Protein Kinase A and Mitogen-Activated Protein Kinase Pathways Antagonistically Regulate Fission Yeast *fbp1* Transcription by Employing Different Modes of Action at Two Upstream Activation Sites

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Received 11 May 2000/Accepted 14 June 2000

A significant challenge to our understanding of eukaryotic transcriptional regulation is to determine how multiple signal transduction pathways converge on a single promoter to regulate transcription in divergent fashions. To study this, we have investigated the transcriptional regulation of the *Schizosaccharomyces pombe* *fbp1* gene that is repressed by a cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway and is activated by a stress-activated mitogen-activated protein kinase (MAPK) pathway. In this study, we identified and characterized two *cis*-acting elements in the *fbp1* promoter required for activation of *fbp1* transcription. Upstream activation site 1 (UAS1), located approximately 900 bp from the transcriptional start site, resembles a cAMP response element (CRE) that is the binding site for the *atf1*-*pcr1* heterodimeric transcriptional activator. Binding of this activator to UAS1 is positively regulated by the MAPK pathway and negatively regulated by PKA. UAS2, located approximately 250 bp from the transcriptional start site, resembles a *Saccharomyces cerevisiae* stress response element. UAS2 is bound by transcriptional activators and repressors regulated by both the PKA and MAPK pathways, although *atf1* itself is not present in these complexes. Transcriptional regulation of *fbp1* promoter constructs containing only UAS1 or UAS2 confirms that the PKA and MAPK regulation is targeted to both sites. We conclude that the PKA and MAPK signal transduction pathways regulate *fbp1* transcription at UAS1 and UAS2, but that the antagonistic interactions between these pathways involve different mechanisms at each site.

Transcriptional regulation is both an essential and a universal biological process. At any given time, a cell must express only a subset of its genes at appropriate levels in order to function properly. The control of transcription in eukaryotes involves the activities of both activators and repressors that directly bind DNA, as well as complexes of coactivators and corepressors that associate with the DNA-binding proteins (33, 41, 54). These activation and repression complexes can change the chromatin structure within a promoter, thus altering the ability of other activators or repressors to bind nearby target sequences (25, 64). An additional layer of control comes from signal transduction pathways that influence the activity of the DNA-binding proteins by altering their cellular location or DNA-binding affinity (23). A signaling pathway can promote transcription by stimulating an activator, inhibiting a repressor, or both. Conversely, a signaling pathway can repress transcription by inhibiting an activator, stimulating a repressor, or both. Thus, the transcriptional regulatory mechanisms of genes controlled by divergently acting signaling pathways may entail any of a large number of “wiring” patterns.

The fission yeast *Schizosaccharomyces pombe* regulates transcription of the *fbp1* gene, encoding fructose-1,6-bisphosphatase, over a 200-fold range in response to changes in carbon source through glucose repression (19, 20, 59). Previous studies have identified two signal transduction pathways that coordinately regulate *fbp1* transcription along with several other

biological processes that are subject to regulation by nutrient monitoring (3, 5, 18, 22, 53, 55).

Glucose detection results in the activation of adenylate cyclase, and the resulting cyclic AMP (cAMP) signal activates a cAMP-dependent protein kinase A (PKA) to repress *fbp1* transcription (3, 18, 21). Elevated PKA activity serves as an indicator of nutrient-rich growth conditions, inhibiting conjugation and sporulation, stationary-phase entry, thermotolerance, and the uptake of gluconate as an alternative carbon source (4, 9, 12, 31, 32, 43). Mutations in genes required for glucose detection and PKA activation cause cells to transcribe *fbp1*, conjugate and sporulate, and transport gluconate while growing in glucose- and nitrogen-rich media. These mutations also confer enhanced thermotolerance and a delay in exit from the stationary phase and spore germination. Mutations in the *cgs1* gene, which encodes the regulatory subunit of PKA, lead to unregulated PKA activity that inhibits *fbp1* transcription, conjugation and sporulation, stationary-phase entry, and gluconate transport (9, 18, 37; J. I. Stiefel and C. S. Hoffman, unpublished observations).

Glucose starvation creates an environmental stress signal that, like nitrogen starvation, osmotic stress, oxidative stress, and heat stress, activates a stress-activated mitogen-activated protein kinase (MAPK) pathway. MAPK cascades are highly conserved signal transduction pathways possessing three protein kinases, the MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). This *S. pombe* pathway is composed of the *win1* and *wis4/wik1/wak1* MAPKKKs, the *wis1* MAPKK, and the *spc1/sty1* MAPK (7, 36, 45, 46, 49–51, 53, 60). A downstream target of the MAPK, a heterodimeric bZIP transcriptional activator encoded by the *atf1/gad7* and the *pcr1* genes, is required to derepress *fbp1* transcription (22, 50, 55,

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TABLE 1. Genotypes of the strains used in this study

