenized with UV radiation of 0–1500 μJ/cm^2 in a Stratalinker (Stratagene, La Jolla, CA). No more than one Ino$^+$ candidate was purified from each patch to ensure that all suppressor candidates were independently derived. Because strains containing *spt15-328* can become polyploid (K. M. Arndt, unpublished observation), we monitored whether the suppressor candidate strains were haploid using a modified canavanine test (Schild *et al.* 1981). Patches of candidate strains were replica plated onto SC-Arg+CAN media, UV irradiated with 9000 μJ/cm^2 in a Stratalinker, and allowed to grow at 30° for 4–5 days. Patches originating from haploid strains are much more likely to contain canavanine-resistant papillations. After purification, ploidy analysis, and passage on 5-FOA, 14 haploid strains were isolated that contained suppressors of the Ino$^-$ phenotype of *spt15-328* in the absence of pPS5.

To determine whether the suppressor mutations were extragenic to *SPT15*, strains containing *spt15-328* with the suppressor mutations were crossed to KY87 or FY630, and the resulting tetrads were analyzed for their pattern of growth on SD-Ino media. None of the 14 candidates were linked to *SPT15*. To determine whether the mutations were dominant or recessive and if the suppression phenotype was caused by a single mutation, double-mutant strains were crossed to either KY214 or KY484. To compensate for the sporulation defect of *spt15-328* homozygous diploids (K. M. Arndt, unpublished observation), the KY214 and KY484 parents were first transformed with pDE28-6, a CEN/ARS plasmid containing *URA3* and *SPT15*$^+$ (Eisenmann *et al.* 1989). Diploids resulting from these crosses were patched and replica plated to 5-FOA media to remove pDE28-6. Recessive mutations were scored as an inability of the diploids to grow on SD-Ino media. Diploids still containing pDE28-6 were allowed to sporulate, and a 2:2 Ino$^+$:Ino$^-$ phenotype in the resulting tetrads, after passage on 5-FOA media, indicated that suppression was caused by a mutation in a single gene. Complementation and linkage analysis among the candidates showed that these suppressors comprised four linkage groups: three represented by only recessive mutations and one represented by only dominant mutations.

Cloning of suppressor genes: A YCp50-based yeast genomic library (Rose *et al.* 1987) was transformed into strains KY486 and KY489, which contain recessive suppressor mutations, and transformants were screened for complementation of the Ino$^+$ phenotype. Four plasmids complemented the Ino$^+$ phenotype of KY486, and two plasmids complemented the Ino$^+$ phenotype of KY489. For each complementing plasmid, the sequence of ~100 bp of genomic DNA was determined, and a search of the Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces/) was performed. The four plasmids that complemented KY486 contained overlapping sequences on chromosome IV (inserts numbered per the Saccharomyces Genome Database): pPS24 (497819–504852), pPS25 (498118–514935), pPS26 (497274–507787), and pPS53 (494534–509421). Both plasmids that complemented KY489 contained overlapping sequences on chromosome VIII (inserts numbered per the Saccharomyces Genome Database): pPS32 (58894–68065) and pPS33 (61812–69897). Subcloning (see below) identified the complementing genes for KY486 and KY489 to be *REG1* and *OPI1*, respectively. Analysis of tetrads sporulated from a cross between KY534 and KY487 showed that *OPI1* is the correct gene.

To identify the dominantly acting suppressor in KY485, a plasmid library of KY485 genomic DNA in vector pRS316 (Sikorski and Hieter 1989) was constructed following the protocol of Thompson *et al.* (1993). One plasmid conferred an Ino$^+$ phenotype when transformed into KY214. The plasmid, pPS34, contained genomic sequences from 285282 to 294950 of chromosome VII. Subcloning (see below) localized the complementing activity to the *SNF4* gene. Analysis of tetrads sporulated from a cross between KY485 and KY501 showed that *SNF4* is the correct gene.

To identify the *SNF4* mutation in KY504, the 7.8-kb *Bgl*II-*Avr*II fragment from pPS34 was transformed into KY504 for gap repair (Orr-Weaver *et al.* 1983). The recovered plasmid, pPS60, contains the *SNF4-313* gene, which encodes a substitution of aspartic acid for glycine at position 145 of Snf4 (Snf4-G145D).

Plasmids: Standard molecular techniques were used for plasmid constructions (Ausubel *et al.* 1998). pKA86 is a derivative of pKA75 (Arndt *et al.* 1995), which contains the *spt15-328* gene in place of *SPT15*$^+$. The 2.4-kb *Xho*I (from the polylinker)-*Bam*HI fragment from pKA86, which contains *spt15-328* sequences, was subcloned into the same sites in pRS426 (Sikorski and Hieter 1989) to create pPS5. To identify the gene encoding the suppressor in KY486, a 5.0-kb *Eco*RI fragment from pPS26, which contains the entire open reading frame (ORF) of *REG1*, was subcloned into the *Eco*RI site in pRS316 (Sikorski and Hieter 1989) to create pPS27. Interestingly, one of the original complementing plasmids, pPS25, lacked the 80 C-terminal amino acids of Reg1. To eliminate the involvement of a second potential ORF, YDR030C, which is also encoded in pPS27, a 1.1-kb *Eco*RI-*Cla*I fragment from pPS24 that encodes only YDR030C was subcloned into the same sites in pRS316 to create pPS29. pPS29 did not encode complementing activity. To identify the gene encoding the suppressor in KY489, a 1.9-kb *Xho*I fragment from pPS32 containing *OPI1* was subcloned into the *Xho*I site in pRS316 to create pPS31. To determine the location of the mutation in *SNF4*, 1.8-kb *Hin*dIII-*Nco*I (blunted) fragments from pPS34 and pFE27-2, which contain wild-type *SNF4* sequences (Celenza *et al.* 1989), were subcloned into pRS316 that was digested with *Hin*dIII and *Xba*I (blunted) to create pPS47 and pPS48, respectively. The 0.8-kb *Xho*I (from the polylinker)-*Eco*RI fragment from pPS48, which encodes the promoter and N-terminal sequences of wild-type Snf4, was substituted for the corresponding sequences in pPS47 to create pPS49. Phenotypic analysis showed that pPS49 still contained the *SNF4-204* mutation. Sequencing confirmed the location of the mutation (see results). pPS52 was constructed by subcloning the 1.8-kb *Kpn*I-*Sac*I fragment containing *SNF4* sequences from pPS48 into the corresponding sites in pRS306 (Sikorski and Hieter 1989).

For the two-hybrid analysis, pGBT9 and pGAD424 were obtained from Clontech (Palo Alto, CA); pGBT9-SNF1 was a gift from M. C. Schmidt (Tillman *et al.* 1995). To facilitate subsequent subcloning, the 1.9-kb *Hin*dIII fragment from pNI12 (Fields and Song 1989), which encodes the entire Snf4-Gal4 activation domain fusion protein, was inserted at the *Hin*dIII site in pRS425 (Sikorski and Hieter 1989) to create pPS50. A 1.0-kb *Sal*I-*Avr*II fragment from pPS50, which contains the N terminus of *SNF4*, was substituted with the related sequence from pPS49 to create pPS51, which encoded Snf4-N177Y fused to the Gal4 activation domain. Most plasmid sequences are available upon request.

