Cell, Vol. 59, 681–686, November 17, 1989, Copyright © 1989 by Cell Press

# Genetic Analysis of Mammalian GAP Expressed in Yeast

**Roymarie Ballester,* Tamar Michaeli,* Kenneth Ferguson,* Hao-Peng Xu,* Frank McCormick,† and Michael Wigler***
* Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724
†Department of Molecular Biology
Cetus Corporation
1400 Fifty-Third Street
Emeryville, California 94608

## Summary

**We have designed a vector to express the mammalian GAP protein in the yeast S. cerevisiae. When expressed in yeast, GAP inhibits the function of the human H-ras$^{gly12}$ protein, but not that of the H-ras$^{val12}$ protein, and complements the loss of *IRA1*. *IRA1* is a yeast gene that encodes a protein with homology to GAP and acts upstream of RAS. Mammalian GAP can therefore function in yeast and interact with yeast RAS. Because expression of GAP complements *ira1*$^-$ mutants, we propose that GAP shares some biochemical functions with IRA1. Other studies indicate that IRA1 controls the level of RAS activity, presumably by regulating GTP hydrolysis. By analogy, we propose that GAP may play a similar role.**

## Introduction

The yeast Saccharomyces cerevisiae has two closely related *RAS* genes (DeFeo-Jones et al., 1983; Powers et al., 1984; Kataoka et al., 1984; Dhar et al., 1984). In yeast, RAS proteins control the activity of adenylate cyclase (Toda et al., 1985; Broek et al., 1985). Although activation of adenylate cyclase does not appear to be the role of vertebrate RAS proteins (Beckner et al., 1985; Birchmeier et al., 1985), there are substantial biochemical similarities between mammalian and yeast RAS proteins (for review, see Barbacid, 1987). Thus, mammalian RAS protein expressed in yeast can supply essential RAS function in that organism and stimulate adenylate cyclase (Kataoka et al., 1985; DeFeo-Jones et al., 1985). Preliminary evidence suggests that mammalian RAS can also interact with the CDC25 gene product, a protein believed to catalyze the exchange of nucleotides bound to yeast RAS proteins (Broek et al., 1987; Powers et al., 1989). Finally, a gene, *IRA1*, has been found in yeast that encodes a protein with a region of homology to mammalian GAP (Trahey et al., 1988; Tanaka et al., 1989). Genetic evidence indicates that IRA1 protein acts upstream of and inhibits wild-type yeast RAS (Tanaka et al., 1989). Cells deficient in *IRA1* function show the same phenotypes as cells with the activating *RAS2*$^{val19}$ mutation: they are sensitive to heat shock and nitrogen starvation and deficient in sporulation (Toda et al., 1985; Sass et al., 1986; Matsumoto et al., 1985; Tanaka et al., 1989).

Mammalian GAP is a ubiquitous cellular protein that catalytically accelerates GTP hydrolysis of the normal mammalian RAS proteins but not that of mutant, "activated" RAS proteins with oncogenic potential (Trahey and McCormick, 1987; Gibbs et al., 1988). Two hypothetical models for the cellular role of GAP have been proposed. In the first model, GAP would function to regulate RAS by virtue of inducing GTP hydrolysis. The failure of GAP to accelerate GTP hydrolysis of mutationally activated forms of RAS could then be invoked to explain the growth transforming properties of oncogenic RAS. In the second model, GAP would be the effector of RAS or part of an effector complex involved in transmitting the signal of activated RAS (Adari et al., 1988; Cales et al., 1988; Sigal, 1988; McCormick, 1989). This model, framed by analogy to the distantly RAS-related GTP binding protein EF-Tu, finds some support in the observation that GAP does not induce GTP hydrolysis in many mutant RAS proteins incapable of stimulating their effector (Adari et al., 1988; Cales et al., 1988; Vogel et al., 1988). To help distinguish the first and second model and gain insight into the possible role of GAP in cells, we have sought to analyze the function of mammalian GAP when expressed in yeast.

