# The Fission Yeast *git5* Gene Encodes a Gβ Subunit Required for Glucose-Triggered Adenylate Cyclase Activation

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ABSTRACT

Fission yeast adenylate cyclase is activated by the gpa2 Gα subunit of a heterotrimeric guanine-nucleotide binding protein (G protein). We show that the *git5* gene, also required for this activation, encodes a Gβ subunit. In contrast to another study, we show that git5 is not a negative regulator of the gpa1 Gα involved in the pheromone response pathway. While 43% identical to mammalian Gβ's, the git5 protein lacks the amino-terminal coiled-coil found in other Gβ subunits, yet the gene possesses some of the coding capacity for this structure 5′ to its ORF. Although both *gpa2* (Gα) and *git5* (Gβ) are required for adenylate cyclase activation, only *gpa2* is needed to maintain basal cAMP levels. Strains bearing a *git5* disruption are derepressed for *fbp1* transcription and sexual development even while growing in a glucose-rich environment, although *fbp1* derepression is half that observed in *gpa2* deletion strains. Multicopy *gpa2* partially suppresses the loss of *git5*, while the converse is not true. These data suggest that Gβ is required for activation of adenylate cyclase either by promoting the activation of Gα or by independently activating adenylate cyclase subsequent to Gα stimulation as seen in type II mammalian adenylate cyclase activation.

HETEROTRIMERIC G proteins are composed of α, β, an γ subunits and regulate many eukaryotic signal transduction pathways. G protein activation involves a receptor-stimulated GDP to GTP exchange of the guanine nucleotide bound to the Gα subunit, followed by the release of the Gα subunit from the Gβγ dimer (Gilman 1984, 1987; Levitzki and Bar-Sinai 1991; Simon *et al.* 1991). Originally, it was proposed that the Gα subunit alone regulates the activity of effector molecules directly; however, this model has undergone considerable revision, as effectors that are stimulated by Gβγ alone, by Gα and Gβγ acting independently, or by Gα in concert with Gβγ have been identified (Clapham and Neer 1993; Sternweis 1994).

Two genes from the fission yeast *Schizosaccharomyces pombe* that encode Gα subunits have been identified. The *gpa1* gene encodes a positive regulator of the pheromone-induced mating pathway (Obara *et al.* 1991). Deletion of *gpa1* results in a mating and sporulation defect, while an activated mutant allele causes a pheromone-independent production of conjugation tubes. The *gpa2* gene encodes a positive regulator of adenylate cyclase acting in a glucose-monitoring pathway (Isshiki *et al.* 1992). Disruption of *gpa2* causes constitutive mating and sporulation in homothallic strains, reduces basal cAMP levels, and eliminates the glucose-induced cAMP response. A single Gβ gene, *gpb1*, has been cloned and was proposed to encode a negative regulator of gpa1 in the pheromone response pathway (Kim *et al.* 1996). Deletion of *gpb1* results in increased mating and sporulation, which is suppressed by loss of either *gpa1* or *ras1*, but has no effect on basal cAMP levels. No Gγ subunits have been identified previously in *S. pombe*.

*S. pombe* regulates intracellular cAMP levels in response to nutritional signals (Maeda *et al.* 1990; Hoffman and Winston 1991; Isshiki *et al.* 1992; Mochizuki and Yamamoto 1992; Byrne and Hoffman 1993), controlling conjugation and sporulation, and the transcription of the *fbp1* gene that encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase. Mutations in the *git2/cyr1* gene encoding adenylate cyclase (Yamawaki-Kataoka *et al.* 1989; Young *et al.* 1989; Maeda *et al.* 1990; Hoffman and Winston 1991) cause constitutive *fbp1* transcription (Hoffman and Winston 1990, 1991) and allow conjugation and sporulation in homothallic ($h^{90}$) strains in the absence of a nitrogen- or glucose-starvation signal (Maeda *et al.* 1990; Kawamukai *et al.* 1991). As mentioned above, deletion of *gpa2* causes similar phenotypes, although the effects of *git2/cyr1* deletions are more pronounced than the effects of a *gpa2* deletion.

Previously, we identified eight *git* (*g*lucose *i*nsensitive *t*ranscription) genes by mutations that confer constitutive *fbp1* transcription (Hoffman and Winston 1990). These genes encode a cAMP-dependent protein kinase (PKA) activation pathway (Hoffman and Winston 1991; Byrne and Hoffman 1993), including the adenyl-

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**TABLE 1**

**Strain list**

| Strain | Genotype |
|---|---|
| 972 | *h*$^{-}$ |
| FWP87 | *h*$^{+}$ *leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ |
| FWP112 | *h*$^{-}$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ |
| CHP311 | *h*$^{-}$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *git5-311* |
| CHP75 | *h*$^{+}$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *git5-75* |
| CHP439 | *h*$^{+}$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *gpa2:ura4*$^{-}$ |
| FWP111 | *h*$^{-}$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *git1-1* |
| CHP200 | *h*$^{-}$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *git3-200* |
| CHP477 | *h*$^{-}$ *ade6-M210 his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *git5-1::his7*$^{+}$ |
| CHP478 | *h*$^{+}$ *his7-366 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *git5-1::his7*$^{+}$ |
| CHP745 | *h*$^{+}$ *ade6-M216 his7-366 leu1-32 gpa1*$^{QL}$ |
| CHP465 | *h*$^{-}$ *ade6-M210 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *git7-235* |
| CHP490 | *h*$^{-}$ *ade6-M210 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *pka1::ura4*$^{-}$ |
| CHP573 | *h*$^{-}$ *ade6-M210 leu1-32 ura4::fbp1-lacZ fbp1::ura4*$^{+}$ *git10-201* |
| FWP94 | *h*$^{-}$ *his7-366 leu1-32 ura4::fbp1-lacZ* |
| CHP463 | *h*$^{-}$ *his7-366 leu1-32 ura4::fbp1-lacZ git5-1::his7*$^{+}$ |
| CHP462 | *h*$^{+}$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ gpa2::ura4*$^{+}$ |
| CHP469 | *h*$^{+}$ *ade6-M216 his7-366 leu1-32 ura4::fbp1-lacZ gpa2::ura4*$^{+}$ *git5-1::his7*$^{+}$ |
| CHP481 | *h*$^{90}$ *ade6-M216 leu1-32 lys1-131 gpa2::ura4*$^{+}$ |
| CHP483 | *h*$^{90}$ *ade6-M216 leu1-32 ura4::fbp1-lacZ* |
| CHP488 | *h*$^{90}$ *ade6-M216 his7-366 leu1-32 lys1-131 ura4::fbp1-lacZ git5-1::his7*$^{+}$ |

