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Isolation and Characterization of Mutants Constitutive for Expression of the *fbp1* Gene of *Schizosaccharomyces pombe*

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ABSTRACT

Transcription of the *fbp1* gene of *Schizosaccharomyces pombe*, encoding fructose-1,6-bisphosphatase, is glucose repressible. We have constructed two hybrid genes, containing the *fbp1* promoter, that allow selection for mutations that alter transcriptional regulation of *fbp1*. Strains carrying *fbp1-ura4* and *fbp1-lacZ* fusions are phenotypically Ura$^-$, resistant to 5-fluoro-orotic acid, and express a low level of β-galactosidase activity when grown under repressing conditions (8% glucose). By selecting for Ura$^+$ strains grown under repressing conditions, we have isolated 187 independent mutants that constitutively express the *fbp1-ura4* fusion. These mutants identify ten complementation groups that represent ten unlinked *git* (*g*lucose *i*nsensitive *t*ranscription) genes. The *git* gene products are required in *trans* for glucose repression of expression from the *fbp1* promoter since these mutations also alter expression of the *fbp1-lacZ* fusion. We have shown that transcription of the wild type *fbp1* gene in most *git* mutants is elevated to a level consistent with the increased expression of the *fbp1-lacZ* hybrid gene. Mutations in some *git* genes confer additional phenotypes such as slow growth, temperature-sensitive lethality and reduced spore viability. Therefore, some of these genes are likely to encode factors that are of general importance for *S. pombe* transcription.

TRANSCRIPTIONAL regulation of gene expression is a common mechanism by which cells control the synthesis of gene products. Much of our understanding of eukaryotic transcriptional regulation comes from the study of gene expression in the yeast *Saccharomyces cerevisiae*. Transcriptional studies in *S. cerevisiae* are facilitated by genetic selections and manipulations that have resulted in the identification of a variety of *trans*-acting factors and *cis*-acting promoter elements (for review, see GUARENTE 1987, 1988; STRUHL 1989). Recent observations that both general and specific transcription factors from *S. cerevisiae* and mammalian cells can function in heterologous systems *in vivo* and *in vitro* suggest that at least some transcriptional mechanisms are conserved between *S. cerevisiae* and higher eukaryotes (for example, see BURATOWSKI *et al.* 1988; CAVALLINI *et al.* 1988; CHODOSH *et al.* 1988; KAKIDANI and PTASHNE 1988; LECH, ANDERSON and BRENT 1988; METZGER, WHITE and CHAMBON 1988; WEBSTER *et al.* 1988).

The fission yeast *Schizosaccharomyces pombe* is amenable to the same genetic selections and manipulations as is *S. cerevisiae*; however, these two yeasts are very different at both the morphological and molecular level (for a review, see RUSSELL and NURSE 1986). Furthermore, certain features of *S. pombe* transcription that differ from *S. cerevisiae* transcription suggest that this organism may also provide a good model system for helping to understand transcription in higher eukaryotes. First, the apparent spacing between TATA boxes and transcription initiation sites in *S. pombe* is 25 to 45 base pairs (bp) (RUSSELL 1985; LOSSON, FUCHS and LACROUTE 1985), similar to that of higher eukaryotes. Second, the SV40 promoter is functional in *S. pombe*, probably due to the presence of an AP-1-like activity in this yeast (JONES *et al.* 1988). In fact, the human collagenase gene promoter AP-1 binding site functions as an upstream activation sequence in *S. pombe*, providing evidence that *S. pombe* transcriptional factors can bind to this mammalian promoter element to activate transcription (JONES *et al.* 1988). We have therefore undertaken the study of transcriptional regulation of an *S. pombe* gene in an effort to further our understanding of eukaryotic gene expression.

The *fbp1* gene, which encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase; VASSAROTTI and FRIESEN 1985), displays dramatic transcriptional regulation, being regulated several hundredfold by glucose repression. FBPase converts fructose-1,6-bisphosphate to fructose-6-phosphate, working at a control point in the gluconeogenic and glycolytic pathways. Therefore, it is important for cells to regulate the level of this activity. FBPases have been observed in a large number of mammalian tissues, especially in liver, kidney and muscle (for a review, see TEJWANI 1983). Mammalian FBPase activity is

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regulated by two metabolic inhibitors, AMP and fructose-2,6-bisphosphate. FBPase activity in *S. cerevisiae* is controlled by glucose repression of transcription and by glucose inactivation of the enzyme (SEDIVY and FRAENKEL 1985; HOLZER 1976). FBPase activity in *S. pombe* is only subject to glucose repression of transcription, and not to glucose inactivation (VASSAROTTI, BOUTRY and COLSON 1982; VASSAROTTI and FRIESEN 1985).

Glucose repression of gene expression is a common regulatory control in both prokaryotes and eukaryotes. In *Escherichia coli*, this process is mediated by the catabolite gene activator protein in conjunction with varying levels of cyclic adenosine monophosphate (cAMP; for a review, see BOTSFORD 1981). In eukaryotes, the mechanism of glucose repression is not understood, but appears to differ from that of *E. coli*. For example, there does not seem to be the same role for cAMP in glucose repression in *S. cerevisiae* (MATSUMOTO *et al.* 1982, 1983; ERASO and GANCEDO 1984; THEVELEIN 1988; CHERRY *et al.* 1989).

Several studies suggest that there are multiple and complex pathways for glucose repression in *S. cerevisiae*. For some *S. cerevisiae* genes, such as the *SUC2* gene, glucose repression is the sole form of transcriptional regulation (CARLSON and BOTSTEIN 1982). For other genes, such as *GAL1*, *GAL7*, and *GAL10*, it is but one aspect of a complex regulatory network (ADAMS 1972; ST. JOHN and DAVIS 1981; JOHNSTON 1987). Genetic selections have identified many genes whose products are required for glucose regulated transcription of the *SUC2* gene and/or of the *GAL* genes (for reviews see, CARLSON 1987; JOHNSTON 1987).

