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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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***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 adenylyl 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 *ras1*$^{Val-17}$ 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 (h^{90}) 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 zygotic sporulation. Diploid cells, formed either by mating or by other means, can be propagated stably, but diploid strains containing both mating type loci will undergo azygotic sporulation upon starvation. The four-spored asci formed by zygotic sporulation look different from the four-spored asci 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). *ste6* 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 *ras1* 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 homozygous 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 *ste8*/*byr2* 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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TABLE 1. *S. pombe* strains used in this study

Strain	Relevant genotype	Source
JM57	h^{90} *leu1 ste2*	U. Leupold
JM66	h^{90} *leu1 ste3*	U. Leupold
JM75	h^{90} *leu1 ste6*	U. Leupold
JM83	h^{90} *leu1 ste7*	U. Leupold
JM86	h^{90} *leu1 ste8*	U. Leupold
SP562[a]	h^{90} *leu1-32 ade6-216 ura4 ras1*:: LEU2 *ras1*$^{Val\text{-}17}$	D. Beach
SP66	h^{90} *leu1-32 ade6-216*	D. Beach
SP826	h^{+N} *leu1-32 ade6-210 ura4-D18*/h^{+N} *leu1-32 ade6-210 ura4-D18*	D. Beach
SP870	h^{90} *leu1-32 ade6-210 ura4-D18*	D. Beach
SPBU	h^{90} *leu1-32 ade6-210 ura4-D18 byr1::ura4*	This study
SPBUD	h^{90} *leu1-32 ade6-210 ura4-D18 byr1::ura4*/h^{90} *leu1-32 ade6-210 ura4-D18 byr1::ura4*	This study
SPR2A	h^{90} *leu1-32 ade6-216 ura4::RAS2*$^{Ala\text{-}22}$	This study
SPRU	h^{90} *leu1-32 ade6-210 ura4-D18 ras1::ura4*	This study
SPRUD	h^{90} *leu1-32 ade6-210 ura4-D18 ras1::ura4*/h^{90} *leu1-32 ade6-210 ura4-D18 ras1::ura4*	This study
SPSL	h^{90} *leu1-32 ade6-210 ura4-D18 byr2::LEU2*	This study
SPSU	h^{90} *leu1-32 ade6-210 ura4-D18 byr2::ura4*	This study
SPSUD	h^{90} *leu1-32 ade6-210 ura4-D18 byr2::ura4*/h^{90} *leu1-32 ade6-210 ura4-D18 byr2::ura4*	This study

[a] In this strain, the wild-type *ras1* gene was replaced with the *S. cerevisiae LEU2* gene and the activated *ras1*$^{Val\text{-}17}$ allele.

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 *Sau*3AI-digested *S. pombe* genomic DNA into the *Bcl*I site of plasmid pWH5 (24), which contains the *LEU2* gene. The plasmid pART-R2A was constructed by inserting a 1.2-kbp *Hpa*I fragment from YIP-OGA (22), which contains the *S. cerevisiae* dominant *RAS2* mutation *RAS2*$^{Ala\text{-}22}$, at the *Sma*I site of *S. pombe* expression vector pART1 (16). pART1 is a pUC118-based vector containing the 2.2-kbp *Hin*dIII fragment of the *S. cerevisiae LEU2* gene cloned into the *Hin*dIII site, a 1.2-kbp *S. pombe* autonomously replicating sequence (ARS) fragment (14) cloned at the *Eco*RI site, and a 0.7-kbp *S. pombe adh* promoter fragment inserted at *Sph*I and *Pst*I sites. The *S. pombe byr1* gene was cloned from yeast genomic DNA by the polymerase chain reaction (PCR) with the oligonucleotide primers 5'-TTGAGAATTTGGCCAATAG and 5'-GATTTTTCTGA AATCCTTCTTT. The PCR product was digested with *Bal*I and *Xmn*I, and the 1.1-kbp *Bal*I-*Xmn*I fragment, which contained the whole coding sequence of *byr1*, was cloned into the *Sma*I 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 pAL was derived from pIRT5 (25) by replacing the *ura4* gene with the 2.2-kbp *Hin*dIII fragment containing the *S. cerevisiae LEU2* gene. Plasmid pALR was constructed by inserting a 1.4-kbp *Bam*HI-*Bgl*II *S. pombe ras1* gene fragment (20) into the *Bam*HI site of pAL. Plasmid pAIS1 was constructed by inserting a *Sal*I-*Sac*I fragment of *byr2* into the *Sal*I-*Sac*I sites of the vector pAIL (13). pAIL contains the *S. pombe ura4* gene, an *S. pombe* ARS element, and the *S. pombe adh* promoter with an oligonucleotide encoding a peptide derived from the hemagglutinin antigen of influenza virus. The *byr2* *Sal*I-*Sac*I fragment was generated by cleavage of the PCR product with the *byr2* gene as the template and the primers 5'-TTATACGTTGTCGACCCACTTTCCTG and 5'-TCATC ATAACTGAGCTCGCTTATGAAAC. A *Sal*I site which is 15 bp upstream of the *byr2* start codon was thus created in the PCR product, and a *Sac*I site which is 3 bp downstream of the stop codon was created. The *byr2* missense allele *byr2*$^{Asp\text{-}534}$ was constructed by site-directed mutagenesis as described by Zoller and Smith (26). The mutagenic oligonucleotide contained a GG to AC double substitution that changed the encoded amino acid from glycine 534 to aspartic acid 534. The mutation was confirmed by dideoxynucleotide sequencing.

