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Multiple regulatory mechanisms control the expression of the *RAS1* and *RAS2* genes of *Saccharomyces cerevisiae*

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Expression of the *RAS1* and *RAS2* genes of *Saccharomyces cerevisiae* has been examined at the transcriptional and translational levels. When dextrose is the carbon source, the steady-state amount of *RAS1* mRNA and the rate of *RAS1* protein synthesis are reduced in parallel as cells approach the mid-exponential phase of growth. *RAS1* mRNA levels and protein synthesis are very low at all stages of growth when ethanol rather than dextrose is provided as the sole carbon source. The rate of *RAS2* protein synthesis is regulated differently. In cells cultured on dextrose, it is lowest in the early exponential phase, increases ~10-fold and remains nearly constant as cells approach stationary phase. By contrast, *RAS2* mRNA is found at uniformly high levels at all phases of exponential growth, suggesting that the translational efficiency of *RAS2* mRNA is repressed during the early exponential phase. This repression is not observed when ethanol is the sole carbon source. Nutrient starvation, resulting in G_1 arrest and sporulation in diploids, leads to greatly decreased amounts of *RAS2* mRNA, accomplished in part by selective repression of *RAS2* transcripts with particular 5′ ends. However, this reduction in *RAS2* mRNA levels has little effect on the rate of *RAS2* protein synthesis, suggesting that the translational efficiency of *RAS2* mRNA is stimulated by nutrient starvation. The combination of transcriptional and translational controls which regulate yeast *RAS* gene expression seems to ensure that one or the other *RAS* proteins will be produced over a wide range of physiological states.

Key words: RAS genes/*Saccharomyces cerevisiae*/gene expression

Introduction

The yeast *Saccharomyces cerevisiae* contains five unlinked genes, *YTP1* (Gallwitz *et al.*, 1986), *RHO1, RHO2* (Madaule *et al.*, 1987), *RAS1* and *RAS2* (DeFeo-Jones *et al.*, 1983; Dhar *et al.*, 1984; Powers *et al.*, 1984) that encode proteins homologous to the *RAS* gene products first identified in the Harvey and Kirsten murine sarcoma viruses (Ellis *et al.*, 1982), and subsequently found in a variety of eukaryotic species as divergent as *Drosophila* and humans (Barbacid, 1987). High level expression or the presence of certain point mutations in the viral and mammalian *RAS* genes has been associated with oncogenic transformation of mammalian cells (Barbacid, 1987). Among the five *RAS*-related genes of *S.cerevisiae, RAS1* and *RAS2* have been studied most extensively. These genes complement one another for a function essential to cellular proliferation: yeast cells containing disruptions in either of the two *RAS* genes are viable under most conditions, but spores carrying disruptions in both *RAS1* and *RAS2* fail to germinate (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984).

The *RAS* products of *S.cerevisiae* share several similarities with their viral and mammalian counterparts. First, all of these *RAS* proteins exhibit GDP−GTP binding capacity and have GTPase activity (Gibbs *et al.*, 1984; McGrawth *et al.*, 1984; Tamanoi *et al.*, 1984; Temeles *et al.*, 1984). Low GTPase activity is observed in yeast *RAS* variants containing amino acid substitutions equivalent to those present in the activated oncogenic form of the mammalian *RAS* proteins (Temeles *et al.*, 1984). Second, *RAS* proteins of *S.cerevisiae* are located at the plasma membrane as a result of a modification involving addition of a fatty acid moiety (Fujiyama and Tamanoi, 1986; Fujiyama *et al.*, 1987; Powers *et al.*, 1986). The same location and mechanism of transport have been shown for the viral and mammalian *RAS* proteins (Buss and Sefton, 1986; Papageorge *et al.*, 1982; Willumsen *et al.*, 1984). Third, human (Kataoka *et al.*, 1985a) or viral (DeFeo-Jones *et al.*, 1985) *RAS* proteins can restore growth to yeast *ras1 ras2* mutants, whereas a modified yeast *RAS1* gene can transform NIH-3T3 cells and such transformed cells lead to metastasis in nude mice (Bradley *et al.*, 1986).

A large amount of genetic and biochemical data indicate that the *RAS* proteins of *S.cerevisiae* positively regulate the activity of adenylate cyclase (Tatchell, 1986). Different classes of suppressor mutations have been isolated that restore growth to *ras*$^-$ cells. These mutations map in genes whose products are involved in the production of cAMP or the activation of cAMP-dependent protein kinase activity (Cannon *et al.*, 1986; Cannon and Tatchell, 1987; De Vendittis *et al.*, 1986; Kataoka *et al.*, 1985b; Tatchell, 1986; Toda *et al.*, 1985). Thus, the *RAS1* and *RAS2* proteins are required for viability in yeast as positive effectors of cAMP-dependent protein kinase activity.

There is evidence to suggest that the *RAS1* and *RAS2* proteins are used differentially in different growth conditions. For example, *RAS1* strains containing a disrupted *ras2* allele fail to grow in media containing non-fermentable carbon sources (Fraenkel, 1985; Tatchell *et al.*, 1985). We showed previously that the inability of *RAS1 ras2* mutants to grow in such media could result from low expression of *RAS1* mRNA in these conditions compared to growth on media containing dextrose. In accord with this idea, extragenic suppressor (*sra6*) that restores growth on non-fermentable carbon sources to *RAS1 ras2* mutants leads to elevated

