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Genetic Analysis of Yeast *RAS1* and *RAS2* Genes

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Summary

We present a genetic analysis of *RAS1* and *RAS2* of S. cerevisiae, two genes that are highly homologous to mammalian *ras* genes. By constructing in vitro *ras* genes disrupted by selectable genes and introducing these by gene replacement into the respective *ras* loci, we have determined that neither *RAS1* nor *RAS2* are by themselves essential genes. However, *ras1*$^-$ *ras2*$^-$ spores of doubly heterozygous diploids are incapable of resuming vegetative growth. We have determined that *RAS1* is located on chromosome XV, 7 cM from *ade2* and 63 cM from *his3;* and *RAS2* is located on chromosome XIV, 2 cM from *met4*. We have also constructed by site-directed mutagenesis a missense mutant, *RAS2*^{val19}, which encodes valine in place of glycine at the nineteenth amino acid position, the same sort of missense mutation that is found in some transforming alleles of mammalian *ras* genes. Diploid yeast cells that contain this mutation are incapable of sporulating efficiently, even when they contain wild-type alleles.

Introduction

Genes found in the acutely pathogenic RNA tumor viruses and in some tumor cells can profoundly alter the proliferative capacity of animal cells (Cooper, 1982). Such transforming genes arise from normal cellular genes by a wide variety of processes. Studies of these cellular genes and their gene families are likely to provide insights into the normal and pathological mechanisms of growth control. For several reasons, the *ras* gene family is a particularly attractive one for study. Activation of the transforming potential of the *ras* genes can result from certain missense mutations and such mutated *ras* genes are frequently found in human and rodent tumors (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982; Yuasa et al., 1983; Taparowsky et al., 1983; Shimizu et al., 1983; Capon et al., 1983b; and Sukumar et al., 1983). Elevated expression of the *ras* proteins can also transform animal cells (Defeo et al., 1981; Chang et al., 1982). Finally, the *ras* genes are highly conserved in evolution (Ellis et al., 1981; Shilo and Weinberg, 1981), and, in particular, the genome of the yeast S. cerevisiae contains genes with strong homology to the mammalian *ras* genes (DeFeo-Jones et al., 1983; Powers et al., 1984). We have begun a study of the biochemical and cellular role of the *ras* genes of S. cerevisiae, using the powerful tools for genetic manipulation that are available in this organism.

In yeast there are two genes, *RAS1* and *RAS2,* with remarkable homology to the mammalian *ras* genes (Powers et al., 1984). *RAS1* and *RAS2* encode proteins of 309 and 322 amino acids, respectively. These two proteins are nearly 90% homologous to each other for the first 180 amino acids, but then diverge radically. In mammals, there are at least three members of the *ras* family, the H-, K-, and N- *ras* genes, encoding proteins of 188 or 189 amino acids (Capon et al., 1983a; Shimizu et al., 1983; McGrath et al., 1983; Taparowsky et al., 1983). In humans, these three proteins are nearly 90% homologous to each other for the first 170 amino acids, but then diverge radically. The region of greatest homology between the mammalian and yeast *ras* protein is the N-terminal domain, with nearly 90% homology between positions 10 through 90 of the yeast *ras* proteins and the corresponding positions 3 through 83 of the mammalian *ras* proteins (Powers et al., 1984; Defeo-Jones et al., 1983). It is in this N-terminal domain, at positions 12, 13, 59, 61, or 63, where amino acid substitutions can activate the transforming potential of the mammalian *ras* proteins (Fasano et al., 1984). At the corresponding positions, the yeast *ras* genes encode the same amino acids as the normal mammalian *ras* genes (Powers et al., 1984). We feel that this homology reflects both a highly conserved biochemical function for *ras* proteins and a highly conserved mechanism for regulating that function.

Here we present a preliminary account of the phenotypic consequences of certain genetic manipulations of the *ras* genes in yeast. In the first series of experiments, we have disrupted either or both *ras* genes in yeast cells. In the second, we have introduced into yeast cells a mutant *RAS2* gene with the type of missense mutation that has been shown to activate the transforming potential of its mammalian counterpart. We also report here the chromosomal location of the yeast *ras* genes.

Results

Disrupting Yeast *ras* Function

To determine if *RAS1* or *RAS2* genes were vital to haploid yeast cells we designed gene replacement experiments (Rothstein, 1983). First, we inserted selectable marker genes into the coding regions of the cloned *ras* genes carried on plasmids p*RAS1* and p*RAS2* (see Figure 1). The resulting plasmids, p*ras1*::*URA3* and p*ras2*::*LEU2* were used to carry out gene replacement experiments by transforming yeast haploid or diploid auxotrophs with DNA fragments spanning the disrupted gene sequences. Transformation by such a procedure most often results from a double crossover recombination event such that the endogenous allele is replaced by the introduced gene disrupted by the selectable marker. For *RAS1* we utilized the Eco RI/Bam HI fragment of p*ras1*::*URA3,* and for *RAS2* the Nco I/Hind III fragment of p*ras2*::*LEU2* (see Figure 1).