Northern hybridization analysis: Cells were grown at 30° to a density of 1–2 × 10^7 cells/ml in the appropriate media and induced as described in the figure legends (see also results). Isolation of RNA and Northern analyses was performed as described (Arndt *et al.* 1995). Hybridization probes for *INO1*, *TUB2*, and *GAL1-GAL10* were prepared from pJH310 (Hirsch and Henry 1986), pYST138 (Som *et al.* 1988), and pGAL1-GAL10, respectively, by nick translation (Boehringer Mannheim, Indianapolis). pGAL1-GAL10 contains the *Eco*RI fragment 4812 (St. John and Davis 1981) subcloned into the same site in pUC18. Quantitation was performed using a FUJIX BAS2000 phosphorimager with MacBAS version 2.4 software.

Invertase assays: Cells were grown at 30° to mid-log phase in YPD. For derepression of *SUC2*, 10 ml of cells was pelleted, washed twice with an equal volume of water, and resuspended in YEP + 0.05% glucose. Cells were allowed to grow for an additional 165 min at 30°. OD_{600} was determined for both repressed and derepressed samples. Invertase assays were performed in duplicate on three isolates of each strain as described (Goldstein and Lampen 1975; Bu and Schmidt 1998). Average values are reported. Standard errors were <17%.

Two-hybrid analysis: Plasmids were transformed into Y153 (Durfee *et al.* 1993) and selected on SC-Trp-Leu media containing 2% glucose. For quantitative β-galactosidase assays, cells were grown to a density of 1–2 × 10^7 cells/ml in either SC-Trp-Leu containing 2% glucose or SC-Trp-Leu containing 2% galactose, 2% glycerol, 2% ethanol, and 0.05% glucose. Extract preparations, β-galactosidase assays, and unit calculations were performed as described (Miller 1972; Rose and Botstein 1983). Values represent the average of two experiments in which three transformants for each plasmid were assayed at two different extract concentrations. Standard errors were <15%.

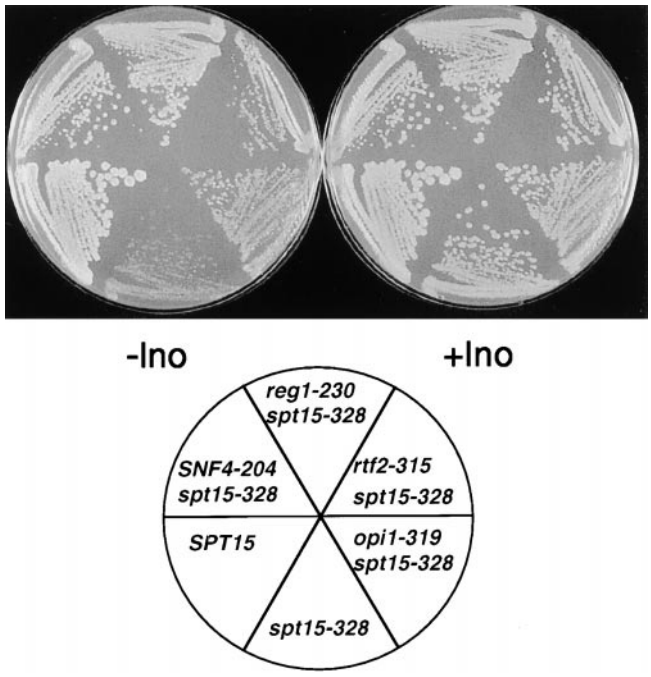

Figure 1.—Suppression of the Ino⁻ phenotype of *spt15-328*. Representatives of four linkage groups that restored growth to KY214 in the absence of inositol were purified on YPD plates and replica plated to SD-Ino media or SD-Ino media supplemented with inositol. Photographs were taken after 2 days of growth at 30°. Strains used were as follows: *SPT15*⁺ (FY630), *spt15-328* (KY214), *SNF4-204 spt15-328* (KY485), *reg1-230 spt15-328* (KY486), *rtf2-315 spt15-328* (KY488), and *opi1-319 spt15-328* (KY489).

RESULTS

Identification of extragenic suppressors of a TBP mutant defective in activated transcription. We previously reported the identification of the TBP mutant TBP-P109A, which is encoded by the *spt15-328* gene. This mutant TBP exhibits promoter-specific defects in activated transcription *in vivo* and greatly reduced affinity for TATA boxes *in vitro* (Arndt *et al.* 1995). We exploited the Ino⁻ phenotype of *spt15-328* strains to identify, through genetic analysis, factors that may regulate TBP function. We reasoned that recessive suppressor mutations might uncover factors that negatively regulate the response of TBP to a particular activator or the binding of TBP to certain promoters, while dominant suppressor mutations might identify factors that assist these functions of TBP.

In our initial selections for suppressors of *spt15-328*, we repeatedly isolated intragenic suppressors that exhibited essentially wild-type phenotypes. Therefore, we modified our approach to enhance our ability to detect extragenic suppressors. In particular, we noticed that overexpression of *spt15-328* has a slight dominant-negative effect in *SPT15*⁺ strains, causing a partial Ino⁻ phenotype compared with strains that do not express *spt15-328* (M. K. Shirra and K. M. Arndt, unpublished observations). Based on this finding, we conducted a selection for Ino⁺ suppressors using strains that expressed *spt15-328* from both a 2μ plasmid (pPS5) and the endogenous chromosomal copy (see materials and methods for details). Twelve recessive and two dominant extragenic suppressors of the Ino⁻ phenotype of *spt15-328*, but no intragenic suppressors, were recovered. These suppressors comprise four linkage groups: three that correspond to previously identified genes (see below), and one that has not been cloned. We named the unidentified gene *RTF2*, for *R*estores *T*BP *F*unction. *RTF1* was reported previously as a suppressor of a different *spt15* allele (Stolinski *et al.* 1997). All suppressor mutations restore growth to *spt15-328* strains on −Ino media (Figure 1). However, they do not significantly affect the temperature sensitivity or Gal⁻ phenotype of *spt15-328* strains. None of the suppressor mutations nor *spt15-328* itself cause an Spt⁻ phenotype.

For the recessive mutations, genes were cloned by complementation of the Ino⁺ phenotype of strains containing *spt15-328* and individual suppressor mutations. Subcloning and DNA sequence analysis of complementing plasmids (see materials and methods) showed that the smallest DNA fragment able to complement the suppressor mutation in KY486 included *REG1*, a gene primarily studied for its role during glucose re-

pression (Matsumoto *et al.* 1983; Niederacher and Entian 1991; reviewed in Johnston and Carlson 1992; Gancedo 1998). Demonstrating that we have cloned the correct gene, tetrad analysis showed that the KY486 suppressor mutation, like *REG1*, is tightly linked to *TRP1* (90 parental ditypes, 0 nonparental ditypes, and 7 tetratypes among 97 complete tetrads). Complementation and linkage tests demonstrated that seven different suppressor strains contain recessive mutations in *REG1*. We found that the suppressor mutation in KY489 can be complemented by a plasmid carrying the *OPI1* gene, a previously identified negative regulator of *INO1* transcription (Greenberg *et al.* 1982b; White *et al.* 1991). The strong Opi$^-$ phenotype of KY489 suggested that the suppressor mutation in this strain is in *OPI1*, and this has been confirmed by linkage analysis (see materials and methods). Four suppressor strains contain recessive mutations in *OPI1*. Repeated efforts to isolate a complementing clone for *rtf2-315*, the sole member of the *RTF2* complementation group, have been unsuccessful, in part because this mutation causes a partially dominant phenotype. Furthermore, in screens to reveal additional phenotypes that might be useful in cloning *RTF2*, neither *SPT15*$^+$ *rtf2-315* nor *spt15-328 rtf2-315* strains exhibited any differences relative to *RTF2*$^+$ strains.

