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Concerted Action of RAS and G Proteins in the Sexual Response Pathways of *Schizosaccharomyces pombe*

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We have shown that the expression of *mam2*, the gene encoding the *Schizosaccharomyces pombe* P-factor pheromone receptor, is dependent upon components of the pheromone signal transduction pathway, including Ras1, Gpa1, Byr1 and Byr2, each of which is required for both conjugation and sporulation. Studies of the expression of *mam2* in mutant *S. pombe* cells confirm previous conclusions, based on the ability of cells to sporulate, that the Byr1 protein kinase acts downstream of the Byr2 protein kinase and that both act downstream of Ras1, the *S. pombe* RAS homolog, and Gpa1, the G_α component that mediates the occupancy of the *mam2* receptor. In addition, our present studies show that Ras1 and Gpa1 each act downstream from the other and hence act in concert. The Spk1 kinase, which is required for conjugation and sporulation and which is a structural and functional homolog of the vertebrate MAP kinases, is not required for *mam2* expression.

RAS proteins are guanine nucleotide binding proteins that are widely conserved among eukaryotes (reviewed in reference 1). In the budding yeast *Saccharomyces cerevisiae*, two RAS proteins, RAS1 and RAS2, activate adenylyl cyclase in a GTP-dependent manner (5, 12, 21, 22, 53, 57). This does not appear to be the function of RAS proteins in higher eukaryotes or Ras proteins in the fission yeast *Schizosaccharomyces pombe* (2, 4, 14, 35). In mammalian cells, RAS proteins participate in pathways that regulate growth and differentiation and are required for signal transduction initiated by extracellular factors. In vertebrates, oncogenic RAS causes the activation of a protein kinase cascade, leading to the activation of MAP/ERK kinases (28, 44, 49, 55, 63).

The single RAS homolog, Ras1, of *S. pombe* is required for the sexual responses induced by mating pheromones, namely, conjugation in haploid cells and sporulation in diploid cells (14, 35). A number of other genes that encode products that are also required for sexual differentiation have been identified (33, 34, 61). We and others have attempted to order the components of the Ras1 mating pheromone response pathway by examining the consequence of combining various genetic perturbations upon sporulation and conjugation. In this way, the actions of two putative protein kinases, Byr1 and Byr2, have been placed downstream of the action of Ras1, with Byr1 acting downstream of Byr2 (33, 34, 61).

The Byr kinases are related to a set of kinases that are conserved in eukaryotes. The Byr1 and Byr2 kinases are structurally related to the STE7 and STE11 kinases, respectively (38), which participate in the sexual response pathway of *S. cerevisiae* (43, 54). Byr1 and STE7 and Byr2 and STE11 can partially complement defects in heterologous yeast strains (38). A third putative kinase, Spk1, is also required for conjugation and sporulation in *S. pombe* (38, 56). Although the functional dependence of Spk1 upon Ras1 or even upon Byr1 has not yet been demonstrated, Spk1 is structurally and functionally homologous to the FUS3 and KSS1 kinases, which function downstream of STE11 and STE7 in *S. cerevisiae* (7, 10, 11, 38, 56).

In many respects, the function of Ras in *S. pombe* resembles the function of RAS in vertebrates (38). In both classes of organisms, RAS function is thought to control the activity of a protein kinase cascade that ultimately regulates a member of the MAP kinase family. FUS3, KSS1, and Spk1 are all structurally homologous to the MAP/ERK kinases of vertebrates (28, 44, 55, 63), and the mammalian ERK2 kinase can partially complement the loss of the *S. pombe* Spk1 kinase (38). Vertebrate MAP kinase kinases that are structurally related to the yeast Byr1 and STE7 protein kinases have been discovered (8, 25, 29), and recently a vertebrate homolog of the Byr2 and STE11 kinases (MAPKK or MEK) was found (27).

There are striking similarities and differences between the pheromone response pathways of the highly diverged yeasts *S. pombe* and *S. cerevisiae*. On the one hand, as we have just discussed, similar kinases are used in both organisms. Both organisms secrete sex-specific pheromones. These act through receptors with seven putative transmembrane domains that are coupled to a heterotrimeric G protein (6, 24, 37, 52). On the other hand, whereas the $G_{\beta\gamma}$ complex (encoded by the *STE4* and *STE18* genes) mediates sexual responses in *S. cerevisiae* (9, 32, 62), the G_α subunit, encoded by *gpa1*, mediates sexual responses in *S. pombe* (40). Moreover, whereas Ras1 is required for sexual responses in *S. pombe* (14, 35), neither RAS1 nor RAS2 appears to affect pheromone responses in *S. cerevisiae*.

Until now, we have been unable to discern clear epistatic relationships among all the components of the sexual differentiation pathway that have been identified by genetic analysis of *S. pombe*. For example, the precise relationship between Ras1 and Gpa1 in the sexual differentiation pathway of *S. pombe* has not been clear, nor has the relationship between Spk1 and Byr1 (38) or the placement of Byr3, another product capable of partly bypassing the function of Ras1 (64). The difficulties of ordering these components may be due, in part, to the complex nature of sporulation and conjugation. We therefore sought a simpler indicator for the Ras1- and Gpa1-dependent signal transduction pathways in *S. pombe*. Others have shown that Ras1 is required for transcription of the *mat1-Pm* gene (39). In this report, we

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TABLE 1. Genotypes of *S. pombe* strains[a]