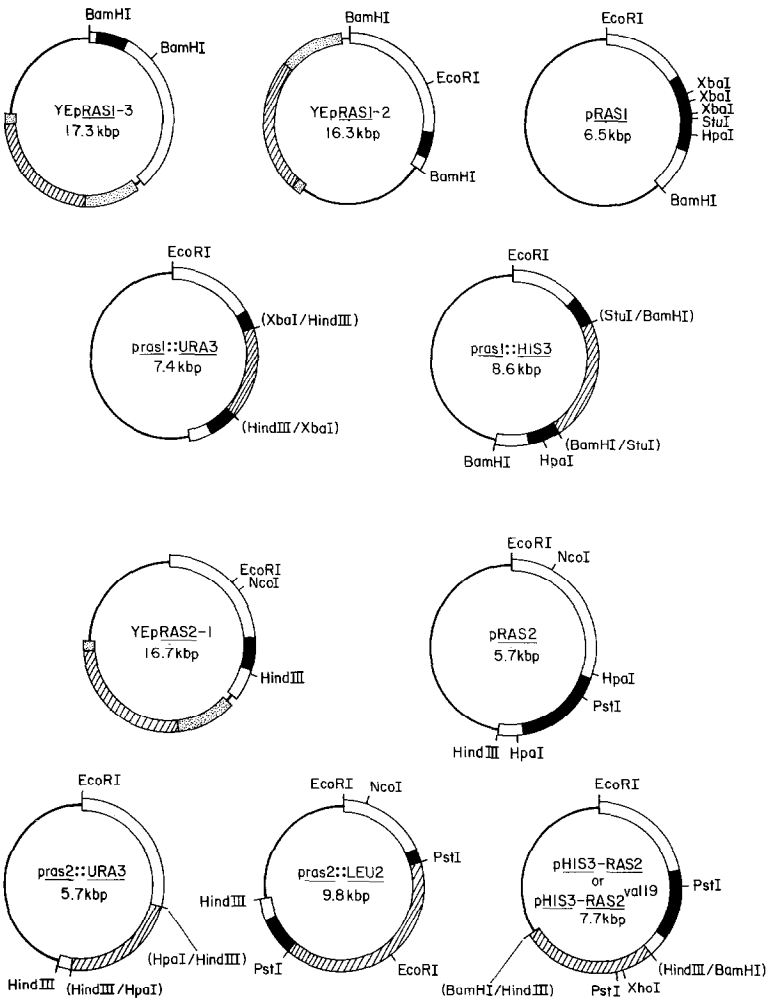

Figure 1. *ras* Plasmids

Maps of plasmids containing DNA sequences from *RAS1* or *RAS2* loci are shown above. YEp*RAS1*-3, YEp*RAS1*-2 and YEp*RAS2*-1 were originally identified in a plasmid library consisting of Sau 3A partial digestion fragments cloned into the unique Bam HI site of the yeast shuttle vector YEp13 as previously described (Powers et al., 1984). YEp13 contains the *LEU2* gene (Broach et al., 1979). p*RAS1* was constructed by subcloning the 2.5 kb Eco RI/Bam HI *RAS1* fragment of YEp*RAS1*-2 into the corresponding sites of pBR322. p*ras1*::*URA3* was obtained from p*RAS1* by inserting the 1.2 kb Hind III fragment of *URA3* (Botstein and Davis, 1981) between the 5′ and 3′ Xba I sites of p*RAS1*. p*ras1*::*HIS3* was obtained by inserting the 2.0 kb Hind III fragment containing *HIS3* (Struhl and Davis, 1980) into the unique Stu I site of p*RAS1*. p*RAS2* was obtained by inserting the 3.0 kb Eco RI/Hind III *RAS2* fragment of YEp*RAS2*-1 into the corresponding sites of pUC8 (Viera and Messing, 1982). p*ras2*::*URA3* was obtained by inserting the 1.2 kb Hind III fragment of *URA3* between the Hpa I sites of p*RAS2*. p*ras2*::*LEU2* was obtained by subcloning the 4.0 kb Pst I fragment containing *LEU2* (Ratzkin and Carbon, 1977) into the Pst I site of p*RAS2*. p*HIS3-RAS2* was obtained by inserting the 2.0 kb fragment containing *HIS3* into the Hind III site of p*RAS2*. p*HIS3-RAS2*^{val19} was obtained from p*HIS3-RAS2* by replacing the Pst I/Eco RI fragment with the mutagenized fragment subcloned into M13mp8 (see Experimental Procedures). The size of the plasmids are indicated in the figure in kb. Where restriction endonuclease sites are indicated for a given plasmid, all sites for the enzyme are indicated, except for the YEp plasmids which contain multiple Eco RI sites. Pairs of restriction endonuclease sites in parentheses refer to novel joints created by blunt-ended ligation of restriction endonuclease fragments after they were made flush-ended by reaction with E. coli polymerase I in the presence of deoxynucleotide triphosphates. Vector sequences are indicated by a thin line; coding sequences from *ras* loci are indicated by a filled-in box; noncoding sequences from the *ras* loci by empty boxes; DNA from the loci of the prototrophic amino acid markers are indicated by boxes with slashes; and DNA from the yeast 2μ circle by stippled boxes. All plasmids are oriented so that transcription from the *ras* loci proceeds clockwise.

After transformant prototrophs were selected, we used Southern hybridization analysis to verify that the appropriate *ras* gene was replaced by the disrupted gene.