To study glucose repression of transcription of the *S. pombe fbp1* gene, we have constructed fusions to the *fbp1* promoter and used them to isolate mutants that are constitutive for *fbp1* expression. We have identified ten genes (designated *git1* through *git10*), mutations in which cause the constitutive expression of an *fbp1-ura4* fusion and an *fbp1-lacZ* fusion. Furthermore, mutations in most of the *git* genes cause the constitutive transcription of the wild type *fbp1* gene (mutations in *git3*, *git4* and *git9* have little or no detectable effect on *fbp1* transcription). Therefore, most *git* genes identified in this search encode factors required in *trans* for glucose repression of *fbp1* expression. Additional *git* mutant phenotypes, such as slow or temperature-sensitive growth, suggest that some *git* gene products may be general transcriptional factors in *S. pombe*.

MATERIALS AND METHODS

Yeast strains: All *S. pombe* strains were derived from the wild type *S. pombe* strains 972 (h^-) and 975 (h^+) and are listed in Table 1. Genetic nomenclature of *S. pombe* follows rules proposed by KOHLI (1987); therefore, we use the gene name *fbp1*, instead of the previous designation of *fbp*. The *fbp1::ura4* allele is a disruption of the *fbp1* gene by the coding region of the *ura4* gene, creating a translational fusion that is under the transcriptional control of the *fbp1* promoter. The *ura4::fbp1-lacZ* allele is a disruption of the *ura4* gene by the *fbp1-lacZ* translational fusion. Both constructions are described in detail below.

Media: Standard rich media YEA and YEL (GUTZ *et al.* 1974) were supplemented with 2% casamino acids. Phloxin B (Sigma) was added at 15 μg/ml for the identification of haploid colonies (GUTZ *et al.* 1974). Cells were also grown on minimal media (SD) supplemented with amino acids and on synthetic complete media (SC) lacking a specific amino acid (SHERMAN, FINK and LAWRENCE 1978). SC media were generally modified by supplementing with an additional 0.3 mM histidine (final concentration of 0.7 mM) and 2.0 mM leucine (final concentration of 3.3 mM). SC-ura medium used to select for *git* mutant strains was supplemented with 0.2 mM lysine (final concentration of 0.7 mM) and contained 8% glucose. Carbon sources were generally present at a concentration of 3% and strains were grown at 30°, unless otherwise specified. Sensitivity to 5-fluoro-orotic acid (5FOA) was determined on SC-ura, SC-ade-ura, SC-his-lys-ura, or SC-ade-lys-ura solid media containing 8% glucose, 50 mg/liter uracil, and 0.4 g/liter 5FOA. Crosses were done on either MEA (GUTZ *et al.* 1974) containing 0.4% glucose or on YPD (with 2% glucose as described by SHERMAN, FINK and LAWRENCE 1978).

Recombinant DNA methodology: Standard recombinant DNA techniques, including DNA restriction digests, ligations, and bacterial transformations, were done according to MANIATIS, FRITSCH and SAMBROOK (1982). *E. coli* strain HB101 (BOYER and ROULLAND-DUSSOIX 1969) was the host strain for bacterial transformations. Yeast transformations were done by the lithium acetate method (ITO *et al.* 1983). Small scale plasmid preparations from *E. coli* were done by the alkaline lysis method (BIRNBOIM and DOLY 1979). DNA fragment isolation was done by electroelution. Restriction endonucleases *Sal*I and *Xho*I were purchased from Boehringer Mannheim Biochemicals. T4 DNA polymerase, the large fragment of DNA polymerase I (Klenow), *Bam*HI 8-mer DNA linkers, and all other restriction enzymes were purchased from New England Biolabs, Inc.

Construction and integration of *fbp1-lacZ* and *fbp1-ura4* fusions: An *fbp1-lacZ* translational fusion (Figure 1), disrupting the *ura4* coding region, was constructed and used to replace the genomic copy of the wild type *ura4* gene. A *Bam*HI-*Xho*I fragment from plasmid pAV04 (VASSAROTTI and FRIESEN 1985) carrying the *fbp1* gene, the *S. cerevisiae LEU2* gene and 2-μm sequences was isolated and ligated with a *Bam*HI-*Xho*I fragment from plasmid pFL20 (LOSSON and LACROUTE 1983), containing the *E. coli bla* gene, the pBR322 *ori* and the *S. pombe ars1*$^+$ to create plasmid pCHY11. A fragment containing the *E. coli lacZ* gene (beginning with codon ten) from plasmid pSEYC102 (EMR *et al.* 1986) was derived by digesting with *Sca*I, which cuts 3′ to *lacZ*, ligating with *Bam*HI 8-mer DNA linkers, and digesting with *Bam*HI. This fragment was inserted into the *Bgl*II site within the *fbp1* coding sequence of pCHY11, creating plasmid pCHY14. The hybrid protein encoded by the *fbp1-lacZ* fusion includes the first four residues of FBPase.

