

## An Adenylate Cyclase from *Saccharomyces cerevisiae* That Is Stimulated by RAS Proteins with Effector Mutations

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**Conservative amino acid substitutions were introduced into the proposed effector regions of both mammalian Ha-ras (residues 32 to 40) and *Saccharomyces cerevisiae* RAS2 (residues 39 to 47) proteins. The RAS2[Ser 42] protein had reduced biological function in the yeast *S. cerevisiae*. A *S. cerevisiae* strain with a second-site suppressor mutation, *SSR2-1*, was isolated which could grow on nonfermentable carbon sources when the endogenous RAS2 protein was replaced by the RAS2[Ser 42] protein. The *SSR2-1* mutation was mapped to the structural gene for adenylate cyclase (*CYR1*), and the gene containing *SSR2-1* was cloned and sequenced. *SSR2-1* corresponded to a point mutation that would create an amino acid substitution of a tyrosine residue for an aspartate residue at position 1547. The *SSR2-1* gene encodes an adenylate cyclase that is dependent on ras proteins for activity, but is stimulated by Ha-ras and RAS2 mutant proteins that are unable to stimulate wild-type adenylate cyclase.**

The *ras* genes encode an evolutionarily conserved family of proteins found in higher and lower eucaryotic cells (15) that share intrinsic biochemical properties with guanine nucleotide-binding regulatory proteins (G proteins) (15, 16, 17, 34). Oncogenic forms of the mammalian *ras* proteins have amino acid substitutions at positions 12, 59, and 61 that reduce the GTPase activity of the proteins (14, 25, 34). Similar substitutions in *Saccharomyces cerevisiae* RAS1 and RAS2 proteins significantly lower the GTPase activities of these proteins (34). These activated forms of the *S. cerevisiae* RAS proteins induce distinct phenotypic changes in yeasts, including reduction in cell viability, glycogen storage, and sporulation (23, 35). Results of <sup>32</sup>P-labeling studies in yeast cells have shown that the forms of both mammalian and *S. cerevisiae* *ras* proteins with impaired GTPase activity immunoprecipitate with more GTP than do the wild-type forms of the *ras* proteins (13). The similarity of mammalian and *S. cerevisiae* *ras* proteins is reflected by the ability of the mammalian *ras* protein to complement RAS disruptions in yeasts and the ability of a modified RAS1 protein to transform NIH 3T3 cells (8, 20).

Whereas the exact function of mammalian *ras* proteins is unknown, *ras* proteins in *S. cerevisiae* have been shown to regulate cell growth by stimulating adenylate cyclase through a yet to be identified mechanism (4, 35). Production of cyclic AMP (cAMP) in turn activates cAMP-dependent protein kinases which promote progression through the cell cycle (5, 24). Deletion of both RAS1 and RAS2 genes is a lethal event which can be bypassed by the presence of mutations that increase adenylate cyclase activity or eliminate the requirement of cAMP for growth (5, 35). Deletion of the RAS2 gene alone prevents cells from growing on nonfermentable carbon sources, apparently because of the repression of RAS1 expression that occurs during growth on nonfermentable carbon sources (3, 5, 10).

We have reported previously (31) the identification of a region of the Ha-ras protein that encompasses residues 32 to 40 in which the introduction of single amino acid substitutions did not affect the known biochemical properties of the *ras* protein (guanine nucleotide binding and membrane local-

ization) but did reduce the ability of the proteins to transform mammalian cells and to stimulate yeast adenylate cyclase. Willumsen et al. (38) have demonstrated that this region could not be deleted without affecting the biological activity of the *ras* proteins. This proposed effector domain is conserved in all *ras* proteins which can function in heterologous systems such as Ha-ras, Ki-ras, N-ras, RAS1, RAS2, and *Schizosaccharomyces pombe* *ras1* (for a review, see reference 31). The amino acid sequences of the corresponding regions in related *ras*-like proteins such as *YPT*, *rho*, and *era* are not conserved (1, 12, 21). The phenotypes associated with the *ras* mutations at residues 32 to 40 suggest that this domain may be critically involved in the interaction of the *ras* protein with its target, either by determining an active conformation or by being a site for protein-protein interaction.

In this study we introduced conservative amino acid substitutions into the effector region of the Ha-ras protein and the analogous region at residues 39 to 47 of the RAS2 protein. The mutant RAS2[Ser 42] protein was observed to have weak effector activity in *S. cerevisiae* cells and was used in a genetic screen to isolate a second-site mutation in the adenylate cyclase gene *CYR1*, which restored the ability of the RAS2[Ser 42] mutant protein to function effectively in *S. cerevisiae*. The ability of this mutant adenylate cyclase to function in vivo with effector mutant *ras* proteins was characterized.

### MATERIALS AND METHODS

**Yeast strains, bacterial strains, and media.** The strains of *S. cerevisiae* used in this study are listed in Table 1. Recombinant DNA manipulations and plasmid maintenance were performed in *Escherichia coli* strains HB101, DH5, and DH5 $\alpha$ . *S. cerevisiae* strains were grown in minimal SD medium supplemented with required amino acids (30) or rich medium (1% yeast extract [Difco Laboratories, Detroit, Mich.], 2% soy peptone [Merck Sharp & Dohme, West Point, Pa.] and 2% dextrose [YEHD], 3% glycerol [YGP], 3% potassium acetate [YPA], or 3% ethanol [YPE]). Bacteria were grown in Luria broth (22) supplemented with 100  $\mu$ g of ampicillin per ml.

**Plasmid construction and transformation.** The construction

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
112.699	$\alpha$ <i>ras2-699(HIS3) leu2-3,112 his3 ura3-1 can1-100 ade2-1</i>	8
112.699-2	$\alpha$ <i>SSR2-1 ras2-699(HIS3) leu2-3,112 his3 ura3-1 can1-100 ade2-1</i>	This study
112.699-6 <sup>a</sup>	$\alpha$ <i>ssrX-1 ras2-699(HIS3) leu2-3,112 his3 ura3-1 can1-100 ade2-1</i>	This study
112.699-8 <sup>a</sup>	$\alpha$ <i>ssrX-2 ras2-699(HIS3) leu2-3,112 his3 ura3-1 can1-100 ade2-1</i>	This study
MM20-6C	$\alpha$ <i>cdc35-10 ras2-699(HIS3) leu2-3,112 his3 ura3-52 trp1</i>	This study
MM41-3A	$\alpha$ <i>SSR2-1 ras2-530(LEU2) leu2-3,112 ura3</i>	This study
RX13-17A	$\alpha$ <i>sra1-18 ras2-530(LEU2) leu2 ura3-52 trp1 his4 lys2</i>	K. Tatchell
JC302-26D	$\alpha$ <i>ras2-530(LEU2) leu2 ura3-52 his4</i>	J. Cannon
JC337-2B	$\alpha$ <i>ras1-545(URA3) leu2-3,112 ura3-52 lys2-801 can1 cyh2</i>	J. Cannon
JC365-5A	$\alpha$ <i>ras2-530(LEU2) leu2-3,112 trp2 met3 ino1</i>	J. Cannon
JC377-16C	$\alpha$ <i>SRA4-6 leu2-3,112 ura3-52 lys2-801 can1</i>	J. Cannon
JC410-24D	$\alpha$ <i>cdc35-10 leu2-3,112 ura3-52 his3-11,15 trp1-1</i>	J. Cannon
JC465-4A	$\alpha$ <i>ras2-699(HIS3) leu2 his3 ura3 trp1 can1</i>	J. Cannon

<sup>a</sup> Although the mutations in strains 112.699-6 and 112.699-8 appear to be in the *sra1-18* complementation group, they have not been genetically mapped and may be unlinked. The *ssrX* designation is only tentative.