In our initial experiments, we transformed the homozygous *leu2*$^-$ diploid strain DC 5/6 with the p*ras2*::*LEU2* fragment described above. 17/24 of the resulting Leu$^+$ diploids were heterozygous at the *RAS2* locus, with one allele disrupted. We call this disrupted *RAS2* allele *ras2*::*LEU2*. These diploids were sporulated and tetrads analyzed. Ninety-eight percent of all spores were viable and in 19/20 tetrads the *LEU2* marker segregated 2:2. From this we conclude that the *RAS2* gene is not essential for vegetative growth. Emboldened by these results, we directly transformed a *ura3*$^-$ haploid strain, SP1, with the *RAS1* gene disrupted by *URA3*. We obtained Ura$^+$ transformants at the expected frequency and demonstrated by Southern hybridization analysis that 3/3 representative transformants resulted from gene replacement of the *RAS1*

gene with the disrupted *ras1* allele. We conclude that the *RAS1* gene is not essential for haploid yeast.

To confirm these results and to test if disruption of both *RAS1* and *RAS2* together was lethal for haploids, we mated a *ras2*$^-$ haploid with a *ras1*$^-$ haploid such that the resulting diploids were heterozygous at each *ras* locus with distinct prototrophic markers at each disrupted allele (see Table 1 for more detail). The *LEU2* marker disrupted *RAS2* and the *URA3* marker disrupted *RAS1*. Upon sporulation and tetrad analysis, we obtained close to the expected numer of parental and nonparental ditypes and tetratypes (Table 1). None of the 32 expected Leu$^+$ Ura$^+$ spores were viable, indicating that spores without a functional *RAS1* or *RAS2* gene cannot resume vegetative growth. Of the remaining 121 spores, 118 were viable. This confirms our previous result that neither *RAS1* nor *RAS2* are by themselves essential genes. These results were repeated for several additional sets of doubly heterozygous diploids. In these experiments, the unviable *ras1*$^-$ *ras2*$^-$ spores were examined microscopically, and only about 50% were judged to have germinated, as determined by their phase lucent appearance and by their increase in size. All the *ras1*$^-$ *ras2*$^-$ germinated spores remained unbudded.

Chromosomal Location of *RAS1* and *RAS2*

RAS1 and *RAS2* were localized to specific chromosomes by the procedure of Falco et al. (1983), which is based on the observation that integration of the 2μ circle *FLP* recombination site into a yeast chromosome induces a marked destabilization of that chromosome in the presence of the *FLP* gene product. Accordingly, we integrated the 2μ circle *FLP* recombination site into the genome of strain SI50-2D [cir^0] at the *RAS1* or *RAS2* locus by site-directed transformation (Orr-Weaver et al., 1981) as described below. We then assessed which chromosome in the resultant strains displayed decreased mitotic stability when mated to [cir$^+$] strains carrying genetic markers on each chromosome.

Plasmid YEp*RAS2*-1 DNA (see Figure 1), which contains the *FLP* site but the not *FLP* gene, was digested to completion with restriction endonuclease Nco I and then used to transform strain SI50-2D [cir^0] to leucine prototrophy. Thirty individual transformants were tested for the stability of the Leu$^+$ phenotype and two stable transformants were retained. Southern blot hybridization analysis of genomic DNA isolated from the two strains indicated that both strains carried a single copy of the plasmid integrated at the *RAS2* locus. These two strains were designated R2-2μ-1 and R2-2μ-2.

Concurrently, plasmid YEp*RAS1*-2 DNA was digested with a limiting amount of restriction endonuculease Bam HI (see Figure 1). Full-length linear fragments were purified by agarose gel electrophoresis and used to transform strain SI50-2D [cir^0] to leucine prototrophy. One strain, which displayed stable leucine prototrophy and was shown by Southern blot hybridization analysis to carry a single copy of the complete plasmid integrated at *RAS1,* was retained and designated R1-2μ-1.

Strains R1-2μ-1, R2-2μ-1, and R2-2μ-2 were each mated with four [cir$^+$] chromosomal tester strains described by Klapholz and Esposito (1982): K382-19D, K381-9D, K393-35C, and K396-22B (see Table 6). These strains carry the *FLP* gene on their 2μ circle. Among these four strains are recessive genetic markers for 16 of the 17 yeast chromosomes. Diploid strains were purified and grown in YPD medium to approximately 5 × 10^7 cells/ml. Appropriate dilutions of the cultures were plated on YPD plates to give approximately 500 colonies per plate. After growth at 30°C, the colonies were replica-plated to appropriate selection or indicator plates. Replica plates were scored after one day's growth at 30°C.

For diploid R1-2μ-1/K396-22B approximately 5% of the colonies tested were Leu$^-$, demonstrating that the chromosome harboring the integrated plasmid is mitotically unstable. In addition, 5% of the diploid R1-2μ-1/K382-19D colonies were Ade$^-$ and 3% of the diploid R1-2μ-1/K381-9D colonies were petite. No other auxotrophic colonies were observed with any of the diploids constructed from strain R1-2μ-1. These data indicate that in strain R1-2μ-1, plasmid YEp*RAS1*-2 is integrated into chromosome XV and therefore that *RAS1* is located on that chromosome.

