Transcriptional Regulators of the *Schizosaccharomyces pombe* fbp1 Gene Include Two Redundant Tup1p-like Corepressors and the CCAAT Binding Factor Activation Complex

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ABSTRACT

The *Schizosaccharomyces pombe* fbp1 gene, which encodes fructose-1,6-bis-phosphatase, is transcriptionally repressed by glucose through the activation of the cAMP-dependent protein kinase A (PKA) and transcriptionally activated by glucose starvation through the activation of a mitogen-activated protein kinase (MAPK). To identify transcriptional regulators acting downstream from or in parallel to PKA, we screened an *adhl* driven cDNA plasmid library for genes that increase fbp1 transcription in a strain with elevated PKA activity. Two such clones express amino-terminally truncated forms of the *S. pombe* tup12 protein that resembles the *Saccharomyces cerevisiae* Tup1p global corepressor. These clones appear to act as dominant negative alleles. Deletion of both *tup12* and the closely related *tup11* gene causes a 100-fold increase in fbp1-lacZ expression, indicating that *tup11* and *tup12* are redundant negative regulators of *fbp1* transcription. In strains lacking *tup11* and *tup12*, the atf1-PCR1 transcriptional activator continues to play a central role in *fbp1*-lacZ expression; however, *spc1* MAPK phosphorylation of atf1 is no longer essential for its activation. We discuss possible models for the role of *tup11* and *tup12*-mediated repression with respect to signaling from the MAPK and PKA pathways. A third clone identified in our screen expresses the php5 protein subunit of the CCAAT-binding factor (CBF). Deletion of *php5* reduces *fbp1* expression under both repressed and derepressed conditions. The CBF appears to act in parallel to atf1-PCR1, although it is unclear whether or not CBF activity is regulated by PKA.

Transcriptional regulation is an important mechanism utilized by cells to control gene expression. In eukaryotes, transcription is regulated by activators and repressors that bind regulatory elements in the DNA as well as coactivators and corepressors that associate with the DNA-binding proteins (Struhl 1995; Ptashne and Gann 1997; Mannervik et al. 1999). These complexes may affect the recruitment of RNA polymerase to a promoter through direct interactions with RNA polymerase and/or its associated protein complexes, or alter the chromatin encompassing the regulatory elements to change the affinity of DNA-binding proteins for these elements.

The *Schizosaccharomyces pombe* fbp1 gene encodes fructose-1,6-bis-phosphatase and is transcriptionally regulated by environmental glucose (Vassarotti and Friesen 1985; Hoffman and Winston 1989, 1990). Various genetic screens have shown that two signaling pathways regulate fbp1 transcription. Glucose triggers the activation of adenylate cyclase, which in turn activates protein kinase A (PKA) to repress fbp1 transcription (Hoffman and Winston 1991; Byrne and Hoffman 1993; Jin et al. 1995). Glucose starvation stimulates a stress-activated, mitogen-activated protein kinase (MAPK) pathway, leading to the derepression of fbp1 transcription (Takeda et al. 1995; Kanoh et al. 1996; Stettler et al. 1996). Major components of this pathway include the spc1/sty1 MAPK (Millar et al. 1995; Shiozaki and Russell 1995, 1996; Degols et al. 1996; Wilkinson et al. 1996), the wis1 MAPK kinase (MAPKK; Warbrick and Fantes 1991), and wis4/wik1/wak1 and win1 MAPKK kinases (MAPKK; Samejima et al. 1997, 1998; Sheeh et al. 1997; Shiozaki et al. 1997). The downstream target of the spc1 MAPK is atf1/gad7 (Takeda et al. 1995; Kanoh et al. 1996; Shiozaki and Russell 1996; Wilkinson et al. 1996), a bZIP phosphoprotein that forms a heterodimer with the pcr1 bZIP protein (Watanabe and Yamamoto 1996). Transcriptional activation by atf1-PCR1 depends upon phosphorylation by the spc1 MAPK, although the role of this phosphorylation is controversial. Several studies have concluded that atf1 is constitutively bound to sequences resembling cAMP response elements (CRE; Hai et al. 1988; Roesler et al. 1988) and that the
phosphorylation of atf1 allows it to activate transcription (Takeda et al. 1995; Wilkinson et al. 1996; Degols and Russell 1997). On the other hand, two recent studies suggest that spc1 phosphorylation of atf1 increases atf1-pcr1 (also known as mts1-mts2) binding affinity for CRE-like elements (Kon et al. 1998; Neely and Hoffman 2000).