ate cyclase *git2* (*cyr1*) gene, six genes required for activation of adenylate cyclase (*git1*, *git3*, *git5*, *git7*, *git8*, and *git10*), and the *pka1* (*git6*) gene that encodes the PKA catalytic subunit (Maeda *et al.* 1994; Jin *et al.* 1995). While mutations in *git1*, *git3*, *git5*, *git7*, *git8*, and *git10* all abolish the glucose-triggered activation of adenylate cyclase, only mutations in *git7* and *git8* reduce basal cAMP levels (Byrne and Hoffman 1993). We showed previously that *git8* is identical to the Gα gene *gpa2* (Nocero *et al.* 1994). In this article, we describe the cloning and characterization of the *git5* gene, which is identical to *gpb1*. We provide evidence that a sequencing error in the *gpb1* study results in the misidentification of the start of the *git5/gpb1* open reading frame (ORF) and that the product lacks the amino-terminal coiled-coil domain found in all other Gβ subunits. Our data suggest that the git5 product functions to activate adenylate cyclase in a glucose-monitoring pathway and is not part of the gpa1-regulated pheromone response pathway. Two possible roles for this Gβ in adenylate cyclase activation are discussed.

## MATERIALS AND METHODS

**Yeast strains and growth media:** Yeast strains used are listed in Table 1. Genetic nomenclature for *S. pombe* follows rules proposed by Kohli (1987). The *fbp1::ura4*$^{+}$ and *ura4::fbp1-lacZ* reporter constructs have been described previously (Hoffman and Winston 1990). Only relevant genotypes are given in the text and legends.

Standard rich media yeast extract agar and yeast extract liquid (Gutz *et al.* 1974) were supplemented with 2% casamino acids. Pombe minimal (PM) media (Watanabe *et al.* 1988) were supplemented with required nutrients at 75 mg/liter, except for leucine, which was added to 150 mg/liter. Glucose was generally present at a concentration of 3%, unless otherwise specified. Sensitivity to 5-fluoroorotic acid (5-FOA) was determined on SC solid media containing 8% glucose as described previously (Hoffman and Winston 1991). Strains were grown at 30°.

**Recombinant DNA methodology:** Standard recombinant DNA techniques, including DNA restriction digests, ligations, and *Escherichia coli* transformations, were performed according to Ausubel *et al.* (1998). Yeast transformations and plasmid rescue into *E. coli* were performed as previously described (Dal Santo *et al.* 1996). DNA sequencing was performed using the CircumVent thermal cycle DNA sequencing kit (New England Biolabs, Beverly, MA) according to the manufacturer's directions using oligonucleotides from National Biosciences Inc.

**Cloning of *git5*$^{+}$:** Constitutive transcription of the *fbp1-ura4*$^{+}$ reporter due to the *git5-311* mutant allele in CHP311 results in a 5-FOA-sensitive phenotype. The *git5*$^{+}$ gene carried on plasmid pMP1 was cloned by its ability to confer 5-FOA resistance upon transformation of strain CHP311 with a *S. pombe* genomic library (Molz *et al.* 1989) from ~20,000 Leu$^{+}$ transformants. Subcloning experiments identified the portion of the insert DNA carrying the *git5*$^{+}$ gene. The sequence of this region (accession no. AF092102), determined on both strands at least once, is identical to the complement of bp 3461–4680 of cosmid c973 (accession no. AB004535) carrying *S. pombe* genomic DNA from chromosome II.

Plasmid pMP1 was linearized with *Bgl*II and was integrated into strain FWP112 (*git*$^{+}$; Hoffman and Winston 1990) by homologous recombination. One integrant was crossed with strain CHP75 (*git5-75*; Hoffman and Winston 1990), and the progeny were examined by tetrad dissection. Progeny displayed tight genetic linkage between the *LEU2* marker carried on plasmid pMP1 and the *git5* mutation (no recombination in 27 tetrads), suggesting that plasmid integration occurred at the *git5* locus.

Plasmid pSL10 carrying a cDNA clone of *git5*$^+$ was identified from a λZAP II *S. pombe* cDNA library (Pidoux *et al.* 1996) using the GeneTrapper kit (Life Technologies) with cloning primer git5-GT 5′ TGTAGATATGTCGATGTCAGGA 3′ according to manufacturer's instructions.