Construction of ras1, byr1, and byr2 mutants of *S. pombe*. The plasmid pRAS contained a 2.5-kbp *S. pombe* genomic DNA fragment containing the *ras1* gene cloned into the *Bam*HI site of pUC118 (20) (provided by D. Beach). This was digested with *Nhe*I and *Bgl*II, blunt-ended, and religated with a 1.8-kbp blunt-ended fragment of the *S. pombe ura4* gene. The resulting plasmid, pRAS::URA, contained a *ras1* gene with the *ura4* gene replacing 60 bp of 3' coding region and about 300 bp of 3'-flanking sequence. *Bam*HI-digested DNA from this plasmid was used to transform wild-type h^{90} 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 *Spe*I-*Ppu*MI 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 conformed by Southern analysis and had the phenotype described previously (18). *byr2* null alleles were made in a similar way. An 840-bp *Nco*I-*Spe*I fragment of the *byr2* coding region was replaced by a 1.8-kbp *S. pombe ura4* gene fragment or a 2.2-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-R2A was digested with *Sph*I and *Sac*I. A 1.7-kbp fragment containing *S. cerevisiae RAS2*$^{Ala\text{-}22}$ 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 *Eco*RV site of the *S. pombe ura4* gene contained in a pUC118-based vector as a 1.8-kbp *Hin*dIII fragment cloned at the *Hin*dIII site. This insertion abolished *ura4* function totally, as proved later. The disrupted *ura4* fragment was released by *Hin*dIII digestion and transformed into the Ura^+ *S. pombe* strain SP66. The Ura^- transformants were selected on plates containing 5-fluoroorotic acid as described previously (8). The integration of *RAS2*$^{Ala\text{-}22}$ 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 $MgCl_2$ [pH 7.4]) at 2×10^7 to 3×10^7 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.

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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 extracts 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 Tetrazolium (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^{90} homothallic 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 × 10^6 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 asci. 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, pWH5S1, containing a gene we call *byr2*. The fourth class is not discussed in this report.

Sequence of *byr2*. pWH5S1 contained a large insert of about 17 kbp. Deletion and subcloning analysis localized the functional gene to a 4-kbp *Bam*HI-*Sma*I 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^{+N}/h^{+N} 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^{90}/h^{+N} 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