Strain	Genotype
Sp870[b]	*h*^{90} *leu1.32 ade6.210 ura4-D18*
Sp65	*h*$^{-S}$ *leu1.32 his2-245*
GP352	*h*$^{-S}$ *leu1.32 ade6-D1*
Sp200	*h*$^{-S}$ *leu1.32 ade6.210 ura4-D18*
Sp813	*h*$^{+N}$ *leu1.32 ade6.210 ura4-D18*
SpBU	*h*^{90} *leu1.32 ade6.210 ura4-D18 byr1::ura4*
SpB2U	*h*^{90} *leu1.32 ade6.210 ura4-D18 byr2::URA4*
SpB3U	*h*^{90} *leu1.32 ade6.210 ura4-D18 byr3::ura4*
Sp525	*h*^{90} *leu1.32 ade6.216 ura4-D18 ras1::LEU2*
SpGL	*h*^{90} *leu1.32 ade6.210 ura4-D18 gpa1::LEU2*
TP114-9C	*h*^{90} *leu1.32 ura4D-18 spk1::ura4*
ST6	*h*^{90} *leu1.32 ade6.210 ura4-D18 ste6::ura4*
JZ396	*h*^{90} *leu1.32 ade6.216 ura4-D18 ste11::ura4*
Sp200GL	*h*$^{-S}$ *leu1.32 ade6.210 ura4-D18 gpa1::LEU2*
MW311	*h*$^{-S}$ *leu1.32 ade6.210 ura4-D18 byr1::ura4*
MW312	*h*$^{-S}$ *leu1.32 ade6.210 ura4-D18 gpa1::LEU2 ras1::ura4-2D*
MW313	*h*$^{-S}$ *leu1.32 ade6.210 ura4-D18 ras1::ura4-2D*
MW304	*h*$^{-S}$ *leu1.32 ade6-D ura4-D18 his2-245*
MW306	*h*$^{-S}$ *leu1.32 ade6-D ura4-D18 his2-245 mam2::ADE2*
MW308	*h*$^{-S}$ *leu1.32 ade6-D ura4-D18 his2-245 mam2::ADE2 ras1::LEU2*
MW310	*h*$^{-S}$ *leu1.32 ade6-D ura4-D18 his2-245 mam2::ADE2 gpa1::LEU2*
MW314	*h*$^{-S}$ *leu1.32 ade6-D ura4-D18 his2-245 mam2::ADE2 byr1::ura4*
MW316	*h*$^{-S}$ *leu1.32 ade6-D ura4-D18 his2-245 mam2::ADE2 gpa1::LEU2 ras1::ura4-2D*

[a] See Materials and Methods for more details.
[b] This strain was a generous gift from David Beach.

demonstrate that the expression of the *mam2* gene, which encodes the P-factor pheromone receptor (24), requires the function of many of the components of the pheromone response pathway, including Gpa1 and Ras1. We have used this dependence to study the relationship among the members of the pheromone response pathway in greater detail than was previously possible and have determined, among other things, that Ras1 and Gpa1 can each regulate *mam2* expression in the absence of the other.

MATERIALS AND METHODS

Yeast strains. *S. pombe* strains were grown in either rich medium (YEA) (35) or synthetic minimal medium (PM) with the appropriate auxotrophic supplements (31). The genotypes of all the strains used are listed in Table 1. Strain Sp200 was a gift from D. Beach. Strains Sp870 (64), SpBU (61), SpB2U (61), SpB3U (64), Sp525 (66), SpGL (38), ST6 (18), and JZ396 (51) have been described elsewhere. *S. pombe* TP114-9C is an *spk1* null strain and was a gift from T. Toda. The following strains were derived from Sp200 by one-step gene transplacement (46) with the lithium acetate transformation protocol (20). Sp200GL is a *LEU2*-containing partial deletion of *gpa1* in Sp200 constructed by use of the *Sal*I-*Sac*I *gpa1::LEU2* fragment from pUC119GPA1::LEU2 (38); MW311 is a *ura4*-containing partial deletion of *byr1* in Sp200 constructed by use of the *Pst*I-*Sac*I *byr1::ura4* fragment from pUC118BYR1::ura4 (61); MW312 is a *ura4*-containing partial deletion of *ras1* in SP200GL constructed by use of the *Bam*HI *ras1::ura4-2D* fragment from pUC118RAS1:: ura4-2D (see plasmid constructions below); and MW313 is a *ura4*-containing partial deletion of *ras1* in SP200 constructed by use of the *Bam*HI *ras1::ura4-2D* fragment from pUC118 RAS1::ura4-2D. MW304 was created through a series of crosses and sporulations by standard genetic methods (15). The parental strain was Sp65 (a gift from D. Beach). The *ura4-D18* allele was introduced from Sp813 (a gift from D. Beach), and the *ade6-D1* allele was introduced from GP352 (42). MW306 is a disruption of *mam2* with the *Sac*I-*Sal*I fragment of pMW59 via one-step transplacement (46) of MW304. MW308 is a *LEU2*-containing deletion of *ras1* in MW306 constructed by use of the *Dra*I *ras1::LEU2* fragment from Spras1ΔHc (35); MW310 is a *LEU2*-containing partial deletion of *gpa1* in MW306 constructed by use of the *Sal*I-*Sac*I *gpa1::LEU2* fragment from pUC119GPA1::LEU2 (38); MW314 is a *ura4*-containing partial deletion of *byr1* in MW306 constructed by use of the *Pst*I-*Sac*I *byr1::ura4* fragment from pUC118BYR1::ura4 (61); and MW316 is a *ura4*-containing partial deletion of *ras1* in MW310 constructed by use of the *Bam*HI *ras1::ura4-2D* fragment from pUC118RAS1::ura4-2D.

