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byr2, a Schizosaccharomyces pombe Gene Encoding a Protein Kinase Capable of Partial Suppression of the ras1 Mutant Phenotype

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Received 8 February 1991/Accepted 10 April 1991

Schizosaccharomyces pombe contains a single gene, ras1, which is a homolog of the mammalian RAS genes. ras1 is required for conjugation, sporulation, and normal cell shape. ras1 has been previously identified as ste5. We report here a gene we call byr2 that can encode a predicted protein kinase and can partially suppress defects in ras1 mutants. ras1 mutant strains expressing high levels of byr2 can sporulate competently but are still defective in conjugation and abnormally round. byr2 mutants are viable and have normal shape but are absolutely defective in conjugation and sporulation. byr2 is probably identical to ste8. In many respects, byr2 resembles the byr1 gene, another suppressor of the ras1 mutation, which has been identified previously as ste1. Our data indicate that if ras1, byr2, and byr1 act along the same pathway, then the site of action for byr2 is between the sites for ras1 and byr1.

RAS proteins are ubiquitous in evolution. They are low-molecular-weight guanine nucleotide-binding proteins that function in signal transduction pathways (1). Mutant activated RAS genes are found in a large number of mammalian tumors, but despite their importance, their function in mammals is unknown. We have studied RAS in the yeast Saccharomyces cerevisiae, in which two RAS proteins, RAS1 and RAS2, regulate the function of adenyl cyclase (23). The latter does not appear to be the function of RAS in vertebrates or even in the fission yeast Schizosaccharomyces pombe (7, 20). We have therefore begun to study RAS function in S. pombe in the hope of learning whether there are general principles which govern the functions of RAS proteins in cells.

S. pombe contains a single RAS gene, ras1 (6, 21). ras1 is not an essential gene but functions in the sexual differentiation pathways of that yeast (7, 20). ras1 mutant cells fail to conjugate and to sporulate. Such cells are also round, unlike wild-type cells, which are elongated. S. pombe cells that contain the activated mutant ras1Val12 allele are also partially sterile. Such cells enter the early phase of conjugation and develop elongated conjugation tubes but fail to enter the subsequent phases.

In S. pombe, there are two mating types, designated h+ and h− (4). Only opposite mating types conjugate, and only upon starvation. Homothallic (h0) haploid strains regularly switch mating type and therefore self-mate. Heterothallic (h+ and h−) strains do not switch mating type and do not self-mate. Conjugation can be divided into an early phase, marked by an increase in cell agglutination and the formation of a conjugation tube, and a later phase, marked by the fusion of cells and karyogamy. Immediately following conjugation, most cells undergo azygotic sporulation. Diploid cells, formed either by mating or by other means, can be propagated sexually, but diploid strains containing both mating type loci will undergo azygotic sporulation upon starvation. The four-spored ascis formed by azygotic sporulation look different from the four-spored ascis formed by azygotic sporulation. This difference forms an essential part of the genetic screen described in the Results section.

Several sterile (ste) mutants of S. pombe have been isolated (17). ras1 is identical to ste5 (15). ste6 is homologous to the S. cerevisiae CDC25 gene, which encodes a protein required to activate S. cerevisiae RAS proteins (11). ste5 appears to act in a similar manner in S. pombe. A single gene, byr1, which is identical to ste1, is known to be capable of the partial phenotypic suppression of rasl mutations (18, 19); ras1 diploid cells containing byr1 on high-copy-number plasmids can sporulate, but overexpression of byr1 fails to suppress the conjugation defects in ras1 haploid cells. Like ras1 mutants, byr1 mutants fail to conjugate or sporulate (18). These results are consistent with the hypothesis that the activity of the byr1 protein is regulated by ras1.

In the present report, we describe a second gene, byr2, capable of the partial phenotypic suppression of ras1 mutations. byr2 resembles byr1 in many respects. The range of their genetic interactions is similar, and both appear to encode protein kinases. If ras1, byr2, and byr1 all act along a common pathway, our data suggest that byr2 acts between the sites of action of ras1 and byr1.

