

Biological activity of the mammalian RAP genes in yeast

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We have screened expression libraries for mammalian cDNAs capable of suppressing defects in *ras1*⁻ *Schizosaccharomyces pombe*. Both the RAP1A and RAP1B genes were identified in this manner. They suppress defects in cell morphology and sporulation, although not conjugation. In contrast, RAP genes do not suppress phenotypes in the yeast *Saccharomyces cerevisiae* that are deficient in RAS. Indeed, expression of RAP1A appears to antagonize the activated *S. cerevisiae* RAS2^{val19} gene. These results indicate that RAP proteins can interact with RAS targets, sometimes productively, sometimes nonproductively.

Introduction

The RAS genes are highly conserved in evolution. Homologs of the mammalian oncogenes are found in simple organisms such as the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*. The two RAS genes of the budding yeast, RAS1 and RAS2, have been studied extensively (Wigler *et al.*, 1988). Despite striking similarities in structure and function between the mammalian and *S. cerevisiae* RAS proteins, the RAS effector signaling systems in the respective hosts probably differ (Birchmeier *et al.*, 1985). In *S. cerevisiae*, RAS proteins regulate adenylyl cyclase (Broek *et al.*, 1985; Toda *et al.*, 1985), although this may not be their only function (Toda *et al.*, 1987b; Wigler *et al.*, 1988). In mammals, the RAS effector pathways are unknown. A single RAS homolog, *ras1*, has been discovered in the fission yeast, *S. pombe* (Fukui and Kaziro, 1985). *Ras1* does not appear to function on the adenylyl cyclase pathway in *S. pombe* (Fukui *et al.*, 1986). The mammalian H-ras gene can complement *ras1*⁻ *S. pombe* (Nadin-Davis *et al.*, 1986).

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We have therefore begun to explore the usefulness of *S. pombe* for the study of the mammalian RAS pathway by searching for other mammalian genes capable of complementing phenotypic defects in *S. pombe* strains lacking the *ras1* gene.

S. pombe cells lacking the *ras1* gene are viable, but are defective in sporulation and conjugation, and have a round rather than an elongated morphology (Fukui *et al.*, 1986). The sporulation phenotype can be readily scored by an iodine staining procedure: colonies containing spores stain brown (Nadin-Davis *et al.*, 1986). We used a *ras1*⁻/*ras1*⁻ diploid strain to screen mammalian cDNA libraries cloned into an *S. pombe* expression vector for cDNAs capable of inducing sporulation. We have isolated several cDNAs by this method, including cDNAs encoding the human RAP1A and RAP1B proteins. The mammalian RAP genes are members of the RAS gene superfamily and were first identified by others by low-stringency hybridization (Pizon *et al.*, 1988). They encode proteins which share extensive amino acid sequence homology with the mammalian oncogenic RAS proteins, particularly in the N-terminal domain. The RAP1A cDNA gene was independently isolated as K-rev-1 by its ability to interfere with malignant transformation by the K-ras oncogene (Kitayama *et al.*, 1989). Our results suggest that the H-ras and RAP proteins can both stimulate some of the same effector pathways in *S. pombe*. Expression of the RAP1A protein in *S. cerevisiae* appears to interfere with the RAS2 function in that organism.

Results

Construction of cDNA libraries

Human cDNAs were made from the poly A⁺ mRNA of the glioblastoma cell line U118 MG, and, after ligation to Not I linkers, were inserted into the λ zap vector (see Materials and methods). To express the cDNAs in *S. pombe*, an *S. pombe* ADH promoter expression vector was used (see Figure 1). This vector, pAAUN, was adapted from the vector pART1, the generous gift of David Beach. pAAUN contains a Not I