**Quantitative oligonucleotide-directed S1 analysis:** Oligonucleotide-directed S1 was performed as described previously (Ausubel *et al.* 1998; using the aqueous hybridization protocol) to measure the level of *git5* transcription and to detect possible splicing events 5′ to the *git5* ORF. A *git5*-specific primer (git5-oligoS1 5′ CATCTTTAGAAATTGAACCAGTAT TCCGTCTAATATTCATACAACAGTAACGCCCA 3′) and a control primer to the *his3* gene (his3-oligoS1 5′ TCCATTC CATGTAGGGTGCTCATT 3′) were used in combination with RNA isolated from exponential phase cells of strain 972 *h*$^-$ grown in PM medium containing either 8% glucose (repressing conditions) or 0.1% glucose plus 3% glycerol (derepressing conditions). RNA was isolated by hot acid phenol extraction (Ausubel *et al.* 1998).

**Construction of a *git5*Δ gene disruption:** The *git5* gene was disrupted by replacing a *Bam*HI to *Xba*I fragment including codons 99–152 of the *git5* ORF with a 1.9-kb *Bgl*II to *Xba*I DNA fragment carrying the *his7*$^+$ gene (Apolinario *et al.* 1993). A linear DNA fragment carrying the *his7*$^+$-marked *git5* disruption was used to replace the *git5*$^+$ gene in strain FWP94 (*gpa2*$^+$; resulting in strain CHP463) and in strain CHP462 (*gpa2*Δ; resulting in strain CHP469). The *git5-1::his7*$^+$ disruption (*git5*Δ) was then crossed into strains carrying the *fbp1-ura4*$^+$ reporter and was shown to confer a 5-FOA-sensitive phenotype, similar to that of *git5* mutant strains. Complementation tests with other *git* mutant strains were performed as previously described (Hoffman and Winston 1990). The 5-FOA-sensitive phenotype of diploids constructed by mating a *git5*Δ strain with a *git5* point mutant places the *git5*Δ allele in the *git5* complementation group.

**Subcloning of the *git5* ORF:** A 1.4-kb *Ssp*I fragment from plasmid pSL10, carrying the *git5* ORF, was blunt-end ligated into the *Sma*I site of plasmid pART1 (McLeod *et al.* 1987). Plasmid pSL11 expresses the *git5* ORF from the *adh* promoter in the vector. Plasmid pSL12 carries the *git5* ORF in the opposite orientation with respect to the *adh* promoter.

**High-copy-number suppression analyses:** High-copy-number suppression was determined by transforming host strains to Leu$^+$ with plasmids expressing *gpa2*$^+$ or *git5*$^+$, along with empty-vector controls. Transformants were tested for sensitivity or resistance to 5-FOA, as an indication of *fbp1-ura4*$^+$ transcription, and assayed for β-galactosidase activity expressed from the *fbp1-lacZ* reporter as described previously (Nocero *et al.* 1994).

**cAMP assays:** Intracellular cAMP levels were measured in glucose-starved cells (basal level) and in the same cultures 1 min after exposure to 100 mm glucose as previously described (Byrne and Hoffman 1993).

## RESULTS

**Cloning and nucleotide sequence of the *git5*$^+$ gene:** The *git5*$^+$ gene was cloned from an *S. pombe* genomic library by its ability to complement a *git5* mutation in strain CHP311 (see materials and methods). Homologous integration of this plasmid and a subsequent linkage analysis demonstrated that the clone carries the *git5*$^+$ gene, rather than a high-copy-number suppressor of the *git5-311* mutation. Our DNA sequence analysis in the region of plasmid pMP1 that carries the *git5* complementing activity (accession no. AF092102) reveals that *git5*$^+$ gene is the same as *gpb1* (Kim *et al.* 1996; accession no. L28061), encoding a Gβ subunit family member. This gene has also been sequenced as part of the *S. pombe* Genome Sequencing Project (http://www.sanger.ac.uk/Projects/S_pombe/) and is present on cosmid c973 (accession no. AB004535). However, while our sequence and the cosmid sequence both predict a 305-amino acid product, the *gpb1* sequence predicts a 317-amino acid product. This is due to a single base pair missing from the *gpb1* sequence relative to the cosmid and our *git5* sequences that shifts an ATG start codon that is upstream from, but out-of-frame with, the *git5* ORF into the same frame as the 305-codon ORF. This A-T base pair is part of an *Ssp*I restriction site that separates the out-of-frame ATG from the 305-codon ORF. *Ssp*I digestion of plasmid pMP1 (*git5*$^+$ genomic clone) produces 1.4- and 2.6-kb fragments as predicted if this *Ssp*I site is present, and not the 4.0-kb fragment predicted if this site is absent (Figure 1). The 4.15- and 7.8-kb bands produced by *Ssp*I digestion of pMP1 are consistent with predictions for the junction fragments

