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 *adh*-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 (MAPKKK; Samejima *et al.* 1997, 1998; Shieh *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

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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 Msn2p and Msn4p (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 (Lutfiyya and Johnston 1996; Lutfiyya *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 *cgs1*. 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* Tup1 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 (*Δ*-429* to *-179)-lacZ* and *ura4::fbp1 (*Δ*-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 3% 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° unless indicated otherwise.

Library screen for regulators of *fbp1* transcription: Strain JSP227 (*cgs1-180*) 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 pLEV3 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 $\sim 6 \times 10^5$ clones, 67% of which carry inserts of 1 kb or more. Library SPLE-2 contains $\sim 1.5 \times 10^5$ clones, 94% of which carry inserts of 1.6 kb or less. RNA used to make cDNA was prepared from an h^- prototrophic strain grown to exponential phase in YEL medium.) Transformants were replica plated to PM-Leu medium containing 3% gluconate as the carbon source. Transformants that grew up within 5–7 days were single colony purified, grown in glucose-rich liquid medium, and assayed for β-galactosidase activity. Plasmids from transformants 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 TTCCGCTCCG 3′ to sequence from the *adh* promoter into the 5′ end of the cDNA.

Deletion of the *tup11* and *tup12* genes: The *tup11* ORF (SPAC18B11.10, accession no. Z50728) was disrupted using a PCR-based approach as described by Bahler *et al.* (1998). Oligonucleotides 5′ KO (5′ ACAAGTTTATTCTTGTACCACA ATTCAAGTGTTGCTATTGTTGTAAAAGGGCGTATATCA ATCGGATTCAGTTTTTGCAATAAGTCTGACGCTTAGCT ACAAATCCCACT 3′) and 3′ KO (5′ ATCAATGCGGTTAA CTATTTCCGAGAGCAAATAGTTATATGTAAACAGGAAC AAAAATTCAAGGAGATGCAGGGTCAATTGACCATATGG GCTCTGACATAAAACGCCTAGG 3′) were used to PCR amplify a 1.6-kb *ura4*-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 SPAC630.14c, accession no. AL109832) was disrupted by inserting the $ura4^+$ gene on an *Sph*I fragment (made blunt by Klenow fill-in) from plasmid pURA4-A (obtained from Dr. Robert Booher) into the *tup12* open reading frame. The *Sph*I fragment replaced a *Bgl*II-*Hpa*I 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 subcultured into YEL medium under repressing or derepressing conditions (0.1% glucose supplemented with 3% glycerol). Cultures were grown overnight to a final cell density of $\sim 1 \times 10^7$ cells/ml. Protein lysates were prepared on ice and assayed for β-galactosidase activity as described previously (Nocero *et al.* 1994).