Strain	Genotype
LAN70	<i>h⁻ ura4::fbp1-lacZ</i>
LAN170	<i>h⁻ ade6-M216 ura4::fbp1(Δ-1399 to -336)-lacZ</i>
LAN171	<i>h⁻ ura4::fbp1(Δ-1399 to -100)-lacZ</i>
LANPac	<i>h⁻ ura4::fbp1(Δ-1399 to -216)-lacZ</i>
LAN6P	<i>h⁻ ura4::fbp1(Δ-429 to -179)-lacZ</i>
LAN6P-Pm1	<i>h⁻ ura4::fbp1(Δ-1399 to -179)-lacZ</i>
LANsca	<i>h⁻ ura4::fbp1(Δ-1399 to -876/Δ-429 to -179)-lacZ</i>
LANSP	<i>h⁻ ade6-M216 ura4::fbp1(Δ-1399 to -876)-lacZ</i>
LAN70mut1	<i>h⁻ ura4::fbp1(-893G → C)-lacZ</i>
LAN170mut2	<i>h⁻ ura4::fbp1(Δ-1399 to -336/-262C → G)-lacZ</i>
LANpka1	<i>h⁻ pka1Δ::ura4⁺ ura4::fbp1-lacZ</i>
LAN170pka1	<i>h⁻ pka1Δ::ura4⁺ ura4::fbp1(Δ-1399 to -336)-lacZ</i>
LAN170atf1	<i>h⁻ atf1Δ::ura4⁺ ura4::fbp1(Δ-1399 to -336)-lacZ</i>
LAN6Ppka1	<i>h⁻ pka1Δ::ura4⁺ ura4::fbp1(Δ-429 to -179)-lacZ</i>
LAN6Pscr1	<i>h⁺ scr1Δ::ura4⁺ leu1-32 his2⁻ ura4::fbp1(Δ-429 to -179)-lacZ</i>
LAN170scr1	<i>h⁻ scr1Δ::ura4⁺ ade6-M216 ura4::fbp1(Δ-1399 to -336)-lacZ</i>
FWP72	<i>h⁻ leu1-32 fbp1::ura4⁺ ura4::fbp1-lacZ</i>
CHP704	<i>h⁺ pka1-261 leu1-32 sty1HA::ura4⁺ ura4::fbp1-lacZ</i>
CHP709	<i>h⁺ cgs1-1 leu1-32 sty1HA::ura4⁺ ura4::fbp1-lacZ</i>
CHP720	<i>h⁻ wis1Δ::LEU2⁺ leu1-32 ade6-M210 ura4::fbp1-lacZ</i>
CHP726	<i>h⁻ styl-1 leu1-32 fbp1::ura4⁺ ura4::fbp1-lacZ</i>
CHP751	<i>h⁺ atf1Δ::ura4⁺ ura4::fbp1(Δ-429 to -179)-lacZ</i>
KS1479	<i>h⁻ atf1HA6H::ura4⁺ leu1-32 ura4-D18</i>
TP367-1C	<i>h⁻ atf1Δ::ura4⁺ leu1-32 his7-366 ad36-M216 ura4-D18</i>
RJP47	<i>h⁺ scr1Δ::ura4⁺ leu1-32 ade6-M216 his2⁻ ura4::fbp1-lacZ</i>

63). The *spc1/sty1* MAPK pathway is required for many processes that are also negatively regulated by PKA, including mating and sporulation, gluconate transport, and thermotolerance. However, data from these studies suggest that these two pathways work in parallel (22, 53, 55) and that the PKA pathway has little or no influence on the function of the *atf1*-*pcr1* activator.

To investigate how the PKA and stress-activated MAPK pathways coordinately regulate *fbp1* transcription, we conducted an analysis of the *fbp1* promoter. Using a combination of promoter deletions and site-directed mutations, we have identified two elements, upstream activation site 1 (UAS1) and UAS2, required for full derepression of *fbp1* transcription. Electrophoretic mobility shift assays (EMSAs) demonstrate that the protein-DNA interactions at UAS1 and UAS2 are regulated by both the MAPK and PKA pathways. However, these signaling pathways accomplish their regulation at these two sites by different mechanisms. We discuss the implications of these results with regard to the ability of signaling pathways to elicit quantitative differences in transcriptional regulation of genes that are qualitatively controlled in a similar fashion.

MATERIALS AND METHODS

Yeast strains and growth media. The *S. pombe* strains used in this study are listed in Table 1. The *ura4::fbp1-lacZ* allele is a disruption of the *ura4* gene by an *fbp1-lacZ* translational fusion (19). This translational fusion includes approximately 1.5 kb of sequence 5' to the *fbp1* transcriptional start site. Changes to the *fbp1* promoter sequence in the derivatives constructed in this study are indicated in parentheses within the *fbp1-lacZ* allele designation (Table 1). Defined PM medium (61) and standard rich yeast extract medium (YEL) (15) were used for culturing cells with glucose present at 8% (repressing conditions), 3% (standard culturing conditions), or 0.1% (derepressing conditions; with 3% glycerol added). Required nutrients were added to PM medium at a concentration of 75 mg/liter, except for leucine, which was present at 150 mg/liter. 5-Fluoroorotic acid (5FOA) solid medium was used to identify homologous insertions of *fbp1-lacZ* constructs into the *ura4* locus (2). Strain constructions were carried out by mating on SPA (15) for tetrad dissection. All yeast strains were grown at 30°C.

***fbp1-lacZ* promoter constructs.** Recombinant DNA manipulations were performed by using restriction enzymes and T4 DNA ligase from New England Biolabs according to the manufacturer's instructions. Synthetic oligonucleotides

were purchased from Integrated DNA Technologies. *Escherichia coli* transformations were performed by using electroporation-competent XL1-Blue cells (Stratagene).

Plasmid pCH150 carries the *fbp1-lacZ* translational fusion inserted at the *SmaI* site within the *S. pombe ura4* gene on a pUC8 vector (19). Digestion of this plasmid with *HindIII* generates an *fbp1-lacZ* fusion flanked by *ura4* sequences that can be used for homologous integration into the *ura4* locus. Promoter variants were constructed as follows. Promoters 170, Pac, SP, and 171 were constructed by digesting plasmid pCH150 with *PmlI* and either *NgoMIV* (for 170), *PacI* (for Pac), *NdeI* (for 171), or *ScaI* (partial digest; for SP) blunting the ends with Klenow fragment, and recircularizing with T4 DNA ligase. The 6P promoter was constructed by digesting pCH150 with *NgoMIV*, followed by treatment with BAL 31 exonuclease. The BAL 31-digested DNA was ligated in the presence of *XbaI* linkers (New England Biolabs). This DNA was digested with *XbaI* and recircularized with T4 DNA ligase to incorporate a unique *XbaI* site at the deletion junction. Deletion endpoints were confirmed by DNA sequencing with the CircumVent Thermal Cycle DNA Sequencing kit (New England Biolabs). The *fbp1-lacZ* fusions were integrated in single copy into the *ura4* genomic locus in *S. pombe* strains by digesting the plasmids with *HindIII* and transforming host cells to 5FOA resistance as previously described (19), with a transformation protocol that enhances the frequency of homologous recombination in *S. pombe* (24). Homologous integration of the *fbp1-lacZ* constructs into *ura4* was verified by Southern blot analysis.

