Characterization of a fission yeast gene, *gpa2*, that encodes a G α subunit involved in the monitoring of nutrition

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The *Schizosaccharomyces pombe gpa2* gene was cloned by hybridization with a cDNA for *Dictyostelium discoideum* G α 1. It encodes a homolog of G-protein α -subunits with 354 amino acids and a predicted molecular mass of 40,522. Disruption of *gpa2* slows cell growth but is not lethal. Cells defective in *gpa2* mate and sporulate readily in the presence of plentiful nutrition, bypassing the requirement of nitrogen starvation for the initiation of sexual development. These phenotypes mimic those of cells defective in *cyr1* encoding adenylyl cyclase. The level of cAMP in *gpa2* null mutants is only one-third of the wild-type level. Mutations in *gpa2* that are likely to inhibit the GTPase activity of the gene product cause a slight increase in intracellular cAMP levels and result in leaky sterility. The cAMP level reaches 20 times as high as the wild-type level if a cell carries both this type of *gpa2* mutation and a null mutation in *pde1* encoding phosphodiesterase. Cells defective in *gpa2* fail to produce cAMP in response to glucose stimulation. These results suggest that Gpa2 is involved in the determination of the cAMP level according to nutritional conditions, most likely as a positive regulator of adenylyl cyclase.

[Key Words: *Schizosaccharomyces pombe*; G protein; cAMP production; glucose; sexual differentiation]

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Cells of the fission yeast *Schizosaccharomyces pombe* initiate sexual development, namely mating, meiosis, and sporulation, in response to nitrogen starvation (Leupold 1970; Egel 1973). Nitrogen starvation reduces the intracellular cAMP level by 50% (Fukui et al. 1986; Mochizuki and Yamamoto 1992). This reduction is a signal for induction of the *ste11* gene, which encodes a transcription factor responsible for the activation of genes involved in mating and meiosis (Watanabe et al. 1988; Sugimoto et al. 1991). Carbon starvation also reduces the cAMP level, even more sharply than nitrogen starvation (N. Mochizuki and M. Yamamoto, unpubl.). However, excessive carbon starvation apparently shuts down any developmental activities. Partial carbon starvation can augment the induction of sexual development by nitrogen starvation. It is thus conceivable that fission yeast has a mechanism that detects the availability of nutrients and regulates the level of intracellular cAMP.

How cells monitor the availability of nutrition is an interesting but poorly understood question. It now appears that our analysis of a G-protein homolog in *S. pombe* has provided a clue to this question.

Heterotrimeric G proteins are generally coupled with

receptors that carry seven membrane-spanning domains. The receptors on the cell membrane bind their specific ligands, and this signal is transmitted into the cell by the G proteins (Gilman 1987; Dohlman et al. 1991). Our previous screen identified the *S. pombe gpa1* gene encoding an α -subunit of a G protein that we concluded to be coupled with the mating pheromone receptors (Obara et al. 1991). We have now identified an *S. pombe* gene that encodes another G α subunit and named it *gpa2*. Genetic analysis of *gpa2* suggests that this gene product is involved in the regulation of the intracellular cAMP level in accordance with nutritional conditions. Its most probable function is modulation of adenylyl cyclase activity. Thus, it is likely that a G α -subunit plays a role in transduction of a signal from nutritional conditions, although the identity of the signal has still not been determined.

Results

Cloning of the *gpa2* gene

S. pombe genomic DNA was analyzed by Southern blotting using cDNAs encoding *Dictyostelium discoideum* G α 1 and G α 2 (Pupillo et al. 1989) as probes. A few restriction fragments hybridized to these probes under low stringency conditions (data not shown), and we cloned an 8.0-kb *EcoRI* fragment that gave a clear hybridization band with the G α 1 cDNA. Subcloning and partial se-

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quence analysis indicated that this fragment carried a gene encoding a $G\alpha$ homolog, hereafter called *gpa2*. Because the 3' end of the *gpa2* gene was not found on the *EcoRI* fragment, we cloned a 2.4-kb *PstI* fragment that covers the missing part of the gene. A restriction map of the *gpa2* locus is shown in Figure 1, and the nucleotide sequence of the entire *gpa2* open reading frame (ORF) and its flanking regions was determined (see Fig. 2). This sequence suggested the existence of a 405-bp-long intron in *gpa2*, which was confirmed by cDNA cloning using the polymerase chain reaction (PCR) method (Saiki et al. 1988) (data not shown). Many mammalian $G\alpha$ genes have an intron at the same position as *gpa2* (Kaziro et al. 1991).