Plasmid constructions. Plasmids pAL (61), pAU (38), pART1 (61), pAAU (61), pAAUN (64), pAALN (64), pAAUNL (64), pAALNL (64), and pAAUCM (38) are *S. pombe* expression vectors which were used for expressing *S. pombe* genes in various probe strains. Plasmid pALRV contains *S. pombe ras1*$^{Val-17}$ in the pAL vector. Plasmids pARTRV and pAAUCMRV contain *S. pombe ras1*$^{Val-17}$ in the pART1 and pAAUCM vectors, respectively (59a). Plasmid pARTBYR1 contains the *S. pombe byr1* gene in the pART1 vector (61). Plasmids pALBYR2 and pAIS1-3 contain the *S. pombe byr2* gene in the pAL and pAAU vectors, respectively (61). Plasmids pALBYR3 and pAAUNLBYR3 contain the *S. pombe byr3* gene in the pAL and pAAUNL vectors, respectively (64). Plasmids pUC118BYR1::ura4 and pUC118BYR2::ura4 were described previously (61). pUC 118RAS1::ura4-2D is the same as pUC118RAS1::ura4 (61), except that the N-terminal coding region (*Hpa*I to *Hin*dIII) of the *ras1* gene was further deleted. pUC119GPA1::LEU2 and pAAUCMBYR1 are described by Neiman et al. (38). The *S. pombe mam2* gene (24) was cloned from *S. pombe* genomic DNA by the polymerase chain reaction (PCR) (47) with the oligonucleotide primers 5′-AAGAA**CTGTCGAC** ACAATAGTAAATC and 5′-CGTAAGGT**GAGCTC**TTG GAGACTCAA (boldfacing indicates *Sal*I and *Sac*I sites, respectively). The resulting 1.7-kb PCR fragment was digested with *Sal*I and *Sac*I and then ligated into the corresponding sites of pUC119, producing plasmid pUC119 MAM2. Plasmid pMW59 contains a PCR clone of *S. cerevisiae ADE2* (50), from nucleotides +3 to +1839 relative to the initiating ATG and with *Nco*I ends, inserted in frame at the *Nco*I site of *mam2* (position +10) in pUC119MAM2. The *gpa1* gene (40) was cloned from *S. pombe* genomic DNA by PCR with the oligonucleotide primers 5′-ATGACCAA**GTC GAC**AAGTTTGCCT and 5′-AGTTACG**GAGCTC**CGAA GAAGCTA (boldfacing indicates restriction endonuclease cleavage sites as described above). The resulting 2.9-kb *gpa1* PCR fragment was digested with *Sal*I and *Sac*I and then ligated into the corresponding sites of pUC119, producing plasmid pUC119GPA1F. pAUGPA1 was constructed by removing an *Sph*I-*Sac*I fragment containing an *adh1* promoter-*byr2* fusion from pAISI (61) and replacing this fragment with an *Sph*I-*Sac*I fragment of *gpa1* isolated from pUC119 GPA1F. When used to transform strain SpGL, pAUGPA1 suppressed the conjugation and sporulation defects resulting from the deletion of *gpa1*.

pSVSPK1 was constructed by cloning a *Hin*dIII fragment of the *spk1* gene (56) into the *Hin*dIII site of pSV (a gift from M. Yamamoto), thus placing *spk1* under the control of the

simian virus 40 early promoter. pSV contains the *S. cerevisiae LEU2* gene and 2μm sequence and can be used to transform *S. pombe leu1*$^-$ strains. The *S. pombe* gene *sir2* (22a) was isolated as a suppressor of *S. cerevisiae RAS2*$^{Ala-22}$-interfering *S. pombe* SpR2A (61). The *sir2* gene encodes a putative RNA helicase and very weakly diminishes the mating defect of strain SpR2A.

Plate assay of *ADE2* expression. Yeast strains were patched on YEA plates (35) and cultured overnight at 30°C. Patches were then replica plated to PM plates with 1% ammonium chloride. After 1 to 2 days of growth at 30°C, the patches were examined for the presence of red pigment.

Northern (RNA) blot analysis. The *S. pombe* strains were cultured in 10 ml of PM and grown overnight at 30°C. On the next morning, 1×10^7 to 2×10^7 cells were transferred to 40 ml of fresh PM and grown for 8 h at 30°C. The cells were then pelleted by centrifugation and washed once with 20 ml of PM lacking ammonium chloride. The cells were resuspended in 30 ml of PM lacking ammonium chloride (about 5×10^7 cells per ml) and grown overnight at 30°C. On the following morning, the cells were pelleted by centrifugation and washed with 20 ml of double-distilled H_2O. The cells were resuspended in 0.3 ml of RGB buffer (1 mM EDTA, 0.1 M LiCl, 0.1 M Tris, 10 mM sodium iodoacetate [pH 7.5]), and then 0.3 ml of phenol-chloroform-isoamyl alcohol (50:50:1) with 0.1% sodium dodecyl sulfate (SDS) and 1 g of glass beads (0.5 mm) were added. The cells were broken by use of a mini-beadbeater (Biospec Products) five times for 80 s each time. Two milliliters of RGB buffer was added and vortexed. The broken cells were centrifuged at 1,500 × *g* for 10 min. The supernatant was transferred to a clean tube and extracted three times with phenyl-chloroform-isoamyl alcohol. The total nucleic acids were precipitated by adding a 1/10 volume of 2 M potassium acetate (pH 5.5) and 2 volumes of ethanol. The mRNAs were purified with mRNA purification kits from Pharmacia LKB Biotechnology Co. Two double-stranded DNA fragments, a 641-bp *Bal*I fragment from the *sir2* coding region and a 1,050-bp *Sac*I-*Sal*I fragment from pUC119MAM2 containing the entire *mam2* coding region, were used as probes for Northern blots. The probes were labeled with random primer labeling kits purchased from Amersham International. Northern blotting was performed as described previously (48), except for the hybridization conditions. Nitrocellulose papers (Schleicher & Schuell) with transferred mRNAs were incubated in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–10 mM EDTA–0.5% SDS–50 μg of salmon sperm DNA per ml for 2 to 4 h at 68°C. The ^{32}P-labeled DNA probes were added directly to the prehybridization buffer and incubated for 12 to 16 h at 68°C. The nitrocellulose papers were rinsed three times with 2× SSC–12 mM Na_2HPO_4–8 mM NaH_2PO_4–1.4 mM $Na_4P_2O_7$–0.5% SDS at 68°C and then washed three times for 20 min each time with the same buffer at 68°C before exposure on Kodak X-OMAT film. For the quantitative analysis, the Northern blots were exposed to a FUJIX screen. The ^{32}P-labeled RNA densities were measured with a FUJIX BAS 2000 Bioimager (Fuji). The relative levels of expression of *mam2* mRNA were calculated in the following way. In each experiment, mRNAs from various strains, grown at the same time and under the same conditions, were quantitated as described above. In all experiments, wild-type strain Sp870 was included. The relative level of *mam2* expression for each strain was calculated as M_iS_o/M_oS_i, where M_i is the absolute reading for *mam2* mRNA from the i^{th} strain, S_i is the absolute reading for *sir2* mRNA from the i^{th} strain, M_o is the absolute reading for *mam2* mRNA from Sp870, and S_o is the absolute reading for *sir2* mRNA from Sp870.