Site-directed mutagenesis of UAS1 was conducted by using the MegaPrimer protocol, as described previously (47). PCR amplification of plasmid pCH150 with primers MutUAS1-1 (5'-GAGTACTAATGCTTTGTGAGGTAGATGAT TGGGAAGTGCTAAAGGTGGG-3'; the altered base is underlined) and UAS1-2 (5'-GCTAATAGGAAGGGCGGG-3') created the megaprimer that was used in a second round of PCR with pCH150 as a template and primer UAS1-3 (5'-TCAACGAAGCCGGCTTAC-3'). This PCR product was digested with *AflII* and *NgoMIV* and ligated with *AflII*-*NgoMIV*-cut pCH150 DNA.

Site-directed mutagenesis of UAS2 was accomplished with oligonucleotides UAS2-1 (5'-GCCGGCTTCGTTGAATTGCAGTATGTCATTGTGTTAGCA GGCTGAACACAGCATTGCCCTG-3') and MutUAS2-2 (5'-CGCTTAAT TAAAAATGCATACACGATAAACCTAATCTTCAAAAAACGATGGG CCTTGCAATGAAACTACAGGGCAATGCTGTTTCAGCCTGC-3'; the altered base is underlined). The oligonucleotides were annealed, filled in with Klenow fragment, digested with *PacI*, and ligated into a *PacI*-*PmlI*-digested pCH150 vector. Base changes in UAS1 and UAS2 were confirmed by DNA sequencing. The fusions were integrated into *S. pombe* strains at the *ura4* locus as described above.

β-Galactosidase assays. Cells were grown for approximately 24 h in PM medium containing 8% glucose (repressing conditions). The cells were counted to determine the cell density, pelleted, washed twice with sterile distilled water, and subcultured into PM medium under repressing (8% glucose) and derepressing (0.1% glucose plus 3% glycerol) conditions. These cultures were grown for 24 h to a density of 5.0×10^6 to 1.0×10^7 cells/ml. β-Galactosidase activity was measured as described previously (18). Results are presented as the mean $\pm$ standard error from two to four independent assays and represent specific activity per milligram of soluble protein.

EMSAs. Yeast cells were grown for 18 to 24 h in YEL (8% glucose) medium. The cells were counted to determine cell density and subcultured into 100 ml of YEL medium under repressing (8% glucose) and derepressing (0.1% glucose plus 3% glycerol) conditions. Exponential-phase cells were rapidly harvested at 4°C. The cells were washed twice in chilled sterile distilled water and resuspended on ice in 50 μ l of chilled lysis buffer (63), although urea was omitted and Triton X-100 was present at 0.2%. Chilled acid-washed glass beads were added to just below the meniscus, and the cells were lysed at 4°C in a mini BeadBeater (Biospec Products, Inc.). When approximately 70% cell lysis was observed, the lysate was removed, the beads were washed with 50 μ l of lysis buffer, and the 100- μ l cell lysate was cleared by centrifugation at $16,000 \times g$ for 15 min. The protein concentration of the supernatant was determined by using a Pierce bicinchoninic acid protein quantitation kit (Pierce Chemical Co.). The supernatant was diluted to 10 μ g/ μ l with lysis buffer. Lysates were aliquoted in 10- μ l volumes, flash frozen in liquid nitrogen, and stored at -80°C.

Synthetic oligonucleotides used in the EMSA studies are listed in Table 2. The annealed oligonucleotides were labeled by filling in with Klenow fragment and deoxynucleoside triphosphates (dNTPs), including [α - 32 P]dATP or [α - 32 P]dCTP, and purified by passage through a Sephadex G-25 column. Binding reaction mixtures containing 10 μ g of protein lysate (unless otherwise indicated), 5 μ g of bovine serum albumin, and 2 to 3 μ g of poly(dI-dC) were preincubated in 20 μ l of binding buffer (25 mM HEPES [pH 7.6], 34 mM KCl, 5 mM MgCl₂) for 10 min on ice, after which 10,000 cpm (~1 ng) of probe was added. The binding reaction mixtures were then incubated for 20 min at room temperature. The reactions were electrophoresed at 25 mA for 2.5 h in a 5% 0.5 $\times$ Tris-borate-EDTA nondenaturing polyacrylamide gel. The binding reaction mixtures used in supershift experiments were the same as those described above, except for the addition of mouse anti-hemagglutinin (anti-HA) antibody (Roche Molecular Biochemicals) or mouse antiactin antibody (as a nonspecific control; Amersham Pharmacia Biotech). For antibody clearing experiments, protein extracts were incubated overnight with anti-atf1 or anti-*pcr1* antibodies (62) coupled to protein A-Sepha-

TABLE 2. EMSA probes used in this study

Probe	Sequence ^a
UAS1-wt.....	5' TTCCCAATCATCTACGTCACAAAGCATTAGtact 3' 3' aaggGTTAGTAGATGCAGTGTTCGTAATCATGA 5'
UAS1-mcore.....	5' TTCCCAATCATCTACCTCACAAGCATTAGtact 3' 3' aaggGTTAGTAGATGGAGTGTTCGTAATCATGA 5'
UAS1-mleft.....	5' TTCCCACTCATCTACGTCACAAGCATTAGtact 3' 3' aaggGTGAGTAGATGCAGTGTTCGTAATCATGA 5'
UAS1-mright.....	5' TTCCCAATCATCTACGTCACACAGCATTAGtact 3' 3' aaggGTTAGTAGATGCAGTGTTCGTAATCATGA 5'
UAS2-wt.....	5' TCATTGCAAGCCCTCGTTTTTGGCGATTAGGTTTa 3' 3' agtaaCGTTCGGGAGCAAAAACTTCTAATCCAAAT 5'
UAS2-mSTRE.....	5' TCATTGCAAGCCCTCGTTTTTGGCGATTAGGTTTa 3' 3' agtaaCGTTCGGGAGCAAAAACTTCTAATCCAAAT 5'

^a Uppercase letters denote the sequence of the oligonucleotides used to make probes for EMSA experiments. Lowercase letters denote nucleotides added to the probe during the labeling reaction. Sequences resembling the consensus CRE (UAS1) and the combined STRE-Mig1 binding site (UAS2) are underlined. Base changes relative to the wild-type probes are indicated in boldface.

rose beads (Sigma) and cleared by centrifugation prior to the addition of probe to the cleared extracts.