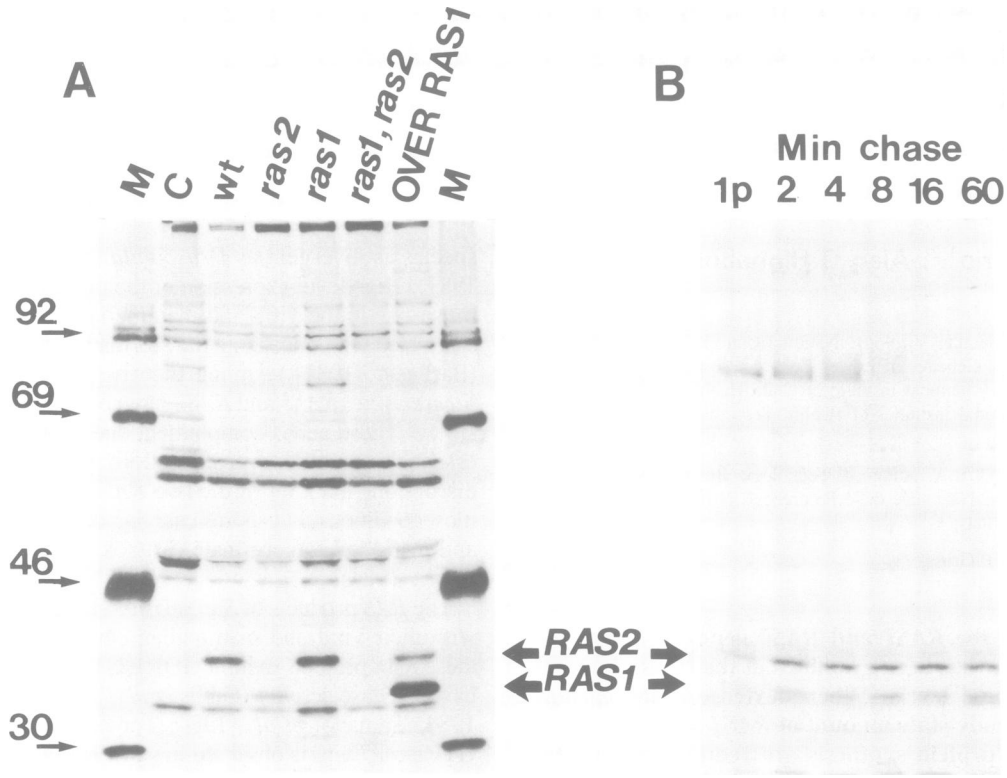

Fig. 1. Determination of size and stability of the *RAS1* and *RAS2* proteins of *S.cerevisiae*. (**A**) Yeast strains containing different alleles of *RAS1* and *RAS2* were grown to early-exponential phase in SD medium. Proteins were labeled with [^{35}S]methionine, extracted and 10^7 c.p.m. of each extract were immunoprecipitated using a monoclonal antibody (Y13-259) directed against a viral *RAS* protein. The strains examined were: wild-type (*RAS1*$^+$ *RAS2*$^+$, (S288C); *ras2* (*RAS1*$^+$ *ras2*$^-$, 112-699); *ras1*1 (*ras1*$^-$ *RAS2*$^+$, JC 308); *ras1 ras2* (*ras1*$^-$ *ras2*$^-$ *sra1-1*, RY31-1A); over *RAS1* (multiple copies of *RAS1*$^+$, XCO26C-485). Mol. wt markers (M) are shown on both sides of the panel. C is a control immunoprecipitation of an S288C lysate using rat pre-immune serum in place of Y13-259 antibody. (**B**) S288C cells were grown to early exponential phase in SD medium. Protein was pulse-labeled with [^{35}S]methionine for 1 min (1p) and then chased for 2, 4, 8, 16 or 60 min following addition of cycloheximide and excess unlabeled methionine. An amount of lysate containing 700 μg of total protein was immunoprecipitated for each time point. The specific activity of total protein remained unchanged throughout the chase period (1.5×10^7 c.p.m./mg of protein).

expression of *RAS1* mRNA in these conditions (Breviario *et al.*, 1986). We extend our previous findings here by showing that the rate of *RAS1* protein synthesis decreases in parallel with the reduction in *RAS1* mRNA levels in wild-type cells cultured with ethanol as the carbon source.

In contrast to *RAS1*, our previous results demonstrated that expression of *RAS2* mRNA is relatively unaffected by the growth phase and carbon source. We wished to further our analysis of *RAS2* gene expression to include measurements of *RAS2* protein synthesis in different conditions. Despite an abundance of *RAS2* mRNA, the rate of *RAS2* protein synthesis is found to be very low in the early exponential phase of growth when dextrose is the carbon source. This is the growth period when *RAS1* protein synthesis is at its highest level, suggesting a reciprocal modulation of expression of the two *RAS* genes. Extending this correlation, we observe a high rate of *RAS2* protein synthesis in the early exponential phase of growth when ethanol is the carbon source. As noted above, the rate of *RAS1* protein synthesis is very low in these conditions.

Another phenotype associated with *RAS1 ras2* mutants is hypersporulation in rich media (Kataoka *et al.*, 1984; Tatchell *et al.*, 1985), a result suggesting that the *RAS2* gene product has a role in preventing sporulation in non-starvation conditions. It was also shown that dominant mutations in *RAS2*, expected to increase the activated GTP-bound form of the *RAS2* protein, lead to reduced viability in starvation conditions (Toda *et al.*, 1985). For these reasons, we wished to determine whether *RAS2* expression is normally down-regulated in starvation conditions. In fact, we show that the steady-state amount of *RAS* mRNA is substantially reduced in starvation conditions. Surprisingly, this response leads to little variation in the rate of *RAS2* protein synthesis. Taken together, our results imply that a combination of transcriptional and translational control mechanisms functions to maintain a relatively constant level of *RAS* protein synthesis in diverse culture conditions.

Results

Molecular weight and stability of the RAS proteins

Previous studies on expression of the *RAS* proteins of *S. cerevisiae* were performed with yeast strains that overproduce these proteins from *RAS* encoding sequences either placed under the control of a strong inducible promoter or carried on a multi-copy plasmid (Fujiyama and Tamanoi, 1986; Fujiyama *et al.*, 1987; Powers *et al.*, 1986; Tamanoi *et al.*, 1984; Temeles *et al.*, 1984). We examined expression of the *RAS* proteins in strains containing a single copy of each gene at its normal chromosomal location. To measure *RAS* protein synthesis, cells grown in dextrose-containing medium were pulse-labeled for 7.5 min and extracts of total

cellular proteins were subjected to immunoprecipitation using a monoclonal antibody directed against a viral *RAS* protein (Furth *et al.*, 1982). By immunoprecipitation of extracts obtained from *RAS1 RAS2* wild-type cells, from *ras1 RAS2*, *RAS1 ras2*, and *ras1 ras2* mutants, and from a strain that overexpresses *RAS1* protein, we identified the *RAS2* protein as a 40 000-dalton species and the *RAS1* protein as a 36 000-dalton species (Figure 1A). Comparison of the proteins immunoprecipitated from the *RAS1 ras2* and *ras1 RAS2* mutant strains showed that the 36-kd protein we identify as RAS1 cannot be a degradation product of the 40-kd *RAS2* gene product. These mol. wts are in agreement with those reported by others (Fujiyama and Tamanoi, 1986; Powers *et al.*, 1984). Precursors of higher mol. wt were not observed, presumably because of rapid processing (see note in Figure 4).

Incorporation of [^{35}S]methionine into the *RAS* proteins was linear during the 7.5-min pulse in every growth condition we examined (data not shown). Figure 1B shows that *RAS* proteins labeled for 1 min during early exponential growth in SD medium are stable during a 1 h chase with unlabeled methionine. The same results were obtained in all other culture conditions we examined (data not shown). Previous results using yeast strains which overexpress the *RAS* proteins demonstrated a turnover time >100 min (Tamanoi *et al.*, 1984; Temeles *et al.*, 1984). Taken together, these data indicate that the amount of ^{35}S incorporated into the *RAS* proteins during a 7.5-min pulse accurately reflects the rate of synthesis of these proteins.

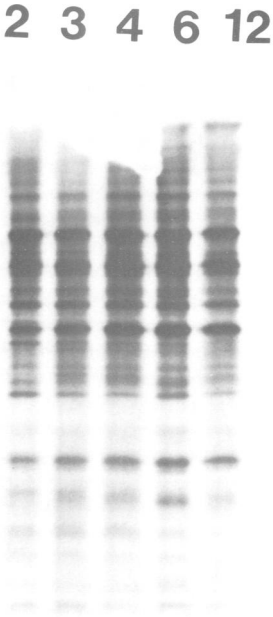

Fig. 2. Total protein synthesis during growth in SD medium. S288C cells from stationary phase cultures were inoculated into fresh SD media and grown at 30°C. Cells were labeled with [^{35}S]methionine for 7.5 min. After 2, 3, 4, 6 and 12 h of growth 10^4 c.p.m. of each cell extract was directly loaded on the gel.

