Glucose repression of transcription of the *Schizosaccharomyces pombe* *fbp1* gene occurs by a cAMP signaling pathway

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Transcription of the *fbp1* gene, encoding fructose-1,6-bisphosphatase, of *Schizosaccharomyces pombe* is subject to glucose repression. Previous work has demonstrated that several genes (*git* genes) are required for this repression. In this report we demonstrate that one of these genes, *git2*, is the same as the *cyr1* gene, which encodes adenylate cyclase, and that loss-of-function mutations in *git2* cause constitutive *fbp1* transcription. Addition of cAMP to the growth medium suppresses the transcriptional defect in *git2* mutants as well as in strains that carry mutations in any of six additional *git* genes. Similarly, exogenous cAMP represses *fbp1* transcription in wild-type cells grown on a derepressing carbon source. Different levels of adenylate cyclase activity in different *git2* mutants, coupled with the result that some *git2* mutants display intragenic complementation, strongly suggest that adenylate cyclase acts as a multimer and that different *git2* mutations alter distinct activities of adenylate cyclase, including catalytic activity and response to glucose. Additional experiments demonstrate that this cAMP signaling pathway is independent of the *S. pombe* *ras1* gene and works by activation of cAMP-dependent protein kinase.

[Key Words: *Schizosaccharomyces pombe*; fructose-bisphosphatase; glucose repression; cAMP; adenylate cyclase; transcription]

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Cells regulate transcription in response to signals from their environment. For example, unicellular organisms transcribe specific genes to metabolize the carbon sources available. If glucose is also present, however, many genes required for the utilization of other carbon sources are no longer expressed. This phenomenon has been termed glucose repression or catabolite repression (Magasanik 1961).

Glucose repression appears to work through different mechanisms in the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. In *E. coli*, glucose causes a reduction in the level of cAMP that binds to and is required for activity of the catabolite gene activator protein (for review, see Botsford 1981). In *S. cerevisiae*, however, cAMP is not required for expression of genes subject to glucose repression (Matsumoto et al. 1982b, 1983) and cAMP levels are not reduced under conditions that derepress expression of such genes (Eraso and Gancedo 1984). In contrast, *S. cerevisiae* *ADH2* transcription appears to be repressed by cAMP through the phosphorylation and inactivation of the transcriptional activator ADRI by cAMP-dependent protein kinase (cAPK; Cherry et al. 1989; Taylor and Young 1990). In *S. cerevisiae*, glucose repression involves a variety of mechanisms, as different mutations have been isolated that

allow constitutive expression of distinct subsets of glucose repressible genes (for review, see Gancedo and Gancedo 1986; Johnston 1987).

In the yeast *Schizosaccharomyces pombe*, the *fbp1* gene, encoding the gluconeogenic enzyme fructose-1,6-bisphosphatase, is subject to glucose repression (Vassarotti and Friesen 1985; Hoffman and Winston 1989, 1990). We have observed that the level of *fbp1* transcription can vary over a range of >200-fold with respect to the carbon source. Using a pair of translational fusions to *fbp1*, we have isolated mutants in which transcription of an *fbp1-ura4* fusion is constitutive. These mutations identified 10 genes, designated *git* (glucose-insensitive transcription) (Hoffman and Winston 1990). Mutations in 8 of these 10 genes cause a significant increase in *fbp1* transcription under normally repressing conditions (see Fig. 2, below; Hoffman and Winston 1990). We have begun a molecular analysis of glucose repression by cloning and analyzing one of these *git* genes.

In this report we demonstrate that the *git2* gene, mutations in which allow high-level constitutive *fbp1* transcription, is identical to the *cyr1* gene, which encodes adenylate cyclase (Yamawaki-Kataoka et al. 1989; Young et al. 1989). This enzyme functions to convert ATP to cAMP in response to external stimuli and is involved in many signaling pathways in eukaryotic cells (Gilman 1984; Levitzki 1988). In these signaling pathways the role of cAMP is to activate cAPK, which is composed of

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a pair of regulatory subunits associated with a pair of catalytic subunits. cAMP binds the regulatory subunits of cAPK, causing them to dissociate from and thereby activate the catalytic subunits of cAPK, which are now able to phosphorylate target proteins (Beebe and Corbin 1986; Edelman et al. 1987). Some known target proteins are enzymes that are inactivated by phosphorylation, whereas others are transcriptional activators (Beebe and Corbin 1986; Roesler et al. 1988).

In *S. cerevisiae*, many genes have been identified that are involved in the adenylate cyclase pathway. Adenylate cyclase is encoded by the *CYR1* gene (Casperson et al. 1985), whereas the upstream activators are encoded by *RAS1* and *RAS2* (Broek et al. 1985; Toda et al. 1985). *CDC25* encodes a positive regulator of RAS activity required for adenylate cyclase activation (Broek et al. 1987; Robinson et al. 1987), whereas *IRA1* (Tanaka et al. 1989) and *IRA2* (Tanaka et al. 1990) encode negative regulators of RAS activity. The *CYR1* gene product is associated with a 70-kD protein encoded by the *CAP/SRV2* gene (Fedor-Chaiken et al. 1990; Field et al. 1990). The 70-kD protein is also required for RAS activation of adenylate cyclase. The regulatory subunit of cAPK is encoded by *BCY1*, mutations in which bypass the requirement for active adenylate cyclase (Matsumoto et al. 1982a). The catalytic subunits of cAPK are encoded by each of the *TPK1*, *TPK2*, and *TPK3* genes (Toda et al. 1987b).

In *S. pombe*, less is known about the adenylate cyclase pathway. The genes that encode adenylate cyclase (*cyr1*; Yamawaki-Kataoka et al. 1989; Young et al. 1989) and the regulatory subunit of cAPK (*cgs1*; M. McLeod, pers. comm.) have been identified. An *S. pombe* RAS gene homolog, *ras1*, has been identified (Fukui and Kaziro 1985; Nadin-Davis et al. 1986a); however, in contrast to *S. cerevisiae*, *ras1* does not appear to stimulate adenylate cyclase activity in *S. pombe* (Fukui et al. 1986; Nadin-Davis et al. 1986b; for review, see Nadin-Davis et al. 1989).