TABLE 1

Strain list

Strain	Genotype
972	*h⁻*
968	*h⁹⁰*
FWP112	*h⁻ ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366*
CHP490	*h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 pka1::ura4⁺*
CHP558	*h⁹⁰ fbp1::ura4⁺ leu1-32 ade6-M216 git2-1::LEU1⁺*
CHP720	*h⁻ leu1-32 ura4::fbp1-lacZ ade6-M210 wis1::LEU2⁺*
JSP227	*h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 his3-D1 git2-2::his7⁺ cgs1-180*
LAN6P	*h⁻ ura4::fbp1(Δ-429 to -179)-lacZ*
LAN170	*h⁻ ade6-M216 ura4::fbp1(Δ-1399 to -336)-lacZ*
RJP8	*h⁺ ura4::fbp1-lacZ leu1-32 his7-366 tup12::ura4⁺*
RJP10	*h⁻ ura4::fbp1-lacZ leu1-32 tup11::ura4⁺*
RJP12	*h⁻ ura4::fbp1-lacZ leu1-32 tup11::ura4⁺ tup12::ura4⁺*
RJP18	*h⁻ ura4::fbp1-lacZ leu1-32 php5::ura4⁺*
RJP25	*h⁺ ura4::fbp1-lacZ leu1-32 ade6-M210 cgs1::ura4⁺*
RJP31	*h⁺ ura4::fbp1-lacZ leu1-32 php5::ura4⁺ pka1::ura4⁺*
RJP33	*h⁺ ura4::fbp1-lacZ leu1-32 wis1::LEU2⁺ tup11::ura4⁺ tup12::ura4⁺*
RJP36	*h⁻ ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 atf1::ura4⁺ tup11::ura4⁺ tup12::ura4⁺*
RJP39	*h⁺ ura4::fbp1-lacZ leu1-32 adeM-216 his7-366 atf1::ura4⁺ php5::ura4⁺*
RJP41	*h⁻ ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 cgs1::ura4⁺ tup11::ura4⁺ tup12::ura4⁺*
RJP46	*h⁻ ura4::fbp1-lacZ leu1-32 ade6-M216 scr1::ura4⁺*
RJP48	*h⁺ ura4::fbp1-lacZ leu1-32 his2⁻ scr1::ura4⁺ tup11::ura4⁺*
RJP51	*h⁺ ura4::fbp1-lacZ leu1-32 his7-366 scr1::ura4⁺ tup11::ura4⁺ tup12::ura4⁺*
RJP52	*h⁹⁰ ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 tup11::ura4⁺ tup12::ura4⁺*
RJP55	*h⁺ ura4::fbp1(Δ-1399 to -336)-lacZ tup11::ura4⁺ tup12::ura4⁺*
RJP57	*h⁻ ura4::fbp1(Δ-429 to -179)-lacZ tup11::ura4⁺ tup12::ura4⁺*
RJP59	*h⁻ ura4::fbp1-lacZ leu1-32 his7-366 pcr1::ura4⁺ tup11::ura4⁺ tup12::ura4⁺*
RJP63	*h⁺ ura4::fbp1-lacZ leu1-32 his7-366 scr1::ura4⁺ tup12::ura4⁺*
RJP66	*h⁻ ura4::fbp1-lacZ leu1-32 php5::ura4⁺ tup11::ura4⁺ tup12::ura4⁺*
RJP67	*h⁺ ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366 atf1::ura4⁺*
RJP72	*h⁻ ura4::fbp1-lacZ his7-366 pka1::ura4⁺*
RJP80	*h⁻ ura4::fbp1(Δ-429 to -179)-lacZ php5::ura4⁺*
RJP82	*h⁺ ura4::fbp1(Δ-1399 to -366)-lacZ php5::ura4⁺*
NT5	*h⁻ ura4-D18 leu1-32 ade6-M216*
SW53	*h⁻ ura4-D18 leu1-32 ade6-M216 tup11::ura4⁺*
SP826	*h⁺/h⁺ ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M216/ade6-M210*
BSP01	*h⁺/h⁺ ura4-D18/ura4-D18 leu1-32/leu1-32 ade6-M216/ade6-M210 tup12⁺/tup12::ura4⁺*

Spot tests: Strains were grown in YEL (3% glucose) to exponential phase, counted, washed twice with water, and adjusted to 5×10^7 cells/ml in water. A total of 0.24 ml of cells was transferred to a microtiter dish. Four fivefold serial dilutions were performed to produce samples of 0.2 ml each. These cultures were spotted to YEA (3% glucose at 30° and 37°), PM (3% glucose), YEA + 1 M KCl, YEA (3% glycerol), and YEA (3% gluconate) media using a microplate replicator.

Conjugation assay: Cells were cultured overnight to exponential phase at 37° in YEL medium (8% glucose), diluted to 1×10^6 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;