For diploid R2-2μ-1/K396-22B approximately 8% of the colonies tested were Leu$^-$, demonstrating that the chromosome into which the plasmid is integrated in strain R2-2μ-1 is mitotically unstable. In addition, 6% of the diploid R2-2μ-1/K393-35C colonies were Met$^-$ and 30% of these Met$^-$ colonies were also petite. No other auxotrophic colonies were observed with any of the diploids formed with

Table 1. Viability of Haploid Progeny from *ras1*$^-$/*RAS1 ras2*$^-$/*RAS2* Diploids

Segregation of *ras1*::*LEU2* and *ras2*::*URA3*	Number of Tetrads	Viability[a]			
		4:0	3:1	2:2	1:3
PD	8	6	2	0	0
T	22	0	21	1	0
NPD	10	0	0	10	0
Unassigned	2	0	0	2	0

Diploids JR10 and JR11 (*leu2*/*leu2 ura3*/*ura3 ras1*::*URA3*/*RAS1 RAS2*/*ras2*::*LEU2*) were sporulated and dissected. These diploids were formed by mating strain JR9-8C with either ST-1 or ST-2. See Table 6 for strain description. The genotypes of the resultant spores were determined by assessing growth on appropriate prototrophic selection plates. When possible, genotypes of nonviable spores were assigned on the basis of the genotypes of the viable spores in the tetrad, assuming normal Mendelian segregation of *RAS1* and *RAS2*. A tetrad was classified as exhibiting parental ditype (PD) segregation of *RAS1* and *RAS2* if the four spores from the tetrad had the phenotypes Leu$^+$ Ura$^-$, Leu$^+$ Ura$^-$, Leu$^-$ Ura$^+$, and Leu$^-$ Ura$^+$. A tetrad was said to exhibit tetratype (T) segregation if the three viable spores had the phenotypes Leu$^-$ Ura$^-$, Leu$^-$ Ura$^+$, and Leu$^+$ Ura$^-$. A tetrad was said to exhibit nonparental ditype (NPD) segregation if the two viable spores had the phenotypes Leu$^-$ Ura$^-$ and Leu$^-$ Ura$^-$. Those tetrads in which the genotypes of nonviable spores could not be unambiguously determined were classified as unassigned.

[a] Viability of spores was determined after four days growth at 30°C on YPD plates. Those tetrads with four viable and no nonviable spores are designated 4:0, and so on. No viable spores with a Leu$^+$ Ura$^+$ phenotype were observed.

strain R2-2μ-1. Diploids obtained with R2-2μ-2 displayed the same pattern of instability. These results indicate that *RAS2* is located on chromosome XIV.

Linkage Relations of *RAS1* and *RAS2*

As discussed in the previous section, we tentatively localized *RAS1* and *RAS2* to chromosomes XV and XIV, respectively. To confirm these assignments and to position the genes on their respective chromosomes, we used tetrad analysis to determine the linkage of the two genes to genetic markers previously located on these chromosomes.

The map position of *RAS1* was determined from tetrad analysis of diploid JR23. This strain is heterozygous for *his3* and *ade2,* both of which lie on chromosome XV. In addition, the strain is homozygous for *ura3* and carries the disrupted *ras1*::*URA3* allele at the *RAS1* locus on one homolog. Thus the segregation of the *ras1*::*URA3* allele in tetrads could be monitored by scoring *URA3*. Analysis of 28 complete tetrads from strain JR23 indicates that the *RAS1* locus is located 7 cM from *ade2* and 63 cM from *his3* (Table 2). From the same set of tetrads we calculate a 44 cM distance between *ade2* and *his3*, a value in good agreement with the published distance of 48 cM (Mortimer and Schild, 1982). Since *his3* is centromere distal to *ade2,* these data place the *RAS1* locus centromere proximal to *ade2*.

The map position of *RAS2* was determined from tetrad analysis of diploids JR12 and JR22. Both strains are heterozygous for *met4* and *pet8,* both of which lie on chromosome XIV. Strain JR12 is homozygous for *leu2* and heterozygous for the disrupted *ras2*::*LEU2* allele. Similarly, strain JR12 is homozygous for *ura3*– and heterozygous for the *ras2*::*URA3* allele. Thus segregation at the *RAS2* locus can be followed by scoring *LEU2* in tetrads from strain JR12 and by scoring *URA3* in tetrads from strain JR22. Eighteen complete tetrads from strain JR12 and nine from strain JR22 were scored and gave essentially identical segregation patterns. The data from these two crosses, which are pooled in Table 2, yield a distance of 2 cM between the *RAS2* locus and *met4*. Using the same set of tetrads, we calculated a distance of 64 cM between *pet8* and *met4,* in reasonable agreement with the published value (Mortimer and Schild, 1982). Consistent with these results, tetrad data from the diploids JR10 and JR11 (strains that were described in Table 1) indicate that *RAS2* lies approximately 80 cM from its centromere (data not presented).