RESULTS

Deletions in the *fbp1* promoter identify two regions required for full derepression. *S. pombe* *fbp1* transcription is tightly regulated over a 200-fold range, depending on the carbon source. To identify the *cis*-acting elements required for this transcriptional regulation, we constructed a series of deletions within the 1.5 kb of sequence 5' to the transcriptional start site of an *fbp1-lacZ* reporter and measured β -galactosidase expression from these constructs when integrated in single copy (Fig.

1A). Data from this deletion series indicate that at least two key sites are required for full derepression of the *fbp1* gene. UAS1 falls within a deletion from -1399 to -876 that causes a fivefold decrease in the β -galactosidase activity measured under derepressing conditions (compare the SP construct with the full-length promoter 70 construct; Fig. 1A). UAS2 lies within a deletion interval from -336 to -216 . Loss of this element from a promoter lacking UAS1 causes an eightfold decrease in *fbp1-lacZ* expression under derepressed conditions (compare the 170 construct with Pac; Fig. 1A). Deletion of both UAS1 and UAS2 (Sca construct; Fig. 1A) causes a 20-fold reduction in *fbp1* derepression. While additional sites of activation and repression are likely present in the *fbp1* promoter, we focused our efforts on identifying the specific sites of activation within the UAS1 and UAS2 deletion intervals due to the importance of these two elements in *fbp1* transcription.

Site-directed mutagenesis identifies *cis*-acting elements in UAS1 and UAS2. The DNA sequences within the UAS1 and UAS2 deletion intervals were analyzed in silico by using the MatInspector program (42) to identify sequences similar to previously characterized *cis*-acting elements. The UAS1 deletion interval (-1399 to -876) contains the sequence TGACG TAG on the complementary strand that resembles the cAMP response element (CRE) consensus sequence TGACGT(C/A)A (1). The *atf1*-*pcr1* heterodimeric activator, required for *fbp1* transcription, has been shown to bind CRE consensus sequences (22, 55, 62). We therefore tested whether or not this sequence is responsible for UAS1-driven *fbp1* transcription by constructing an *fbp1-lacZ* fusion carrying a base change within the core ACGT sequence of this CRE-like element. To our surprise, this change, creating the 70mut1 promoter construct, reduces *fbp1* expression to a greater degree (27-fold; Fig. 1B)

FIG. 1. Schematic of *fbp1-lacZ* constructs and β -galactosidase assay data. (A) A deletion series was constructed within the *fbp1* promoter of the *fbp1-lacZ* reporter to identify *cis*-acting elements involved in the regulation of *fbp1* transcription. β -Galactosidase activity was determined in exponentially growing cells in both glucose-rich (repressed) and glucose-limited (derepressed) media (see Materials and Methods). β -Galactosidase activity is expressed as specific activity per milligram of protein $\pm$ standard deviation. (B) Site-directed mutagenesis of UAS1 (70mut1) and UAS2 (170mut2; see Materials and Methods) identifies two elements important for *fbp1* derepression. The sequences of the wild-type elements and the mutated elements are given.

FIG. 2. EMSA studies of UAS1. (A) Whole-cell extracts prepared from cells grown in glucose-rich (8% glucose) or glucose-limited (0.1% glucose plus 3% glycerol) media were incubated with a double-stranded oligonucleotide probe (Table 2 [UAS1-wt]) identical in sequence to the -908 to -875 region of the *fbp1* promoter. The following protein extracts were used (by lane): 1, no protein added; 2, strain FWP72 (wild type) repressed; 3, strain FWP72 (derepressed); 4, strain KS1479 (*atf1HA6H*) repressed; 5, KS1479 (*atf1HA6H*) derepressed. An arrow indicates the position of the starvation-induced complex. (B) Binding reactions were carried out by using protein extracts from derepressed cells of strain FWP72 (wild type) and various probes to determine binding site specificity. The probes (Table 2) were as follows (by lane): 1, UAS1-wt; 2, UAS1-mcore; 3, UAS1-mleft; 4, UAS1-mright. An arrow indicates the position of the starvation-induced complex. (C) A supershift experiment was conducted with protein extracts from derepressed cells of strain KS1479 (*atf1HA6H*). Additions to the binding reactions were as follows (by lane): 1, 1 μ g of a mouse antiactin antibody per ml (nonspecific control); 2, no addition; 3, 0.5 μ g of a mouse anti-HA antibody per ml. Arrows indicate the positions of the starvation-induced complex (S) and the supershifted complex (SS). (D) Antibody clearing of UAS1 binding activity. Antibodies to *atf1* and *pcr1* (62) were used to clear the UAS1 binding activity from extracts of derepressed FWP72 cells. Binding reactions with UAS1-wt probe included the following extracts (by lane): 1, no protein; 2, untreated protein extract; 3, anti-*atf1* antibody-cleared protein extract; 4, anti-*pcr1* antibody-cleared protein extract. An arrow indicates the position of the starvation-induced complex.

than deletions of this region do. Relative to the 70mut1 construct, the SP deletion causes a fivefold increase in *fbp1* derepression, while the larger 170 deletion causes an eightfold increase in *fbp1* derepression (Fig. 1A). Therefore, both of these deletions appear to remove UAS1 and additional negatively acting elements within the *fbp1* promoter.

The UAS2 deletion interval (-336 to -216) contains the sequence AAAAAACGAGGGG on the complementary strand, which resembles an *S. cerevisiae* stress response element (STRE), AGGGG (34, 48), that is bound by the stress-induced activators Msn2 and Msn4 (11). This sequence also resembles the binding site for three *S. cerevisiae* glucose repressors: Mig1, which binds the sequence (A/T)₄AT(G/C)(C/T)GGGG (28, 39); Mig2, which acts as a redundant repressor with Mig1 (29, 30); and Nrg1, which recognizes the sequence AGGGG and/or GAGGG (40). A base change was introduced into the shortened 170 promoter, changing a base pair that is absolutely conserved in the STRE and Mig1-like consensus sequences. This single-base change reduces the β -galactosidase activity under derepressing conditions eightfold (Fig. 1B). We have therefore identified *cis*-acting elements within the UAS1 and UAS2 deletion intervals required for derepression of *fbp1* transcription.