of YCp50-RAS2 and AAH5-Ha-*ras* has been fully described in detail elsewhere (8, 23). The plasmid pUC13-CYR1-11 was derived from pUC13 (Pharmacia Fine Chemicals, Piscataway, N.J.) and pAC2 (a gift from G. Casperson and H. Bourne), which contains the *CYR1* gene. Plasmid pAC2 was digested with *AvrII* such that only a single cleavage was introduced into the plasmid. The 5' overhang was made flush with the Klenow fragment of DNA polymerase I, and *BamHI* linkers were attached with T4 DNA ligase. The DNA was then digested to completion with *BamHI*, and the 7,161-base-pair (bp) fragment was purified and ligated with *BamHI*-digested pUC13. YCpCYR1 was constructed by subcloning the 7,161-bp *BamHI* fragment from pUC13-CYR1-11 into the *BamHI* site of YCp50. YCpCYR1 $\Delta$ , which contained a 3.6-kilobase-pair internal deletion in the *CYR1* coding region, was constructed by digesting YCpCYR1 with *SacI*, isolating the vector fragment, and religating the DNA with T4 DNA ligase. An *SSR2-1* mutant genomic library was made by partial digestion of yeast strain 112.699-2 genomic DNA with *MboI*, isolating 10- to 15-kilobase-pair fragments from a 10 to 40% sucrose gradient (22), and ligating the fragments into the *BamHI* site of pBR322. Competent DH5 and DH5 $\alpha$  cells were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and transformed according to the protocol recommended by the manufacturer. *S. cerevisiae* cells were transformed by the lithium acetate method described by Ito et al. (18). Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England BioLabs, Inc. (Beverly, Mass.). DNA polymerase I Klenow fragment was purchased from Bethesda Research Laboratories. Standard methodologies for manipulating recombinant DNA were followed (22).

**Mutagenesis of RAS2 and Ha-*ras*.** Site-specific mutations were introduced into the *RAS2* and *Ha-ras* genes by oligonucleotide-directed mutagenesis as described previously (14, 32). Each mutation was confirmed by dideoxynucleotide DNA sequencing (see below). In addition, the entire coding region of the *RAS2*[Ser 42] mutant gene was also sequenced.

**DNA sequencing.** Dideoxynucleotide DNA sequencing was performed on alkali-denatured supercoiled plasmids according to a modification of the method described by Chen and Seeburg (7). Reactions were performed with a <sup>35</sup>S-sequencing kit (Pharmacia); the DNA polymerase I Klenow fragment (Bethesda Research Laboratories) and [ $\alpha$ -<sup>35</sup>S]dATP (>600 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). Oligonucleotide primers were designed from the published *CYR1* sequence (19).

**Yeast genetic techniques.** The standard genetic manipulations described by Sherman et al. (30) were followed. Meiotic map distances were calculated by using the formulas summarized by Mortimer and Schild (26). Marker rescue of genomic DNA by homologous recombination with linearized plasmids was based on the methodology of Orr-Weaver et al. (27) and Rothstein (29). To isolate the *CYR1* gene by marker rescue from the *SSR2-1* mutant strain 112.699-2, plasmid YCpCYR1 $\Delta$  was digested with *SacI* and either *SacII* or *SnaBI* and then heated to 65°C for 5 min, followed by immediate chilling on ice. The linearized DNA was then used immediately to transform strain 112.699-2. Transformants were picked and used to prepare plasmid DNA by the method described by Volkert and Broach (37). The *CYR1* marker rescue plasmids were then transformed into *E. coli* and screened for the presence of a full-sized *CYR1* gene. By this criterion, marker rescue occurred in greater than 90% of the original yeast transformants derived from the linearized DNA.

**Biological determination of RAS function.** The *RAS1 ras2 S. cerevisiae* strain 112.699 was transformed with plasmids that expressed mutant *RAS2* and *Ha-ras* genes. The ability of mutant *RAS2* and *Ha-ras* proteins to function in vivo was determined by scoring the transformed strain for growth on nonfermentable carbon sources at 37°C (5, 8).

**Selection of second-site suppressors of *ras* effector mutations.** Mutations able to complement the *RAS2*[Ser 42] protein were isolated by screening for spontaneous mutations in strain 112.699, which promoted growth on nonfermentable carbon sources only in the presence of the YCp50-RAS2[Ser 42] plasmid. The *RAS1 ras2* strain 112.699 was transformed with YCp50-RAS2[Ser 42]. Single colony isolates were inoculated into 2-ml cultures of SD broth lacking uracil containing 5% glucose and incubated with aeration at room temperature to a cell density of 10<sup>6</sup> to 10<sup>7</sup> cells per ml. The cells were washed with water, and 10<sup>6</sup> to 10<sup>7</sup> cells were plated onto YPA or YPE plates followed by incubation at 37°C. After 3 to 4 days, three independent colonies were picked from each plate and streaked onto a fresh plate of the same medium. The cells were then grown again at 37°C. To test for plasmid dependence, the mutants were cured of the YCp50-RAS2[Ser 42] plasmid by inoculating a single colony from each mutant strain into 5 ml of YEHD broth followed by growth for 2 days at room temperature. The liquid cultures were then plated onto YEHD plates at a density of about 300 cells per plate, followed by a 2-day incubation at 30°C. The YEHD plates were then replica plated onto supplemented SD plates lacking uracil; these plates were

then incubated again at 30°C. The duplicate rich and uracil-selective plates were then aligned, and colonies which grew only on YEHD (without plasmid) or on both YEHD and SD without uracil (with plasmid) were picked. These same yeast mutants, with and without plasmid, were patched onto YPA or YPE plates and incubated at 37°C. Those mutants which required the YCp50-*RAS2*[Ser 42] plasmid for growth on YPA or YPE were further characterized. In the secondary screening, the plasmid-cured clones of the yeast mutants that required YCp50-*RAS2*[Ser 42] for growth on YPA or YPE were retransformed with the YCp50-*RAS2*[Ser 42] plasmid. The transformants were patched onto YPA or YPE plates and incubated at 37°C. Those mutants which grew on YPA or YPE after the plasmid was reintroduced were saved for the third screening. In the third screening the secondary transformants of each plasmid-dependent mutant were grown again in YEHD broth to segregate the plasmid. Cells which lost the plasmid were identified as described above. Those mutants which had once again been cured of plasmid were again patched onto YPA or YPE plates and incubated at 37°C. Those mutants which did not grow after this second round of plasmid curing were considered true second-site suppressors of *RAS* (*SSR* mutants).

**Adenylate cyclase assays.** The procedure followed for measuring adenylate cyclase activity in *S. cerevisiae* membrane fractions has been described in detail elsewhere (23). In a typical experiment, appropriate cell cultures were grown in selective liquid medium (SD without uracil or SD without leucine) at 30°C to an  $A_{660}$  of 0.5 to 1.0. Membrane fractions were prepared by glucuronidase digestion of the cells; Dounce homogenization in buffer containing 50 mM NaMES [2-(*N*-morpholino)ethanesulfonic acid; pH 6.0], 0.1 mM EGTA [ethylene glycol-bis( $\beta$ -aminoethylether)-*N,N,N',N'*-tetraacetic acid], 0.1 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol; and then centrifugation at 105,000  $\times$  *g* for 30 to 40 min at 4°C. Adenylate cyclase assays in which the conversion of [ $\alpha$ -<sup>32</sup>P]ATP to [<sup>32</sup>P]cAMP was measured were performed in a 100- $\mu$ l reaction volume at 30°C for 30 to 40 min, in the presence of 10 mM MgCl<sub>2</sub> or 1 mM MnCl<sub>2</sub>. Duplicate determinations varied by <10%. The assay was linear over the concentration range of membrane protein used (26 to 71  $\mu$ g). When stated, 100  $\mu$ M guanosine  $\beta$ , $\gamma$ -imidotriphosphate [Gpp(NH)p] (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was included in the reactions.