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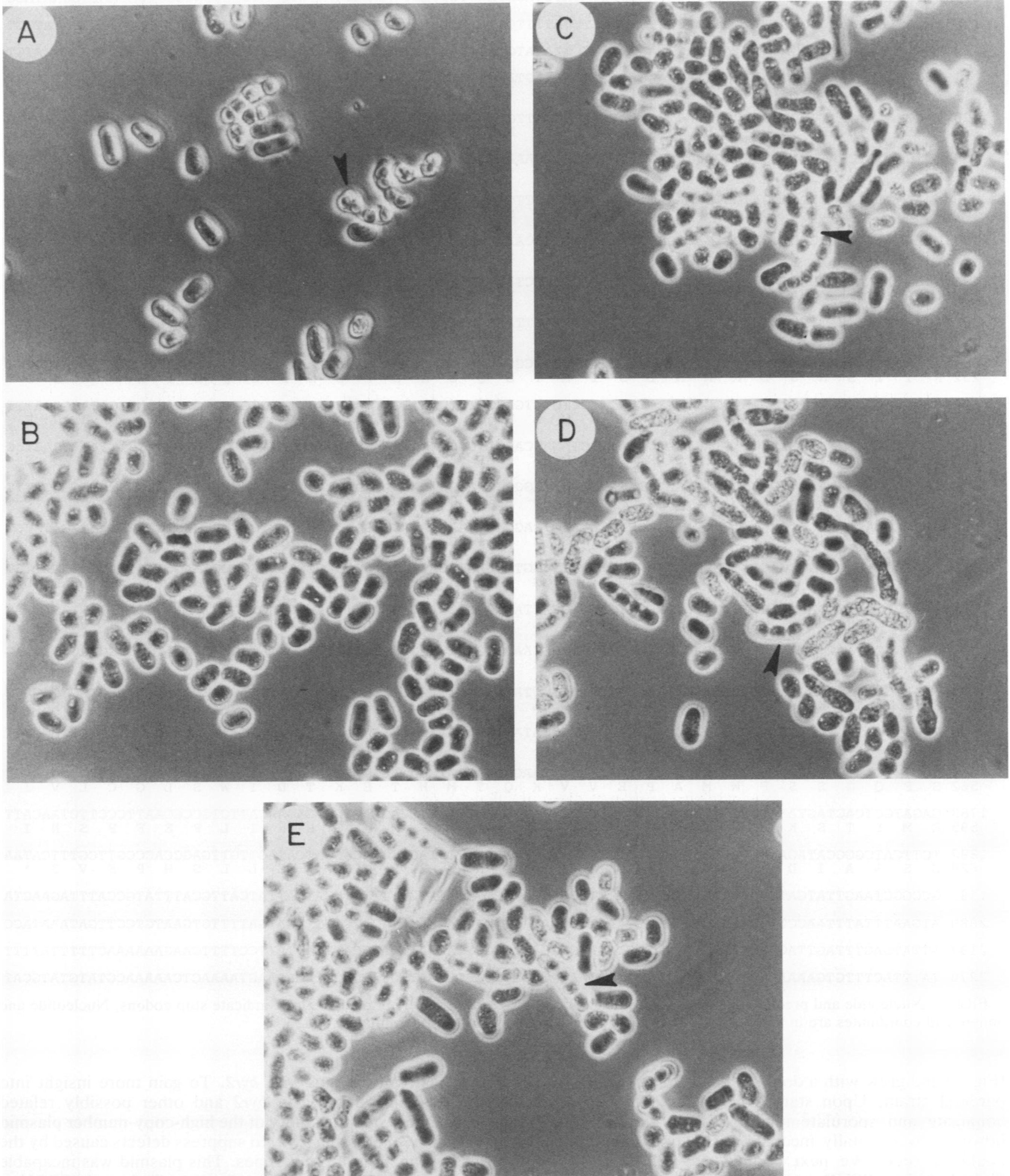

FIG. 1. Phenotype conferred by expressing the *S. cerevisiae* $RAS2^{Ala-22}$ gene in *S. pombe*. Cells were grown on PM plates with appropriate auxotrophic supplements, and phase-contrast micrographs were taken after 2 days of incubation at 30°C. (A) SP66, a wild-type *S. pombe* strain. (B) SPR2A, derived from SP66 by transformation and expressing $RAS2^{Ala-22}$ (see text). (C) A strain derived from SPR2A by transformation with pALR, which expresses the *ras1* gene. (D) A strain derived from SPR2A by transformation with pART-BYR1, which expresses the *S. pombe byr1* gene. (E) A strain derived from SPR2A by transformation with pWH5S1, which expresses the *S. pombe byr2* gene. Arrowheads indicate asci, evident in each panel except B.

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-297 ATTTCTCGTCGATATTTAGACTCCTTGTGGAACTCGTATATGTACGTAAGTGTTATTTAATAGATATCTGAATCTGTGCCGGGTGAGAATTGCAATACC

-198 TATATAAACAAGCAGCCTTTACGAATTTTTTGGCAGCTTCATTTTGGTAATGAGATTTAGTGAAACTAATATCGGTTATAAAGTGTATAGTTATATTGT

 -99 TACGTCAAAGAGCACGAGACTAAGGGAGGCAGGTTAAACGTAAATGCTACCAACTTTGAACTTAGAAGTTATACGTTGTACAACCACTTTCCTGCATTT

   1 ATGGAATATTATACCTCGAAGGAGGTAGCAGAATGGTTGAAAAGTATCGGATTGGAGAAGTATATCGAACAATTTTCACAAAATAACATTGAAGGACGA
   1 M  E  Y  Y  T  S  K  E  V  A  E  W  L  K  S  I  G  L  E  K  Y  I  E  Q  F  S  Q  N  N  I  E  G  R