## Results

To express mammalian GAP in yeast we constructed a high copy yeast extrachromosomal plasmid in which the coding sequences of the full-length human GAP cDNA gene were transcribed from the powerful yeast *ADH1* promoter (Figure 1). This plasmid, pADGAP, also contains the *LEU2* gene. We chose first to introduce GAP into yeast cells that depend upon the expression of mammalian H-ras protein for viability. Two yeast strains were transformed: TTRB-G1, which is *cdc25*$^-$ and expresses the wild-type human H-ras protein; and TTRB-V2, which is *cdc25*$^-$ and expresses the mutant H-ras$^{val12}$ protein. In both strains, the H-*ras* genes are expressed under the control of the *ADH1* promoter and are contained in a high copy plasmid that also contains the *HIS3* gene (see Experimental Procedures). The *CDC25* gene encodes an essential yeast protein, thought to be required for the activation of wild-type yeast RAS proteins, by catalyzing nucleotide exchange (Broek et al., 1987; Robinson et al., 1987; Camonis et al., 1986). Cells deficient in *CDC25* can be rendered viable if they express the mammalian H-*ras* or H-*ras*$^{val12}$ genes (Marshall et al., 1987). Such cells are not temperature sensitive. However, *cdc25*$^-$ cells containing the wild-type H-*ras* plasmid become temperature sensitive for growth if transformed by the GAP plasmid (Figure 2A). Of a total of 36 independent transformants, 31 containing a control plasmid (vector with auxotrophic marker) are able to grow at 37°C, whereas all 36 transformants containing the GAP plasmid are temperature sensitive (Table 1). In contrast, *cdc25*$^-$ cells containing the mutant H-*ras*$^{val12}$ plasmid are not affected by the presence of the GAP plasmid (Figure 2B). Of 36 independent

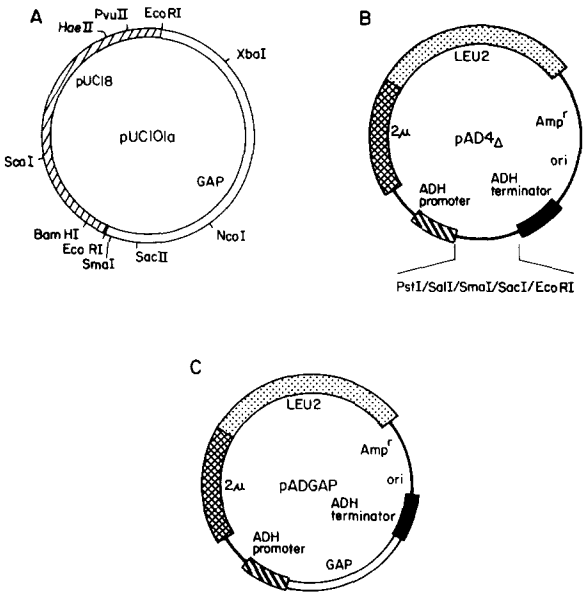


Figure 1. Construction of the Plasmid pADGAP

(A) The entire coding sequence of the human GAP gene was isolated from pUC101a as a 4.0 kb SmaI–PvuII endonuclease fragment. This fragment contains nucleotides from position −38 to 3864 of clone 101 (Trahey et al., 1988) and 180 nucleotides from pUC18 at the 3′ end. pUC18 sequences are indicated by a hatched bar, GAP sequences by an open bar.

(B) pAD4Δ, a 2μm-based *LEU2* plasmid that contains the yeast alcohol dehydrogenase (*ADH1*) promoter and terminator (see Experimental Procedures) was used as the vector to express mammalian GAP in yeast. This plasmid was used previously to express the H-*ras* (Powers et al., 1989), *SCH9* (Toda et al., 1988), *CDC25* (T. M., unpublished data), and *CYR1* (Field et al., 1988) genes in yeast.

(C) The 4.0 kb fragment isolated from pUC101a was inserted into the SmaI site of pAD4Δ. The direction of the GAP insert was confirmed by digestion with the restriction enzymes SalI (a single site is present in the polylinker region) and NcoI (a single site is present in the GAP-containing fragment). The resulting plasmid pADGAP was used to transform yeast strains.

transformants, 35 containing the control plasmid and 35 containing the GAP plasmid grow at 37°C (Table 1). These experiments suggest that mammalian GAP can be expressed in yeast and inhibits the activity of wild-type but not that of mutationally activated H-ras protein.

We next determined whether the expression of GAP had an appreciable phenotype in yeast that did not express mammalian *RAS* genes. The plasmid pADGAP was placed into three strains: SP1, a wild-type yeast strain; IR-1, a strain isogenic to SP1 that contains a deletion of the *IRA1* gene; and TK161-R2V, a strain isogenic to SP1 that contains the *RAS2*$^{val19}$ allele (see Experimental Procedures). *RAS2*$^{val19}$ has a mutation analogous to that found in H-*ras*$^{val12}$ (Kataoka et al., 1984; Toda et al., 1985). Strains were also transformed with "control" plasmids containing only the appropriate auxotrophic marker. The resulting transformed strains were tested for temperature-sensitive growth, ability to grow on various carbon sources, and ability to mate. In this respect, all were normal.