Table 2. Linkage Relations of *RAS1* and *RAS2*

Strain[a]	Interval	PD	T	NPD	Linkage[b]
JR23	*RAS1-ade2*	24	4	0	7
	RAS1-his3	8	15	3	63
	ade2-his3	8	17	1	44
JR12 and JR22	*RAS2-met4*	26	1	0	2
	met4-pet8	5	11	2	64

[a] Strain JR12 was obtained by mating strains JR7-6B and JR9-7C. The pertinent genetic markers are *leu2/leu2 met4/+ pet8/+ +/ras2::LEU2*. Strain JR22 was obtained by crossing strains K393-35C and ST-1. The pertinent genetic markers are *ura3/ura3 met4/+ pet8/+ +/ras2::URA3*. Strain JR23 was obtained by mating strains JR10-13B and K382-19D. The pertinent genetic markers are *ura3/ura3 ade2/+ +/his3 +/ras1::URA3*.

[b] Linkage is given in centiMorgans (cM) and is calculated from the formula cM = 100 (T + 6 (NPD))/2 (PD + T + NPD), where PD is the number of parental ditypes, T the number of tetratypes, and NPD the number of nonparental ditypes.

Phenotype of Yeast Cells Containing *RAS2*^{val19}

Dramatic changes in growth properties result when transforming mutants of the mammalian *ras* genes are introduced into mammalian cells. Mutations that substitute valine for glycine at the 12th N-terminal amino acid position, for example, strongly activate the transforming potential of the mammalian H-*ras* gene (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982). The corresponding nineteenth amino acid position in the yeast *RAS2* protein is also glycine. In order to observe the phenotype of yeast cells expressing a *RAS2* gene encoding valine in position 19, we used oligonucleotides to site direct this mutation in a clone of the *RAS2* gene. To introduce the mutant gene, which we call *RAS2*^{val19}, or the corresponding wild-type gene into yeast cells, we utilized plasmids p*HIS3-RAS2*^{val19} or p*HIS3-RAS2,* both of which contain the *HIS3* gene (Struhl and Davis, 1980) (see Figure 1). By transforming *his3*$^-$ haploid strains SP1 and SX50-1C with these plasmids linearized at Xho I, we were able to site direct plasmid integration into the *HIS3* locus (Orr-Weaver et al., 1981). These transformed haploid cells therefore still contained their wild-type *RAS1* and *RAS2* genes at their respective loci.

We obtained His$^+$ transformants with p*HIS3-RAS2*^{val19} or p*HIS3-RAS2* at equal frequency. Blot hybridization indicated that 4/4 p*HIS3-RAS2* transformants and 15/16 p*HIS3-RAS2*^{val19} transformants contained one or more copies of the plasmids integrated at the *HIS3* locus. Haploid yeast cells containing the *RAS2*^{val19} gene are therefore viable. However, these haploid cells did not appear to grow as vigorously as haploid cells containing the *RAS2* gene at the *HIS3* locus. In particular, their plating efficiency in rich medium was reduced and their viability was reduced upon storage at 4°C.

We next attempted to find a definite phenotype associated with the expression of the mutant *RAS2*^{val19}. We found that haploids of either mating type containing the mutant *ras* gene could mate efficiently with strains XC13-3B or XC13-2A to form diploid cells. However, when the resulting diploids were plated onto sporulation medium, very few asci were observed, at least 100- to 1000-fold fewer than were observed in control diploids. Rather, the diploids were found to arrest in an unbudded state.

In order to measure the sporulation defect, we performed quantitative spore recovery experiments. For this purpose, we utilized the recessive drug resistance marker *can1* which renders haploid cells resistant to canavanine

Table 3. Sporulation Competence of Diploids Containing $RAS2^{val19}$

Diploid Strains	Crosses	*RAS2* Allele at *HIS3* Locus	Cloning Efficiency	
			Expt. 1	Expt. 2
ES141A	TK141 × XC13-3B	*RAS2*	6%	20%
ES141C	TK141 × XC13-3B	*RAS2*	4%	40%
ES241A	TK241 × XC13-2A	*RAS2*	5%	nt
ES241B	TK241 × XC13-2A	*RAS2*	5%	nt
ES132A	TK132 × XC13-3B	$RAS2^{val19}$	.01%	.09%
ES121A	TK121 × XC13-3B	$RAS2^{val19}$	.01%	.04%
ES131A	TK131 × XC13-3B	$RAS2^{val19}$	<.01%	.06%
ES232A	TK232 × XC13-2A	$RAS2^{val19}$	<.01%	nt
ES221A	TK221 × XC13-2A	$RAS2^{val19}$	.01%	nt
ES222A	TK222 × XC13-2A	$RAS2^{val19}$	.003%	nt

The indicated crosses were performed, creating new diploid strains with either the *RAS2* or the $RAS2^{val19}$ alleles integrated into the *HIS3* locus as indicated. See Table 6 and text for details. Cloning efficiency is calculated as the percentage of Can^R colonies formed per total number of cells that were treated with glusulase and plated onto canavanine plate as described in Experimental Procedures. In Experiment 1, sporulation was performed on plates containing potassium acetate with limiting amounts of glucose and yeast extract. In Experiment 2, sporulation was performed in aerated liquid cultures (1% potassium acetate) after preculture in rich medium containing potassium acetate. See Experimental Procedures for more detail. nt = not tested.