Additional experiments in this report demonstrate that *git2* (*cyr1*), other *git* genes, and *cgs1* are required for normal glucose-mediated regulation of *fbp1* transcription. As expected, *ras1* does not play a role in *fbp1* regulation. In addition, intragenic complementation between various *git2* mutant alleles and differential effects on adenylate cyclase activity (as assayed in vitro) and cAMP levels by these mutations indicate that adenylate cyclase is a multimer and that *git2* mutations cause constitutive *fbp1* transcription by altering distinct functions of the protein. These results lead to the conclusion that *fbp1* transcription is regulated by a cAMP signaling pathway, independent of *ras1*, and that the role of the cAMP signal in *S. pombe* glucose repression is to activate cAPK.

Results

Cloning of the *git2* gene as a high copy suppressor of a *git1* mutation

Previous work had identified a large number of *git* genes required for glucose repression of *fbp1* transcription (Hoffman and Winston 1990). To initiate a molecular

analysis of the *git* genes, we set out to clone the *git1* gene. Mutations in *git1* allow constitutive transcription of the wild-type *fbp1* gene, as well as constitutive expression of both *fbp1-ura4* and *fbp1-lacZ* translational fusions, under normally repressing conditions (Hoffman and Winston 1990). Because cells that express the *fbp1-ura4* fusion are sensitive to the pyrimidine analog 5-fluoro-orotic acid (5FOA; Boeke et al. 1984; Hoffman and Winston 1990), *git1*[−] mutants that contain the *fbp1-ura4* fusion are 5FOA sensitive (5FOA^S; *Git*[−] phenotype) when grown under repressing conditions (8% glucose as the carbon source); *git1*⁺ strains are 5FOA resistant (5FOA^R; *Git*⁺ phenotype). We therefore screened an *S. pombe* genomic library in a high copy number vector for plasmids that could transform a *git1*[−] *fbp1-ura4* strain (*Git*[−]) to *Git*⁺ (see Materials and methods). After screening 17,000 transformants, we obtained one plasmid (pCHY26) that conferred a strong *Git*⁺ (5FOA^R) phenotype.

Three pieces of evidence demonstrated that pCHY26 encodes *git2* rather than *git1*. First, when pCHY26 was integrated into a *git1*[−] strain, the integrant unexpectedly remained *Git*[−], demonstrating that the cloned DNA, when present in low copy number, could not complement the *git1-1* mutation. Second, by tetrad analysis, the *LEU2* gene on pCHY26 was unlinked to *git1*, demonstrating that the plasmid had not integrated at the *git1* locus. Third, additional crosses by other *git* mutants demonstrated that pCHY26 had directed integration to the *git2* locus (see Materials and methods). To rule out the possibility that plasmid pCHY26 had integrated by nonhomologous recombination at a site fortuitously linked to *git2*, we also integrated a smaller derivative of pCHY26, pCHY27 (Fig. 1), and demonstrated by Southern hybridization analysis and linkage analysis that it had integrated by homologous recombination at the *git2* locus (data not shown). Therefore, pCHY26 carries the *git2* gene, and *git2* is a high copy number suppressor of a *git1* mutation.

Because *git2* is a high copy number suppressor of a *git1* mutation, we transformed strains carrying mutations in *git1-git10* (FWP113, CHP210, CHP200, CHP17, CHP75, CHP261, CHP235, CHP60, CHP232, CHP201) with pCHY26 to determine whether other *git* mutations could be suppressed by *git2* in high copy number. Because each strain also contained the *fbp1-ura4* fusion, suppression was assayed by monitoring the 5FOA phenotype of each transformant. Strains that carry a mutation in *git1*, *git2*, *git3*, *git5*, *git7*, *git8*, or *git10* were suppressed by high copy number *git2* (5FOA^R), whereas transformants carrying a mutation in *git4*, *git6*, or *git9* were not suppressed (5FOA^S). These results suggest that the *git2* gene product may act downstream of the gene products of *git1*, *git3*, *git5*, *git7*, *git8*, and *git10*. The gene products of *git4*, *git6*, and *git9* may act either downstream of or in a separate pathway from the *git2* gene product.

The *git2* gene encodes adenylate cyclase

Molecular analysis of the cloned *git2* gene has shown

Figure 1. Restriction map, transcription units, and subclones of the *git2* clone. The insert DNA present in the original *git2* clone, pCHY26, and two nonfunctional subclones are shown. The insert DNA in pCHY26 is an ~10.5-kb *HindIII* fragment, with additional internal *HindIII* sites not shown. *SalI* (S), *BamHI* (B), *BglII* (Bg), and *EcoRI* (R) sites are marked. Function was determined by the ability of the plasmid to transform a *git2* mutant strain from 5FOA^S to 5FOA^R. The size, orientation, and approximate positions of the two transcripts that hybridize to pCHY26 were determined by a series of Northern hybridization analyses (data not shown). The longer transcript (right) is the *git2* transcript.

that it is identical to the previously identified *cyr1* gene, which encodes adenylate cyclase (Yamawaki-Kataoka et al. 1989; Young et al. 1989). Plasmid pCHY28 (Fig. 1), a derivative of pCHY26 in which a 2.8-kb *BglII* fragment has been removed, was unable to transform *git2* mutant strains to Git⁺. We, therefore, assumed that the *git2* gene must overlap some, if not all, of this portion of the clone and initiated DNA sequencing around the left-most *BglII* site (Fig. 1).

Determination of 340 bp of DNA sequence gave a sequence identical to that of part of the coding region for the adenylate cyclase catalytic domain obtained by Yamawaki-Kataoka et al. (1989; EMBL/GenBank file name Yspcyr1a.pl; base pairs 4084–4235 and 4261–4448) and by Young et al. (1989; EMBL/GenBank file name Yspad-c.pl; base pairs 5485–5636 and 5662–5849). Restriction mapping of pCHY26 with nine restriction endonucleases (data not shown) was consistent with our conclusion that *git2* and *cyr1* are the same gene.

To verify that *git2* and *cyr1* are the same gene and to determine whether the Git⁻ phenotype can be caused by loss of adenylate cyclase activity, we constructed *git2* (*cyr1*) null strains. In these *S. pombe* strains, FWP190 and FWP191, the wild-type gene is replaced by DNA that contains a deletion of a *BglII* fragment internal to *git2* (see Materials and methods; Fig. 1). This deletion mutation, designated *git2-1*, removes part of the coding region for the catalytic domain of adenylate cyclase and should cause the remainder of the gene to be translated out of frame. Northern hybridization analysis of RNA from strain FWP191 indicates that *git2-1* only alters the 5400-nucleotide *git2* (*cyr1*) transcript and has no effect on a 1600-nucleotide transcript > 700 bp from the *BglII* deletion (data not shown). Therefore, the *git2* (*cyr1*) gene is the only gene affected by the *BglII* deletion.