To construct an *fbp1-lacZ* disruption of the *ura4* gene, plasmid pCHY14 was digested with *Hin*dIII and *Sal*I and the ends were made blunt using Klenow. This 5.1 kilobase pairs (kb) fragment, containing the *fbp1-lacZ* fusion and 1500 bp of the *fbp1* 5′ noncoding region, was inserted into the *Stu*I site in the *ura4* gene present on pUC8-*ura4* (BACH

TABLE 1

Strains

Strain	Genotype
FWP46	*h^+ ade6-M216 lys1-131 leu1-32*
FWP70	*h^+ ade6-M216 lys1-131 leu1-32 ura4::fbp1-lacZ*
FWP71	*h^+ ade6-M216 lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4*
FWP36	*h^- ura4-D6 $leu1^-$ fbp1-16*
FWP75	*h^- fbp1-16*
FWP87	*h^+ leu1-32 ura4::fbp1-lacZ fbp1::ura4*
FWP101	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*
FWP112	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*
CHP207	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git1-207*
CHP210	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-210*
CHP200	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git3-200*
CHP203	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git4-203*
CHP311	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git5-311*
CHP261	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git6-261*
FWP145	*h^- ade6-M216 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git7-235*
CHP235	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git7-235*
CHP276	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git8-276*
CHP232	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git9-232*
CHP201	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git10-201*
FWP113	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git1-1*
CHP7	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-7*
CHP14	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git3-14*
CHP17	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git4-17*
CHP75	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git5-75*
CHP107	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git6-107*
CHP93	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git7-93*
CHP60	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git8-60*
FWP149	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git9-232*
FWP134	*h^+ ade6-M210 leu1-32 ura4::fbp1-lacZ git1-1*
FWP135	*h^- his7-366 leu1-32 ura4::fbp1-lacZ git2-210*
FWP136	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ git3-200*
FWP137	*h^- leu1-32 ura4::fbp1-lacZ git4-203*
FWP138	*h^+ leu1-32 ura4::fbp1-lacZ git5-311*
FWP139	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ git6-261*
FWP140	*h^- leu1-32 ura4::fbp1-lacZ git7-235*
FWP141	*h^+ ade6-M216 leu1-32 ura4::fbp1-lacZ git8-276*
FWP142	*h^+ leu1-32 ura4::fbp1-lacZ git9-232*
FWP143	*h^+ leu1-32 ura4::fbp1-lacZ git10-201*
FWP107	*h^- lys1-131 ade6-M216 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git1-1*
FWP150	*h^- lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-7*
FWP151	*h^- lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git3-14*
FWP152	*h^- lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git4-17*
FWP153	*h^- lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git5-5*
FWP154	*h^- lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git6-8*
FWP155	*h^- lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git7-27*
CHP1	*h^+ lys1-131 ade6-M216 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git1-1*
FWP156	*h^+ lys1-131 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-7*
FWP157	*h^+ lys1-131 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git3-14*
FWP158	*h^+ lys1-131 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git4-17*
FWP159	*h^+ lys1-131 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git5-5*
FWP146	*h^+ lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git10-201*
FWP147	*h^+ his7-366 lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-7*
CHP61	*h^+ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-61*
FWP114	*h^- ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-7*

1987), creating plasmid pCH150. Transcription of the *fbp1-lacZ* fusion is in the opposite direction from that of the *ura4* gene. *S. pombe* strain FWP46 (Table 1) was transformed to 5FOA resistance (5FOA^R; loss of *ura4* activity) by a 6.8-kb *Hin*dIII fragment from pCH150 that contains the fusion plus 0.8 kb to 0.9 kb of *ura4* flanking sequence. Transformants were screened for β-galactosidase activity by filter assay (Breeden and Nasmyth 1987). Positive candidates were analyzed by Southern hybridization analysis (Southern 1975; Hoffman and Winston 1987). A strain carrying a single copy of the *fbp1-lacZ* disruption of the *ura4* gene (*ura4::fbp1-lacZ*) was designated FWP70.

To construct an *fbp1-ura4* translational fusion, plasmid pCHY11 was linearized with a partial *Bgl*II digestion,

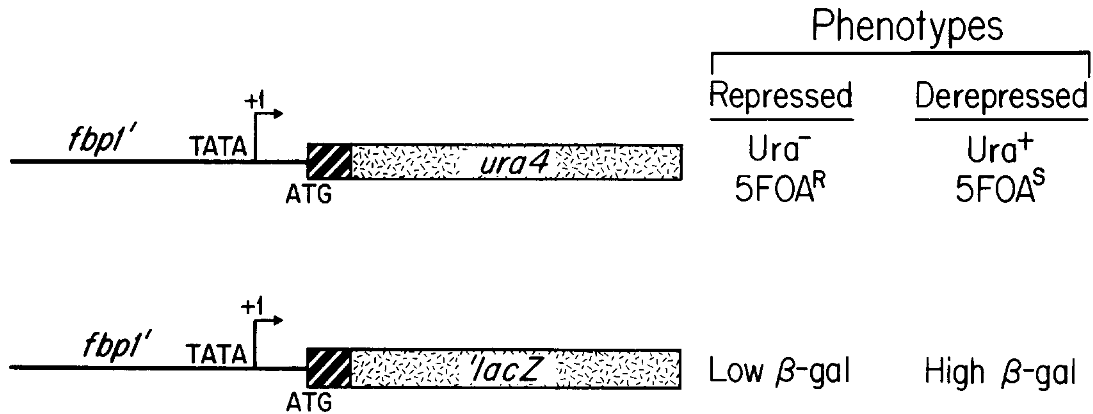

FIGURE 1.—Structure of the *fbp1-ura4* and *fbp1-lacZ* fusions and their associated phenotypes. The *fbp1-ura4* fusion is integrated at *fbp1*. The *fbp1-lacZ* fusion, including 1.5 kb of the *fbp1* 5′ noncoding region, is integrated at *ura4*. These are both translational fusions which include the first four codons of the *fbp1* open reading frame (see MATERIALS AND METHODS). The *S. pombe ura4* gene encodes OMP decarboxylase which is required for uracil prototrophy and for sensitivity to 5FOA (BOEKE, LACROUTE and FINK 1984); the *E. coli lacZ* gene encodes β-galactosidase.

