CELL REGULATION, Vol. 2, 453–465, June 1991

Sar1, a gene from *Schizosaccharomyces pombe* encoding a protein that regulates ras1

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Proper ras1 function is required for normal sexual function in the yeast *Schizosaccharomyces pombe*. We have found a gene in *S. pombe*, *sar1*, that encodes a product capable of regulating ras1 function. sar1 is a member of an expanding family of RAS GTPase-activating proteins (GAPs) that includes mammalian GAP, the yeast *Saccharomyces cerevisiae* IRA proteins, and the product of the human neurofibromatosis locus, NF1 sar1, like these other proteins, can complement the loss of IRA function in *S. cerevisiae*. Computer analysis shows that the highest degree of sequence conservation is restricted to a very small number of diagnostic residues represented by the motif Phe-Leu-Arg-X-X-X-Pro-Ala-X-X-X-Pro. We find no evidence that sar1 is required for the effector function of ras1.

Introduction

The mammalian *RAS* genes were first discovered as the oncogenes contained in acutely transforming retroviruses. They are found activated by mutation in a wide variety of tumors (Barbacid, 1987). The biochemical function of RAS proteins remains an enigma, although they have been studied intensively. Much is known about RAS structure, processing, biological effects, and biochemical properties (Bourne *et al.*, 1990). RAS proteins bind GTP and GDP and slowly hydrolyse GTP to GDP. This process is greatly accelerated by a protein called GTP-activating protein (GAP). GAP fails to induce GTP hydrolysis by oncogenic RAS (Trahey and McCormick, 1987; Gibbs *et al.*, 1988). It is the GTP-bound form of RAS that is active (Field *et al.*, 1987; Trahey and McCormick, 1987), and hence GAP can down-regulate wild-type RAS, but not oncogenic, activated RAS (Ballester *et al.*, 1989; Zhang *et al.*, 1990). Recently the product of the von Recklinghausen Neurofibromatosis locus, *NF1*, has been discovered to have structural and biochemical similarities to GAP (Ballester *et al.*, 1990; Buchberg *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990a,b). Much attention has been devoted to GAP, and now NF1, in part because GAP-like molecules are the only proteins known with certainty to interact directly with RAS and in part because some scientists have postulated that these molecules may be required for the effector function of RAS. For mammalian RAS, the latter hypothesis has received some experimental support, but is still unproven (Adari *et al.*, 1988; Cales *et al.*, 1988; Tatani *et al.*, 1990).

RAS proteins are highly conserved in evolution, and some insights into mammalian RAS function may be gained by the study of RAS in yeast. In the most extensively studied yeast, *Saccharomyces cerevisiae*, the main function of RAS proteins is to stimulate adenylyl cyclase (Wigler *et al.*, 1988). Although this is not the function of RAS in vertebrates, or even in the yeast *Schizosaccharomyces pombe*, many features of RAS protein interactions have been conserved in evolution. Mammalian RAS proteins can stimulate yeast adenylyl cyclase and appear able to interact with the product of the *CDC25* gene that is required to activate yeast RAS proteins. Of particular relevance to the experiments described below, yeast have *IRA1* and *IRA2* genes, which encode proteins that are structurally and functionally related to both GAP and NF1 (Tanaka *et al.*, 1989, 1990a). The IRA proteins down-regulate yeast RAS and accelerate GTP hydrolysis by RAS. Loss of either *IRA* gene leads to a phenotype that resembles the phenotype of cells containing activating mutations of RAS. Expression of either NF1 or GAP can complement the loss of IRA function (Ballester *et al.*, 1989, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990).

S. pombe contains a single gene, *ras1*, that is homologous to the mammalian *RAS* genes (Fukui and Kaziro, 1985; Nadin-Davis *et al.*, 1986b). The biochemical function of ras1 is unknown, but ras1 does not appear to act through

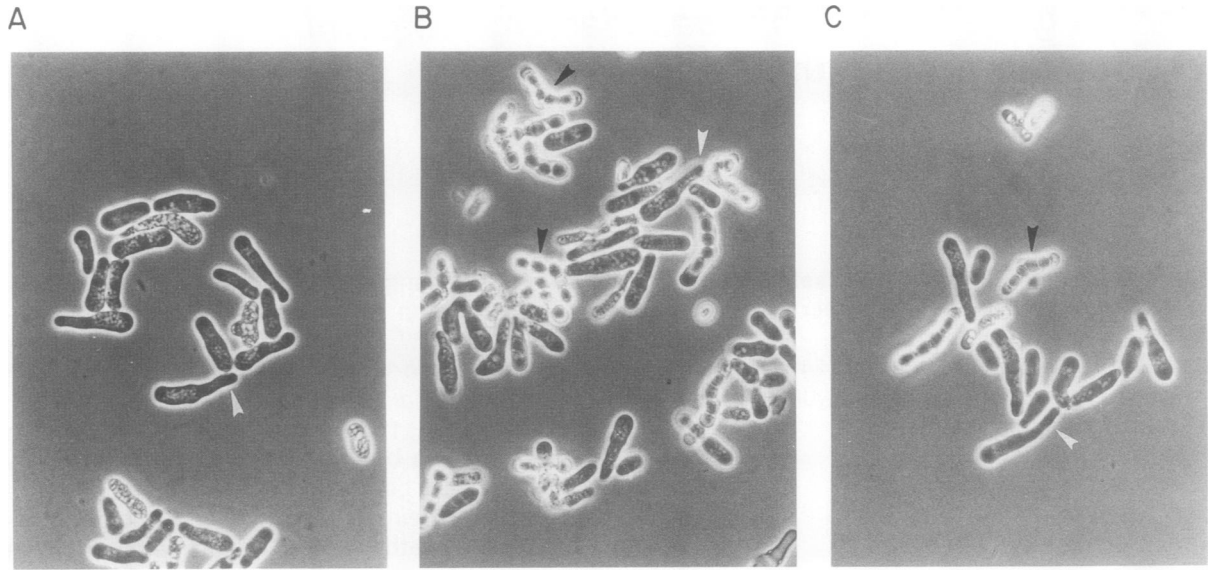

Figure 1. **The *ras1* and *sar1* genes complement the conjugation defect conferred by *ras1*^{val17}.** Cells were grown on PM plates with appropriate auxotrophic supplements and phase-contrast micrographs were taken after 2-d incubation at 30°C. (A), SP593, a *ras1*^{val17} strain. (B), A strain derived from SP593 by transformation with pALR, which expresses the *ras1* gene. (C), A strain derived from SP593 by transformation with pWH5SAR1, which expresses the *sar1* gene. Empty arrow heads indicate elongated conjugation tubes. Solid arrow heads indicated zygotic asci, evident in panels B and C but not in panel A.

adenylyl cyclase. We have been studying *S. pombe* ras1 for the insights it may provide into the function of mammalian RAS. In *S. pombe, ras1* is not an essential gene, but *ras1*$^-$ cells fail to initiate conjugation, are defective in sporulation, and have a round shape, rather than the normal elongated shape (Fukui *et al.,* 1986; Nadin-Davis *et al.,* 1986a). Cells with the activated *ras1*^{val17} allele have an exaggerated early sexual response, but are greatly impaired in conjugation. They are otherwise normal (Fukui *et al.,* 1986; Nadin-Davis *et al.,* 1986a).

We have sought genes present on high-copy plasmids that can suppress the conjugation defects of cells containing *ras1*^{val17}. Two such genes were so identified: *ras1* itself and a gene we call *sar1* (suppressor of activated *ras1*^{val17}). The sar1 protein has highly significant similarities to the human NF1, mammalian GAP, and IRA proteins of *S. cerevisiae.* Complementation experiments using portions of GAP and NF1 (Ballester *et al.,* 1989, 1990; Martin *et al.,* 1990; Xu *et al.,* 1990a) have localized functionally important regions of these sequences to a "catalytic" domain that corresponds to the most highly-conserved region of sar1. Detailed multiple alignment studies of the catalytic domains suggest that the critical determinants of function may be restricted to a very small number of amino acid residues, and a diagnostic "motif" for GAP-related proteins is proposed. Genetics analysis indicates that, like the IRA proteins, sar1 down-regulates ras1. sar1 is not required for the effector function of ras1.