UAS1 is bound by the *atf1*-*pcr1* transcriptional activator. To characterize the UAS1 binding activity from *S. pombe* whole-cell extracts, we conducted EMSAs by using a double-stranded oligonucleotide probe identical in sequence to the -908 to -875 region of the *fbp1* promoter (see Table 2 for probe sequences). We observed a slowly migrating complex in binding reactions using extracts from cells grown under glucose-starved conditions that is not present in similar reactions using extracts from cells grown under glucose-rich conditions (Fig. 2A). This novel band is not present when the probe carries a base change within the CRE-like core sequence (the same change that inhibits transcriptional activation; Fig. 1B),

but is present when using probes carrying a base change to either side of the CRE-like element (Fig. 2B).

Since the *atf1*-*pcr1* heterodimeric transcriptional activator is required for *fbp1* transcription and has been shown to bind the CRE consensus sequence (22, 55, 62), we tested whether this activator is physically present in any of the UAS1-binding complexes. Extracts from a strain expressing an HA-tagged form of *atf1* produce the same band shift pattern with the UAS1 probe as extracts from a wild-type strain (Fig. 2A). The tagged protein is functional and is expressed from a construct that replaces the wild-type *atf1*⁺ allele in the *S. pombe* genome (50). The addition of anti-HA antibody to the binding reaction causes a supershift of the starvation-specific band (Fig. 2C). Conversely, anti-*atf1* and anti-*pcr1* antibodies (62) are able to deplete wild-type extracts of the starvation-induced UAS1 binding activity (Fig. 2D). Therefore, the starvation-induced complex contains *atf1* and *pcr1* and binds in a sequence-specific manner to the UAS1 CRE-like element. Additional higher-mobility complexes are eliminated by anti-*atf1* and anti-*pcr1* clearing of the protein extracts (Fig. 2D, lanes 3 and 4). Since these complexes are not supershifted by the anti-HA antibody (Fig. 2C, lane 3), they may represent complexes containing *pcr1* and *atf21*, a bZIP protein related to *atf1* (50) which may cross-react with the anti-*atf1* antibodies.

PKA and MAPK pathways regulate *atf1*-*pcr1* binding to UAS1. Having shown that the *atf1*-*pcr1* activator is present in the starvation-induced UAS1-binding complex, we examined the effects of mutations in the MAPK and PKA pathways on this interaction. As expected, the starvation-induced complex is not present in cells lacking the *atf1* gene (Fig. 3, lanes 4 and 5) or the *pcr1* gene (data not shown). Extracts prepared from a *wis1* MAPKK deletion strain also lack this activity (Fig. 3, lanes 6 and 7). Conversely, this activity is increased in extracts from a *pka1* mutant strain and is even present in cells growing under repressed conditions (Fig. 3, lanes 8 and 9). Finally, extracts prepared from a *cgs1* deletion strain produce a shift pattern similar to that seen with wild-type extracts (Fig. 3, lanes

FIG. 3. Starvation-induced UAS1 binding activity is altered by mutations in the PKA and MAPK pathways. The binding reaction mixtures contained 10 μ g of extract prepared from strains grown in glucose-rich (lanes 2, 4, 6, 8, and 10) or glucose-limited (lanes 3, 5, 7, 9, and 11) media. Lanes: 1, no protein; 2 and 3, strain FWP72 (wild type); 4 and 5, strain TP367-1C (*atf1* Δ); 6 and 7, strain CHP720 (*wis1* Δ); 8 and 9, strain CHP704 (*pka1*-261); and 10 and 11, strain CHP709 (*cgs1*-1).

FIG. 4. EMSA studies of UAS2. (A) Changes in the growth conditions and the sequence of the probe alter the relative intensity of four protein-DNA complexes (indicated on the left as A, B, C, and D). Protein extracts prepared from wild-type strain FWP72 grown in glucose-rich (lanes 1 and 3) or glucose-limited (lanes 2 and 4) media were incubated with double-stranded oligonucleotide probes that are either identical in sequence to the -272 to -235 region of the *fbp1* promoter (lanes 1 and 2 [Table 2; UAS2-wt]) or carry the C-to-G base change that eliminates transcriptional activation by UAS2 in the 170mut2 construct shown in Fig. 1B (lanes 3 and 4 [Table 2; UAS2-mSTRE]). (B) The *scr1* repressor is required for maintaining the relative intensities of UAS2-binding complexes A through D. The binding reaction mixtures contained $10 \mu\text{g}$ of extract prepared from strains grown in glucose-rich (lanes 1 and 3) or glucose-limited (lanes 2 and 4) media. Lanes: 1 and 2, strain FWP72 (wild type); 3 and 4, strain RJP47 (*scr1* Δ).

10 and 11). These data suggest that glucose-rich conditions cause PKA to inhibit *atf1*-*pcr1* binding to UAS1, while glucose starvation conditions cause the *spc1*/*sty1* MAPK to promote *atf1*-*pcr1* binding to UAS1 (see Discussion).

UAS2 is bound by multiple factors. To characterize the UAS2 binding activity from *S. pombe* whole-cell extracts, we conducted EMSAs using probes representing the -272 to -235 region of the *fbp1* promoter (see Table 2 for probe sequences). Binding reactions using wild-type protein extracts and the UAS2 probe reveal at least four complexes designated A, B, C, and D (Fig. 4A, lanes 1 and 2). Glucose starvation causes an increase in the intensity of complex D, little change in complexes B and C, and a reduction in the intensity of complex A. In addition, upon glucose starvation, complex A becomes a doublet with the appearance of a slower-migrating band. Binding reactions using a probe that contains the same base change that eliminates transcriptional activation by UAS2 (UAS2-mut; Table 2 and Fig. 1B) result in a significant reduction in complexes A and B under both glucose-rich and glucose starvation conditions (Fig. 4A, lanes 3 and 4).