RESULTS

Expression of *mam2* requires components of the pheromone response pathway. In preliminary experiments (data not shown), we tested the expression of several genes for dependence upon the presence of functional *ras1* and *byr2*. In these experiments, we used an h^{90} host strain, Sp870. h^{90} cells switch mating type frequently and therefore release mating pheromones of both types into the medium. Gene disruptions were constructed in this host background. Poly(A)$^+$ mRNA was extracted from cultures after 15 h of starvation, because starvation induces sexual responsiveness in *S. pombe*. mRNAs were subjected to Northern blot analysis, with expression of *sir2* or *ade6* as a control. Of the genes tested, including *sar1* (60), *byr1* (33), *byr2* (61), *spk1* (56), *gpa1* (40), *hcs* (65), *Pi* (23), *Pc* (23), and *mei3* (30), only *mam2* showed a clear requirement for *ras1* and *byr2* for expression. Much of our subsequent work focused on the expression of *mam2* in the Sp870 host strain or derivatives of this strain.

We examined the dependence of *mam2* expression upon the following genes: *byr1*, *byr2*, *byr3*, *gpa1*, *ras1*, *spk1*, *ste11*, and *ste6*. *byr3* encodes a protein with seven putative zinc finger binding domains. It was previously identified as a gene that, upon overexpression, could bypass the sporulation defects of *ras1*$^-$ cells and the conjugation defects of cells expressing interfering mutations of *S. cerevisiae RAS2* in *S. pombe* (64). *ste11* encodes a protein that is required for the expression of the starvation-inducible components of the sexual response pathway (51). *ste6* encodes a putative Ras1 GTP/GDP exchange protein (18). *ste11* is required for conjugation and sporulation, *ste6* is required for conjugation only, while *byr3* is required for neither.

In each case, the test gene of the host strain was disrupted by homologous recombination with plasmid DNA, and cultures were grown to an equivalent cell density (~10^7 cells per ml). Cells were transferred to starvation medium (see Materials and Methods). Poly(A)$^+$ RNA was harvested, size fractionated by gel electrophoresis, blotted, and probed with *mam2* and *sir2* sequences, with the latter being used as a control. *sir2* is a gene, previously cloned by us, that encodes a protein homologous to RNA helicases (22b). Previous studies indicated that the ratio of *sir2* to *ade6* transcripts was constant in all genetic backgrounds (data not shown). *sir2* RNA was used as our standard, since *ade6* RNA has the same mobility as *mam2* RNA. The results of several experiments are summarized in Table 2, and the outcomes of individual experiments are illustrated in Fig. 1 and 2.

The results of these experiments clearly indicate that the expression of *mam2* is dependent on *byr1*, *byr2*, *ras1*, *gpa1*, *ste11*, and *ste6*. Ten- to 100-fold-lower levels of expression are seen in strains lacking these genes. On the other hand, expression is lowered at most twofold in strains lacking *byr3* or *spk1*. The outcome with *byr3* is not surprising, because the disruption of *byr3* produces only a mild defect in conjugation. The outcome with *spk1* is surprising, because the disruption of *spk1*, like the disruption of *byr1* and *byr2*, leads to an absolute block in conjugation and sporulation. This result suggests that *mam2* expression does not require *spk1* even though *spk1* is essential for other aspects of the mating response.

Epistasis relationships with the protein kinases. The lack of *mam2* expression in *byr1*$^-$ and *byr2*$^-$ cells enabled us to test

TABLE 2. Induction of *mam2* expression in Sp870 h^{90} wild-type and null strains

Genotype	Relative expression of *mam2*[a] in expt:														
	1	2	3	4	6	7	8	9	11	13	14	15	16	17	Avg[b]
Wild type	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
byr1$^-$	0.00	—	0.08	—	—	0.07	0.17	—	0.15	—	0.15	—	—	0.13	0.09
byr2$^-$	0.08	0.06	—	—	—	0.02	—	0.13	0.10	—	—	0.13	—	0.07	0.08
byr3$^-$	—	—	—	—	—	0.53	—	0.62	—	—	—	—	—	0.56	0.57
gpa1$^-$	0.03	0.06	—	0.01	—	0.00	—	—	0.02	0.10	—	—	—	0.02	0.03
ras1$^-$	0.03	0.05	0.06	—	0.00	0.00	—	—	0.02	—	—	—	0.02	0.01	0.02
spk1$^-$	0.92	0.30	—	—	—	0.45	—	—	—	—	—	—	—	0.51	0.55
ste11$^-$	—	0.02	—	—	—	—	—	—	—	—	—	—	—	0.00	0.01
ste6$^-$	0.20	—	—	—	—	—	—	—	—	—	—	—	—	0.11	0.16

[a] All values are normalized to wild-type values by the methods described in Materials and Methods. —, not determined.
[b] Average value for all experiments.

whether the overexpression of other genes could restore *mam2* expression. The *byr1*$^-$ strains were thus transformed with multicopy plasmids carrying *byr1*, *byr2*, *byr3*, *spk1*, *gpa1*, or the activated *ras1*$^{Val-17}$ gene. Except for the plasmids carrying *byr1*, none could restore *mam2* expression to *byr1*$^-$ strains (Table 3), and only the multicopy plasmids carrying *byr1* or *byr2* could restore *mam2* expression to the *byr2*$^-$ strains (Table 4). These results are completely consistent with previous epistasis experiments, based upon the restoration of sporulation, that indicated that the Byr kinases act downstream of Ras1 and Gpa1 and that Byr1 acts downstream of Byr2.