We previously identified two cis-acting elements in the fbp1 promoter necessary for fbp1 transcriptional activation (Neely and Hoffman 2000). UAS1 contains a CRE-like element and is the binding site for atf1-pcr1. Mobility shift data suggest that binding of atf1-pcr1 to UAS1 is stimulated by glucose starvation and is dependent upon the spc1 MAPK pathway, while PKA inhibits this binding. UAS2 resembles the Saccharomyces cerevisiae stress response element (STRE) that is bound by transcriptional activators Msnt2p and Msnt4p (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). The UAS2 sequence also resembles the binding site for the zinc finger glucose repressors Mig1p, Mig2p, and Nrg1p (Lutfyya and Johnston 1996; Lutfyya et al. 1998; Wu and Trumbly 1998; Park et al. 1999). Both PKA and MAPK pathways regulate UAS2-binding activities; however, atf1 is not present in the UAS2-specific protein-DNA complexes (Neely and Hoffman 2000). Thus, the MAPK and PKA pathways regulate fbp1 transcription at both UAS1 and UAS2, but these interactions involve different mechanisms at each site.

Here, we describe a screen designed to identify downstream targets of PKA by selecting for genes that, when overexpressed, partially suppress the loss of fbp1 derepression due to high PKA activity in a strain carrying a mutation in the PKA regulatory subunit gene ena1. This screen led to the identification of a pair of redundant corepressors of fbp1 transcription, tup11 and tup12, that resemble the S. cerevisiae Tup1p global corepressor. We show that in the absence of tup11 and tup12-mediated repression, atf1 remains a key activator of fbp1 transcription, while atf1 activation by the MAPK pathway is no longer required for atf1-dependent transcriptional activation. We also present evidence that increased PKA activity represses fbp1 transcription independent of tup11- and tup12-mediated action. Finally, the screen led to the identification of the CCAAT-binding factor (CBF) as a positive regulator of fbp1 transcription, although it is unclear whether or not CBF acts directly at the fbp1 promoter or is a direct target of PKA.

MATERIALS AND METHODS

Yeast strains and growth media: S. pombe strains used in this study are listed in Table 1. Distinct nomenclature rules for S. cerevisiae vs. S. pombe proteins are used such that the S. cerevisiae Tup1p protein is referred to as Tup1p, while the S. pombe tup12 protein is referred to as tup12. The ura4::fbp1-lacZ allele is a disruption of the ura4 gene by a fbp1-lacZ translational fusion (Hoffman and Winston 1990) and includes ∼1.5 kb of sequence 5′ to the fbp1 transcriptional start site. The ura4::fbp1 (∆-429 to -179)-lacZ and ura4::fbp1 (∆-1399 to -336)-lacZ alleles carry overlapping deletions of the fbp1 promoter driving expression of the fbp1-lacZ fusion (Neely and Hoffman 2000). Defined pombe medium (PM; Watanabe et al. 1988) and standard rich yeast extract medium (YEL; Gutz et al. 1974) containing 8% glucose (repressing conditions), 3% glucose (standard conditions), or 0.1% glucose plus 5% glycerol (derepressing conditions) were used to culture the cells. PM media were supplemented with 75 mg/liter of required nutrients, except for leucine, which was present at 150 mg/ml. Yeast strains were grown at 30°C unless indicated otherwise.

Library screen for regulators of fbp1 transcription: Strain JSP227 (ena1-180) was transformed to Leu+ on PM-Leu with either of two S. pombe cDNA libraries. These libraries contain size-selected cDNAs expressed from the constitutive adh promoter in vector pLEY3 that utilizes the S. cerevisiae LEU2 selectable marker, and the S. pombe adh promoter and actin terminator (H. Prentice and R. Kingston, unpublished data). (Library SPLE-1 contains ∼6 × 106 clones, 67% of which carry inserts of 1 kb or more. Library SPLE-2 contains ∼1.5 × 106 clones, 94% of which carry inserts of 1.6 kb or less. RNA was used to make cDNA was prepared from an h+ prototrophic strain grown to exponential phase in YEL medium.) Transforms were replica plated to PM-Leu medium containing 3% glucose as the carbon source. Transforms that grew within 5–7 days were single colony purified, grown in glucose-rich liquid medium, and assayed for β-galactosidase activity. Transforms from transforms expressing ≥ 90 units of β-galactosidase activity were rescued into Escherichia coli (Hoffman and Winston 1987). Plasmid DNA was sequenced at the Beth Israel Deaconess Medical Center Sequencing Facility (Boston) using oligonucleotide adh-forward 5′ CATTGGTC TCCCGTCCCG 3′ to sequence from the adh promoter into the 5′ end of the cDNA.