## RESULTS

**Effect of conservative amino acid substitutions in the effector region of *ras* proteins.** Previously (31), amino acid substitutions within the region encompassing residues 32 to 40 of the mammalian *Ha-ras* protein have been shown to reduce its ability to stimulate yeast adenylate cyclase. To more precisely define the residues that are required for effector function, we created additional conservative substitutions within this region of the *Ha-ras* protein and the analogous region at residues 39 to 47 of the *S. cerevisiae* *RAS2* protein (Fig. 1). The ability of these mutant *ras* proteins to complement the *ras2* phenotype of *S. cerevisiae* 112.699 was tested. In strain 112.699 the chromosomal *RAS2* gene has been inactivated by the insertion of the *HIS3* gene into the *RAS2* coding region, leaving only the *RAS1* gene functional (33). Cells that lacked a functional *RAS2* protein were unable to grow on nonfermentable carbon sources at elevated temperatures. Expression of either *RAS2* or *Ha-ras* restored the ability to grow on glycerol at 37°C (Fig. 1A and

B, lanes 2). The *RAS2* protein was expressed by subcloning the gene with its own promoter into the single-copy plasmid YCp50. For expression of *Ha-ras* proteins, the *Ha-ras* gene variants were subcloned into AAH5, a high-copy-number expression plasmid. Substituting the Thr at position 42 of the *RAS2* protein (corresponding to the Thr at position 35 in *Ha-ras*) with an Ala or a Ser residue eliminated or greatly reduced, respectively, the ability of the protein to promote growth on glycerol medium at 37°C (Fig. 1A, lanes 3 and 4). Only after an extended incubation of 4 days was growth observed for the *RAS2*[Ser 42] strain under these conditions. The *RAS2*[Ser 42] mutant protein was completely unable to promote growth on either ethanol or acetate medium (see Fig. 2A, lane 3). The conservative replacements of a Val residue for Ile at position 43 and a Leu residue for Phe at position 47 had only moderate effects on *RAS2* protein function (Fig. 1A, lanes 5 and 6). These results were reproduced in a different *ras2* strain, JC465-4A. Conservative amino acid substitutions in the *Ha-ras* protein at positions 33 (Asn for Asp) and 35 (Ser for Thr) eliminated its ability to complement the *ras2* defect (Fig. 1B, lanes 3 and 4). When Asp at position 38 of the *Ha-ras* protein was substituted with an Asn residue, there was only a slight reduction in biological activity (Fig. 1B, lane 5). As was reported previously for other substitutions in the region at positions 32 to 40 of *Ha-ras*, the expression of the *Ha-ras*[Ser 35] protein had a profound growth-retarding effect on the host strain when grown on glucose media (31). In contrast, expression of the *Ha-ras*[Asn 33] protein had little effect on the growth rate of the host. The ability of the *RAS2* proteins to couple with adenylate cyclase in vivo was examined by using iodine staining to monitor intracellular glycogen levels. The amount of glycogen that accumulated in the *RAS1 ras2* strain 112.699 when the *RAS2*[Ser 42] protein was expressed was less than that which accumulated when only the vector was present but more than that which accumulated when the *RAS2* protein was expressed.

The validity of the glycerol assay to screen for the *ras* function was tested by expressing the *RAS2*[Ser 42] protein in a *ras1 ras2* background and scoring its ability to promote spore outgrowth. *S. cerevisiae* with the *ras1 ras2* YCp50-*RAS2*[Ser 42] was obtained by sporulating diploids made by crossing the *ras2* strain 112.699 transformed with YCp50-*RAS2*[Ser 42] with the *ras1* strain JC337-2B. The *RAS1* gene in JC337-2B was inactivated by insertion of the *URA3* gene (33), allowing the meiotic segregation of both the *ras1::URA3* and *ras2::HIS3* alleles to be followed. Although the YCp50-*RAS2*[Ser 42] plasmid also had a *URA3* marker, *ras1 ras2* spores could be identified in some cases by following marker segregation in all four spores of a single tetrad. Viable *ras1 ras2* spores were assumed to contain the

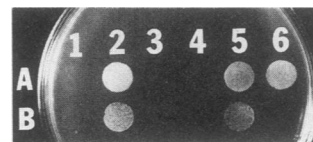


FIG. 1. Effect of *ras* effector region mutations on function. The *RAS1 ras2* strain 112.699 was transformed with *RAS2* derivatives on YCp50 and with *Ha-ras* derivatives on AAH5 and spotted from overnight liquid cultures onto YPG plates followed by incubation for 2 days at 37°C. (Row A) Lane 1, YCp50; lane 2, *RAS2*; lane 3, *RAS2*[Ala 42]; lane 4, *RAS2*[Ser 42]; lane 5, *RAS2*[Val 43]; lane 6, *RAS2*[Leu 47]. (Row B) Lane 1, AAH5; lane 2, *Ha-ras*; lane 3, *Ha-ras*[Asn 33]; lane 4, *Ha-ras*[Ser 35]; lane 5, *Ha-ras*[Asn 38].

YCp50-RAS2[Ser 42] plasmid. A control cross in the absence of the YCp50-RAS2[Ser 42] plasmid yielded no viable *ras1 ras2* spores. Of nine asci, two viable *ras1 ras2* spores were identified. The colonies resulting from these spores grew to only one-third the size of their sister colonies that contained wild-type *RAS1* or *RAS2* genes, presumably because of complementation by the *RAS2*[Ser 42] gene. These results were later reproduced with a diploid strain that was heterozygous for *ras1* and *ras2* gene disruptions constructed with markers other than *URA3*, allowing segregation of the YCp50-RAS2[Ser 42] plasmid to be followed (data not shown). These results confirm the usefulness of the *ras2* complementation assay on glycerol for determining the biological potency of *ras* proteins.

**Second-site suppressor of *ras* (*SSR*) mutants.** The procedure for selecting suppressors involved the isolation of spontaneous mutants of the *RAS1 ras2* strain 112.699 which grew on ethanol or acetate media only when the *RAS2*[Ser 42] plasmid was present. Of the 61 individual plasmid-requiring mutations that were identified, all but 11 of these mutations were located in the YCp50-RAS2[Ser 42] plasmid. These 11 *SSR* mutant strains were then cured of the YCp50-RAS2[Ser 42] plasmid and transformed with both YCp50-RAS2 and YCp50-RAS2[Ser 42]. Of the *SSR* mutants, six were observed to grow more vigorously on acetate or ethanol plates when transformed with the mutant *RAS2*[Ser 42] gene than with the wild-type *RAS2* gene (data not shown). Since a suppressor mutation that influences a protein-protein interaction probably would be dominant, we determined whether the mutations in these six strains were dominant or recessive. Each strain that was transformed with YCp50-RAS2[Ser 42] was mated with the *RAS1 ras2* strain JC465-4A under conditions that were selective for the plasmid. Of the resulting diploids, only those from the cross with the *SSR* strain 112.699-2 (YCp50-RAS2[Ser 42]) were able to grow on ethanol plates, indicating that 112.699-2 contained a dominant mutation. The other five strains, 112.699-1, -4, -6, -7, and -8, all contained recessive mutations. When assayed for the ability to grow on glycerol plates at 37°C in the absence of any *RAS2* plasmid, only the 112.699-6 and -8 strains showed no growth. The ability of several of the *SSR* strains to grow on various carbon sources in the absence or presence of the *RAS2* or *RAS2*[Ser 42] genes is summarized in Table 2.