The UAS2 sequence resembles the binding sites for both transcriptional activators and repressors in *S. cerevisiae*. Because these proteins share similar zinc finger DNA-binding motifs, we tested the effect of a deletion of the *scr1* gene (56), encoding the closest *S. pombe* homolog to the *S. cerevisiae* Mig1 repressor, on both *fbp1* transcription and on the formation of UAS2-specific complexes. Loss of *scr1* causes a ninefold increase in *fbp1* expression in glucose-repressed cells (Table 3), as well as a decrease in the formation of complexes A, B, and C, and an increase in complex D in EMSAs using extracts from glucose-repressed cells (Fig. 4B, lane 3). Therefore, *scr1* appears to act as a repressor of *fbp1* transcription; however, we cannot establish whether *scr1* directly binds to UAS2 at this time. In addition, since the loss of *scr1* results in the derepression of both UAS1- and UAS2-driven *fbp1*-*lacZ* fusions (Table 3), *scr1* must exert its effect through multiple sites in the *fbp1* promoter, either directly or indirectly.

PKA and MAPK pathways regulate UAS2 binding activities. To study the relationship between UAS2 activation and the

PKA and MAPK signaling pathways, we examined the effects of mutations in these pathways on UAS2 binding activities. Unexpectedly, extracts from a *pka1* mutant strain show only a modest reduction in complexes B and D under glucose starvation conditions (Fig. 5A, lanes 3 and 4). On the contrary, extracts from a *cgs1* mutant strain display significant changes in the UAS2 binding activity in glucose-starved cells, with the loss of complexes B and C, as well as the upper band of the complex A doublet (Fig. 5A, lane 6). An additional band of slightly lower mobility than complex D is also present. Finally, extracts from a *cgs1* mutant strain grown under glucose-rich conditions show a reduction in complex D (Fig. 5A, lane 5).

We have also examined the effects of mutations in genes encoding the *wis1* MAPKK, the *spc1*/*sty1* MAPK, and the *atf1* transcriptional activator on UAS2 binding activities. Mutations affecting MAPKK and MAPK produce similar results. These include the loss of complex B in both glucose-repressed and glucose-starved cells, the loss of complex C and the upper band of the complex A doublet in glucose starved cells, a reduction in complex D in glucose-starved cells, and an increase in complex D in glucose-repressed cells (Fig. 5B, lanes 1, 2, 5, and 6). Complex formation using extracts from cells lacking *atf1* does not resemble that of either wild-type cells or MAPK pathway mutants. Complex A appears to be migrating slightly faster, while complexes B and C are replaced by a band of slightly slower mobility than complex C (Fig. 5B, lanes 3 and 4). While these data do not lend themselves to a simple model of how the protein-DNA interactions at UAS2 regulate *fbp1* transcription, it is clear that all four complexes are affected by mutations in the PKA and/or MAPK pathway.

***atf1* is not a component of the UAS2 binding activities.** Since loss of either *atf1* or signaling from the MAPK pathway alters the UAS2 binding activity (Fig. 5B), we questioned whether or not *atf1* is physically present in any of the UAS2 binding complexes, as had been shown with UAS1. Contrary to the results obtained with the UAS1 probe (Fig. 2C), we were unable to supershift UAS2 complexes formed by extracts from repressed or derepressed cells by using HA-*atf1* and anti-HA antibodies (Fig. 5C). Therefore, *atf1* is not physically part of the UAS2 binding complexes.

PKA and MAPK pathways regulate transcriptional activation from both UAS1 and UAS2. The EMSA data (Fig. 2 to 5) suggest that signaling through the PKA and MAPK pathways regulates both UAS1 and UAS2 binding activities. To determine if in vivo activation of *fbp1* transcription by each element

TABLE 3. Effects of gene deletions on transcriptional activation by UAS1 and UAS2^a

Strain	Pro-moter	UAS1	UAS2	Relevant genotype	Mean $\pm$ SE β -galactosidase activity ^b	
					Repressed	Derepressed
LAN70	70	+	+	Wild type	14 $\pm$ 3	3,031 $\pm$ 192
RJP47	70	+	+	<i>scr1</i> Δ	124 $\pm$ 1	2,875 $\pm$ 179
LAN6P	6P	+	–	Wild type	32 $\pm$ 8	1,733 $\pm$ 115
LAN6Pscr1	6P	+	–	<i>scr1</i> Δ	167 $\pm$ 0	2,037 $\pm$ 148
LAN6Ppka1	6P	+	–	<i>pka1</i> Δ	2,034 $\pm$ 294	2,516 $\pm$ 231
CHP751	6P	+	–	<i>atf1</i> Δ	8 $\pm$ 3	10 $\pm$ 3
LAN170	170	–	+	Wild type	15 $\pm$ 0	902 $\pm$ 81
LAN170scr1	170	–	+	<i>scr1</i> Δ	175 $\pm$ 30	1,268 $\pm$ 86
LAN170pka1	170	–	+	<i>pka1</i> Δ	753 $\pm$ 13	844 $\pm$ 58
LAN170atf1	170	–	+	<i>atf1</i> Δ	10 $\pm$ 1	50 $\pm$ 1

^a Schematics of the 70, 170, and 6P promoter constructs are shown in Fig. 1.

^b β -Galactosidase activity was determined from at least two independent cultures as described in Materials and Methods. The values are given as specific activity per milligram of soluble protein $\pm$ standard error.

FIG. 5. UAS2 binding activities are regulated by the MAPK and PKA pathways, yet do not contain atf1 itself. (A) Binding reactions were carried out with the UAS2-wt probe (Table 2) and extracts prepared from strains grown in glucose-rich (lanes 1, 3, and 5) or glucose-limited (lanes 2, 4, and 6) media. Lanes: 1 and 2, strain FWP72 (wild type); 3 and 4, strain CHP704 (*pka1-261*); and 5 and 6, strain CHP709 (*cgs1-1*). The mobilities of complexes A through D are indicated on the left. (B) Binding reactions were performed as in panel A with extracts from strains grown in glucose-rich (lanes 1, 3, and 5) or glucose-limited (lanes 2, 4, and 6) media. Lanes: 1 and 2, strain CHP720 (*wis1*Δ); 3 and 4, strain TP367-1C (*atf1*Δ); 5 and 6, strain CHP726 (*sty1-1*). (C) A supershift experiment was conducted as in Fig. 2C with protein extract from strain KS1479 (*atf1HA6H*) grown in glucose-rich (lanes 1 to 3) or glucose-limited (lanes 4 to 6) media. Additions to the binding reactions were as follows (by lane): 1 and 3, no addition; 2 and 4, 1 µg of a mouse antiactin antibody per ml (as a nonspecific control); 3 and 6, 0.5 µg of a mouse anti-HA antibody per ml.

is also regulated by both pathways, we crossed *fbp1-lacZ* constructs containing only UAS1 (6P) or UAS2 (170) into *pka1*Δ and *atf1*Δ backgrounds and assayed β-galactosidase activity in repressing and derepressing cultures (Table 3). The results of these assays corroborate the data from the in vitro binding experiments. Both the UAS1-driven (6P) and the UAS2-driven (170) promoters become fully derepressed by the loss of PKA. As expected, the UAS1-driven promoter is almost totally inactive in cells lacking the atf1 transcriptional activator.