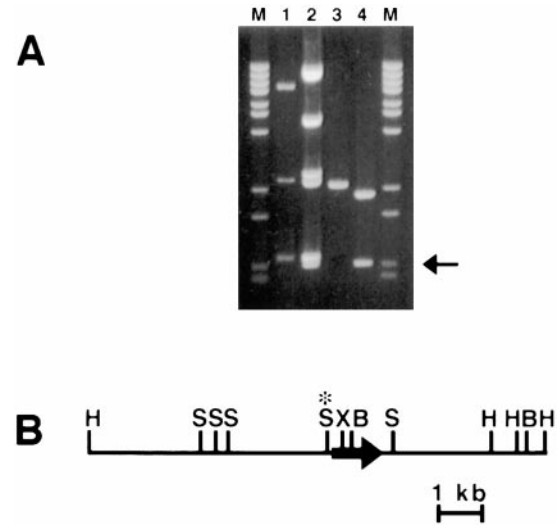


Figure 1.—*Ssp1* restriction digestion of genomic and cDNA clones of *git5*$^+$. (A) Plasmids pWH5 (lane 1), pMP1 (*git5*$^+$ genomic clone in pWH5; lane 2), pBluescript SK(−) (lane 3), and pSL10 [*git5*$^+$ cDNA clone in pBluescript SK(−); lane 4] were digested with *Ssp*I and electrophoresed on a 1.2% agarose gel. Size standards (M) contain λ *Bst*EII DNA. The 1.4-kb fragment common to pMP1 and pSL10 (indicated by an arrow) demonstrates the presence of an *Ssp*I site immediately 5′ to the *git5* ORF, as well as a second *Ssp*I site 0.46 kb 3′ to the *git5* ORF. (B) Schematic of the 10.6-kb insert in pMP1. Plasmid pMP1 carries the region of chromosome 2 from base pair 34,804 of cosmid c1750 (accession no. AB004534) to base pair 10267 of cosmid c973 (accession no. AB004535). The position and orientation of the *git5*$^+$ ORF is indicated as an arrow. *Hin*dIII (H), *Ssp*I (S), *Bam*HI (B), and *Xba*I (X) sites are marked. The *Ssp*I site in question, indicated with an asterisk, contributes to the production of the 2.6- and 1.4-kb *Ssp*I restriction fragments in lane 2 of A and of the 1.4-kb *Ssp*I restriction fragment in lane 4 of A.

of vector and insert DNA. *Ssp*I digestion of plasmid pSL10 (*git5*$^+$ cDNA clone; see materials and methods) results in the same 1.4-kb *Ssp*I restriction fragment that carries the *git5* ORF (Figure 1). The presence of this *Ssp*I site proves that the ATG identified as the start codon in the *gpb1* sequence is not in-frame with the 305-codon Gβ-encoding ORF.

**Analysis and subcloning of the *git5* ORF:** The putative 305-residue *git5* product is ~44% identical and 62% similar to Gβ subunits from other organisms (Figure 2). However, for the 16 residues identified as contacting the Gα subunit from a crystal structure analysis (Sondek *et al.* 1996), git5 displays 63% identity and 75% similarity to members of this family.

The most unusual characteristic of the predicted 305-residue product is the absence of 36 residues at the amino terminus relative to other members of this family (Figure 2). This region of other Gβ subunits forms a coiled-coil that appears to be required for the formation of the Gβγ dimer (Garritsen *et al.* 1993; Wall *et al.* 1995; Garcia-Higuera *et al.* 1996; Lambright *et al.* 1996; Sondek *et al.* 1996; Pellegrino *et al.* 1997). According to Sondek *et al.* (1996), the bovine Gβ (Figure 2, bosGb1) has an amino-terminal 47-residue coiled-coil preceding the classic seven-bladed β propeller structure formed by WD repeat proteins. Of these 47 residues, 20 form contacts with the Gγ subunit. Modeling of the 305-residue git5 protein predicts only 14 residues preceding the β propeller, suggesting a loss of 15 Gγ contact residues (data not shown). Of the remaining 34 Gγ contact residues of bosGb1, 19 (56%) are conserved in git5 (Figure 2). However, the location of these conserved residues is strikingly restricted to the central blades of the Gβ that contact Gγ. In blades 5, 6, 7, and 1, 16 of 21 (76%) Gγ contact residues are conserved, while in blades 2 and 4, as well as the 14 residues preceding the β propeller, only 3 of 13 (23%) Gγ contact residues are conserved.

**The *git5* gene contains a single 305-codon ORF:** The coding capacity of the DNA immediately upstream of the *git5* ORF displays characteristics of a classical Gβ subunit amino terminus, as residues defined by the 9 "codons" prior to the start codon are highly conserved with respect to other Gβ subunits (Figure 2). In addition, the sequence TAG, which commonly identifies a 3′ splice site in *S. pombe*, is present 3′ to the in-frame STOP codon preceding the git5 ORF but 5′ to these codons. This led us to assume that one or more exons must be present 5′ to this ORF, such that the true git5 product possesses a standard amino-terminal coiled-coil. However, several lines of evidence suggest that there is no splicing and that the 305-codon ORF encodes the complete and functional git5 product.