Plasmids carrying *spk1* also failed to induce *mam2* expression in *byr*$^-$ strains. Indeed, plasmids carrying *spk1* did not induce *mam2* expression in any subsequent experiments, even though the plasmids correct the conjugation and sporulation defects of *spk1*$^-$ strains. These experiments are consistent with the result of deleting *spk1* and indicate that *mam2* expression is not dependent on *spk1*.

Epistasis relationships with Ras1 and Gpa1 in h^{90} strains. We tested plasmids carrying *byr1*, *byr2*, *byr3*, *spk1*, and *gpa1* for the ability to induce *mam2* expression in the absence of *ras1* in the h^{90} host strain background (Table 5 and Fig. 1). As expected from previous experiments on the suppression of the sporulation defect in *ras1*$^-$ cells, the *byr1*, *byr2*, and *byr3* genes on multicopy plasmids were able to bypass the *ras1* defect. As before, *spk1* had no effect. Previous experiments showed that *gpa1* cannot induce sporulation in *ras1*$^-$/ras1$^-$ diploids (28a). Hence, it is significant that *gpa1* on a multicopy plasmid is capable of inducing *mam2* expression even in the absence of *ras1*. This result suggests that Gpa1 can act on the signal transduction pathway at a point downstream from Ras1.

Similar experiments were performed on *gpa1*$^-$ cells (Table 6 and Fig. 2). As expected, the *byr1* and *byr2* genes could induce *mam2* expression even in the absence of *gpa1*, while *spk1* could not. Of interest were two results. Neither activated *ras1*$^{Val-17}$ nor *byr3* on a multicopy plasmid and transcribed from its natural promoter was able to induce *mam2* in the absence of *gpa1*. However, when *ras1*$^{Val-17}$ was transcribed from a stronger promoter, we observed some induction of *mam2* expression. Although this effect was small, a similar relationship between *ras1* and *gpa1* was concluded from the results presented below. Possible interpretations of the results with *byr3* will be discussed later.

Concerted action of Ras1 and Gpa1: evidence from h^- strains. h^{90} strains undergo mating type switching and consequently produce P factor. The dependence of *mam2* expression upon Gpa1 and other components of the mating pheromone response pathway would suggest that *mam2*

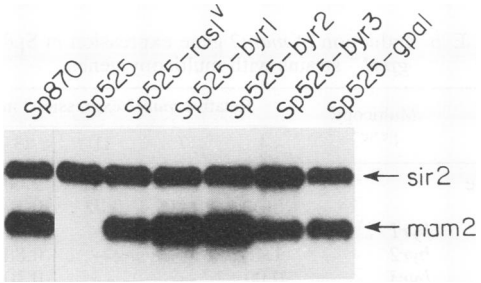

FIG. 1. Expression of *mam2* in *ras1* null strains. Sp525, an *S. pombe* h^{90} *ras1* null strain, was transformed with different *S. pombe* genes. Sp525-ras1^V, Sp525-byr1, Sp525-byr2, Sp525-byr3, and Sp525-gpa1 are the Sp525 strains transformed with the *S. pombe* *ras1*$^{Val-17}$ gene in pAAUCMRV, the *byr1* gene in pAAUCMBYR1, the *byr2* gene in pA1S1-3, the *byr3* gene in pAAUNLBYR3, and the *gpa1* gene in pAUGPA1, respectively (see Materials and Methods). Sp870 is an *S. pombe* h^{90} wild-type strain that served as a positive control. The arrows show the *mam2* and *sir2* mRNA bands. The RNA samples were derived from experiment 16 (Table 5).

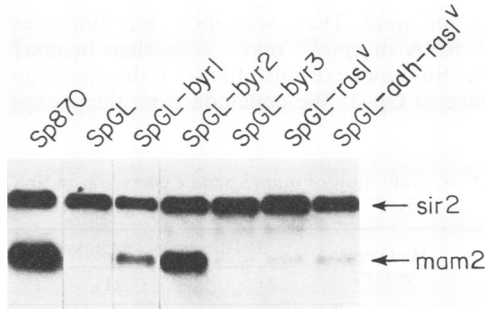

FIG. 2. Expression of *mam2* in *gpa1* null strains. SpGL, an *S. pombe* h^{90} *gpa1* null strain, was transformed with different *S. pombe* genes. SpGL-byr1, SpGL-byr2, SpGL-byr3, SpGL-ras1^V, and SpGL-adh-ras1^V are the SpGL strains transformed with the *S. pombe* *byr1* gene in pARTBYR1, the *byr2* gene in pALBYR2, the *byr3* gene in pALBYR3, the *ras1*$^{Val-17}$ gene in pALRV, and the *ras1*$^{Val-17}$ gene in pARTRV, respectively. In the last plasmid, *ras1*$^{Val-17}$ is controlled by the *S. pombe adh* promoter. Sp870 served as a positive control. The arrows show the *mam2* and *sir2* mRNA bands. The RNA samples were derived from experiment 13 (Table 6).