To clone the dominant suppressor mutation in KY485, a genomic library was constructed from this strain and transformed into an *spt15-328* mutant. One plasmid that suppressed the Ino$^-$ phenotype of KY214 was isolated. Subcloning and DNA sequencing of this plasmid, followed by linkage analysis (see materials and methods), showed that the dominant suppressor mutation in KY485 lies in the gene for Snf4, a component of the Snf1 kinase complex that is required for derepression of glucose-repressible genes (Schüller and Entian 1988; Celenza *et al.* 1989; reviewed in Johnston and Carlson 1992; Gancedo 1998). Moreover, sequencing of the mutant *SNF4* gene showed that the mutation (*SNF4-204*) encodes a substitution of tryptophan for asparagine at position 177 (Snf4-N177Y). This asparagine is conserved between *S. cerevisiae* and *Schizosaccharomyces pombe SNF4* homologs (GenBank accession no. 2130248), although it is not strictly conserved in homologs of other species (Wilson *et al.* 1994; Gao *et al.* 1996; Piosik *et al.* 1996; Woods *et al.* 1996). Gap rescue followed by DNA sequence analysis showed that *SNF4-313*, a second isolate from this linkage group, encodes Snf4-G145D (see materials and methods). This glycine is not conserved in homologs of other species, and no further characterization of this mutant protein has been performed.

Further evidence that the *reg1*, *opi1*, *rtf2*, and *SNF4* mutations are involved in transcriptional control was obtained from their suppression of another activation-defective TBP mutant encoded by *spt15-341* (Arndt *et al.* 1995). Similar to *spt15-328* strains, *spt15-341* strains are inositol auxotrophs and inefficiently use galactose as a carbon source (Arndt *et al.* 1995). In addition, the mutant TBP encoded by *spt15-341* is severely impaired in TATA box binding *in vitro* (Arndt *et al.* 1995). Using genetic crosses, we found that members of each of the four linkage groups could also suppress the Ino$^-$ phenotype of *spt15-341* (data not shown), demonstrating that suppression is not specific to the *spt15-328* allele.

Suppressor mutations restore transcription of *INO1* in TBP mutant strains: To determine if the Ino$^+$ phenotype of the suppressed strains correlated with an increase in *INO1* transcription, we performed Northern analyses on the double-mutant strains (Figure 2A). A low concentration of inositol was used in the derepression media to allow partial derepression of *INO1* while permitting growth of strains severely defective in *INO1* transcription (Greenberg *et al.* 1982a; Hirsch and Henry 1986). Cells were induced for 10 hr, a time sufficient for maximal induction of *INO1* transcription in the wild-type background (K. M. Arndt, unpublished results). Under these conditions, all suppressor mutations restored transcription of *INO1*, from 10- to 30-fold, in the *spt15-328* background (Figure 2A). These results suggest that the suppressor mutations affect the ability of the mutant TBP to support transcription initiation at the *INO1* promoter.

Although the suppressor mutations do not significantly affect the Gal$^-$ phenotype of *spt15-328* strains, we asked whether any subtle effects on *GAL* gene transcription could be detected by Northern analysis (Figure 2, B and C). Strains were grown in media (2% raffinose or 3% glycerol/2% lactate) that are nonrepressing and noninducing for *GAL* gene expression. Galactose was added subsequently to activate transcription of *GAL1* and *GAL10*. [*spt15-328 SNF4-204* double mutants are unable to grow in media containing raffinose as the sole carbon source (M. K. Shirra and K. M. Arndt, unpublished results), necessitating the use of the alternative glycerol/lactate media.] No more than a twofold effect on *GAL1* or *GAL10* transcription was observed for any of the suppressor mutations in *spt15-328* strains.

To examine the transcriptional effects of the suppressor mutations in a wild-type TBP background, Northern analysis of *INO1* transcription was performed with *SPT15*$^+$ strains containing the suppressors (Figure 3). Because *SPT15*$^+$ strains will grow in the absence of inositol, induction of *INO1* in these strains was achieved without the addition of low levels of inositol and was monitored for 4 hr; we have seen that maximal derepression of *INO1* transcription in the absence of inositol occurs in 3–4 hr (for example, see Figure 4, lanes 2–5). Although strains containing *SNF4-204* or *reg1-230* show slightly reduced levels of *INO1* transcription in derepressing conditions, *rtf2-315* and *opi1-319* have little effect on derepressed levels of *INO1* mRNA. As seen for other *opi1* mutants (Greenberg *et al.* 1982a; White *et al.* 1991; Ashburner and Lopes 1995), the *INO1* gene