treated with Klenow to blunt the ends, and recircularized to create plasmid pCHY12, which has a unique *Bgl*II site in the *fbp1* coding region. To form plasmid pCHY15, which encodes the *fbp1-ura4* fusion, a fragment from pUC8-*ura4* carrying the *ura4* coding region was inserted into the *Bgl*II site of plasmid pCHY12. The *ura4* fragment was obtained by digesting pUC8-*ura4* with *Dra*I which cuts 12 bp upstream of the *ura4* open reading frame, ligating with *Bam*HI 8-mer linkers, and digesting with *Bam*HI. The protein encoded by this fusion includes four amino acids of FBPase, two amino acids encoded by the linker, four amino acids encoded by the *ura4* 5′ noncoding region, and the entire *ura4* gene product. A 4.3-kb *Hpa*I-*Mlu*I fragment of pCHY15, carrying the *fbp1-ura4* fusion plus 3′ sequences of *fbp1*, was used to transform FWP70 (Table 1) to uracil prototrophy (Ura^+) on SD+leu+lys+ade with 3% raffinose as a carbon source. Raffinose had previously been shown to allow partial derepression of the *fbp1-lacZ* fusion; therefore, it was assumed that the *fbp1-ura4* fusion would be similarly expressed on this medium. To detect transformants in which the *fbp1-ura4* fusion disrupted the wild type *fbp1* gene, Ura^+ candidates were screened for their ability to grow on YEA with glycerol as the carbon source, as *fbp1*⁻ strains are unable to utilize glycerol as their sole carbon source. Transformants unable to utilize glycerol were examined by Southern hybridization analysis. A strain carrying a single copy of the *fbp1-ura4* fusion (*fbp1::ura4*) was designated FWP71.

Isolation of *git* mutants: Mutants constitutive for *fbp1-ura4* expression (*git* mutants), were isolated from strains FWP101 and FWP112 on the basis of their ability to express the *fbp1-ura4* fusion under repressing conditions (8% glucose). These mutants were selected as Ura^+ colonies on SC-ura plates containing 8% glucose. The *his7-366* allele aided the selection, as it reduced the growth rate of these strains, even in the presence of 0.7 mM histidine, and thereby reduced the background growth of the git^+ cells on the selective medium. Candidates were single colony purified on selective (SC-ura) and then on nonselective (YEA) media, and then tested for the presence of β-galactosidase activity by filter assay (BREEDEN and NASMYTH 1987). Approximately 50% of the Ura^+ strains lacked β-galactosidase activity, presumably due to a gene conversion of the *ura4::fbp1-lacZ* allele to wild type *ura4* by recombination with sequences from *fbp1::ura4* allele. Ura^+ candidates that still contained β-galactosidase activity were screened for sensitivity to 5FOA. The 5FOA-sensitive ($5FOA^S$) strains were designated as *git* mutant strains and were frozen as 15% glycerol stocks.

TABLE 2

Predicted dominance/recessiveness and complementation test results

Parents	Diploid phenotype on 5FOA	Conclusion
A. Dominance test		
$git^- \times git^+$	Resistant	Recessive git^-
$git^- \times git^+$	Sensitive	Dominant git^-
B. Complementation test		
$git^- \times git^-$	Resistant	Complementation
$git^- \times git^-$	Sensitive	Noncomplementation

A total of 86 *git* mutants were isolated from FWP101 and 100 *git* mutants were isolated from FWP112. In addition, a single *git* mutant, CHP1, was isolated from strain FWP71 (Table 1) and designated as carrying the *git1-1* mutant allele. This allele was crossed into an FWP101 background to produce strain FWP113 which was then subjected to genetic analyses along with other FWP101 *git* mutant derivatives.

Dominance/recessiveness tests and complementation analysis: Dominance/recessiveness tests were performed by selecting git^-/git^+ diploids and determining if they were sensitive or resistant to 5FOA. Specifically, all *git* mutant derivatives of FWP101 were mated to strain FWP112, and all *git* mutant derivatives of FWP112 were mated to strain FWP101. Cells were mixed on MEA plates and then transferred to SC-ade (containing 8% glucose) after 2 days at room temperature to select for Ade^+ diploid strains (the Ade^+ phenotype results from intragenic complementation by the *ade6-M210* and *ade6-M216* alleles; GUTZ *et al.* 1974). The patches of cells contained many small Ade^+ colonies after 3 days at 30°. At this time, before too many cells within the colonies entered stationary phase, thus derepressing expression from the *fbp1* promoter (HOFFMAN and WINSTON 1989), these colonies were replica plated to SC-ade media containing 8% glucose with and without 5FOA. A *git* allele was scored as recessive if the diploids exhibited the wild type $5FOA^R$ phenotype (Table 2). Alternatively, a diploid $5FOA^S$ phenotype (Git^-) would indicate that the *git* allele was dominant.

Complementation analyses were carried out by determining the sensitivity or resistance to 5FOA of diploids formed between *git* mutant derivatives of FWP101 and *git* mutant derivatives of FWP112. The procedure was the same as for the dominance/recessiveness test. Complementation between two recessive mutant alleles would restore regulated expression from the *fbp1* promoter, causing the diploids to be 5FOA^R (Table 2). Noncomplementation would result in constitutive expression from the *fbp1* promoter, causing the diploids to be 5FOA^S.

