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Six *git* genes encode a glucose-induced adenylate cyclase activation pathway in the fission yeast *Schizosaccharomyces pombe*

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SUMMARY

An important eukaryotic signal transduction pathway involves the regulation of the effector enzyme adenylate cyclase, which produces the second messenger, cAMP. Previous genetic analyses demonstrated that glucose repression of transcription of the *Schizosaccharomyces pombe fbp1* gene requires the function of adenylate cyclase, encoded by the *git2* gene. As mutations in *git2* and in six additional *git* genes are suppressed by exogenous cAMP, these 'upstream' *git* genes were proposed to act to produce a glucose-induced cAMP signal. We report here that assays of cAMP levels in wild-type and various mutant *S. pombe* cells, before and after exposure to glucose, show that this is the case. The data suggest that the cAMP signal results from the activation of adenylate cyclase. Therefore these 'upstream' *git* genes appear to encode a glucose-induced adenylate cyclase activation pathway. Assays of cAMP on a strain carrying a mutation in the *git6* gene, which acts downstream of adenylate cyclase, indicate that *git6* may function to feedback regulate adenylate cyclase activity. Thus *git6* may encode a cAMP-dependent protein kinase.

Key words: *Schizosaccharomyces pombe*, fission yeast, adenylate cyclase, cAMP, signal transduction

INTRODUCTION

Eukaryotic cells commonly respond to environmental signals by regulating adenylate cyclase activity, thus altering intracellular cAMP levels. This process has been the subject of intensive biochemical studies in mammalian cells and genetic and biochemical studies in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In mammalian cells, heterotrimeric guanine-nucleotide binding proteins (G proteins) regulate the activity of adenylate cyclases (Gilman, 1984; Levitzki, 1988; Simon et al., 1991). In *S. cerevisiae*, monomeric guanine-nucleotide binding Ras proteins activate adenylate cyclase (Broek et al., 1985; Toda et al., 1985). In *S. pombe*, little is known about the mechanism of activation of adenylate cyclase. Although *S. pombe* adenylate cyclase shares considerable homology with the *S. cerevisiae* enzyme (Young et al., 1989; Yamawaki-Kataoka et al., 1989), it is not regulated by the Ras homolog encoded by *ras1* (Nadin-Davis et al., 1986; Fukui et al., 1986; Hoffman and Winston, 1991). Furthermore, researchers have been unable to activate *S. pombe* adenylate cyclase in vitro with guanine nucleotides (Yamawaki-Kataoka et al., 1989; Engelberg et al., 1990), preventing the use of standard biochemical approaches for the study of this process. In this report, we present evidence that glucose triggers the activation of adenylate cyclase in *S. pombe*. Six genes, required for glucose repression of the *fbp1* gene, encode components of this activation pathway.

The *S. pombe fbp1* gene, encoding fructose-1,6-bisphosphatase, is transcriptionally repressed by glucose (Vassarotti and Friesen, 1985; Hoffman and Winston, 1989, 1990). We have previously identified mutations in ten *git* genes (*git*= ***g***lucose ***i***nsensitive ***t***ranscription) that interfere with glucose repression of two hybrid genes possessing the *fbp1* promoter (Hoffman and Winston, 1990). Mutations in eight of these genes, including the *git2* gene, cause increased transcription of the *fbp1* gene in cells grown in the presence of glucose, as judged by northern hybridization analysis (Hoffman and Winston, 1990, 1991).

The *git2* gene was cloned (Hoffman and Winston, 1991) and shown to be identical to the *cyr1* gene (Young et al., 1989; Yamawaki-Kataoka et al., 1989; Maeda et al., 1990), which encodes adenylate cyclase. We have demonstrated that *fbp1* transcription is repressed by exogenous cAMP in wild-type cells grown in the absence of glucose and in *git1, git2, git3, git5, git7, git8*, and *git10* mutant strains, but not in a *git6* mutant strain (Hoffman and Winston, 1991).