Strains that contain the *git2-1* null allele display a Git⁻ phenotype and constitutively express the *fbp1-lacZ* fusion (FWP191; Table 1). Membrane extracts made from strain FWP191 (*git2-1*) lack any detectable adenylate cyclase activity (Table 2). Therefore, loss of *git2* function (adenylate cyclase) causes a Git⁻ phenotype. This null allele fails to complement other *git2* mutant alleles, confirming that the other *git2* mutations are in the *cyr1* gene. In contrast to *S. cerevisiae*, *S. pombe* haploid strains that contain a null mutation in *git2* (*cyr1*) are viable in the absence of cAMP. This result has also been observed by Maeda et al. (1990) and Kawamukai et al. (1991).

cAMP represses *fbp1* transcription in *git* mutant and wild-type strains

Because adenylate cyclase acts to convert ATP to cAMP, and as mutations in the gene that encodes adenylate cyclase cause constitutive transcription of the *fbp1* gene, we examined the effect of exogenous cAMP on transcription of the *fbp1* gene. Our results demonstrate that in most *git* mutants and in wild-type strains, cAMP alters *fbp1* transcription. The addition of 5 mM cAMP represses transcription of the *fbp1* gene in a *git2* mutant strain as well as in *git1*, *git3*, *git5*, *git7*, *git8*, or *git10* mutants (Fig. 2). Exogenous cAMP, however, has no detectable effect on *fbp1* transcription in a *git6* mutant (Fig. 2). Because the *git4* and *git9* mutations do not cause a detectable increase in *fbp1* transcription, we were unable to determine whether there is an effect of addition of exogenous cAMP on *fbp1* transcription in *git4* or *git9* mutants.

Expression of an *fbp1-lacZ* fusion in most *git* mutants is also repressed by cAMP (Table 1). β -Galactosidase assay results demonstrate that the addition of cAMP sup-

Table 1. *fbp1-lacZ* expression in *git* mutants $\pm$ cAMP

Strain	<i>git</i> genotype	β -Galactosidase activity in 8% glucose ^a	
		- cAMP	+ cAMP
FWP70	<i>git</i> ⁺	17 $\pm$ 5	13 $\pm$ 3
FWP134	<i>git1-1</i>	1590 $\pm$ 121	34 $\pm$ 3
FWP191	<i>git2-1</i>	3486 $\pm$ 432	24 $\pm$ 4
FWP135	<i>git2-210</i>	1994 $\pm$ 354	27 $\pm$ 5
FWP136	<i>git3-200</i>	505 $\pm$ 107	37 $\pm$ 15
FWP173	<i>git4-17</i>	39 $\pm$ 11	36 $\pm$ 3
FWP174	<i>git5-75</i>	848 $\pm$ 161	21 $\pm$ 3
FWP139	<i>git6-261</i>	2396 $\pm$ 71	1674 $\pm$ 114
FWP140	<i>git7-235</i>	1672 $\pm$ 301	94 $\pm$ 11
FWP175	<i>git8-60</i>	859 $\pm$ 195	20 $\pm$ 3
FWP142	<i>git9-232</i>	100 $\pm$ 27	103 $\pm$ 12
FWP143	<i>git10-201</i>	1106 $\pm$ 321	20 $\pm$ 2

^aThe values given represent β -galactosidase sp. act. $\pm$ S.E. for two to four independent cultures. Strains were grown overnight to 1×10^7 cells/ml in YEL (8% glucose) in the presence or absence of 5 mM cAMP and assayed.

Table 2. Adenylate cyclase activity and cAMP levels in *git2* mutants

Strain	<i>git2</i> allele	Adenylate cyclase activity ^a	cAMP levels ^b	
			repressed	derepressed
FWP70	<i>git2</i> ⁺	10.4 ± 0.3	3.6 ± 0.1	3.4 ± 0.6
FWP135	<i>git2-210</i>	8.5 ± 1.5	2.5 ± 0.3	ND
FWP182	<i>git2-7</i>	8.6 ± 0.8	5.7 ± 0.1	ND
FWP183	<i>git2-13</i>	0.3 ± 0.2	0.9 ± 0.1	ND
FWP184	<i>git2-61</i>	1.4 ± 0.5	2.8 ± 0.2	ND
FWP191	<i>git2-1</i>	<0.1	0.4 ± 0.1	ND

^aThe values given represent sp. act. ± S.E. for three to four independent cultures assayed as described in Materials and methods. One unit of adenylate cyclase activity represents the production of 1 pmole of cAMP/min per mg of protein.

^bcAMP levels were measured as described in Materials and methods for cultures grown in PM medium under repressing (8% glucose) or derepressing (0.1% glucose + 3% glycerol) conditions. Values given represent pmoles of cAMP/mg of total protein ± S.E. for two independent cultures. The low value for cAMP measurements in the *git2-1* strain was still observed after treatment of the extract with phosphodiesterase (data not shown). Therefore, this strain does not produce any detectable cAMP. (ND) Not determined.

presses the constitutive expression of the *fbp1-lacZ* fusion in the same set of *git* mutants as indicated by the Northern blot analysis of the *fbp1* transcript. Exogenous cAMP has little or no effect on β-galactosidase activity in *git4*, *git6*, or *git9* mutant strains.