 100 CACCTAAATCATTTAACCTTGCCTTTACTGAAAGACCTAGGCATTGAAAACACCGCCAAAGGCAAACAATTTTTAAAACAAAGAGACTATTTACGAGAG
  34 H  L  N  H  L  T  L  P  L  L  K  D  L  G  I  E  N  T  A  K  G  K  Q  F  L  K  Q  R  D  Y  L  R  E

 199 TTTCCACGTCCATGTATACTGAGGTTTATTGCCTGTAACGGGCAAACTCGAGCAGTTCAATCACGAGGAGATTACCAGAAAACACTCGCTATTGCTTTG
  67 F  P  R  P  C  I  L  R  F  I  A  C  N  G  Q  T  R  A  V  Q  S  R  G  D  Y  Q  K  T  L  A  I  A  L

 298 AAAAAGTTTAGCCTTGAAGATGCTTCAAAATTCATTGTATGCGTTTCACAGAGTAGTCGAATTAAGCTGATTACTGAAGAAGAATTTAAACAAATTTGT
 100 K  K  F  S  L  E  D  A  S  K  F  I  V  C  V  S  Q  S  S  R  I  K  L  I  T  E  E  E  F  K  Q  I  C

 397 TTTAATTCATCTTCACCGGAACGCGACAGGTTAATTATTGTGCCAAAAGAAAAGCCTTGTCCATCGTTTGAAGACCTCCGCCGTTCTTGGGAGATTGAA
 133 F  N  S  S  S  P  E  R  D  R  L  I  I  V  P  K  E  K  P  C  P  S  F  E  D  L  R  R  S  W  E  I  E

 496 TTGGCTCAACCGGCAGCATTATCATCACAGTCCTCCCTTTCTCCTAAACTTTCCTCTGTTCTTCCCACGAGCACTCAGAAACGAAGTGTCCGCTCAAAT
 166 L  A  Q  P  A  A  L  S  S  Q  S  S  L  S  P  K  L  S  S  V  L  P  T  S  T  Q  K  R  S  V  R  S  N

 595 AATGCGAAACCATTTGAATCCTACCAGCGACCTCCTAGCGAGCTTATTAATTCTAGAATTTCCGATTTTTTCCCCGATCATCAACCAAAGCTACTGGAA
 199 N  A  K  P  F  E  S  Y  Q  R  P  P  S  E  L  I  N  S  R  I  S  D  F  F  P  D  H  Q  P  K  L  L  E

 694 AAAACAATATCAAACTCTCTTCGTAGAAACCTTAGCATACGTACGTCTCAAGGGCACAATCTTGGTAATTTTGGTCAAGAAATTTTGCCAAGAAGCTCT
 232 K  T  I  S  N  S  L  R  R  N  L  S  I  R  T  S  Q  G  H  N  L  G  N  F  G  Q  E  I  L  P  R  S  S

 793 CGGCGGGCTCGCCCTTCCGAATTGGTTTGTCCTCTTTCCTCTCTGAGGATTTCTGTTGCTGAAGATGTTAACCGACTTCCTAGGATTGATAGGGGATTT
 265 R  R  A  R  P  S  E  L  V  C  P  L  S  S  L  R  I  S  V  A  E  D  V  N  R  L  P  R  I  D  R  G  F

 892 GATCCTCCACTTACTGTATCTTCTACTCAGCGTATTTCTAGACCACCATCGCTTCAAAAATCCATAACCATGGTTGGTGTGGAACCTTTATACCAGTCC
 298 D  P  P  L  T  V  S  S  T  Q  R  I  S  R  P  P  S  L  Q  K  S  I  T  M  V  G  V  E  P  L  Y  Q  S

 991 AACGGAAATGAAAAATCGTCGAAGTATAATGTTTTTTCAGAAAGCGCGCATGGCAACCATCAAGTACTTTCCTTTTCACCTGGTTCATCCCCATCATTT
 331 N  G  N  E  K  S  S  K  Y  N  V  F  S  E  S  A  H  G  N  H  Q  V  L  S  F  S  P  G  S  S  P  S  F

1090 ATTGAGCAACCATCCCCAATTTCTCCAACTTCCACTACAAGTGAGGACACAAATACACTTGAAGAGGACACTGATGATCAGTCTATCAAATGGATACGT
 364 I  E  Q  P  S  P  I  S  P  T  S  T  T  S  E  D  T  N  T  L  E  E  D  T  D  D  Q  S  I  K  W  I  R