TABLE 3. Induction of *mam2* gene expression in Sp870 h^{90} *byr1*$^-$ strains with multicopy genes

Strain	Multicopy gene[a]	Relative *mam2* expression[b] in expt:			
		8	11	14	Avg[c]
Wild type		1.00	1.00	1.00	1.00
byr1$^-$		0.17	0.12	0.15	0.15
byr1$^-$	*byr2*	—	0.08	0.04	0.06
byr1$^-$	*byr3*	0.02	—	0.01	0.02
byr1$^-$	*ras1*$^{Val-17d}$	—	0.04	0.01	0.03
byr1$^-$	*spk1*	—	—	0.00	0.00

[a] The indicated genes were present on multicopy plasmids and were transformed into SpBU, a *byr1*$^-$ strain.
[b] See Table 2, footnote *a*.
[c] See Table 2, footnote *b*.
[d] The activated *ras1*$^{Val-17}$ gene is expressed from the *adh1* promoter in this and all other experiments, except as indicated otherwise. All other genes are expressed from their natural promoters.

TABLE 5. Induction of *mam2* gene expression in Sp870 h^{90} *ras1*$^-$ strains with multicopy genes

Strain	Multicopy genes[a]	Relative *mam2* expression[b] in expt:				
		3	6	11	16	Avg[c]
Wild type		1.00	1.00	1.00	1.00	1.00
ras1$^-$		0.06	0.00	0.02	0.02	0.03
ras1$^-$	*byr1*	1.68	1.58	—	1.18	1.48
ras1$^-$	*byr2*	1.90	1.34	—	1.04	1.43
ras1$^-$	*byr3*	0.45	0.32	—	0.41	0.39
ras1$^-$	*ras1*$^{Val-17d}$	0.70	0.54	—	0.44	0.56
ras1$^-$	*gpa1*	—	—	0.60, 0.65[e]	0.58, 0.35[e]	0.47
ras1$^-$	*spk1*	—	0.01	—	0.01	0.01

[a] See Table 3, footnote *a*.
[b] See Table 2, footnote *a*.
[c] See Table 2, footnote *b*.
[d] See Table 3, footnote *d*.
[e] Two independent transformants were examined.

expression is stimulated in h^{90} cells by an interaction with P factor. We were therefore puzzled by the observation reported by Kitamura and Shimoda, who first described the cloning of *mam2*, that the levels of expression of *mam2* in h^- and h^{90} strains did not appreciably differ (24). h^- strains do not undergo mating type switching and do not produce P factor. These results suggested that signalling in h^- cells might differ from signalling in cells that switch mating type and are chronically stimulated by mating pheromones.

To explore this question further, we examined by Northern blotting *mam2* transcripts in h^- strains that carried disruptions in the *gpa1*, *ras1*, or *byr1* gene (Table 7). Parental strain Sp200 expressed about twofold-lower levels of *mam2* than did h^{90} wild-type strain Sp870, consistent with the results of Kitamura and Shimoda (24). The level of *mam2* expression in the h^- strain was dependent upon *byr1*, consistent with the requirement for basal activity of some components of the signal transduction pathway. Levels of *mam2* expression were only marginally decreased by disrupting *gpa1*, consistent with the absence of stimulation by mating pheromones in h^- cells. This result is in contrast to the strong dependence on *gpa1* in h^{90} cells. *mam2* expression in h^- cells was more clearly dependent upon *ras1* but, again, not to the extent found in h^{90} cells. Levels of *mam2* expression were still further diminished in h^- cells lacking both *ras1* and *gpa1*. There was about four times less *mam2* poly(A)$^+$ RNA in *gpa1*$^-$ *ras1*$^-$ cells than in *gpa1*$^-$ cells. Therefore, Ras1 can exert its effects on this pathway even in the absence of Gpa1. We conclude from these experiments and the experiments reported in Table 6 that Ras1 and Gpa1 are at least partly independent, each capable of acting downstream from the other.

The observed quantitative difference in *mam2* expression between *gpa1*$^-$ h^- cells and *ras1*$^-$ *gpa1*$^-$ h^- cells was small, as was the effect of overexpressing *ras1*$^{Val-17}$ in *gpa1*$^-$ h^{90} cells. We therefore sought to confirm this interaction in a qualitative, intact-cell assay. Such an assay allows the analysis of multiple independent strains without the statistical noise introduced by cumbersome biochemical manipulations. To monitor *mam2* expression, we exploited the observations that strains deficient in *ade6* require adenine for optimal growth and develop a red pigment and that the *S. cerevisiae ADE2* gene complements the *ade6* mutation in *S. pombe* (28a). We thus joined a copy of the *S. cerevisiae ADE2* gene, lacking its endogenous promoter, to the *mam2* promoter at position +10 relative to the start codon of *mam2*. This construct was integrated into the *mam2* locus of an h^- *S. pombe* strain that was *ade6*$^-$ (MW304), producing strain MW306, in which *S. cerevisiae ADE2* is under the control of the *S. pombe mam2* promoter. Strains with a deletion mutation of *byr1*, *ras1*, or *gpa1* and strains with deletion mutations of both *ras1* and *gpa1* were constructed by transforming MW306 with the appropriate DNA fragments (see Materials and Methods). Eight independent

TABLE 4. Induction of *mam2* gene expression in Sp870 h^{90} *byr2*$^-$ strains with multicopy genes

Strain	Multicopy genes[a]	Relative *mam2* expression[b] in expt:				
		7	9	11	15	Avg[c]
Wild type		1.00	1.00	1.00	1.00	1.00
byr2$^-$		0.02	0.13	0.08	0.13	0.09
byr2$^-$	*byr1*	0.49	0.52	—	0.66	0.56
byr2$^-$	*byr3*	—	0.04	—	0.12	0.08
byr2$^-$	*gpa1*	—	—	0.02	0.11	0.07
byr2$^-$	*ras1*$^{Val-17d}$	—	—	0.05	0.17	0.11
byr2$^-$	*spk1*	—	0.01	—	0.11	0.06

[a] See Table 3, footnote *a*.
[b] See Table 2, footnote *a*.
[c] See Table 2, footnote *b*.
[d] See Table 3, footnote *d*.