Linkage analyses: Two forms of linkage analysis were carried out to determine allelism of *git* mutations. Random spore analysis was performed to support much of the complementation studies. In this analysis, representative *git* mutant alleles were crossed into a background containing *lys1-131*. Lawns of these strains were replica plated to MEA along with patches of all of the *git* mutants of the opposite mating type carrying *his7* and *ade6* mutations. Mating and sporulation were allowed to occur for three to five days at room temperature. Spores were then replica plated to SC-lys-ade or SC-lys-his containing 8% glucose at 30° to select for the recombinant progeny. After 3 days, these recombinants were replica plated to SC-lys-ade or SC-lys-his containing 8% glucose plus and minus 5FOA to identify *git*$^+$ recombinant progeny. The absence of such progeny indicated allelism between the mutation in the tester lawn and that in the patch.

Possible allelism of the *git* mutations in different complementation groups was also tested by tetrad dissection of crosses between *git* mutant strains representing all pairwise combinations of the ten complementation groups. In this analysis, at least ten tetrads were dissected per cross. However, due to poor spore viability in some crosses, as few as two tetrads with four viable progeny may have been examined. The phenotypes of progeny in tetrads that produced fewer than four viable progeny were also determined to detect possible double mutant inviability. The appearance of 5FOA^R *git*$^+$ progeny was taken to indicate that the *git* mutations in the cross were not allelic.

β-Galactosidase assays: Strains were grown in YEL to 1–2 × 10^7 cells/ml. Carbon sources used were 8% glucose for repressing conditions and either 3% glycerol (only for wild-type *fbp1* strain FWP70), 3% maltose, or 0.1% glucose+3% glycerol for derepressing conditions. β-Galactosidase assays were carried out as described by ROSE and BOTSTEIN (1983) and the specific activity per milligram of protein was determined. For each culture, two different volumes of protein extract were assayed. Protein concentrations were determined by BRADFORD (1976) assays using bovine serum albumin as a standard.

Northern RNA hybridization analysis: Strains were grown in YEL with either 8% glucose (repressing conditions) or 0.1% glucose+3% glycerol (derepressing conditions) as the carbon source to a concentration of 1–2 × 10^7 cells/ml. Total yeast RNA was isolated by the method of CARLSON and BOTSTEIN (1982). Approximately 5 μg of RNA was loaded per lane of a 1% agarose-formaldehyde gel. RNA amounts were standardized by hybridization to plasmid pYK311 which carries the *leu1* gene (KIKUCHI *et al.* 1988). Electrophoresis was for 750 volt-hours to enhance separation of the *leu1* and *fbp1* RNAs. Blotting and hybridization were carried out on GeneScreen using the dextran-sulfate method described by the manufacturer (New England Nuclear Corp.). The RNA was UV-cross-linked onto GeneScreen (1200 μW/cm^2 for 2 min; CHURCH and GILBERT 1984). DNA probes were labeled with ^{32}P by nick translation (RIGBY *et al.* 1977). Plasmid pAV06 (VASSAROTTI and FRIESEN 1985) was used as a probe for the *fbp1* transcript.

RESULTS

The *fbp1* clone and *fbp1* mutant alleles identify the same gene: The *fbp1* gene was originally identified in *S. pombe* by mutant alleles *fbp1-6* and *fbp1-16*, isolated *in vivo*, which lead to the loss of FBPase activity and the inability to utilize glycerol as a carbon source (VASSAROTTI, BOUTRY and COLSON 1982). Putative *fbp1* clones from an *S. pombe* genomic library were identified by their ability to complement these mutations (VASSAROTTI and FRIESEN 1985). We wanted to verify that these clones carried the *fbp1* gene. Therefore, tetrad analysis was used to determine that the *fbp1-16* mutation is allelic to a mutation created by integration of a disrupted copy of the cloned gene.

To disrupt the cloned *fbp1* gene, we constructed a translational fusion of the cloned gene to *ura4* and recombined this DNA into the *S. pombe* genome, replacing the wild type copy of the *fbp1* gene (see MATERIALS AND METHODS). Cells that carry the integrated fusion are unable to utilize glycerol as a carbon source. In two separate crosses, a strain carrying the integrated fusion was crossed with a strain carrying the *fbp1-16* allele (FWP71 × FWP36 and FWP87 × FWP75) and the progeny were examined for the ability to utilize glycerol. If the integrated fusion and the *fbp1-16* mutation are allelic, such a cross should not generate any progeny able to grow on glycerol. For 73 tetrads examined in the two crosses, there were no progeny able to grow on glycerol. Therefore, the clone isolated by VASSAROTTI and FRIESEN (1985) contains the *fbp1* gene identified by the *fbp1-6* and *fbp1-16* mutations.

Isolation of constitutive mutants using *fbp1-ura4* and *fbp1-lacZ* fusions: To isolate mutants that express *fbp1* constitutively under repressing conditions, we constructed *fbp1-ura4* and *fbp1-lacZ* fusions and integrated them into the *S. pombe* genome (see MATERIALS AND METHODS). To verify that these fusions are regulated the same as the *fbp1* gene, strains that contain them were characterized with respect to their ability to grow in the absence of uracil, their sensitivity to 5FOA, and their β-galactosidase expression when grown on various carbon sources (Figure 1). As expected, cells grown on SC-ura containing 8% glucose displayed a leaky Ura$^-$ phenotype (formed only tiny colonies after more than a week at 30°) and were resistant to 0.4 mg/ml 5FOA, due to glucose repression of the *fbp1-ura4* fusion. Cells grown on medium containing maltose, raffinose or fructose, were Ura$^+$. Cells grown on medium containing maltose were also sensitive to 0.4 mg/ml 5FOA. Sensitivity to 5FOA was not tested on other carbon sources.