We have cloned a cDNA copy of *git5* (see materials

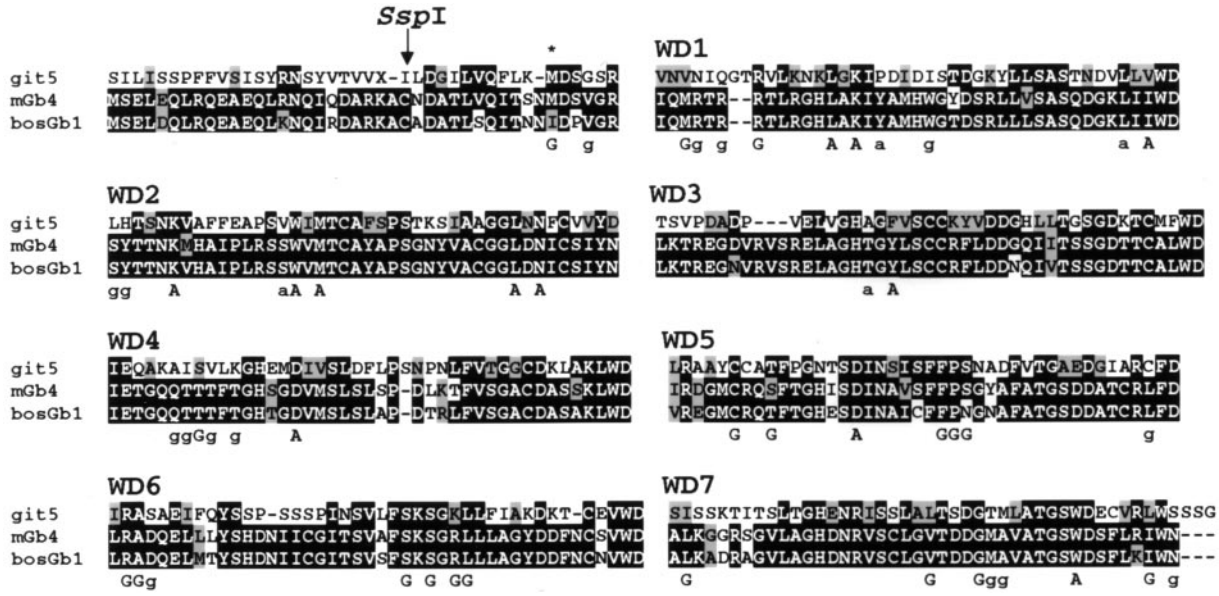


Figure 2.—The amino acid sequence alignment between the predicted git5 protein and two other Gβ subunits. Included in the git5 protein sequence are the hypothetical residues encoded by the 34 codons upstream from the translational start, including 1 stop codon (X). This sequence has been aligned with the murine Gβ4 (mGb4; P29387) and the bovine Gβ1 (bosGb1; 1942174) subunits using the CLUSTAL W version 1.7 sequence alignment program (Thompson *et al.* 1994) and was displayed using BOXSHADE. The *Ssp*I site used to subclone the *git5* ORF breaks the DNA between the second and third base pairs of the isoleucine (I) codon indicated by an arrow. The starting methionine of the git5 protein is indicated by an asterisk. Identical residues are shaded in black, while conserved residues are shaded in gray. The positions of contact residues between the bosGb1 Gβ subunit and the bovine Gα and Gγ subunits determined by Sondek *et al.* (1996) are indicated by A and G, respectively. Uppercase lettering identifies sites where git5 possesses an identical or conserved residue to mGb4 or bosGb1, and lowercase lettering identifies sites where git5 residues are not conserved.

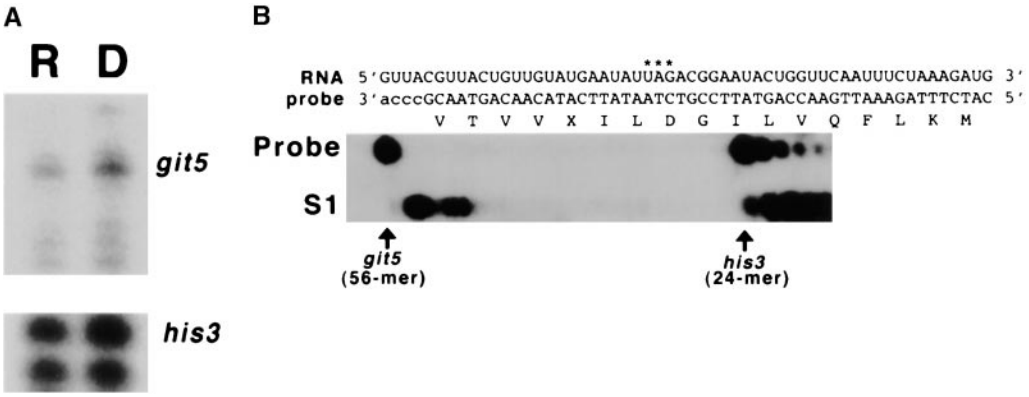


Figure 3.—Oligonucleotide-directed S1 analysis of *git5* transcript levels and of a candidate splice junction in the *git5* transcript. (A) A 56-nt probe, complementary to the *git5* transcript, and a 24-nt probe, complementary to the *his3* transcript (a loading control), were hybridized with RNA from 972 ($h^-$) cells grown under glucose-rich (R) and glucose-starved (D) conditions and digested with exonuclease S1 (see materials and methods). The relative intensities of the S1-protected fragments indicate that *git5* transcription is increased modestly by glucose starvation (less than fourfold). (B) Alignment of a schematic of the *git5* S1 analysis with an overexposed S1 digest shows no sign of splicing 5′ to the 305-codon *git5* ORF. Two lanes of an S1 digest are presented horizontally with the gel run left to right. The top lane (Probe) contains the untreated *git5* 56-mer probe and *his3* 24-mer probe as size standards. The bottom lane (S1) contains the S1-protected products of an analysis using these probes. The *git5* probe produces a major 52-nt band, along with minor bands of 47, 48, and 49 nt. The *his3* probe produces bands of 21 and 20 nt, along with bands of 24, 23, and 22 nt representing incompletely digested probe. The bands are roughly colinear with the schematic representation of the probe and RNA alignment. There is no detectable band at 29 nt that would be produced by hybridization of the probe to a transcript that was spliced at the UAG sequence (indicated by asterisks) in the RNA. The 47-, 48,- and 49-nt bands, along with several fainter bands, are likely due to partial melting of the A-T-rich 3′ end of the probe from the RNA during S1 treatment.

and methods) and found its sequence to be colinear to the genomic DNA. The cloned sequence in plasmid pSL10 initiates 122 bp upstream from the start codon of the 305-residue ORF. The cDNA sequenced in the *gbp1* study (Kim *et al.* 1996) initiated 324 bp upstream from the ATG of the 305-codon ORF and also showed no evidence of splicing. Therefore, at this crude level, it does not appear that the 305-codon ORF is connected to an upstream exon by splicing.