Addition of exogenous cAMP also represses *fbp1* transcription in both *git2*⁺ and *git2-210* strains grown under derepressing conditions (Fig. 3). Similarly, cAMP addition represses expression of an *fbp1-lacZ* fusion in a *git*⁺ strain (FWP70) grown under derepressing conditions: This strain contained 1549 ± 47 units of β-galactosidase activity (sp. act. ± S.E. for four independent liquid cultures) in the absence of cAMP and only 122 ± 25 units in the presence of cAMP. In addition, cAMP caused cell death when FWP70 was grown in the presence of 0.1% glucose and 3% glycerol (data not shown), probably due to the inability of the cells to use glycerol as a carbon source owing to the simulation of glucose repression by cAMP. These results demonstrate that exogenous cAMP is sufficient for repression of transcription from the *fbp1* promoter in a wild-type strain grown under normally derepressing conditions.

git2 intragenic complementation

We determined previously that diploids formed from strains carrying the *git2-7* and *git2-61* alleles have a *Git*⁺ phenotype, indicating intragenic complementation (Hoffman and Winston 1990). Therefore, strains that contain the *git2-7* and *git2-61* alleles have been tested for their ability to complement strains carrying 31 independent *git2* mutations. Diploids carrying either the *git2-7* or *git2-61* allele on one homolog and each of the 31 *git2* alleles on the other homolog were tested for complemen-

tation as described in Materials and methods. We also tested all combinations of the 17 original *h*[−] *git2* isolates by the 14 original *h*⁺ *git2* isolates for intragenic complementation.

On the basis of these complementation tests, 11 of the 31 *git2* mutants display at least some intragenic complementation. Eight members form one group, and three members form the second group. The remaining 20 spontaneous *git2* mutant alleles, as well as the *git2-1* null allele, do not complement any *git2* allele. The first complementation group consists of three strongly complementing alleles, *git2-7*, *git2-110*, and *git2-266*, four moderately complementing alleles, *git2-18*, *git2-68*, *git2-216*, and *git2-226*, and one weakly complementing allele, *git2-210*. The second complementation group consists of the strongly complementing allele *git2-61* and two moderately complementing alleles, *git2-29* and *git2-83*. An example of the complementation analysis is shown in Figure 4. Because most spontaneous *git2* mutant alleles do not complement alleles of either complementation group, these groups represent intragenically complementing alleles and not mutations in two tightly linked genes.

Adenylate cyclase activity and cAMP levels in *git2* mutant strains

To determine whether all *git2* mutations cause a loss or reduction of adenylate cyclase activity, adenylate cyclase activity was assayed in membrane extracts from *git*⁺ and a set of *git2* mutant strains. These assays were performed in the presence of Mn²⁺, as in vitro activation of *S. pombe* adenylate cyclase in the presence of Mg²⁺ has not been observed (Yamawaki-Kataoka et al. 1989; Engelberg et al. 1990; C.S. Hoffman and F. Winston, unpubl.). The results of the assays (Table 2) show that the *git2-61* and *git2-13* mutations significantly reduce adenylate cyclase activity, whereas the *git2-1* null allele causes the complete loss of adenylate cyclase activity. The *git2-7* and *git2-210* mutations, however, have little or no effect on adenylate cyclase activity as measured in vitro. Although the four spontaneous *git2* mutations have different effects on adenylate cyclase activity, they all increase *fbp1-lacZ* expression to a similar high constitutive level (data not shown). Therefore, *git2* alleles that confer very different levels of adenylate cyclase activity as measured in vitro all confer a similar *Git*[−] phenotype in vivo.

To further analyze the different classes of *git2* mutants, we also measured steady-state cAMP levels in wild-type and *git2* mutant strains (Table 2). The *git2-1* (null) and *git2-13* mutants both have greatly reduced levels of cAMP. (The low value that we obtained for cAMP measurements in the *git2-1* strain was still observed even after treatment of the extract with phosphodiesterase (data not shown). Therefore, this strain does not produce any detectable cAMP.) The other *git2* mutants tested do not have significantly reduced cAMP levels. In addition, steady-state cAMP levels in a *git2*⁺ strain are similar for growth under conditions repressing or dere-

Figure 2. Exogenous cAMP represses *fbp1* transcription in most *git* mutant strains. Northern hybridization analysis was performed on RNA from a *git*⁺ strain (FWP70) and from strains carrying mutations in *git1*–*git10* (FWP134, FWP135, FWP136, FWP173, FWP174, FWP139, FWP140, FWP175, FWP142, and FWP143; see Table 1 for corresponding *fbp1*–*lacZ* expression), grown under repressing conditions (8% glucose) in the presence and absence of 5 mM cAMP. The filter was hybridized to ³²P-labeled plasmids pAV06 (to detect *fbp1* RNA; Vassarotti and Friesen 1985) and pYK311 (to detect *leu1* RNA as an internal standard; Kikuchi et al. 1988), and exposed to X-ray film.

pressing for *fbp1* expression. Therefore, although some *git2* alleles abolish or reduce cAMP levels and cause a *Git*[−] phenotype, some other *git2* alleles cause a *Git*[−] phenotype, although they cause little change in steady-state cAMP levels.

fbp1 transcription is controlled by a cAMP-dependent protein kinase

In other eukaryotic cAMP signaling pathways, the role of cAMP is to activate cAMP-dependent protein kinase (cAPK), which then phosphorylates target proteins (Beebe and Corbin 1986). To determine whether the role of cAMP in *S. pombe* glucose repression is to activate cAPK, we examined *fbp1* transcription in a strain containing a mutation in the *cgs1* gene that encodes the regulatory subunit of cAPK (M. McLeod, pers. comm.). On the basis of constitutive transcription of *fbp1* in a strain lacking adenylate cyclase activity, if *cgs1* is in the same pathway, a *cgs1* mutation would be expected to reduce or eliminate *fbp1* expression in derepressed cells. For this experiment we used a mutant that contains the spontaneous *cgs1*–1 mutation (M. McLeod, pers. comm.). The results demonstrate that *cgs1*–1 reduces *fbp1* transcription in a strain grown under derepressing conditions (0.1% glucose and 3% glycerol; Fig. 5). Furthermore, the *cgs1*–1 mutation greatly reduces *fbp1*–*lacZ* expression in

strains grown under derepressing conditions (3% maltose; Table 3). These results strongly suggest that cAMP represses *fbp1* transcription by the activation of cAPK. The more dramatic effect of the *cgs1* mutation observed in Table 3, relative to Figure 5, could be due to the fact that 3% maltose is a less derepressing carbon source than 0.1% glucose plus 3% glycerol.

