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byr2, a Schizosaccharomyces pombe Gene Encoding a Protein Kinase Capable of Partial Sporulation of the rasl Mutant Phenotype

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Schizosaccharomyces pombe contains a single gene, rasl, which is a homolog of the mammalian RAS genes. rasl is required for conjugation, sporulation, and normal cell shape. rasl 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 rasl mutants. rasl 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 rasl mutation, which has been identified previously as ste1. Our data indicate that if rasl, byr2, and byr1 act along the same pathway, then the site of action for byr2 is between the sites for rasl 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, rasl (6, 21). rasl is not an essential gene but functions in the sexual differentiation pathways of that yeast (7, 20). rasl 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 raslVal17 mutant 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 zygotic 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 ascus formed by zygotic sporulation look different from the four-spored ascus 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). rasl 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 rasl mutations (18, 19); rasl diploid cells containing byr1 on high-copy-number plasmids can sporulate, but overexpression of byr1 fails to suppress the conjugation defects in rasl haploid cells. Like rasl 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 rasl.

In the present report, we describe a second gene, byr2, capable of the partial phenotypic suppression of rasl mutations. byr2 resembles byr1 in many respects. The range of their genetic interactions is similar, and both appear to encode protein kinases. If rasl, byr2, and byr1 all act along a common pathway, our data suggest that byr2 acts between the sites of action of rasl 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-
TABLE 1. _S. pombe_ strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM57</td>
<td>h²⁰ leu1 ste2</td>
<td>U. Leupold</td>
</tr>
<tr>
<td>JM66</td>
<td>h²⁰ leu1 ste3</td>
<td>U. Leupold</td>
</tr>
<tr>
<td>JM75</td>
<td>h²⁰ leu1 ste6</td>
<td>U. Leupold</td>
</tr>
<tr>
<td>JM83</td>
<td>h²⁰ leu1 ste7</td>
<td>U. Leupold</td>
</tr>
<tr>
<td>JM86</td>
<td>h²⁰ leu1 ste8</td>
<td>U. Leupold</td>
</tr>
<tr>
<td>SP56²⁴</td>
<td>h²⁰-leu1-32 ade6-216 ura4 ras1∗</td>
<td>LEU2 rasI¹⁶⁻¹⁷</td>
</tr>
<tr>
<td>SP66</td>
<td>an leu1-32 ade6-216</td>
<td>D. Beach</td>
</tr>
<tr>
<td>SP826</td>
<td>h⁻¹⁷ leu1-32 ade6-210 ura4-D18h⁻¹⁷</td>
<td>D. Beach</td>
</tr>
<tr>
<td>SP870</td>
<td>h²⁰-leu1-32 ade6-210 ura4-D18</td>
<td>D. Beach</td>
</tr>
<tr>
<td>SPBU</td>
<td>h²⁰-leu1-32 ade6-210 ura4-D18 byr1::ura4</td>
<td>This study</td>
</tr>
<tr>
<td>SPBUD</td>
<td>ura4h²⁰ leu1-32 ade6-210 ura4-D18 byr1::ura4</td>
<td>This study</td>
</tr>
<tr>
<td>SPR2A</td>
<td>h²⁰-leu1-32 ade6-216 ura4::RAS2²⁸⁻²²</td>
<td>This study</td>
</tr>
<tr>
<td>SPRU</td>
<td>h²⁰-leu1-32 ade6-210 ura4-D18 ras1::ura4</td>
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<tr>
<td>SPSL</td>
<td>h²⁰-leu1-32 ade6-210 ura4-D18 byr2::LEU2</td>
<td>This study</td>
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<tr>
<td>SPSU</td>
<td>h²⁰-leu1-32 ade6-210 ura4-D18 byr2::ura4</td>
<td>This study</td>
</tr>
<tr>
<td>SPUSD</td>
<td>ura4h²⁰ leu1-32 ade6-210 ura4-D18 byr2::ura4</td>
<td>This study</td>
</tr>
</tbody>
</table>

a In this strain, the wild-type ras1 gene was replaced with the _S. cerevisiae_ LEU2 gene and the activated rasI¹⁶⁻¹⁷ allele.