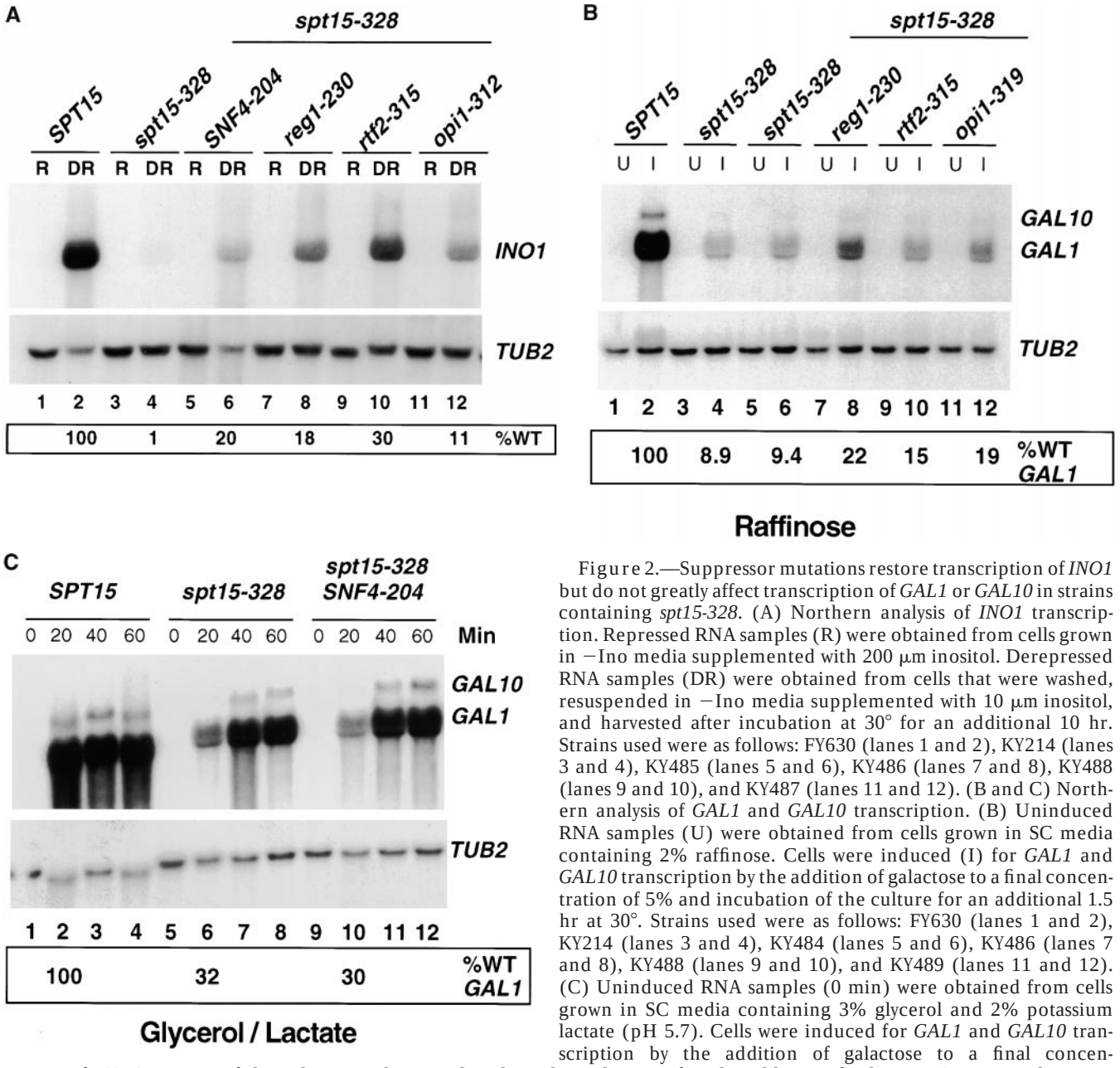

Figure 2.—Suppressor mutations restore transcription of *INO1* but do not greatly affect transcription of *GAL1* or *GAL10* in strains containing *spt15-328*. (A) Northern analysis of *INO1* transcription. Repressed RNA samples (R) were obtained from cells grown in −Ino media supplemented with 200 μm inositol. Derepressed RNA samples (DR) were obtained from cells that were washed, resuspended in −Ino media supplemented with 10 μm inositol, and harvested after incubation at 30° for an additional 10 hr. Strains used were as follows: FY630 (lanes 1 and 2), KY214 (lanes 3 and 4), KY485 (lanes 5 and 6), KY486 (lanes 7 and 8), KY488 (lanes 9 and 10), and KY487 (lanes 11 and 12). (B and C) Northern analysis of *GAL1* and *GAL10* transcription. (B) Uninduced RNA samples (U) were obtained from cells grown in SC media containing 2% raffinose. Cells were induced (I) for *GAL1* and *GAL10* transcription by the addition of galactose to a final concentration of 5% and incubation of the culture for an additional 1.5 hr at 30°. Strains used were as follows: FY630 (lanes 1 and 2), KY214 (lanes 3 and 4), KY484 (lanes 5 and 6), KY486 (lanes 7 and 8), KY488 (lanes 9 and 10), and KY489 (lanes 11 and 12). (C) Uninduced RNA samples (0 min) were obtained from cells grown in SC media containing 3% glycerol and 2% potassium lactate (pH 5.7). Cells were induced for *GAL1* and *GAL10* transcription by the addition of galactose to a final concentration of 5%. A portion of the culture was harvested at the indicated times after the addition of galactose. Strains used were as follows: FY630 (lanes 1–4), KY214 (lanes 5–8), and KY485 (lanes 9–12). In each experiment, the filter from the top panel was reprobed for *TUB2* mRNA. Quantitation is presented as the percentage of induced mRNA levels in the wild-type control, normalized to *TUB2*. Results from representative experiments are shown.

is expressed in an *SPT15*$^{+}$*opi1-319* strain in the presence of inositol (Figure 3, lane 9). In contrast, *spt15-328* strains that contain the *opi1-312* mutation, which is phenotypically similar to the *opi1-319* mutation, do not express *INO1* under repressing conditions (Figure 2A, lane 11, and data not shown). Taken together with the *in vitro* DNA-binding defect of the TBP mutant, these results argue for an additional *OPI1*-independent mechanism of inositol-mediated repression that may involve TATA box accessibility. As we describe below, this *OPI1*-independent repression appears to be regulated, at least in part, by the Snf1-Snf4 kinase pathway.

The *SNF4-204* mutation alters the interaction of Snf4 with Snf1: As an initial characterization of the mutation in *SNF4-204*, we examined the ability of the *SNF4-204* gene product, Snf4-N117Y, to interact with Snf1 in the two-hybrid system. The interaction between Snf1 and Snf4, which has been well documented with this assay (Fields and Song 1989), is regulated by glucose levels such that low-glucose conditions significantly enhance formation of the Snf1-Snf4 complex (Jiang and Carlson 1996). Therefore, we performed a two-hybrid analysis of Snf1 and Snf4-N177Y in media containing 2 or 0.05% glucose (Table 2). Strikingly, unlike Snf1 and

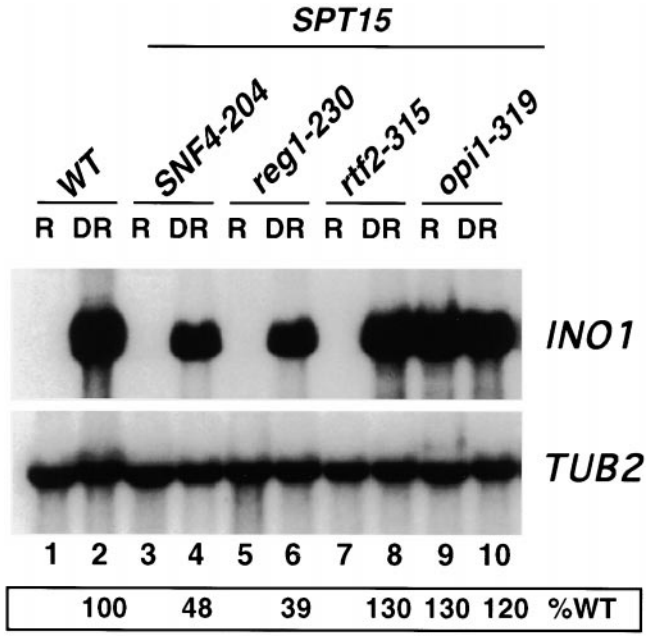

Figure 3.—In strains containing *SPT15*$^{+}$, the suppressor mutations have little effect on derepressed levels of *INO1* transcription. Northern analysis of *INO1* transcription is shown. Repressed RNA samples (R) were obtained from cells grown in −Ino media supplemented with 200 μm inositol. Derepressed RNA samples (DR) were obtained from cells that were pelleted, washed, resuspended in −Ino media, and harvested after incubation at 30° for an additional 4 hr. The filter in the top panel was reprobed for *TUB2* mRNA. Quantitation is presented as the percent of derepressed *INO1* mRNA levels in the wild-type control, normalized to *TUB2*. Strains used were as follows: FY630 (lanes 1 and 2), KY491 (lanes 3 and 4), KY490 (lanes 5 and 6), KY493 (lanes 7 and 8), and KY492 (lanes 9 and 10). Results from a representative experiment are shown.