Deletion of tup11 and tup12 genes: The tup11 ORF (SPAC18B11.10, accession no. Z50728) was disrupted using a PCR-based approach as described previously by Bahler et al. (1998). Oligonucleotides 5′ KO (5′ ACAAGTATTACCTTGCACACA ATTCAGGTGTGCAATTGTTGTA AAAAAGGCGATATACA ATCCGATTCTGATTTTTGCAATAAGTCTGAGCCTTAGCT ACAATTCCACG 3′) and 3′ KO (5′ ATCAATGCGCCTTTTCTATTTTCCAGAGAAGTTTATGGAATAGCCGG AAAAAATCAGAGGAGTAGGCGGTAGTCAATGCATATGGGT GCTTCGACATAAAAACCTCTTGG 3′) were used to PCR amplify a 1.6-kb ura4-k-containing fragment from pREP42. The amplified fragment was used to transform S. pombe strain NT5 to Ura−, creating strain SW53. Integration at the tup11+ locus, which was confirmed by PCR analysis and Southern blotting, resulted in the replacement of tup11+ sequence from −10 to +2140 relative to the tup11 start codon with the ura4+ cassette. The tup12 ORF (formerly tup1, accession no. U92792; also SPAC650.14c, accession no. AL109832) was disrupted by inserting the ura4+ gene on an SpbI fragment (made blunt by Klenow fill-in) from plasmid pURA4-A (obtained from Dr. Robert Boorer) into the tup12 open reading frame. The SpbI fragment replaced a BglII-BsaI fragment that carried codons 179–548 on plasmid pBB306-5-1 to create plasmid pBB312-B1. A linear DNA fragment carrying the ura4+ gene flanked by tup12 sequences was used to transform strain SP826 to Ura−, creating strain BSP01.

β-Galactosidase assays: Strains were cultured overnight under repressing conditions (8% glucose) in YEL medium. Cells were washed twice with sterile water and resuspended into YEL medium under repressing or derepressing conditions (0.1% glucose supplemented with 5% glycerol). Cultures were grown overnight to a final cell density of ∼1 × 106 cells/ml. Protein lyases were prepared on ice and assayed for β-galactosidase activity as described previously (Nocero et al. 1994).
Spot tests: Strains were grown in YEL (3% glucose) to exponential phase, counted, washed twice with water, and adjusted to 5 x 10^6 cells/ml in water. A total of 0.24 ml of cells was transferred to a microtiter dish. Four-fold serial dilutions were performed to produce samples of 0.2 ml each. These cultures were spotted to YEA (5% glucose at 30° and 37°), PM (5% glucose), YE A + 1 m KCl, YEA (5% glycerol), and YEA (3% glucose) media using a microplate replicator.

Conjugation assay: Cells were cultured overnight to exponential phase at 37° in YEL medium (8% glucose), diluted to 1 x 10^6 cells/ml in YEL (8% glucose) in the presence or absence of 5 mM cAMP, and grown overnight at 30° without shaking, before photographing.

RESULTS

cDNA library screen for suppressors of a cgs1 mutant allele: To investigate how the PKA and MAPK pathways interact to regulate fbp1 transcription, we screened for genes encoding regulatory factors that may be targets of PKA. Strain JSP227 (cgs1-180) fails to derepress fbp1 transcription and utilize gluconate as a carbon source, presumably due to PKA repression of gluconate uptake (Caspari 1997; J. Stiefel and C. S. Hoffman, unpublished results). To screen for multicopy suppressors that alleviate these cgs1-180 conferred phenotypes, JSP227 was transformed to Leu^+ with either of two adh-driven cDNA libraries (H. Prentice and R. Kingston, personal communication; see MATERIALS and METHODS). Of 250,000 transformants, 118 displayed growth on a gluconate-based medium and were screened further by assaying β-galactosidase expressed from an integrated fbp1-lacZ reporter. A total of 23 transformants expressed >90 units of β-galactosidase activity in glucose-grown cells, as compared with ~9 units for empty vector pLEV3 transformants. Sequence analysis of these clones revealed that two express tup12, a homolog of the S. cerevisiae Tup1p corepressor protein (Keleher et al. 1992;
Figure 1.—The amino acid sequence alignment of the *S. pombe* *tup11* and *tup12* proteins and the *S. cerevisiae* Tup1p corepressor. The *tup12* protein (accession no. T38992) was aligned with the *tup11* (accession no. CAA90594) and Tup1p (accession no. NP_010007) proteins using the Clustal W (version 1.8) sequence alignment program (Thompson et al. 1994) and displayed using BOXSHADE. Identical residues are boxed in black with white letters, while conserved residues are boxed in gray with black letters. The first residues of each of the truncated *tup12* proteins encoded by the cDNA library plasmids *ptup12a* and *ptup12b* are indicated by arrows. The Ssn6p-protein-binding domain and WD repeats of the Tup1p protein are indicated beneath the sequence.