The ability of exogenous cAMP to suppress the mutant phenotype in some, but not all, *git* mutants leads us to propose the following model (Fig. 1). Mutations suppressed by cAMP identify genes whose function is to create a cAMP signal. These include *git2* and the six 'upstream' *git* genes,

git1, git3, git5, git7, git8 and *git10.* cAMP levels are largely controlled by two events: the conversion of ATP to cAMP by adenylate cyclase, and the conversion of cAMP to AMP by cAMP phosphodiesterase. While a rise in cAMP levels can be created either by stimulating adenylate cyclase activity or by inhibiting cAMP phosphodiesterase activity, our data (see below) support an adenylate cyclase activation mechanism. The 'upstream' *git* genes may encode proteins that act to detect glucose and activate adenylate cyclase in response to glucose detection (Fig. 1). One such gene, *gpa2*, has recently been identified by Isshiki and coworkers (1992). The *gpa2* gene encodes a protein homologous to the alpha subunit of heterotrimeric guanine nucleotide-binding proteins (G proteins), which appears to act as a positive regulator of adenylate cyclase. We have determined that *gpa2* is identical to the *git8* gene (Nocero and Hoffman, unpublished). Other *git* genes may encode proteins known to interact with G proteins. These may include a G protein-coupled receptor (presumably glucose would be the ligand), beta or gamma subunits of the G protein, proteins responsible for activating the G alpha subunit, or proteins responsible for post-translational modifications of the alpha or gamma subunits of the G protein. Alternatively, intracellular glucose or some derivative may directly activate the G protein, bypassing the need for an external receptor.

Mutations insensitive to cAMP identify genes whose function is to respond to the cAMP signal or is independent of the cAMP signal pathway. (The *git4* and *git9* genes are not included in Fig. 1, since mutations in these genes do not confer an obvious defect in *fbp1* transcription.) The *git6* gene is proposed to encode the catalytic domain of a cAMP-dependent protein kinase (cAPK) for reasons described below. The kinase acts to repress *fbp1* transcription. This could be accomplished by phosphorylating, and thus inactivating, a protein that activates transcription at the *fbp1* promoter (not shown in Fig. 1).

MATERIALS AND METHODS

Strains

All strains used in these studies are listed in Table 1 with full genotypes according to the nomenclature rules for *S. pombe* proposed by Kohli (1987). The *ura4::fbp1-lacZ* allele is a disruption of the *ura4* gene by an *fbp1-lacZ* translational fusion (Hoffman and Winston, 1990). Only the relevant genotype with respect to *git* or *cgs* genes is presented in the text and figure legends.

cAMP assays

Cells were first grown in YEL (yeast extract liquid medium; Gutz et al., 1974) and then starved for glucose by subculturing them in PM liquid medium (0.1% glucose + 3% glycerol; Beach et al., 1985), with the necessary supplements. Strain FWP143 (*git10-201*) was subcultured in PM liquid medium (0.2% glucose + 3% glycerol). (The higher concentration of glucose was required to establish growth in minimal medium. Wild-type cells grown under these conditions were still responsive to glucose (see text).) PM liquid cultures were grown to log phase (6.0×10^6 to 1.5×10^7 cells/ml) overnight at 30°C. cAMP extracts were prepared from 10 ml of cells, two minutes and one minute before addition of glucose to establish the basal level of cAMP. Glucose was then added to a final concentration of 100 mM and additional extracts were prepared at the times indicated in the figure. cAMP was released from cells by the method of Fedor-Chaiken et al. (1990) using 1 M formic acid, and measured using a radioimmune assay kit from Amersham. Total protein extracts were prepared and assayed from separate 10 ml samples from the same culture to standardize the cAMP levels (Hoffman and Winston, 1991). cAMP levels are given as pmoles cAMP/mg total protein.