RAS1 and RAS2 expression during growth in SD medium

Either the *RAS1* or *RAS2* gene product is required for cell growth and division in media containing glucose as the carbon source (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984). We wished to determine whether *RAS* gene expression varies as cells enter and exit the exponential phase of growth in such a medium. Towards this end, we determined the steady-state amounts of the *RAS* mRNAs and rates of *RAS* protein synthesis in wild-type cells at different times during their growth in SD medium. Figure 2 shows that the amount of [^{35}S]methionine incorporation into total proteins was fairly constant throughout the growth curve. (The amount of [^{35}S]methionine incorporated in stationary phase is about one half that seen for other time points; data not shown.) By contrast, the relative rates of synthesis of *RAS1* and *RAS2* proteins varied during growth in a reciprocal fashion. The rate of synthesis of *RAS1* protein was highest at the beginning of exponential growth (Figure 3C, 2 and 6 h points) and declined as the culture proceeded into late exponential growth (Figure 3C, 12 h point). [Note that *RAS1* is the upper band in the doublet shown in Figure 3C. *RAS1* in Figure 3D has migrated with the lower band in this gel. In other experiments (data not shown), *RAS1* synthesis was found to be constant between 2 and 6 h of growth.] This pattern of *RAS1* protein synthesis parallels the abundance profile of *RAS1* mRNA observed in the same conditions (Figure 3B). By contrast, the rate of *RAS2* protein synthesis was very low in early-exponential growth, increased 5- to 10-fold during mid-exponential growth and remained relatively high as cells entered late exponential phase (Figure 3C and D). The abundance profile of *RAS2* mRNA varied from this pattern of *RAS2* protein synthesis. The level of *RAS2* mRNA was highest in early-exponential growth and decreased as cells approached stationary phase (Figure 3B and D).

Equal amounts of the total RNA samples analyzed by blot-hybridization in Figure 3B were translated *in vitro* in a rabbit reticulocyte lysate. The results in Figure 4A show that roughly equal amounts of *RAS2* protein were synthesized *in vitro* from RNA samples collected at 2, 6 and 12 h of growth. These data more closely parallel the abundance profile of *RAS2* mRNA shown in Figure 3B than the *in vivo* pattern of *RAS2* protein synthesis shown in Figure 3C and D. Taken together, these results suggest that translatable *RAS2* mRNA is present in abundance in the early exponential period but is translated at a low efficiency compared to later times in the growth curve. By contrast with the data for *RAS2*, there is much closer agreement between the rates of protein synthesis measured *in vivo* and *in vitro* for the *RAS1* gene (Figures 3C and 4A). These findings suggest that regulation of *RAS1* expression in these conditions occurs primarily by controlling mRNA abundance.

The blot-hybridization analysis of *RAS2* mRNA shown in Figure 3B and D suggests that a change in the size of *RAS2* mRNA occurs during growth in SD medium. To investigate this possibility, we used S1 endonuclease digestion of DNA–RNA hybrids to map the 5′ ends of *RAS2* mRNA (Figure 5). These data demonstrate the existence of multiple *RAS2* mRNA species with major 5′ ends mapping between 45 and 235 bp upstream from the presumptive *RAS2* ATG initiation codon. Based upon the pattern of their differential enrichment in different culture conditions, we grouped these transcripts into three major clusters with 5′ ends mapping around positions −45 bp (I), −180 bp (II) and −235 bp (III). All three clusters of *RAS2* mRNA are present in cells during the exponential phase of growth in SD (Figure

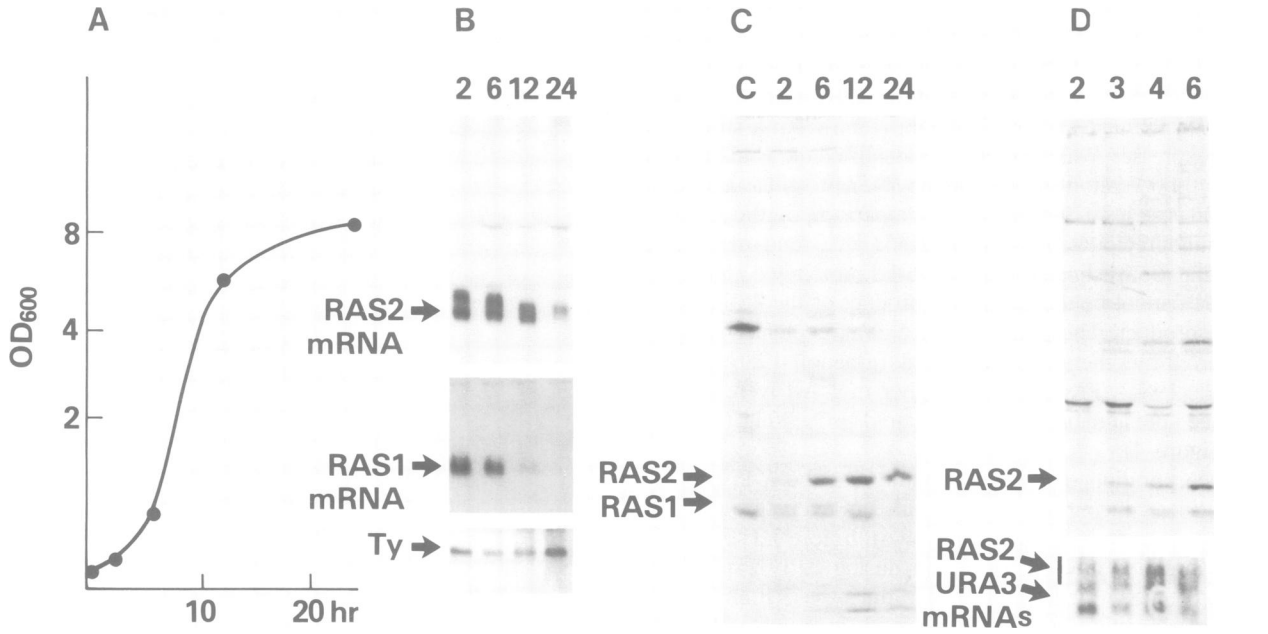

Fig. 3. *RAS1* and *RAS2* gene expression along an SD growth curve. (**A**) Growth curve of S288C cells in SD medium. Cells taken from an SD stationary phase culture were inoculated into fresh medium and growth at 30°C was monitored. (**B**) RNA blot hybridization analysis of *RAS1* and *RAS2* mRNA. Total RNA was extracted at 2, 6, 12 or 24 h from cells growing in SD protein and hybridized against *RAS1* or *RAS2* radiolabeled DNA probes. The sizes of the heterogeneous population of *RAS2* mRNAs vary from 1.3 to 1.1 kb. *RAS1* mRNA is 1.15 kb. (**C**) Immunoprecipitation of *RAS1* and *RAS2* proteins synthesized at different times along an SD growth curve. S288C cells were labeled with 7.5-min pulses of [^{35}S]methionine after 2, 6, 12 or 24 h of growth. 10^7 c.p.m. of each cell extract were used for immunoprecipitation with Y13-259 antibody. C is a control immunoprecipitation using rat pre-immune serum. Note the presence of an abundant species that migrates slightly faster than *RAS1* protein in these gels (see also Figure 1). This species is precipitated by pre-immune serum and therefore is unrelated to *RAS1* proteins. (**D**) Immunoprecipitation of *RAS* proteins synthesized at early times in an SD growth curve. S288C cells were labeled (as in **C**) after 2, 3, 4 or 6 h of growth. 10^7 c.p.m. of each lysate were used per immunoprecipitation reaction. Total RNA was also extracted at the same time points and hybridized against *RAS2* and *URA3*-specific radiolabeled probes.