(Siddiqi, 1971; Whelan et al., 1979). Six independent diploids with the genotype *can1/+ his3/his3*::p*HIS3-*$RAS2^{val19}$ and four with the genotype *can1/+ his3/ his3*::p*HIS3-RAS2* were sporulated, treated with 10% glusulase in H_2O, and plated onto medium containing canavanine. Plating efficiency on this medium reflects sporulation efficiency since the diploids are phenotypically sensitive to canavanine. The results of this experiment (Table 3) suggest that sporulation efficiency in diploids containing the $RAS2^{val19}$ gene is at least 500-fold lower than controls. A disproportionate number of these colonies that grew on canavanine were histidine auxotrophs (data not shown) suggesting that the few diploids which could sporulate may have deleted the $RAS2^{val19}$ gene inserted at the *HIS3* locus.

In order to confirm the linkage between a sporulation defective phenotype and the presence of the $RAS2^{val19}$ gene in a more rigorous manner, a single yeast haploid strain, TK232, which had integrated a single copy of the p*HIS3-*$RAS2^{val19}$ at the *HIS3* locus, was grown for several generations in rich medium and His^+ and His^- subclones were obtained. From the His^+ subclones, additional His^- subclones were derived. All of these subclones were tested for the ability to sporulate when mated with the yeast haploid strain XC13-2A. In addition, DNAs were prepared and the presence of the $RAS2^{val19}$ gene at the *HIS3* locus was determined by Southern blot hybridization. The results, shown in Table 4, clearly show that diploids sporulate well if and only if they do not contain the $RAS2^{val19}$ gene.

Since diploids containing the $RAS2^{val19}$ gene failed to sporulate, we tested whether such diploids might fail to arrest properly in G1 when deprived of essential nutrients (Johnston et al., 1977). However, such diploids were indistinguishable from control diploids in that they arrested in the unbudded G1 state when deprived of sources for either nitrogen, sulfate, or phosphate (Table 5).

Table 4. Linkage of Sporulation Defect to Presence of $RAS2^{val19}$

Strain	Parent Strain	His Phenotype	Sporulation Phenotype	$RAS2^{val19}$ Insert
SX50-1C		−	+	−
TK232	SX50-1C	+	−	+
H-11	TK232	−	+	−
H-12	TK232	−	+	−
H-13	TK232	−	+	−
H-A	TK232	+	+	−
H-B	TK232	+	−	+
H-C	TK232	+	+	−
H-D	TK232	+	−	+
W#1	H-B	−	+	−
X#1	H-D	−	−	+

Strains H-11, H-12, and H-13 are His^- subclones of the p*HIS3-*$RAS2^{val19}$ transformant TK232; strains H-A, H-B, H-C and H-D are His^+ subclones of TK232. W#1 is a His^- revertant of H-B, and X#1 is a His^- revertant of H-D. X#1 presumably arose from H-D by gene conversion of *HIS3*. The other His^- revertants probably arose by deletion of p*HIS3-*$RAS2^{val19}$. All strains had the other prototrophic markers of SX50-1C. Sporulation phenotype was determined by first mating the strains to XC13-2A. The resulting diploids were sporulated for 36–48 hr on potassium acetate plates containing limiting amounts of glucose and yeast extract, and then examined microscopically. Strains were considered Spo^+ if >10% of the cells had formed asci; Spo^- if <0.1% of the cells had formed asci. Presence of the $RAS2^{val19}$ insert was determined by Southern blot hybridization analysis.

Discussion

The *ras* genes, which were first discovered as the transforming elements of the Harvey and Kirsten sarcoma

Table 5. Growth Arrest of Diploids Containing *RAS2*^{val19}

Diploid Strains	*RAS2* Gene at *HIS3* Locus	Percentage of Unbudded Cells				Sporulation Efficiency
		$-NH_4$	$-SO_4$	$-PO_4$	−Mg	
ES141A	*RAS2*	99%	>99%	97%	75%	20%
ES241A	*RAS2*	>99%	99%	99%	63%	13%
ES121A	*RAS2*^{val19}	96%	>99%	98%	52%	<0.1%
ES221A	*RAS2*^{val19}	99%	98%	98%	60%	<0.1%

Prototrophic diploid strains are described in the legend to Table 3. Logarithmically growing cultures, with 57% to 76% budded cells, were washed extensively and were either resuspended into synthetic medium lacking either ammonium ions, sulfate ions, phosphate ions, or magnesium ions, or plated into sporulation medium. Twenty-four hours later, cells were observed by phase microscopy at 400× magnification and the percentage of budded and unbudded cells was determined. Magnesium deprivation leads to random cell-cycle arrest (Pringle and Hartwell, 1982). Percentage sporulation was determined as described in the legend to Table 4.