**TABLE 2**

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<th>Derepressed</th>
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<td>1586 ± 83</td>
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<td><em>scr1Δ</em></td>
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β-Galactosidase activity was determined from six to eight independent cultures as described in MATERIALS AND METHODS. The average ± SE represents specific activity per milligram of soluble protein.

*S. pombe* *tup11* and *tup12* are redundant negative regulators of *fbp1* transcription: While our screen was designed to identify *fbp1* transcriptional activators, *tup12*, along with *tup11*, is homologous to the *S. cerevisiae* Tup1p global corepressor. Tup1p, *tup11*, and *tup12* are highly conserved in the two functional domains of Tup1p, an amino-terminal Ssn6p-binding domain and a carboxy-terminal WD repeat domain (Figure 1). Deletion of *tup11* or *tup12* alone derepresses *fbp1* transcription slightly, while deletion of both genes causes a 100-fold increase in *fbp1-lacZ* expression in glucose-grown cells (Table 2), demonstrating that these genes act as redundant negative regulators. While these results seem inconsistent with the multicopy effect of *tup12* in increasing *fbp1* transcription, both *tup12*-containing clones obtained in this screen lack some of the coding region for the Ssn6p-binding domain (Figure 1). The insert in *ptup12a* lacks 33 codons of this region, while
the insert in ptp12b lacks 46 codons. We therefore presume that these truncated clones are acting as weak dominant negative alleles in our screen. Consistent with this hypothesis, transformation of a tup11Δ tup12Δ double mutant with the ptp12a plasmid has no effect (Table 3). However, the effect of ptp12a is restricted to strains that have high PKA activity due to a mutation in the cgs1 gene and low cAMP levels, either due to the deletion of the gtl adenylate cyclase gene (as was the case in the original screen) or due to growth under derepressing conditions (strain RJP25, Table 3). There is no effect of introducing ptp12a into either a wild-type strain or a strain carrying a deletion of the pka1 gene that encodes the catalytic subunit of PKA (Table 3; Maeda et al. 1994).

<table>
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<td>CHP490</td>
<td>pka1Δ</td>
<td>3612 ± 62</td>
<td>2091 ± 80</td>
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β-Galactosidase activity was determined from three to four independent cultures as described in MATERIALS AND METHODS. The average ± SE represents specific activity per milligram of soluble protein.

### TABLE 3

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<th>Strain</th>
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<td>2667 ± 325</td>
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<td>RJP25</td>
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<td>CHP490</td>
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<td>4064 ± 118</td>
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### TABLE 4

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<td>1586 ± 83</td>
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<td>RJP41</td>
<td>cgs1Δ tup11Δ tup12Δ</td>
<td>72 ± 2</td>
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β-Galactosidase activity was determined from four to six independent cultures as described in MATERIALS AND METHODS. The average ± SE represents specific activity per milligram of soluble protein.
subunit, reduces fbp1-lacZ expression in a tup11Δ tup12Δ strain (Table 4). Thus, PKA repression does not appear to operate through the stimulation of tup11 and tup12 activity.

**Other phenotypes associated with the tup11Δ tup12Δ double deletion:** Strains carrying the tup1Δ tup12Δ double deletion display other phenotypes in addition to the derepression of fbp1-lacZ expression. Similar to S. cerevisiae tup1Δ strains (Lipke and Hull-Pillsbury 1984), S. pombe tup11Δ tup12Δ strains are extremely flocculent growing as large aggregates in liquid culture (data not shown). In addition, these strains grow poorly on PM defined medium. Finally, tup11Δ tup12Δ strains display an osmotic-sensitive phenotype (Figure 2), although these cells do not have the highly elongated morphology of wis1Δ cells grown under similar conditions (data not shown).