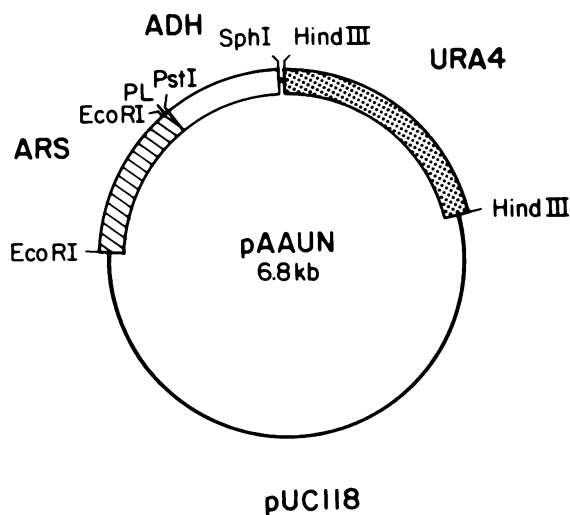


Figure 1. Map of the *S. pombe* expression vector. The expression vector pAAUN was derived from pART1 (McLeod *et al.*, 1987) by replacing the *leu2* gene with a 1.8-kbp *Hind*III fragment containing the *S. pombe* *ura 4* gene and adding Not I linkers at the *Sma* I site in the polylinker (PL) derived from pUC118 (Viera and Messing, 1987). The thin line indicates pUC118 sequence. The slashed box indicates an 1.2-kbp ARS sequence of *S. pombe*. The empty box indicates the 700-bp *S. pombe* ADH promoter. The direction of transcription from this promoter is counterclockwise. The prototrophic amino acid marker is indicated by the stippled boxes.

restriction endonuclease cloning site just 3' to the *S. pombe* ADH promoter. The vector also contains the *ura4* gene for selection in *ura4*⁻ yeast, and the bacterial marker for ampicillin resistance for selection in *Escherichia coli*. cDNA inserts from the λ zap library were excised by cleavage with Not I and size fractionated on sucrose velocity sedimentation gradients. cDNAs in the size range of 1.5–4 kb were collected, ligated into pAAUN at the Not I site, and transformed into *E. coli* strain DH5 α . A library of complexity of $\sim 10^6$ was thus obtained. For optimum expression in *S. pombe*, cDNA inserts in this library must provide a methionine codon for translation of the proper open reading frame.

Screening in *S. pombe* and identification of cDNA candidates

A *ras1*⁻/*ras1*⁻ diploid strain, Sp565, was obtained from D. Beach. This strain was made by the self fusion of *ras1*⁻ haploid strain Sp525. DNA from the cDNA expression library was prepared and used to transform Sp565 by the lithium acetate method (Ito *et al.*, 1983). About 10^6 transformants were screened and colonies capable of sporulating were identified by iodine staining (Nadin-Davis *et al.*, 1986). The few

staining colonies were picked and the plasmids they contained were rescued into *E. coli*. Most of the plasmids so obtained were capable of efficiently inducing sporulation when transformed back into Sp565. Several of these were grouped by restriction endonuclease mapping and representatives of various groups were sequenced. Two representative clones, pMRS1 and pMRS9, were sequenced in their entirety, and contained cDNAs which were identical in their coding sequences to the published coding sequences of cDNAs for RAP1B and RAP1A, respectively. The cDNA of RAP1A initiated 49 bp upstream of its ATG, and the cDNA for RAP1B initiated 27 bp upstream of its ATG.