RESULTS

Wild-type *S. pombe* cells elevate intracellular cAMP levels in response to glucose

To directly test the model shown in Fig. 1, we have assayed cAMP levels in wild-type and mutant strains before and after exposure to glucose. In strain FWP77 (git^+; see Table 1 for full genotypes of all strains), cAMP levels rise from a basal level of 4.5 pmoles/mg protein to a level of 13.8 pmoles/mg protein within one minute of glucose addition, and peak at 26 pmoles/mg protein 15 minutes after glucose addition (Fig. 2A). The cAMP level then falls to 13 pmoles/mg protein two hours after glucose addition, indicating that the rise in cAMP is transient. A similar response curve has been observed in the prototrophic strain 972 (h^-) (data not shown). Exposure of *S. cerevisiae* cells to glucose results in a similar elevation in cAMP that is Ras-dependent. The peak of the *S. cerevisiae* response occurs one minute after glucose addition (Mbonyi et al., 1988).

The cAMP response to glucose involves adenylate cyclase activation, not phosphodiesterase inactivation

To test whether the rise in cAMP levels in wild-type cells exposed to glucose is caused by adenylate cyclase stimu-

Table 1. *S. pombe* strains

Strain		Genotype			
FWP77	h^-	*leu1-32*	*ura4::fbp1-lacZ*		
FWP134	h^+	*leu1-32*	*ura4::fbp1-lacZ*	*ade6-M210*	*git1-1*
FWP182	h^-	*leu1-32*	*ura4::fbp1-lacZ*	*git2-7*	
FWP184	h^-	*leu1-32*	*ura4::fbp1-lacZ*	*git2-61*	
FWP136	h^-	*leu1-32*	*ura4::fbp1-lacZ*	*ade6-M216*	*his7-366 git3-200*
FWP174	h^-	*leu1-32*	*ura4::fbp1-lacZ*	*git5-75*	
FWP139	h^-	*leu1-32*	*ura4::fbp1-lacZ*	*ade6-M216*	*his7-366 git6-261*
FWP140	h^-	*leu1-32*	*ura4::fbp1-lacZ*	*git7-235*	
FWP175	h^-	*leu1-32*	*ura4::fbp1-lacZ*	*git8-60*	
FWP143	h^+	*leu1-32*	*ura4::fbp1-lacZ*	*git10-201*	
SP578	h^{90}	*leu1-32*	*ade6-M216*	*cgs2-2*	

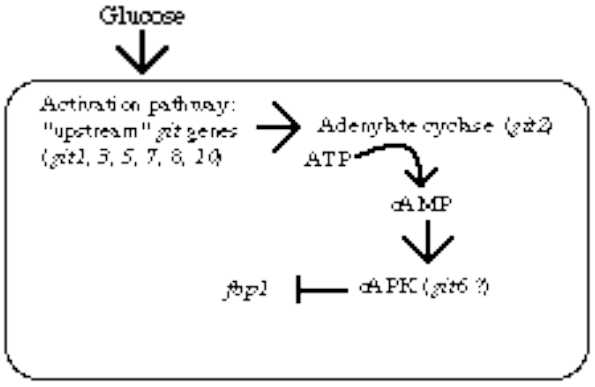

Fig. 1. A model for the transcriptional regulation of the *S. pombe fbp1* gene. The upstream *git* genes encode proteins that detect glucose and activate adenylate cyclase, encoded by *git2*. The *git8* gene encodes the alpha subunit of a G protein. Adenylate cyclase produces cAMP, that then activates a cAMP-dependent protein kinase (cAPK), possibly encoded by *git6*. The kinase acts to inhibit *fbp1* transcription. See text for more detail.

lation or by cAMP phosphodiesterase inhibition, we examined cAMP levels in strains that are defective in these activities. Two strains with a defective adenylate cyclase, FWP182 (*git2-7*) and FWP184 (*git2-61*), were used in this study as these mutations appear to affect different functions of adenylate cyclase. Haploid strains possessing either the *git2-7* or the *git2-61* mutant allele had been shown to display a mutant Git$^-$ phenotype as judged by their constitutive expression of an *fbp1-ura4* hybrid gene (producing a phenotype of sensitivity to the pyrimidine analog 5-fluoroorotic acid (5FOA; Boeke et al., 1984; Hoffman and Winston, 1990)). A diploid strain carrying these two mutant *git2* alleles displayed a wild-type Git$^+$ phenotype (5FOA-resistant; Hoffman and Winston, 1990, 1991). This example of intragenic complementation indicates that adenylate cyclase is composed of at least two copies of the *git2* gene product and that the two mutations identify functionally discrete domains of the protein. Adenylate cyclase activity, assayed in vitro, is similar in strain FWP182 (*git2-7*) and strain FWP77 (*git2*$^+$), but is sevenfold lower in strain FWP184 (*git2-61*) (Hoffman and Winston, 1991). These data are consistent with the hypothesis that the *git2-61* mutation alters the catalytic domain and the *git2-7* mutation alters a regulatory domain of adenylate cyclase. It is important to note that the in vitro assay of adenylate cyclase activity is not a measurement of the in vivo activity present in the cells from which the extract was made.