MATERIALS AND METHODS

Microbial manipulation and analysis. Yeast strains (Table 1) were grown in either the rich medium YEA or the synthetic medium PM, with appropriate auxotrophic supplements (20). Sporulation was detected by iodine vapor staining as described previously (9). The lithium acetate procedure (12) was used to transform S. pombe cells. Plasmids in S. pombe cells were recovered by transforming Escherichia coli DH5α with crude DNA extracts prepared from transformed yeast cells. The homozigous diploid strains used in this study were generated during the transformation process and isolated from plates containing phloxin B. Ploidy was confirmed by microscopic examination of cell size and the presence of azygotic sporulation. The ste8byr2 heterozygous diploid mutant strain was constructed by protoplast fusion as described previously (19). The byr2 mutant SPSL (Ura−) and ste8 mutant JM86 (Leu−) were used as parental strains. The heterozygous diploids were selected on sorbitol-

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containing minimum medium plates lacking leucine and uracil.

Nucleic acid manipulation and analysis. An S. pombe genomic bank, provided by D. Beach, was constructed by inserting partially Sau3A1-digested S. pombe genomic DNA into the BclI site of plasmid pWHS (24), which contains the LEU2 gene. The plasmid pART-2A was constructed by inserting a 1.2-kbp HpaI fragment from YIP-OGA (22), which contains the S. cerevisiae dominant RAS2 mutation RAS2<sup>Ala-22</sup> at the Smal site of S. pombe expression vector pART1 (16). pART1 is a pUC118-based vector containing the 2.2-kbp HindIII fragment of the S. cerevisiae LEU2 gene cloned into the HindIII site of a 1.2-kbp S. pombe autonomously replicating sequence (ARS) fragment (14) cloned at the EcoRI site, and a 0.7-kbp S. pombe adh promoter fragment inserted at Spal and Psfl sites. The S. pombe byr1 gene was cloned from yeast genomic DNA by the polymerase chain reaction (PCR) with the oligonucleotide primers 5'--TTCAGAATTCTGGAATAG and 5'-GATTCTTCTGAAATCCTTTT. The PCR product was digested with BclI and XmnI, and the 1.1-kbp Bcll-XmnI fragment, which contained the whole coding sequence of byr1, was cloned into the Smal site of pART1. A functional clone was selected by its ability to restore normal sexual differentiation to a byr1 mutant (see below). The S. pombe expression vector pPAL was derived from pRIT5 (25) by replacing the ura4 gene with the 2.2-kbp HindIII fragment containing the S. cerevisiae LEU2 gene. Plasmid pPALR was constructed by inserting a 1.4-kbp BamHI-BglII S. pombe ras1 gene fragment (20) into the BamHI site of pAL. Plasmid pAIS1 was constructed by inserting a SalI-SacI fragment of byr2 into the SalI-SacI sites of the vector pAIL (15). pAIL contains the S. pombe ura4 gene, the AUS2 element, and the S. pombe adh promoter with an oligonucleotide encoding a peptide derived from the hemagglutinin antigen of influenza virus. The byr2

**Table 1. S. pombe strains used in this study**

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<th>Strain</th>
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<th>Source</th>
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*S. pombe* strain Sp870. One resultant ras1 transformant was verified by its phenotype (7, 20) and by Southern blot analysis and named SPRU.

A byr1 null allele was created by replacing a 167-bp SpeI-PpuMI fragment from the byr1 coding region with a 1.8-kbp fragment of the *S. pombe* ura4 gene. A linear fragment of this DNA containing the byr1 null allele was transformed into SP870. Gene disruptions were confirmed by Southern analysis and had the phenotype described previously (18). byr2 null alleles were made in a similar way. An 840-bp NcoI-SpeI fragment of the byr2 coding region was replaced by a 1.8-kbp S. cerevisiae LEU2 gene fragment (see Fig. 4), resulting in a byr2 null allele contained on a fragment that could be transformed into appropriate strains of *S. pombe*.