Properties of *S. pombe* expressing mammalian RAP genes

We compared RAP1A and RAP1B genes to the human H-ras, *S. pombe* *ras1* and *S. pombe* *byr1* genes for the ability to suppress the phenotypic defects in *ras1*⁻ haploid and *ras1*⁻/*ras1*⁻ diploid *S. pombe* strains. The *byr1* gene encodes a protein kinase that has been reported to suppress the sporulation defects of *ras1*⁻/*ras1*⁻ diploid strains, but not suppress the conjugation defects of *ras1*⁻ strains, nor revert the morphological abnormalities of either *ras1*⁻ or *ras1*⁻/*ras1*⁻ strains (Nadin-Davis and Nasim, 1988). We have repeated all these results with our strains (data not shown). In contrast to *byr1*, the mammalian H-ras and the *S. pombe* *ras1* gene each suppressed all of the phenotypic defects in *ras1*⁻ and *ras1*⁻/*ras1*⁻ strains (see Figures 2 and 3). The RAP genes, like H-ras and *ras1*, suppressed defects in sporulation, as shown in Figure 2, and restored elongated morphology, as seen in Figures 2 and 3. Unlike H-ras and *ras1* genes, however, the RAP genes were not able to restore defects in conjugation. This is seen in Figure 3. The *ras1*⁻ homothallic haploid strain Sp525 was transformed with various plasmids expressing RAS or RAP genes, and transformed colonies, grown on synthetic medium, were examined microscopically. When Sp525 was transformed with vector alone (panel A), it remained round and showed no evidence of conjugation or sporulation. When Sp525 was transformed with plasmids expressing either H-ras or *ras1*, normal morphology was restored and multiple sporulating zygotic cells were observed (panels D and E), evidence of conjugal activity. When Sp525 was transformed with plasmids expressing either RAP1A or RAP1B, morphology was restored, but no sporulating zygotes were observed, indicating that conjugation had not occurred.

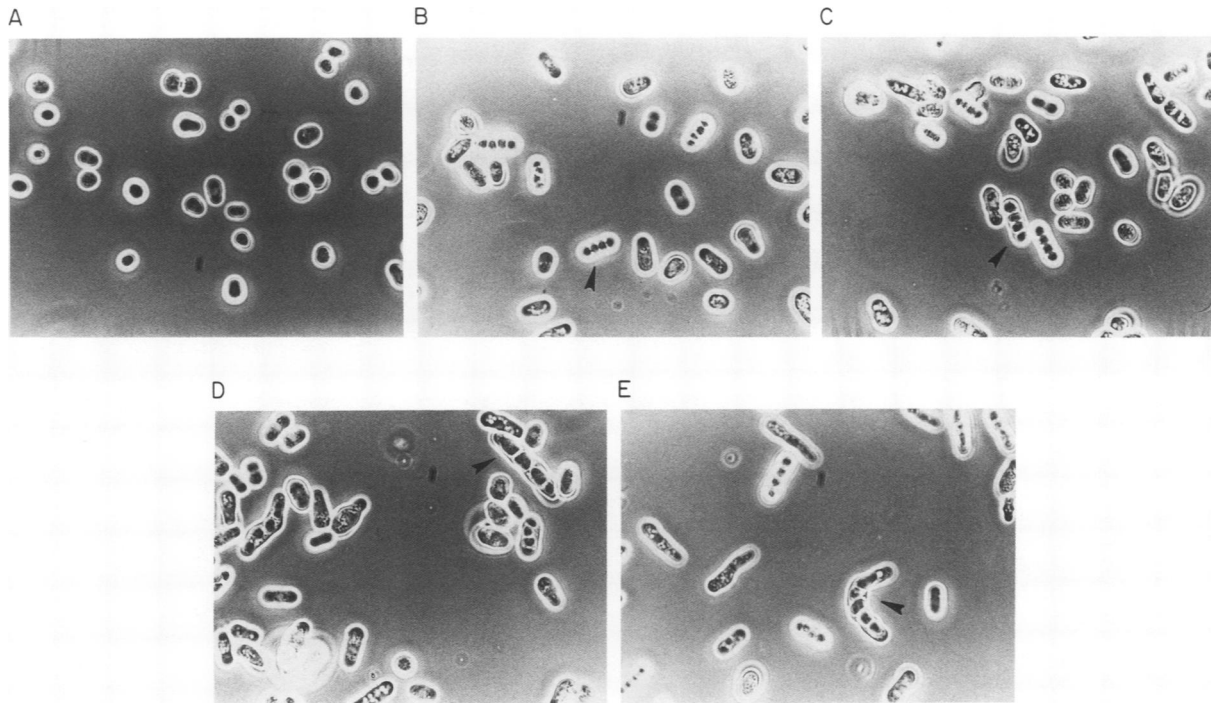


Figure 2. Morphology and sporulation induced by different genes in the *S. pombe ras1⁻/ras1⁻* diploid strain Sp565. The strain Sp565 was transformed with (A) pAAUN, a *S. pombe* expression vector; (B) pMRS1, a plasmid containing the human RAP1B gene; (C) pMRS9, a plasmid containing the human RAP1A gene; (D) pAAUHR, a plasmid containing the human H-ras gene; and (E) pAUR, a plasmid containing the *S. pombe ras1* gene. The arrows indicate sporulated cells.