**Genetic analysis of *SSR* strains.** Although recessive, the mutations in the 112.699-6 and -8 strains were considered interesting because their phenotypes were totally dependent on *RAS2*. To assign these mutations to a known complementation group, the 112.699-6 and -8 strains, with and without the YCp50-RAS2[Ser 42] plasmid, were mated with the *sral-18* strain RX13-17A. The resulting diploids were capable of growth on ethanol plates at 37°C only when the *RAS2*[Ser 42] plasmid was also present. Since the mutations present in strains 112.699-6 and -8 were recessive, the plasmid-dependent growth observed in these diploids suggests that these mutations are in the *sral* complementation group. The *sral-18* mutation was originally isolated as a recessive *ras2* suppressor that was capable of promoting cell growth on ethanol plates at 35°C, even in the total absence of the *ras* gene product (5). The *sral-18* mutation is believed to be in the gene that encodes the regulatory subunit of the cAMP-dependent protein kinase (5). As determined by iodine staining of storage carbohydrates (23), strain 112.699-8 has increased responsiveness to *RAS2*. It was also observed that the 112.699-6 and -8 strains grew more vigorously on ethanol plates in the presence of the *RAS2*[Ser 42] plasmid

than with the wild-type *RAS2* plasmid (data not shown). Since poor growth on ethanol medium is also a phenotype associated with the expression of the activated *RAS2*[Val 19] allele (unpublished data), we suspect that the *RAS2* allele specificity displayed by both the 112.699-6 and -8 strains may reflect higher than normal cAMP-dependent protein kinase activity. Additionally, mutant strain 112.699-6 was unable to grow on ethanol after transformation with the *Ha-ras*[Asn 33] gene. This implies that there is a requirement for *ras* proteins that are capable of some detectable activity.

The mutation present in strain 112.699-2 was of particular interest in that it was dominant and showed preference for the *RAS2*[Ser 42] protein during cell growth on acetate. The ability of 112.699-2 to grow on glycerol at 37°C in the absence of *RAS2* provided a plasmid-independent phenotype by which the mutation could be scored. This dominant phenotype was shown to be the result of a single mutation. The diploid resulting from the cross between 112.699-2 and the *ras2* strain JC465-4A was sporulated and subjected to tetrad analysis. The *SSR* mutation was followed through meiosis by scoring the ability of the spore progeny to grow on glycerol in the absence of *RAS2*. The ability to promote growth on glycerol in the absence of *RAS2* segregated 2<sup>+</sup>:2<sup>-</sup>. The plasmid-dependent *SSR* phenotype cosegregated with the ability to grow on glycerol, indicating that these two phenotypes are the result of a single mutation. The mutation in 112.699-2 was designated *SSR2-1*.

To test its responsiveness to other *ras* effector mutant proteins, the *SSR2-1* strain was transformed with the *Ha-ras*, *Ha-ras*[Asn 33], and *Ha-ras*[Ser 35] genes subcloned into the alcohol dehydrogenase (ADH) expression plasmid AAH5. On acetate medium at 37°C, the wild-type *Ha-ras* protein promoted better growth of the *SSR2-1* strain 112.699-2 than of the 112.699 control (Fig. 2A and B, lanes 5). The *Ha-ras*[Asn 33] protein, which was not able to complement the *ras2* defect in the 112.699 control, promoted growth of 112.699-2 on acetate (Fig. 2A and B, lanes 6). The *Ha-ras*[Ser 35] protein did not complement the *ras2* defect in either 112.699 or 112.699-6 (Fig. 2A and B, lanes 7). However, in contrast with strain 112.699, transformation of strain

TABLE 2. Relative growth of *SSR* mutant strains on nonfermentable carbon sources<sup>a</sup>

Strain	Plasmid	Colony diameter (mm) after growth on:		
		YEHD	YPG	YPA
112.699	YCp50	1.5	0.0	0.0
	YCp50-RAS2	2.0	1.2	0.7
	YCp50-RAS2[Ser 42]	2.0	1.0	0.0
112.699-2	YCp50	2.2	0.8	0.0
	YCp50-RAS2	2.0	1.2	0.4
	YCp50-RAS2[Ser 42]	2.0	1.5	0.6
112.699-6	YCp50	1.2	0.0	0.0
	YCp50-RAS2	2.0	1.2	0.4
	YCp50-RAS2[Ser 42]	2.2	1.2	0.2
112.699-8	YCp50	1.2	0.0	0.0
	YCp50-RAS2	2.2	1.2	0.5
	YCp50-RAS2[Ser 42]	2.2	1.2	0.2

<sup>a</sup> The parental *ras2* strain 112.699 and the *SSR* strains 112.699-2, -6, and -8 were transformed with the plasmids YCp50, YCp50-RAS2, and YCp50-RAS2[Ser 42]. Transformants were streaked for single-colony isolation on solid YEHD, YPG, or YPA medium. Following incubation for 4 days at 37°C, the average colony diameter for each strain was determined.

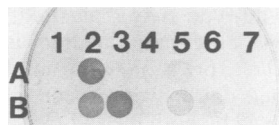


FIG. 2. *RAS* allele-dependent growth of *SSR2-1*. The *ras2* strains 112.699 and 112.699-2 (*SSR2-1*) were transformed with wild-type and mutant alleles of both *RAS2* (on YCp50) and *Ha-ras* (on AAH5). Suppression of defective *ras* alleles was determined by spotting stationary-phase liquid cultures onto a YPA plate followed by incubation at 37°C for 4 days. (Row A) 112.699. (Row B) 112.699-2. Lanes 1, YCp50; lanes 2, YCp50-*RAS2*; lanes 3, YCp50-*RAS2*[Ser 42]; lanes 4, AAH5; lanes 5, AAH5-*Ha-ras*; lanes 6, AAH5-*Ha-ras*[Asn 33]; lanes 7, AAH5-*Ha-ras*[Ser 35].

112.699-2 with *Ha-ras*[Ser 35] did not drastically reduce its rate of growth on glucose media. As observed in the original mutant screen, expression of the *RAS2*[Ser 42] protein in the *SSR2-1* strain promoted slightly more vigorous growth on acetate medium at 37°C than did expression of the wild-type *RAS2* protein (Fig. 2B, lanes 2 and 3). Strain 112.699-2 was also transformed with the *RAS2*[Ala 42] gene, which was unable to complement the *ras2* defect in strain 112.699 (Fig. 1A, lane 3). The *RAS2*[Ala 42] protein did not complement the *ras2* defect in the *SSR2-1* strain 112.699-2 (data not shown).

Qualitative measurement of glycogen levels by iodine staining of 112.699-2 revealed that there were lower levels of glycogen in the *SSR2-1* strain than in 112.699 (data not shown). Expression of the wild-type *RAS2* protein particularly lowered the amount of glycogen in the cell, which was indicative of increased *RAS2* activity. Curiously, expression of the activated *RAS2*[Ala 18, Val 19] protein in 112.699-2, while lowering glycogen levels at 30°C, actually increased the amount of glycogen in the cell when assayed after growth at 37°C. This result is in sharp contrast with that observed for the parental 112.699 control strain transformed with the same *RAS2*[Ala 18, Val 19] plasmid (23). A subsequent shift of 112.699-2 transformed with YCp50-*RAS2*[Ala 18, Val 19] from growth at 37 to 30°C followed by iodine staining demonstrated that the *RAS2*[Ala 18, Val 19] plasmid was stably maintained under the conditions of the assay.