The *gpa2* gene has a coding capacity of 354 amino acids. The deduced Gpa2 protein exhibits 39.3% amino acid identity with *D. discoideum* $G\alpha_1$, whose cDNA was used as the probe. Gpa2 is 40.7% identical with *D. discoideum* $G\alpha_2$, 40.1% with human $Gi2\alpha$, and only 36.2% with *S. pombe* Gpa1. It is most similar to *Saccharomyces cerevisiae* Gpa2 (42.4%) among G-protein α -subunits identified so far (Fig. 3).

Disruption of the *gpa2* gene

One of the two *gpa2* alleles in a diploid strain, JY765, was disrupted, as described in Materials and methods. The structure of the disrupted allele, in which nucleotides -22 to +1514 in Figure 2 were deleted and replaced by a *ura4⁺* cassette, is schematically shown in Figure 1. Heterothallic haploid cells carrying the disrupted *gpa2* allele were obtained by tetrad dissection of the diploid strain. They were viable but were smaller in cell size (Fig. 4E) and grew 1.4 times slower than the wild type. Homothallic *gpa2*-disrupted cells were constructed

Figure 1. A restriction map and schematic illustration of disruption of the *gpa2* gene. A pUC119-based plasmid, pGPE13H, carries a 4.8-kb *HindIII*-*EcoRI* fragment. Using the *EcoRI* terminal region of this insert (solid bar), a 2.4-kb *PstI* fragment was cloned in pUC119 and the resulting plasmid was named pGPR. A restriction map of the *gpa2* locus was deduced from the inserts of these two plasmids and is shown at the top. The open arrow indicates the translated region of *gpa2* interrupted by an intron. The structure of the linear fragment used to disrupt the *gpa2* gene is shown at the bottom. The hatched box represents an *S. pombe ura4⁺* cassette. Restriction sites are abbreviated as follows: (B) *BamHI*; (E) *EcoRI*; (H) *HindIII*; (Hc) *HincII*; (K) *KpnI*; (P) *PstI*; and (S) *ScaI*.

Figure 2. The nucleotide and deduced amino acid sequences of *gpa2*. Numbering starts at the presumed initiation codon. Underlined regions match the consensus sequence for splicing in *S. pombe* (Russell 1989). The region between a *KpnI* site and a *ScaI* site is shown, which has been sequenced in both directions. The nucleotide sequence data reported here have been submitted to the DNA Data Bank of Japan, EMBL, and GenBank nucleotide sequence data bases under accession number D13366.

by genetic crosses. The homothallic derivatives mated and sporulated readily in nitrogen-rich medium (Fig. 4B) and grew very poorly. These phenotypes of the *gpa2*-disrupted cells were reminiscent of those of the *cyr1*-disrupted cells, which have no measurable activity of adenylyl cyclase and no measurable amount of cAMP (Maeda et al. 1990; Kawamukai et al. 1991). Addition of 2 mM cAMP and 5 mM caffeine to the medium, the latter of which is a phosphodiesterase inhibitor, could suppress the observed phenotypes of the *gpa2*-disrupted cells: Heterothallic cells resumed normal cell size and growth rate (Fig. 4F), and homothallic cells did not enter ectopic sexual development (Fig. 4C). These observations suggested that loss of function of *gpa2* could cause a decrease in the level of intracellular cAMP, and measurement of the amount of cAMP in the cell demonstrated that this was the case (Table 1, experiment 1).