We show here that strains FWP182 (*git2-7*) and FWP184 (*git2-61*) possess cAMP basal levels similar to that of strain FWP77 (*git*$^+$); however, there is a less than twofold rise in cAMP in either strain after glucose addition (Fig. 2A). The altered response to glucose observed in the *git2-7* mutant strain, which possesses normal adenylate cyclase catalytic activity and basal cAMP levels, supports the hypothesis that adenylate cyclase is the target of regulation by glucose. If phosphodiesterase activity were the target, it would be unlikely that a mutation in *git2* could interfere with the cAMP signal without also altering adenylate cyclase catalytic activity.

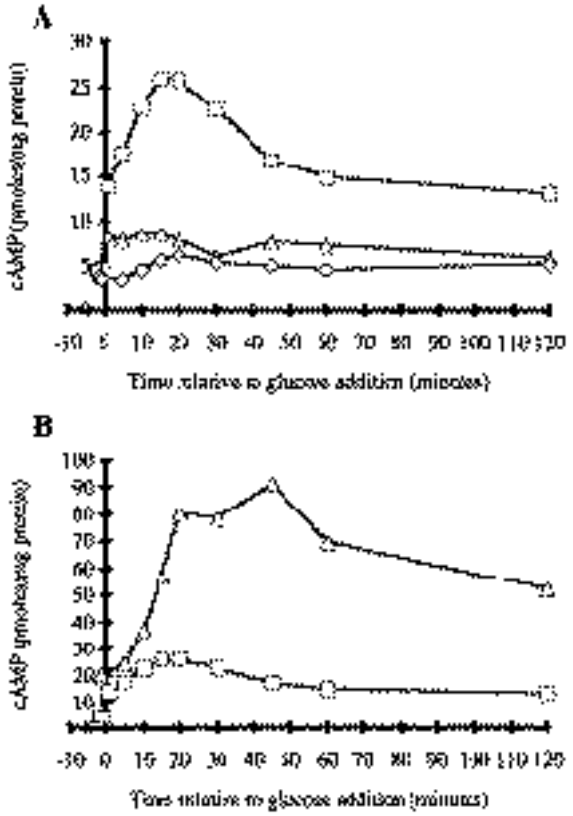

Fig. 2. cAMP levels in wild type, *git2* mutants and a *cgs2* mutant strain before and after exposure to glucose. Assays were performed as described in Materials and Methods. (A) Four independent cultures of FWP77 (*git*$^+$) were assayed. The average value at each timepoint was used to produce an average curve (□) that is included in subsequent figures as a reference curve. Standard errors were less than 30% of the value. Also shown are average curves generated from assays of four independent cultures of FWP182 (*git2-7*; Δ) and of three independent cultures of FWP184 (*git2-61*; ◊). (B) Average curve generated from assays of four independent cultures of SP578 (*cgs2-2*; Δ). Note that the scales of cAMP concentrations differ between A and B.