TABLE 6. Induction of *mam2* gene expression in Sp870 h^{90} *gpa1*$^-$ strains with multicopy genes

Strain	Multicopy gene[a]	Relative *mam2* expression[b] in expt:				
		4	7	11	13	Avg[c]
Wild type		1.00	1.00	1.00	1.00	1.00
gpa1$^-$		0.01	0.00	0.02	0.10	0.03
gpa1$^-$	*byr1*	1.17	—	—	0.46	0.82
gpa1$^-$	*byr2*	1.48	—	—	0.88	1.18
gpa1$^-$	*byr3*	0.00	—	—	0.10	0.05
gpa1$^-$	*ras1*$^{Val-17d}$	0.01	0.04	—	0.08	0.04
gpa1$^-$	*ras1*$^{Val-17e}$	—	—	0.15	0.15	0.15

[a] See Table 3, footnote *a*.
[b] See Table 2, footnote *a*.
[c] See Table 2, footnote *b*.
[d] The activated *ras1*$^{Val-17}$ gene is expressed from its natural promoter. The plasmid carrying *ras1*$^{Val-17}$ expressed from its natural promoter was as effective as a plasmid expressing *ras1*$^{Val-17}$ from the *adh1* promoter in restoring function to *ras1*$^-$ strains (data not shown).
[e] See Table 3, footnote *d*.

TABLE 7. Induction of *mam2* gene expression in h^- strains with various deletions

Genotype[a]	Transformant[b]	Relative *mam2* expression[c] in expt:			
		18	19	20	Avg[d]
h^{90}		1.0	1.0	1.0	1.0
h^-		0.32	0.50	0.53	0.45
h^- *gpa1*$^-$	1	0.50		0.32	0.38
	2		0.32	0.36	
h^- *ras1*$^-$	1	0.26		0.17	0.20
	2		0.10	0.13	
	3			0.24	
	4			0.22	
h^- *gpa1*$^-$ *ras1*$^-$	1	0.09		0.11	0.09
	2		0.08	0.09	
h^- *byr1*$^-$	1	0.08			0.08
	2		0.07		

[a] The h^{90} strain was Sp870, and the h^- strain was Sp200.
[b] Several independent transformants were analyzed.
[c] See Table 2, footnote *a*.
[d] The average for strains of the same genotype is given.

transformants were isolated from each transformation. We examined the effect of these mutations on the expression of the *mam2-ADE2* fusion protein by first patching the strains on rich medium (YEA) and then replica plating them (after culturing overnight) on minimal medium (PM) lacking adenine. After 1 to 2 days of growth at 30°C, the patches were examined for the presence of the red pigment produced as an intermediate in adenine biosynthesis in cells lacking sufficient enzyme produced from the *ade6* or *mam2* locus (45). The results of these experiments are described below.

Patches from MW306 produced sufficient ADE2 fusion protein on the PM plates to be white. All eight *ras1*$^-$ derivatives of this strain produced light pink patches on PM plates, an indication of decreased expression of the ADE2 fusion protein from the *mam2* locus. All eight *gpa1*$^-$ strains also reproducibly produced light pink patches on PM plates, demonstrating that *gpa1*, as well as *ras1*, is required for full expression from the *mam2* locus under these culture conditions. The reduction of expression from the *mam2* locus in *gpa1*$^-$ cells is qualitatively the same as the reduction that results from a deletion of *ras1*. All eight strains containing deletions of both *ras1* and *gpa1* produced dark pink patches on PM plates, indicating that the double deletion results in a stronger reduction of *mam2-ADE2* expression than either single deletion alone. Similarly, all eight strains lacking *byr1* produced dark pink patches. These results are in good agreement with the Northern blot analysis of *mam2* expression in h^- cells and support the conclusion that *ras1* and *gpa1* can act independently and in concert to maintain *mam2* expression in h^- cells.

DISCUSSION

We have found that the expression of the *S. pombe* P-factor pheromone receptor-encoding gene, *mam2*, is dependent upon the components of the pheromone response pathway. Recently, others similarly showed that the expression of *map3*, encoding the M-factor receptor, is dependent upon Gpa1 (52). A similar relationship is observed in *S. cerevisiae*, in which basal-level expression of pheromone receptors is likewise partly dependent upon the STE7 and STE11 protein kinases (13), which mediate pheromone responses in that organism. This arrangement may seem peculiar at first but is consistent with an arousal response in which the sensing of a pheromone induces the organism to enhance its responsiveness to the pheromone by the production of more receptor. This response has been demonstrated for *S. cerevisiae* (3, 16, 17), but we have not demonstrated that a pheromone can induce the expression of its own receptor in *S. pombe*. Nevertheless, we have used the expression of *mam2* as a "molecular phenotype" to help delineate the order of action of the components of the pheromone response pathway. We have also noted that the expression of *mam2* in h^- cells is less dependent upon Ras1 and Gpa1 than that in h^{90} cells. We do not know at present whether this result reflects intrinsic differences between h^{90} and h^- cells or merely differences in the particular strains that we have examined.