In contrast to the effect on fbp1-lacZ expression, deletion of tup11 and tup12 does not suppress the temperature- or osmotic-sensitive growth conferred by a wis1 deletion (Figure 2). In fact, while a tup11Δ tup12Δ double deletion strain does not have an obvious effect on growth at 37°C, deletion of the tup genes enhances the temperature-sensitive phenotype conferred by a wis1 deletion (Figure 2). The tup1Δ tup12Δ wis1Δ triple-deletion strain displays a synthetic sickness leading to extremely poor growth on YE medium and an almost total loss of growth on defined PM medium (Figure 2). Finally, the tup11Δ tup12Δ double deletion suppresses the wis1Δ conferred defect in the utilization of glycerol, but not gluconate, as a carbon source.

There is a general correlation between the control of fbp1 transcription and of conjugation and sporulation since both processes are regulated by nutrient conditions. Mutations that reduce PKA activation both inhibit glucose repression of fbp1 transcription and allow cells to mate and sporulate in nutrient-rich medium (Maeda et al. 1990, 1994; Isshiki et al. 1992; Landry et al. 2000; Welton and Hoffman 2000). We therefore examined the effect of the tup11Δ tup12Δ double deletion in a homothallic h0 strain (such strains undergo mating-type switching to create mating partners within a purified culture) with respect to regulation of conjugation and sporulation. Wild-type h0 cells grow vegetatively as long as they do not receive a glucose or nitrogen starvation signal (Figure 3, A and B). An h0 git2Δ (adenylate cyclase) strain will grow vegetatively at 37°C (data not shown), but will efficiently conjugate and sporulate upon shifting to 30°C, even in nutrient-rich conditions (Figure 3C). This unregulated sexual development is blocked by the addition of 5 mM cAMP to the medium (Figure 3D). The h0 tup11Δ tup12Δ strain displays a unique set of phenotypes. As with the git2Δ strain, little or no conjugation is seen in cells grown at 37°C (data not shown). Upon shifting to 30°C, the h0 tup11Δ tup12Δ cells conjugate to form zygotes in the presence or absence of cAMP (Figure 3, E and F). However, most of these zygotes appear to be blocked in meiosis, failing to form four-spored asci. Thus, while deletion of tup11 and tup12 creates a defect in repression of both fbp1 transcription and conjugation, it is not a phenocopy of mutations that inhibit PKA activation.

**Cloning of the CCAAT box binding factor gene php5:** A third clone identified from the cDNA library screen encodes php5, a component of the S. pombe CBF (McNabb et al. 1997). A php5 deletion decreases fbp1-lacZ expression under both repressed and derepressed conditions, although the fold regulation is not reduced (Table 5). A php2 deletion, affecting a second CBF subunit, causes a similar reduction in fbp1-lacZ expression (data not shown). The php5 deletion reduces fbp1-lacZ expression in tup11Δ tup12Δ and in pka1Δ strains that are defective in glucose repression (Table 5). Finally, a php5Δ atf1Δ strain is completely defective in fbp1 derepression, suggesting that atf1-pcr1 and CBF work in parallel to activate fbp1 transcription (Table 5).

**Effect of tup11Δ tup12Δ and php5Δ on fbp1 promoter derivatives:** To study whether tup11 and tup12 or CBF act at a unique site within the fbp1 promoter, we examined the effect of the tup11Δ tup12Δ double deletion and the php5Δ deletion on lacZ expression from a pair of fbp1 promoter variants that represent two overlapping deletions covering >1.2 kb of the fbp1 promoter (Neely and Hoffman 2000). The fbp1 (Δ-429 to -179) promoter variant lacks UAS2, but contains UAS1, which is the binding site for the atf1-pcr1 activator (Neely and Hoffman 2000). The fbp1 (Δ-1399 to -336) promoter variant lacks UAS1, but contains UAS2 that includes a
Figure 3.—Starvation-independent sexual development in a tup11Δ tup12Δ homothallic strain. Homothallic strains 968 (wild type, A and B), CHP-558 (git2 Δ, C and D), and RJP59 (tup11Δ tup12Δ, E and F) were grown to exponential phase in YEL medium (at 37°C to inhibit conjugation) and then diluted to 10^6 cells/ml in YEL in the absence (A, C, and E) or presence (B, D, and F) of 5 mM cAMP. These cells were incubated for 24 hr at 30°C without shaking, and were then photographed.