Diploid strains carrying *git2-7* and *git2-61* failed to show intragenic complementation as judged by these assays (data not shown). Intracellular cAMP levels in such strains before and after glucose addition resembled those of the haploid strains FWP182 (*git2-7*) and FWP184 (*git2-61*) (Fig. 2A) or of diploids homozygous for either *git2* mutant allele. The failure to observe a cAMP response in these heterozygous diploids suggests that the resistance to 5FOA, due to transcriptional repression of an *fbp1-ura4* fusion, seen in similar diploids (Hoffman and Winston, 1991), is a more sensitive assay of function and that adenylate cyclase activity is not fully restored to wild-type levels.

cAMP assay data from a strain with a defective cAMP phosphodiesterase (*cgs2-2*) indicate that cAMP phosphodiesterase is not regulated by glucose (Fig. 2B). The *cgs2* gene displays some homology to a phosphodiesterase gene from *Dictyostelium discoideum*, and *cgs2* mutant strains possess elevated cAMP levels that are only slightly lower than the peak levels observed in wild-type cells exposed to glucose (DeVoti et al., 1991; Fig. 2B). The wild-type

cgs2 gene was cloned by complementation of the sporulation-deficient phenotype of the *cgs2-2* mutant SP578. Therefore, *cgs2-2* is apparently a loss-of-function mutation affecting cAMP phosphodiesterase. When strain SP578 (*cgs2-2*) cells were exposed to glucose, cAMP levels rose from 20.1 pmoles/mg protein to a peak of 91.3 pmoles/mg protein 45 minutes after glucose addition. This 4.5-fold increase in cAMP after glucose addition indicates that the cgs2 protein is not the target of glucose regulation, since its activity is already compromised by the mutation. However, the observed reduction in cAMP levels from 45 minutes to 120 minutes following glucose addition could indicate the presence of a second phosphodiesterase activity. While it is possible that a second phosphodiesterase activity is regulated by glucose, the presence of a redundant phosphodiesterase is inconsistent with the high cAMP levels observed in *cgs2* mutant strains. We believe that cAMP levels fall in this strain due to excretion or nonspecific destruction of cAMP.

Strains carrying mutations in the 'upstream' *git* genes lack a cAMP response to glucose

cAMP assay data from strains carrying *git* mutations that are repressed by exogenous cAMP indicate that these genes encode components of the glucose-induced cAMP signal pathway. Strains FWP134 (*git1-1*), FWP136 (*git3-200*), FWP174 (*git5-75*), FWP140 (*git7-235*), FWP175 (*git8-60*) and FWP143 (*git10-201*) are all defective in their ability to raise intracellular cAMP levels in response to glucose (Fig. 3A,B). Basal levels range from a low of 2.0 pmoles/mg protein in strain FWP175 (*git8-60*) to a high of 6.8 pmoles/mg protein in strain FWP174 (*git5-75*). Peak levels range from a low of 3.2 pmoles/mg protein in strain FWP140 (*git7-235*) to a high of 8.4 pmoles/mg protein in strain FWP174 (*git5-75*). The failure of these strains to increase intracellular cAMP in response to glucose indicates that the role of these six 'upstream' *git* genes is to encode an adenylate cyclase activation pathway as depicted in Fig. 1.

Complementation and linkage studies have demonstrated that the *git8* gene is identical to the *gpa2* gene (Isshiki et al., 1992), which encodes the alpha subunit of a G protein (Nocero and Hoffman, unpublished). The low basal cAMP level and defective response to glucose observed in strain FWP175 (*git8-60*) suggest that the G protein is involved in establishing both basal and stimulated levels of adenylate cyclase activity.

While strain FWP143 (*git10-201*) was starved for glucose by growing overnight in PM liquid medium with 0.2% glucose and 3% glycerol as the carbon source, the higher concentration of glucose is not responsible for the failure of these cells to elevate cAMP levels in response to glucose. This higher glucose concentration (the medium for other strains contained only 0.1% glucose with 3% glycerol) was needed to establish growth under low glucose conditions in minimal medium. FWP77 (git^+) cells grown under identical conditions respond to glucose in the same manner as shown in Fig. 2A. Therefore, it is the mutation in *git10*, not the growth conditions, that is responsible for the low cAMP levels in FWP143 after glucose addition.