Mutations affecting the GTPase activity of Gpa2

Mutations at either of the two amino acid residues in mammalian $G\alpha$, namely Arg-201 and Gln-227, are able to inhibit the GTPase activity of the protein and bring oncogenic characteristics to it (Landis et al. 1989). Mutations in the corresponding positions of $Gi2\alpha$ also cause a similar effect (Lyons et al. 1990). The two residues are conserved in Gpa2 as Arg-176 and Gln-202. We mutated Arg-176 to His and Gln-202 to Leu according to the procedure described by Kunkel (1985). These mutations

were termed R176H and Q202L. We then constructed three types of plasmids that express the wild type, the R176H allele, and the Q202L allele, respectively, from the *adh1* promoter, by inserting a 2.4-kb *Pst*I fragment carrying each allele into the *Nde*I site of an expression vector, pART3 (Kelly et al. 1988). They were named pAGWT (wild type), pAGH (R176H), and pAGL (Q202L). A homothallic haploid strain, JY450, was transformed with these plasmids or the vector pART3. Compared with the control cells carrying pART3 (vector), cells transformed with pAGWT (*gpa2*⁺) were less efficient in mating and sporulation (Fig. 5B). Cells transformed with either pAGH (*gpa2*^{R176H}) or pAGL (*gpa2*^{Q202L}) were much less efficient (Fig. 5C,D). Thus, these transformants mimicked strains that have high levels of intracellular cAMP (Maeda et al. 1990; DeVoti et al. 1991; Mochizuki and Yamamoto 1992). The cAMP level was elevated in these transformants (Table 1, experiment 3); however, the elevation was not striking, although these transformants showed unambiguous phenotypes in mating. This probably reflects that the cAMP level is strongly feedback regulated in fission yeast cells and that even a small increase or decrease in this level can significantly affect cell physiology, as was discussed previ-

Figure 1 consists of six panels (A-F) showing fluorescence micrographs of *C. elegans* expressing GFP::GFP-1. Panel A shows two worms: the top one has diffuse cytoplasmic GFP-1, while the bottom one has punctate localization. Panel B shows a cluster of worms with punctate localization. Panel C shows several worms with punctate localization. Panel D shows a cluster of worms with punctate localization. Panel E shows a cluster of worms with punctate localization. Panel F shows two worms with punctate localization, including a scale bar in the bottom right corner.

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Table 1. *cAMP level in various strains*

Strain	Relevant genotype	Plasmid	cAMP level (pmole/mg of protein)
Experiment 1			
JY333	$h^- gpa2^+$		3.6 ± 0.1
JZ393	$h^- \Delta gpa2$		1.3 ± 0.1
			1.2 ± 0.1
Experiment 2			
JY450	$h^{90} gpa2^+$		3.4 ± 0.1
JZ692	$h^{90} gpa2^{R176H}$		4.4 ± 0.1
JZ593	$h^{90} gpa2^{Q202L}$		3.8 ± 0.2
Experiment 3			
JY450	$h^{90} gpa2^+$	pART3	2.5 ± 0.1
			1.9 ± 0.1
JY450	$h^{90} gpa2^+$	pAGWT($gpa2^+$)	2.5 ± 0.1
			2.5 ± 0.1
JY450	$h^{90} gpa2^+$	pAGH($gpa2^{R176H}$)	4.0 ± 0.3
			2.9 ± 0.3
JY450	$h^{90} gpa2^+$	pAGL($gpa2^{Q202L}$)	3.2 ± 0.3
			2.7 ± 0.1
Experiment 4			
JY450	$h^{90} gpa2^+ pde1^+$		2.3 ± 0.2
JZ666	$h^{90} gpa2^+ \Delta pde1$		8.0 ± 1.2
JZ655	$h^{90} \Delta gpa2 \Delta pde1$		2.9 ± 0.1
JZ656			3.2 ± 0.1
JZ657	$h^{90} gpa2^{R176H} \Delta pde1$		96.6 ± 4.3
JZ658			56.1 ± 4.1
JZ659	$h^{90} gpa2^{Q202L} \Delta pde1$		59.1 ± 2.8
JZ660			52.7 ± 12.1
Experiment 5			
JZ666	$h^{90} gpa2^+ \Delta pde1$		10.7 ± 0.1
JZ667			14.5 ± 0.3
JZ655	$h^{90} \Delta gpa2 \Delta pde1$		2.6 ± 0.4
JZ656			2.6 ± 0.2
JZ657	$h^{90} gpa2^{R176H} \Delta pde1$		34.2 ± 0.9
JZ658			53.4 ± 2.5
JZ659	$h^{90} gpa2^{Q202L} \Delta pde1$		64.9 ± 0.7
JZ660			46.5 ± 0.2