To measure the range over which the *fbp1* promoter is regulated, β-galactosidase activity was assayed in exponential phase cultures of fusion-containing

TABLE 3

Regulation of an *fbp1-lacZ* integrated fusion

Carbon source	Strain	
	FWP101	FWP70
8% Glucose	11 ± 2	10 ± 2
0.1% Glucose + 3% glycerol	2171 ± 530	2114 ± 583
3% Maltose	637 ± 45	580 ± 93
3% Glycerol	ND	6474 ± 735

β-Galactosidase activity was assayed in at least three independent cultures, as described in MATERIALS AND METHODS. The values given represent specific activity ± standard error. FWP101 carries the *fbp1::ura4* disruption of *fbp1*, and therefore cannot grow on glycerol as the sole carbon source. ND, not determined.

strains grown in the presence of various carbon sources (Table 3). These results demonstrate that expression from the *fbp1* promoter can vary over a greater than 600-fold range depending upon the carbon source, and are consistent with the previously studied regulation of *fbp1* (VASSAROTTI and FRIESEN 1985; HOFFMAN and WINSTON 1989). Therefore, these results demonstrate that the fusions are regulated the same as *fbp1*.

Since the fusion-bearing strains are Ura$^-$ when grown in the presence of 8% glucose, we were able to isolate mutants that constitutively express the fusions by selecting for Ura$^+$ colonies on SC-ura plates containing 8% glucose (see MATERIALS AND METHODS). From this selection, we isolated 187 spontaneous mutants from strains FWP71, FWP101 and FWP112. These mutants are Ura$^+$ and 5FOA^S when grown under normally repressing conditions; therefore, the *fbp1-ura4* fusion is being aberrantly expressed. We have designated these mutants as *git* mutants (*git* = *g*lucose *i*nsensitive *t*ranscription).

Dominance/recessiveness analysis of *git* mutations: To test whether the *git* mutant alleles are dominant or recessive, *git* mutants derived from FWP101 (h^+) were crossed with strain FWP112 (h^- git^+) and the resulting diploid colonies were tested for sensitivity to 5FOA. Similarly, diploids arising from crosses of *git* mutant derivatives of FWP112 by strain FWP101 (git^+) were tested for sensitivity to 5FOA. If a *git* mutant allele is dominant to wild type, the diploid will be 5FOA^S due to constitutive expression of the *fbp1-ura4* fusion (Table 2). If a *git* mutant allele is recessive to wild type, the diploid will be 5FOA^R when grown in the presence of 8% glucose, due to repression of expression of the *fbp1-ura4* fusion. By this analysis, all 187 *git* mutant alleles are recessive, since in every case the diploids were 5FOA^R.

Complementation analysis of *git* mutants: The *git* mutations were placed into complementation groups by determining the 5FOA phenotype of diploids formed between h^+ *git* mutants and h^- *git* mutants. Diploids that carry complementing mutations are 5FOA^R strains, while diploids that carry noncomplementing mutations are 5FOA^S (Table 2). One hundred eight-five of the 187 *git* mutations identify 8 complementation groups represented by three or more mutant alleles (Table 4). The two unassigned mutations, *git9-232* and *git10-201*, were both present in the FWP112 (h^-) background; therefore, crosses were done to obtain h^+ and h^- strains carrying these mutations, to be used in complementation and recombination analyses. These analyses demonstrated that these two mutations identify two separate complementation and linkage groups.

TABLE 4

Distribution of *git* mutations into complementation groups

Complementation group	No. in group	No. ts alleles
1	73	0
2	31	0
3	22	0
4	28	0
5	6	0
6	18	0
7	3	1
8	4	0
9	1	1
10	1	0

Complementation was determined by the sensitivity of diploids to 5FOA as described in MATERIALS AND METHODS. Strains carrying alleles *git2-210*, *git4-17* or *git10-201* displayed very slow growth at 30°.

A single case of intragenic complementation was observed for two *git2* mutant alleles. Random spore analysis and tetrad analysis (see MATERIALS AND METHODS) indicated that *git2-7* and *git2-61* were allelic as judged by the absence of git^+ recombinant progeny. However, a diploid strain heterozygous for these two mutations was phenotypically Git$^+$ (5FOA^R on 8% glucose), indicating that these mutations complemented each other. Complementation tests with a third *git2* mutant allele, *git2-210* (also shown to be allelic with *git2-7*), showed that both *git2-7/git2-210* and *git2-61/git2-210* diploids failed to complement (5FOA^S on 8% glucose). Therefore, we conclude that *git2-7* and *git2-61* are *git2* alleles that complement intragenically. Since complementation tests with every pairwise combination of mutations within each *git* complementation group have not been done, other cases of intragenic complementation may exist among *git* mutants.

Linkage analysis: Representatives from each complementation group were crossed with each other and with a git^+ strain, and tetrads were dissected and analyzed to determine if each complementation group represents a different gene. All ten complementation groups represent different genes as judged by the appearance of git^+ (5FOA^R) recombinant progeny. Furthermore, in crosses with git^+ strains, all *git* mu-

TABLE 5

β-Galactosidase expression in *git* mutants

Strain	*git* allele	Repressed	Derepressed
FWP101	*git*⁺	11 ± 2	2171 ± 530
FWP112	*git*⁺	15 ± 4	2687 ± 366
FWP113	*git1-1*	2553 ± 398	7360 ± 2185
CHP210	*git2-210*	2598 ± 631	4443 ± 81
CHP200	*git3-200*	432 ± 80	6738 ± 1882
CHP17	*git4-17*	144 ± 45	3076 ± 720
CHP75	*git5-75*	729 ± 104	4530 ± 1067
CHP261	*git6-261*	3435 ± 668	9820 ± 4697
CHP235	*git7-235*	1118 ± 206	2417 ± 1003
CHP60	*git8-60*	808 ± 146	3241 ± 681
CHP232	*git9-232*	164 ± 21	1409 ± 96
CHP201	*git10-201*	1009 ± 255	4564 ± 839

The values given represent specific activity ± standard errors of at least two independent cultures per strain. Cells were grown under repressed (8% glucose) and derepressed (0.1% glucose + 3% glycerol) conditions and assayed as described in MATERIALS AND METHODS.

tations tested segregated 2:2 and are therefore single nuclear mutations. Random spore analyses (see MATERIALS AND METHODS) were also performed to determine linkage between members of the various complementation groups, although representatives of all ten complementation groups were not included in these experiments. These analyses confirmed the gene assignments determined by complementation and tetrad dissection analyses for 150 of the 187 *git* mutations. By both tetrad and random spore analysis of different alleles within a single complementation group, we saw no evidence for unlinked noncomplementation (mutations within the same complementation group that are not allelic), although not all possible combinations of alleles were tested.