The strains described above were transformed with various plasmids and tested for sensitivity to heat shock as described by Sass et al. (1986) and for sensitivity to nitrogen starvation as described by Toda et al. (1985). Wild-type yeast strains become extremely resistant to heat shock when they enter stationary phase (Pringle et al., 1981) (see Figure 3A). This response is blocked when the RAS/adenylyl cyclase pathway is activated (Sass et al., 1986). Both the *ira1*$^-$ strains (Tanaka et al., 1989) (Figure 3, B1) and the *RAS2*$^{val19}$ strains (Sass et al., 1986; Nikawa et al., 1987) (Figure 3, C1) are heat shock sensitive. The sensitivity to heat shock of both strains is suppressed by a high copy plasmid containing *PDE2*, the gene encoding a high affinity cAMP-phosphodiesterase, (Tanaka et al., 1989; Sass et al., 1986) (Figure 3, B3 and C3). When mammalian GAP is expressed in these strains, only the *ira1*$^-$ strain becomes resistant to heat shock (Figure 3, B2); the

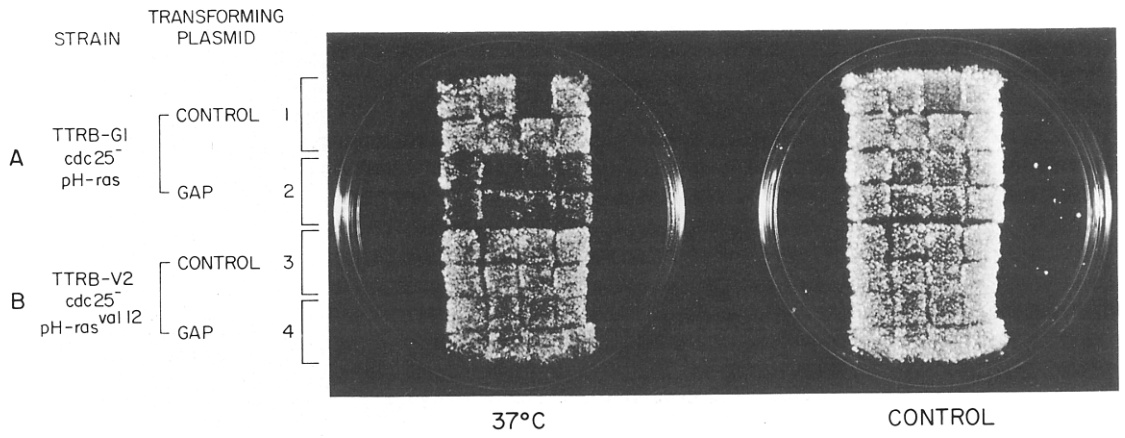


Figure 2. Effect of GAP on the Temperature Sensitivity of Strains Lacking *CDC25* and Expressing the Wild-Type Human H-ras or the Mutant H-ras$^{val12}$ Proteins

Cells completely lacking the *CDC25* gene (see Experimental Procedures for description of strains) but containing pAHRG-H1, a plasmid with the *HIS3* gene and the wild-type H-*ras* (A, strain TTRB-G1), or the plasmid pAHRV-H2, containing the *HIS3* gene and H-*ras*$^{val12}$ (B, strain TTRB-V2), were transformed with a control plasmid pAD4Δ (*LEU2*; rows 1 and 3), or a plasmid expressing GAP, pADGAP (*LEU2*; rows 2 and 4 ), and selected on SC-Leu-His plates. Independent transformants were patched onto selective plates and incubated at room temperature for 3 days and then replica-plated onto YPD plates and incubated at room temperature (control) or 37°C for 2 days. Each set (1–4) shows eight independent transformants.

Table 1. Effect of GAP on the Growth at 37°C of Yeast Strains Lacking *CDC25* and Expressing H-*ras* or H-*ras*$^{val12}$

| Strain | Genotype[a] | Transforming Plasmid[b] | Growth at 37°C (number of transformants growing/total number of transformants tested)[c] |
|---|---|---|---|
| TTRB-G1 | *HIS3*, *cdc25*$^-$, H-*ras* | pAD4Δ | 31/36 |
| TTRB-G1 | *HIS3*, *cdc25*$^-$, H-*ras* | pADGAP | 0/36 |
| TTRB-V2 | *HIS3*, *cdc25*$^-$, H-*ras*$^{val12}$, | pAD4Δ | 35/36 |
| TTRB-V2 | *HIS3*, *cdc25*$^-$, H-*ras*$^{val12}$, | pADGAP | 35/36 |

[a] See Experimental Procedures for the complete genotype of the yeast strains.
[b] See Figure 1 for description of the plasmids.
[c] The different strains were transformed with a control plasmid (pAD4Δ) or GAP-containing plasmid (pADGAP). Thirty-six independent transformants were tested for temperature sensitivity, as described in Figure 2, and scored for the ability to grow at 37°C in YPD.