We have looked directly for evidence of splicing at the 5′ end of the *git5* ORF by oligonucleotide-directed S1 analysis. A 56-nucleotide (nt) probe complementary to the *git5* transcript and extending past the in-frame STOP codon preceding the ORF was used both to measure levels of *git5* transcription and to detect splice junctions within this region. The probe terminates in 4 nt that are not complementary to the *git5* sequence. End-labeled probe was hybridized to RNA from cells grown under repressing and derepressing conditions and treated with exonuclease S1 to degrade any unannealed probe, along with single-stranded portions of the annealed probe. The appearance of a 52-nt protected fragment indicates that *git5* transcription initiates upstream from the region complementary to this probe (Figure 3, A and B). An overexposed S1 analysis (Figure 3B) shows that the UAG sequence in this region of the mRNA does not serve as a 3′ splice site. If such splicing occurred, it would have produced a transcript that only protects 29 nt at the 5′ end of the probe. In addition, the ratio of the *git5*-specific product to a *his3*-specific product that serves as a loading control reveals a modest increase in *git5* transcription under derepressing conditions (Figure 3A).

To test whether the 305-codon ORF encodes a functional git5 protein, we subcloned the 1.4-kb *Ssp*I fragment encompassing this ORF from pSL10 into the pART1 expression vector. Both plasmids pSL11 and pSL12, which carry this fragment in either orientation relative to the vector-provided promoter, complement *git5* mutations as they restore glucose repression of *fbp1*

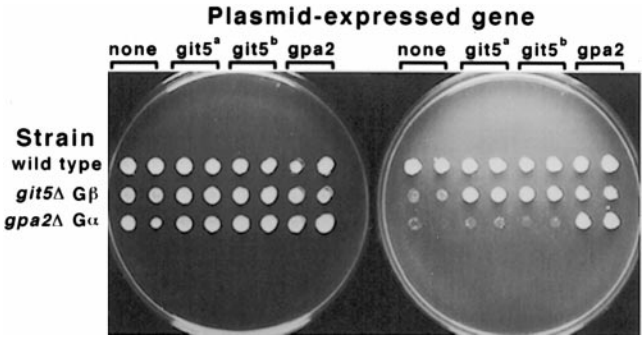


Figure 4.—High-copy-number suppression by *gpa2*$^+$ (Gα) and *git5*$^+$ (Gβ). Two independent transformants of strains FWP112 (*git*$^+$, wild type), CHP477 (*git5*Δ), and CHP439 (*gpa2*Δ) transformed to Leu$^+$ with pART1 (empty vector), pMP1 (*git5*$^a$; genomic *git5* clone), pSL12 (*git5*$^b$, *Ssp*I subclone of *git5* into pART1), or pGMSK1 (*gpa2*; Nocero *et al.* 1994) were tested for growth in the presence (right) and absence (left) of 5-FOA. Growth in the presence of 5-FOA reflects the ability to repress *fbp1* transcription. Overexpression of either *git5*$^+$ or *gpa2*$^+$ suppresses the 5-FOA-sensitive phenotype of the *git5*Δ strain, while only overexpression of *gpa2* suppresses the 5-FOA-sensitive phenotype of the *gpa2*Δ strain. Plasmid pSL11 (not shown) also confers 5-FOA resistance to strain CHP477 (*git5*Δ); however, pSL11 transformants display a general slow growth phenotype regardless of the host strain.

**TABLE 2**

**β-Galactosidase activity from *fbp1-lacZ* reporter**

| Host genotype | Plasmid-expressed gene | | | |
|---|---|---|---|---|
| | Empty vector | *gpa2* | *git5*[a] | *git5*[b] |
| *git*$^+$ | 8 | 8 | 15 | 24 |
| *gpa2*Δ | 2079 | 13 | 1905 | 1802 |
| *git5*Δ | 922 | 180 | 15 | 39 |

Strains FWP112 (*git*$^+$), CHP439 (*gpa2*Δ), and CHP477 (*git5*Δ) were transformed to Leu$^+$ with pART1 (empty vector; McLeod *et al.* 1987), pGMSK1 (*gpa2*; Nocero *et al.* 1994), pMP1 (*git5*[a]; genomic *git5* clone), or pSL11 (*git5*[b]; *Ssp*I subclone of *git5* into pART1). Two independent transformants from each transformation were purified and grown to exponential phase under glucose-rich (8%) conditions. β-Galactosidase expressed from the *fbp1-lacZ* fusion was determined from two to four independent cultures of each transformant as described previously (Nocero *et al.* 1994). Standard errors were <20% of the mean.

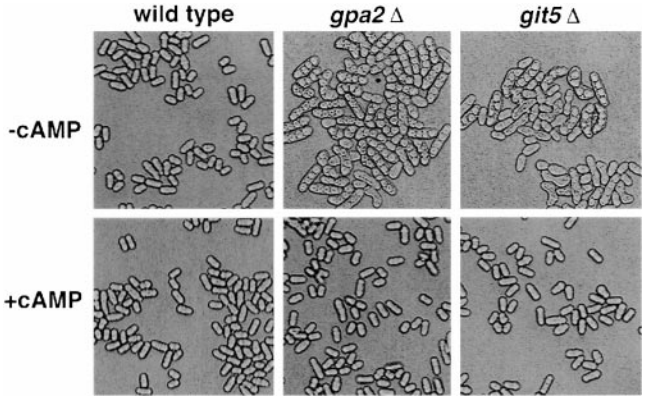


Figure 5.—Starvation-independent sexual development in *git5*Δ strains. Cells of homothallic strains CHP483 (wild type), CHP481 (*gpa2*Δ), and CHP488 (*git5*Δ) were grown to log phase in PM liquid medium (at 37° to inhibit conjugation) and then diluted to $10^6$ cells/ml in PM liquid medium with or without 5 mm cAMP. These cells were incubated for 24 hr at 30° without shaking and photographed.

transcription to the same degree as the original genomic clone (Figure 4 and Table 2), although pSL11 (expressing *git5* from the plasmid-borne *adh* promoter) reduces the general growth rate of transformants. The ability of pSL12 to complement suggests that transcription must also occur from *ars1* adjacent to the cloning polylinker to allow transcription of *git5*. Since these subclones separate the *git5* ORF from all upstream *git5* sequences including any other possible start codons, the product of this single exon is functional.