To characterize the role of *cgs1* in this pathway in greater detail, we carried out epistasis tests with *cgs1* and *git2*, and with *cgs1* and *git6*. As expected, the *cgs1*–1 *git2*–1 double mutant resembled the *cgs1*–1 mutant (Table 3), that is, the *cgs1*–1 mutation suppressed the constitutive expression of the *fbp1*–*lacZ* fusion conferred by the *git2*–1 allele. This result supports the idea that glucose repression of *fbp1* is mediated by cAPK. Interestingly, the *cgs1*–1 *git6*–261 double mutant is constitutive for expression of *fbp1*–*lacZ*, consistent with the observation that the *git6*–261 mutation is not suppressed by exogenous cAMP. This epistasis test suggests that the

Figure 3. Exogenous cAMP represses *fbp1* transcription in a *git2*⁺ and a *git2*[−] mutant strain grown on a derepressing carbon source. Northern hybridization analysis was performed on RNA from FWP70 (*git2*⁺) and from FWP135 (*git2*–210) grown on a repressing carbon source (8% glucose) and a derepressing carbon source (3% maltose), in the presence and absence of 5 mM cAMP, and probed as described in Fig. 2.

Figure 4. Certain *git2* mutant alleles display strong intragenic complementation. Complementation analyses were performed as described in Materials and methods. Two diploids from each cross were colony-purified and replica-plated to media with and without 5FOA at 30°C. Diploids that contain a *git2*⁺ allele are 5FOA^R, demonstrating that the *git2* mutations are recessive. Diploids that contain two *git2* mutations are usually 5FOA^S (noncomplementation), except those that contain *git2*–7 with *git2*–61, which are 5FOA^R (growth on 5FOA by day 3; strong complementation) and those that contain *git2*–210 with *git2*–61, which are weakly 5FOA^R (growth on 5FOA by day 7; weak complementation). Rare, individual 5FOA^R colonies that appear in 5FOA^S patches likely represent events other than complementation, such as mitotic recombination at *git2*, second site suppressor mutations, or reversion events.

Figure 5. *fbp1* transcription is reduced in a *cgs1* mutant strain and is normal in strains carrying *ras1* mutations. Northern hybridization analysis was performed on RNA from strains FWP180 (*cgs1*⁺ *ras1*⁺), FWP177 (*cgs1*⁻¹), FWP179 (*ras1*⁻), and FWP181 (*ras1*^{val17}; activated form) grown under repressing (8% glucose) and derepressing (0.1% glucose and 3% glycerol) conditions. Additional bands present in lanes containing FWP180, FWP179, and FWP181 RNAs are probably due to transcription of the *LEU2* gene integrated adjacent to *ras1*.

git6 gene product does not normally participate in the stimulation of adenylate cyclase but, rather, acts more directly in repression of *fbp1* transcription.

Mutations in *ras1* do not alter *fbp1* transcription

S. cerevisiae adenylate cyclase is activated by the products of the *RAS1* and *RAS2* genes (Toda et al. 1985). In *S. pombe*, however, experiments indicate that the *ras1* gene does not carry out a similar function (Fukui et al. 1986; Nadin-Davis et al. 1986b, 1989). To investigate further the role of *ras1* with respect to adenylate cyclase activity, we examined *fbp1* transcription in strains that contain either a *ras1*⁻ or a *ras1*^{val17} (activated) mutation (Nadin-Davis et al. 1986b). Northern hybridization analysis (Fig. 5) demonstrates that there is wild-type regulation of *fbp1* transcription in both strains. This result demonstrates further that *S. pombe* *ras1* does not regulate adenylate cyclase activity.

Discussion

Our previous work identified a set of genes required for glucose repression of *fbp1* transcription in *S. pombe*

Table 3. *fbp1-lacZ* expression in *cgs1*-1, *cgs1*-1 *git2*-1 and *cgs1*-1 *git6*-261 mutants

Strain	Relevant genotype	β-Galactosidase activity ^a	
		8% glucose	3% maltose
FWP189	<i>cgs1</i> ⁺ <i>git</i> ⁺	12 ± 0	1508 ± 212
FWP191	<i>git2</i> -1	3486 ± 432	5444 ± 1043
FWP139	<i>git6</i> -261	2396 ± 71	7400 ± 2413
FWP192	<i>cgs1</i> -1	21 ± 6	118 ± 8
FWP193	<i>cgs1</i> -1 <i>git2</i> -1	18 ± 5	116 ± 14
FWP194	<i>cgs1</i> -1 <i>git6</i> -261	3417 ± 264	4621 ± 1078

^aThe values given represent β-galactosidase sp. act. ± S.E. for two or three independent cultures. Strains were grown overnight in YEL to 1 × 10⁷ cells/ml, with either 8% glucose (repressing) or 3% maltose (derepressing) as the carbon source, and assayed.

(Hoffman and Winston 1990). Our present work demonstrates that one of these genes, *git2*, is the same as the previously identified *cyr1* gene, encoding adenylate cyclase, and that cells that lack this activity are unable to repress *fbp1* transcription. Our experiments also demonstrate that exogenous cAMP represses *fbp1* transcription in both wild-type and many, but not all, *git* mutant strains. These results strongly suggest that *S. pombe* responds to glucose by activating adenylate cyclase as part of a cAMP signaling pathway.

The adenylate cyclase in vitro assays performed here almost certainly represent the unstimulated activity of adenylate cyclase and do not reflect the ability of adenylate cyclase to respond to in vivo stimuli. Adenylate cyclase activity from *S. pombe* is not stimulated by the addition of guanine nucleotides in vitro, and activity has only been observed when assayed in the presence of Mn²⁺; activity in the presence of Mg²⁺ has not been detected (Yamawaki-Kataoka et al. 1989; Engelberg et al. 1990; C.S. Hoffman and F. Winston, unpubl.). Our results suggest that the addition of glucose may cause a post-translational modification of adenylate cyclase that stimulates activity in vivo but that is not detectable by current in vitro assays. Clearly, an in vitro assay that reflects more clearly in vivo activity and stimulation will be necessary to allow a more accurate assessment of the adenylate cyclase defects in the different *git2* mutants.

Differences in the adenylate cyclase levels among *git2* mutants and the observation of *git2* intragenic complementation suggest that *S. pombe* adenylate cyclase contains separable domains and that it functions in a complex containing two or more copies of the *git2* (*cyr1*) gene product. Genetic evidence for such a complex in *S. cerevisiae* has been obtained by Field et al. (1990b). That mutations in each *git2* intragenic complementation group represent alterations of different functional domains of adenylate cyclase is consistent with results of adenylate cyclase assays for different *git2* mutants (Table 2). Two strains that contain mutations in the first complementation group (*git2*-7 and *git2*-210) possess virtually wild-type levels of adenylate cyclase activity as measured in vitro and are therefore unlikely to affect directly the adenylate cyclase catalytic domain. In contrast, a strain that contains a mutation in the second complementation group (*git2*-61) possesses significantly reduced adenylate cyclase activity as does a strain that contains a mutation that fails to complement members of either group (*git2*-13).