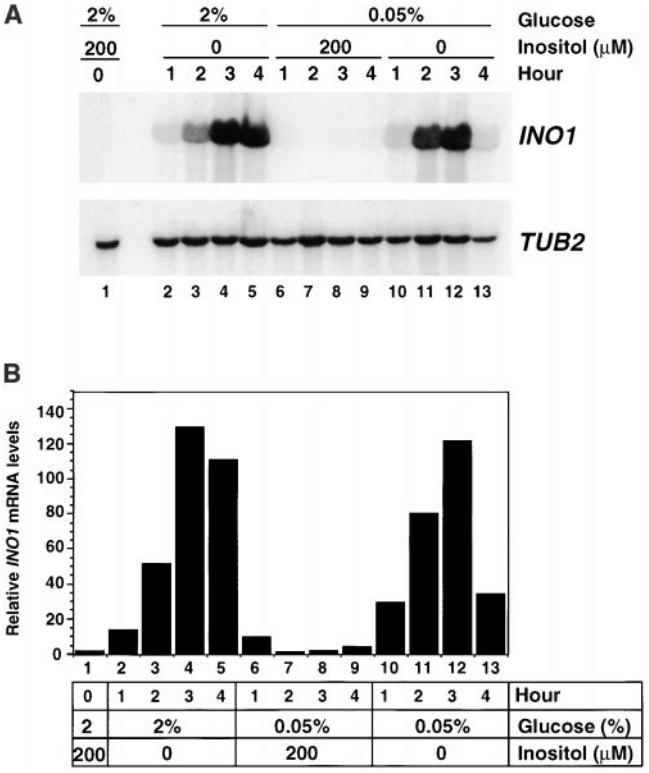

Figure 4.—*INO1* transcription is not greatly affected by glucose levels. (A) Northern analysis of *INO1* transcription. *SPT15* (FY630) cells were grown in −Ino media supplemented with 200 μm inositol (lane 1). Portions of the culture were centrifuged, and cell pellets were washed and resuspended in −Ino media containing 2% glucose (lanes 2–5), −Ino media containing 0.05% glucose and 200 μm inositol (lanes 6–9), or −Ino media containing 0.05% glucose (lanes 10–13). Samples were harvested at the indicated times after incubation at 30°. The filter in the top panel was reprobed for *TUB2* mRNA as a normalization control. (B) Quantitation of Northern analysis shown in A. Normalized *INO1* mRNA levels are shown relative to the lowest level detected (lane 7), which was arbitrarily set to 1. Results from a representative experiment are shown.

wild-type Snf4, the interaction between Snf1 and Snf4-N177Y, as measured in β-galactosidase units, was readily detected in the presence of high glucose. Furthermore, under low-glucose conditions, β-galactosidase levels were twofold greater for the Snf1-Snf4-N177Y interaction pair than for Snf1 and wild-type Snf4. These results indicate that the dominant *SNF4-204* mutation increases the affinity of Snf4 for Snf1 and renders the interaction independent of glucose levels.

Analysis of *INO1* transcription in response to glucose: The identification of *opi1* in our selection for *spt15* suppressors was not surprising. We expected to uncover negative regulators of *INO1* transcription, since mutations in these factors might relieve a block in transcriptional activation to which the mutant TBP is particularly sensitive. However, the identification of mutations in *REG1* and *SNF4* was unanticipated. Although these genes have been implicated in a number of biological processes, they have been most extensively studied for their roles in glucose regulation of gene expression. The isolation of recessive alleles in *REG1* and dominant alleles in *SNF4* in our selection may indicate that these mutations function by relieving glucose repression of *INO1* transcription. To test this idea, we have examined the effect of glucose levels on *INO1* expression.

In the case of glucose-repressed genes such as *SUC2*, derepression of transcription occurs when the levels of glucose are reduced. To determine if a similar mechanism is involved in the regulation of *INO1*, we examined the levels of *INO1* transcription by Northern analysis in high (2%) and low (0.05%) glucose (Figure 4). We found that *INO1* transcription in low-glucose conditions is reproducibly elevated only twofold relative to high-glucose conditions 1 and 2 hr after shifting cells from repressing (high inositol) to inducing (no inositol) media (Figure 4, compare lanes 2 and 10 and lanes 3 and 11). However, the maximal levels of *INO1* mRNA are unaffected by the glucose concentration (Figure 4, compare lanes 4 and 12). Note that in low-glucose conditions, *INO1* transcription levels drop between the 3- and

TABLE 2

Two-hybrid analysis of *SNF4-204*

DNA-binding hybrid[a]	Activation hybrid[a]	β-Galactosidase activity[b]	
		High glucose[c]	Low glucose[d]
Gal4(1-147)	GAD	<1	<1
Gal4(1-147)	GAD-Snf4	<1	<1
Gal4(1-147)	GAD-Snf4-N177Y	<1	<1
Gal4-Snf1	GAD	<1	<1
Gal4-Snf1	GAD-Snf4	<1	44
Gal4-Snf1	GAD-Snf4-N177Y	47	81

[a] Plasmids used are as follows: pGBT9 [Gal4(1-147)], pGBT9-SNF1 (Gal4-Snf1), pGAD424 (GAD), pPS50 (GAD-Snf4), and pPS51 (GAD-Snf4-N177Y).
[b] Expressed in Miller units.
[c] 2% glucose.
[d] 2% galactose, 2% glycerol, 2% ethanol, and 0.05% glucose.

4-hr time points, presumably because of the depletion of the carbon source (Henry and Patton-Vogt 1998). These data also show that in the presence of inositol, growth in low glucose is not sufficient to derepress *INO1* (Figure 4, lanes 6–9).

Although these results indicate that glucose levels do not greatly affect *INO1* expression in *SPT15*$^+$ cells, we investigated whether a defect in the glucose repression pathway could be responsible for suppression of the Ino$^-$ phenotype in *spt15-328* strains. In addition, we wanted to compare any effect of glucose derepression to the effect of a suppressor mutation on *INO1* transcription in an *spt15-328* strain (Figure 5). We chose *reg1-230* for this comparison because it showed a small but reproducible effect on *GAL1* transcription in *spt15-328* strains and, therefore, might be impaired in glucose repression. To mimic the growth conditions typically used to study glucose repression, we used a scheme similar to that used in Figure 4. Clearly, the small derepressing effect of low glucose on *INO1* transcription in the *spt15-328* background (less than twofold effect; compare Figure 5, lanes 5 and 9) is substantially less than the degree of suppression by *reg1-230* under either low- or high-glucose conditions. These results suggest that suppression of *spt15-328* by mutations in *REG1* and *SNF4* may not arise from the release of *INO1* transcription from glucose repression.

***reg1-230* and *SNF4-204* strains still exhibit glucose repression:** To evaluate whether the *reg1* and *SNF4* mutations we isolated are indeed defective in glucose repression, we examined the expression of a gene known to be regulated primarily through this pathway, *SUC2*. We measured the activity of invertase, the *SUC2* gene product, in strains containing wild-type TBP and either *reg1-230* or *SNF4-204* (Table 3). As a control, we constructed a *reg1* null allele in our strain background. In agreement with previous results (Tu and Carlson 1995; Frederick and Tatchell 1996), a *reg1*Δ mutation caused derepression of *SUC2* in the presence of high-glucose concentrations. Strikingly, our suppressor mutations did not relieve glucose repression of *SUC2*, even though the *SNF4-204* product interacts with Snf1 in the presence of glucose (Table 2). These data also show that the *reg1-230* mutation is not equivalent to a null allele. The *SNF4-204* strain may be somewhat defective in *SUC2* derepression, in agreement with the inability of *spt15-328 SNF4-204* strains to grow in raffinose media. Importantly, independent of any effect on derepression, strains containing the *reg1-230* and *SNF4-204* alleles are still capable of repressing *SUC2* expression under high-glucose conditions, suggesting that these mutations do not generally alleviate glucose repression.