Results

Isolation of sar1

Wild-type *S. pombe* h^{90} strains conjugate and sporulate on starvation, and therefore colonies of h^{90} strains stain black with iodine vapor. Strains containing the activated *ras1*^{val17} allele have increased sexual agglutination and elongated conjugation tubes but fail to conjugate when starved (Fukui *et al.,* 1986; Nadin-Davis *et al.,* 1986) (Figure 1). Thus colonies of h^{90} *ras1*^{val17} strains do not form spores on starvation and do not stain black with iodine vapor. We screened banks of genomic clones contained on high-copy shuttle vectors for plasmids that can overcome the conjugation defects in the *leu1*$^-$ *ras1*^{val17} strain, SP593. SP593 does not contain a wild-type *ras1* allele. Leu$^+$ transformants were screened for black colonies by iodine vapor staining. The black staining colonies were then examined by microscopy for the presence of zygotic spores. From 10^5 Leu$^+$ transformants we identified 11 colonies containing zygotic spores, and plasmids were rescued from all 11. We grouped these 11 plasmids into two classes based on restriction endonuclease analysis.

One class of plasmids with three members contained *ras1.* This was determined by restriction endonuclease mapping and confirmed by transformation into *ras1*$^-$ strains. To verify this directly, we transformed the *ras1*^{val17} strain SP593 with plasmid pALR that expresses the wild-type *ras1* allele. pALR efficiently suppressed the mating defect in SP593, and like the other three suppressors, failed to have effect on either the increased agglutinability or deformed cell shape of this strain (Figure 1). The significance of this result will be discussed later.

The other class of plasmids, with eight members, contained the gene we call *sar1.* One plasmid, pWH5SAR1, was used for further studies. Like plasmids containing wild-type *ras1,* pWH5SAR1 can efficiently correct the conjugation defect of the *ras1*^{val17} strain SP593, but has no detectable effect on cell agglutinability or the presence of conjugation tubes (Figure 1).

Nucleotide and predicted amino acid sequence of sar1

Deletion and subcloning analysis of pWH5SAR1 localized the functional gene to a 4.2 kb *Bam*HI/*Bal* I fragment. This fragment was subcloned into pUC118 and pUC119 and sequenced. The nucleotide sequence contains an open reading frame of 2298 bp with the capacity to encode a protein of 766 amino acids (Figure 2). No other significant open reading frames were found within 1830 bp upstream of this open reading frame. To confirm that we had defined the entire coding potential of *sar1,* we cloned *sar1* from an *S. pombe* cDNA library. The cDNA clone we isolated was colinear with the genomic clone both upstream and downstream from the large open reading frame. Our initial computer searches of GenBank and EMBL data banks, using the FASTDB program, failed to show any convincing relationship between sar1 and other known proteins. However, after initial genetic experiments suggested that sar1 had GAP-like functions (see below), these searches were redone using more advanced techniques, and statistically significant similarities were found between sar1 and the "catalytic" domains of human NF1, mammalian GAP, and yeast IRA sequences.

The new BLAST family of programs (Altschul *et al.,* 1990) was used to search the major protein and nucleic acid sequence databases (see Materials and Methods) for homologies to sar1 (Table 1). BLAST incorporates a new statistical theory (Karlin and Altschul, 1990) for assessing the significance of sequence similarities. Interestingly, sar1 appears to be more closely related to human NF1 ($p \sim 10^{-5}$) and mammalian GAP ($p \sim 10^{-4}$) than to the *S. cerevisiae* IRA1/2 proteins (Table 1), but in all cases the most significant similarities are confined to rather small subsequences (<250 residues) of these proteins in contrast to the rather large sizes (765–3079 residues) of the latter. Residues 170–379 of sar1 consistently matched regions of the other proteins that have been previously defined by complementation experiments (Ballester *et al.,* 1990; Tanaka *et al.,* 1990a,b) as the "catalytic domains" (see below). A more detailed analysis of conserved and invariant residues among these catalytic domains is presented in the subsequent section on comparative sequence analysis.

Phenotypes of sar1$^-$ *strains*

Plasmids pUCSAR::ura4 and pUCSAR::LEU2 containing the *sar1* gene disrupted by *ura4* or *LEU2,* respectively, were constructed (Figure 3). These disruptions removed parts of the *sar1* gene-encoding sequences N-terminal to the GAP-like catalytic domain, and DNA fragments from these plasmids were transformed into various strains to cause disruptions at the *sar1* locus. We used *sar1* disrupted by *ura4* to transform the h^+/h^+ diploid strain SP826. Stable Ura$^+$ transformants were selected, and disruption of one copy of *sar1* was confirmed by Southern blotting. h^{90}/h^+ revertants were selected by picking black colonies after iodine vapor staining. Tetrad analysis revealed 2:2 segregation of the Ura$^+$ phenotype, and blotting analysis confirmed these haploid progeny contained a disrupted *sar1* allele. The *sar1*$^-$ haploid strains displayed normal morphology and growth rate when cultured on rich medium. Thus *sar1* is not an essential gene.

Two h^{90} haploid strains containing disrupted *sar1* alleles were made by transforming SP870. SPSAU and SPSAL contained *sar1* disrupted by *ura4* and *LEU2,* respectively. These genotypes were confirmed by Southern blotting. Each had normal morphology and growth rates when cultured on rich medium. On nitrogen starvation, however, the *sar1*$^-$ strains displayed strong cell agglutination, elongated conjugation tubes, and a dramatic decrease in zygotic asci formation (Figure 4). h^{90} *sar1*$^-$/h^{90} *sar1*$^-$ strains, formed by diploidization of these strains, formed azygotic asci at high frequencies. Thus *sar1*$^-$ strains displayed the same phenotypes as *ras1*^{val17} strains. The phenotypes of *sar1*$^-$ strains could all be readily restored to normal by trans-

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-297 CTTTTCTTTTTTCTTTGGAAGAATTTCGCTTTCTTTAATCGTTTAATCGGGCGCCAGTGGGTCTTAATACTGTGTTTTGCTAATAGAACCAATAGGTTC

-198 TGTCCGATCTCCTCTAATAACGTTCTTTTGATGATTTTCTCTTTTATTTTATTTTTTTTTTGTTTGAAAACTTAGTATAATATCCATCCTTGTTTTCTT

 -99 CTTTTACACTTTGACTTGATCCCTACACTCTTTCTTTTTTTTTTCTACCCTCTCTTGTTCATTAGCTACTTAGGCAATTTTTAGCTATTGAAATTTATA

   1 ATGACTAAGCGGCACTCTGGTACCCTATCTTCGTCGGTGCTTCCGCAAACAAATCGATTGTCCTTGTTAAGGAATCGTGAAAGCACCTCTGTGCTTTAT
   1 M  T  K  R  H  S  G  T  L  S  S  S  V  L  P  Q  T  N  R  L  S  L  L  R  N  R  E  S  T  S  V  L  Y

 100 ACAATCGATCTTGATATGGAGTCCGACGTTGAGGATGCCTTTTTCCACTTGGATCGTGAATTGCATGATCTCAAACAACAAATATCCAGTCAGTCCAAA
  34 T  I  D  L  D  M  E  S  D  V  E  D  A  F  F  H  L  D  R  E  L  H  D  L  K  Q  Q  I  S  S  Q  S  K

 199 CAAAACTTCGTCCTCGAGAGGGATGTACGCTACCTTGATTCCAAAATTGCACTTCTTATCCAAAATCGAATGGCACAAGAAGAACAGCATGAGTTTGCA
  67 Q  N  F  V  L  E  R  D  V  R  Y  L  D  S  K  I  A  L  L  I  Q  N  R  M  A  Q  E  E  Q  H  E  F  A