The cAMP levels in some *git2* mutants are reduced relative to that of a wild-type strain (Table 2), supporting the hypothesis that these mutations cause a reduction in the ability of the cell to produce cAMP in response to glucose. The facts that a *git2* null mutant, which makes no detectable cAMP, causes derepression of *fbp1* transcription, and that addition of exogenous cAMP restores repression, demonstrate that cAMP causes *fbp1* repression. The result that some *git2* mutants do not have reduced cAMP levels may reflect feedback control of cAMP levels as in *S. cerevisiae* (Nikawa et al. 1987). In

addition, wild-type cultures grown under repressing and derepressing conditions possess similar steady-state levels of cAMP. These results suggest that the steady-state level of cAMP is not altered by glucose repression. Rather, the cAMP signal is likely to be transient in cells exposed to glucose, as is the case in *S. cerevisiae* (Mbonyi et al. 1988; Thevelein 1988). Measurement of cAMP levels immediately after glucose addition in wild-type and *git* strains will test whether cAMP levels are altered in response to glucose.

These studies provide us with likely candidates for genes required for the activation of adenylate cyclase in *S. pombe*. These candidates include the genes *git1*, *git3*, *git5*, *git7*, *git8*, and *git10*, as the *Git*[−] phenotype caused by mutations in these genes is suppressed by exogenous cAMP and by the *git2* gene in high copy number. One or more of these *git* genes may encode subunits of a trimeric guanine nucleotide-binding protein (G protein) that stimulates adenylate cyclase activity, as has been observed for mammalian cells (Gilman 1984). Because the G protein may have to assemble and/or couple to the glucose receptor before stimulation of adenylate cyclase, mutations in any of the genes encoding G protein subunits could produce a *Git*[−] phenotype. One or more *git* genes may also encode a glucose receptor, proteins that modulate G protein activity [analogous to *CDC25* of *S. cerevisiae* (Broek et al. 1987; Robinson et al. 1987)], or additional subunits of the active adenylate cyclase complex [analogous to the 70-kD adenylate cyclase-associated protein of *S. cerevisiae* (Fedor-Chaiken et al. 1990; Field et al. 1990a)]. Alternatively, stimulation of adenylate cyclase in *S. pombe* may occur by a different type of pathway.

Glucose repression of *fbp1* in *S. pombe* may be analogous in some ways to glucose repression of *ADH2* in *S. cerevisiae*. In *S. cerevisiae*, studies have demonstrated that glucose induces a transient cAMP signal and that this response requires a functional *RAS1* or *RAS2* gene (Mbonyi et al. 1988; Thevelein 1988). The cAMP signal activates cAPK; for *ADH2*, glucose repression occurs via this pathway, as cAPK appears to phosphorylate and inactivate the *ADH2* transcriptional activator ADR1 (Cherry et al. 1989; Taylor and Young 1990). Cherry et al. (1989) have also shown that mutations in *BCY1* (encoding the regulatory subunit of cAPK) reduce expression of *ADH2*. The result that a mutation in *S. pombe cgs1* (encoding the regulatory subunit of cAPK) reduces *fbp1* derepression indicates that the role of cAMP in glucose repression of *fbp1* is to stimulate cAPK. (Interestingly, the ability of a *git2-1 cgs1-1* strain to show mild derepression of *fbp1-lacZ* expression (Table 3) suggests that glucose also causes repression of *fbp1* transcription by a cAMP-independent mechanism.)

From our results thus far, we cannot conclude that cAPK acts to repress *fbp1* transcription by the same mechanism as seen for *ADH2* in *S. cerevisiae*. Mutations in *git6* are not suppressed by exogenous cAMP or by a mutation in *cgs1* (Fig. 3; Table 3). Possibly, *git6* encodes the catalytic subunit of cAPK that inactivates the transcriptional activator of *fbp1*. However, the *git6* gene

could also encode a transcriptional repressor that is activated by cAPK. The cAMP-responsive element-binding protein (CREB) from rat can act as either a transcriptional activator or a repressor, depending on its phosphorylation state, at the *c-jun* promoter (Lamph et al. 1990).

Previous work has shown that transcription of the *mei2* gene of *S. pombe* is also repressed by cAMP (Watanabe et al. 1988). This gene is required for entry into meiosis and is inducible by nitrogen starvation; however, the role of cAMP in meiosis is still unclear (for review, see McLeod 1989). Therefore, *S. pombe* may regulate cAMP levels with respect to both the carbon source and the presence of a nitrogen source. It is likely that additional factors are involved that distinguish between response to glucose and response to a nitrogen source.

Our future work will address how adenylate cyclase is activated in *S. pombe* and how cAPK acts to repress *fbp1* transcription. By continued molecular and genetic analyses of *git* genes, and by analysis of the *fbp1* promoter, we hope to further our understanding of the role of signal transduction and transcriptional repression of *fbp1* expression.

Materials and methods

Yeast strains and media

All *S. pombe* strains used in this study are listed in Table 4. Genetic nomenclature of *S. pombe* follows rules proposed by Kohli (1987). The *fbp1::ura4* allele is a translational fusion that disrupts the *fbp1* gene, and the *ura4::fbp1-lacZ* allele is a disruption of the *ura4* gene by the *fbp1-lacZ* translational fusion (Hoffman and Winston 1990). Standard rich media YEA and YEL (Gutz et al. 1974) were supplemented with 2% casamino acids. Minimal media (SD) supplemented with amino acids and synthetic complete media (SC) lacking a specific amino acid (Sherman et al. 1978) were used with modifications described previously (Hoffman and Winston 1990). PM medium (defined minimal medium; Watanabe et al. 1988) was supplemented with required amino acids. Carbon sources were generally present at a concentration of 3%, and strains were grown at 30°C, unless otherwise specified. Sensitivity to 5FOA (PCR, Inc.) was determined on SC, SC-leu, and SC-ade solid media containing 8% glucose, 50 mg/liter of uracil, and 0.4 g/liter of 5FOA. When indicated, cAMP (Aldrich Chemical Company, Inc.) was added to a final concentration of 5 mM. Crosses were done on either MEA (Gutz et al. 1974) containing 0.4% glucose or on YPD (Sherman et al. 1978).