5A, lane 7) whereas in stationary phase, the 5′ proximal transcripts are considerably reduced relative to the 5′ distal species and a specific enrichment in cluster II is observed (Figure 5A, lane 8). Other variations in the pattern of *RAS2* mRNA 5′ end utilization were observed in different growth conditions and are discussed below.

Expression of RAS1 and RAS2 genes during growth in SE medium

ras2 mutants are unable to grow in minimal media containing a non-fermentable carbon source (Fraenkel, 1985; Tatchell *et al.*, 1985). We showed previously that *RAS1* mRNA expression is significantly reduced in these culture conditions relative to growth in glucose-containing medium. The data shown in Figure 6C extend this finding by demonstrating that synthesis of *RAS1* protein was detectable only in the early exponential phase of growth in cultures of wild-type cells grown on ethanol medium (Figure 6C, 11 h point). By contrast, *RAS2* protein synthesis occurred almost constitutively throughout the SE growth curve (Figure 6C). In direct comparisons, we found that the constitutive level of *RAS2* protein synthesis in SE medium is comparable to that observed at mid-exponential growth in SD medium (data not shown). Unlike the results obtained in SD medium, the translational efficiency of *RAS2* mRNA does not appear to be low in the early phase of exponential growth on SE medium. In fact, it seems to be highest at the earliest time point and to decline somewhat as growth proceeds. Figure 6B shows that the amount and 5′ end distribution of *RAS2* mRNA during growth in SE medium was similar to that described for cells grown in SD medium.

RAS2 expression during sporulation

ras2/ras2 diploids hypersporulate in rich medium (Kataoka *et al.*, 1984; Tatchell *et al.*, 1985), suggesting that the *RAS2* product is a negative effector of sporulation. To investigate whether initiation of sporulation is accompanied by a reduction in *RAS2* expression, we measured *RAS2* mRNA levels and the rate of *RAS2* protein synthesis in diploid strains subjected to a standard sporulation regime in medium lacking a nitrogen source and containing acetate as the carbon source. When cells were shifted to sporulation medium, the amount of *RAS2* mRNA was considerably reduced (Figure 7) and a shift from transcript clusters I and II to transcript cluster I was observed (Figure 5A, lanes 1−6). This response is independent of the sporulation pathway since it occurs in an isogenic, asporogenous *a/a* strain. Addition of ammonium sulfate to cultures incubated for 2 h in sporulation medium restored high levels of total *RAS2* mRNA and, in particular, transcript cluster II (Figure 7, lane −2+4 and Figure 5, lane 6). Despite these changes at the transcriptional level, little or no variation in the rate of *RAS2* protein synthesis was observed in cells cultured in sporulation medium compared to cells grown in nutritionally complete medium (Figure 7). (The rate of total protein remained relatively constant in these experiments.) By contrast, the amount of *RAS2* protein synthesized *in vitro* from RNA extracted from cells cultured in sporulation medium was significantly lower than that observed for RNA extracted from cells maintained in SA medium (data not shown). This result is in agreement with the reduction in *RAS2* mRNA observed by blot-hybridization when cells are shifted into sporulation medium (Figure 7). Taken together, these data indicate that no significant

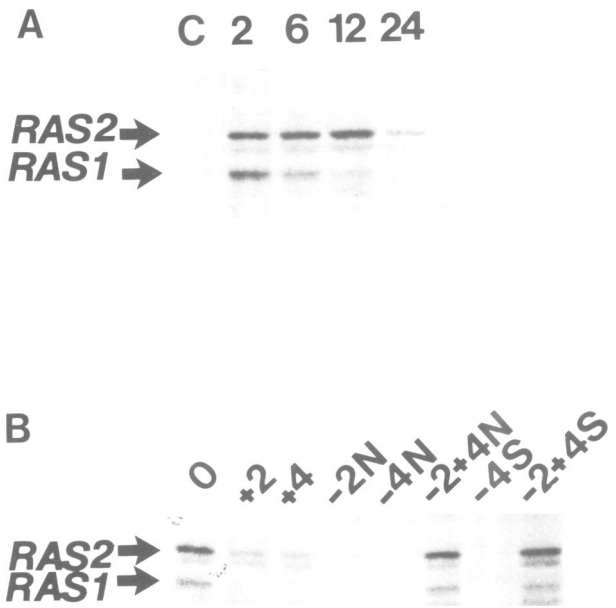

Fig. 4. Immunoprecipitation analysis of *RAS1* and *RAS2* proteins synthesized *in vitro* in a rabbit reticulocyte lysate. (**A**) SD growth curve: equal amounts of total RNA extracted from cells after 2, 6, 12 or 24 h of growth in SD medium were translated in a rabbit reticulocyte lysate, after which immunoprecipitation was performed with Y13-259 antibody. C is a control immunoprecipitation using rat pre-immune serum. (**B**) Nitrogen and sulfur starvation: equal amounts of total RNA extracted from cells growing in MIN medium or starved for nitrogen or sulfur were translated *in vitro*; immunoprecipitation was performed as in **A**. 0 corresponds to the time of the shift; +2 and +4 designate 2 and 4 h respectively, after a shift to fresh non-starvation medium; −2N and −4N designate 2 and 4 h respectively, in nitrogen starvation medium. −2 + 4N designates 2 h of nitrogen starvation followed by 2 h in non-starvation conditions for a total elapsed time of 4 h after the shift. −4S and −2 + 4S are defined as above except that starvation for sulfur was imposed. *RAS* proteins synthesized *in vivo* comigrated with the lower bands of the doublets obtained for both *RAS1* and *RAS2* proteins in the *in vitro* reactions (data not shown). This is probably the result of rapid processing of the precursor *RAS* proteins in the yeast strains we used plus inefficient processing in the reticulocyte lysate.