 298 AAACGACTGAATGATAATTACAATGCTGTAAAAGGATCATTTCCTGACGATCGTAAGCTTCAGTTGTATGGAGCGTTGTTTTTTTTACTTCAGTCTGAA
 100 K  R  L  N  D  N  Y  N  A  V  K  G  S  F  P  D  D  R  K  L  Q  L  Y  G  A  L  F  F  L  L  Q  S  E

 397 CCAGCCTATATCGCTAGCCTTGTTCGTCGCGTCAAGCTTTTTAACATGGATGCACTTCTACAAATCGTTATGTTTAATATATACGGAAACCAATACGAG
 133 P  A  Y  I  A  S  L  V  R  R  V  K  L  F  N  M  D  A  L  L  Q  I  V  M  F  N  I  Y  G  N  Q  Y  E

 496 AGTAGAGAAGAGCATCTTTTATTATCTCTTTTTCAAATGGTATTAACCACCGAATTTGAGGCCACTTCTGACGTTTTGTCTCTGCTTAGGGCTAATACT
 166 S  R  E  E  H  L  L  L  S  L  F  Q  M  V  L  T  T  E  F  E  A  T  S  D  V  L  S  L  L  R  A  N  T

 595 CCGGTATCTAGAATGCTTACAACTTATACACGTCGTGGACCCGGACAAGCATATCTTCGTAGTATCCTTTACCAATGCATTAATGACGTTGCTATCCAT
 199 P  V  S  R  M  L  T  T  Y  T  R  R  G  P  G  Q  A  Y  L  R  S  I  L  Y  Q  C  I  N  D  V  A  I  H

 694 CCTGATTTGCAGCTTGACATCCATCCTCTTTCAGTTTATCGCTATTTGGTGAATACCGGTCAATTATCACCATCTGAAGATGATAATTTATTAACAAAC
 232 P  D  L  Q  L  D  I  H  P  L  S  V  Y  R  Y  L  V  N  T  G  Q  L  S  P  S  E  D  D  N  L  L  T  N

 793 GAGGAAGTTTCAGAGTTTCCTGCTGTAAAAAATGCAATTCAAGAGCGTTCTGCTCAATTATTGCTTTTGACAAAACGATTTTTAGATGCTGTTCTTAAC
 265 E  E  V  S  E  F  P  A  V  K  N  A  I  Q  E  R  S  A  Q  L  L  L  L  T  K  R  F  L  D  A  V  L  N

 892 AGCATCGACGAAATTCCATATGGTATTCGCTGGGTCTGTAAGTTAATTCGCAATCTGACAAATCGTCTGTTTCCTAGTATTTCAGACAGCACTATTTGC
 298 S  I  D  E  I  P  Y  G  I  R  W  V  C  K  L  I  R  N  L  T  N  R  L  F  P  S  I  S  D  S  T  I  C

 991 TCTTTAATAGGTGGATTTTTCTTTCTTCGTTTCGTTAATCCAGCTATTATTTCGCCACAAACTTCTATGCTTTTAGACAGTTGTCCATCTGATAACGTC
 331 S  L  I  G  G  F  F  F  L  R  F  V  N  P  A  I  I  S  P  Q  T  S  M  L  L  D  S  C  P  S  D  N  V

1090 CGCAAAACACTTGCTACTATTGCAAAAATTATTCAAAGTGTTGCCAATGGCACAAGTTCTACGAAGACACATTTGGATGTTTCTTTTCAACCTATGTTG
 364 R  K  T  L  A  T  I  A  K  I  I  Q  S  V  A  N  G  T  S  S  T  K  T  H  L  D  V  S  F  Q  P  M  L

1189 AAAGAGTATGAAGAAAAAGTTCACAATCTTTTGCGTAAACTTGGAAACGTCGGTGACTTTTTCGAGGCTTTGGAACTTGACCAATATATAGCTCTATCA
 397 K  E  Y  E  E  K  V  H  N  L  L  R  K  L  G  N  V  G  D  F  F  E  A  L  E  L  D  Q  Y  I  A  L  S

1288 AAGAAGAGCTTAGCACTTGAGATGACCGTCAATGAAATATACCTTACACACGAAATCATTTTGGAGAATTTGGATAATTTATATGATCCCGATAGTCAT
 430 K  K  S  L  A  L  E  M  T  V  N  E  I  Y  L  T  H  E  I  I  L  E  N  L  D  N  L  Y  D  P  D  S  H

1387 GTTCATTTAATTCTTCAAGAATTAGGCGAGCCGTGTAAATCTGTTCCACAGGAAGACAATTGCCTTGTCACACTTCCTCTTTATAATCGATGGGATTCT
 463 V  H  L  I  L  Q  E  L  G  E  P  C  K  S  V  P  Q  E  D  N  C  L  V  T  L  P  L  Y  N  R  W  D  S

1486 TCAATTCCTGATTTAAAACAAAATTTAAAGGTAACCCGCGAAGATATTTTATACGTCGATGCCAAAACGCTTTTCATTCAACTTTTACGTCTTCTGCCA
 496 S  I  P  D  L  K  Q  N  L  K  V  T  R  E  D  I  L  Y  V  D  A  K  T  L  F  I  Q  L  L  R  L  L  P

1585 TCGGGCCATCCAGCTACAAGGGTGCCTCTAGACCTTCCACTAATTGCTGACAGTGTTTCGTCCTTAAAAAGTATGTCATTAATGAAAAAGGGCATACGT
 529 S  G  H  P  A  T  R  V  P  L  D  L  P  L  I  A  D  S  V  S  S  L  K  S  M  S  L  M  K  K  G  I  R

1684 GCAATAGAGCTTTTAGATGAGTTGTCAACCTTACGTTTGGTTGACAAGGAGAACCGTTATGAGCCTTTAACTTCTGAGGTCGAAAAGGAATTTATAGAC
 562 A  I  E  L  L  D  E  L  S  T  L  R  L  V  D  K  E  N  R  Y  E  P  L  T  S  E  V  E  K  E  F  I  D

1783 CTCGATGCCCTTTATGAAAGGATACGTGCAGAACGTGATGCTTTACAGGATGTTCATCGTGCTATATGTGACCATAATGAGTATCTTCAAACTCAATTG
 595 L  D  A  L  Y  E  R  I  R  A  E  R  D  A  L  Q  D  V  H  R  A  I  C  D  H  N  E  Y  L  Q  T  Q  L

1882 CAAATTTATGGGAGCTATTTGAACAATGCCCGTTCTCAGATTAAACCCAGTCATAGTGACAGCAAAGGATTTTCTAGAGGCGTTGGCGTTGTCGGAATT
 628 Q  I  Y  G  S  Y  L  N  N  A  R  S  Q  I  K  P  S  H  S  D  S  K  G  F  S  R  G  V  G  V  V  G  I

1981 AAGCCCAAAAATATTAAGTCATCAAATACTGTAAAGCTTTCTTCGCAGCAATTGAAAAAAGAGTCTGTCCTGCTCAATTGTACCATTCCTGAATTTAAC
 661 K  P  K  N  I  K  S  S  N  T  V  K  L  S  S  Q  Q  L  K  K  E  S  V  L  L  N  C  T  I  P  E  F  N

2080 GTGTCTAACACATATTTTACATTTTCTTCTCCTTCTACGGATAATTTTGTTATTGCCGTTTACCAACGTGGACATTCAAAAGTCCTTGTTGAAGTTTGC
 694 V  S  N  T  Y  F  T  F  S  S  P  S  T  D  N  F  V  I  A  V  Y  Q  R  G  H  S  K  V  L  V  E  V  C

2179 ATATGCCTTGATGATGTATTGCAACGACGATATGCAAGCAACCCTGTCGTCGATTTAGGATTTTTGACATTTGAGGCTAATAAGTTATACCATTTATTT
 727 I  C  L  D  D  V  L  Q  R  R  Y  A  S  N  P  V  V  D  L  G  F  L  T  F  E  A  N  K  L  Y  H  L  F

2278 GAACAATTGTTTTTACGAAAGTAAATGTTCTACCAAGCATCAGTGACAACACTGGAAC
 760 E  Q  L  F  L  R  K  *
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Figure 2. **Nucleotide and predicted amino acid sequences of the *sar1* gene.** Letters in bold type indicate stop codons. Nucleotide and amino acid coordinates are in the left-hand margin. The coordinates begin with the initiating codon.