**Construction of S. pombe SPR2A.** Plasmid pART-2A was digested with SpeI and SacI. A 1.7-kbp fragment containing *S. cerevisiae* RAS2<sup>Ala-22</sup> under the control of the *S. pombe* adh promoter was released. This fragment was blunt-ended with DNA polymerase, purified by agarose gel electrophoresis, and inserted into the EcoRV site of the *S. pombe* ura4 gene contained in a pUC118-based vector as a 1.8-kbp HindIII fragment cloned at the HindIII site. This insertion abolished ura4 function totally, as proved later. The disrupted ura4 fragment was released by HindIII digestion and transformed into the Ura<sup>+</sup> *S. pombe* strain SP66. The Ura<sup>-</sup> transformants were selected on plates containing 5-fluoroorotic acid as described previously (8). The integration of RAS2<sup>Ala-22</sup> was proven by Southern blotting.

**Cell agglutination test.** Cellular agglutination is an early step in conjugation. In order to measure changes in agglutination, we developed a microtiter well test. *S. pombe* cells, starved as patches on minimum plates for 2 to 3 days, are suspended in testing buffer (phosphate-buffered saline containing 10 mM MgCl<sub>2</sub> [pH 7.4]) at 2 x 10<sup>6</sup> to 3 x 10<sup>6</sup> cells per ml. Cell clumps are broken down by pipetting up and down. Aliquots of 50-μl cell suspensions are inoculated into U-bottomed 96-well plates (Dynatech Laboratories, Inc.) and kept at room temperature. Cells which do not agglutinate settle down as a spot at the center of the bottom of the well within 30 min, while cells which agglutinate scatter at the bottom and slowly form a spot. The time required for cell spot formation is highly reproducible.
Western immunoblotting. Yeast cells harboring the hemagglutinin antigen epitope-byr2 plasmids were suspended in 50 mM Tris (pH 7.4)-250 mM NaCl-1 mM EDTA-2 mM phenylmethylsulfonyl fluoride at a concentration of about 10^9 cells per ml. An equal volume of glass beads (0.5 mm diameter) was added, and the cells were vortexed at 4°C for 5 min. The samples were centrifuged for 3 min at 200 × g, and the cell extract was collected as the supernatant. Cell extract were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose filters, and probed with the monoclonal antibody 12CA5 (5) raised against the peptide epitope. Goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Bio-Rad Laboratories) was used to identify the immunoreactive bands, which were subsequently visualized with the color development reagents 5-bromo-4-chloro-3-indolylphosphate toluidinium and Nitro Blue Tetroxidum (both from Bio-Rad).

Nomenclature. In general, S. pombe genes and proteins are designated by lowercase letters, while S. cerevisiae genes and proteins are designated by uppercase letters. Genes are italicized, and proteins are not. Phenotypes have only the initial letter capitalized and are not italicized.

RESULTS

Phenotype of S. pombe cells expressing S. cerevisiae RAS2^{Ala-22}. We have previously described a dominant interfering mutation of the yeast S. cerevisiae RAS2 gene, RAS2^{Ala-22} (22). The product of this gene appears to interfere with the activation of wild-type RAS proteins by blocking the product of the S. cerevisiae CDC25 gene. Since interactions between CDC25- and RAS-like proteins are probably conserved in evolution, we expected that expression of RAS2^{Ala-22} in S. pombe would interfere with ras1 function. To pursue this possibility, we designed the plasmid pART-R2A (see Materials and Methods), which expresses the RAS2^{Ala-22} gene from the S. pombe adh promoter. The h^0 homoallelic mating type strain SP870 was transformed with pART-R2A. The resulting transformants grew normally but showed a very low level of sporulation, as visualized by iodine vapor staining of nutrient-starved colonies. Microscopic examination showed that cells were of normal size and shape, but there were fewer than 1% zygotic spores (Fig. 1). In contrast, 50% of the cells in control transformant colonies had undergone zygotic sporulation. Thus, expression of RAS2^{Ala-22} interferes with the ras1 functions required for conjugation but not with the ras1 functions required to maintain normal cell shape. In diploid cells, expression of RAS2^{Ala-22} did not interfere with sporulation (data not shown). Hence, RAS2^{Ala-22} only partially blocks the action of ras1 in S. pombe, rendering cells defective in conjugation but not in sporulation or shape.