The *SSR2-1* mutation was tested for its ability to promote cell growth in the complete absence of both *RAS1* and *RAS2* gene products. The 112.699-2 strain was crossed with JC302-26D, a strain that contains a *RAS2* gene disrupted by the insertion of *LEU2*. The resulting diploid was sporulated, and *SSR2-1 ras2::leu2* spores were identified. One such *SSR2-1 ras2::LEU2* strain, MM41-3A, was crossed with JC337-2B, a strain in which the *RAS1* gene is disrupted with *URA3*. The resulting diploid was sporulated, and the progeny were scored for the *Leu<sup>+</sup> Ura<sup>+</sup>* phenotype that is indicative of *ras1::URA3 ras2::LEU2* spores. Since disruption of both *RAS* genes in a haploid cell is lethal (33), survival of any *ras1 ras2* spores would have occurred only if a secondary mutation had been present which could have bypassed the need for *RAS* in the cell. Of 44 tetrads analyzed, no *Ura<sup>+</sup> Leu<sup>+</sup>* spores were observed. Of the asci that contained four viable spores, five of six were parental ditypes. Of the asci that contained three viable spores, 30 of 31 were tetratypes. Of the six asci that contained two viable spores, all were nonparental ditypes. Since the *SSR2-1* mutation is not linked to either *RAS1* or *RAS2* (data not shown), these results demonstrate that the *SSR2-1* mutation is *ras* dependent and cannot independently promote cell growth.

**Mapping of the *SSR2-1* mutation.** The location of the

*SSR2-1* mutation in the yeast genome was determined by meiotic gene mapping. When the *SSR2-1* mutant strain 112.699-2 was crossed with strains that contained the centromere-linked gene *trp1*, a strong centromere linkage was shown and the *SSR2-1* mutation was found within 1 centimorgan of an unidentified centromere (Table 3). Additional gene mapping placed the location of *SSR2-1* within 2.1 centimorgans of *met3*, a centromere-linked gene on chromosome X (Table 3). Earlier mapping data (2, 5, 24) for the structural gene of adenylate cyclase (*CYR1*, *CDC35*, *SRA4*) has placed its chromosomal location within 1.1 centimorgans of *met3* and within 3 centimorgans of centromere X. When strain 112.699-2 was crossed with a *cdc35-10* strain (Table 3), it became apparent that the *SSR2-1* mutation was most likely allelic with *CDC35* (*CYR1*). It was observed that the *SSR2-1* phenotype segregated 2:2 with the *cdc35-10* phenotype. Of 47 asci scored, all 47 were parental ditypes, locating the *SSR2-1* mutation less than 1 centimorgan away from *CDC35* (Table 3).

**Cloning of the *CYR1* gene containing the *SSR2-1* mutation.** Since the *SSR2-1* mutation was found to be either allelic or tightly linked to the *CYR1* gene locus, the *CYR1* gene from the *SSR2-1* strain 112.699-2 was cloned. A size-selected library of 112.699-2 genomic DNA was screened with an oligonucleotide probe that was specific for the wild-type *CYR1* gene. Seven separate positive clones were identified. Clone pUC13-*SSR2-11.1* was found to contain the entire *CYR1* gene as well as extensive flanking sequences and was used for further studies. Additionally, the *CYR1* gene from strain 112.699-2 was cloned independently by the marker rescue technique. In the marker rescue technique, homologous recombination is used to replace plasmid-borne gene sequences with chromosomal homologs (29). Two segments of the *SSR2-1* adenylate cyclase gene were obtained by marker rescue into YCp*CYR1*Δ (Fig. 3). The plasmid YCp*SSR2-1A* contained *CYR1* sequences derived from the *SSR2-1* mutant strain 112.699-2 that extended from the *SacI* site at base position 744 to the *SacII* site at base position 4805. Plasmid YCp*SSR2-1B* contained sequences rescued from strain 112.699-2 that extended from the same *SacI* site at position 744 to the *SnaBI* site at position 6866. The *SSR2-1* chromosomal sequences were recovered as complete and functional *CYR1* genes by being recombined into the wild-type *CYR1* gene sequences in YCp*CYR1*Δ. Plasmids YCp*SSR2-1A*, YCp*SSR2-1B*, and the wild-type YCp*CYR1* were transformed into the parental *CYR1* strain 112.699, and the resulting transformants were screened for

TABLE 3. Meiotic mapping of the *SSR2-1* mutation<sup>a</sup>

Gene pair	Segregation (PD:NPD:T) <sup>b</sup>	Map distance (cM) <sup>c</sup>
<i>SSR2-1</i> × <i>trp1</i> <sup>d</sup>	43:37:1	1 <sup>e</sup>
<i>SSR2-1</i> × <i>met3</i> <sup>f</sup>	68:0:3	2.1
<i>SSR2-1</i> × <i>cdc35-10</i> <sup>g</sup>	47:0:0	<1.0

<sup>a</sup> Yeast strain 112.699-2 was crossed with a variety of marker strains to determine the centromere linkage of *SSR2-1* and its map distance from *met3* and *cdc35*. The *SSR2-1* mutation was followed by the ability of *ras2 SSR2-1* strains to grow on glycerol at 37°C. The *cdc35* mutation was followed by scoring of the temperature-sensitive phenotype.

<sup>b</sup> PD, Parental ditypes; NPD, nonparental ditypes; T, tetratypes.

<sup>c</sup> cM, Centimorgans.

<sup>d</sup> *trp1* data were obtained from 112.699-2 × JC465-4A, 112.699-2 × JC410-24D, and 112.699-2 × MM20-6C.

<sup>e</sup> Distance from centromere.

<sup>f</sup> *met3* data were obtained from 112.699-2 × JC365-5A.

<sup>g</sup> *cdc35-10* data were obtained from 112.699-2 × JC410-24D and 112.699-2 × MM20-6C.

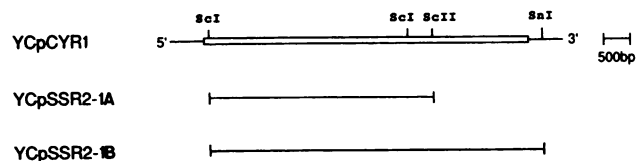


FIG. 3. Cloning of the *SSR2-1* adenylate cyclase gene by homologous recombination. Schematic diagram of the *CYR1* gene compared with the corresponding regions of the *CYR1* gene cloned from strain 112.699-2 by marker rescue. Cloned sequences are in YCp50 plasmids containing wild-type *CYR1* 5' and 3' DNA sequences. Restriction endonuclease cleavage sites are abbreviated as follows: *SacI*, *SacI*; *SacII*, *SacII*; *SnaI*, *SnaBI*.

phenotypes that are characteristic of the dominant *SSR2-1* mutation. Only cells transformed with YCp*SSR2-1B* had the *SSR2-1* phenotype of promoting growth on glycerol and not on acetate in a *ras2* background, exactly as was seen in the *SSR2-1* control strain (Table 4). To test for responsiveness to a *ras* effector mutant, the AAH5-Ha-*ras*[Asn 33] plasmid was cotransformed with YCp*CYR1* or YCp*SSR2-1B* into 112.699. The Ha-*ras*[Asn 33] plasmid was used for this experiment because the AAH5 *LEU2* marker could be coselected with the YCp50 *URA3* marker. Growth on acetate was observed only in the presence of both YCp*SSR2-1B* and AAH5-Ha-*ras*[Asn 33] plasmids, as was seen with the *SSR2-1* strain 112.699-2 that was also transformed with the AAH5-Ha-*ras*[Asn 33] plasmid (Table 4). These results indicate that the cloned gene in YCp*SSR2-1B* reproduced the *SSR2-1* phenotype and that the mutation resided in the adenylate cyclase structural gene *CYR1* between the *SacII* and *SnaBI* restriction sites.