site resembling both an S. cerevisiae stress response element (STRE; Estruch and Carlson 1993; Martinez-Pastor et al. 1996; Schmitt and McEntee 1996) and the binding site for the S. cerevisiae glucose repressors Mig1p, Mig2p, and Nrg1p (Nehlin and Ronne 1990; Trumbly 1992; Lundin et al. 1994; Park et al. 1999). Both of the fbp1-lacZ promoter derivatives are derepressed by the tup11Δ tup12Δ double deletion (Table 6), indicating that there is not a unique site of action for tup11 and tup12 within these deletion intervals (see Discussion). Similarly, transcriptional derepression by glucose starvation of both constructs is reduced by the php5 deletion (Table 6), although the fold reduction is significantly greater for the UAS2-driven [fbp1 (Δ-1399 to -336)] promoter than for the UAS1-driven [fbp1 (Δ-429 to -179)] promoter. As with the tup11Δ tup12Δ data, these results suggest that the CFB does not act at a unique site within these deletion intervals, although CBF appears to be essential for UAS2-driven transcription (Table 6).

**DISCUSSION**

Transcription of the S. pombe fbp1 gene is regulated by the activity of both a negatively acting PKA pathway that is activated by glucose and a positively acting MAPK pathway that is activated by glucose starvation (Hoffman and Winston 1990, 1991; Byrne and Hoffman 1993; Takeda et al. 1995; Dal Santo et al. 1996; Stettler et al. 1996). These pathways regulate transcriptional activation from at least two positively acting elements, UAS1 and UAS2, through multiple mechanisms (Neely and Hoffman 2000). We previously showed both direct and indirect roles for the transcriptional activator atf1-pcr1 (Neely and Hoffman 2000). In this study, we identify a pair of redundant negative regula-

**TABLE 5**

<table>
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<th>Strain</th>
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<th>β-Galactosidase activity</th>
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<td>RJP67</td>
<td>atf1Δ</td>
<td>8 ± 0</td>
</tr>
<tr>
<td>RJP59</td>
<td>atf1Δ php5Δ</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

β-Galactosidase activity was determined from four to six independent cultures as described in MATERIALS AND METHODS. The average ± SE represents specific activity per milligram of soluble protein. ND, not determined.
tors, tup11 and tup12, along with a second activation complex, CBF, that works in parallel to atf1-pcr1.

The tup11 and tup12 proteins are homologous to the *S. cerevisiae* Tup1p protein, a WD repeat protein involved in transcriptional repression. Tup1p is physically associated to Ssn6p/Cyc8p and this complex negatively regulates the transcription of many diversely regulated genes (Williams et al. 1991; Keleher et al. 1992; Tzamarias and Struhl 1994). The N-terminal region of Ssn6p consists of 10 degenerate repeats of the (tetratrico peptide repeat TPR) motif (Schultz et al. 1990). Some of the TPR repeats interact with Tup1p while others mediate recruitment of the Tup1p-Ssn6p complex to different promoters through interactions with structurally different DNA-bound repressor proteins (Tzamarias and Struhl 1995; Smith and Johnson 2000).

Mutations in *TUP1* or *SSN6* cause derepression of several genes, including genes regulated by cell type (Mukai et al. 1991; Keleher et al. 1992), glucose (Schultz and Carlson 1987; Trumbly 1992), oxygen (Zitomer and Lowry 1992), osmotic stress (Marquez et al. 1998; Proft and Serrano 1999), or DNA damage (Elledge et al. 1993). The Tup1p-Ssn6p complex appears to work via two distinct mechanisms. It is able to bind the RNA polymerase II holoenzyme (Wahi et al. 1998; Papamichos-Chronakis et al. 2000). Tup1p also binds monoaetylated or unacetylated histones H3 and H4 in vitro (Edmondson et al. 1996, 1998), forming a hairpin structure containing 10 nucleosomes encompassing the STE6 gene (Ducker and Simpson 2000). Thus, Tup1p-Ssn6p may initially interact with the basal transcriptional machinery to inhibit transcription. This repressed state may be maintained by Tup1p-Ssn6p binding to histones H3 and H4 to alter the chromatin structure, making these regulatory elements less accessible to transcriptional activators.