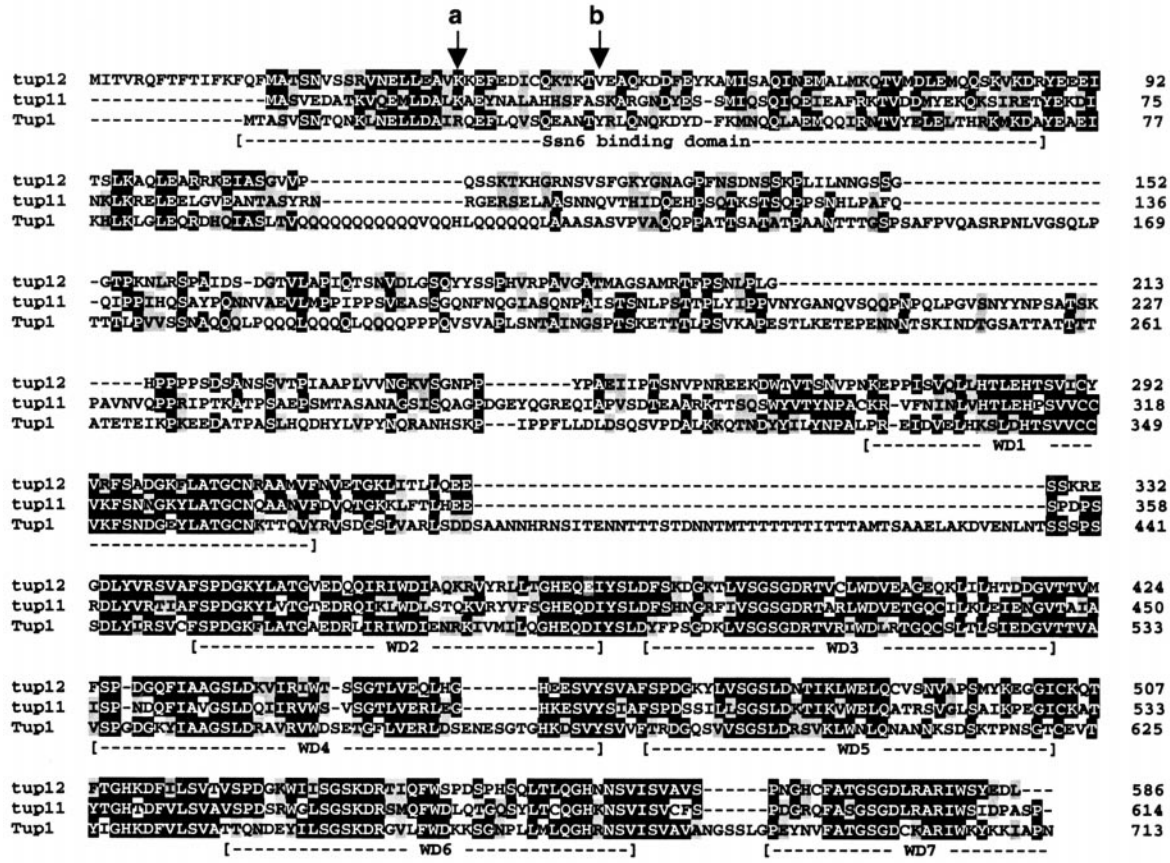

FIGURE 1.—The amino acid sequence alignment of the *S. pombe* tup11 and tup12 proteins and the *S. cerevisiae* Tup1p corepressor. The tup12 protein (accession no. T38992) was aligned with the tup11 (accession no. CAA90594) and Tup1p (accession no. NP_010007) proteins using the Clustal W (version 1.8) 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 p*tup12*a and p*tup12*b are indicated by arrows. The Ssn6p-protein-binding domain and WD repeats of the Tup1p protein are indicated beneath the sequence.

TRUMBLY 1992; MUKAI *et al.* 1999). A third clone expresses php5, a homolog of the *S. cerevisiae* Hap5p CBF subunit (MCNABB *et al.* 1995, 1997).

***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 p*tup12*a lacks 33 codons of this region, while

TABLE 2

Effects of *tup11*Δ, *tup12*Δ, and *scr1*Δ deletions on *fbp1-lacZ* expression

		β-Galactosidase activity	
Strain	Relevant genotype	Repressed	Derepressed
FWP112	Wild type	9 ± 1	1586 ± 83
RJP8	*tup12*Δ	83 ± 11	1730 ± 73
RJP10	*tup11*Δ	40 ± 3	1127 ± 54
RJP12	*tup11*Δ *tup12*Δ	956 ± 53	1780 ± 95
RJP46	*scr1*Δ	138 ± 23	1886 ± 163
RJP63	*scr1*Δ *tup12*Δ	610 ± 122	3023 ± 336
RJP48	*scr1*Δ *tup11*Δ	385 ± 59	1704 ± 129
RJP51	*scr1*Δ *tup11*Δ *tup12*Δ	743 ± 258	2441 ± 157

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.