*RAS2*$^{val19}$ strain remains heat shock sensitive (Figure 3, C2). Similar results were observed when we scored resistance to nitrogen starvation (data not shown). These results indicate that expression of mammalian GAP can functionally complement *IRA1* deficiency and suggest that GAP can inhibit the wild-type yeast RAS2 protein but not the activated RAS2$^{val19}$ mutant protein.

## Discussion

GAP accelerates the GTP hydrolysis catalyzed by wild-type mammalian RAS proteins (Trahey and McCormick, 1987; Gibbs et al., 1988). From this, one predicts that GAP has the potential to inhibit the activity of RAS proteins. Our studies in yeast lead us to conclude that GAP can indeed inhibit wild-type H-ras protein. Within the limits of the genetic system we have tested, GAP does not appear to inhibit the activity of mutant H-ras$^{val12}$ protein. Thus, GAP neither effectively down regulates H-ras$^{val12}$ protein nor effectively competes for the binding of H-ras$^{val12}$ protein to its target effector in yeast.

GAP expression can restore the heat shock and nitrogen starvation resistance of *ira1*$^-$ mutant strains. GAP expression has no discernible effects on these phenotypes in *RAS2*$^{val19}$ strains. These results suggest that mammalian GAP can effect the function of wild-type yeast RAS protein but not that of the mutationally activated yeast RAS protein. Thus, GAP protein interaction with yeast RAS proteins appears similar to its interaction with mammalian RAS proteins. GAP expression does not complement *ira1*$^-$ mutant strains perfectly, as such strains are somewhat more heat shock sensitive than wild-type strains (data not shown).

We cannot infer that GAP and IRA proteins have the same biochemical function. It is reasonable to infer, from the ability of GAP to effect wild-type but not mutationally activated RAS proteins, that GAP effects are due to its induction of GTP hydrolysis. IRA proteins, however, need not act through this mechanism. For example, IRA proteins might block nucleotide exchange on RAS. However, since both GAP and IRA proteins can interact with yeast RAS and share a domain with clear sequence homology, both

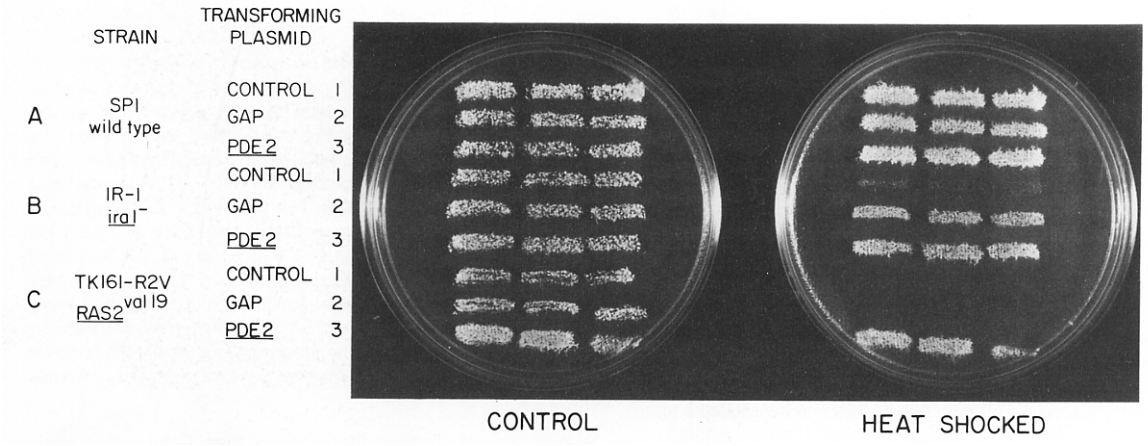


Figure 3. Effect of GAP on Heat Shock Sensitivity in Strains Lacking *IRA1* and Strains Containing *RAS2*$^{val19}$

Yeast strains were transformed with various plasmids containing the *LEU2* gene and plated onto SC-Leu plates. Independent transformants were patched onto SC-Leu plates, incubated at 30°C for 2 days, replica-plated onto SC-Leu plates, and heat-shocked (at 55°C) for 10 min or 0 min (control). After heat shock treatment the plates were incubated at 30°C for 2 days. The strains used in this experiment were: (A) SP1, a wild-type strain; (B) IR-1, a strain that contains a disrupted chromosomal *IRA1* gene; and (C) TK161-R2V, a strain that contains the *RAS2*$^{val19}$ allele (see Experimental Procedures for description of the strains). The different strains were transformed with: (1) pAD4Δ, a control plasmid; (2) pADGAP, a plasmid containing the human GAP gene; and (3) Yep*PDE2*, a plasmid containing a yeast cAMP-phosphodiesterase gene.

may bind to the same site on RAS proteins. The homologous region may represent a common site for RAS protein interactions.