We have shown that tup11 and tup12 act as redundant regulators of *fbp1* transcription (Table 2), which is similar to the observations of Mukai et al. (1999). In addition, genetic interactions between an *scr1* deletion and deletions in either *tup11* or *tup12* suggest that *scr1* acts in concert with *tup11* and *tup12* (Table 2). However, as the loss of *scr1* is not equivalent to the combined loss of *tup11* and *tup12*, other proteins may recruit *tup11* and *tup12* to the *fbp1* promoter. Several *S. pombe* proteins contain a pair of *scr1*-like zinc fingers that could allow them to bind similar DNA sequences (Neely and Hoffman 2000). As both UAS1- and UAS2-driven *fbp1* promoter derivatives are derepressed by the *tup11Δ tup12Δ* double deletion (Table 6), *tup11* and *tup12* may act through multiple sites in the *fbp1* promoter. Alternatively, a unique site of *tup11* and *tup12* recruitment may lie outside of the deletion intervals, from base pairs −1508 to −1400 or downstream from −178.

As the loss of *tup11* and *tup12* derepresses *fbp1* transcription, one might infer that *tup11* and *tup12* activity is negatively regulated by the MAPK pathway and/or positively regulated by the PKA pathway. However, such models cannot fully explain the roles of *tup11*, *tup12*, and the signaling pathways. In the absence of *tup11* and *tup12*, the *atf1* and *pcr1* proteins remain key activators of *fbp1* transcription (Table 4). Therefore, *atf1-pcr1* is not simply a negative regulator of *tup11* and *tup12* activity. In addition, since an *atf1* deletion causes a greater reduction in *fbp1-lacZ* expression than does a *per1* deletion, *atf1* appears to function, albeit less effectively, as either a homodimer or as a heterodimer with other bZIP proteins such as *atf21* (Shiozaki and Russell 1996). Conversely, since *fbp1-lacZ* expression in a *tup11Δ tup12Δ* strain is reduced by PKA activation due to a *cgs1* deletion (Table 4), *PKA* must be able to repress *fbp1* transcription independently of *tup11* and *tup12* action. While these data demonstrate that the MAPK and PKA pathways continue to regulate *fbp1* expression in the absence of *tup11* and *tup12*, we cannot discount the possibility that one of the effects of signaling from these pathways in a wild-type cell is to regulate *tup11* and *tup12*-mediated repression.

Surprisingly, a deletion of the *wis1* MAPKK gene has

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**TABLE 6**

Effects of *tup11Δ tup12Δ* and *php5Δ* deletions on *fbp1* promoter variants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Promoter</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Repressed</td>
</tr>
<tr>
<td>FWP112</td>
<td>Wild type</td>
<td><em>fbp1</em>^+</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>LAN6P</td>
<td>Wild type</td>
<td><em>fbp1</em> (Δ-429 to -179)</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>RJP57</td>
<td><em>tup11Δ tup12Δ</em></td>
<td><em>fbp1</em> (Δ-429 to -179)</td>
<td>528 ± 62</td>
</tr>
<tr>
<td>RJP80</td>
<td><em>php5Δ</em></td>
<td><em>fbp1</em> (Δ-429 to -179)</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>LAN170</td>
<td>Wild type</td>
<td><em>fbp1</em> (Δ-1399 to -336)</td>
<td>17 ± 0</td>
</tr>
<tr>
<td>RJP55</td>
<td><em>tup11Δ tup12Δ</em></td>
<td><em>fbp1</em> (Δ-1399 to -336)</td>
<td>441 ± 15</td>
</tr>
<tr>
<td>RJP82</td>
<td><em>php5Δ</em></td>
<td><em>fbp1</em> (Δ-1399 to -336)</td>
<td>2 ± 0</td>
</tr>
</tbody>
</table>

β-Galactosidase activity was determined from two to four independent cultures as described in MATERIALS AND METHODS. The average ± SE represents specific activity per milligram of soluble protein. The *fbp1*^+* promoter contains sequences from −1508 to +284 relative to the *fbp1* transcriptional start site. ND, not determined.