Table 6. Yeast Strains Used In This Study

Strain	Genotype
DC5/6[a]	*MATa/MATα leu2/leu2 his3/+ his4/+ can1/can1*
SP1[a]	*MATa leu2 ura3 trp1 his3 ade8 can1*
SX50-1C[a]	*MATα leu2 ura3 trp1 his3*
XC13-3B[a]	*MATα his3*
XC13-2A[a]	*MATa his3 can1*
SI50-2D[cir^0][b]	*MATa leu2 ura3 trp1 his3 ade8 can1* [cir^0]
K382-19D[c]	*MATα spo11 ura3 can1 cyh2 ade2 his7 hom3 tyr1*
K381-9D[c]	*MATα spo11 ura3 ade6 arg4 aro7 asp5 met4 lys2*
K393-35C[c]	*MATα spo11 ura3 his2 leu1 lys1 met4 pet8*
K396-22B[c]	*MATα spo11 ura3 ade1 his1 leu2 lys7 met3 trp5*
JR7-6B[d]	*MATa leu2 met4 pet8 his(2,4) ura3 lys1*
ST-1, ST-2[e]	*MATa leu2 ura3 trp1 his3 ade8 can1 ras1::URA3*
TK141[f]	*MATa leu2 ura3 trp1 ade8 can1 his3::pHIS3-RAS2*
TK132, TK121, TK131[f]	*MATa leu2 ura3 trp1 ade8 can1 his3::pHIS3-RAS2*^{val19}
TK241[f]	*MATα leu2 ura3 trp1 his3::pHIS3-RAS2*
TK232, TK221, TK222[f]	*MATα leu2 ura3 trp1 his3::pHIS3-RAS2*^{val19}
X2-2-4C[g]	*MATa leu2 can1 ras2::LEU2*
JR9-8C[h]	*MATα leu2 ura3 ras2::LEU2*
JR9-7C[h]	*MATα leu2 his3 trp1 can1 ras2::LEU2*
JR10-13B[i]	*MATa leu2 ura3 his3 trp1 ras1::URA3 can1*

[a] These strains are from the collection at Cold Spring Harbor Laboratory.
[b] SI50-2D was obtained from D. Botstein, and cured of the 2μ circle by J. Broach.
[c] These chromosomal tester strains are described by Klapholz and Esposito (1982).
[d] This strain was constructed from a cross between K392-35C and a *MATa leu2 his4* strain from CSH. The strain is phenotypically His$^-$, but it is not known whether the genotype is *his4, his2,* or *his4 his2.*
[e] These Ura$^+$ strains were obtained after transformation of SP1 with the Eco RI/Bam HI fragment of p*ras1::URA3.*
[f] These His$^+$ strains were obtained after transformation of either SP1 or SX50-1C with the plasmid p*HIS3-RAS2* or p*HIS3-RAS2*^{val19}.
[g] This strain is a haploid progeny from a DC5/6 diploid transformant containing one *RAS2* allele disrupted by *LEU2.*
[h] These strains are haploid progeny from diploids arising by crosses between X2-2-4C and SX50-1C.
[i] This strain is a haploid progeny from a diploid arising by a cross between JR9-8C and ST-1.

viruses, probably play an important role in the control of cellular proliferation. Several members of the *ras* gene family are found in mammals, and the genes have been found in organisms as distant as man and yeast. Little is known, however, of the precise cellular function of these genes. It is not known, for example, whether the *ras* genes are essential genes or whether the different members of the family have distinguishable physiological roles. Although it is known that the *ras* proteins bind guanine nucleotides and are bound to the internal cell membrane, their other biochemical interactions are largely unknown. Analysis of gene function in higher vertebrates is complicated by the large size of the cellular genome and the lack of the full repertoire of tools for genetic manipulation that

is available in some of the simpler, faster growing single cell eucaryotes. For that reason, we have begun an analysis of the function of the *ras* genes in the yeast S. cerevisiae.

In yeast there are at least two closely related but distinct members of the *ras* gene family, *RAS1* and *RAS2*. We have clearly demonstrated that neither the *RAS1* nor the *RAS2* gene is in itself essential for the vegetative growth of haploid yeast cells. However, by performing tetrad analysis of doubly heterozygous diploids, we have determined that spores which contain neither intact *ras* gene never resume vegetative growth. Thus some *ras* function is essential for cellular growth and *RAS1* and *RAS2* form a complementary set. Hence the *YP2* gene described by Gallwitz et al. (1983), which encodes a protein weakly homologous to the mammalian and yeast *ras* proteins, is not a member of this set. As yet we have no phenotype that corresponds to disruption of *RAS1* and *RAS2* alone, and so we cannot distinguish their physiological roles. This is somewhat of a puzzle since *RAS1* and *RAS2* proteins show little sequence homology for over 100 amino acid positions at their carboxy ends (Powers et al., 1984).

Nearly half the spores that are *ras1*$^-$ *ras2*$^-$ do germinate, but these germinated spores never bud. It thus appears that *ras* gene function is required during some portion of the cell cycle prior to S phase. Some *ras* function could also be required for other portions of the cell cycle. It may also be that *ras* function is required for germination and that those spores that do germinate do so with residual *ras* protein inherited from their parental diploids.

We previously noted strong sequence homology between the N-terminal domains of the mammalian and yeast *ras* proteins. This homology is particularly strong at all the positions where missense mutations are known to activate the mammalian *ras* protein (Powers et al., 1984). To test the extent of functional homology between mammalian and yeast *ras* proteins, we constructed in vitro the yeast *RAS2*^{val19} allele which is analogous to one of the transforming alleles of mammalian *ras* genes. Yeast diploids which contain a single copy of this allele sporulate very inefficiently, if at all, even when they also contain wild-type *ras* genes. Thus like its mammalian counterpart, the mutant yeast *ras* gene acts in a dominant way to alter the growth properties of diploid yeast cells. These results are compelling evidence that, in their molecular interactions, the yeast and mammalian *ras* proteins behave similarly.