Table 1. Results of database searching with the sar1 amino acid sequence

Related sequence	Karlin score	p value	No. of segments matched
Human NF1	96	0.000011	3
Human GAP	86	0.00032	1
Yeast IRA1	69	0.0044	3
Yeast IRA2	58	0.28	2

The BLASTP and TBLASTN programs (Altschul *et al.,* 1990) were used to search protein and DNA sequence databases, respectively. Databases searched included: NBRF/PIR (release 26.0), SWISS-PROT (release 16.0), GenPept (release 64.3), GenPept (daily update, 1/28/91), the GenInfo Backbone (prerelease version 1/28/91), and GenBank (release 66.0) translated in all six reading frames. Entries in the table represent a summary of the significant results with redundant matches to the same protein in different databases excluded. The Karlin scores and p values were automatically computed according to Karlin and Altschul (1990) for an effective database size of 7 348 950 residues (size of NBRF/PIR release 26.0, which is the largest of the protein sequence databases). Thus the p values indicate how surprising it is to find such similarities in a sequence collection of this size. The Karlin score given represents the highest-scoring segment and the p value is that of the most significant segment (when multiple, nonoverlapping matching segments were identified). Significant matches between the sar1 and the other proteins were limited to residues 170–379 of sar1 and equally limited subsequences of the other proteins (Figure 6). These local similarities generally correlated with the homology blocks subsequently defined by multiple alignment analyses (Figure 6). Retrieval codes for the sequences referred to in this table are as follows: NF1 (GenInfo Backbone sequid 19609), GAP (GenBank locus HUMGAPA), IRA (NBRF/PIR code A30135), and IRA2 (NBRF/PIR code A35656).

formation with plasmids expressing *sar1* (Figure 4).

After discovering that the sar1 protein is related to GAP-like molecules, we repeated all the above experiments with strains carrying more extensive disruptions of *sar1,* which removed the entirety of the N-terminal protein through the catalytic region (Figure 3). These strains had the same phenotypes as described above. Thus *sar1* is not an essential gene, and its disruption leads to phenotypes that are indistinguishable from the phenotypes that result from the mutant, activated *ras1*^{val17} allele.

Transformation of S. pombe *strains with plasmids expressing GAP and NF1*

To test if GAP and NF1 were functionally related to sar1, two *S. pombe* expression vectors containing the entire human GAP protein sequence (pARTGAP) or the proposed catalytic domain of NF1 (pARTNF1) were constructed (see Materials and Methods). Three strains, SP870 (wild type), SPSAU (*sar1*$^-$), and SP593 (*ras1*^{val17}) were transformed with pARTGAP, pARTNF1, pWH5SAR1, which expresses *sar1,* and a control plasmid, pART1. Leu$^+$ transformants were nitrogen-starved and then checked for asci formation by iodine vapor staining and microscopy. Agglutination was tested by a sedimentation assay. Plasmids expressing GAP, NF1, or SAR1 were able to suppress efficiently the conjugation defects and to correct the exaggerated agglutinability and conjugation tubes of the *sar1*$^-$ strains (Figure 4), suggestive of a functional relationship between the three. In wild-type cells, expression of GAP had no discernible phenotype, but expression of either NF1 or sar1 diminished agglutination and sporulation. Thus both of the latter appear to diminish the activity of ras1. Only the plasmid expressing sar1 was able to correct the conjugation defect of the *ras1*^{val17} strain.

Expression of sar1 *in* S. cerevisiae

To test further the relatedness of sar1 to GAP-like molecules, we expressed sar1 in strains of *S. cerevisiae* lacking IRA function. For this purpose we constructed plasmid pAD4ΔSAR1, designed to express full-length sar1 protein under

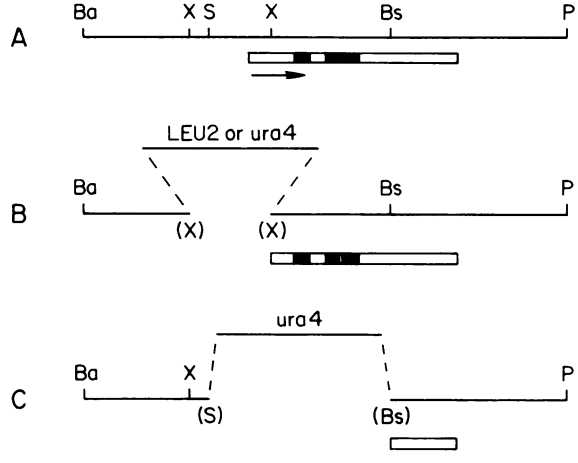

Figure 3. **Construction of *sar1* null alleles.** (A), The DNA fragment containing the *sar1* gene. The bar represents the open reading frame. The solid portions of the bar encode the homologous region between *sar1* and other GAP-like proteins. The arrow shows the direction of transcription. The relevant restriction enzyme sites are also shown. They are: Ba, *Bam*HI; Bs, *Bst*EII; P, *Pst* I; S, *Spe* I; and X, *Xho* I. The *Pst* I site is derived from the vector. (B), the *sar1* null alleles in strain SPSAU or SPSAL, which contain the *LEU2* or *ura4* markers cloned between the *Xho* I sites. (C), The more extensive deletion of *sar1* that was used in some disruptants.