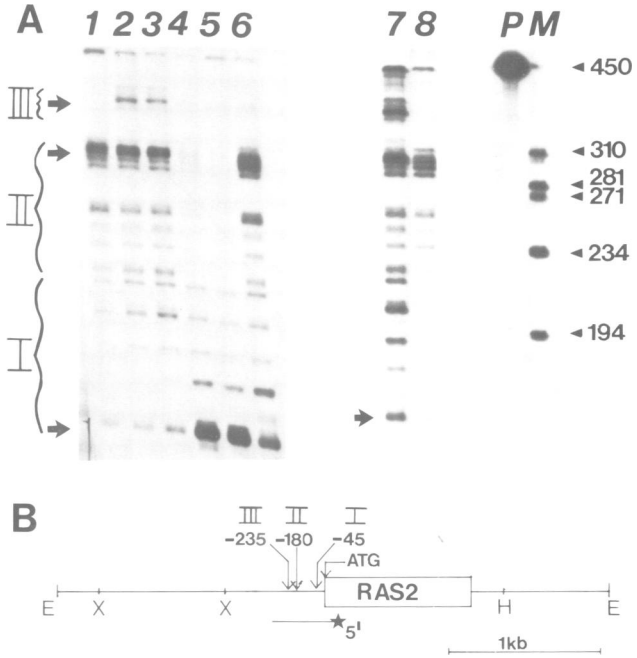

Fig. 5. 5′ termini of *RAS1* mRNA mapped by S1 endonuclease protection analysis. (**A**) 50 μg of total RNA extracted from either AP3 *a*/α (**lanes 1−6**) or S288C (**lanes 7** and **8**) were hybridized with a ^{32}P-end-labeled, single-stranded probe and then digested with S1 endonuclease. The protected fragments were resolved on a 6% polyacrylamide, 8 M urea gel and visualized by autoradiography. **Lanes 1−6** refer to the experiment of Figure 6 and define the pattern of *RAS2* mRNA 5′ ends in AP3 *a*/α cells at the time of the shift into sporulation medium (**lane 1**), 2 and 4 h after a transfer back to fresh SA medium (**lanes 2** and **3**), 2 and 4 h after the shift to sporulation medium (**lanes 4** and **5**) 2 and 4 h after the addition of ammonium sulfate to cells cultured for 2 h in sporulation medium (**lane 6**). **Lanes 7** and **8** represent the 5′ end patterns of *RAS2* mRNA in S288C grown exponentially in SD medium or in stationary phase respectively. Mol. wt markers (φX DNA *Hae*III restriction fragments) are shown at the right end of the panel (M) as well as the end-labeled probe DNA used in the analysis (P). I, II and III represent major clusters of *RAS2* mRNA molecules. No protection of the probe DNA was observed in mock hybridizations containing *Escherichia coli* tRNA in place of yeast RNA. (**B**) The 5′ ends of *RAS2* mRNA. The positions of the major transcripts within each of the three clusters shown in **A** (I, II, III) are indicated with respect to the presumptive *RAS2* ATG initiation codon. The rectangle symbolizes the protein coding sequence of *RAS2*. The line with the star symbolizes the 5′ end-labeled single-stranded *Sau*3AI fragment used as probe in the experiments shown in **A**. Restriction sites for *Eco*RI (E), *Xba*I (X) and *Hin*dIII (H) are indicated.

reduction in *RAS2* protein synthesis occurs in wild-type diploid cells entering meiosis, despite extensive changes in the amount and 5′ end distribution of *RAS2* mRNA.

RAS2 expression in cells starved for nitrogen or sulfur

The data just presented for sporulation conditions suggest that expression of *RAS2* mRNA is altered by nitrogen starvation. To extend these observations, we examined *RAS2* expression in haploid yeast cells subjected to starvation for either nitrogen or sulfur. Figure 8A shows that a dramatic reduction (~10-fold) in the amount of *RAS2* mRNA occurred in cells cultured in the absence of either nitrogen or sulfur (−N and −S). In addition, nitrogen starvation resulted in a shift from all three transcript clusters to cluster I alone. By contrast, in cells starved for sulfur, only transcript clusters II and III were observed (data not shown). Addition of the missing nutrient after 2 h of starvation restored high levels of *RAS2* mRNA and all three clusters of *RAS2* transcripts (Figure 8A, −2+4 and −2+8 points).

The results in Figure 4B demonstrate a marked reduction in the amount of translatable *RAS2* mRNA in total RNA samples extracted from cells starved for nitrogen or sulfur, compared to unstarved cells. This reduction parallels the decrease in *RAS2* mRNA measured by blot-hybridization (Figure 8A). However, immunoprecipitation of pulse-labeled *RAS2* protein from lysates prepared after 4 h of nitrogen or sulfur starvation showed no significant difference in the rate of *RAS2* protein synthesis compared to that measured in unstarved cells (Figure 8B). These data suggest that *RAS2* mRNA is translated more efficiently in cells starved for nitrogen or sulfur than in unstarved cells.

RAS1 protein synthesis remained at a relatively high level in cells subjected to nitrogen or sulfur starvation compared to unstarved cells (Figure 8B). This finding is consistent with our previous measurements of *RAS1* mRNA accumulation in the same conditions (Breviario *et al.*, 1986) and demonstrates that G_1 arrest brought about by nutrient deprivation