***SSR2-1* is a point mutation in the *CYR1* gene.** Using oligonucleotide primers based on the published sequence of

TABLE 4. General localization of the *SSR2-1* mutation<sup>a</sup>

Strain	Plasmid	Growth at 37°C on:	
		Glycerol	Acetate
112.699	None	-	-
	AAH5-Ha- <i>ras</i> [Asn 33]	-	-
112.699-2 ( <i>SSR2-1</i> )	None	++	-
	AAH5-Ha- <i>ras</i> [Asn 33]	+++	+
112.699	YCp <i>CYR1</i>	-	-
	YCp <i>CYR1</i> plus AAH5-Ha- <i>ras</i> [Asn 33]	±	-
	YCp <i>SSR2-1A</i>	-	-
	YCp <i>SSR2-1A</i> plus AAH5-Ha- <i>ras</i> [Asn 33]	±	-
	YCp <i>SSR2-1B</i>	++	-
	Ycp <i>SSR2-1B</i> plus AAH5-Ha- <i>ras</i> [Asn 33]	+++	+

<sup>a</sup> Wild-type *CYR1* and *CYR1/SSR2-1* hybrid genes containing marker rescued sequences were transformed alone or cotransformed with AAH5-Ha-*ras*[Asn 33] into 112.699 cells. As described in the text, YCp*SSR2-1A* contains *SSR2-1* DNA sequences from nucleotides 744 to 4805, and YCp*SSR2-1B* contains *SSR2-1* sequences from nucleotides 744 to 6866. The *SSR2-1* strain 112.699-2 and wild-type *CYR1* strain 112.699 were transformed with AAH5-Ha-*ras*[Asn 33] as controls. Transformants and controls were patched onto YPG and YPA plates and scored for growth after 2 to 4 days of incubation at 37°C.

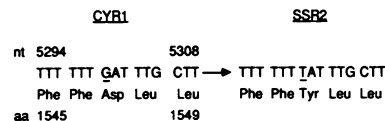


FIG. 4. *SSR2-1* is a point mutation in the *CYR1* structural gene. The nucleotide (nt) and predicted amino acid (aa) sequence of the wild-type *CYR1* gene and the *SSR2-1* mutant gene are compared. Coordinates are those given by Kataoka et al. (19).

the *CYR1* gene, the *SacII-SnaBI* fragment of the genomic *SSR2-1* clone pUC13-*SSR2-11.1* and the wild-type *CYR1* clone pUC13-*CYR1-11* were sequenced. Only one difference was found between the wild-type *CYR1* and *SSR2-1* DNA sequences. There was a single G to T transversion at base position 5300 that changed the predicted amino acid residue from an Asp to a Tyr residue (Fig. 4). The corresponding DNA fragment obtained independently from the marker rescue plasmid YCp*SSR2-1B* was sequenced in the same region, and the predicted sequence change at position 5300 was again observed. An oligonucleotide was synthesized which contained the sequence for the *SSR2-1* point mutation and was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP. Plasmids YCp*CYR1*, YCp*SSR2-1B*, pUC13-*CYR1-11*, and pUC13-*SSR2-11.1*, as well as genomic DNA from strains 112.699 and 112.699-2, were digested with restriction endonucleases; transferred to nitrocellulose; and hybridized with the *SSR2-1*-specific probe. The filter was washed at the melting temperature of the oligonucleotide (61.5°C) in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Under these specific washing conditions, probe hybridization was observed only with YCp*SSR2-1B*, pUC13-*SSR2-11.1*, and 112.699-2 genomic DNA (Fig. 5, lanes 2, 4, and 6). To confirm that this point mutation was responsible for the *SSR2-1* phenotype, a 205-bp *XbaI* to *NcoI* restriction fragment containing the mutation from YCp*SSR2-1B* was subcloned into the *XbaI-NcoI* sites of the wild-type *CYR1* gene in pUC13-*CYR1-11*, replacing the wild-type fragment. When subcloned into YCp50, this hybrid *CYR1/SSR2-1* gene produced an *SSR2-1* phenotype identical to that observed on transformation of 112.699 with YCp*SSR2-1B* (data not shown).

**In vitro adenylate cyclase measurements.** Membranes were prepared from both the wild-type *CYR1* strain 112.699 and

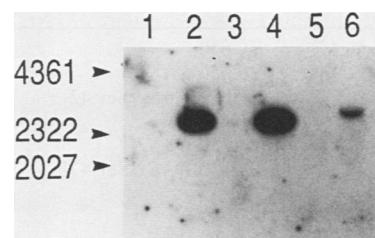


FIG. 5. *SSR2-1*-specific oligonucleotide hybridization analysis. An oligonucleotide specific for the *SSR2-1* point mutation was synthesized (5'-GGGTTTTTTTATTTGCTTCCC-3') and used as a <sup>32</sup>P-labeled hybridization probe against wild-type *CYR1* and *SSR2-1* DNA. The hybridized filter was washed in 6× SSC at 61.5°C for 3 min. Lanes 1 to 4, DNAs were digested with endonuclease *SpeI*; lanes 5 and 6, DNAs were digested with endonuclease *EcoRI*. Lane 1, YCp*CYR1*; lane 2, YCp*SSR2-1B*; lane 3, pUC13-*CYR1-11*; lane 4, pUC13-*SSR2-11.1*; lane 5, 112.699 genomic DNA; lane 6, 112.699-2 genomic DNA. Under nonstringent wash conditions (room temperature), the oligonucleotide probe hybridized as strongly with wild-type *CYR1* sequences as with *SSR2-1* sequences (data not shown). Numbers to the left of the gel indicate fragment sizes (base pairs).



the *SSR2-1* mutant strain 112.699-2 that were transformed with vector, wild-type *RAS2*, and *Ha-ras* plasmids or mutant *RAS2* and *Ha-ras* plasmids. Cells were grown at 30°C in selective medium. For the *RAS2* series, activities were measured in the presence of  $Mg^{2+}$ ;  $Mg^{2+}$  plus a nonhydrolyzable GTP analog, Gpp(NH)p; or  $Mn^{2+}$ . The activities of *RAS* proteins are dependent on bound GTP (13, 23, 36). The maximum *RAS*-dependent activities as seen in the presence of Gpp(NH)p reflect both the presence of the *RAS2* variants and the endogenous *RAS1* protein. To account for the variation in the amount of adenylate cyclase for each cell type, these values were normalized with respect to the adenylate cyclase activities measured in the presence of  $Mn^{2+}$ . Mn-ATP uncouples the catalytic activity of adenylate cyclase from regulation by guanine nucleotides and reflects the amount of cyclase present (6). Values for a typical series are shown in Table 5, together with the average normalized activities for several series resulting from different transformations. The twofold stimulation observed for both the strain 112.699 and the *SSR2-1* adenylate cyclases on the addition of Gpp(NH)p indicates a functional coupling between the *RAS* proteins and the wild-type and mutant adenylate cyclases. The *ras*-dependent nature of the *SSR2-1* mutation was also evident by a >70% inhibition of adenylate cyclase activity (assayed with  $Mg^{2+}$ ) in membranes prepared from the *SSR2-1* strain 112.699-2 expressing *RAS2* or *RAS2*[Ser 42] on the addition of the neutralizing *ras*-specific monoclonal antibody Y13-259 (data not shown) (11). In strain 112.699, the expression of the *RAS2* protein increased the adenylate cyclase activity close to that of the Mn-induced level. An intermediate level of *RAS*-dependent adenylate cyclase stimulation was observed on the expression of the *RAS2*[Ser 42] protein. A similar pattern was apparent for the *RAS* proteins expressed in the *SSR2-1* strain. The increased specific activity observed for the vector alone in the *SSR2-1* strain reflects a greater sensitivity of the *SSR2-1* adenylate cyclase toward *RAS1*.