**Disruption of *git5* confers phenotypes associated with a defect in glucose monitoring, but not pheromone signaling:** Disruption of the *git5* ORF (*git5*Δ; see materials and methods) confers Git$^-$ mutant phenotypes, including elevated *fbp1-lacZ* expression in cells grown under glucose-rich conditions (Table 2) and 5-FOA-sensitive growth due to constitutive expression of the *fbp1-ura4*$^+$ reporter (Figure 4; strain CHP477 carrying empty vector). Complementation analyses place this gene disruption in the *git5* complementation group, as a *git5-75*/*git5*Δ diploid strain displays the Git$^-$ 5-FOA-sensitive phenotype (data not shown).

The *git5* disruption also stimulates conjugation and sporulation of an $h^{90}$ homothallic strain growing under nutrient-rich conditions that can be suppressed by exogenous cAMP, similar to the phenotype of a *gpa2*Δ strain (Figure 5). Derepression of sexual development was also attributed to the null allele in the *gpb1* study, although the authors of that study concluded that this is due to the activation of the gpa1 protein that positively regulates the pheromone response pathway (Kim *et al.* 1996). However, this interpretation is unjustified since conjugation and sporulation in the *git5*Δ mutants is starvation independent, but not pheromone independent.

As shown in Figure 6, a heterothallic *git5*Δ strain does not produce conjugation tubes when starved in the absence of a mating partner, as is seen in a strain carrying the activated allele of *gpa1* (*gpa1*$^{QL}$; Obara *et al.* 1991). Therefore, git5 does not negatively regulate gpa1.

**High-copy suppression by *gpa2*$^+$ and *git5*$^+$:** While multicopy *git5*$^+$ expression suppresses *git5* point mutations and a *git5* deletion, it has no effect upon mutations in the other five genes required for adenylate cyclase activation (*gpa2*, *git1*, *git3*, *git7*, or *git10*; data shown for only the *gpa2* mutant strain; Figure 4 and Table 2). In contrast to this, *gpa2*$^+$ (Gα) overexpression suppresses the mutant phenotypes associated with the *git5* disruption (Figure 4 and Table 2). However, the suppression of a *git5* deletion by *gpa2*$^+$ overexpression is only partial compared to suppression by *git5*$^+$ itself (Table 2), as indicated by the inability of Gα overexpression to fully repress *fbp1-lacZ* expression in a *git5* mutant strain. This genetic relationship is consistent with a model in which git5 acts in the same heterotrimeric G protein complex as gpa2 (see discussion).

**cAMP levels in *gpa2* and *git5* disruption strains:** We showed previously that mutations in *gpa2* (*git8*) or in *git5* inhibit the glucose-triggered elevation of cAMP levels in

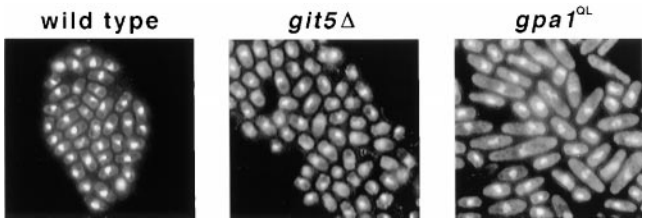


Figure 6.—Conjugation tube formation remains pheromone-dependent in *git5*Δ strains. Cells of heterothallic $h^+$ strains FWP87 (*git5*$^+$ *gpa1*$^+$), CHP478 (*git5*Δ), and CHP745 (*gpa1*$^{QL}$) were pregrown on PM solid medium, transferred to SPA medium for 2 days at 30°, and photographed. Only the CHP745 (*gpa1*$^{QL}$) cells display conjugation tubes in the absence of a pheromone-producing mating partner.

**TABLE 3**

**Basal cAMP levels in *S. pombe* strains**

| Strain | Relevant genotype | Basal cAMP | Glucose-induced cAMP |
|---|---|---|---|
| Experiment 1[a] | | | |
| FWP94 | *gpa2*$^+$*git5*$^+$ | 3.3 ± 0.5 | ND |
| CHP463 | *git5*Δ | 3.2 ± 0.6 | ND |
| CHP462 | *gpa2*Δ | 2.0 ± 0.2 | ND |
| CHP469 | *gpa2*Δ *git5*Δ | 1.9 ± 0.4 | ND |
| Experiment 2[b] | | | |
| FWP94 | *gpa2*$^+$*git5*$^+$ | 3.0 ± 0.7 | 11.2 ± 3.9 |
| CHP463 | *git5*Δ | 4.3 ± 0.8 | 4.7 ± 0.9 |

[a] From five to nine independent cultures of each strain were grown to logarithmic phase under low glucose conditions (0.1% glucose, 3% glycerol) and assayed as previously described (Byrne and Hoffman 1993) using a RIA assay kit (Amersham, Piscataway, NJ).