To investigate the possibility of interactions between *git* gene products, pairwise crosses between strains containing representative alleles of the ten *git* genes were examined for double mutant inviability. Tetrads that gave rise to four viable spores and that contained one or two *git*⁺ progeny (TT and NPD tetrads), thus also possessing *git* double mutants, were observed in all crosses. Therefore, we did not observe any instances of double mutant inviability.

β-Galactosidase activity in *git* mutants: To determine the degree to which mutations in the ten *git* genes affected expression from the *fbp1* promoter, we assayed β-galactosidase activity in representative *git* mutants grown to exponential phase under repressing (8% glucose) and derepressing (0.1% glucose+3% glycerol) conditions. Results (Table 5) demonstrate that all *git* mutants tested have elevated levels of β-galactosidase activity when grown under repressing conditions, varying from approximately 10-fold to greater than 200-fold above wild-type repressed levels. Mutations in *git1*, *git2* and *git6* have the greatest effect, mutations in *git5*, *git7*, *git8* and *git10* have a moderate effect, and mutations in *git3*, *git4* and *git9* have the weakest effect upon expression of the *fbp1-lacZ* fusion under repressing conditions. The alleles listed in Table 5 represent those having the strongest effects on *fbp1-lacZ* expression among mutant alleles tested for each *git* gene. β-Galactosidase levels under derepressing conditions were generally higher in the *git* mutants than in the *git*⁺ parents; however, the difference was never greater than four-fold. From these results, we conclude that the major effect of *git* mutations on *fbp1* expression is to allow elevated expression under normally repressing conditions.

Additional phenotypes conferred by *git* mutations: Some *git* mutants displayed other phenotypes in addition to effects on expression of the fusions. Strains carrying the *git7-235* or the *git9-232* allele were temperature sensitive for growth on YEA at 37°, while strains carrying the *git2-210*, *git4-17*, or *git10-201* allele grew very slowly at 30° (Table 4). *git4* mutants displayed a sporulation and/or germination defect: few tetrads produced four viable progeny when either parent carried a *git4* mutation. Poor spore viability was associated with all *git4* mutant alleles as evidenced by low numbers of progeny observed in random spore analyses. There was also poor spore viability in crosses when one parent carried the *git10-201* allele.

Effect of *git* mutations on wild-type *fbp1* transcription: To determine if the *git* mutations, isolated on the basis of their effect upon expression of the two fusions, altered transcript levels for the wild-type *fbp1* gene, we crossed mutant alleles of *git1* through *git10* into an *fbp1* background. These strains still possessed the *fbp1-lacZ* fusion to identify strains carrying the mutant *git* alleles, since many of the *git* mutations do not otherwise have an obvious phenotype. Northern hybridization analysis of these strains demonstrated that there are elevated levels of *fbp1* transcripts under repressing conditions for most classes of *git* mutants tested (Figure 2). These results are generally consistent with the effects seen on β-galactosidase expression shown in Table 5. Mutations in *git1*, *git2*, *git6*, *git7* and *git10* had the most dramatic effect, mutations in *git5* and *git8* had a more moderate effect, and mutations in *git3*, *git4* and *git9* had little or no detectable effect upon *fbp1* transcription.

DISCUSSION

By selecting for mutations that allow expression of an *fbp1-ura4* fusion under repressing conditions, we have identified ten genes (*git1*–*git10*) required for glucose repression of the *fbp1* gene of *S. pombe*. Since *git9* and *git10* are identified by single mutant alleles, it seems likely that we have not identified all of the genes involved in glucose repression of *fbp1*. Northern hybridization analysis demonstrates that *fbp1*

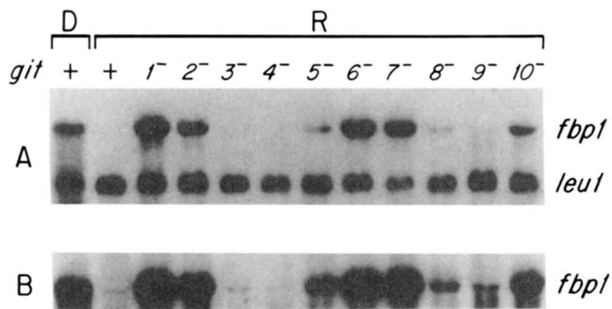

FIGURE 2.—Northern hybridization analysis of *fbp1* RNA in *git* mutants. Northern hybridization analysis of *fbp1* transcripts in a *git*$^{+}$ strain (FWP70) growing under derepressing (D) and repressing (R) conditions, and in *git1* through *git10* mutants (FWP134 through FWP143) grown under repressing conditions was performed as described in MATERIALS AND METHODS. The filter was hybridized to ^{32}P-labeled probes to detect *fbp1* (pAV06) and *leu1* (pYK311; as an internal standard) RNA. A, The filter was exposed to X-ray film for 24 hr. B, The filter was exposed to X-ray film for six days. Only the *fbp1* transcripts are shown in panel B.

transcript levels are elevated in *git* mutants, with the possible exceptions of *git3*, *git4* and *git9* mutants. Therefore, at least seven *git* gene products are required for glucose repression of transcription of the *fbp1* gene.