Cells grown in MM at 30°C were harvested at a concentration of 5×10^6 to 1×10^7 cells/ml and processed for cAMP assay. Each sample was assayed in duplicate. Two values are given for one strain when two independent samples were assayed.

dation. To differentiate between these two possibilities, we combined R176H and Q202L mutations with a null mutation in *pde1* (Mochizuki and Yamamoto 1992), also called *cgs2* (DeVoti et al. 1991), which encodes cAMP phosphodiesterase. Disruption of *pde1* resulted in elevation of the intracellular cAMP level (Mochizuki and Ya-

mamoto 1992; Table 1, JZ666 and JZ667). If Gpa2 were a negative regulator of this enzyme, the R176H or the Q202L mutation would cause no additional elevation in the cAMP level in the *pde1*-disrupted cells. Alternatively, if Gpa2 were involved in the regulation of cAMP production, further elevation might have occurred. Anal-

Figure 5. Morphology of homothallic cells carrying mutant Gpa2 with low GTPase activity. The following plasmids were introduced into JY450 (h^{90} wild type), and the cells were incubated on SSA plates at 30°C for 5 days to induce mating and sporulation. pART3 is the vector, as a control. pAGWT carries the wild-type allele of *gpa2*. pAGH and pAGL carry mutant alleles R176H and Q202L, respectively. Construction of these mutant alleles is described in Materials and methods. Phase-contrast micrographs of JY450 cells transformed with each plasmid are shown in A–D. (A) The vector pART3; (B) pAGWT ($gpa2^+$); (C) pAGH ($gpa2^{R176H}$); (D) pAGL ($gpa2^{Q202L}$). Bar, 10 μ m.

Table 2. Mating efficiency of *gpa2* mutants

Strains	<i>gpa2</i> allele	Mating efficiency (%)
JY450	<i>gpa2</i> ⁺	92.1 ± 1.8
JZ692	<i>gpa2</i> ^{R176H}	8.7 ± 1.2
JZ593	<i>gpa2</i> ^{Q202L}	10.2 ± 1.9

ysis of the double mutants showed that the latter is the case (Table 1, experiments 4 and 5). A remarkable increase in the level of cAMP was observed in both *gpa2*^{R176H} *pde1*⁻ strains (JZ657, JZ658) and *gpa2*^{Q202L} *pde1*⁻ strains (JZ659, JZ660). Furthermore, the cAMP level in *gpa2*⁻ *pde1*⁻ strains (Table 1, JZ655 and JZ656) was much lower than that of the parental *pde1*⁻ strains (JZ666, JZ667) and was comparable to that of the wild type. They were competent in mating. These results indicate that Gpa2 does not regulate the *pde1*-encoded phosphodiesterase. Thus, Gpa2 is most likely to be involved in the regulation of cAMP production in *S. pombe*, although we cannot completely rule out the possibility that *S. pombe* has another phosphodiesterase, yet undetected, and that its activity is regulated by Gpa2.

Gpa2 is required for the response to glucose stimulation

It has been shown that stimulation of quiescent *S. cerevisiae* cells by glucose results in a transient increase in the intracellular cAMP level (Nakafuku et al. 1988). To test whether Gpa2 is involved in this type of response in *S. pombe*, *gpa2*⁺, *gpa2*⁻, and *gpa2*^{R176H} cells were starved for both glucose and nitrogen and then stimulated by the addition of glucose to a final concentration of 2%. The amount of cAMP in the cell was assayed at intervals, and the results are given in Figure 6. The cAMP level in the *gpa2*⁺ cells reached a peak 4 min after stimulation and then decreased and stayed around the value of 2.0 pmoles/mg of protein. The *gpa2*⁻ cells, however, showed no peak, and the level increased gradually and reached only 1.1 pmoles/mg of protein. The R176H mutant showed a sharp increase in the level of cAMP, which then stayed at ~2.7 pmoles/mg of protein. These results indicate that Gpa2 has a role in the assessment of glucose availability.