Properties of *S. cerevisiae* expressing mammalian RAP genes

We next tested if expression of the mammalian RAP genes could function on the RAS pathway in *S. cerevisiae*. For this purpose we inserted RAP cDNAs into an *S. cerevisiae* ADH1 expression vector, pADNS. A similar vector was used to express the human H-ras gene (Powers *et al.*, 1986). These plasmids were then tested for the ability to alter the phenotype of the *S. cerevisiae* strain SP1 (Kataoka *et al.*, 1984), which is wild type at RAS loci (*MAT α leu2 his3 ura3 his3 trp1 ade8 can1*), the strain STS1 (Powers *et al.*, 1986), which is *ras1⁻ ras2^{ts}* (*MAT α his3 leu2 ura3 trp1 ras1⁻ ras2^{ts8}*), the strain S28.4B which is *cdc25^{ts}* (*MAT α his3 leu2 ura3 trp1 ade8 can1^R cdc25-1*) (Hartwell *et al.*, 1973) and the strain TK161-R2V (Kataoka *et al.*, 1984), which expresses *RAS2^{val19}* (*MAT α leu2 his3 ura3 trp1 ade8 can1 RAS2^{val19}*). The STS1 strain first shows a temperature-sensitive growth defect at 36.5°C. CDC25 encodes a protein required for *S. cerevisiae* RAS function (Camonis *et al.*, 1986; Broek *et al.*, 1987). Strains that are *RAS2^{val19}*, such as TK161-R2V, are typically sensitive to heat shock and nitrogen starvation, due to the activation of RAS targets by the con-

stitutively activated mutant *RAS2^{val19}* gene (Kataoka *et al.*, 1984; Sass *et al.*, 1986).

Neither RAP1A nor RAP1B clones were able to restore wild-type growth at 36.5°C to the *ras2^{ts}* strain STS1, although the H-ras cDNA clone was (data not shown). The temperature sensitivity of another *ras2^{ts}* strain and a *cdc25^{ts}* strain were similarly unaffected by RAP clones, although wild-type growth in both was restored by H-ras. Moreover, neither RAP1A nor RAP1B clones made wild-type cells heat-shock sensitive, although the H-ras cDNA clone did (see Figure 4). The RAP1A clone, but not the RAP1B clone, was able to slightly diminish the heat-shock-sensitive phenotype of the *RAS2^{val19}* strain (see Figure 5). Expression of RAP1A had no effect on the nitrogen starvation sensitivity of this strain.

These results suggest that RAP1A proteins can antagonize the action of *RAS2^{val19}*, perhaps by interacting nonproductively with yeast RAS2 target proteins. However, from this data we cannot exclude the possibility that RAP proteins act on a pathway that opposes RAS. To test this hypothesis, we transformed the RAP1A expression vector into the strain RTF1.5prC, which has the genotype *bcy1⁻ tpk1^{wc} tpk2⁻ tpk3⁻*.