Suppression of *spt15-328* can be achieved by mutations in some but not all genes implicated in glucose repression: To determine if any defect in the glucose-repression pathway could suppress *spt15-328*, we constructed double mutants between *spt15-328* and null mutations in *REG1*, *MIG1*, *TUP1*, *SSN6*, and *HXK2*. Because a deletion of *GLC7* is lethal (Clotet *et al.* 1991; Tu and Carlson 1994), we introduced the *glc7-T152K* mutation into the *spt15-328* background. The *glc7-T152K* mutation partially relieves glucose repression of gene expression (Neigeborn and Carlson 1987) and diminishes the interaction of Glc7 with Reg1 (Tu and Carlson 1995). Double-mutant strains were examined for their ability to grow in the absence of inositol (Figure 6). To varying degrees, *glc7-T152K*, *tup1*Δ, *ssn6*Δ, and *reg1*Δ restore growth of *spt15-328* on −Ino media. Preliminary Northern analyses showed that this suppression is occurring at the level of *INO1* transcription (M. K. Shirra and K. M. Arndt, unpublished observations). However, null alleles of *MIG1* and *HXK2* do not significantly suppress the Ino$^-$ phenotype conferred by *spt15-328*. Together with the previous data, these findings suggest that suppression of the TBP mutant by *reg1-230* and *SNF4-204* is unrelated to the glucose-repression pathway and may represent a distinct role for these genes in *INO1* transcription.

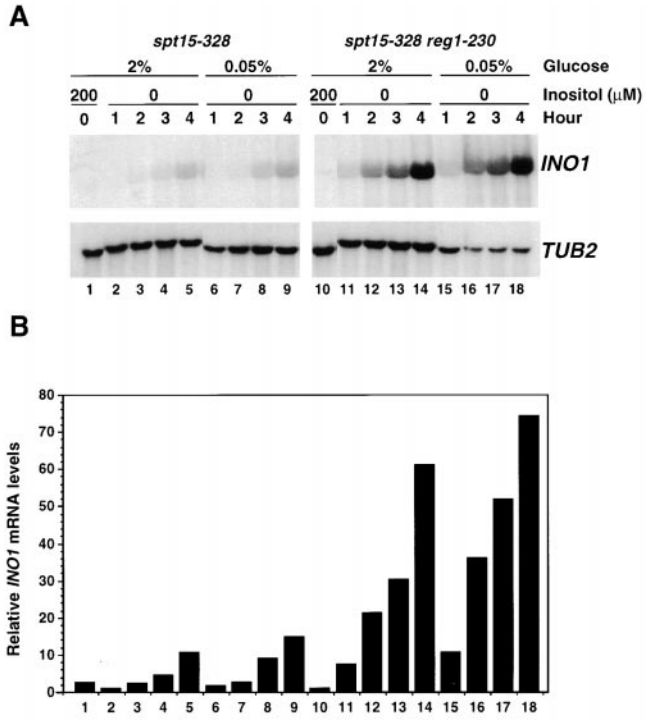

Figure 5.—*INO1* transcription in *spt15-328* strains is not greatly affected by glucose levels. Northern analysis of *INO1* transcription is shown. KY214 (lanes 1–9) or KY486 (lanes 10–18) cells were grown in −Ino media supplemented with 200 μm inositol (lanes 1 and 10). Portions of the culture were centrifuged, and cell pellets were washed and resuspended in −Ino media containing 2% glucose (lanes 2–5 and 11–14) or −Ino media containing 0.05% glucose (lanes 6–9 and 15–18). Samples were harvested at the indicated times after incubation at 30°. All lanes are from the same autoradiogram, but they have been rearranged for clarity of presentation. (B) Quantitation of Northern analysis shown in A. These values represent *INO1* mRNA levels that have not been normalized to *TUB2* mRNA levels, because we consistently noticed a decrease in *TUB2* mRNA levels in *spt15-328 reg1-230* double mutants grown in low glucose. However, ribosomal RNA levels in these samples were approximately equivalent, as determined by ethidium bromide staining. *INO1* mRNA levels are shown relative to the lowest level detected (lane 10), which was arbitrarily set to 1. Results from a representative experiment are shown.

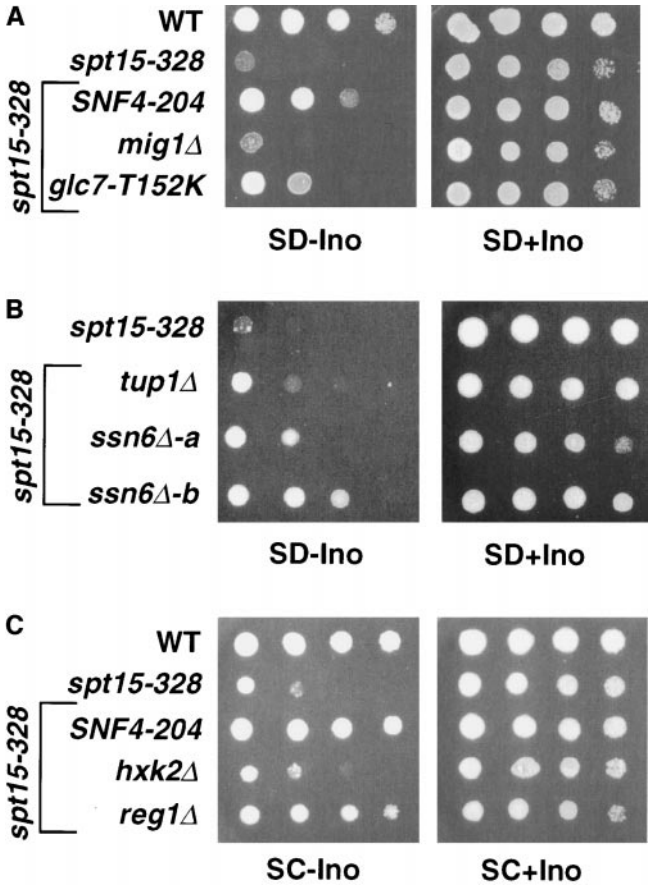

Figure 6.—The Ino⁻ phenotype conferred by *spt15-328* is suppressed by mutations in some but not all genes involved in glucose repression. Cells were grown in YPD to saturation, washed, and diluted to 1×10^8 cells/ml. A total of 3 μl of 10-fold serial dilutions (A and C) or 3-fold serial dilutions (B) were spotted onto solid media as indicated. (C) Because *spt15-328 hxk2*Δ and *spt15-328 reg1*Δ double mutants are unable to grow on minimal media, we supplemented our standard −Ino media (SD-Ino) with all 20 amino acids (SC-Ino) to examine suppression in these strains. Photographs were taken after (A and C) 3 days or (B) 5 days of growth at 30°. Strains used were as follows: FY630 (WT), KY214 (*spt15-328*), KY485 (*SNF4-204*), KY499 (*mig1*Δ), KA20 (*glc7-T152K*), KY500 (*tup1*Δ), KY529 (*ssn6*Δ*-a*), KY530 (*ssn6*Δ*-b*), KY502 (*hxk2*Δ), and KY501 (*reg1*Δ).