Discussion

This study has shown that *S. pombe* has a second G homolog. The first homolog, encoded by *gpa1*, was characterized previously and concluded to be coupled with the mating pheromone receptors (Obara et al. 1991). The second homolog, encoded by *gpa2*, is not closely related to the first, structurally or functionally.

Analysis of *gpa2* mutants suggested that its gene product Gpa2 is likely to be involved in regulation of cAMP production. The level of cAMP in *S. pombe* cells varies, depending on nutritional conditions. Gpa2 apparently

plays an important role in the assessment of these conditions. We presented direct evidence that Gpa2 is required for sensing glucose stimulation. Judging from the phenotypes of *gpa2* mutants, it is also possible that Gpa2 detects the availability of nitrogen.

Despite the above conclusions, however, our study is still too preliminary to answer questions regarding how cells monitor nutritional conditions. For instance, one may ask which kind of molecule represents nutritional conditions, or whether such a molecule exists at all. If so, does a single molecule represent the availability of both nitrogen and carbon? Is Gpa2 coupled to a receptor that binds a ligand? Is the receptor membrane bound? We presently have no answer to these fundamental questions. Further analysis of the Gpa2 pathway will help to solve these problems.

It has been established that G proteins coupled to hormone receptors regulate adenylyl cyclase in mammalian cells (for review, see Gilman 1987). Our conclusion is that Gpa2 is likely to regulate adenylyl cyclase in fission yeast. This analogy is not as simple as it may appear. *S. pombe* adenylyl cyclase (Yamawaki-Kataoka et al. 1989; Young et al. 1989) resembles *S. cerevisiae* adenylyl cyclase (Kataoka et al. 1985), but these yeast enzymes have only limited homology with mammalian adenylyl cyclases and the two groups apparently differ in their means of association with the plasma membrane (Krupinski et al. 1989; for review, see Tang and Gilman 1992). Furthermore, *S. cerevisiae* adenylyl cyclase is regulated by Ras proteins (Toda et al. 1985), but the only Ras homolog in *S. pombe*, encoded by *ras1*, does not regulate adenylyl cyclase activity (Fukui et al. 1986).

Figure 6. Glucose-stimulated cAMP formation in *gpa2* mutants. Cells of JY333 (*h*⁻ wild type), JZ393 (*h*⁻ *gpa2::ura4*⁺), and JZ693 (*h*⁻ *gpa2*^{R176H}) were grown in MM to the stationary phase and collected. They were resuspended in MM-C-N and incubated for 6–7 hr. These cells were stimulated with glucose (final concentration, 2%), and the cAMP level was measured at short intervals. Only one sample was assayed at each point, but essentially the same pattern was reproduced in a separate experiment. (●) JY333 (*gpa2*⁺); (▲) JZ393 (*gpa2::ura4*⁺); (■) JZ693 (*gpa2*^{R176H}).

Thus, *S. pombe* adenylyl cyclase appears to occupy a curious intermediate position in terms of regulation by the GTP-binding protein.

An interesting observation has been made in *S. cerevisiae* relative to the above point. In the budding yeast, two genes have been shown to encode G α homologs. The first homolog, encoded by *GPA1/SCG1*, is coupled to the mating pheromone receptors (Dietzel and Kurjan 1987; Miyajima et al. 1987). The role of Gpa2, the second homolog encoded by the *GPA2* gene, is less clear, because the disruption of *GPA2* did not cause any obvious phenotype (Nakafuku et al. 1988). Overexpression of *GPA2* elevated the cAMP level and could suppress a temperature-sensitive *RAS2* mutation. Although this observation was interpreted in several ways by Nakafuku et al. (1988), our finding that *S. pombe* Gpa2 is likely to regulate adenylyl cyclase suggests that *S. cerevisiae* Gpa2 may also be capable of activating adenylyl cyclase. The similarity observed between *S. pombe* Gpa2 and *S. cerevisiae* Gpa2 supports this notion. These considerations lead to the following concept. Both Ras proteins and a G protein regulate the activity of adenylyl cyclase in *S. cerevisiae*, but the contribution of the G protein is unimportant under physiological conditions. In contrast, the Ras protein is not involved in the regulation of adenylyl cyclase in fission yeast (Fukui et al. 1986) and a G protein has established itself as the major regulator of the enzyme.