A *git6* mutant possesses an elevated basal cAMP level and is defective in restoration of the basal cAMP level after exposure to glucose

cAMP assay results from a *git6* mutant strain demonstrate that the lack of a cAMP response observed in the 'upstream' *git* mutants is not a nonspecific defect present in all *git* mutants. Strain FWP139 (*git6-261*) possesses an elevated basal cAMP level of 11.9 pmoles/mg protein. Upon addition of glucose, this level rises to a peak of 19.3 pmoles/mg protein (Fig. 4). While this peak is not as high as that observed in the wild-type strain, it is significantly higher than those seen in the 'upstream' *git* mutants. We conclude that the low cAMP levels observed in the 'upstream' *git* mutants accurately reflect defects in the adenylate cyclase activation pathway. Interestingly, cAMP levels remain at peak values for at least two hours after glucose addition.

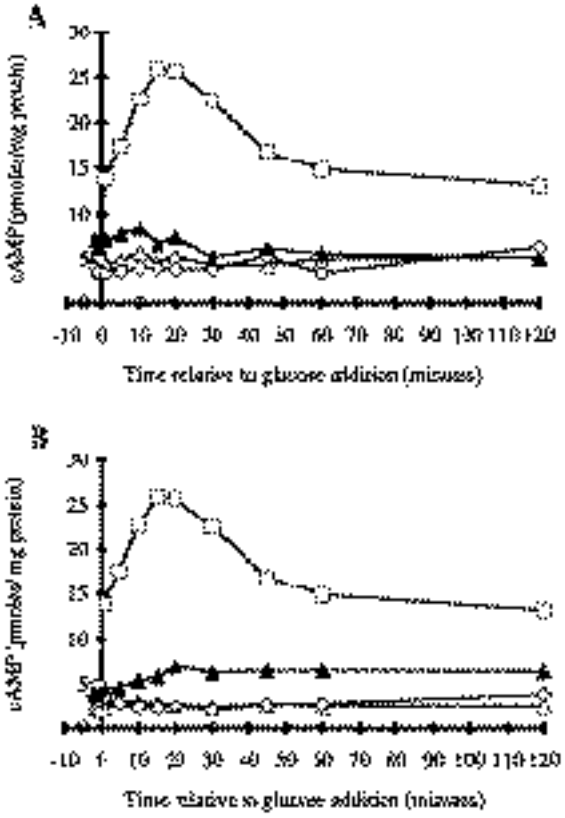

Fig. 3. cAMP levels in *git* mutant strains before and after exposure to glucose. Assays were performed as described in Materials and Methods. The FWP77 (git^+) average curve from Fig. 2A (□) is displayed as a reference curve. (A) Average curves generated from assays of three independent cultures of FWP134 (*git1-1*; Δ), of three independent cultures of FWP136 (*git3-200*; ◊), and of three independent cultures of FWP174 (*git5-75*; ▲). (B) Average curves generated from assays of four independent cultures of FWP140 (*git7-235*; △), of three independent cultures of FWP175 (*git8-60*; ◇), and of three independent cultures of FWP143 (*git10-201*; ▲). Strain FWP143 was grown in PM containing 0.2% glucose and 3% glycerol as a carbon source (see text).

Fig. 4. cAMP levels in a *git6* mutant strain before and after exposure to glucose. Assays were performed as described in Materials and Methods. The FWP77 (*git*$^+$) average curve from Fig. 2A (□), is displayed as a reference curve. The average curve generated from assays of three independent cultures of FWP139 (*git6-261*; ◇) is shown.

DISCUSSION

Previous studies involving suppression of *git* mutant phenotypes by exogenous cAMP led to the suggestion that these genes act to create a cAMP signal in *S. pombe* cells exposed to glucose (Hoffman and Winston, 1991). This present work provides direct evidence that when *S. pombe* cells are exposed to glucose, the proteins encoded by the *git1, git3, git5, git7, git8* and *git10* genes function to activate adenylate cyclase.

The ability of strain SP578 (*cgs2-2*) to respond to glucose with a 4.5-fold increase in cAMP levels suggests that the cgs2 cAMP phosphodiesterase is not the target for the glucose-induced cAMP response. However, this does not address the question of whether cAMP phosphodiesterase activity is regulated by other factors, such as intracellular cAMP concentration. Such regulation may explain why FWP184 (*git2-61*) cells, which possess 13% of the adenylate cyclase activity found in FWP77 cells (Hoffman and Winston, 1991), possess a basal cAMP level only 20% lower than that of FWP77 cells.