1189 GGTGCGTTAATAGGTTCAGGATCGTTTGGACAAGTTTATCTCGGTATGAATGCATCTAGTGGTGAGTTGATGGCTGTTAAGCAAGTGATCTTAGATTCT
 397 G  A  L  I  G  S  G  S  F  G  Q  V  Y  L  G  M  N  A  S  S  G  E  L  M  A  V  K  Q  V  I  L  D  S

1288 GTTTCTGAATCAAAAGACCGCCATGCCAAACTTTTAGATGCATTAGCGGGTGAAATTGCTCTTCTACAGGAGTTGTCTCATGAGCATATTGTCCAATAT
 430 V  S  E  S  K  D  R  H  A  K  L  L  D  A  L  A  G  E  I  A  L  L  Q  E  L  S  H  E  H  I  V  Q  Y

1387 CTCGGTTCTAACTTAAATTCGGACCACTTGAACATTTTTTTGGAATATGTTCCTGGGGGGTCGGTTGCCGGATTGCTTACAATGTATGGAAGCTTTGAG
 463 L  G  S  N  L  N  S  D  H  L  N  I  F  L  E  Y  V  P  G  G  S  V  A  G  L  L  T  M  Y  G  S  F  E

1486 GAAACGCTAGTCAAGAACTTTATTAAGCAAACCCTTAAAGGCTTAGAATACCTTCATTCACGCGGCATTGTACACCGTGATATAAAAGGTGCGAACATC
 496 E  T  L  V  K  N  F  I  K  Q  T  L  K  G  L  E  Y  L  H  S  R  G  I  V  H  R  D  I  K  G  A  N  I

1585 TTGGTTGATAATAAAGGGAAGATTAAAATTTCGGACTTTGGGATATCGAAGAAACTTGAACTAAATAGTACCTCTACGAAGACGGGTGGTGCTCGCCCT
 529 L  V  D  N  K  G  K  I  K  I  S  D  F  G  I  S  K  K  L  E  L  N  S  T  S  T  K  T  G  G  A  R  P

1684 TCTTTTCAAGGCTCTTCTTTTTGGATGGCTCCCGAAGTGGTGAAGCAAACTATGCATACGGAGAAAACAGATATTTGGTCTTTGGGATGTTTGGTAATC
 562 S  F  Q  G  S  S  F  W  M  A  P  E  V  V  K  Q  T  M  H  T  E  K  T  D  I  W  S  L  G  C  L  V  I

1783 GAGATGCTCACTAGTAAGCACCCTTATCCGAATTGTGATCAAATGCAGGCCATTTTCAGAATTGGGGAGAACATTCTGCCGGAATTCCCTTCTAACATT
 595 E  M  L  T  S  K  H  P  Y  P  N  C  D  Q  M  Q  A  I  F  R  I  G  E  N  I  L  P  E  F  P  S  N  I

1882 TCTTCATCGGCCATAGATTTCCTTGAGAAAACGTTTGCCATAGATTGTAATCTTAGACCTACAGCATCAGAGTTGTTGAGCCACCCGTTCGTTTCATAA
 628 S  S  S  A  I  D  F  L  E  K  T  F  A  I  D  C  N  L  R  P  T  A  S  E  L  L  S  H  P  F  V  S  *

1981 GCCGGCTAAGTTATGATGAAAACAGAAACATTATTTTACGATCTATCTTTTTTATGTTTTTTTGCATCATTATCATTGCATTTATGCCATTTAGAACTA

2080 ATGAATTTATTTAACCTATAAACCGTATGATTTAAGTTGCTATATTTTTCTACAATATTCATGTTTTTAATGATTTTGTGAATGTCCTTGATAAATACC

2197 ATTATGAGTTTAGTTACTAAAAAAATACTATTTTACATGTTCAGTTTGTTTAAAAAATATCCATTGATTATTCCTTTTCAAGAAAAAACTTTTTATTTT

2278 TATTTACTTTGTGAAAACGATAACATAAATATATAATAACACATATCTACGGGAATTCTATCACTTAAACAAGTAAAACTCAAAAACGTATGTATGCAT
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FIG. 2. Nucleotide and predicted amino acid sequences of the *byr2* gene. Letters in boldface type indicate stop codons. Nucleotide and amino acid coordinates are in the left-hand margin. The coordinates begin with the initiating codon.