Since GAP can complement *ira1*$^-$ mutants, it is reasonable to speculate that GAP and the IRA1 protein have a conserved cellular role. In yeast, cAMP production is under feedback control (Nikawa et al., 1987). Wild-type yeast cells show a rapid biphasic change in cAMP levels in response to glucose (Thevelein and Beullens, 1985). However, if cells are *ira1* mutants (Tanaka et al., 1989), or if they contain attenuated cAMP-dependent protein kinase activity, cAMP levels rise and remain elevated in response to glucose (Wigler et al., 1988). Thus, an argument can be made that the IRA1 protein is a component of a feedback pathway that controls RAS protein activity. In yeast, RAS2$^{val19}$ protein is refractory to feedback inhibition (Nikawa et al., 1987). In mammalian cells there is also evidence from microinjection experiments that wild-type H-ras protein activity is subject to feedback control, while the H-ras$^{val12}$ protein is not (Bar-Sagi and Feramiso, 1986). These considerations lead us to suggest that, in mammalian cells, GAP is a component of a feedback pathway down regulating RAS proteins by accelerating hydrolysis of RAS-bound GTP. This model is not particularly consonant with GAP, also serving as an effector for RAS proteins.

## Experimental Procedures

### Yeast and Escherichia coli Strains, Media, Genetic Manipulations, and Nomenclature

The S. cerevisiae strains used for this study are: SP1 (*MATa his3 leu2 ura3 trp1 ade8 can1*; Toda et al., 1985); TT1A-1 (*MATα his3 leu2 ura3 trp1 ade8 cdc25::URA3 pCDC25(TRP1)*-1; Broek et al., 1987); and TK161-R2V (*MATa leu2 ura3 his3 trp1 ade8 can1 RAS2*$^{val19}$; Toda et al., 1985). Yeast was grown in YPD (2% peptone, 1% yeast extract, and 2% glucose) or in synthetic medium (0.67 g/l Yeast Nitrogen Base, 2% glucose, and appropriate auxotrophic supplements). Standard yeast genetic methods were followed as described previously (Sherman et al., 1986). The lithium acetate method was used for transformation of yeast cells (Ito et al., 1983). The E. coli HB101 strain was used for plasmid propagation and isolation (Boyer and Roulland-Dussoix, 1969). E. coli transformation was performed by standard methods (Maniatis et al., 1982). Wild-type alleles and dominant mutations are denoted by capital italicized letters, recessive mutations by lowercase italicized letters, and gene disruptions by lowercase letters, which represent the disrupted gene, followed by two colons and the auxotrophic gene marker used for the disruption. For example, *cdc25::URA3* indicates the *CDC25* gene disrupted by the *URA3* marker. In the text, gene disruptions are abbreviated by lowercase italicized letters representing the gene followed by a superscript minus sign, such as *cdc25*$^-$.

### DNA Manipulation

Plasmid DNA was isolated from E. coli using the alkali–lysis method (Maniatis et al., 1982). DNA restriction endonucleases, polymerases, and ligases were used under conditions recommended by suppliers (New England Biolab or Bethesda Research Laboratory). For gene disruption experiments, suitable linear DNA fragments were isolated and used for transformation of yeast cells as described (Rothstein, 1983).

### Plasmids

The cloning vector pAD4Δ is similar to the plasmid pADNS previously described (Colicelli et al., 1989). It contains the *LEU2* gene, an HpaI–HindIII fragment from 2μm including the origin of replication, an SspI–EcoRI fragment containing the ampicillin resistance gene from the plasmid pUC18, a HindIII–BamHI fragment containing the S. cerevisiae alcohol dehydrogenase (*ADH1*) promoter, and a HindIII–BamHI fragment containing the *ADH1* terminator sequences. The promoter and terminator sequences are separated by a polylinker from pUC18 that lacks the SphI and HindIII sites (Figure 1). The human GAP gene was inserted into the SmaI site in the polylinker region pAD4Δ as described in Figure 1.