It has been proposed that GTP-binding proteins are unlikely to be involved in the regulation of adenylyl cyclase in *S. pombe*, based on the observation that it has been impossible to reproduce enzymatic activity in vitro in a GTP-dependent manner (Engelberg et al. 1990). Our findings urge more careful investigation of this point, especially because it is now possible to use genetically engineered Gpa2 protein, with or without a mutation, to establish the in vitro assay system.

Materials and methods

Strains, media, genetic methods, and transformation of *S. pombe*

S. pombe strains used in this study are listed in Table 3. Media for routine cultures and general genetic methods are according to Gutz et al. (1974). Cells subjected to the analysis of cAMP were grown in minimal liquid medium (MM) and its derivatives. MM is the same as EMM, described by Moreno et al. (1990) except that the concentration of molybdic acid is 0.13 mg/liter. MM-N lacks NH₄Cl, and MM-C-N lacks glucose and NH₄Cl. Mating and sporulation efficiency were examined on SSA plates (Egel and Egel-Mitani 1974). Transformation of *S. pombe* was described previously (Beach et al. 1982; Okazaki et al. 1990).

Cloning of *gpa2*

S. pombe genomic DNA was digested with *EcoRI* and electrophoresed in a 0.9% agarose gel. DNA fragments of ~8 kb in length were recovered from a piece of the gel by using a silica matrix (GeneClean II Kit, Bio101, Inc.) and cloned in λ ZAP (Stratagene). One thousand recombinant phages were screened

Table 3. *S. pombe* strains used in this study

Strain	Genotype
JY333	<i>h</i> ⁻ <i>ade6-M216 leu1</i>
JY450	<i>h</i> ⁹⁰ <i>ade6-M216 leu1</i>
JY765	<i>h</i> ⁺ / <i>h</i> ⁻ <i>ade6-M216/ade6-M210 leu1/leu1 ura4-D18/ura4-D18</i>
JZ393	<i>h</i> ⁻ <i>ade6-M216 leu1 ura4-D18 gpa2::ura4⁺</i>
JZ415	<i>h</i> ⁹⁰ <i>ade6-M216 leu1 ura4-D18 gpa2::ura4⁺</i>
JZ593	<i>h</i> ⁹⁰ <i>ade6-M216 leu1 gpa2^{Q202L}</i>
JZ655	<i>h</i> ⁹⁰ <i>ade6-M216 leu1 ura4-D18 pde1::ura4⁺ gpa2::ura4⁺</i>
JZ656	<i>h</i> ⁹⁰ <i>ade6-M210 leu1 ura4-D18 pde1::ura4⁺ gpa2::ura4⁺</i>
JZ657	<i>h</i> ⁹⁰ <i>ade6-M216 leu1 ura4-D18 pde1::ura4⁺ gpa2^{R176H}</i>
JZ658	<i>h</i> ⁹⁰ <i>ade6-M210 leu1 ura4-D18 pde1::ura4⁺ gpa2^{R176H}</i>
JZ659	<i>h</i> ⁹⁰ <i>ade6-M216 leu1 ura4-D18 pde1::ura4⁺ gpa2^{Q202L}</i>
JZ660	<i>h</i> ⁹⁰ <i>ade6-M210 leu1 ura4-D18 pde1::ura4⁺ gpa2^{Q202L}</i>
JZ666	<i>h</i> ⁹⁰ <i>ade6-M216 leu1 ura4-D18 pde1::ura4⁺</i>
JZ667	<i>h</i> ⁹⁰ <i>ade6-M210 leu1 ura4-D18 pde1::ura4⁺</i>
JZ692	<i>h</i> ⁹⁰ <i>ade6-M216 leu1 gpa2^{R176H}</i>
JZ693	<i>h</i> ⁻ <i>ade6-M216 leu1 gpa2^{R176H}</i>