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(Fig. 5) and grew with a doubling time similar to that of the parental strain. Upon starvation, cultures of SP870 cells conjugate and sporulate readily. SPSU and SPSL cells, however, were totally incapable of mating, and no spores could be seen. We next created a *byr2*::*ura4*/*byr2*::*ura4* diploid strain, SPSUD, by diploidizing SPSU. SPSUD was completely unable to sporulate upon starvation. Transformation of these *byr2* mutants with pWH5S1 completely restored these sexual functions (Fig. 5). These results demonstrate that the *byr2* gene is absolutely required for both conjugation and sporulation and is thus functionally closely related to the *ras1* and *byr1* genes.

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 *ras1* null allele *ras1*::*ura4*. pWH5S1 was, however, able to suppress the defective sporulation of a *ras1*::*ura4*/*ras1*::*ura4* diploid strain. Thus, *byr2* is capable of reversing some but not all of the defects in *ras1* null strains. In this respect, *byr2* resembles *byr1* (18). We next tested the ability of pWH5S1 to suppress defects in strains carrying

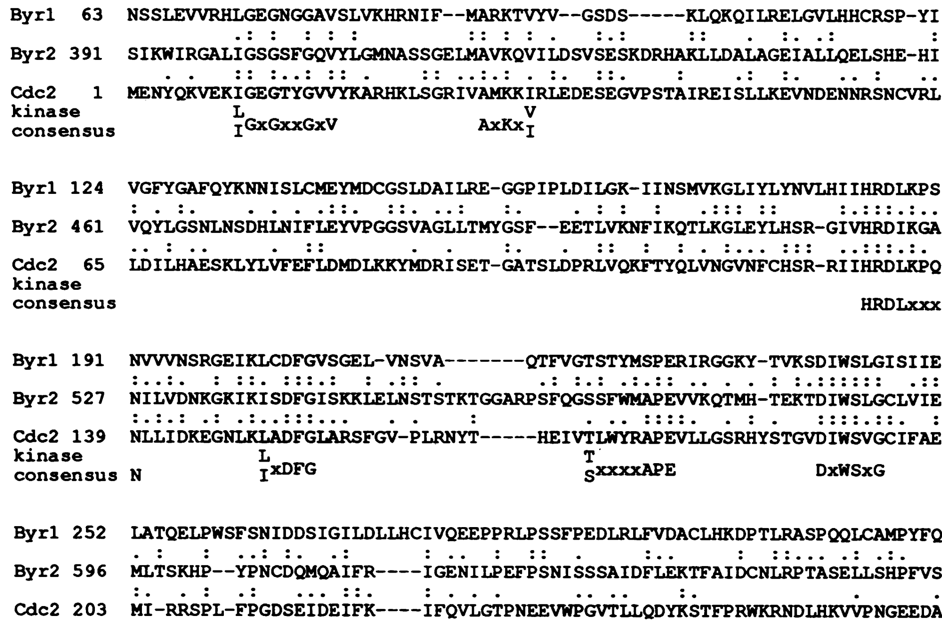

FIG. 3. Sequence comparison of the byr2 protein and two other protein kinases, *S. pombe* byr1 and *S. pombe* cdc2. Amino acid coordinates are in the left-hand margin. The bottom line represents a protein kinase consensus sequence deduced from 65 protein kinases (16). Double dots between sequences indicate 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.

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. pWH5S1 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); *ste6* is required for *ras1* function (11); and *byr1* has been identified as the locus of *ste1* (19). We therefore tested the plasmid pWH5S1, 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.

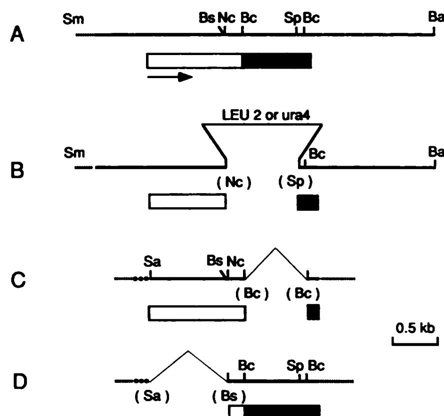

FIG. 4. Construction of *byr2* null alleles and *byr2* deletion mutants. (A) Restriction map of the *byr2* gene. The bar represents the open reading frame. The solid portion of the bar is the putative protein kinase catalytic region. (B) *byr2* null alleles, containing the *LEU2* or *ura4* marker between the *Nco*I and *Spe*I sites. (C) *byr2* plasmid pAIS1-BD, which contains the indicated deletion. (D) *byr2* plasmid pAIS1-SBD, which contains the indicated deletion. Restriction enzymes: Ba, *Bam*HI; Bc, *Bcl*I; Bs, *Bst*XI; Nc, *Nco*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Spe*I. 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.