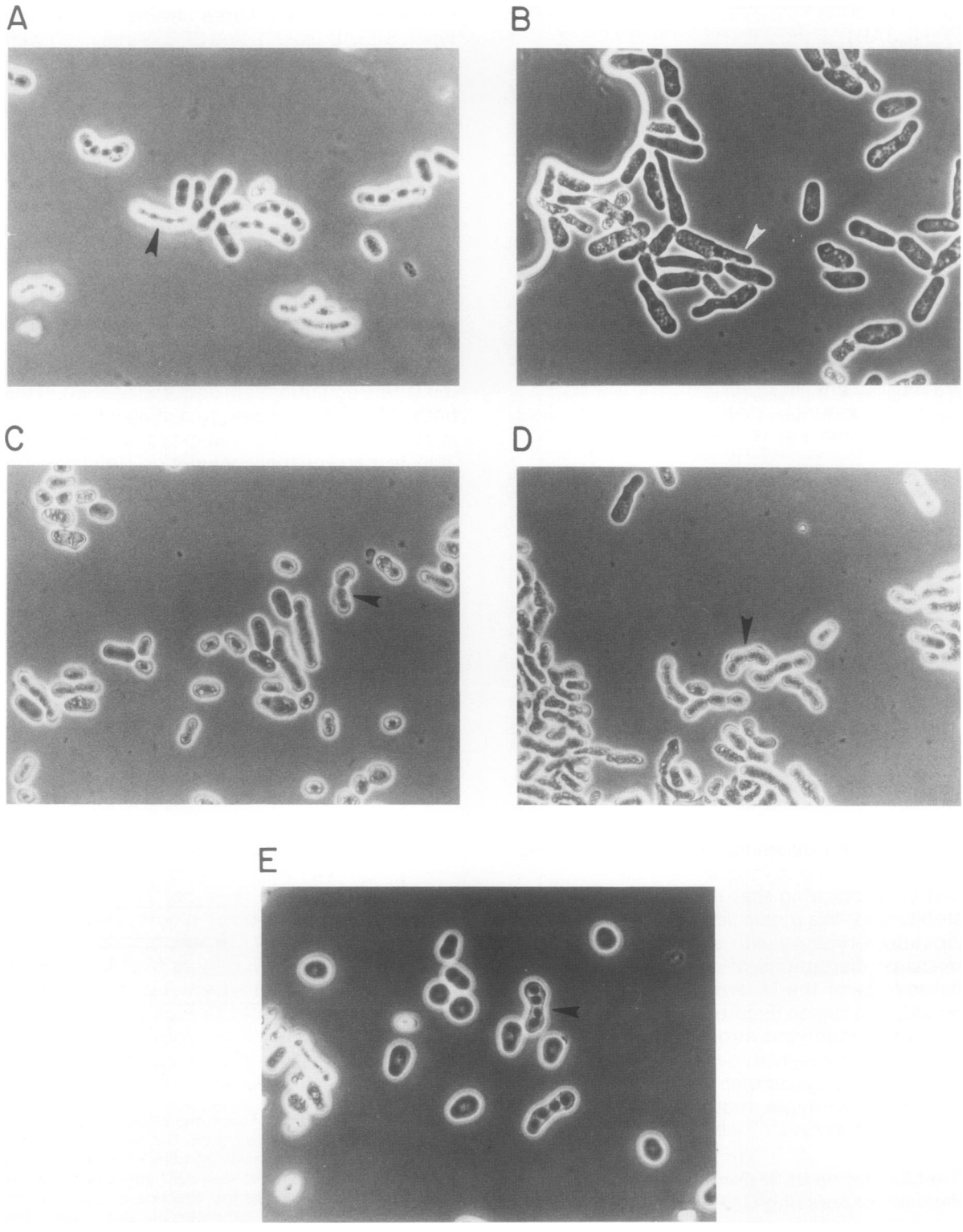

Figure 4. **Phenotype of *sar1*$^-$ strains of *S. pombe*.** Yeast cells were grown on PM plates with appropriate auxotrophic supplements and phase-contrast micrographs were taken after 2-d incubation at 30°C. (A), SP870, a wild type *S. pombe* strain. (B), SPSAU, a *sar1*$^-$ strain. (C), A strain derived by transformation of SPSAU with pWH5SAR1, which expresses the *sar1* gene. (D), A strain derived by transformation of SPSAU with pARTGAP, which expresses the intact human *GAP* gene driven by the *S. pombe adh* promotor. (E), A strain derived by transformation of SPSAU with pARTNF1, which expresses the

the control of the yeast *ADH1* promoter. pAD4ΔSAR1, along with control plasmids pAD4 and YEpPDE2, were transformed into the *S. cerevisiae* strain IR-1, in which the *IRA1* gene is disrupted, and into TK161-R2V, which contains the activated $RAS2^{val19}$ allele. Both IR-1 and TK161-R2V are heat-shock sensitive because of the activation of the RAS pathways. The plasmid YEpPDE2 suppresses this phenotype in both strains because it expresses the yeast high affinity cAMP phosphodiesterase (Sass *et al.,* 1986). Expression of sar1 suppressed the heat-shock sensitivity of the $ira1^-$ strain, but not the heat-shock sensitivity of the $RAS2^{val19}$ strains (Figure 5). Thus sar1 can suppress the wild-type *S. cerevisiae* RAS2, but not the mutant activated $RAS2^{val19}$. Thus sar1 resembles the human GAP and NF1 in these respects (Ballester *et al.,* 1989).

Disruption of ste6 *in* sar1$^-$ *strains*

The yeast *S. cerevisiae* contains two known *GAP*-like genes, *IRA1* and *IRA2.* Disruption of either results in a phenotype that is due to activation of RAS. *S. cerevisiae* contains a single gene, *CDC25,* that encodes a product required for the activation of RAS. Disruption of *CDC25* in either an $ira1^-$ or $ira2^-$ background restores a normal phenotype (Tanaka *et al.,* 1989, 1990b). The yeast *S. pombe* contains a gene homologous to *CDC25* called *ste6,* which appears to be functionally homologous as well (Hughes *et al.,* 1990). $ste6^-$ cells do not agglutinate and fail to conjugate, but are otherwise normal. We therefore examined the phenotype of *S. pombe* strains disrupted in both *ste6* and *sar1.* We disrupted *ste6* in the wild-type strain SP870, creating strain SPSTE6. We next disrupted *sar1* in this strain using a DNA fragment containing a *LEU2* insertion in *sar1,* as described before. Leu^+ transformants were picked, and gene disruptions of *ste6* and *sar1* were confirmed by Southern blotting. On starvation, the $ste6^-$ $sar1^-$ strains displayed increased cell agglutination, elongated conjugation tubes, very few zygotic asci, and normal azygotic sporulation in homozygous diploids, precisely the phenotypes observed in $sar1^-$ strains. Thus disruption of *ste6* has no apparent effect in $sar1^-$ strains.

Disruption of ras1 *in* sar1$^-$ *strains*

The results just described are compatible with a number of different hypotheses. Most important among these is that *ras1* function is not required for the $sar1^-$ phenotype. We tested this directly by constructing $sar1^-$ $ras1^-$ strains. The $ras1^-$ strain SP525 was transformed with a DNA fragment from the plasmid pUCSAR::ura4 containing *sar1* disrupted with *ura4,* as was done in creating the strain SPSAU. Disruption of *sar1* was confirmed by Southern blotting. The phenotypes of the $ras1^-$ $sar1^-$ strains were identical to those of $ras1^-$ strains: round shape, no agglutination, total conjugation deficiency, and very little sporulation in homozygous diploids. To confirm these results, we disrupted the *ras1* gene in the $sar1^-$ strain SPSAL using a *ura4* disruption of *ras1* as described previously (Wang *et al.,* 1991). Again the $ras1^-$ $sar1^-$ strains had the same phenotypes as $ras1^-$ strains. We conclude that the phenotype of $sar1^-$ strains is dependent on *ras1* function.

Comparative sequence analysis

The genetic experiments we have presented led us to examine more carefully the sequence relationships between sar1 and other GAP-related proteins. These proteins range in length from 765 residues for sar1 to 3079 residues for IRA2. Only IRA1 and IRA2 appear to be "globally" homologous, i.e., they have significant sequence similarities along their entire lengths. Our previous analysis of human NF1, mammalian GAP, and yeast IRA1/2 proteins (Ballester *et al.,* 1990) indicated that the catalytic domain was rather limited in extent (223–232 residues).

The MACAW program (Schuler *et al.,* 1991) was used to locate, analyze, and assess the significance of homology blocks among sar1, NF1, GAP, and IRA1/2. Four significant regions (blocks 1, 2, 3A and B; Figure 6) of five-way sequence conservation were detected, and these were correlated with the significant pairwise homologies identified by database searching (Table 1). Homology blocks 1 through 3 in the present analysis correspond to portions of blocks 8 through 10 in Figures 1 and 2 of Ballester *et al.* (1990).

For optimal placement of gaps (representing insertion/deletion mutations), a full dynamic programming alignment (Lipman *et al.,* 1989)

NF1 gene encoding the catalytic domain, driven by *S. pombe adh* promotor. The empty arrow head indicate the elongated conjugation tubes, which can be seen only in panel B. Solid arrow heads indicate zygotic asci, evident in each panel except B.