Identification of suppressors of RAS2^{Ala-22}. In order to find suppressors of RAS2^{Ala-22}, we first integrated the adh promoter-driven mutant gene into S. pombe genomic DNA at the ura4 locus. The resulting strain, SPR2A, showed the same phenotype as SP870 cells carrying the high-copy-number plasmid pART-R2A, i.e., very little conjugation upon starvation. We next tested the effect of high-copy-number plasmids expressing various known genes on SPR2A (Fig. 1). Plasmid pALR, which expresses the S. pombe ras1 gene, and pART-BYR1, which expresses the byr1 gene, were both capable of restoring conjugal efficiency. To our surprise, pST6, which expresses ste6, an S. pombe homolog of the CDC25 gene (11), was unable to restore conjugal efficiency to SPR2A.

To search for unknown suppressors of RAS2^{Ala-22}, we screened plasmid libraries of S. pombe genomic DNA cloned into shuttle vectors for plasmids conferring conjugal efficiency to SPR2A upon transformation. Conjugation in Leu+ transformants was scored indirectly by staining colonies for spores. A total of 2,992 positive-staining colonies were found among 5 × 10^9 transformants examined. Only 26 of these colonies contained zygotic spores. The majority of the remainder contained azygotic spores, the products, we presume, of the diploid cells which commonly arise during DNA-mediated transformation of haploid S. pombe strains. A few colonies displayed the haploid pattern of sporulation. When haploid cells sporulate, they yield two spored asc. This is a very rare event in wild-type cells. When diploid cells sporulate, four spored asci result. Plasmids were recovered only from colonies containing zygotic spores, and all such plasmids could confer conjugal efficiency to SPR2A upon retransformation. The pattern of restriction enzyme cleavage indicated that a total of four loci had been cloned. One class of plasmids contained the ras1 gene, and another contained byr1. A third class was represented by a single plasmid, pWHSS1, containing a gene we call byr2. The fourth class is not discussed in this report.

Sequence of byr2. pWHSS1 contained a large insert of about 17 kbp. Deletion and subcloning analysis localized the functional gene to a 4-kbp BamHI-Smal fragment. This fragment was subcloned into pUC118 and pUC119 for nucleotide sequencing. The nucleotide sequence revealed an intronless open reading frame of 1,977 bp, with the capacity to encode a protein of 659 amino acids (Fig. 2).

Amino acid sequence similarity searches of different data banks revealed that a region of about 200 residues near the carboxyl terminus of the byr2 product had significant homology to a large number of proteins, all of them known or suspected to be protein kinases. In fact, all of the conserved amino acid residues deduced from 65 protein kinases (10) are present in the byr2 product. The serine at position 566 suggests that byr2 encodes a threonine/serine kinase, since proline is most commonly found at that position for tyrosine kinases and threonine or serine for threonine/serine kinases (10). There was no kinase especially homologous to the product of byr2, but the two most similar appear to be the products of the byr1 and cdc2 genes of S. pombe. The sequence comparison of these proteins is shown in Fig. 3.

Phenotypes conferred by the null allele of byr2. Plasmids with disruptions of byr2 were constructed by replacing an 840-bp fragment of the byr2 gene with the ura4 or the LEU2 marker (see Materials and Methods) (Fig. 4). A DNA fragment containing the disrupted byr2 gene was transformed into the h^0/h^− diploid strain SP826. Stable Ura+ transformants were selected, and the disruption of one copy of the endogenous byr2 gene by ura4 was confirmed by Southern blotting (data not shown). h^0/h^− revertants of these disruptants were detected by iodine vapor staining. Tetrad analysis of several revertants revealed viable Ura+ spores at the expected frequency, indicating that byr2 is not an essential gene and is not required for germination. byr2 haploid mutant strains showed no alterations in either morphology or growth rate in comparison to wild-type strains.