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 $ras1^{Val-17}$ 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 $ras1^{Val-17}$ 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 *leu1* (data not shown).

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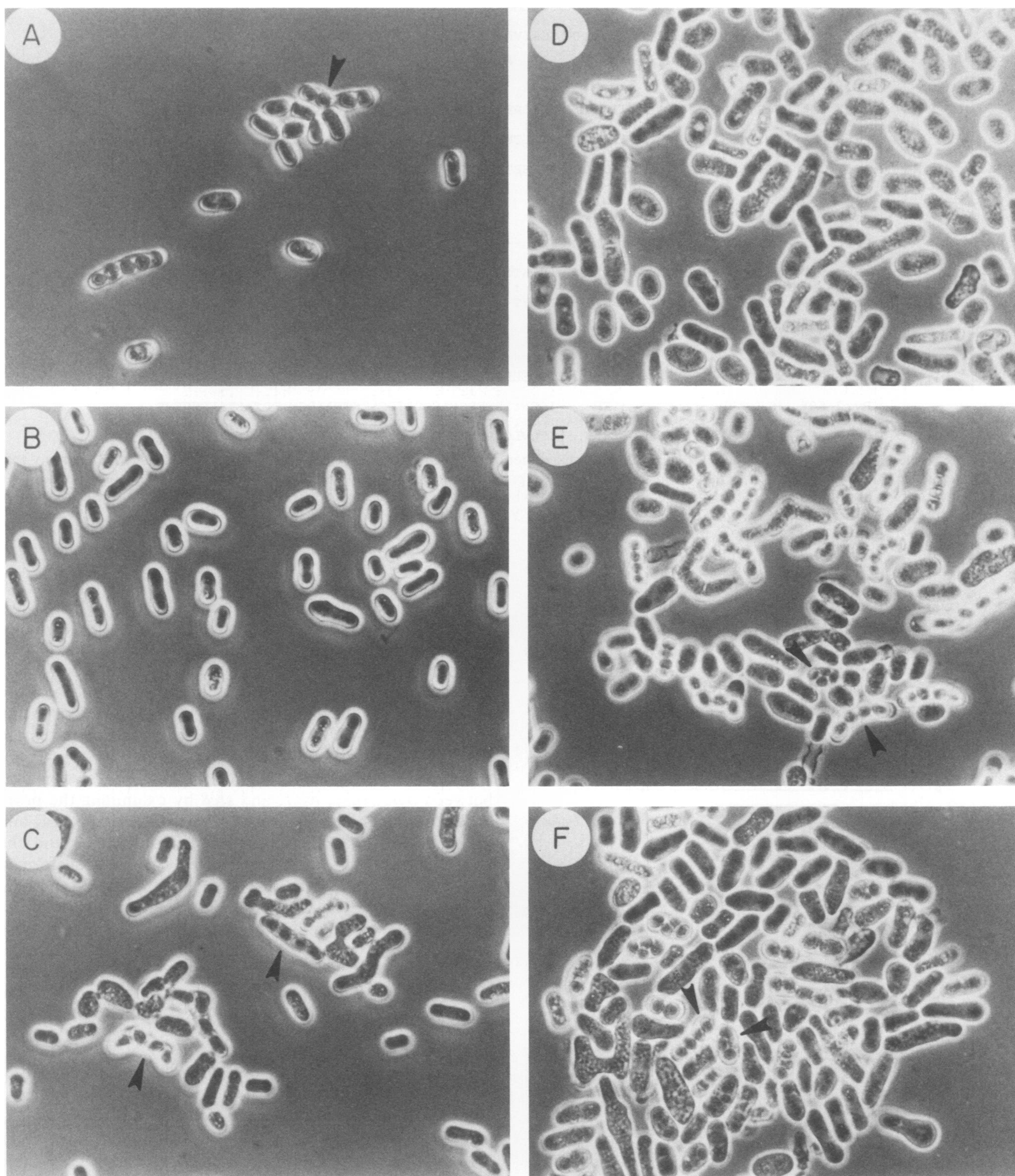

FIG. 5. Phenotype of *byr2* mutants of *S. pombe*. Cells were grown on PM plates with appropriate auxotrophic supplements, and phase-contrast micrographs were taken after 2 days of incubation at 30°C. (A) SP870, a wild-type *S. pombe* strain. (B) SPSU, a *byr2* mutant. (C) A strain derived by transformation 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.