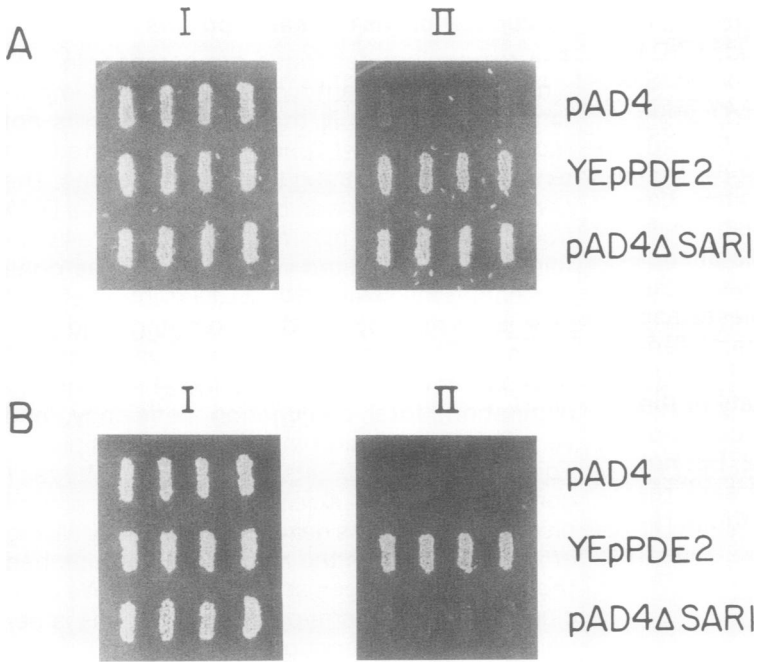

Figure 5. **The effect of *sar1* expression plasmids on the heat-shock response of *S. cerevisiae* strains.** Yeast strains, either IR-1, an *ira1*$^-$ strain (A) or TK161-R2V, a *RAS2*^{val19} strain (B), were transformed with the plasmids indicated at the right of the Figure and patched on synthetic medium plates. After 2-d incubation at 30°C the plates were replica plated onto cold plates (I) or onto preheated plates and incubated at 55°C for 5 min before returning to 30°C (II). The plasmids are: pAD4, an *S. cerevisiae* expression vector used as a negative control; pAD4ΔSAR1, which expresses the *sar1* gene in *S. cerevisiae;* and YEpPDE2, a plasmid expressing the *S. cerevisiae* PDE2 gene (Sass *et al.,* 1986).

was performed on the MACAW-derived subsequences (Figure 6), and this alignment was used to calculate the degrees of pairwise sequence identity for the catalytic domains (Table 2). As expected, IRA1 and IRA2 are most closely related with 54% sequence identity. In contrast, the pairwise identities among all of the other sequences cluster in a lower range of 22–33%.

Sequence identities are not uniformly distributed along the lengths of the catalytic domains but rather cluster into discrete homology blocks as identified by MACAW (Figure 6). Blocks 3A and B are extremely well conserved among all five sequences. Blocks 1 and 2 show lesser, but still significant, degrees of conservation. Additionally, sar1 contains two sizeable insertion mutations (8–11 and 18 residues, respectively) between blocks 1 and 2, relative to the other four proteins (Figure 6). Block 1 contains two invariant, charged residues, glutamic acid and arginine, suggesting the conservation of some electrostatic interaction (either intra- or intermolecular). There are two invariant residues in block 2, and the fact that these are both turn-inducing prolines may indicate that they delimit some discrete structural element. Block 3A contains the invariant tripeptide Phe-Leu-Arg, the invariant dipeptide Pro-Ala, and another invariant proline residue. Block 3B has invariant lysine and glutamine residues followed by the invariant dipeptide Ala-Asn. The conserved arginine and lysine in blocks 3A and B imply an important function for positive charges; the two conserved proline residues in 3A may represent some structural demarcations; and the conserved glutamine and asparagine in 3B indicate a role for uncharged, yet distinctly hydrophilic, residues in these positions.

Sequence motifs

With the cloning of sar1, there are now enough examples of the GAP catalytic domain to propose a diagnostic motif for this entity. We investigated both PROSITE signatures (Bairoch, 1991) and Gribskov profiles (Gribskov *et al.,* 1990) of blocks 3A and B as quantitative representations of the GAP catalytic domain (not shown). A very simple motif, FLR...PA...P (where . = any residue), derived from block 3A was necessary and sufficient to discriminate known GAP-related proteins from all other known sequences (see Materials and Methods). In other words, this particular 12-residue sequence (with 6 invariant positions) is present in sar1, NF1, GAP, and IRA1/2 and absent from all other known proteins. In contrast, a second motif, K..Q..AN, derived from block 3B, was present in >100 proteins unrelated to GAP (data not shown).

Discussion

We identified a gene in *S. pombe* we call *sar1* that encodes a protein that is structurally and functionally related to human GAP, NF1, and

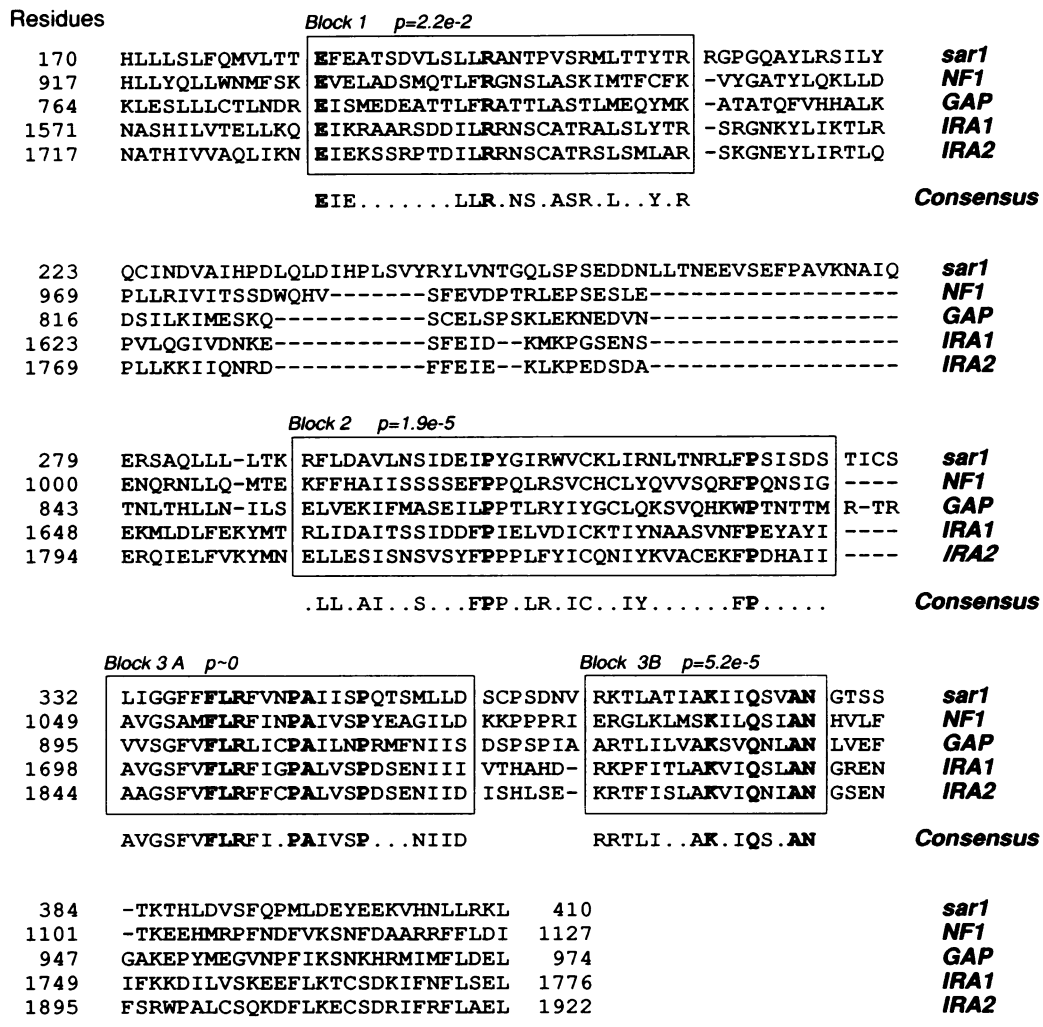

Figure 6. **The MACAW program (Schuler *et al.*, 1991) was used to locate, analyze, and assess the statistical significance of local similarities with the requirement that they be present in all five sequences.** Thus the extent of pairwise, 3-way, and 4-way alignments are not necessarily represented in the analysis. All five sequences were used to find significant homology blocks. However, to eliminate overestimation of statistical significance due to the substantial pairwise similarity of IRA1 and IRA2 (see Table 2), IRA2 was excluded from calculations of the p values displayed with the various homology blocks. The p values here are based on a multiple alignment search space confined to this set of sequences, whereas p values for the BLAST searches (Table 1) assume a search space that includes the entire database. The complete sequences of all five proteins were employed with the MACAW program but, to optimally place gaps (represented by dashes) between the various homology blocks, only the displayed subsequences were used with program MSA (Lipman *et al.*, 1989) for reasons previously discussed (Ballester *et al.*, 1990). Consensus sequences are based on at least 60% sequence identity per column; dots represent more variable positions even though individual columns may be well conserved with respect to hydrophobicity, etc. More quantitative methods to represent the degree of sequence variation are discussed in the text. The sources of sequence data and residue numbering are: sar1 (this paper), NF1 (Xu *et al.*, 1990), GAP (GenBank locus HUMGAPA), IRA1 (NBRF/PIR code A30135), and IRA2 (NBRF/PIR code A35656).