The _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 pWH5 (24), which contains the _LEU2_ gene. The plasmid pART-R2A was constructed by inserting a 1.2-kbp _HpaI_ fragment from YIP-OGA (22), which contains the _S. cerevisiae_ dominant _RAS2_ mutation _RAS2²¹⁻²²_ at the _SmaI_ 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 _SpI_ and _PstI_ 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'-GATTTTCTGAATCTCTTCTT. The PCR product was digested with _BglII_ and _XmnI_, and the 1.1-kbp _BglII-XmnI_ fragment, which contained the whole coding sequence of _byr1_, was cloned into the _SmaI_ 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 _pA_ was derived from pIRT5 (25) by replacing the _ura4_ gene with the 2.2-kbp _HindIII_ fragment containing the _S. cerevisiae_ _LEU2_ gene. Plasmid _pALR_ was constructed by inserting a 1.4-kbp _BamH1-BglII_ _S. pombe_ _ras1_ gene fragment (20) into the _BamH1_ 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. An _ASIS_ element, and the _S. pombe adh_ promoter with an oligonucleotide encoding a peptide derived from the hemagglutinin antigen of influenza virus. The _byr2_ fragment was generated by cleavage of the PCR product with the _byr2_ gene as the template and the primers 5'-TATACGTTTGCAACCAGTCGCTTTTGTG and 5'-TCATCATACTGAGTCTCGATTGAAAC. A _SaiI_ site which is 15 bp upstream of the _byr2_ start codon was thus created in the PCR product, and a _SacI_ site which is 3 bp downstream of the stop codon was created. The _byr2_ missense allele _byr2²¹⁻²²_ 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 _BamH1_ site of pUC118 (20) (provided by D. Beach). This was digested with _NheI_ and _BglII_, 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 a 0.9-kbp of _3' coding region_ and about 300 bp of _3'-flanking sequence_. _BamH1_-digested DNA from this plasmid was used to transform wild-type _h²⁰_ 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 _SpI-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. 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 _SpI_ and _SalI_. A 1.7-kbp fragment containing _S. cerevisiae RAS2²¹⁻²²_ 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 _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- S. pombe_ strain _Sp66_. The _Ura- transformants were selected on plates containing 5-fluoroorotic acid as described previously (8). The integration of _RAS2²¹⁻²²_ 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 MgCl₂ [pH 7.4]) at 2 × 10⁶ to 3 × 10⁶ 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.
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^{30} 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). Plasmids pALR, which expresses the S. pombe ras1 gene, and pART-BYR1, which expresses the byr1 gene, were both capable of restoring conjugational efficiency. To our surprise, pST6, which expresses ste6, an S. pombe homolog of the CDC25 gene (11), was unable to restore conjugational 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 conjugational efficiency to SPR2A upon transformation. Conjugation in Leu1 transfectants was scored indirectly by staining colonies for spores. A total of 2,992 positive-staining colonies were found among 5 \times 10^9 transformants examined. Only 26 of these colonies contained zygotic spores. The majority of the remainder contained zygotic 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 ascus result. Plasmids were recovered only from colonies containing zygotic spores, and all such plasmids could confer conjugational 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 kb. 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 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^{+} h^{+}/h^{−} h^{−} diploid strain SP826. Stable Ura1 transfectants were selected, and the disruption of one copy of the endogenous byr2 gene by ura4 was confirmed by Southern blotting (data not shown). h^{30} h^{+}/h^{−} revertants of these disruptants were detected by iodine vapor staining. Tetrads were then dissected to confirm the presence of 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 *S. cerevisiae* RAS2<sup>Ala<sub>22</sub></sup> 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<sup>Ala<sub>22</sub></sup> (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 ascus, evident in each panel except B.
and demonstrate that the byr2 gene is absolutely required for both conjugation and sporulation of both strains. 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::ura4ras1::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. 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 SPLS 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.
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Byr1  63  NSSLEVVRHLGEANNGAVALVHRMIF---MARKTVVY---GSDS-----KLQKQRELGEVHLRCSRE-YI