The plasmid pAHRG-H1 was constructed by inserting an SphI fragment that contains the human H-*ras* cDNA driven by the ADH1 promoter into the vector pHV1. pHV1 is a high copy number vector that contains the polylinker from pUC18, the *HIS3* gene, and an HpaI–HindIII fragment from 2μm, including the origin of replication but lacking *REP3* (Broach et al., 1983). The plasmid pAHRV-H2 is the same as pAHRG-H1 except that it contains the mutant H-*ras*$^{val12}$ instead of the wild-type H-*ras*. This mutation was obtained by oligonucleotide-directed mutagenesis performed by a modification of a procedure of Zoller and Smith (1984), which utilizes uracil-containing template DNA (Kunkel, 1985). The oligonucleotide 5′-GGTGGGCGCCGTCGGTG was used to change codon 12 to encode valine. Mutagenesis was confirmed by sequencing.

The YepPDE2 plasmid contains the yeast *PDE2* gene on the high-copy *LEU2* plasmid YEp13 (Sass et al., 1986). The *PDE2* gene encodes a cAMP-phosphodiesterase. The plasmid pUC101a contains an EcoRI fragment of clone 101 of the GAP gene (Trahey et al., 1988) inserted into pUC18 (Figure 1). This fragment contains the entire coding region of the human GAP.

### Construction of Yeast Strains

TT1A-1 is a strain that lacks a functional chromosomal *CDC25* gene but is viable because it has the *CDC25* gene in a multicopy plasmid containing the *TRP1* marker (pCDC25(TRP1)-1; Broek et al., 1987). Cells deficient in *CDC25* are viable if they express the mammalian H-*ras* or H-*ras*$^{val12}$ genes (Marshall et al., 1987). To construct a strain dependent upon the expression of mammalian H-ras proteins, TT1A-1 was transformed with pAHRG-H1 or pAHRV-H2, and His$^+$ transformants were selected. These were grown without selection in YPD medium for 48 hr and plated onto SC–His plates. The resulting colonies were replica plated onto SC–His, YPD, and SC–Trp plates. Colonies that had lost the pCDC25(TRP)-1 plasmid but that were His$^+$ were presumed to contain the plasmid with the wild-type H-*ras* or H-*ras*$^{val12}$ gene. This "plasmid exchange" procedure has been used successfully to suppress the loss of *CDC25* by the *RAS2*$^{val19}$, the *TPK1*, and the *CYR1* genes (Broek et al., 1987). The *cdc25*$^-$ strain TTRB-G1 (*MATα his3 leu2 ura3 trp1 ade8 cdc25::URA3* pAHRG-H1) contains the normal H-*ras* gene, whereas the strain TTRB-V2 (*MATα his3 leu2 ura3 trp1 ade8 cdc25::URA3* pAHRV-H2) expresses the mutant H-ras$^{val12}$ protein. To test whether the GAP gene carried on a multicopy plasmid has an effect on mammamlian H-ras proteins expressed in yeast, we transformed these strains with the plasmids pAD4Δ and pADGAP (see Figure 1). His$^+$– Leu$^+$ transformants were selected and tested for temperature sensitivity.

The yeast strain IR-1 (*MATa his3 leu2 ura3 trp1 ade8 can1 ira1::HIS3*) contains a disruption in the *IRA1* gene. This strain was constructed as follows: an XbaI DNA fragment from YIp-pd13 (a plasmid containing the entire *IRA1* sequence; Tanaka et al., 1989) was inserted into pUC118 (Vieira and Messing, 1987), which had been digested with XbaI. The resulting plasmid was digested with BglII and ligated to a BamHI fragment of the *HIS3* gene. This results in a 3.2 kb deletion of *IRA1* coding sequences. (A similar deletion/insertion was used previously by Tanaka and co-workers, 1989, to generate a mutant *ira1* allele.) The resulting plasmid was used to carry out gene replacement experiments (Rothstein, 1983). The yeast haploid auxotroph SP1 was transformed with the XbaI fragment of the deleted *IRA1* gene, and transformants were selected by histidine prototrophy. Southern hybridization analysis was used to verify that the *IRA1* gene was replaced by the disrupted gene.

### Heat Shock and Nitrogen Starvation of Yeast Cells

Heat shock sensitivity and nitrogen starvation sensitivity were determined as described previously (Toda et al., 1985; Sass et al., 1986). Heat shock was performed by replica plating cells to a plate preheated for 1 hr at 55°C. This plate was then incubated for 10 min at 55°C and transferred to 30°C for 2 days. Yeast cells were starved for nitrogen by replica plating cells onto medium lacking a source of nitrogen. This replica was incubated for 9 days at 30°C and then replica plated onto YPD or synthetic media and grown for 2 days at 30°C.