Recombinant DNA methodology

Standard recombinant DNA techniques (including DNA restriction digests, ligations, and bacterial transformations) were done according to Maniatis et al. (1982). *E. coli* strains HB101 (Boyer and Roulland-Dussoix 1969) and JM101 (Messing 1979) were the host strains 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). Small-scale DNA preparations from yeast to recover plasmids in *E. coli* were done as described previously (Hoffman and Winston 1987). DNA fragment isolation was done by electroelution. Restriction endonucleases *Sall* and *XhoI* were purchased from Boe-

Table 4. *Strains*

Strain	Genotype
CHP1	<i>h⁺ ade6-M216 lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git1-1</i>
FWP148	<i>h⁺ lys1-131 leu1-32 ura4::fbp1-lacZ fbp1::ura4 [pCHY26]</i>
FWP167	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 [pCHY27]</i>
FWP101	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4</i>
FWP112	<i>h⁺ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4</i>
FWP113	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git1-1</i>
CHP210	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-210</i>
FWP189	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2⁺ int::LEU2</i>
FWP190	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-1 int::LEU2</i>
CHP200	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git3-200</i>
CHP17	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git4-17</i>
CHP75	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git5-75</i>
CHP261	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git6-261</i>
CHP235	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git7-235</i>
CHP60	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git8-60</i>
CHP232	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git9-232</i>
CHP201	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git10-201</i>
FWP70	<i>h⁺ ade6-M216 lys1-131 leu1-32 ura4::fbp1-lacZ</i>
FWP134	<i>h⁺ ade6-M210 leu1-32 ura4::fbp1-lacZ git1-1</i>
FWP135	<i>h⁻ his7-366 leu1-32 ura4::fbp1-lacZ git2-210</i>
FWP136	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ git3-200</i>
FWP173	<i>h⁻ leu1-32 ura4::fbp1-lacZ git4-17</i>
FWP174	<i>h⁻ leu1-32 ura4::fbp1-lacZ git5-75</i>
FWP139	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ git6-261</i>
FWP140	<i>h⁻ leu1-32 ura4::fbp1-lacZ git7-235</i>
FWP175	<i>h⁻ leu1-32 ura4::fbp1-lacZ git8-60</i>
FWP142	<i>h⁺ leu1-32 ura4::fbp1-lacZ git9-232</i>
FWP143	<i>h⁺ leu1-32 ura4::fbp1-lacZ git10-201</i>
FWP182	<i>h⁻ leu1-32 ura4::fbp1-lacZ git2-7</i>
FWP183	<i>h⁻ leu1-32 ura4::fbp1-lacZ git2-13</i>
FWP184	<i>h⁻ leu1-32 ura4::fbp1-lacZ git2-61</i>
FWP191	<i>h⁻ ade6-M210 leu1-32 ura4::fbp1-lacZ git2-1 int::LEU2</i>
CHP7	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-7</i>
CHP13	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-13</i>
CHP61	<i>h⁺ ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-61</i>
FWP114	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-7</i>
FWP169	<i>h⁻ ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4 git2-61</i>
FWP177	<i>h⁹⁰ ade6-M210 leu1-32 cgs1-1</i>
FWP180	<i>h⁺ ade6-M216 leu1-32 ras1⁺ int::LEU2BglII</i>
FWP179	<i>h⁺ ade6-M216 leu1-32 ura4⁻ ras1⁻ int::LEU2HincII</i>
FWP181	<i>h⁹⁰ ade6-M210 leu1-32 ras1^{val17} int::LEU2BglII</i>
FWP192	<i>h⁹⁰ ade6-M216 leu1-32 ura4::fbp1-lacZ cgs1-1</i>
FWP193	<i>h⁺ ade6-M216 leu1-32 his7-366 ura4::fbp1-lacZ cgs1-1 git2-1 int::LEU2</i>
FWP194	<i>h⁺ ade6-M216 leu1-32 his7-366 ura4::fbp1-lacZ cgs1-1 git6-261</i>

hringer Mannheim Biochemicals. T4 DNA polymerase, the large fragment of DNA polymerase I (Klenow), DNA primers, and all other restriction enzymes were purchased from New England Biolabs.

Cloning of the *git2* gene

The *git2* gene was cloned as a high copy number suppressor of the *git1-1* mutation in strain CHP1 (Table 4). CHP1 was transformed to Leu⁺ on SC-leu (8% glucose) with an *S. pombe* genomic library (partial *Hind*III-digested DNA cloned into the *Hind*III site of pWH5; Wright et al. 1986; P. Young and D. Beach, unpubl.), and transformants were replica-plated to SC-leu and SC-leu with 5FOA to screen for plasmids that restored regulation of the *fbp1-ura4* fusion. Plasmids were isolated from 10

5FOA^R transformants and used to transform *E. coli* strain HB101 to Amp^R. Plasmid preparations from the *E. coli* transformants were used for restriction analyses and retransformation of CHP1. A single candidate plasmid, pCHY26 (Fig. 1), conferred a strong 5FOA^R phenotype on retransformation of CHP1. Other candidate plasmids conferred a weak 5FOA^R phenotype and have not been examined further.

Plasmid pCHY26 was integrated into the *S. pombe* genome by transformation of CHP1 with *Pml*I-digested pCHY26. Analysis of transformants indicated that pCHY26 did not carry the *git1* gene, as originally anticipated. In a cross, the Git⁻ phenotype associated with the *git1-1* mutation and the Leu⁺ phenotype associated with the *LEU2* gene from pCHY26 segregated independently in tetrads. Additional genetic crosses with a progeny from this cross, FWP148 (Git⁺ Leu⁺), however, demonstrated

that these phenotypes did cosegregate (nine of nine PD tetrads) when crossed with the *git2* mutant CHP210. Although this genetic analysis indicated that pCHY26 carried the *git2* gene, we were unable to determine whether pCHY26 had integrated by homologous recombination (as judged by Southern hybridization analysis) owing to the large size of the insert DNA in this plasmid. To determine whether the plasmid insert directs homologous integration at *git2*, we constructed plasmid pCHY27 (Fig. 1) by digesting pCHY26 with *Bam*HI and recircularizing the large restriction fragment. Plasmid pCHY27 contains most of the vector DNA plus ~4 kb of *S. pombe* insert DNA. Homologous integration into strain FWP101 (*Git*⁺ *Leu*⁻), creating strain FWP167 (*Git*⁺ *Leu*⁺), was confirmed by Southern hybridization analysis (Southern 1975) of both *Eco*RI and *Eco*RV digests of transformant DNA. In a cross of FWP167 with strain CHP210, the *Git*⁺ and *Leu*⁺ phenotypes cosegregated (25 PD tetrads and one TT tetrad). Therefore, pCHY27 contains genomic DNA from the *git2* locus.