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As the loss of *tup11* and *tup12* derepresses *fbp1* transcription, one might infer that *tup11* and *tup12* activity is negatively regulated by the MAPK pathway and/or positively regulated by the PKA pathway. However, such models cannot fully explain the roles of *tup11*, *tup12*, and the signaling pathways. In the absence of *tup11* and *tup12*, the *atf1* and *pcr1* proteins remain key activators of *fbp1* transcription (Table 4). Therefore, *atf1-pcr1* is not simply a negative regulator of *tup11* and *tup12* activity. In addition, since an *atf1* deletion causes a greater reduction in *fbp1-lacZ* expression than does a *per1* deletion, *atf1* appears to function, albeit less effectively, as either a homodimer or as a heterodimer with other bZIP proteins such as *atf21* (Shiozaki and Russell 1996). Conversely, since *fbp1-lacZ* expression in a *tup11Δ tup12Δ* strain is reduced by PKA activation due to a *cgs1* deletion (Table 4), *PKA* must be able to repress *fbp1* transcription independently of *tup11* and *tup12* action. While these data demonstrate that the MAPK and PKA pathways continue to regulate *fbp1* expression in the absence of *tup11* and *tup12*, we cannot discount the possibility that one of the effects of signaling from these pathways in a wild-type cell is to regulate *tup11*- and *tup12*-mediated repression.

Surprisingly, a deletion of the *wis1* MAPKK gene has
little effect on fbp1-lacZ expression in a tup11Δ tup12Δ double deletion strain (Table 4). The discrepancy between the effect of an atf1 deletion and a wis1 deletion suggests that in the absence of the tup11- and tup12-mediated repression, phosphorylation of atf1 by the MAPK pathway is not required for atf1 activity. Alternatively, the MAPK pathway may regulate tup11- and tup12-mediated repression in an atf1-independent manner. While some studies indicate that atf1 binding to DNA is independent of phosphorylation (Wilkinson et al. 1996; Degols and Russell 1997), our study of fbp1 UAS1 binding in vitro suggests that phosphorylation by the MAPK pathway increases the binding while phosphorylation by the PKA pathway inhibits binding (Neely and Hoffman 2000). (The conclusions from these various studies do not necessarily conflict as they may represent different requirements for binding of atf1-pecr1 to nonequivalent cis-acting elements.) Given the known mechanisms of Tup1p-mediated repression in S. cerevisiae, tup11 and tup12 may modify the chromatin at the fbp1 promoter such that the spc1 MAPK must phosphorylate atf1 for it to bind UAS1. Alternatively, or additionally, tup11 and tup12 could interfere with transcriptional activation by atf1-pecr1 in a manner that is sensitive to the phosphorylation state of atf1.

We have also shown here that S. pombe CBF plays an important role in fbp1 transcriptional activation. CBF is a multimeric DNA-binding complex that binds to promoter elements containing the CCAAT sequence. In S. cerevisiae, CBF contains four subunits, Hap2p, Hap3p, Hap4p, and Hap5p, that are required for the transcriptional activation of nuclear genes whose products are involved in mitochondrial functions (Olesen et al. 1987; Forsburg and Guarente 1989; McNabb et al. 1995). Disruption of S. pombe homologs to HAP2 and HAP5, php2 and php5, was shown to confer defects in growth on glycerol-containing medium, presumably due to a defect in respiration (Olesen et al. 1991; McNabb et al. 1997). However, reduced fbp1 expression would also contribute to a loss of glycerol utilization (Table 5).

While php5 was identified in a screen designed to identify PKA targets, it is unclear whether CBF is regulated by PKA or acts as a basal factor in fbp1 transcription. A php5 deletion reduces fbp1 transcription in cells grown in either repressed or derepressed conditions, but has little effect on the ratio of the two levels of expression (Table 5). It is also unclear whether the CBF is acting directly at the fbp1 promoter or acts indirectly by regulating expression of other transcriptional activators. We have not been able to identify a unique site of CBF action, although it appears to be essential for UAS2-driven transcription (Table 6). This result is consistent with the dramatic loss of fbp1-lacZ expression from the full-length promoter in an atf1Δ php5Δ strain (Table 5), as we have previously shown that atf1 is required for UAS1-driven transcription.

The results described here share a common theme with those of our earlier study identifying UAS1 and UAS2 of the fbp1 promoter (Neely and Hoffman 2000), namely that individual regulatory components play multiple roles in fbp1 transcriptional regulation. This may explain our inability to identify unique sites of action of tup11 and tup12 or of CBF within the fbp1 promoter. Such multiplicity of action may also explain the wide range over which fbp1 transcript levels vary with respect to carbon source conditions. Genes whose promoters are less intricately “wired” could be subject to regulation by the same signaling pathways and yet have transcript levels vary by only a few fold. This allows a single input signal that feeds into a finite number of signaling pathways to produce a wide range of transcriptional outputs from different genes.

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