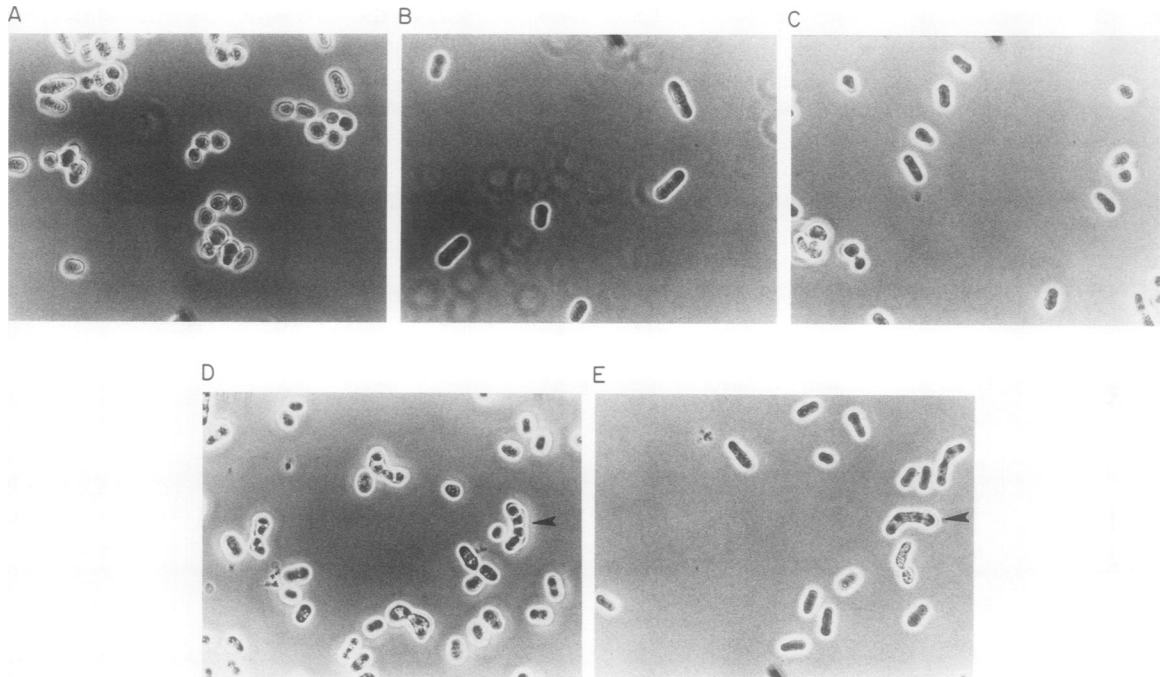


Figure 3. Morphology and conjugation induced by different genes in the *S. pombe ras1*⁻ haploid strain Sp525. The strain Sp525 was transformed with (A) pAAUN, a *S. pombe* expression vector; (B) pMRS1, a plasmid containing the human RAP1B gene; (C) pMRS9, a plasmid containing the human RAP1A gene; (D) pAAUHR, a plasmid containing the human H-ras gene; and (E) pAUR, a plasmid containing the *S. pombe ras1* gene. The arrows indicate sporulated cells, evidence of conjugation activity.

This strain is heat-shock sensitive since it lacks the *BCY1* gene, which encodes the regulatory component of the cAMP dependent protein kinase (Toda *et al.*, 1987a). This strain contains disruptions of two of the three TPK genes, which encode catalytic components of the cAMP-dependent protein kinase (Toda *et al.*, 1987b), and an attenuating mutation in the third TPK gene (Cameron *et al.*, 1988). Hence RTF1.5prC is less heat-shock sensitive than the *RAS2*^{val19} strain, TK161-R2V, but more sensitive than wild-type strains. The strain TRF1.5prC is insensitive to activated *RAS2*^{val19}: it is not made more heat-shock sensitive by the expression of *RAS2*^{val19} (S. Cameron, unpublished data). This is presumably because the disruption of the *BCY1* locus "uncouples" the major pathway by which RAS proteins influence the cell. Expression of RAP1A in this strain also has no effect on heat-shock sensitivity. This result suggests that RAP1A protein functions in *S. cerevisiae* only in strains which are sensitive to RAS proteins and thus does so by competing with *RAS2*^{val19} protein.