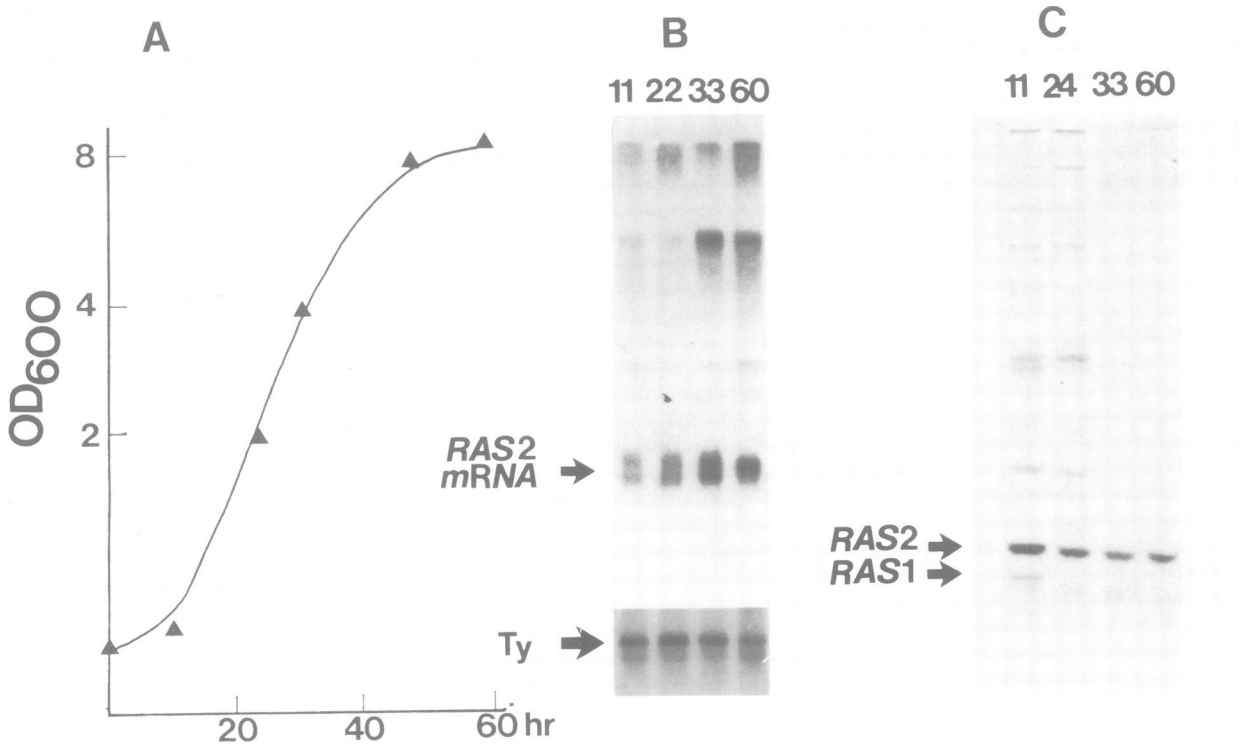

Fig. 6. *RAS1* and *RAS2* gene expression along an SE growth curve. (**A**) Growth curve of S288C cells in SE medium. Cells were taken from an SD stationary phase culture, washed with SE medium and inoculated into SE medium at an OD_{600} of 0.3. Growth at 30°C was monitored over a period of 60 h. (**B**) RNA blot-hybridization analysis of *RAS2* mRNA. Total RNA was extracted at 11, 22, 33 and 60 h from cells growing in SE medium and hybridized against a *RAS2*-specific radiolabeled DNA probe. (**C**) Immunoprecipitation of *RAS1* and *RAS2* proteins synthesized at different times along an SE growth curve. S288C cells were labeled with 7.5-min pulses of [^{35}S]methionine after 11, 24, 33 and 60 h of growth. 10^7 c.p.m. of each lysate were used per immunoprecipitation reaction.

is associated with an elevated level of *RAS2* gene expression. This response was observed even when cells were starved in media containing acetate as the carbon source (Figure 7, −2 and −6 h) despite the fact that *RAS1* mRNA and protein synthesis is normally reduced in the presence of this non-fermentable carbon source.

Discussion

The *RAS1* and *RAS2* proteins of *S.cerevisiae* are differentially expressed under a variety of culture conditions. In cells grown with dextrose as the carbon source, *RAS1* protein synthesis is at its highest level at early times in the growth curve and is nearly undetectable after mid-exponential phase. By contrast, the rate of *RAS2* protein synthesis is low at the beginning of exponential growth, increases to a maximum by mid-exponential phase and remains relatively high into stationary phase. Regulation of *RAS1* protein synthesis can be accounted for by modulation of the *RAS1* mRNA level. By contrast, the mechanism controlling *RAS2* protein expression is complex. Our data suggest that at the beginning of the exponential phase of growth, *RAS2* mRNA is present at high levels but is inefficiently translated. The relative translational efficency of *RAS2* mRNA increases as cells proceed to mid-exponential growth. This occurs with no change in the 5′ end distribution of *RAS2* mRNA. Therefore, the translational mechanism responsible for modulating *RAS2* expression in early exponential growth appears to be independent of changes in the 5′ end distribution of *RAS2* mRNA.

Since *RAS1* protein synthesis ceases by the mid-exponential phase in SD, it might be expected that *RAS1 ras2* mutants would be unable to grow in this medium to the same high cell density as wild-type cells. Contrary to this expectation, we observed that *RAS1 ras2* strains saturate at higher stationary phase densities than isogenic wild-type strains in SD (data not shown). One way to explain this finding derives from our previous observation that *RAS1* mRNA expression remains abnormally elevated well into stationary phase in *RAS1 ras2* mutant cells (Breviario *et al.*, 1986). On the other hand, we have observed reduced viability in stationary phase cultures of a strain containing many copies of *RAS1*. These cells show elevated rates of *RAS1* protein synthesis in late-exponential growth compared to isogenic wild-type cells (data not shown). This finding suggests that greatly elevated expression of *RAS1* protein in stationary phase cells can be deleterious.

In cultures grown on a non-fermentable carbon source such as ethanol, *RAS1* protein synthesis is very low, even in early exponential growth, compared to cells grown with glucose as the carbon source. We showed previously that *RAS1* mRNA expression is reduced in the presence of these carbon sources compared to glucose (Breviario *et al.*, 1986) and this regulation may explain the inability of *RAS1 ras2* mutants to grow on media containing ethanol and acetate as carbon sources. *RAS2* protein synthesis is constitutively elevated in cells grown on ethanol at levels comparable to the highest level seen during growth on glucose. The total amount of *RAS2* mRNA is fairly constant and may even increase somewhat throughout the growth curve in SE medium.

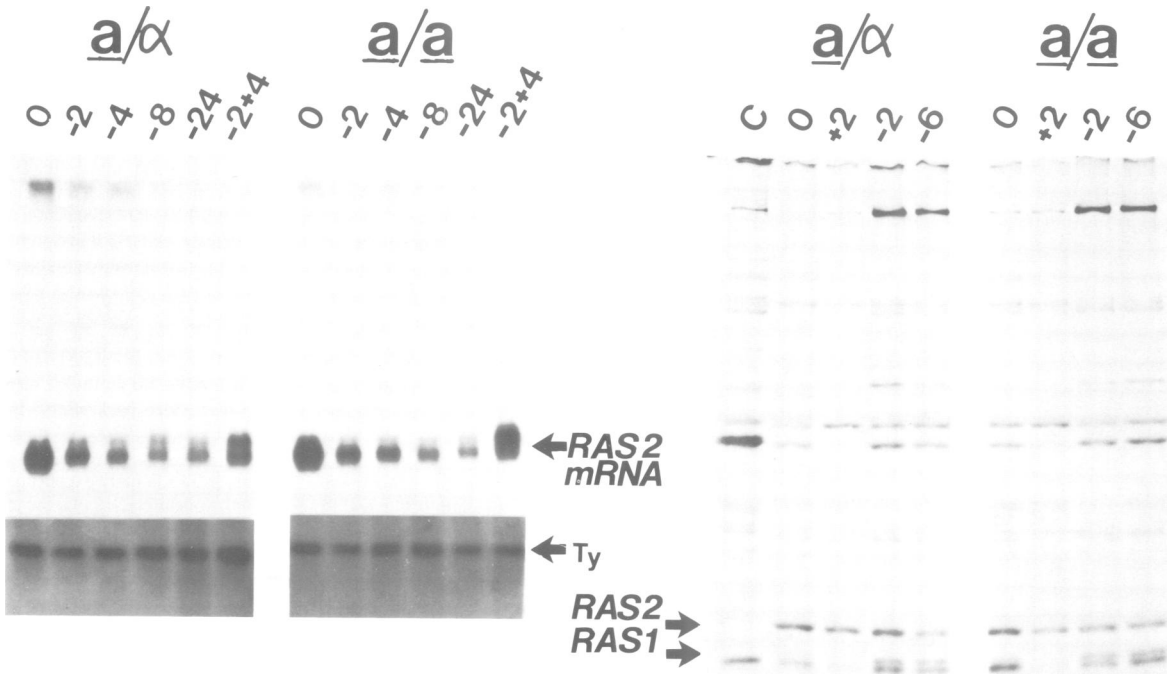

Fig. 7. Sporulation and *RAS* gene expression. **Left panel**: RNA blot hybridization analysis of *RAS2* mRNA. Total RNA was extracted from AP3 *a*/α and AP3 *a*/*a* isogenic diploid cells at the time of the shift from cells in SA medium (0) and from cells cultured in sporulation (Spo) medium for 2 (−2), 4 (−4), 8 (−8) and 24 (−24) h. Lanes −2/+4 refer to cultures in which ammonium sulfate was added after 2 h of incubation in Spo medium and total RNA was prepared 2 h later. Filter-bound RNA was hybridized with a *RAS2*-specific radiolabeled DNA probe. Similar data were obtained for AP3 (α/α). **Right panel**: immunoprecipitation of *RAS1* and *RAS2* proteins synthesized in sporulation medium. AP3 (*a*/α) and AP3 (*a*/*a*) diploid cells were grown to early exponential phase in SA medium and shifted to either fresh SA or Spo medium. Proteins were labeled with [^{35}S]methionine, extracts were prepared and immunoprecipitation was performed as already described. Immunoprecipitations were performed on extracts prepared at the time of the shift (0), 2 h later from cells cultured in fresh SA (+2) or Spo (−2) medium and 6 h after the shift to Spo medium (−6). C is a control immunoprecipitation done without Y13-259 antibodies. 10^7 c.p.m. of each lysate were immunoprecipitated for each extract.