Some of the *git* mutants display phenotypes that cannot be simply due to overexpression of the *fbp1* gene, otherwise all constitutive mutants would display these phenotypes. Temperature sensitivity and reduced spore viability observed for some of the mutants indicate that certain *git* genes may also be involved in the transcription of other *S. pombe* genes. Therefore, while some *git* gene products may act only to regulate *fbp1* expression, some others may function as general transcription factors, while still others may function in a global glucose repression pathway. Since mutations in *git* genes lead to constitutive expression, the most likely role of *git* gene products is to repress *fbp1* transcription. These genes may encode actual repressors, modifiers or regulators of repressors, or they may encode proteins which act to establish a transcriptionally inactive chromatin structure at the *fbp1* promoter. Conceivably, some *git* mutations could affect mRNA stability. However, since *git* mutations alter expression of the two *fbp1* fusions as well as *fbp1* itself, any effect would have to be via the *fbp1* mRNA leader still present in the fusion constructs.

We assayed invertase activity in wild type and *git* mutant *S. pombe* strains to determine if any of these mutations caused an increase in invertase expression under repressing conditions, since invertase activity in *S. cerevisiae* is glucose repressed at the transcriptional level (CARLSON and BOTSTEIN 1982). Preliminary results indicate that invertase activity is regulated approximately 90-fold by glucose repression in *S. pombe* (8% glucose grown cells versus 0.1% glucose+3% glycerol grown cells), but that there is no more than a sixfold increase in invertase activity in any of the representative *git* mutants (the same strains as shown in Table 5) under repressing conditions (C. S. HOFFMAN and F. WINSTON, unpublished). However, until the gene(s) encoding invertase in *S. pombe* is cloned, we cannot conclude that any of the *git* mutations affect transcription in this system, or even that regulation of invertase in wild type *S. pombe* strains is at the transcriptional level.

Some *git* genes may function in cellular processes other than transcription and may therefore alter *fbp1* expression in different ways. Mutations that affect glucose uptake could lead to an apparent transcriptional defect. However, we doubt that any of the *git* mutations have a significant effect on glucose transport, since the *git* mutants are able to grow in a low glucose medium. Similarly, mutations that inhibit the cell's ability to detect glucose or create the glucose repression signal would alter transcription of *fbp1*. Mutations in the *HXK2* gene of *S. cerevisiae*, encoding hexokinase II, lead to constitutive expression of multiple glucose repressed systems (ENTIAN and ZIMMERMANN 1980; MA and BOTSTEIN 1986; NEIGEBORN and CARLSON 1987), although the actual role for hexokinase II in this pathway is not clear.

Transcription of *fbp1* in *git3*, *git4* and *git9* mutant strains grown under repressing conditions was similar to that in a *git*$^{+}$ strain (Figure 2). One possibility is that there is increased transcription in these mutants, but that the transcript levels are still too low for accurate detection. Alternatively, the Ura^{+} phenotype and the increased β-galactosidase activity observed in these mutants may not be due to a change in the level of transcription. An increase in translation of the *fbp1* open reading frame would lead to a Git^{-} phenotype, since both are translational fusions. An increase in stability of the fusion proteins in these mutants could also account for this phenotype. In any case, our results leave open the possibility that some components of glucose repression of *fbp1* expression do not operate at the transcriptional level.

Mutant searches for *S. cerevisiae* strains that constitutively express the *SUC2* gene have identified mutations in five genes (CARLSON *et al.* 1984; TRUMBLY 1986; NEIGEBORN and CARLSON 1987). Possibly, some *git* genes have similar functions to the genes identified in the *SUC2* studies. However, it may be misleading to look for similarities between *fbp1* regulation in *S. pombe* and *SUC2* regulation in *S. cerevisiae*. The fact that both genes are regulated by glucose repression at the transcriptional level does not dictate that the mechanism of regulation will be similar. For example, both yeasts display the phenomenon of mating type switching in which only one of the two cells resulting from cell division is competent to undergo the switch, yet the mechanisms for this asymmetry are very dif-

ferent in these two organisms (for a review, see KLAR 1989).

In this work, we have developed a system to identify *trans*-acting factors involved in regulation of transcription of the *fbp1* gene of *S. pombe*. The two fusions described are subject to glucose repression to a similar degree as the *fbp1* gene. The *fbp1-ura4* fusion allows selection for mutations that cause constitutive expression of the fusion under repressing conditions or the inability to express the fusion under derepressing conditions. Fusions to the *S. cerevisiae ura4* homologue, *URA3*, were previously described as a means to select for regulatory mutations (ALANI and KLECKNER 1987). The *fbp1-lacZ* fusion allows easy quantitation of the effects of the mutations and the determination of whether the mutations act in *cis* or in *trans*. In addition to the isolation of constitutive mutants, we can use this system to isolate mutants defective in derepression of *fbp1* transcription by isolating $5FOA^R$ strains grown under derepressing conditions and screening for reduced β-galactosidase activity.

Future efforts will include the molecular analysis of the *git* genes. We have recently cloned the *git2* gene by complementing the $5FOA^S$ phenotype of a mutant strain (C. S. HOFFMAN and F. WINSTON, unpublished data). Through the genetic and molecular analysis of *git* genes, along with a structural analysis of the *fbp1* promoter and the isolation and characterization of mutants defective in derepression of *fbp1* expression, we hope to elucidate the mechanism(s) of glucose repression in *S. pombe*.

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