Discussion

There is a large family of low molecular weight proteins that resemble the RAS proteins in their

GTP-binding domains. Some members of this superfamily are also highly homologous to RAS proteins in other domains (Chardin and Tavitan, 1986; Lowe *et al.*, 1987; Pizon *et al.*, 1988). The RAP proteins (RAP1A, RAP1B, and RAP2) are among these, since they share >50% identity with RAS overall (Pizon *et al.*, 1988). The RAP1A gene has been independently isolated many times in the recent past: once by low-stringency hybridization with RAS gene probes (Pizon *et al.*, 1988); once as a low molecular weight GTP-binding protein from brain (Kawata *et al.*, 1988); once as a component of a purified neutrophil cytochrome complex (Quinn *et al.*, 1989); once as a cDNA with the ability to partially suppress transformation by the K-ras oncogene (Kitayama *et al.*, 1989); and in the present report. In our functional genetic screen, based on complementation in the yeast *S. pombe*, we have isolated cDNAs for RAP1A and RAP1B. In a continuation of this project, we have also isolated the RAP2 cDNA (work in progress).

Our results suggest that the mammalian RAP proteins can share effector function with the *S. pombe ras1* protein. It is of interest to us that although expression of human H-ras can suppress all the phenotypic defects of *ras1*⁻ *S. pombe* strains, expression of human RAP genes

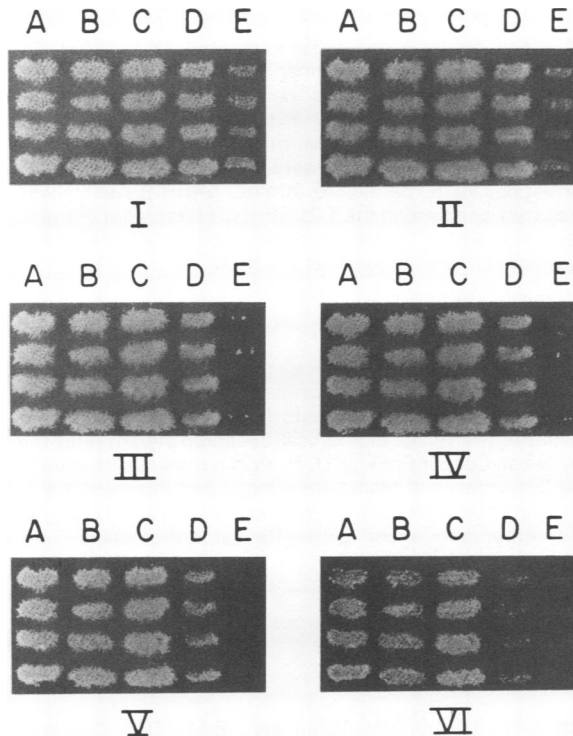


Figure 4. The effects of RAS and RAP expression plasmids on the heat-shock response of the *S. cerevisiae* wild-type strain SP1. Yeast cells were patched on synthetic medium plates (SC). Heat-shock experiments were performed by replica plating as described in Materials and methods. Plate I was replica plated onto a cold plate and not subjected to heat shock. Plates II, III, IV, V, and VI were replica plated onto preheated SC plates and subjected to further incubation at 55°C for 0, 15, 30, 45, and 60 min, respectively. In columns A, B, C, D, and E, SP1 was transformed with plasmids: (A) YEp13M4, a *S. cerevisiae* expression plasmid (Field *et al.*, 1990); (B) pADNSRAP1B, a plasmid expressing the human RAP1B gene; (C) pADNSRAP1A, a plasmid expressing the human RAP1A gene; (D) pADH-H-ras, a plasmid expressing the human H-ras gene (Powers *et al.*, 1986); and (E) pADH-RAS2^{val19}, a plasmid expressing the *S. cerevisiae* RAS2^{val19} gene.

fails to correct defects in conjugation. One possible explanation of these observations is that RAS proteins have at least two independent effector functions in *S. pombe*, as we have found they do in *S. cerevisiae* (Wigler *et al.*, 1988). RAP proteins might stimulate one effector function (required for morphology and sporulation) but not the other (required for conjugation). Other explanations are also possible.