**Acknowledgments**

We are grateful to Drs. K. Matsumoto and K. Tanaka for the *IRA1* plasmid and to P. Bird for preparing this manuscript. This work was supported by grants from the National Institutes of Health, the American Cancer Society, and by the Pfizer Biomedical Research Award. R. B. is supported by an NIH fellowship. T. M. is supported by the Damon Runyon–Walter Winchell Cancer Fund Fellowship (DRG-923). H-P. X. is a graduate student from the Department of Biochemistry and Molecular Biology at the State University of New York at Stony Brook. M. W. is an American Cancer Society Research Professor.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore by hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 27, 1989; revised September 7, 1989.

**References**

Adari, H., Lowy, D. R., Willumsen, B. M., Der, C. J., and McCormick, F. (1988). Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. Science *240*, 518–521.

Barbacid, M. (1987). *ras* genes. Annu. Rev. Biochem. *56*, 779–827.

Bar-Sagi, D., and Feramisco, J. R. (1986). Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by *ras* proteins. Science *233*, 1061–1068.

Beckner, S. K., Hattori, S., and Shih, T. Y. (1985). The ras oncogene product p21 is not a regulatory component of adenylate cyclase. Nature *317*, 71–72.

Birchmeier, C., Broek, D., and Wigler, M. (1985). *RAS* proteins can induce meiosis in Xenopus oocytes. Cell *43*, 615–621.

Boyer, H., and Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. *41*, 459–472.

Broach, J., Li, Y., Wu, L., and Jayaram, M. (1983). Vectors for high-level inducible expression of cloned genes in yeast. In Experimental Manipulation of Gene Expression, M. Inouye, ed. (New York: Academic Press), pp. 83–177.

Broek, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J., and Wigler, M. (1985). Differential activation of yeast adenylate cyclase by wild-type and mutant *ras* proteins. Cell *41*, 763–769.

Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S., and Wigler, M. (1987). The S. cerevisiae *CDC25* gene product regulates the *RAS*/adenylate cyclase pathway. Cell *48*, 789–799.

Cales, C., Hancock, J. F., Marshall, C. J., and Hall, A. (1988). The cytoplasmic protein GAP is implicated as the target for regulation by the *ras* gene product. Nature *332*, 548–551.

Camonis, J. H., Kalekine, M., Bernard, G., Garreau, H., Boy-Marcotte, E., and Jacquet, M. (1986). Characterization, cloning and sequence analysis of the *CDC25* gene which controls the cyclic AMP level of *Saccharomyces cerevisiae*. EMBO J. *5*, 375–380.

Colicelli, J., Birchmeier, C., Michaeli, T., O'Neill, K., Riggs, M., and Wigler, M. (1989). Isolation and characterization of a mammalian gene encoding a high-affinity cAMP phosphodiesterase. Proc. Natl. Acad. Sci. USA *86*, 3599–3603.

DeFeo-Jones, D., Scolnick, E. M., Koller, R., and Dhar, R. (1983). *ras*-related gene sequences identified and isolated from *Saccharomyces cerevisiae*. Nature *306*, 707–709.

DeFeo-Jones, D., Tatchell, K., Robinson, L. C., Sigal, I. S., Vass, W. C., Lowy, D. R., and Scolnick, E. M. (1985). Mammalian and yeast *ras* gene products: biological function in their heterologous systems. Science *228*, 179–184.

Dhar, R., Nieto, A., Koller, R., DeFeo-Jones, D., and Scolnick, E. M. (1984). Nucleotide sequence of two $ras^H$-related genes isolated from the yeast *Saccharomyces cerevisiae*. Nucl. Acids Res. *12*, 3611–3618.

Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988). Purification of a *RAS*-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. *8*, 2159–2165.

Gibbs, J. B., Schaber, M. D., Allard, W. J., Sigal, I. S., and Scolnick, E. M. (1988). Purification of *ras* GTPase activating protein from bovine brain. Proc. Natl. Acad. Sci. USA *85*, 5026–5030.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

Kataoka, T., Powers, S., McGill, C., Fasano, O., Strathern, J., Broach, J., and Wigler, M. (1984). Genetic analysis of yeast *RAS1* and *RAS2* genes. Cell *37*, 437–445.

Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J., and Wigler, M. (1985). Functional homology of mammalian and yeast *RAS* genes. Cell *40*, 19–26.