The cAMP response curve for FWP139 (*git6-261*) differs significantly from that of strain FWP77 (*git*$^+$) in that cAMP levels do not drop from peak levels within two hours of glucose addition (Fig. 4). In FWP77 cells, cAMP levels at the two-hour timepoint are approximately half of the peak levels. The high basal cAMP level and the defect in restoration of the basal level after exposure to glucose in strain FWP139 indicate that *git6* may be required for feedback regulation of adenylate cyclase activity. In *S. cerevisiae*, the catalytic subunits of the cAMP-dependent protein kinase (cAPK) carry out a similar role (Mbonyi et al., 1990; Nikawa et al., 1987). There is much circumstantial evidence that *git6* encodes the catalytic subunit of cAPK. The role of cAMP in the *fbp1* regulatory pathway is to activate cAPK. A mutation in the *cgs1* gene (DeVoti et al., 1991), which encodes the regulatory subunit of cAPK, reduces *fbp1* transcription in derepressed cells (Hoffman and Winston, 1991) and blocks meiosis (DeVoti et al., 1991). *git6* mutants have the opposite phenotype. These cells constitutively transcribe *fbp1* (Hoffman and Winston, 1990; Hoffman and Winston, 1991) and homothallic (h^{90}) strains enter meiosis in the absence of a nutritional starvation signal (unpublished data). Double mutants (*cgs1-1 git6-261*) resemble *git6* mutants for both phenotypes (Hoffman and Winston, 1991; unpublished data), as expected if *git6* encodes the catalytic subunit of cAPK.

This pathway is particularly interesting, due to its similarities to and differences from related pathways in other organisms. Adenylate cyclases from *S. pombe* and *S. cerevisiae* display considerable homology to each other, but very little homology to the mammalian adenylate cyclases identified to date. Yet, the mechanism of *S. pombe* adenylate cyclase activation is not like that of *S. cerevisiae*. Ras proteins activate adenylate cyclase in *S. cerevisiae* (Broek et al., 1985; Toda et al., 1985), but not in *S. pombe* (Nadin-Davis et al., 1986; Fukui et al., 1986; Hoffman and Winston, 1991). Mammalian adenylate cyclase activity is regulated by heterotrimeric G proteins, and thus is also responsive to guanine nucleotides (Gilman, 1984; Levitzki, 1988; Simon et al., 1991). Activation of *S. pombe* adenylate cyclase by guanine nucleotides has been unsuccessful to date (Yamawaki-Kataoka et al., 1989; Engelberg et al., 1990), probably due to the disruption of the activation mechanism by in vitro manipulation rather than to the absence of an activator that binds guanine nucleotides.

Recently, an *S. pombe* gene, *gpa2*, was cloned, based on its homology to the alpha subunit of heterotrimeric G proteins (Isshiki et al., 1992). Studies of *gpa2* suggest that this gene acts to positively regulate adenylate cyclase activity. We have shown that *gpa2* is identical to *git8* (Nocero and Hoffman, unpublished). The present study suggests a requirement for *git8/gpa2* for both basal and activated adenylate cyclase activity. FWP175 (*git8-60*) cells possess less than half the basal cAMP level of FWP77 cells and show little or no rise in cAMP following glucose addition (Fig. 3B).

The involvement of a heterotrimeric G protein in this pathway suggests that cloning of additional *git* genes may reveal an adenylate cyclase activation pathway in *S. pombe* that strongly resembles that of mammalian cells. However, at this time we cannot rule out the possibility that the G protein is activated by a mechanism that does not involve a classical ligand-activated receptor. The cloning and characterization of the other 'upstream' *git* genes will help to elucidate this genetically pliable model system for the study of adenylate cyclase activation that may promote our understanding of this process in mammalian cells and in *S. cerevisiae*, as well as in *S. pombe*.

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