Construction of the *git2-1* null allele

A *git2* null allele was constructed by digesting pCHY26 with *Bgl*II and recircularizing the large *Bgl*II fragment, resulting in plasmid pCHY28 (Fig. 1). This plasmid is unable to complement mutations in *git2*. On the basis of DNA sequence analysis of the *git2* gene (*cyr1*; Yamawaki-Kataoka et al. 1989; Young et al. 1990) this *Bgl*II deletion removes part of *git2* that encodes the adenylate cyclase catalytic domain and shifts the remainder of the coding region out of frame with the amino-terminal coding region. Plasmid pCHY28 was digested with *Clal*I, followed by ligation of the large restriction fragment, resulting in removal of much of the vector DNA. This plasmid, pCHY29, contains a single *Sal*I site, present in the insert DNA. pCHY29 was then digested with *Sal*I and ligated with a 2.2-kb *Sal*I-*Xho*I fragment from YEp13 (Broach et al. 1979), which carries the *LEU2* gene of *S. cerevisiae*, to form plasmid pCHY30. (The *LEU2* gene of *S. cerevisiae* complements the *leu1-32* mutation in *S. pombe*.) Strain FWP101 was transformed to *Leu*⁺ with a *Hind*III fragment from pCHY30 that carries the *git2 Bgl*II deletion (*git2-1*) and the *LEU2* insertion. One transformant (FWP190) was confirmed to contain a single copy of the *git2-1* null allele and the adjacent *LEU2* insertion by Southern hybridization analysis. In a cross between FWP190 (*Git*⁻ *Leu*⁺) and FWP112 (*Git*⁺ *Leu*⁻), the *Git*⁻ phenotype cosegregates with the *Leu*⁺ phenotype. The *git2-1* mutation is also unable to complement other *git2* mutations (Fig. 4). Strain FWP189, which contains the *LEU2* insertion, but adjacent to *git2*⁺, is *Git*⁺. In addition, integration of the *LEU2* gene adjacent to the *git2*⁺ gene has no effect on expression of an *fbp1-lacZ* fusion (Table 3; strain FWP189); therefore, it is the *git2-1* deletion and not the *LEU2* insertion that causes the *Git*⁻ phenotype.

Complementation analysis

Complementation analyses were carried out by determining the sensitivity or resistance to 5FOA of diploids formed between *git2* mutant strains, as described previously (Hoffman and Winston 1990). Complementation between two recessive *git2* alleles restores regulated expression of the *fbp1-ura4* fusion, resulting in a 5FOA^R phenotype when grown under repressing conditions (8% glucose). Noncomplementation results in constitutive expression of the *fbp1-ura4* fusion, resulting in a 5FOA^S phenotype. Diploids formed between any two members of the two groups that displayed *git2* intragenic complementation showed partial to complete 5FOA^R (complementation),

whereas diploids formed between members of the same groups were 5FOA^S (noncomplementation).

β-Galactosidase assays and Northern hybridization analysis

β-Galactosidase assays and Northern hybridization analysis were done as described previously (Hoffman and Winston 1990). Plasmid pAV06 (Vassarotti and Friesen 1985) was used as a probe for the *fbp1* transcript. RNA amounts were standardized by hybridization to plasmid pYK311, which carries the *leu1* gene (Kikuchi et al. 1988).

cAMP assays

Extracts for cAMP assays were prepared from cultures grown to 1×10^7 cells/ml in PM medium according to the method of Fedor-Chaikin et al. (1990) and assayed using the radioimmune assay kit available from Amersham. cAMP levels were standardized to total protein concentration using the BCA protein assay (Smith et al. 1985). Total protein extracts were made in 0.2 N NaOH by vortexing cells in the presence of glass beads.

Adenylate cyclase assays

Adenylate cyclase activity was assayed in membrane extracts as described by Caspersen et al. (1985) with some modifications. Strains were grown in 250 ml of YEL (8% glucose) to a density of $\sim 1 \times 10^7$ cells/ml. The cultures were poured over an equal volume of ice and subsequently maintained at 0°C to optimize recovery of active adenylate cyclase. Cells were pelleted and washed twice, once with 30 ml water and once with 10 ml of buffer A [50 mM Mes (pH 6.2), 1 mM MgCl₂, 1 mM EGTA, 1 mM *β*-mercaptoethanol], and resuspended in 0.4 ml of buffer A with 1 mM PMSF. Glass beads were added to the meniscus, and cells were lysed by vortex-mixing for 4 min (done in 30-sec intervals followed by at least 1 min on ice). Lysates were transferred to Eppendorf tubes (glass beads were washed with 1 ml of buffer A and pooled with the lysates) and centrifuged for 15 min in a microfuge. Membrane extracts were gently collected by resuspending the translucent section of the pellet in 100 μ l of buffer A with 10% glycerol and were stored at -70°C.

Each reaction mix contained 20–25 μ g of membrane extracts in 100 mM Mes (pH 6.2), 5 mM MnCl₂, 0.1 mg/ml of BSA, 20 mM creatine phosphate, 20 units/ml of creatine phosphokinase, 0.1 mM EGTA, 2 mM *β*-mercaptoethanol, 1.3 mM [α -³²P]ATP (20–65 cpm/pmol), and 0.5 mM ³H-labeled cAMP (5000 cpm). Each reaction was initiated by the addition of the membrane extract, proceeded for 30 min at 30°C, and was terminated by the addition of 1 ml of 0.2% SDS, 0.1 mM cAMP, and 1.2 mM ATP, followed by boiling for 3 min. The amount of ³²P-labeled cAMP produced was determined by the procedure of Salomon et al. (1974). Assays were linear with respect to time and membrane extract concentration under these conditions.

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