yeast IRA proteins. These latter proteins all have been shown to accelerate the GTPase activity of RAS proteins (Trahey and McCormick, 1987; Gibbs *et al.*, 1988; Tanaka *et al.*, 1990a; Xu *et al.*, 1990). We have not shown this for the sar1 protein, but it is a reasonable hypothesis based on the ability of *sar1* to complement *S. cerevisiae* lacking *IRA1* or *IRA2* function and the inability of *sar1* to suppress the phenotype of *S. cerevisiae* containing the mutant, activated $RAS2^{val19}$ allele. All the proteins from this group are capable of down-regulating RAS proteins.

In terms of sequence similarity, sar1 appears most closely-related to the human NF1 protein,

Table 2. Pairwise identities among GAP catalytic domains

	NF1	GAP	IRA1	IRA2
sar1	29	22	31	27
NF1		33	29	31
GAP			23	27
IRA1				54

The percentage of identical residues between each of the sequence pairs was calculated for the catalytic domains only based on the alignments in Figure 6.

encoded by the von Recklinghausen Neurofibromatosis locus, although the significant homology is very localized in extent. The *S. cerevisiae* proteins, IRA1 and IRA2, are even more closely-related to NF1 and, in this case, the homology extends over a much greater length of sequence (Ballester *et al.,* 1990; Buchberg *et al.,* 1990; Xu *et al.,* 1990). GAP itself is actually not the most characteristic example of this expanding protein family. As each new member of this family is discovered, the structural criteria for relatedness appear to dwindle; all known examples are large proteins, yet significant sequence similarities reside within a small catalytic domain functionally defined as that region of a particular sequence sufficient to cause accelerated GTP hydrolysis by RAS proteins. The conclusion, rather striking in retrospect, is that there need not be a great deal of conservation in primary structure for this group of proteins to interact with RAS proteins and, presumably, to change the conformation of RAS in response to this interaction. We can speculate with some confidence that the structural requirements will be equally limited for the group of proteins that are sites of RAS action.

Some have speculated that GAP-like molecules are in fact the sites of action of RAS (Adari *et al.,* 1988; Cales *et al.,* 1988; Tatani *et al.,* 1990). The evidence for this is largely circumstantial, but the hypothesis has received some direct support in the case of mammalian GAP. In the yeast *S. cerevisiae,* the evidence is largely, but not conclusively, against the hypothesis. Disruption of either *IRA1* or *IRA2* enhances RAS activity (Tanaka *et al.,* 1989, 1990b). Thus neither *IRA* gene could be the sole gene able to encode the major RAS effector. However, *IRA1* and *IRA2* could each encode RAS effectors. This possibility has not been properly addressed by previously published experiments. Although disruption of both *IRA* genes leads to a phenotype identical to that resulting from activation of RAS (Tanaka *et al.,* 1990a), no one has yet shown that this phenotype is RAS dependent. It is conceivable that at least one IRA protein is required to "harness" adenylyl cyclase to RAS. Moreover, there may be yet undiscovered GAP-like molecules in *S. cerevisiae* that might mediate RAS effects on adenylyl cyclase.

A strong case can be made against the proposition that *sar1* alone encodes the *S. pombe* RAS effector. Disruption of *sar1,* like the disruption of the *IRA* genes, leads to a phenotype identical to that produced by activated mutant *ras1*^{val17}. This phenotype is dependent on the presence of *ras1.* In extensive genetic screens, we have found no *S. pombe* genes capable of complementing *sar1* (data not shown). However, we cannot rule out the possibility that other highly diverged GAP-like molecules exist in *S. pombe* that might mediate RAS effects.

The GAP-related sequences represent large proteins and, with a predicted molecular mass of >87.5 kDa, sar1 is the smallest. Previous analyses have indicated that the catalytic domain is comparatively small (<250 residues), although this is still about the size of an average globular protein. Quantitative sequence analysis, however, indicates that the critical determinants of function may be limited to several short "homology blocks" or even just a handful of key invariant residues. Block 3A, e.g., is exceedingly well-conserved among these evolutionarily diverse proteins, and just a 12-residue sequence motif derived from this block is absolutely diagnostic for known GAP-related sequences. If GAP really does have a catalytic domain, this motif may represent its "active site." This does not imply that this short motif encompasses all the determinants of function, and it may be necessary to expand and modify this pattern as additional examples of sequences with GAP activity accrue. But at the present time, the pattern FLR...PA...P is characteristic of GAP proteins and no others. Genetic and biochemical evidence summarized by Bourne *et al.* (1991) indicates that GAP interacts with the so-called "G-2" region of Ras proteins. This region contains at least one, and usually two or three, acidic (negatively charged) residues in most members of the protein superfamily (see Table 1 in Bourne *et al.,* 1991). It is interesting to note that there are two invariant basic residues in the most highly-conserved region among GAP-related proteins (Block 3 in Figure 6), and this suggests that a specific electrostatic interaction may be the critical determinant of GAP function.

Table 3. Yeast strains used in this study

Strain	Genotype	Source
S. Pombe strains		
SP525	*h*^{90} *leu1.32 ade6.216 ura4- ras1::LEU2*	D. Beach
SP593*	*h*^{90} *leu1.32 ade6.216 ras1*^{val17}	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
SPSAL	*h*^{90} *leu1.32 ade6.210 ura4-D18 sar1::LEU2*	This study
SPSAU	*h*^{90} *leu1.32 ade6.210 ura4-D18 sar1::ura4*	This study
SPSTE6	*h*^{90} *leu1.32 ade6.210 ura4-D18 ste6::ura-4*	This study
S. Cerevisiae strains		
IR-1	*MATa his3 leu2 ura3 trp1 ade8 can1 ira1::HIS3*	Ballester *et al.*, 1989
TK161-R2V	*MATa leu2 ura3 his3 trp1 ade8 can1 RAS2*^{val19}	Ballester *et al.*, 1989

* In this strain the wild-type *ras1* allele was replaced by the *ras1* activated allele, *ras1*^{val17}.

What are the functions of divergent sequences outside of the catalytic domain? One can speculate that these regions of the various proteins have effector functions, or that they integrate information needed to regulate RAS activity, or that they aid the assembly of multisubunit protein complexes, or that they are silent. It is unlikely that these potential functions are reflected in any way in our phenotypic testing, because both GAP and NF1, which have essentially no primary structural similarity to sar1 outside the common homology regions, are both able to complement loss of sar1 function.

There are some minor discrepancies in the properties of sar1, IRA, GAP, and NF1 proteins. First, high-level expression of sar1 can suppress the phenotype of *ras1*^{val17} in *S. pombe* strains, whereas GAP and NF1 proteins cannot. On the other hand, high level expression of NF1 can suppress the phenotypic effects of the activated human H-ras^{val12} when it is expressed in *S. cerevisiae* strains (Ballester *et al.*, 1990), whereas sar1, GAP, and IRA proteins cannot. We think these differences reflect variations in the affinity of GAP-like proteins for the various species of activated RAS proteins. Thus NF1 might bind H-ras^{val12} and sar1 might bind ras1^{val17}, each sufficiently tightly to inhibit these mutant proteins.