Haploid strains SPSU and SPSL were constructed by replacing the normal byr2 gene in the haploid strain SP870 with the ura4-disrupted or LEU2-disrupted byr2 gene, respectively, as described above. Genotypes were confirmed again by Southern blotting. These strains had normal shapes
FIG. 1. Phenotype conferred by expressing the \textit{S. cerevisiae} RAS2\textsuperscript{Aha-22} gene in \textit{S. pombe}. Cells were grown on PM plates with appropriate auxotrophic supplements, and phase-contrast micrographs were taken after 2 days of incubation at 30°C. (A) SP66, a wild-type \textit{S. pombe} strain. (B) SPR2A, derived from SP66 by transformation and expressing RAS2\textsuperscript{Aha-22} (see text). (C) A strain derived from SPR2A by transformation with pALR, which expresses the \textit{ras1} gene. (D) A strain derived from SPR2A by transformation with pART-BYR1, which expresses the \textit{S. pombe} \textit{byr1} gene. (E) A strain derived from SPR2A by transformation with pWH5S1, which expresses the \textit{S. pombe} \textit{byr2} gene. Arrowheads indicate asci, evident in each panel except B.
amino acid parental strain. Upon and demonstrate that the byr2 gene is absolutely required for sporulation and is thus capable of reversing some but not all of the defects in rasl null strains. In this respect, byr2 resembles byr1 (18). We next tested the ability of pWH5S1 to suppress defects in strains carrying

Genetic interactions with byr2. To gain more insight into the relationship between byr2 and other possibly related genes, we tested the ability of the high-copy-number plasmid pWH5S1, expressing byr2, to suppress defects caused by the loss of function of other genes. This plasmid was incapable of restoring defects in conjugation or cell shape in SPRU, a strain carrying the rasl null allele rasl::ura4. pWH5S1 was, however, able to suppress the defective sporulation of a rasl::ura4::ura4 diploid strain. Thus, byr2 is capable of reversing some but not all of the defects in rasl null strains.
**S. POMBE PROTEIN KINASE-ENCODING GENE byr2**

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**byr1 null mutations.** We transformed the haploid strain SPBU (byr1::ura4), which is sterile, and the diploid strain SPBUD (byr1::ura4/byr1::ura4), which is defective in sporulation. pH551 could suppress the phenotypic defect in neither. A series of *S. pombe* sterile (ste) alleles have been isolated by previous investigators. *ras1* has been identified as the locus of ste5 (15); *ste5* is required for *ras1* function (11); and *byr1* has been identified as the locus of *ste1* (19). We therefore tested the plasmid pH551, expressing *byr2*, for suppressor activity on a panel of haploid ste strains, including JM57 (ste2), JM66 (ste3), JM75 (ste6), JM83 (ste7), and JM86 (ste8) (17). Normal conjugation and sporulation were restored only in the ste8 mutant. Neither pALR, expressing *ras1*, nor pART-BYR1, expressing *byr1*, was able to restore these functions in the ste8 mutant.

We next investigated some further functional relationships between *byr2*, *ras1*, *byr1*, and *ste8* by examining the properties of strains with *byr2* disruptions. Plasmids pALR and pALRV, which contain the wild-type *ras1* gene and the activated *ras1Val-17* gene, respectively, were transformed separately into SPSU and SPSUD. Neither wild-type *ras1* nor activated *ras1* could overcome the conjugation or sporulation deficiencies conferred by the *byr2* null allele in these strains. Expression of *ras1Val-17* did not induce the typical morphological abnormalities in the *byr2* mutants. We conclude that *byr2* function is absolutely necessary for the sexual differentiation functions of *ras1*. *byr1* resembles *byr2* in this respect as well. The plasmid pART-BYR1, which contains the adh promoter-driven *byr1* gene, could induce azygotic sporulation in the diploid strain SPSUD but could not induce conjugation in the haploid strain SPSU.