TABLE 3

Invertase activity in strains containing *reg1-230* and *SNF4-204*

Yeast strain	Relevant genotype	Invertase activity[a]	
		Repressed	Derepressed
KY87	WT	2	379
KY498	*reg1*Δ	832	635
KY497	*reg1-230*	3	377
KY495	*SNF4-204*	12	126

[a] Expressed as μmol glucose/min/OD_{600} cells assayed.

***REG1* and *SNF4* regulate *INO1* transcription in a manner independent of *OPI1*:** To begin to address the mechanism by which the Snf1 kinase regulates *INO1* transcription, we examined the genetic interactions between *SNF1* and *OPI1*. *snf1*Δ mutants are inositol auxotrophs, while *opi1* mutants overproduce inositol. We performed a genetic cross between KY531 and FY1193 to examine the epistatic relationship between these two genes. If *snf1*Δ mutations are unaffected by *opi1* mutations, we would expect 2:2 segregation of the inositol auxotrophy in the resulting tetrads. Instead, we found 5 tetrads that show 2:2 segregation of Ino^+:Ino^- phenotypes, 4 tetrads that show 4:0 segregation, and 24 tetrads that show 3:1 segregation. Among these tetrads were spores that could

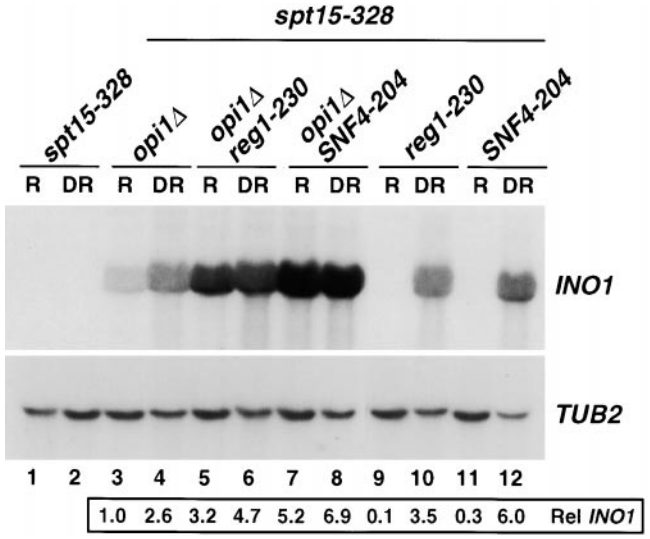

Figure 7.—In *opi1*Δ strains, *reg1* and *SNF4* mutations elevate the level of *spt15-328* suppression. Northern analysis of *INO1* transcription is shown. Repressed RNA samples (R) were obtained from cells grown in −Ino media supplemented with 200 μm inositol. Derepressed RNA samples (DR) were obtained from cells that were washed, resuspended in −Ino media supplemented with 10 μm inositol, and harvested after incubation at 30° for an additional 10 hr. Strains used were as follows: KY214 (lanes 1 and 2), KY535 (lanes 3 and 4), KY536 (lanes 5 and 6), KY537 (lanes 7 and 8), KY532 (lanes 9 and 10), and KY533 (lanes 11 and 12). The filter from the top panel was reprobed for *TUB2* mRNA. Quantitation is presented as *INO1* transcription levels relative to lane 3, after normalization to *TUB2*. Results from representative experiments are shown.

not efficiently use raffinose as the sole carbon source (Snf$^-$ phenotype) but could grow on media lacking inositol. These data show that the *opi1-319* mutation is epistatic to a *snf1*Δ mutation. This has been confirmed by Northern blot analysis, in which we found that *INO1* is transcribed under repressing conditions in a *snf1*Δ *opi1-319* strain (data not shown). These results could indicate that Snf1 and Opi1 function in the same pathway to regulate *INO1* transcription. Alternatively, the *opi1* mutation may phenotypically bypass the effect of the *snf1* mutation.

To test the hypothesis that Snf1 operates through Opi1, we asked whether the degree of *spt15-328* suppression caused by an *opi1* mutation is affected by our *reg1* and *SNF4* mutations. If Reg1 and Snf4 modulate *INO1* transcription solely through an effect on Opi1, then *reg1* and *SNF4* mutations, when combined with an *opi1* mutation, should not increase the level of *spt15-328* suppression relative to an *opi1* mutation alone. To rule out any effect of Reg1 or Snf4 on Opi1 activity, we performed this analysis with an *opi1*Δ mutation. As shown in Figure 7, the *opi1*Δ mutation, like our original *opi1-312* suppressor mutation, significantly restores *INO1* transcription in the TBP mutant strain (lanes 1–4). Unlike the *opi1-312* allele, however, the *opi1*Δ mutation renders *INO1* transcription partially derepressed in the mutant TBP background in the presence of high levels of inositol (Figure 7, compare lanes 3 and 4). Introduction of the *reg1-230* and *SNF4-204* mutations into the *spt15-328 opi1*Δ background leads to further increases in *INO1* transcription in both repressing and derepressing conditions (Figure 7, lanes 3–8). These results argue that the Snf1 kinase and the Opi1 repressor operate through different pathways to regulate *INO1* transcription. In addition, the residual, Opi1-independent repression observed in high-inositol conditions (Figure 7, compare lanes 3 and 4) is alleviated by the *reg1* and *SNF4* mutations (compare lanes 3, 5, and 7), suggesting that the Snf1 kinase pathway counters a repressive mechanism that is inositol mediated but Opi1 independent. Finally, because the *opi1* mutations derepress *INO1* transcription to a greater extent in *SPT15*$^+$ strains (Figure 3 and data not shown) compared with *spt15-328* strains (Figures 2A and 7), the mutant TBP appears to be more sensitive than wild-type TBP to this additional layer of *INO1* repression.

DISCUSSION

To identify factors that regulate TBP function *in vivo*, we performed a genetic selection for suppressors of the *spt15-328* gene product. We specifically searched for extragenic suppressors of the Ino$^-$ phenotype of this TBP mutant because *INO1* transcription is particularly sensitive to mutations that affect components of the RNA polymerase II transcription machinery (Nonet and Young 1989) and chromatin remodeling factors (Gansheroff *et al.* 1995; Roberts and Winston 1996; Grant *et al.* 1997). In this way, we identified four genes that directly or indirectly affect TBP function at the *INO1* promoter: *OPI1*, *REG1*, *SNF4*, and *RTF2*.

Identification of suppressor mutations in *OPI1*: One model to explain the transcriptional properties of the TBP mutant argues that promoter-specific factors negatively control TATA box accessibility at the most highly affected genes. Therefore, we expected to isolate mutations in genes, such as *OPI1*, that encode repressors of *INO1* transcription. Although the biochemical activity of Opi1 remains elusive, our genetic results suggest that Opi1 operates, at least in part, by impairing TBP function at the *INO1* promoter.

The identification of an *opi1* mutation in our selection suggested the possibility that disruption of any negative regulator of *INO1* transcription could suppress the Ino$^-$ phenotype of the TBP mutant. Other negative regulators of *INO1* transcription include *UME6* (Jackson and Lopes 1996); *HHF1*, which encodes histone H4 (Santisteban *et al.* 1997); and *SIN3* (Hudak *et al.* 1994). We constructed deletions of *SIN3* and *RPD3* in our genetic background and tested their ability to suppress *spt15-328*. Instead of suppression, we found that double-mutant strains containing *spt15-328* and either *sin3*Δ or

*rpd3*Δ are inviable (M. K. Shirra and K. M. Arndt, unpublished observations). While this synthetic lethality has intriguing implications for the functions of *SIN3* and *RPD3*, it prevented an analysis of *INO1* transcription in the double-mutant strains. Further tests of the specificity of suppression by *opi1* mutations will require the use of mutations in genes that are less pleiotropic.