The sporulation defect we observe in diploid yeast cells may not be the most informative or important phenotypic consequence of cells containing the *RAS2*^{val19} allele. Indeed, it is apparent that this allele induces other changes in the growth of yeast cells as well, but we have not precisely defined these changes. Nevertheless, the sporulation defect may provide us with a clue to *ras* function. Sporulation has often been taken as a model system for differentiation, and has been extensively studied both in yeast and in bacteria. In the yeast S. cerevisiae, sporulation is under very complex genetic controls and is normally induced only under precise conditions of nutritional stress (Esposito and Klapholz, 1982; Freese et al., 1982). Despite this complexity, it may nevertheless be of value to speculate that *ras* proteins are involved in the process by which diploids assess their nutritional status and decide whether to arrest in G1, sporulate, or progress through the next round of cell division. From this point of view, it is of interest that sporulation in B. subtilis can be induced by decreases in guanine nucleotide levels (Ochi et al., 1982). Since the mammalian *ras* proteins bind guanine nucleotides (Shih et al., 1980, 1982; Papageorge et al., 1982), it is amusing to speculate that the signals for sporulation, if not the actual proteins, have been conserved from bacteria to yeast. How this response may have been adapted during the evolution of the metazoa is a still more intriguing question.

None of the previously mapped yeast cell cycle or sporulation mutant genes have the chromosomal location we find for the yeast *RAS1* and *RAS2* loci. In a survey of the literature, we have found descriptions of dominant acting sporulation defective mutants but they are largely uncharacterized and their chromosomal positions have not yet been assigned (Esposito and Klapholz, 1982; Esposito et al., 1972). Since neither inactivation of *RAS1* or *RAS2* alone induces a noticeable phenotype it is unlikely that defective *ras* mutants would have been previously isolated as recessive mutations, such as temperature-sensitive lethals.

Experimental Procedures

Growth, Growth Arrest, and Sporulation Conditions

Routine culturing of yeast was performed on YPD plates ("rich medium") (2% Bacto-peptone, 1% yeast extract, 2% glucose, and 2% agar). Selection of transformants and diploids was carried out with synthetic medium (2% glucose, 0.67% Yeast Nitrogen Base, and appropriate amino acids and nucleic acid bases). Sporulation was performed in two different ways. The first method utilized overnight cultures of diploid cells on YPD plates which were then transferred to plates containing 2% potassium acetate, 0.1% yeast extract, and 0.05% glucose. Thirty-six to 48 hours later, the cells were either examined microscopically or assayed for sporulation competence as described below. The second method of sporulation involved logarithmic growth of diploid cells in 2% potassium acetate, 2% Bacto-peptone, and 1% yeast extract to an OD_{600} of 1.0, washing, and resuspension at the same density in 1% potassium acetate. The cells were then incubated for 36 hr at 30°C with shaking, and then either observed microscopically or assayed for sporulation competence. Sporulation competence of diploids heterozygous for the recessive drug marker *can1* (Siddiqi, 1971; Whelan et al., 1979) was assayed by treating sporulated cultures with 10% glusulase in H_2O, sonicating to disrupt asci, and plating onto canavanine plates (synthetic medium with appropriate amino acids and nucleic acid bases plus 60 μg/ml canavanine). For growth arrest experiments, we used the synthetic medium described by Johnston et al. (1977).

Site-Directed Mutagenesis

A 15-mer oligonucleotide 5′AACACCAACACCACC-3′ that contained one transversion (C → A) at the codon of the nineteenth amino acid residue of the *RAS2* gene was synthesized by the modified phosphate triester method (Efimov et al., 1982) and used for site-directed mutagenesis of the corresponding position of the *RAS2* gene as described by Zoller and Smith (1983). We used single-stranded DNA of an M13mp8 phage containing a 1.9 kb Eco RI–Pst I fragment of the *RAS2* gene as a template. Mutagenized phages were identified by in situ plaque hybridization (Benton and Davis,

1977) with the oligonucleotide labeled by polynucleotide kinase and γ-^{32}P-ATP, and confirmed by DNA sequencing (Sanger et al., 1977). The Eco RI/Pst I fragment of the mutant phage was transferred to the corresponding position of p*HIS3-RAS2,* replacing the Eco RI/Pst I fragment of the normal *RAS2* gene. This was also confirmed by DNA sequencing (Maxam and Gilbert, 1980).

Other Methods

General genetic manipulations of yeast were carried out essentially as described (Mortimer and Hawthorne, 1969). Yeast transformations were done according to the methods of Beggs (1978). Yeast DNAs were prepared essentially as described (Struhl et al., 1979). Plasmid DNAs were prepared by centrifugation in ethidium bromide-cesium chloride gradients (Tanaka and Weisblum, 1975). DNA restriction endonuclease digestions were performed under conditions recommended by suppliers (New England Biolabs or Bethesda Research Laboratories). Nitrocellulose filter blot hybridization was performed as described by Southern (1975). The filters were hybridized with appropriate DNA fragments or plasmids labeled by nick translation (Maniatis et al., 1975). Chromosomal mapping by integration of the 2μ circle *FLP* site was carried out essentially as described (Falco et al., 1983). See the text for more detail.

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