To test further the relatedness of *byr2* and *ste8*, we made *byr2*/*ste8* diploid mutant strains by haploid cell fusion (see Materials and Methods). The resulting diploids were unable to sporulate. Thus, these genes are in the same complementation class. From this result and the ability of plasmids expressing *byr2* but not *ras1* or *byr1* to suppress conjugation defects in *ste8* mutants, it seems likely that *byr2* corresponds to *ste8*. This conclusion is supported by linkage analysis studies, which indicate that both *ste8* and *byr2* are linked to *lea1* (data not shown).

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**FIG. 3.** Sequence comparison of the *byr2* protein and two other protein kinases, *S. pombe* *byr1* and *S. pombe* cdc2. Amino acid coordinates are in the left-hand margin. The bottom line represents a protein kinase conserved sequence deduced from 65 protein kinases (16). Double dots between sequences indicate identical amino acids, while single dots indicate conservative amino acids. The conservative groupings are: A, G, P, S, T; L, I, V, M; D, E, N, Q; K, H, R; F, Y, W; and C.

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**FIG. 4.** Construction of *byr2* null alleles and *byr2* deletion mutants. (A) Restriction map of the *byr2* gene. The bar represents the open reading frame. The solid portion of the bar is the putative protein kinase catalytic region. (B) *byr2* null alleles, containing the LEU2 or *ura4* marker between the *NcoI* and *SpeI* sites. (C) *byr2* plasmid pAIS1-BD, which contains the indicated deletion. (D) *byr2* plasmid pAIS1-SBD, which contains the indicated deletion. Restriction enzymes: *BamHI*, *BcI*, *Bell*, *Bs*, *BstXI*, *Nc*, *NcoI*, *Sa*, *SalI*, *Sm*, *SmaI*, *Sp*, *SpeI*. Sites in parentheses were destroyed during vector construction. The dashes represent sequences from the vector, and the dots represent sequences encoding the peptide epitope. The arrow indicates the direction of transcription.
FIG. 5. Phenotype of byr2 mutants of *S. pombe*. Cells were grown on PM plates with appropriate auxotrophic supplements, and phase-contrast micrographs were taken after 2 days of incubation at 30°C. (A) SP870, a wild-type *S. pombe* strain. (B) SPSU, a byr2 mutant. (C) A strain derived by transformation of SPSU with pWH5S1, which expresses the byr2 gene. (D) SPSUD, a diploid byr2/byr2 mutant strain. (E) A strain derived by transformation of SPSUD with pWH5S1, which expresses the byr2 gene. (F) A strain derived by transformation of SPSUD with pART-BYR1, which expresses the *S. pombe* byr1 gene. Arrowheads indicate asci, evident in each panel except B and D.
Mutational analysis of byr2. In an effort to develop tools for studying the role and regulation of the protein kinase encoded by byr2, we have attempted to create dominant acting and dominant interfering forms of byr2. The organization of the byr2 kinase resembles that of the protein kinases C and the cyclic GMP-dependent protein kinases (3). In the latter two kinases, the catalytic portions are C-terminal and the regulatory domains are N-terminal. The byr2 kinase also resembles the S. pombecdc2 kinase, and dominant activated alleles of the latter are known (1). These considerations led us to make the byr2 mutants described below.

Plasmid pAlSI contains the entire byr2 coding region fused in frame to sequences encoding an N-terminal oligopeptide epitope transcribed from the adh promoter. The epitope, derived from the hemagglutinin protein of the influenza virus, is useful for monitoring the presence of the byr2 fusion protein. pAlSI, like pWHSS1, was able to fully complement the phenotypic defects of byr2 mutants. A 77-kDa protein, of the expected size, was detected in cells containing pAlSI by Western blotting with monoclonal antibodies directed against the peptide epitope. Further mutations were made in this plasmid.