Identification of suppressor mutations in *REG1* and *SNF4*: In addition to the well-established importance of *REG1* and *SNF4* in glucose repression, various genetic results have indicated an involvement of these genes in other biological processes, such as RNA processing (Pearson *et al.* 1982; Tung *et al.* 1992; Maddock *et al.* 1994), glycogen accumulation (Tu and Carlson 1995; Huang *et al.* 1996), and sporulation (Celenza *et al.* 1989). In our study, we have uncovered a role for *REG1* in the regulation of *INO1* transcription. The inositol auxotrophy of *snf1* mutant strains previously suggested a requirement for the Snf1-Snf4 complex in *INO1* induction (Hirschhorn *et al.* 1992). In the accompanying article a mutation in *REG1* was also identified in a search for suppressors of a mutant Ino4 transactivator, and a *reg1*Δ strain was shown to constitutively express *INO1* (Ouyang *et al.* 1999).

Several results suggest that the functions of *REG1* and *SNF4* in *INO1* regulation may be unrelated to their roles in glucose repression. First, glucose levels do not affect the maximal, induced levels of *INO1* transcription and do not bypass the normal induction signal for this gene. Second, our *reg1* and *SNF4* mutations are not generally defective in glucose repression, as indicated by the high level of repression seen at *SUC2*. Third, mutations in *MIG1* and *HXK2*, two other genes with well-known roles in glucose repression, do not suppress our TBP mutant. Fourth, we have found that unlike the singly mutated strains, *spt15-328 reg1*Δ double-mutant strains grow extremely slowly on rich media and are unable to grow on minimal media and that *spt15-328 reg1-230 SNF4-204* triple mutants are inviable (M. K. Shirra and K. M. Arndt, unpublished observations). Such synthetic growth defects suggest more global roles for *REG1* and *SNF4* in gene regulation. In agreement with this interpretation, others have noted functions for *REG1* that are apparently distinct from its involvement in glucose repression (Tung *et al.* 1992; Frederick and Tatchell 1996; Huang *et al.* 1996).

An alternative explanation for our results is that *INO1* transcription is subject to a modest level of glucose repression (*i.e.*, twofold), and that alleviation of this repression by our *reg1* and *SNF4* alleles is sufficient to overcome the transcriptional defect of the TBP mutant. At promoters that are more strongly repressed by glucose, such as *SUC2*, our *reg1* and *SNF4* mutations may be too weak to relieve repression. Because *hxk2* and *mig1* mutations do not relieve repression of all glucose-repressed genes (Marykwas and Fox 1989; Moehle and Jones 1990; Lundin *et al.* 1994), our genetic results cannot completely eliminate the possibility that glucose levels are regulating the *INO1* promoter. Importantly, independent of the actual signal that is transduced by the Reg1-Glc7 phosphatase and the Snf1-Snf4 kinase, our data strongly suggest that this pathway directly or indirectly regulates the activity of TBP at the *INO1* promoter.

Connections to the RNA polymerase II holoenzyme and chromatin: At glucose-repressed promoters, the Snf1-Snf4 kinase regulates phosphorylation of Mig1 (Treitel *et al.* 1998), negating the effects of the tethered Ssn6-Tup1 corepressor complex (Treitel and Carlson 1995; Lutfiyya and Johnston 1996). Our observation that *tup1*Δ and *ssn6*Δ mutations can moderately suppress the Ino$^-$ phenotype conferred by *spt15-328* suggests that a similar mechanism is operating at *INO1*. Since a *mig1*Δ does not suppress the Ino$^-$ phenotype of our TBP mutant, we postulate that some other protein tethers the Ssn6-Tup1 complex to the *INO1* promoter. Consistent with reports that Opi1 lacks DNA binding activity (S. A. Henry, personal communication), our genetic results suggest that this protein is unlikely to be Opi1. In addition we have found that *tup1*Δ mutants do not have a strong Opi1$^-$ phenotype (M. K. Shirra and K. M. Arndt, unpublished observations), providing further evidence that Tup1 does not act through Opi1.

Based on previous results, two principal mechanisms can be proposed to explain suppression of the activation- and DNA-binding-defective TBP mutants by mutations in *REG1*, *SNF4*, *SSN6*, and *TUP1*. The promoter-specific effects of the TBP mutants suggest that TATA box accessibility may be more constrained at some promoters, such as *INO1*, than at others. Nucleosome positioning may be critical for this distinction. A combination of genetic, molecular, and biochemical data strongly support a role for the Ssn6-Tup1 complex in regulating chromatin structure (Cooper *et al.* 1994; Edmondson *et al.* 1996; Gavin and Simpson 1997). Although one analysis of *INO1* chromatin structure did not show any gross differences in the presence or absence of inositol (Santisteban *et al.* 1997), it would be interesting to examine nucleosome positioning at this promoter in our mutant strains.

Alternatively, our suppressor mutations may affect the RNA polymerase II holoenzyme, enabling it to compensate for a defective TBP. In support of this idea, truncations of the heavily phosphorylated C-terminal domain of the large subunit of RNA polymerase II result in inositol auxotrophy (Nonet and Young 1989). Selections for suppressors of these C-terminal domain truncations have identified a class of *SRB* genes that appear to play negative roles in gene regulation (Carlson 1997). Interestingly, mutations in these same *SRB* genes suppress the effects of an *snf1* mutation at *SUC2* (Song *et al.* 1996). Whether mutations in these holoenzyme

components can restore *INO1* transcription to our TBP mutant strains remains to be determined.

What is the target of the Snf1 kinase for *INO1* regulation? Our results imply that activation of the Snf1 kinase, either by inactivating Reg1 or by stimulating the Snf1-Snf4 interaction, is responsible for suppression of our TBP mutant. Thus, phosphorylation of some target appears to bypass the need for a completely functional TBP at the *INO1* promoter. The identity of this target is unknown. We found that an *opi1* mutation is epistatic to *snf1*Δ for *INO1* transcription, suggesting that *OPI1* acts downstream of the kinase. However, Northern analysis on double- and triple-mutant strains (Figure 7) revealed that the Snf1-Snf4 kinase does not act solely through Opi1 to suppress our TBP mutant. In the accompanying article, Ouyang *et al.* (1999) showed that a *reg1* mutation suppresses the inositol auxotrophy of *ino4-8*, but not *ino4*Δ or *ino2*Δ, mutant strains. This necessity for residual Ino4 function may indicate that the Ino2-Ino4 complex is a target of the Snf1-Snf4 kinase. In addition, components or regulators of chromatin or the RNA polymerase II transcription machinery may be substrates for the Snf1 kinase at the *INO1* promoter.

In summary, by searching for suppressors of an activation-defective TBP mutant, we have implicated a pathway that includes the Reg1-Glc7 phosphatase and the Snf1-Snf4 kinase in transcription initiation at the highly regulated *INO1* promoter. Together with our previous results, our current findings support a model in which the formation or stability of the TBP-TATA complex at the *INO1* promoter, and perhaps at other promoters, may be regulated by a substrate of the Snf1 kinase.

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