In contrast to our results in the fission yeast, RAP proteins do not appear to stimulate the RAS pathways of the budding yeast, *S. cerevisiae*. They fail to complement temperature sensitive alleles of RAS2, and do not induce heat-shock sensitivity, as do both mammalian H-ras

and activated *S. cerevisiae* RAS2^{val19} proteins. On the contrary, expression of RAP1A actually reduces somewhat the heat-shock sensitivity of RAS2^{val19} strains. Expression of RAP1A does not reduce the heat-shock sensitivity of yeast with activated cAMP-dependent protein kinases. The simplest interpretation of these results is that the RAP1A protein retains the ability to interact with RAS targets in *S. cerevisiae*, but that this interaction is ineffective, and thus RAP1A competes with activated RAS2^{val19} protein. Such nonproductive interactions with effectors might explain why RAP1A can partially block transformation of oncogenic RAS proteins in mammalian systems (Kitayama *et al.*, 1989). We can only speculate on the reason why RAP1A fails to activate RAS pathways in *S. cerevisiae*. One possibility is that the RAP1A protein interacts with RAS targets but fails to stimulate them. Another possibility is that RAP1A interacts with RAS targets but causes their aberrant localization.

Recently, Frech *et al.* (1990) have reported that RAP1A protein can interact with mammalian GAP. GAP is a protein that interacts with mammalian RAS proteins to promote GTP hydrolysis (Trahey and McCormick, 1987). It is unknown if GAP is a target or regulator of RAS interactions (Adari *et al.*, 1988; Hall, 1990). Our results indicate that mammalian RAP proteins can indeed interact with RAS targets. In some cases, they can stimulate these targets; in others, they fail to interact productively. In both budding and fission yeasts, the mammalian H-ras protein functions more like the endogenous RAS proteins than do the mammalian RAP pro-

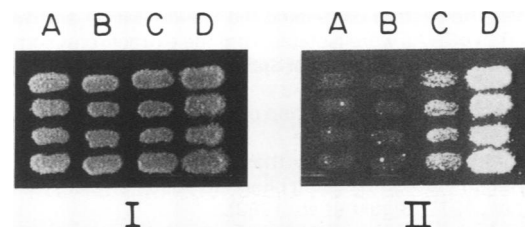


Figure 5. Suppression of heat-shock sensitivity in the *S. cerevisiae* RAS2^{val19} strain TK161-R2V by expression of RAP genes. Yeast patches were grown on YPD plates for 2 d and then replica plated to fresh plates at 30°C (I) or to preheated plates and incubated at 55°C for 10 min before returning to 30°C (II). Recovery time at 30°C was 48 h. In columns A, B, C, and D, TK161-R2V was transformed with: (A) YEp13M4, a *S. cerevisiae* expression plasmid (Field *et al.*, 1984); (B) pADNSRAP1B, a plasmid expressing the human RAP1B gene; (C) pADNSRAP1A, a plasmid expressing the human RAP1A gene; and (D) YEpPDE2, a plasmid expressing the *S. cerevisiae* cAMP phosphodiesterase gene (Sass *et al.*, 1986).

teins, but it is quite clear that members of the RAS superfamily can be promiscuous in their target interactions.