Two deletion mutations and one point mutation were made. An N-terminal deletion, carried on plasmid pAlSI-SBD, lacked the 960-bp SalI-BstX fragment encoding 320 residues from 1 to 320. It encoded an intact catalytic domain in frame with the peptide epitope (Fig. 4). A C-terminal deletion carried on plasmid pAlSI-BD lacked the 654-bp BclI-BclII fragment encoding 217 amino acids from positions 389 to 606 (Fig. 4). The plasmid pAlSI<sup>Asp-534</sup> contained a single point mutation which directed the synthesis of aspartic acid rather than glycine at codon 534 of byr2. This is one of the highly conserved residues in protein kinases (10), and in the cdc2 kinase this substitution leads to a dominantly activated protein (1). All three plasmids directed the synthesis of proteins of the expected mobilities, detected in Western blots with monoclonal antibodies (data not shown).

As expected, the plasmid pAlSI-BD, which lacks the kinase catalytic region, could not restore functions to byr2 mutant haploid and diploid strains. The plasmid pAlSI-SBD, which contains the catalytic domain, could complement the loss of the byr2 mutation, although complementation was not as strong as with pAlSI itself. The plasmid pAlSI<sup>Asp-534</sup> was unable to replace byr2 function. This last result was the reverse of expectations from studies of the cdc2 kinase but consonant with the observation that this residue is highly conserved among protein kinases.

These plasmids were next transformed into wild-type cells (Fig. 6). To see the effects on wild-type cells, we monitored cellular agglutination, an early step in the conjugation process. Both pAlSI and pAlSI-SBD increased cell agglutination, while both pAlSI-BD and pAlSI<sup>Asp-534</sup> decreased agglutination. These results suggest that high-level expression of byr2 kinase catalytic function deregulates a step in conjugation, while the expression of catalytically inactive byr2 protein dominantly interferes with wild-type byr2 function.

To test these observations further, the same plasmids were transformed into the strain SP562, which contains an activated ras<sup>1</sup><sup>V</sup><sup>^a1-17</sup> allele. The presence of this ras<sup>1</sup> allele increases cell agglutination, induces an elongated conjugation tube, and causes partial sterility (7, 20). Both pAlSI-BD and pAlSI<sup>Asp-534</sup> reduced cell agglutination (Fig. 6) and diminished the presence of elongated conjugation tubes. There was no improvement in conjugation in ras<sup>1</sup><sup>V</sup><sup>^a1-17</sup> strains carrying these plasmids (data not shown).

DISCUSSION

We have used genetic approaches to identify components of RAS pathways in two yeasts. In this study, we have sought genes that, on high-copy-number plasmids, can overcome deficiencies in S. pombe cells expressing the S. cerevisiae mutant RAS2<sup>3</sup><sup>Ab-22</sup> gene. In S. cerevisiae, expression of this gene blocks the function of CDC25, which is to activate wild-type RAS proteins (22). In S. pombe, expression of RAS2<sup>3</sup><sup>Ab-22</sup> mildly interferes with normal ras1 function but apparently by a different mechanism, since overexpression of ste6, the homolog of the S. cerevisiae CDC25 gene (11), has no salutary effect. One of the genes we isolated by our selection procedure we have called byr2. The genetic interactions between byr2 and ste8 suggest that they are the same gene. If so, this brings to four the number of previously identified "sterile" genes thought to act on the ras1 pathway.

byr2 has the potential to encode a serine/threonine protein kinase, but one which is not especially similar in sequence to any of the previously identified protein kinases. The functional organization of the byr2 protein kinase resembles that of many other serine/threonine protein kinases, such as protein kinase C and the cyclic GMP protein kinase, in that the catalytic function is C-terminal (3). We have therefore tested whether the N-terminal domain of the byr2 mutant kinase has a regulatory role by examining the properties of byr2 genes that cannot, or are not expected to, encode a catalytically active kinase. Such mutant genes appear to interfere with the wild-type byr2 function. We conclude that it is likely that the N-terminal domain of byr2 interacts with a protein, present in limiting amount, that is necessary for byr2 function. Such a protein could be a positive regulatory factor, a substrate of the byr2 kinase, or the kinase itself.