Also included in Table 5 are adenylate cyclase activities in both wild-type and *SSR2-1* strains that express *Ha-ras* proteins. Since *Ha-ras*-stimulated adenylate cyclase shows

full activity without the addition of exogenous GTP (23), we examined adenylate cyclase activities in the presence of  $Mg^{2+}$  alone. Under these conditions activity from endogenous *RAS1* protein was reduced since the *RAS1* protein present in these strains requires GTP for full activity (23). The normalized  $Mg^{2+}$  activity measured in membranes prepared from the *SSR2-1* strain 112.699-2 transformed with AAH5 alone showed an increase two times greater than that in membranes prepared from the wild-type 112.699 strain transformed with AAH5. This increase may reflect the greater sensitivity of the *SSR2-1* adenylate cyclase to *RAS1* which, while diminished, is still measurable in the absence of exogenous GTP. Membranes prepared from both strains 112.699 and 112.699-2, which express wild-type *Ha-ras* protein, had high levels of adenylate cyclase activity. Expression of the *Ha-ras*[Asn 33] protein, which gave low stimulation of adenylate cyclase in the 112.699 membranes, stimulated 112.699-2 adenylate cyclase to the same level as that of the wild-type *Ha-ras* protein. The expression of the *Ha-ras*[Ser 35] protein in both 112.699 and 112.699-2 resulted in adenylate cyclase activities similar to or slightly greater than those of the vector controls.

## DISCUSSION

We made conservative amino acid substitutions in the putative *Ha-ras* and *RAS2* effector domains that resulted in reduced *ras* biological activity. Previously (31), we showed that substitution of *Ha-ras* residues Thr at position 35, Ile at position 36, and Asp at position 38 with an Ala residue and Tyr at position 40 with a Lys residue eliminates the biological activity of the protein. Initially, we postulated that the region of the *Ha-ras* protein at positions 32 to 40 was an  $\alpha$  helix, and amino acid substitutions were chosen which would conserve the integrity of an  $\alpha$  helix. Recent computer modeling has raised the possibility that this region of the *ras* protein might be an extended coil (I. S. Sigal, G. M. Smith, F. Journak, J. D. Marsico-Ahern, J. S. D'Alonzo, E. M. Scolnick, and J. B. Gibbs, *Anti-Cancer Drug Design*, in press). In this study amino acid substitutions were made

TABLE 5. Biochemical analysis of the *SSR2-1* mutation<sup>a</sup>

Strain	Plasmid	Adenylate cyclase activity (pmol of cAMP/min per mg)			Normalized activity <sup>b</sup>
		$Mg^{2+}$	$Mg^{2+}$ , Gpp(NH)p	$Mn^{2+}$	
112.699 (wild type)	YCp50	6	13	110	0.12 (0.25 ± 0.12)
	YCp50- <i>RAS2</i>	19	35	39	0.90 (0.82 ± 0.13)
	YCp50- <i>RAS2</i> [Ser 42]	8	17	38	0.45 (0.62 ± 0.37)
112.699-2 ( <i>SSR2-1</i> )	YCp50	10	14	38	0.37 (0.42 ± 0.05)
	YCp50- <i>RAS2</i>	14	24	28	0.86 (0.90 ± 0.16)
	YCp50- <i>RAS2</i> [Ser 42]	11	24	36	0.67 (0.60 ± 0.11)
112.699 (wild type)	AAH5	9		34	0.26 (0.16 ± 0.10)
	AAH5- <i>Ha-ras</i>	36		48	0.75 (1.00 ± 0.51)
	AAH5- <i>Ha-ras</i> [Asn 33]	10		23	0.43 (0.42 ± 0.06)
	AAH5- <i>Ha-ras</i> [Ser 35]	17		51	0.33 (0.35 ± 0.03)
112.699-2 ( <i>SSR2-1</i> )	AAH5	8		15	0.53 (0.43 ± 0.10)
	AAH5- <i>Ha-ras</i>	50		43	1.16 (0.84 ± 0.37)
	AAH5- <i>Ha-ras</i> [Asn 33]	23		18	1.28 (0.94 ± 0.31)
	AAH5- <i>Ha-ras</i> [Ser 35]	22		44	0.50 (0.66 ± 0.16)

<sup>a</sup> The *RAS1 ras2* strain 112.699 and the isogenic *SSR2-1* strain 112.699-2 were transformed with the indicated plasmids. Membrane fractions were prepared and adenylate cyclase assays were performed as described in the text. The data are from one complete experimental series, which was representative of multiple experiments performed in duplicate with membrane fractions prepared from three to four independent plasmid transformants of each strain. Normalized activities were determined by calculating the ratio of  $Mg^{2+}$  + Gpp(NH)p activity to  $Mn^{2+}$  activity for YCp50-transformed strains and the ratio of  $Mg^{2+}$  activity to  $Mn^{2+}$  activity for AAH5-transformed strains.

<sup>b</sup> Values in parentheses represent the average normalized activity ± standard deviation measured in membranes prepared from three independently derived transformed clones (four in the case of YCp50-*RAS2*- and YCp50-*RAS2*[Ser 42]-transformed strains).

which were more structurally compatible with an extended coil. The phenotypes observed on expression of these mutant *ras* proteins in *S. cerevisiae* indicate that the most critical region for *ras* biological activity includes residues 33 and 35 of the Ha-*ras* and residue 42 of the *RAS2* proteins.

If the Ha-*ras* domain at positions 32 to 40 is involved in determining a direct interaction with a target protein, then a slight modification of the *ras* protein in this region might compromise the *ras*-target interaction. Using such a mutant *ras* protein, we sought a second-site mutation in *S. cerevisiae* that would compensate for the reduced biological activity of the mutant *ras* protein. The *RAS2*[Ser 42] protein displayed some biological activity, but was incapable of complementing the *ras2* phenotype during growth on acetate or ethanol medium. A second-site suppressor mutation in a gene encoding a product which physically interacted with *ras* proteins was expected to be dominant and capable of interacting more strongly with the *RAS2*[Ser 42] mutant protein than with the wild-type target protein.

There were three general categories of *RAS2*[Ser 42]-dependent mutations that were isolated. The most common mutations that were observed which increased the biological activity of the *RAS2*[Ser 42] protein were those located on the YCp50-*RAS2*[Ser 42] plasmid. These mutations were probably a result of intragenic reversion of either the original point mutation or another codon which allowed complementation of the Ser substitution at position 42. The next most abundant class of mutations found were those which were recessive second-site suppressor mutations. The most interesting mutants in this case were strains 112.699-6 and -8. The mutations in these two strains appeared to be allelic with the *sra1-18* mutation, the gene that is believed to encode the regulatory subunit of the cAMP-dependent protein kinase (5). The 112.699-6 and -8 strains appeared to be sensitive to *RAS2* function, and were found to be responsive only to *ras* alleles that showed some biological activity in the *ras2* complementation assay. These mutations were *ras* dependent.

The most interesting of all the mutants isolated had the dominant *RAS2*[Ser 42]-dependent suppressor mutation *SSR2-1*, which appeared to show some preferential activity with the *RAS2*[Ser 42] protein. We have shown here that the *SSR2-1* mutation is allelic with *CYR1*, the structural gene for adenylate cyclase. The region of the *CYR1* protein affected by the *SSR2-1* point mutation (amino acid positions 1533 to 1555) shares similarity with a number of procaryotic and eucaryotic integral membrane proteins (Fig. 6). The similar

region of bacteriorhodopsin is helix 7, a transmembrane segment that contains the retinal attachment site (9). The significance of this conserved region in the other proteins is unknown. The amino acid that is altered by the *SSR2-1* mutation, Asp at position 1547, lies 66 amino acids N terminal to the catalytic domain (19). This region might be involved in modulating the activity of the catalytic site.

Some of the phenotypes observed for the *SSR2-1* mutant cyclase gene suggest that the *SSR2-1* mutation alters the ability of adenylate cyclase to interact with *ras* proteins in a direct manner. The *SSR2-1* strain 112.699-2 grew slightly better on acetate medium at 37°C in the presence of the *RAS2*[Ser 42] protein than it did with the wild-type *RAS2* protein. The *RAS2*[Ala 42] protein was nonfunctional in both strains. In addition, the *SSR2-1* strain was able to grow on acetate at 37°C in the presence of the biologically inactive Ha-*ras*[Asn 33] protein. A second-site mutation in an actual target protein gene would be expected to promote a productive interaction with a biologically inactive *ras* protein.