Therefore, in contrast to growth on SD, the translational efficiency of *RAS2* mRNA is not repressed during early exponential growth in SE medium.

These data suggest that a combination of transcriptional and translational controls exist to modulate the levels of *RAS1* and *RAS2* gene expression according to the carbon source. When dextrose is the carbon source, *RAS1* protein is synthesized during early exponential growth. *RAS2* mRNA is also expressed at this stage but is translated with a relatively low efficiency. As cells enter mid-exponential growth, *RAS1* transcription and protein synthesis are suppressed, whereas the rate of *RAS2* protein synthesis increases. The pattern of *RAS1* and *RAS2* gene expression observed at the mid-exponential point in SD medium is similar to that seen in SE medium. This correspondence may exist because ethanol is generated during growth on dextrose, in which case the presence of ethanol rather than the absence of dextrose would be the key regulatory signal. It is not clear at present what purpose is served by modulating the levels of the two *RAS* gene products in this complex fashion.

Homozygous *ras2* diploids sporulate in rich medium even in the presence of mutations that result in high levels of *RAS1* expression (Breviario *et al.*, 1986). This suggests that a requirement exists for *RAS2* function to repress sporulation in non-starvation conditions that cannot be supplied by *RAS1*. It was of interest to determine whether initiation of sporulation was accompanied by a reduction in *RAS2* gene expression. We observed no change in the rate of synthesis or the stability of *RAS2* protein during sporulation. These data suggest that nutrient starvation leads to a reduction in the specific activity of *RAS2* protein rather than a reduction in its amount. Alternatively, the function of the *RAS2* product may be overridden in sporulating cells.

Despite the constitutivity of *RAS2* protein synthesis, significant modulation of the amount and 5′ end distribution of *RAS2* mRNA is observed in diploid and haploid cells starved for nitrogen. A 5- to 10-fold reduction in the amount of *RAS2* mRNA is observed in starved cells compared to unstarved cells, accompanied by a shift to transcripts in cluster I. These data suggest that the translational efficiency of cluster I transcripts is increased in cells starved for nitrogen. Similar data from sulfur starvation experiments suggest an increased translational efficiency for cluster III transcripts in these starvation conditions.

The purpose behind the complex transcriptional modulation of *RAS2* expression is unclear. Perhaps positive transcriptional factors needed for *RAS2* transcription are repressed or less active in starvation conditions. In order to offset this circumstance and maintain a fairly constant level of *RAS2* protein synthesis, *RAS2* mRNA may be optimized for efficient translation in nutrient-starved cells. Alternatively, the features of *RAS2* mRNA that contribute to translational control in other culture conditions might lead to unacceptably high levels of *RAS2* protein synthesis in the

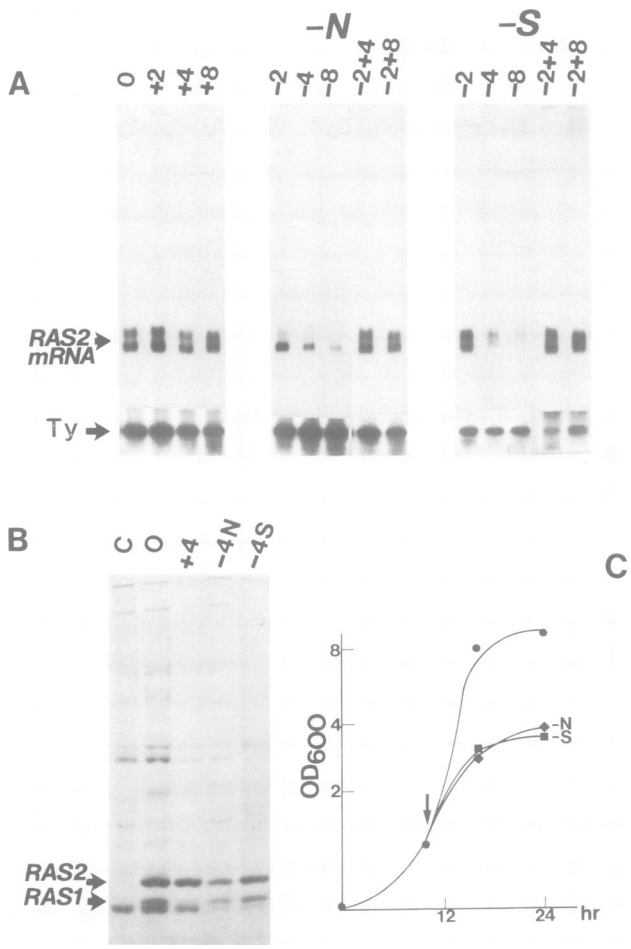

Fig. 8. *RAS1* and *RAS2* expression in nitrogen and sulfur starved cells. (**A**) RNA blot hybridization analysis of *RAS2* mRNA. Total RNA was extracted from cells grown in MIN medium or starved for nitrogen (−N) or sulfur (−S). Hybridization was conducted with a specific *RAS2* radiolabeled DNA probe. Time points refer to Figure 7C. 0 represents the pattern of *RAS2* mRNA at the time of the shift from MIN medium (arrow in Figure 7C). +2, +4 and +8 indicate the number of hours elapsed after shifting from MIN medium to fresh MIN (left panel); −2, −4 and −8 indicate hours of incubation in media lacking nitrogen or sulfur; −2 + 4 and −2 + 8 indicate 2 h of starvation for the particular nutrient plus the total elapsed time from the shift (4 or 8 h). (**B**) Immunoprecipitation of *RAS1* and *RAS2* proteins synthesized during sulfur or nitrogen starvation. S288C cells were labeled, total protein extracts prepared and immunoprecipitations were performed with Y13-259 antibodies. Extracts were prepared at the times indicated as explained in **A**. 10^7 c.p.m. of each lysate were used per reaction with the exception of time 0 where twice as many c.p.m. were used. C is a control immunoprecipitation without Y13-259 antibodies. (**C**) Growth curves in the nutritional deprivation experiments. Cells were grown to early exponential phase in MIN medium and shifted into either fresh MIN or MIN lacking nitrogen (−N) or sulfur (−S). The arrow indicates the time of the shift (time 0 in panels **A** and **B**).

absence of a mechanism for down-regulation of *RAS2* transcription. Whatever the explanation, it seems clear that a combination of transcriptional and translational controls are employed to maintain a constant supply of the *RAS* proteins under a wide range of physiological states.