The genetic interactions of byr2 bear striking resemblance to those of byr1, a gene previously isolated as a suppressor in ras1/ras1 mutant diploid cells, which also encodes a predicted serine/threonine protein kinase (18). Both byr2 and byr1 are absolutely required by normal cells for conjugation...
and for sporulation but are otherwise not essential genes. Both genes can suppress the sporulation defects of ras1la/ras1 diploids and the conjugation defects of S. pombe strains expressing RAS2Ala22, but not the conjugation defects of ras1 haploid strains. High-copy expression of the activated mutant ras1Val11 gene fails to suppress the sporulation defects of either byr1/byn1 or byr2/byn2 mutant diploid cells. If we assume that ras1, byr2, and byr1 encode proteins that act on the same pathway and are not redundant, then the sites of action of these proteins can be unambiguously ordered. Overexpression of byr1 induces sporulation in ras1la/ras1 mutant diploids, and with the assumptions stated above, it therefore cannot act through ras1. The site of action of byr1 must lie downstream of that of ras1, as others have proposed (18). Consistent with the idea that ras1 must act through the site of action of byr1, we have shown that expression of the activated mutant ras1Val17 gene cannot bypass the sporulation defect of byr1/byn1 mutant diploids. By the same reasoning, byr2 must act downstream of ras1. Finally, we can conclude that the site of action of byr1 lies downstream of that of byr2, because overexpression of byr1 can overcome the sporulation defects of byr2/byn2 mutant diploids and because expression of byr2 cannot induce sporulation in byr1/byn1 mutant diploids. Thus, byr2 protein may be closer to ras1 protein in the chain of command than is the byr1 protein.

The above conclusions are no stronger than the starting assumptions of the model. We do not rule out the possibility that byr1 and/or byr2 operate on pathways parallel to ras1. Expression of neither byr2 nor byr1 can overcome the conjugation defects of ras1 mutants, nor does byr1 overcome the conjugation defects of byr2 mutants. These observations are readily explained by any of three plausible hypotheses. First, the dynamics of the activation of the three gene products may be critical for achieving conjugation. Substitution of one component for another would be unlikely to restore this critical temporal order of activation. This may be particularly true for ras1, as discussed below. Second, multicopy or promoter fusion genes may not produce sufficient levels of byr1 or byr2 activity in ras1 mutant cells to induce conjugation. Third, the pathway controlled by one component may branch upstream of the pathway controlled by another component. There is clear evidence for this in the case of ras1; specifically, ras1 mutant cells are round, while byr1 and byr2 mutant cells have a normal, elongated shape. Hence, ras1 has other functions.

Conjugation in S. pombe is a complex process. One of the first discernible stages in the conjugation process is increased cellular agglutination and the development of conjugation tubes (4). Cells which carry the ras1 mutation are virtually sterile and do not undergo even the early phases of conjugation. Cells which carry the activated ras1Val17 mutation are nearly sterile but undergo a pronounced, or exaggerated, first phase (7, 20). This observation suggests that ras1 activity controls entry into the first phase of conjugation but that diminution of ras1 activity is required for the ensuing phases. Our work helps to define the role of byr2 in the entry into these phases. Overexpression of byr2 increases cell agglutinability, and interfering forms of byr2 block the increased agglutinability and diminish the elongated conjugation tubes of a ras1Val17 strain. Moreover, ras1Val17 byr2 mutants do not display the typical ras1Val17 phenotype. Therefore, byr2 function appears to be required for the first phase of conjugation. On the other hand, interfering alleles of byr2 do not increase the conjugation of ras1Val17 mutants, and hence it is not the diminution of byr2 function that is required for entry into the ensuing phases of conjugation. It is probable that byr2 function is not itself sufficient for the first phase of conjugation, since multiple copies of byr2 in a ras1 mutant background induce increased agglutination but do not induce the formation of conjugation tubes. We cannot determine at present whether both or either ras1 and byr2 are required in later phases of conjugation.

ACKNOWLEDGMENTS

We thank M. McLeod, D. Beach, and M. Yamamoto for useful discussions and for providing plasmids and yeast strains. We thank U. Leupold for providing sterile strains. We thank P. Bird for help in preparing the manuscript. This work was supported by grants from the National Cancer Institute and the American Cancer Society. M.W. is an American Cancer Society Research Professor.

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