TABLE 3

p*tup12*a partial suppression of a *cgs1* deletion

		β-Galactosidase activity			
		pLEV3 transformant		p*tup12*a transformant	
Strain	Relevant genotype	Repressed	Derepressed	Repressed	Derepressed
FWP112	Wild type	10 ± 0	1095 ± 126	15 ± 1	1218 ± 78
RJP12	*tup11*Δ *tup12*Δ	511 ± 30	2585 ± 263	602 ± 36	2667 ± 325
RJP25	*cgs1*Δ	3 ± 0	90 ± 10	3 ± 1	721 ± 233
CHP490	*pka1*Δ	3612 ± 62	2091 ± 80	4064 ± 118	2730 ± 284

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.

the insert in p*tup12*b 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 *tup11*Δ *tup12*Δ double mutant with the p*tup12*a plasmid has no effect (Table 3). However, the effect of p*tup12*a 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 *git2* 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 p*tup12*a 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).

Genetic interactions between *tup11* and *tup12*, and the *MIG1*-like *scr1* gene: *S. cerevisiae* Tup1p is brought to various promoters through interactions with DNA-binding proteins (KELEHER *et al.* 1992; TRUMBLY 1992). The most important DNA-binding partner of the Ssn6p-Tup1p corepressor with respect to glucose repression is Mig1p (NEHLIN and RONNE 1990; TRUMBLY 1992). The closest *S. pombe* homolog of Mig1p is scr1 (TANAKA *et al.* 1998). Similar to our previous results (NEELY and HOFFMAN 2000), an *scr1* deletion (*scr1*Δ) increases *fbp1-lacZ* expression in glucose-repressed cells >10-fold (Table 2). This effect is enhanced by deletion of either *tup11* or *tup12*. However, deletion of *scr1* has no effect in a strain lacking both *tup11* and *tup12*, suggesting that scr1 is a DNA-binding protein that brings tup11 and tup12 to the DNA, but that other proteins can carry out a similar function in the absence of scr1.

Genetic relationship between the *tup* genes and the PKA and MAPK pathways: To study the genetic relationship between *tup11* and *tup12*, and the PKA and MAPK pathways, the *tup11*Δ *tup12*Δ double deletion was combined with mutations affecting these signaling pathways that either increase or reduce *fbp1* transcription. Deletion of either *atf1* or *pcr1*, encoding subunits of the bZIP transcriptional activator that is activated by the spc1 MAPK, significantly reduces *fbp1-lacZ* expression (Table 4). These results are consistent with our previous data showing that the atf1-pcr1 activator directly binds one element within the *fbp1* promoter (UAS1) and is indirectly required for transcriptional activation from a second element (UAS2; NEELY and HOFFMAN 2000). On the other hand, *fbp1-lacZ* expression is only modestly reduced by a deletion of the *wis1* MAPKK gene that is normally required for activation of the atf1-pcr1 heterodimer (Table 4). Since *fbp1-lacZ* expression in *tup11*$^+$ *tup12*$^+$ strains is equally strongly reduced by deletion of either *wis1* or *atf1* (Table 4), we infer that in the absence of the tup11 and tup12 proteins, the MAPK signaling pathway is not required to activate atf1-pcr1.

Since loss of PKA activity strongly derepresses *fbp1* transcription, it is possible that PKA repression operates through tup11 and tup12, leading to derepression when either system is absent. The work of STETTLER *et al.* (1996), however, shows that constitutive expression due to lack of PKA is dependent on *wis1*, contrary to what we see in the *tup11*Δ *tup12*Δ mutant cells (Table 4). Likewise, deletion of *cgs1*, encoding the PKA regulatory

TABLE 4

Genetic interactions between the *tup12*Δ *tup11*Δ double deletion and mutations in the MAPK or PKA pathways

		β-Galactosidase activity	
Strain	Relevant genotype	Repressed	Derepressed
FWP112	Wild type	9 ± 1	1586 ± 83
RJP12	*tup11*Δ *tup12*Δ	956 ± 53	1780 ± 95
RJP67	*atf1*Δ	8 ± 0	147 ± 20
CHP720	*wis1*Δ	8 ± 0	79 ± 9
RJP36	*atf1*Δ *tup11*Δ *tup12*Δ	105 ± 11	430 ± 28
RJP59	*pcr1*Δ *tup11*Δ *tup12*Δ	370 ± 20	778 ± 147
RJP33	*wis1*Δ *tup11*Δ *tup12*Δ	572 ± 6	1140 ± 101
RJP41	*cgs1*Δ *tup11*Δ *tup12*Δ	72 ± 2	279 ± 7

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.