Kunkel, T. (1985). Rapid and effective site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA *82*, 488–492.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Marshall, M. S., Gibbs, J. B., Scolnick, E. M., and Sigal, I. S. (1987). Regulatory function of the *Saccharomyces cerevisiae RAS* C-terminus. Mol. Cell. Biol. *7*, 2309–2315.

Matsumoto, K., Uno, I., Kato, K., and Ishikawa, T. (1985). Isolation and characterization of a phosphatase protein phosphatase deficient mutant in yeast. Yeast *1*, 25–38.

McCormick, F. (1989). *ras* GTPase activating protein: signal transmitter and signal terminator. Cell *56*, 5–8.

Nikawa, J., Cameron, S., Toda, T., Ferguson, K. M., and Wigler, M. (1987). Rigorous feedback control of cAMP levels in *Saccharomyces cerevisiae*. Genes Dev. *1*, 931–937.

Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. (1984). Genes in S. cerevisiae encoding proteins with domains homologous to the mammalian *ras* proteins. Cell *36*, 607–612.

Powers, S., Michaelis, S., Broek, D., Anna-A., S. S., Field, J., Herskowitz, I., and Wigler, M. (1986). *RAM*, a gene of yeast required for a functional modification of *RAS* proteins and mating pheromone **a**-factor. Cell *47*, 413–422.

Powers, S., O'Neill, K., and Wigler, M. (1989). Dominant yeast and mammalian *RAS* mutants that interfere with the *CDC25*-dependent activation of wild-type *RAS* in *S. cerevisiae*. Mol. Cell. Biol. *9*, 390–395.

Pringle, J. R., and Hartwell, L. H. (1981). The *Saccharomyces cerevisiae* cell cycle. In The Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance, J. N. Strathern, E. W. Jones, and J. R. Broach, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp 97–142.

Robinson, L. C., Gibbs, J. B., Marshall, M. S., Sigal, I. S., and Tatchell, K. (1987). CDC25: a component of the RAS-adenylate cyclase pathway in *Saccharomyces cerevisiae*. Science *235*, 1218–1221.

Rothstein, R. J. (1983). One-step gene disruption in yeast. Meth. Enzymol. *101*, 202–211.

Sass, P., Field, J., Nikawa, J., Toda, T., and Wigler, M. (1986). Cloning and characterization of the high affinity cAMP phosphodiesterase of *S. cerevisiae*. Proc. Natl. Acad. Sci. USA *83*, 9303–9307.

Sherman, F., Fink, G. R., and Hicks, J. B. (1986). Laboratory Course Manual for Methods in Yeast Genetics. F. Sherman, G. R. Fink, and J. B. Hicks, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Sigal, I. S. (1988). The *ras* oncogene: a structure and some functions. Nature *332*, 485–486.

Tanaka, K., Matsumoto, K., and Toh-e, A. (1989). IRA1, an inhibitory regulator of the *RAS*-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *9*, 757–768.

Thevelein, J. M., and Beullens, M. (1985). Cyclic AMP and the stimulation of trehalase activity in the yeast *Saccharomyces cerevisiae* by carbon sources, nitrogen sources, and inhibitors of protein synthesis. J. Gen. Microbiol. *131*, 3199–3209.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, *RAS* proteins are controlling elements of adenylate cyclase. Cell *40*, 27–36.

Toda, T., Cameron, S., Sass, P., and Wigler, M. (1988). *SCH9*, a gene

of *S. cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP dependent protein kinase catalytic subunits. Genes Dev. *2*, 517–527.

Trahey, M., and McCormick, F. (1987). A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science *238*, 542–545.

Trahey, M., Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G. A., Ladner, M., Long, C., Crosier, W. J., Watt, K., Koths, K., and McCormick, F. (1988). Molecular cloning of two types of GAP complementary DNA from human placenta. Science *242*, 1697–1700.

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. Meth. Enzymol. *153*, 3–11.

Vogel, U. S., Dixon, R. A., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., and Gibbs, J. B. (1988). Cloning of bovine GAP and its interaction with oncogenic *ras* p21. Nature *335*, 90–93.

Wigler, M., Field, J., Powers, S., Broek, D., Toda, T., Cameron, S., Nikawa, J., Michaeli, T., Colicelli, J., and Ferguson, K. (1988). Studies of *RAS* function in the yeast *S. cerevisiae*. Cold Spring Harbor Symp. Quant. Biol. *LIII*, 649–655.

Zoller, M., and Smith, M. (1984). Oligonucleotide directed mutagenesis: a simple method using two oligonucleotide primers and a single stranded DNA template. DNA *3*, 479–488.