The second discrepancy is that, in *S. cerevisiae,* disruption of *CDC25* restores a normal phenotype to *ira1*$^-$ or *ira2*$^-$ cells (Tanaka *et al.*, 1989, 1990a), whereas in *S. pombe* disruption of *ste6,* the homolog of *CDC25,* does not restore a normal phenotype to *sar1*$^-$ strains. We have experimentally excluded the most significant possible explanation, that the phenotype of *sar1*$^-$ cells is not dependent on *ras1.* A more likely explanation is that in the absence of an element that down-regulates ras1 function, ras1 protein remains predominantly in its active, GTP-bound form even in the absence of an activating element. It is also possible that there is another *CDC25* homolog in *S. pombe.*

It has not been clear why strains with the mutant *ras1*^{val17} allele are blocked in conjugation. One possibility is that, while ras1^{val17} is a constitutively active form of ras1, it might not be capable of the full functional activity required for conjugation. Our data effectively rules out this possibility, because expression of high-level sar1 can restore the conjugation capability of ras1^{val17} strains that do not contain a wild-type ras1 allele. Moreover, disruption of *sar1* in a strain that contains wild-type *ras1* produces a phenotype identical to that of strains containing a *ras1*^{val17} allele. Thus, it is safe to conclude that, while activation ras1 is required to initiate conjugation, diminution of ras1 activity is required to complete conjugation. Two distinct models can be envisaged to explain the requirement of diminished ras1 activity for conjugation. The first and, most likely, is that activation of ras1 inhibits a cellular activity required to complete conjugation. The second, and more interesting, hypothesis is that ras1 in its GDP-bound form has an effector function required to complete conjugation. This hypothesis receives some support from the observation that high-copy plasmids expressing wild-type ras1 can suppress the conjugation block in *ras1*^{val17} strains. Although we cannot rule out this interesting hypothesis, it is unlikely to be correct in our view, because the *ras1*^{val17} phenotype dominates over the presence of a single chromosomal copy of wild-type *ras1* (Nadin-Davis *et al.*, 1986a). Rather, we believe it more likely that high levels of wild-type ras1 inhibit ras1^{val17} by competition for some limiting cellular component.

Materials and methods

Nomenclature

In general, *S. pombe* genes and proteins are designated by lower case letters, while *S. cerevisiae* genes, dominant mutant and wild-type alleles, and proteins are designated by upper-case letters. Lower-case letters are used to refer to recessive alleles in *S. cerevisiae* strains. The initial letter of phenotypes are upper case. Genes and alleles are italicized, while proteins and phenotypes are not. Genes with superscript − or + refer to disrupted or wild-type alleles, respectively.

Sequence determination and analysis

Nucleotide sequencing was performed by the dideoxynucleotide chain-termination method by using oligonucleotide primers (Sanger *et al.*, 1977; Biggin *et al.*, 1983). Database searching was carried out using the GenInfo BLAST Network Service of NCBI as described in Altschul *et al.* (1990) and the legend to Table 1. Multiple sequence alignment was performed using the MACAW (Schuler *et al.*, 1991) and MSA (Lipman *et al.*, 1989) programs as described previously (Ballester *et al.*, 1990). Database searches with the GAP motifs were carried out using the fpat program (resembling the UNIX grep utility) of the NIH Molecular Biology Users Group. A composite database (with redundant sequences removed) derived from translated GenBank, NBRF/PIR, SWISS-PROT, and the GenInfo Backbone (see legend to Table 1) was used. This database contains ~12 million residues in 43 000 unique sequences (W. Gish, NCBI, personal communication).

Microbial manipulation and analyses

Yeast strains (Table 3) were grown in either rich medium (YEA for *S. pombe*, YPD for *S. cerevisiae*) or synthetic medium with appropriate auxotrophic supplements (PM for *S. pombe*, SC for *S. cerevisiae*) (Mortimer and Hawthorne, 1969; Nadin-Davis *et al.*, 1986a). The detection of sporulation by iodine vapor staining was performed as previously described (Gutz *et al.*, 1974). The lithium acetate procedure (Ito *et al.*, 1983) was used to transform *S. pombe* and *S. cerevisiae* cells. Plasmids in *S. pombe* cells were recovered by transforming *E. coli* strain DH5α with crude DNA extracts prepared from transformed yeast cells. The homozygotic diploid strains used in this study were generated by treatment with the lithium acetate transformation protocol and isolated from plates containing phloxin B. Ploidy was confirmed by microscopic examination of cell size and the presence of azygotic asci (Gutz *et al.*, 1974). Heat-shock experiments were performed by replica plating onto preheated plates as previously described (Sass *et al.*, 1986). Cell agglutination was tested by a sedimentation assay described previously (Wang *et al.*, 1991).

Plasmid construction

pLAR is an *S. pombe* expression plasmid containing the *S. pombe* ras1 gene as described before (Wang *et al.*, 1991). pAD4ΔSAR was constructed by inserting a *Sal* I-*Sac* I fragment containing the entire *sar1* coding region into the *S. cerevisiae* expression vector pAD4Δ (Ballester *et al.*, 1989) at the *Sal* I-*Sac* I sites. The *Sal* I and *Sac* I sites of the *sar1* fragment were introduced by various cloning procedures. The fragment contains nucleotides − 1 to 2333 from the *sar1* gene (see Figure 2). In pAD4ΔSAR the *sar1* gene is under the control of the *S. cerevisiae ADH1* promotor. pARTGAP was constructed by cloning the 4 kbp *Bam*HI-*Pst* I fragment that was derived from plasmid pADGAP (Ballester *et al.*, 1989) and that contains the entire coding sequence of human *GAP* gene into pART1 at *Bam*HI and *Pst* I sites. pART1 is a *S. pombe* expression vector containing the *S. cerevisiae LEU2* marker, a *S. pombe* ARS element, and the *S. pombe adh* promotor, as described before (Wang *et al.*, 1991). pARTNF1 was constructed by inserting the *Ssp* I-*Sph* I fragment containing the catalytic domain of the *NF1* gene into the *Sma* I site of pART1. The *Ssp* I-*Sph* I fragment of *NF1* was derived from pADNS-NF1 (Ballester *et al.*, 1989). It contains the amino acids 791 through 1203 in the coordinates of Xu *et al.* (1990b).

Construction of yeast strains

To construct a *sar1* null allele, a 5.5 kb *Bam*HI-*Pst* I fragment from pWH5SAR1 containing entire *sar1* gene was subcloned into pUC118 at the *Bam*HI-*Pst* I sites. A 885 bp *Xho* I-*Xho* I fragment that contains the sequence encoding 72 N-terminal codons and 647 bp upstream of the first codon was replaced by a 1.8 kbp fragment of the *S. pombe ura4* gene or a 2.2 kbp fragment of the *S. cerevisiae LEU2* gene (Figure 3), forming plasmids pUCSAR::ura4 or pUCSAR::LEU2, respectively. The *Bam*HI-*Pst* I fragments containing the disrupted *sar1* gene were transformed into the appropriate strains. A more extensive deletion of *sar1* was constructed by substituting the *S. pombe ura4* gene for the 2 kbp *Spe* I-*Bst*EII *sar1* fragment that contains 500 bp of upstream sequences and the sequences encoding the first 505 codons. This deletion removes the sequences encoding the proposed catalytic domain (Figure 3).

Plasmid pUCSTE6 was a gift from Dr. M. Yamamoto and contains the *ste6* gene as a 3.27 kbp *Eco*RI fragment in pUC118 (Hughes *et al.*, 1990). A 1014 *Nhe* I-*Spe* I fragment of the *ste6* coding region was replaced by the *S. pombe ura4* gene. Linear DNA fragments containing the disrupted *ste6* gene were transformed into the h^{90} wild-type strain SP870. The disruptants were confirmed by Southern blotting. One of the transformants, named SPSTE6, showed the conjugation defects expected for a $ste6^-$ strain (Hughes *et al.*, 1990). These defects could be restored by transformation with a plasmid carrying the *ste6* gene.

Acknowledgments

We thank Drs. D. Beach and M. Yamamoto for useful discussions and for providing plasmids and yeast strains. We thank P. Bird for help in preparing this 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.

Received: March 7, 1991.
Accepted: April 3, 1991.

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