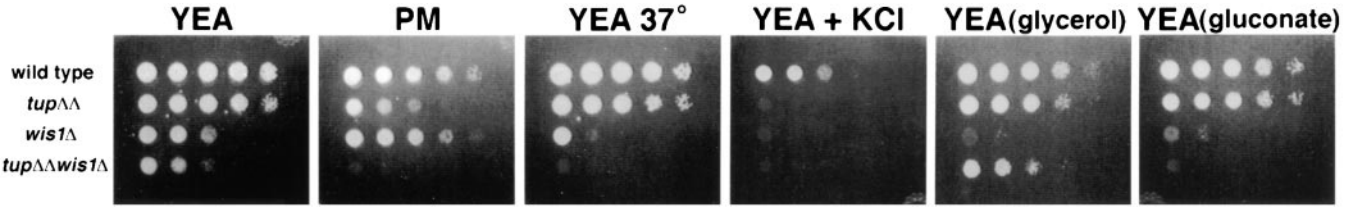

FIGURE 2.—Growth characteristics conferred by deletion of *tup11* and *tup12*. Strains 972 (wild type), RJP12 (*tup11*Δ *tup12*Δ, designated *tup*ΔΔ), CHP720 (*wis1*Δ), and RJP33 (*tup11*Δ *tup12*Δ *wis1*Δ, designated *wis1*Δ *tup*ΔΔ) were scored for their growth characteristics by spot test onto various media as indicated. Cells spotted to YEA (at 30°), PM, YEA (at 37°), and YEA + 1 M KCl were photographed after 2 days growth. Cells spotted to YEA (3% glycerol) and YEA (3% gluconate) were photographed after 5 days growth.

subunit, reduces *fbp1-lacZ* expression in a *tup11*Δ *tup12*Δ strain (Table 4). Thus, PKA repression does not appear to operate through the stimulation of tup11 and tup12 activity.

Other phenotypes associated with the *tup11*Δ *tup12*Δ double deletion: Strains carrying the *tup11*Δ *tup12*Δ double deletion display other phenotypes in addition to the derepression of *fbp1-lacZ* expression. Similar to *S. cerevisiae tup1*Δ strains (LIPKE and HULL-PILLSBURY 1984), *S. pombe tup11*Δ *tup12*Δ 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, *tup11*Δ *tup12*Δ strains display an osmotic-sensitive phenotype (Figure 2), although these cells do not have the highly elongated morphology of *wis1*Δ 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 *tup11*Δ *tup12*Δ double deletion strain does not have an obvious effect on growth at 37°, deletion of the *tup* genes enhances the temperature-sensitive phenotype conferred by a *wis1* deletion (Figure 2). The *tup11*Δ *tup12*Δ *wis1*Δ triple-deletion strain displays a synthetic sickness leading to extremely poor growth on YEA medium and an almost total loss of growth on defined PM medium (Figure 2). Finally, the *tup11*Δ *tup12*Δ double deletion suppresses the *wis1*Δ-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 *tup11*Δ *tup12*Δ double deletion in a homothallic h^{90} 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 h^{90} cells grow vegetatively as long as they do not receive a glucose or nitrogen starvation signal (Figure 3, A and B). An h^{90} *git2*Δ (adenylate cyclase) strain will grow vegetatively at 37° (data not shown), but will efficiently conjugate and sporulate upon shifting to 30°, 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 h^{90} *tup11*Δ *tup12*Δ strain displays a unique set of phenotypes. As with the *git2*Δ strain, little or no conjugation is seen in cells grown at 37° (data not shown). Upon shifting to 30°, the h^{90} *tup11*Δ *tup12*Δ 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 *tup11*Δ *tup12*Δ and in *pka1*Δ strains that are defective in glucose repression (Table 5). Finally, a *php5*Δ *atf1*Δ strain is completely defective in *fbp1* derepression, suggesting that atf1-pcr1 and CBF work in parallel to activate *fbp1* transcription (Table 5).