Another possible explanation for the *SSR2-1* phenotype is that the *SSR2-1* adenylate cyclase is more sensitive to *RAS* function in general than is the wild-type enzyme. The observation that the *SSR2-1* strain 112.699-2 grew on glycerol at 37°C with only the endogenous *RAS1* protein being expressed is consistent with the interpretation that the *SSR2-1* adenylate cyclase is overly sensitive to *ras*. However, the proposed effector region of the *RAS1* protein differs at residues 34 and 38 (*RAS1* positions) from the corresponding regions of the *RAS2* and Ha-*ras* proteins (28). These changes could alter the conformation of the protein so that the effector region of the *RAS1* protein could be structurally different from the same region of *RAS2* and Ha-*ras* proteins. Consequently, the *SSR2-1* adenylate cyclase might be more responsive to the *RAS1* effector domain than is the wild-type enzyme. If the *SSR2-1* adenylate cyclase is overly *ras* responsive, the slower growth observed for strain 112.699-2 on acetate in the presence of wild-type *RAS2* protein relative to that observed in the presence of the *RAS2*[Ser 42] protein might reflect toxicity that is caused by too large an increase in cAMP levels.

Although the biological assays were done at 37°C, biochemical measurements of membrane adenylate cyclase were performed at 30°C because of the lability of the adenylate cyclase at 37°C in vitro. Under these conditions the *RAS2*[Ser 42] protein, although it had some activity, was less effective in stimulating the adenylate cyclase than was the wild-type *RAS2* protein. This result agrees with the reduced ability of the *RAS2*[Ser 42] protein to alter glycogen levels in wild-type cells grown at this temperature. The *SSR2-1* strain demonstrated an increase in *RAS1*-stimulated adenylate cyclase activity that was consistent with the slight reduction in glycogen levels observed for the *SSR2-1* strain grown at 30°C. Similar to the results for the wild-type *CYR1* strain, expression of the *RAS2*[Ser 42] protein in the *SSR2-1* strain gave an increase in the adenylate cyclase levels that was less than that observed for the *RAS2* wild-type protein. Although the presence of the endogenous *RAS1* gene makes measurement of the effect of the *RAS2* variants less definitive, it appears that at 30°C the *SSR2-1* adenylate cyclase has a similar allele specificity for the *RAS2* variants, as does the wild-type adenylate cyclase. The data for the Ha-*ras* mutants most conclusively demonstrates that the *SSR2-1* adenylate cyclase can be stimulated by *ras* mutants that have little activity against the wild-type *CYR1* adenylate cyclase. In particular, the Ha-*ras*[Asn 33] protein which could only weakly stimulate the *CYR1* gene product was able to

CYR1-1547 region	1533	<u>L</u> <u>D</u> <u>G</u> <u>V</u> <u>V</u> <u>D</u> <u>V</u> <u>S</u> <u>R</u> <u>A</u> <u>V</u> <u>G</u> <u>F</u> <u>F</u> <u>D</u> <u>L</u> <u>L</u> <u>P</u> <u>H</u> <u>I</u> <u>H</u> <u>A</u> <u>S</u>	1555
Bacteriorhodopsin	207	<u>L</u> <u>F</u> <u>M</u> <u>V</u> <u>L</u> <u>D</u> <u>V</u> <u>S</u> <u>A</u> <u>K</u> <u>V</u> <u>G</u> <u>F</u> <u>G</u> <u>L</u> <u>I</u> <u>L</u> <u>L</u> <u>R</u> <u>S</u> <u>R</u> <u>A</u> <u>I</u>	229
E. coli K <sup>+</sup> ATPase	66	<u>I</u> <u>L</u> <u>Q</u> <u>L</u> <u>N</u> <u>M</u> <u>L</u> <u>G</u> <u>L</u> <u>A</u> <u>V</u> <u>L</u> <u>F</u> <u>F</u> <u>M</u> <u>L</u> <u>L</u> <u>G</u> <u>O</u> <u>H</u> <u>Y</u> <u>L</u> <u>P</u>	88
Bovine H <sup>+</sup> ATPase	14	<u>I</u> <u>L</u> <u>Q</u> <u>L</u> <u>P</u> <u>L</u> <u>V</u> <u>T</u> <u>L</u> <u>I</u> <u>V</u> <u>L</u> <u>F</u> <u>P</u> <u>S</u> <u>L</u> <u>L</u> <u>F</u> <u>P</u> <u>T</u> <u>S</u> <u>N</u> <u>R</u>	36
Oxi 3 intron 4 protein	346	<u>I</u> <u>K</u> <u>L</u> <u>T</u> <u>K</u> <u>D</u> <u>N</u> <u>S</u> <u>W</u> <u>F</u> <u>V</u> <u>G</u> <u>F</u> <u>F</u> <u>D</u> <u>A</u> <u>D</u> <u>G</u> <u>T</u> <u>I</u> <u>N</u> <u>Y</u> <u>S</u>	368

FIG. 6. Peptide sequences that are similar to the *CYR1* region at position 1547. The amino acid sequence of the *CYR1* region at positions 1541 to 1563 was used to search the Protein Identification Resource data bank for proteins similar to *CYR1* in this region. Significant similarities were found in four proteins, three of which are known integral membrane proteins. The *CYR1* amino acid sequence from positions 1533 to 1555 is aligned with the sequences of the four proteins most similar to this region. Amino acids conserved with *CYR1* are underlined. The beginning and ending residue numbers for each protein similarity are shown. The asterisk denotes the location of the Asp residue changed by the *SSR2-1* point mutation to a Tyr residue.



strongly activate the *SSR2-1* adenylate cyclase. This activation was reflected by the ability of the *SSR2-1* strain to grow on acetate with the expression of the Ha-*ras*[Asn 33] protein.

Recently, Vendittis et al. (36) isolated a point mutation in the *CYR1* gene which bypassed the G1 arrest phase of a temperature-sensitive *RAS2* gene product, as well as the lethality associated with a double *RAS* gene disruption. This adenylate cyclase mutation, *CR14*, was shown to encode a constitutively active adenylate cyclase which, like the *SRA4-6* mutation, was largely independent of *RAS* function (5). In contrast, the *SSR2-1* adenylate cyclase was entirely *ras* dependent, and its activity in vitro was stimulated by GTP and was inhibited by a *ras*-specific monoclonal antibody. In vitro adenylate cyclase activity measurements in the presence of  $Mn^{2+}$ , which is indicative of *ras*-independent activity (5, 6, 36), gave no indication of elevated catalytic activity in the *SSR2* adenylate cyclase or increased enzyme levels.

This study was initiated with the goal of genetically determining the immediate target of the *RAS2* protein in *S. cerevisiae* by isolating second-site suppressors of the *RAS2* mutant proteins. Effector mutations in *RAS2* were used because they appeared to alter the ability of the *RAS2* protein to function without affecting the known intrinsic biochemical properties of the protein. The dominant second-site suppressor strain that was isolated had a mutant adenylate cyclase gene and, under certain growth conditions, demonstrated allele-specific phenotypes. Whether these biological properties also reflect allele specificity toward the mutant *RAS* proteins at the biochemical level under these conditions remains to be determined by experiments that involve reconstitution of the *RAS* protein-adenylate cyclase complex in vitro with purified mutant and wild-type *RAS* and adenylate cyclase proteins. The identification here of the adenylate cyclase *CYR1* gene as the locus for the *SSR2-1* mutation supports further site-directed mutagenesis experiments of the *CYR1* gene, with the goal of isolating mutants that demonstrate allele specificity for mutant *RAS* proteins.

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