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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. pombe* cdc2 kinase, and dominant activated alleles of the latter are known (1). These considerations led us to make the *byr2* mutants described below.

Plasmid pAIS1 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. pAIS1, like pWH5S1, was able to fully complement the phenotypic defects of *byr2* mutants. A 77-kDa protein, of the expected size, was detected in cells containing pAIS1 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 pAIS1-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 pAIS1-BD lacked the 654-bp *BclI-BclI* fragment encoding 217 amino acids from positions 389 to 606 (Fig. 4). The plasmid pAIS1$^{Asp\text{-}534}$ 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 pAIS1-BD, which lacks the kinase catalytic region, could not restore functions to *byr2* mutant haploid and diploid strains. The plasmid pAIS1-SBD, which contains the catalytic domain, could complement the loss of the *byr2* mutation, although complementation was not as strong as with pAIS1 itself. The plasmid pAIS1$^{Asp\text{-}534}$ 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 pAIS1 and pAIS1-SBD increased cell agglutination, while both pAIS1-BD and pAIS1$^{Asp\text{-}534}$ 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 *ras1*$^{Val\text{-}17}$ allele. The presence of this *ras1* allele increases cell agglutination, induces an elongated conjugation tube, and causes partial sterility (7, 20). Both pAIS1-BD and pAIS1$^{Asp\text{-}534}$ reduced cell agglutination (Fig. 6) and diminished the presence of elongated conjugation tubes. There was no improvement in conjugation in *ras1*$^{Val\text{-}17}$ strains carrying these plasmids (data not shown).

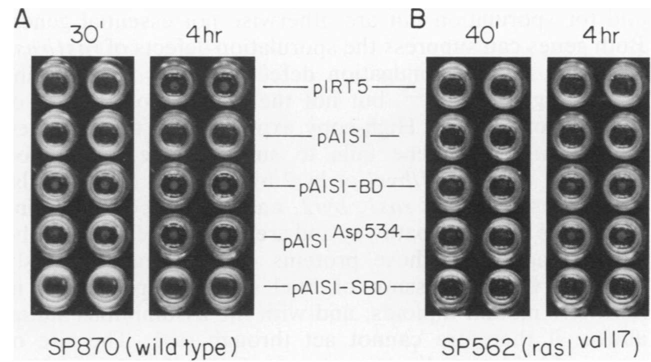

FIG. 6. Effect of *byr2* mutations on cell agglutination of SP870 (wild type) and SP562 (*ras1*$^{Val\text{-}17}$). The cell agglutination test (see Materials and Methods) was carried out after cells containing the indicated plasmids were cultured on minimal medium plates for 2 days. (A) SP870 cells transformed with various plasmids. pIRT5 is a control plasmid. The pictures were taken after 30 min or 4 h of incubation. Cells expressing interfering forms of *byr2* (pAIS1-BD and pAIS1$^{Asp\text{-}534}$) formed spots at the center within 30 min, while cells expressing catalytically active protein (pAIS1 and pAIS1-SBD) could not form spots even after 4 h. (B) SP562 cells transformed with the same plasmids. The pictures were taken after 40 min or 4 h of incubation. Only cells containing pAIS1-BD or pAIS1$^{Asp\text{-}534}$ formed spots, indicative of a decrease in cell agglutination.

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*$^{Ala\text{-}22}$ 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*$^{Ala\text{-}22}$ 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

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and for sporulation but are otherwise not essential genes. Both genes can suppress the sporulation defects of *ras1/ras1* diploids and the conjugation defects of *S. pombe* strains expressing *RAS2*$^{Ala-22}$, but not the conjugation defects of *ras1* haploid strains. High-copy expression of the activated mutant *ras1*$^{Val-17}$ gene fails to suppress the sporulation defects of either *byr1/byr1* or *byr2/byr2* 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 *ras1/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 *ras1*$^{Val-17}$ gene cannot bypass the sporulation defect of *byr1/byr1* 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/byr2* mutant diploids and because expression of byr2 cannot induce sporulation in *byr1/byr1* 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 *ras1*$^{Val-17}$ 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 *ras1*$^{Val-17}$ strain. Moreover, *ras1*$^{Val-17}$ *byr2* mutants do not display the typical *ras1*$^{Val-17}$ 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 *ras1*$^{Val-17}$ 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.

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