Byr2  391  SIKWIRGLAIGSSGFFVYQLYNMQASSGEIMAVQVIVDWSESKDRHAKLALAGELALQELSHE-HI

Cdc2  1  MERRYKVEKGETGVYKVRKLSGRIVAKKIDESEGVPSATAIREISILLEVDDERHNRSCVRL

Byr1  124  VQFGQAFQNYKNNILSCHEYMCDG ddlAI-EGPPIPLDLGK-INSMVGYGLTYNVHHIHRLDKEPS

Cdc2  65  L011NAESKSLTVFELMDLKYERYDISET-QATSDPRLVRQTTLYNGVNYCSGR-31HIDLRIFQ

Byr1  191  NNVVNSREIKGLCDFVGSEVL-VNSAVA------QTFVGSTYSMSREIRGGRTY-4VKSDDWLSIGSIE

Byr2  527  NILVONKKI1SDPGISSKLEINSTSKTGARSPFGSSVMWAVEV-KQM1-TEKWDWELGCMIE

Cdc2  139  NLLIDKEGNLLKALDGFGRASFGV-PLANYT-----HEIVTLYXPEVLGLSRTYGDIVLWGICFAE

Byr1  252  LTQGELPSFNSIDOSGILDLHCVIQEERPLPPSSFPEDLRLVDAACLHDTDPLRSAPOQLCMPYFQ

Byr2  596  MLTKSH-PYPCDQAIFR---IGENILEPEFENISSAIDFLKAPLCHLMPLSTATELLHPVS

Cdc2  203  W1-RRSPL-FPGDSISEIFK------IFQGVTNPEWNVGYSSLQKSTRFRMKMLDHLKVVPMGEEDA

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.

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 ras1Val17 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 ras1Val17 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 leul (data not shown).

FIG. 4. Construction 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 Nc1 and Spel sites. (C) byr2 plasmid pAIS1-BD, which contains the indicated deletion. (D) byr2 plasmid pAIS1-SBD, which contains the indicated deletion. Restriction enzymes: Ba, BamHI; Bc, BclI; Bs, BstXI; Nc, NcoI; Sa, SalI; Sm, Smal; 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.

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**S. POMBE PROTEIN KINASE-ENCODING GENE byr2**

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.

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 ras1Val17 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 ras1Val17 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 leul (data not shown).
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. 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 pA1S1 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. pA1S1, like pWH551, was able to fully complement the phenotypic defects of byr2 mutants. A 77-kDa protein, of the expected size, was detected in cells containing pA1S1 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 pA1S1-BSD, 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 pA1S1-BCD lacked the 654-bp BclI-BclII fragment encoding 217 amino acids from positions 389 to 606 (Fig. 4). The plasmid pA1S1-ASP-534 contained a single point mutation which directed the synthesis of aspar- tic 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 pA1S1-BCD, which lacks the kinase catalytic region, could not restore functions to byr2 mutant haploid and diploid strains. The plasmid pA1S1-BSD, which contains the catalytic domain, could complement the loss of the byr2 mutation, although complementation was not as strong as with pA1S1 itself. The plasmid pA1S1-ASP-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 pA1S1 and pA1S1-BSD increased cell agglutination, while both pA1S1-BCD and pA1S1-ASP-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 ras1V34-A7 allele. The presence of this ras1 allele increases cell agglutination, induces an elongated conjugation tube, and causes partial sterility (7, 20). Both pA1S1-BCD and pA1S1-ASP-534 reduced cell agglutination (Fig. 6) and diminished the presence of elongated conjugation tubes. There was no improvement in conjugation in ras1V34-A7 strains carrying these plasmids (data not shown).

**FIG. 6.** Effect of byr2 mutations on cell agglutination of SP870 (wild type) and SP562 (ras1V34-A7). 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 (pA1S1-BCD and pA1S1-ASP-534) formed spots at the center within 30 min, while cells expressing catalytically active protein (pA1S1 and pA1S1-BSD) 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 pA1S1-BCD or pA1S1-ASP-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_A422 allele. 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_A422 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 ste6 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 essentially 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 ras1rasi 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 ras1Val17 gene fails to suppress the sporulation defects of either byr1/yr1 or byr2/yr2 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 ras1rasi 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/yr1 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/yr2 mutant diploids and because expression of byr2 cannot induce sporulation in byr1/yr1 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.

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