Materials and methods

Microbial manipulations and analysis

E. coli strain DH5 α (Hanahan, 1983) was used for plasmid preparation, isolation, transformation, and maintenance of the cDNA library (Hanahan, 1983). We used *S. pombe* strains kindly provided by D. Beach, Sp525 (*h⁹⁰ade6⁻leu1⁻ura4⁻ras1⁻*) (Nadin-Davis *et al.*, 1986) and Sp565, the Sp525/Sp525 diploid formed by the fusion of two haploid spheroplasts during a transformation procedure (Nadin-Davis and Nasim, 1988). The *S. cerevisiae* strains used were TK161-R2V (*MAT α leu2 his3 ura3 trp1 ade8 can1 RAS2^{val19}*) (Toda *et al.*, 1985), STS1 (*MAT α his3 leu2 ura3 trp1 ade8 can1 ras1::URA3 ras2⁹⁸*) (Powers *et al.*, 1986), SP1 (*MAT α leu2 his3 ura3 trp1 ade8 can1*) (Kataoka *et al.*, 1984) and RTF1.5 prC (*MAT α his3 leu2 ura3 trp1 ade8 tpk^{mc}tpk2::HIS3 tpk3::TRP1 bcy1::URA3*) (S. Cameron, unpublished data). Yeast strains were grown in either rich medium (YEA, dextrose/yeast extract/adenine, for *S. pombe*; YPD, yeast extract/peptone/dextrose, for *S. cerevisiae*) or synthetic medium with appropriate auxotrophic supplements (PMA for *S. pombe*; SC for *S. cerevisiae*) (Nadin-Davis *et al.*, 1986; Mortimer and Hawthorne, 1969). The iodine vapor staining was performed as previously described (Nadin-Davis *et al.*, 1986). Heat-shock experiments were performed by replica plating onto preheated plates as previously described (Ito *et al.*, 1983). *S. pombe* and *S. cerevisiae* strains were transformed by the lithium acetate procedure (Ito *et al.*, 1983).

Nucleic acid manipulation and analysis

Poly A⁺ mRNA was purified from the human glioblastoma cell line U-118 MG as previously described (Watson and Jackson, 1984). Double stranded cDNAs were prepared, ligated to Not I linkers, and cloned into the Not I sites of λ zap. This library will be described in more detail in another publication (Colicelli *et al.*, 1990; in preparation). The cDNAs were excised from the λ zap library by cleavage with Not I and size fractionated by velocity sedimentation in sucrose gradients. The 1.5- to 4.0-kbp fragments were collected and ligated into the Not I site in the *S. pombe* expression vector, pAAUN. The vectors containing the 1.8-kbp MRS1 and 1.3-kbp MRS9 cDNAs were isolated from the extrachromosomal DNA of transformed strains of Sp565 as described (Nasmyth and Reed, 1980). These DNAs were transformed into *E. coli* (Holmes and Quigley 1981), and plasmid DNA was obtained from individual *E. coli* (Katz *et al.*, 1973). Nucleotide sequencing was performed by the dideoxynucleotide chain-termination method by using oligonucleotide primers (Sanger *et al.*, 1977; Biggin *et al.*, 1983).

The cloning vector pAAUN was derived from plasmid pART1 (McLeod *et al.*, 1987) by replacing the *leu2* gene with a 1.8-kbp *HindIII* *ura4* fragment from *S. pombe* and adding Not I linkers at the *Sma* I site (Figure 1). Plasmid pAUR was constructed by insertion of a 1.4 kbp *Bam*HI-*Bgl*II *S. pombe* *ras1* gene fragment (Nadin-Davis *et al.*, 1986) into the *Bam*HI site of pIRT5. pIRT5 is a vector containing a 1.2-kbp *S. pombe* ARS fragment at the *Eco*RI site and a 1.8-kbp *S. pombe* *ura4* fragment at the *Hind*III site of pUC118. Both pIRT5 and the plasmid containing *S. pombe* *ras1* gene were kind gifts from D. Beach. pAAUHR was constructed by inserting a 0.7-kbp *Spe*I-*Sal*I human H-ras cDNA fragment from plasmid pOFSP-2 (Kataoka *et al.*, 1985) into

the *Sma* I site of pART1. The *S. cerevisiae* expression vector pADNS (Colicelli *et al.*, 1989) was used for constructing expression vectors for the human RAP1A and RAP1B cDNAs in *S. cerevisiae* and was named pADNSRAP1A and pADNSRAP1B, respectively. YEp13M4 (Field *et al.*, 1990), YEpPDE2 (Sass *et al.*, 1986), pADH-H-ras (Powers *et al.*, 1986), and pADH-RAS2^{val19} were used as controls in transformations of *S. cerevisiae* strains. pADH-RAS2^{val19} was prepared by inserting the 1.25-kbp *Hpa* I fragment of the *S. cerevisiae* RAS2^{val19} gene into the blunt ended *Hind*III site of plasmid pLD95 (Powers *et al.*, 1986).

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