While expression from the UAS2-driven promoter is reduced 18-fold by the loss of atf1, β-galactosidase activity is still induced 5-fold by glucose starvation (strain LAN170atf1; Table 3). This reduction in expression is consistent with in vitro data showing that atf1 is indirectly required for UAS2 binding activity in glucose-starved cells (Fig. 5B); however, the remaining induction suggests that glucose repression by PKA involves both atf1-dependent and atf1-independent mechanisms. Further support for this proposal comes from our observation that a *pka1* deletion causes a sixfold increase in *fbp1-lacZ* (using the full-length 70 promoter) expression in an *atf1* deletion background (data not shown). Thus, both the PKA and MAPK pathways regulate *fbp1* transcription by at least two mechanisms each.

DISCUSSION

Transcription of the *S. pombe* *fbp1* gene is under the control of PKA and MAPK signaling pathways that are largely responsible for glucose repression and glucose starvation-induced derepression, respectively. In this study, we examined the *fbp1* promoter and identified two elements required for transcriptional activation. Both of the signaling pathways regulate protein-DNA interactions at each element, although the mechanisms by which these pathways exert their control differ at the two sites.

UAS1, located 900 bp upstream from the *fbp1* transcriptional start site, resembles a CRE that is bound by members of the ATF and CREB family of bZIP transcriptional activators (16, 44). The discovery of this site as a key element in *fbp1* transcription is not surprising, since the atf1-pcr1 bZIP transcriptional activator is required for *fbp1* derepression (22, 55, 62). A base change in the core of this sequence greatly reduces *fbp1* transcriptional activation in vivo (170mut2 promoter con-

struct; Fig. 1B) and eliminates the in vitro binding by a starvation-induced protein complex (Fig. 2A and B). This base change reduces *fbp1* derepression to a greater extent than do deletions of this region (170 and SP deletion constructs; Fig. 1A), suggesting that additional sequences in this region negatively regulate *fbp1* transcription. As expected, the atf1-pcr1 heterodimer is responsible for the starvation-induced UAS1 binding activity (Fig. 2C and D).

Further characterization of the UAS1 binding activity shows that both the MAPK and PKA pathways regulate this protein-DNA interaction. Deletion of the *wis1* MAPKK gene results in a loss of UAS1 binding activity similar to that to that conferred by an *atf1* deletion (Fig. 3). Furthermore, loss of PKA activity allows for binding in the absence of a starvation signal, while PKA activation by a mutation affecting the *cgs1* regulatory subunit of PKA has little effect. These data suggest that PKA activity inhibits atf1-pcr1 binding to UAS1, while the *spc1/sty1* MAPK activity overcomes this inhibitory effect. Therefore, activation of the MAPK pathway is not required for atf1-pcr1 binding to UAS1 in a *pka1* mutant, while the elevated PKA activity in a *cgs1* mutant does not prevent starvation-induced MAPK stimulation of atf1-pcr1 binding to UAS1. Yet, we previously showed that *cgs1* mutants are grossly defective in derepression of *fbp1* transcription (18), indicating that activation of the MAPK pathway is not sufficient to overcome the elevated PKA activity. This apparent contradiction may be explained by our discovery here that both the MAPK and PKA pathways exert multiple regulatory effects on *fbp1* transcription. These varied interactions might also explain why a mutation that eliminates the only consensus PKA phosphorylation site within *atf1* (*gad7*) was seen to have only a moderate effect on mating efficiency (22).

While the *S. pombe* PKA and MAPK pathways have been previously shown to antagonistically regulate a wide range of biological processes (4, 9, 12, 31, 32, 43), this is the first demonstration of a direct interaction between these pathways and control of atf1 activity at the level of DNA binding. Previous studies have suggested that the atf1-pcr1 heterodimer is constitutively bound to DNA (6, 63) and that mutations in the PKA pathway do not affect transcriptional activation by this complex (55). One possible explanation for the discrepancy between those studies and this one comes from the fact that we are examining binding to an endogenous site rather than a

FIG. 6. Alignment of zinc fingers from related *S. cerevisiae* and *S. pombe* proteins. Pairs of zinc fingers from *S. cerevisiae* proteins Msn2 (6323680), Mig1 (6321403), Mig2 (6321229), and Nrg1 (6320248) and *S. pombe* proteins C3H1.11 (Q10076), scr1 (CAB10978), rsv1 (AAB87047), SPAC11D3.17 (Q10096), and SPAC6G10.12c (O14258) were aligned by using the Clustal W version 1.7 sequence alignment program (57) and displayed by using BOXSHADE. Asterisks indicate the positions of conserved cysteines and histidines in the two zinc fingers predicted for each sequence.

consensus CRE. Signaling through the MAPK and PKA pathways might only confer a detectable change in atf1-pcr1 binding affinity to suboptimal binding sites. The atf1-pcr1 heterodimer may bind a consensus CRE site with such a high affinity that this interaction is insensitive to PKA action. Loss of regulation due to the optimization of a protein binding site has been observed for the *E. coli* cAMP receptor protein (CRP), which activates transcription of a large number of operons subject to glucose repression. The CRP binds an optimized palindromic sequence with a 450-fold greater affinity than it binds the endogenous sequence from the *lac* operon promoter (10). In vivo, a *galP1* promoter bearing an optimized CRP binding site is no longer subject to glucose repression (13).