by Southern analysis (Southern 1979) under low stringency conditions using *D. discoideum* Gal1 cDNA as a probe. The stringency conditions employed in this study are as follows. Hybridization was done in 6 $\times$ SSC (0.9 M NaCl/0.09 M sodium citrate) containing 0.1% *N*-lauroylsarcosinate, 0.02% sodium lauryl sulfate, 3% blocking reagent (Boehringer Mannheim), and 20% formamide for 15 hr at either 42°C or 49°C. Washing was carried out in 2 $\times$ SSC (0.3 M NaCl/0.03 M sodium citrate) containing 0.1% sodium lauryl sulfate. Washing for 5 min was repeated three times at room temperature, and washing for 30 min was repeated three times at 53°C. Two positive clones were eventually obtained, which turned out to have the same insert. Subclones of the insert were made in pUC119. Because the original insert was found to carry only part of the *gpa2* gene, a 2.4-kb *PstI* fragment that carried the missing 3'-terminal region of *gpa2* was cloned in pUC119 using a 0.1-kb *ScaI-EcoRI* fragment (Fig. 1) as the probe.

DNA sequence determination

Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger et al. 1977). DNA fragments to be sequenced were cloned in either pUC119 or pBluescript (Stratagene), and subclones for sequencing were generated by progressive deletion with exonuclease III and S1 nuclease (Henikoff 1984). Single-stranded DNA was used as the template for sequence determination. The region shown in Figure 2 has been sequenced in both directions at least once.

Disruption of *gpa2*

One-step gene disruption of *gpa2* was done essentially as described (Rothstein 1983; Fukui et al. 1986). A pUC119-based plasmid was constructed by ligating the following three fragments: a 1.1-kb *BamHI-PstI* fragment carrying the immediate 5' upstream element to the *gpa2* ORF, of which the *PstI* end was

blunted; a 1.8-kb *S. pombe ura4⁺* cassette (Grimm et al., 1988) with both ends blunted; and a *HincII*–*PstI* fragment (0.9 kb) carrying the immediate 3' downstream element to the *gpa2* ORF and being connected to the vector pUC119 linearized with *PstI* and *BamHI*. Digestion of this plasmid with *BamHI* and *PstI* gave a 3.7-kb *BamHI*–*PstI* fragment that covered the *gpa2* locus but in which the 1.5-kb *PstI*–*HincII* fragment carrying the entire *gpa2* ORF was replaced by *ura4* (Fig. 1). An *S. pombe* diploid strain, JY765 (*h⁺/h⁻ ade6-M216/ade6-M210 leu1/leu1 ura4-D18/ura4-D18*), was transformed with this fragment, and *Ura⁺* transformants were selected. Disruption of one allele of *gpa2* was confirmed by Southern blotting in these transformants.

Construction of mutant *gpa2* alleles

In vitro site-directed mutagenesis of *gpa2* was performed as described (Kunkel 1985). The synthetic oligonucleotide used for construction of the R176H mutation was 5'-ACCCAGAGTAC-TATTCTGAGACCGGAGAATGTC-3', and that for Q202L was 5'-CCTTCGTTCCGTTCTTAAGCCACCAACGTCGAA-3'. Nucleotides that were changed from the wild-type sequence are underlined. The R176H mutation eliminated an *XbaI* recognition site, whereas the Q202L mutation created an *AflIII* recognition site. Successful construction of the mutant alleles was confirmed by sequence analysis. These mutant alleles were either borne on a multicopy plasmid or integrated in the chromosome. For integration, a 3.4-kb *BamHI*–*Sall* (*PstI*) fragment carrying the mutated allele was introduced into an *h⁹⁰ gpa2* disruptant, JZ415, in which the *gpa2* ORF was replaced by a *ura4⁺* cassette. Substitution of the disrupted allele with the mutant allele was expected to result in uracil auxotrophy. *Ura⁻* transformants were chosen by 5-FOA selection (Boeke et al. 1984; Grimm et al. 1988), and successful substitution was confirmed in some of them by Southern blotting.

cAMP assay

Preparation of samples for cAMP assay was described previously (Fukui et al. 1986; Mochizuki and Yamamoto 1992). We used a cAMP assay kit (Amersham TRK.432) and followed the protocol provided by the supplier. Samples to be compared were assayed simultaneously using a single batch of the kit; otherwise, the results were not sufficiently reproducible (Mochizuki and Yamamoto 1992). The amount of protein was determined according to Lowry et al. (1951).

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