Effect of *tup11*Δ *tup12*Δ and *php5*Δ 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 *tup11*Δ *tup12*Δ double deletion and the *php5*Δ 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

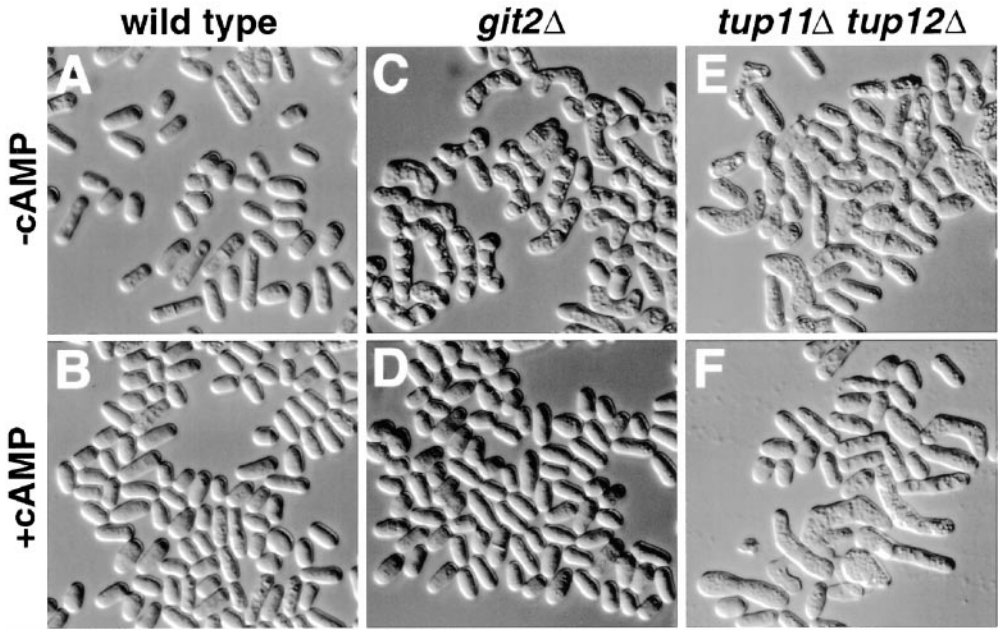

FIGURE 3.—Starvation-independent sexual development in a *tup11*Δ *tup12*Δ homothallic strain. Homothallic strains 968 (wild type, A and B), CHP-558 (*git2*Δ, C and D), and RJP52 (*tup11*Δ *tup12*Δ, E and F) were grown to exponential phase in YEL medium (at 37° to inhibit conjugation) and then diluted 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° 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 *tup11*Δ *tup12*Δ 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 *tup11*Δ *tup12*Δ 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

***S. pombe php5* is important for *fbp1* transcription**

Strain	Relevant genotype	β-Galactosidase activity	
		Repressed	Derepressed
FWP112	Wild type	9 ± 1	1586 ± 83
RJP18	*php5*Δ	3 ± 0	690 ± 95
RJP12	*tup11*Δ *tup12*Δ	956 ± 53	1780 ± 95
RJP66	*tup11*Δ *tup12*Δ *php5*Δ	110 ± 5	483 ± 18
RJP72	*pka1*Δ	1864 ± 114	ND
RJP31	*pka1*Δ *php5*Δ	1264 ± 83	ND
RJP67	*atf1*Δ	8 ± 0	147 ± 20
RJP39	*atf1*Δ *php5*Δ	1 ± 0	11 ± 0

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.

TABLE 6

Effects of *tup11*Δ *tup12*Δ and *php5*Δ deletions on *fbp1* promoter variants

Strain	Relevant genotype	Promoter	β-Galactosidase activity	
			Repressed	Derepressed
FWP112	Wild type	$fbp1^+$	9 ± 1	1586 ± 83
LAN6P	Wild type	*fbp1* (Δ-429 to -179)	18 ± 1	916 ± 12
RJP57	*tup11*Δ *tup12*Δ	*fbp1* (Δ-429 to -179)	528 ± 62	ND
RJP80	*php5*Δ	*fbp1* (Δ-429 to -179)	6 ± 1	221 ± 27
LAN170	Wild type	*fbp1* (Δ-1399 to -336)	17 ± 0	559 ± 19
RJP55	*tup11*Δ *tup12*Δ	*fbp1* (Δ-1399 to -336)	441 ± 15	ND
RJP82	*php5*Δ	*fbp1* (Δ-1399 to -336)	2 ± 0	9 ± 0

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.

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 monoacetylated 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 *tup11*Δ *tup12*Δ 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 *pcr1* 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 *tup11*Δ *tup12*Δ 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 *tup11*Δ *tup12*Δ 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 atf-pcr1 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-pcr1 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 *atf1*Δ *php5*Δ 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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