The one study that has shown a positive role for the spc1/sty1 MAPK on DNA binding by atf1-pcr1 (also known as mts1-mts2) (26) examined a site involved in meiotic hot spot recombination. While a mutation in the *spc1/sty1* gene reduced binding to a similar DNA element at the *ade6-M26* recombination hot spot, this element is not involved in transcriptional regulation (27). There is no evidence for a role of the PKA pathway in this interaction.

UAS2, located 250 bp upstream from the transcriptional start site, contains overlapping consensus sequences for both transcriptional activators and repressors. A base change in the STRE-like sequence CCCCT eliminates the in vivo derepression of *fbp1* transcription from this element (compare the Fig. 1A Pac construct with the Fig. 1B 170 and 170mut2 constructs) and alters in vitro binding to a probe carrying this sequence by *S. pombe* extracts (Fig. 4A). While the CCCCT element is clearly required for transcriptional activation, adjacent sequences may also positively or negatively regulate *fbp1* transcription.

The pattern of protein-DNA complexes present in EMSAs using a UAS2-containing probe is surprisingly complex. At least four distinct complexes appear to be affected by changes in the sequence of the probe (Fig. 4A), the loss of the scr1 glucose repressor (Fig. 4B), mutations affecting the PKA pathway (Fig. 5A), or mutations affecting the MAPK pathway or atf1 (Fig. 5B). However, unlike the UAS1 binding activity, atf1 does not appear to be a component of the UAS2 binding activity (Fig. 5C). The most likely candidates for the UAS2-specific activators and repressors are members of a family of proteins containing two zinc fingers that resemble the DNA binding domains of the *S. cerevisiae* Msn2 and Msn4 transcriptional activators, as well as the Mig1, Mig2, and Nrg1 repressors (35, 39, 40). These transcriptional regulators are all associated with either stress-induced transcription or glucose repression and bind to sequences similar to that of UAS2.

Analysis of the *S. pombe* genome reveals at least seven gene products with similar zinc fingers, including the scr1 repressor (56), the rsv1 activator (17), and several uncharacterized proteins. Figure 6 displays an alignment of the zinc fingers from five of these proteins, along with those of the *S. cerevisiae* transcriptional regulators mentioned above. To further complicate matters, two pairs of these *S. pombe* proteins, one that includes scr1, differ in length by only three residues. Further work will be needed to determine what if any redundant functions are carried out by these proteins.

Since the EMSA studies conducted here utilize whole-cell extracts, it is quite possible that we are detecting binding activities that are cytoplasmic at the time of extract preparation. This complicates our ability to assign roles for any of the UAS2 complexes in either transcriptional activation or repression. It has been shown that phosphorylation of the *S. cerevisiae* Mig1 repressor and Msn2 activator determines whether these proteins are nuclear or cytoplasmic (8, 14, 52). However, even though similar regulation is likely to occur in *S. pombe*, this does not detract from our evidence that UAS2 is the target of both transcriptional activators and repressors. Introduction of a base change in the set of four C's results in the loss of transcriptional activation from this site (Fig. 1B) and dramatically reduces complex A and B formation (Fig. 4A). Conversely, loss of the Mig1-like scr1 repressor also alters the protein-DNA interactions at UAS2 (Fig. 4B) and causes an increase in expression from both the full-length promoter and a UAS2-driven promoter (Table 3). The Mig1 protein, responsible for glucose repression of many *S. cerevisiae* genes, binds to a sequence resembling the UAS2 element and recruits the corepressor Ssn6-Tup1 (58). The scr1 repressor may function in a similar manner, because we and others have observed that *S. pombe* possesses two *TUP1*-like genes that encode redundant repressors of *fbp1* transcription (38; R. Janoo and C. S. Hoffman, unpublished observations). However, since a promoter lacking the UAS2 sequence is also partially derepressed by the loss of scr1 (Table 3), UAS2 cannot represent a unique site for scr1 action within the *fbp1* promoter.

Our data suggest that the PKA and MAPK pathways each employ at least two independent mechanisms to antagonistically regulate *fbp1* transcription. PKA negatively regulates transcriptional activation from both UAS1 and UAS2. At UAS1, PKA inhibits atf1-pcr1 binding; however, PKA must have additional roles, since we have observed that an *atf1Δ* strain is partially derepressed for *fbp1-lacZ* expression by the subsequent deletion of *pka1* (data not shown). PKA could stimulate the activity of a repressor such as scr1 and/or inhibit the activity of a UAS2-specific activator. The MAPK pathway stimulates atf1-pcr1 binding at UAS1 and promotes the expres-

sion or function of other activators at UAS2. These two distinct roles for *atf1* are confirmed by the in vivo activation data. UAS1-driven expression is absolutely dependent upon *atf1* (Table 3), as expected, since the UAS1 site is an *atf1*-*pcr1* binding site (Fig. 2). On the other hand, while UAS2-driven expression is largely *atf1* dependent, some regulation is still evident in an *atf1* Δ strain, consistent with an indirect role for *atf1*.

While it seems unnecessarily complicated that both the PKA and MAPK pathways employ multiple mechanisms to regulate *fbp1* transcription, such mechanistic diversity would allow cells to regulate a collection of genes by the same two pathways, but with different degrees of sensitivity. The *fbp1-lacZ* constructs (Fig. 1) are all subject to glucose regulation, yet display regulation over a 5-fold to 200-fold range, with a 3-fold range in absolute repressed levels (excluding the virtually inactive 171 construct). As with these different constructs, the *S. pombe* genes controlled by the PKA and MAPK pathways in response to nutrient and stress conditions may utilize only subsets of the mechanisms observed here. This is likely to be a general theme of eukaryotic transcriptional regulation, allowing a finite number of signaling pathways to exert qualitatively different outcomes in gene expression over a large number of genes that are subject to the same environmental signals.

ACKNOWLEDGMENTS

We thank Kazuhiro Shiozaki, Peter Fantes, Takashi Toda, Maureen McLeod, and Kaoru Takegawa for providing strains and Masayuki Yamamoto for providing antibodies to *atf1* and *pcr1*. We thank Steve Buratowski, Thomas Chiles, and Steve Howes for insightful discussions and critical evaluation of the manuscript.

This work was supported by NIH grant R01 GM46226 and a Research Expense grant from Boston College to C.S.H.

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