With some exceptions, the proteins required for conjugation and sporulation are also required for *mam2* expression in h^{90} cells. This list includes Ras1, Gpa1, Byr2, Byr1, and Ste11. Ste6, a putative Ras1 GTP/GDP exchange protein that is required for conjugation but not sporulation, is also required for optimal *mam2* expression. One notable exception is *spk1*, which is required for both conjugation and sporulation but is not required for *mam2* expression (56). Only a small effect on *mam2* expression was observed when *spk1* was deleted, and no induction of *mam2* expression was observed with multicopy plasmids containing *spk1*. It is not clear whether in this respect *S. pombe* differs from *S. cerevisiae*. Spk1 shares structural and functional homology with FUS3 and KSS1. At least one of the latter two protein kinases is required for pheromone-induced gene expression in *S. cerevisiae* (10), but it has not been shown, to our knowledge, that basal-level expression of *S. cerevisiae* pheromone receptors is dependent on either kinase. Thus, the expression of pheromone receptors may not be dependent upon the activity of a MAP kinase homolog. Alternatively, *S. pombe* may contain another protein kinase that is related to Spk1, FUS3, KSS1, and the MAP/ERK kinases, that is under the control of Byr1, and that has specialized functions in sexual responses.

byr3, which is not required for conjugation or sporulation (64), is also not required for *mam2* expression. However, the overexpression of *byr3* bypasses the requirement for Ras1 for *mam2* expression, as it does the requirement for Ras1 for sporulation. Hence, Byr3 influences events downstream of Ras1. The relationship of Byr3 to Gpa1 is more difficult to assess. The overexpression of *byr3* cannot induce sporulation or *mam2* expression in *byr1*$^-$, *byr2*$^-$, or *gpa1*$^-$ cells. This result is consistent with several possibilities. One possibility is that Byr3 acts upstream of Gpa1. Another is that Byr3 acts downstream of all of these components but that its overexpression produces too weak an effect for us to monitor in the absence of *byr1*, *byr2*, or *gpa1*. We cannot effectively decide between these possibilities at present.

Previous studies of Ras1 and Gpa1 indicated that both are required for sexual differentiation and act upstream of Byr2 and Byr1. However, those studies failed to demonstrate the relative order of action of Ras1 and Gpa1. In the present experiments, we clearly demonstrated that the overexpression of *gpa1* induces *mam2* expression in h^{90} cells that lack *ras1*. Thus, Gpa1 acts downstream of Ras1. Our Northern blot analysis also suggested that Ras1 can act downstream of

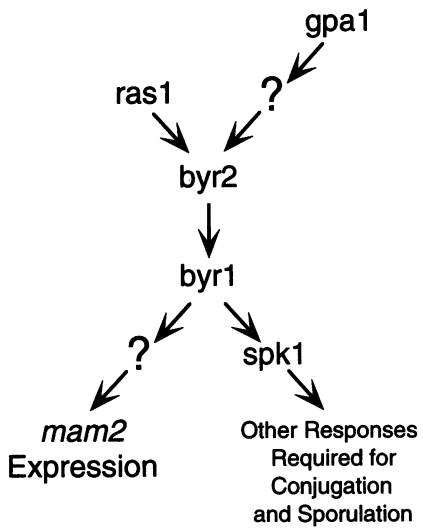

FIG. 3. Portion of the sexual differentiation signal transduction pathway of *S. pombe*. See the Discussion for details.

Gpa1. The overexpression of *ras1*$^{Val-17}$ can increase *mam2* expression in h^{90} cells that lack *gpa1*, and the deletion of *ras1* in *gpa1*$^-$ h^- cells reduces *mam2* expression. The effects that we observed are small, and our Northern blot analyses represent too small a sample size for us to be completely confident in them. However, a highly reproducible, intact-cell assay also indicated that *ras1* is required to maintain *mam2* expression in h^- cells that lack *gpa1*. Overall, therefore, we have considerable confidence that Ras1 acts downstream of Gpa1. Hence, we conclude that Gpa1 and Ras1 act independently and in concert to control the activity of the Byr kinases.

Our composite data have allowed us to draw the "wiring diagram" shown in Fig. 3. We have omitted Byr3 because of uncertainty over its roles. We have assumed a direct interaction between Byr2 and Byr1 because the experiments of Neiman et al. (38) demonstrated their cooperativity. We have depicted a direct interaction between Ras1 and Byr2 because recent experiments demonstrated that these proteins can form a complex (58). We have thus depicted Byr2 as the first downstream target common to both Gpa1 and Ras1. We do not know if the interaction between Gpa1 and Byr2 is direct or mediated through yet another protein. We speculate that the latter possibility is correct and have drawn our diagram this way on the basis of the observation that Byr2 function appears to be pheromone regulatable when it is expressed in *S. cerevisiae* (38). In *S. cerevisiae*, other components map between the G proteins and STE11, the homolog of Byr2.

The relationship between Ras and G proteins that we have noted for *S. pombe* may be found for other organisms as well. In *S. cerevisiae*, the Ras homologs, RAS1 and RAS2, regulate cyclic AMP levels through their effects on adenylyl cyclase (57). A G_α homolog, GPA2, also regulates cyclic AMP levels (36). It has not been demonstrated that GPA2 acts on adenylyl cyclase, but this seems likely in view of the observation that a similar protein in *S. pombe*, Gpa2, acts on a similar form of adenylyl cyclase (19). For vertebrates, investigators recently showed that thrombin and bombesin, which act through receptors coupled to G proteins, can activate MAP kinases (41, 59), as can RAS. Moreover, recent data suggest that the full cellular effects of thrombin require RAS proteins (26). Thus, vertebrate signal pathways controlled by G proteins and controlled by RAS converge, perhaps in a manner not unlike what we have proposed for *S. pombe*.

A recent paper by Lange-Carter and coworkers (27) described the identification of a mammalian MEK kinase (MEKK) homologous to Byr2 and STE11. MEK is a MAP kinase kinase that is homologous to Byr1 (8, 25, 29). They argued, on the basis of homology to STE11, that MEKK mediates RAS-independent, G protein-dependent signalling to the MAP kinases. However, to our knowledge, no one has shown that the activation of MAP kinases that is induced by factors that act through receptors coupled to G proteins is independent of RAS. This question merits closer investigation. If MEKK is closer in function to Byr2 than to STE11, then MEKK will be downstream of RAS and represent a juncture with another signal cascade, perhaps one that is controlled by a G protein.

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