[b] Three independent cultures of each strain were grown as in experiment 1. cAMP levels were determined from cells collected prior to, and 1 min after, the addition of glucose to a final concentration of 100 mm. Values indicate mean ± standard error of the pmol cAMP/mg total protein.

fission yeast (Byrne and Hoffman 1993). This cAMP response is due to the activation of adenylate cyclase rather than the inactivation of cAMP phosphodiesterase, which converts cAMP to AMP. In that study, we also showed that the basal cAMP level was reduced in the *gpa2*/*git8* (Gα) mutant, but not in the *git5* (Gβ) mutant. We show here that deletion of *git5* has no effect on basal cAMP levels in strains that either possess or lack the *gpa2* gene (Table 3, experiment 1), in agreement with other deletion studies of these two genes (Isshiki *et al.* 1992; Kim *et al.* 1996). As with the point mutation in *git5* (Byrne and Hoffman 1993), the *git5* disruption inhibits the cAMP response to glucose (Table 3, experiment 2), confirming a role for *git5* in the glucose-triggered adenylate cyclase activation pathway.

## DISCUSSION

We have shown that the fission yeast *git5*$^+$ gene encodes a Gβ subunit and is identical to the previously cloned *gpb1*$^+$ gene (Kim *et al.* 1996). While the *gpb1* study concluded that this gene acts in the gpa1-regulated pheromone response pathway, and not in the gpa2-regulated adenylate cyclase activation pathway, we come to the opposite conclusions. Our work clearly shows that while *git5* is not required for maintaining basal cAMP levels, it is needed to activate adenylate cyclase in response to glucose detection (Table 3). As the *gpb1* study only examined basal cAMP levels, there were no direct data to indicate a defect in the cAMP pathway. We have shown previously that while *git1*$^+$, *git3*$^+$, *git5*$^+$, *git7*$^+$, *git10*$^+$, and *gpa2*$^+$ are all required for generating a cAMP response to glucose, only *git7*$^+$ and *gpa2*$^+$ are required for maintenance of basal cAMP levels (Byrne and Hoffman 1993).

The *gpb1* study described the effects of a *gbp1* deletion as stimulating conjugation and sporulation under nutrient-rich conditions, which we have also observed. While the authors of that study attributed this to the activation of the gpa1 protein of the pheromone response pathway, our observations are more consistent with a reduction in activation of gpa2 in the glucose-triggered adenylate cyclase activation pathway. If loss of git5/gpb1 activated gpa1, we would expect to see a pheromone-independent stimulation of conjugation as seen by the production of conjugation tubes in a heterothallic strain, similar to that seen in strains carrying an activated allele of *gpa1* (*gpa1*$^{QL}$; Obara *et al.* 1991). Since *git5*/*gpb1* deletion strains conjugate in a nutrient-independent, but pheromone-dependent, fashion (Figures 5 and 6), it appears that this Gβ works in concert with gpa2, but not antagonistically to gpa1.

The data presented here show that the git5 Gβ is required for adenylate cyclase activation. The git5 Gβ may simply be required for the efficient activation of the gpa2 Gα, facilitating the delivery of the Gα to a G-protein-coupled receptor. Alternatively or in addition, git5 may act more directly to stimulate adenylate cyclase as seen for type II mammalian adenylate cyclase (Gao and Gilman 1991; Tang and Gilman 1991; Federman *et al.* 1992). For these enzymes, both the Gα subunit and the Gβγ dimer act to stimulate adenylate cyclase in an ordered fashion with the Gα subunit acting first. Our data to date cannot distinguish between these two possibilities.

As described above, the git5 Gβ subunit is unusual in its lack of an amino-terminal coiled-coil found in all other members of this protein family. Complicating this observation is the presence of DNA sequences in the 5′ untranslated region of the gene that have the capacity to encode a portion of this domain. At first, we assumed that a splicing event must occur to create a transcript that encodes a standard-length Gβ. However, there is only one potential 3′ splice junction between an upstream in-frame stop codon and the conserved LVQ codons immediately 5′ to the large ORF (Figures 2

and 3B). We show that this site is not utilized in splicing as determined by both DNA sequence analysis of cDNA clones and by the more sensitive oligonucleotide-directed S1 analysis (Figure 3B). Most importantly, a subclone containing only this single ORF is fully functional (Figure 4 and Table 2). Therefore, it appears that the genomic sequence immediately upstream from the 305-codon ORF is an evolutionary remnant from a gene that encoded a standard-length Gβ subunit possessing an amino-terminal coiled-coil.

The absence of an amino-terminal coiled-coil in the git5 protein brings into question the existence of a Gγ subunit in this G protein, since binding to Gγ is the major function attributed to this region of Gβ (Garritsen *et al.* 1993; Wall *et al.* 1995; Garcia-Higuera *et al.* 1996; Lambright *et al.* 1996; Sondek *et al.* 1996; Pellegrino *et al.* 1997). However, we have preliminary evidence that suggests that the git5 Gβ is part of a classical heterotrimeric G protein. We have isolated a cDNA clone in a two-hybrid screen using a git5 bait that appears to encode a Gγ subunit. The 71-codon ORF present on the cDNA is part of a 72-codon gene whose putative product displays characteristics of a Gγ, including a lysine-rich carboxy terminus and a carboxy-terminal CAAX box (S. Landry and C. S. Hoffman, unpublished results).

The regulation of *S. pombe* adenylate cyclase provides us with a genetically pliable model system for the study of G protein signal transduction that is significantly different from that of the well-studied *Saccharomyces cerevisiae* pheromone response pathway. In this latter system, the Gβγ dimer is responsible for activation of a mitogen-activated protein kinase cascade, while the Gα subunit is a negative regulator of pheromone response (Whiteway *et al.* 1988; Nomoto *et al.* 1990). Given the cooperative functional nature of the G protein subunits in *S. pombe* adenylate cyclase activation and the lack of the amino-terminal coiled-coil in the Gβ subunit, further studies of the relationship between G protein subunit interactions and effector activation in this system may provide us with new insights regarding mechanisms of G protein signal transduction.

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