Materials and methods

Growth conditions

Yeast cells were grown in minimal media [yeast nitrogen base (YNB) plus ammonium sulfate (Sherman *et al.*, 1985)] containing the necessary nutritional requirements and glucose (SD), ethanol (SE) or acetate (SA) as the carbon source at a concentration of 2%. The acetate medium was buffered to pH 5 with phthalic acid. Growth at 30°C was monitored by measuring cell density at 600 nM and by cell counting with a hemocytometer.

Sporulation. Cells taken from a saturated SD culture were pre-adapted to respiratory metabolism by growth in SA medium. An early-exponential phase culture (10^7 cells/ml) was collected on a 0.5 μM nitrocellulose filter, washed once with sporulation (Spo) medium [1% potassium acetate, pH 7 (Kurtz and Lindquist, 1984)] and resuspended in either sporulation medium or fresh SA medium at the original cell density. After 2 h in Spo medium, additions of YNB, ammonium sulfate and the nutritional requirements were made to one-half of the culture to restore growth. All cultures were maintained with shaking at 30°C. The appearance of asci and the yield of sporulation were determined after 24 h by phase contrast microscopy at 400× magnification. By this time, 70% of *a*/α AP3 cells had formed asci whereas no asci were seen in the asporogenous *a*/*a* AP3. No asci were seen in the *a*/α cultures where ammonium sulfate was added back after 2 h in Spo medium.

Nitrogen and sulfur starvation. Cells were grown to early-exponential phase in MIN medium (Lillie and Pringle, 1980) containing 2% glucose at 30°C, then shifted to nitrogen- or sulfur-deficient MIN medium and cultured for up to 24 h. After 2 or 6 h, the missing nutrient was added to half of the culture. Growth and terminal phenotype of the cells were assessed as described above.

Strains

The following *RAS* mutant strains were generously provided by Kelly Tatchell and, with the exception of JC308, were previously described (Cannon *et al.*, 1986; Tatchell *et al.*, 1985).

112-699:α *ras2-699 leu2-112 ura3-1 can1-100 ade2-1 his3*;
RY31-1A:α *trp1 his3 lys1 ura3 leu2 ras1-545 ras2-530 sra1-1*;
JC308:*a leu2 his4-539 ura3-52 lys2-801 ras1-545*;
XCO26C-485:α *ste11 leu2-3 leu2-112 his4-480 his3 trp1 RAS1-485* (the *RAS1-485* allele was constructed by integration of ~40 copies of YRp7-*RAS1*$^+$ at the *RAS1* locus).

Diploid strains AP3 (*a*/α), AP3 (*a*/*a*) and AP3 (α/α), kindly provided by Susan Lindquist and Sandford Silverman, have the following genotype: *ade1*/+ *ade2*/*ade2 gal1*/+ *tyr1*/+ *lys2*/+ *his7*/+ *ura1*/+ +/*ura3* +/*can1* +/*cyh2* +/*leu1*. S288C (α, *gal2*) was employed as a *RAS1 RAS2* strain.

RNA blot-hybridization analysis

Total RNA was extracted according to published procedures (Carlson and Botstein, 1982). Ten μg of each RNA sample were denatured and electrophoresed in the presence of formaldehyde according to Maniatis *et al.* (1982). RNA species were blotted to a Gene Screen Plus membrane and hybridized with radiolabeled DNA probes according to the supplier's directions (New England Nuclear). For *RAS1*-specific hybridization, a 625-bp *Acc*I−*Hin*dIII fragment containing the 3′ end of the *RAS1* gene (Dhar *et al.*, 1984) was nick-translated (Rigby *et al.*, 1977) and used to probe the RNA blots. For *RAS2*-specific hybridization, a nick-translated 498-bp *Rsa*I fragment containing the 3′ end of the *RAS2* gene (Dhar *et al.*, 1984) was used as the probe. No cross-hybridization between *RAS1* and *RAS2* mRNAs was detected with either probe (data not shown). All RNA blots were also hybridized with a DNA sequence containing either the yeast transposable element Ty (Elder *et al.*, 1983) or the *URA3* gene (Botstein *et al.*, 1979) to verify that the RNA gels were evenly loaded (data not shown). Quantitation of the relative levels of hybridization was performed by scanning autoradiographs with an LKB laser scanning densitometer.

S1 endonuclease protection analysis

A 450-bp *Sau*3AI fragment from position +100 within the coding region of the *RAS2* gene to position −350 bp upstream of the *RAS2* ATG codon was dephosphorylated and radiolabeled at its 5′ end using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase, as described (Maxam and Gilbert, 1980). The complementary strands of this fragment were separated by electrophoresis as described and eluted from the gel by homogenization of the gel slice followed by isolation of the DNA on NACS columns (Bethesda Research Laboratories). 2 × 10^4 Cerenkov c.p.m. of the non-coding DNA strand were mixed with 50 μg of total RNA and incubated for 12 h at 65°C in 25 μl of 0.8 M NaCl, 0.5 M Pipes pH 7.8, 0.01 M EDTA. After hybridization, samples were added to an S1 endonuclease digestion mixture (S1 endonuclease 300 U/ml, 4 mM $ZnSO_4$, 30 mM sodium acetate pH 4.6, 250 mM Nacl and 20 μg/ml of denatured calf thymus DNA) at 0°C and then transferred to 37°C for 30 min. After alcohol precipitation of the digested fragments, the pellet was resuspended in 5 μl of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 120 μg/

ml of xylene cyanole and bromophenol blue), denatured for 4 min at 95°C and resolved on a 6% polyacrylamide:bis (20:1), 8 M urea gel.

Protein-labeling conditions and preparation of total cellular extracts

Proteins were pulse-labeled for 7.5 min with [^{35}S]methionine (800 Ci/mmol) at a final concentration of 20 μCi/ml. In all conditions, incorporation of the labeled precursor into *RAS1* and *RAS2* proteins was linear during the 7.5-min labeling period. At the end of the pulse, cells were rapidly chilled and a mixture of protease inhibitors (TLCK, TCPK, PMSF) was added, each to a final concentration of 100 μg/ml. After one wash with phosphate-buffered saline (PBS), cellular pellets were collected and frozen at −70°C. In pulse−chase experiments, cycloheximide was added to 50 μg/ml and unlabeled methionine was added to 2 mM at the end of the pulse and incubation was continued for the appropriate time. Frozen cells (from 20 ml of culture) were resuspended in 1.2 ml of modified RIPA buffer (20 mM Mops, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 1% Aprotinin, pH 7; a gift from J.Bolen) and disrupted by vigorous mixing on a vortex mixer in the presence of 0.45 μm glass beads. Undisrupted cells, cellular debris and glass beads were removed by centrifugation at 1000 r.p.m. for 5 min. Total cellular extracts were clarified by centrifugation at 40 000 r.p.m. for 60 min in a Ti50 Beckman rotor. Total incorporation of [^{35}S]methionine into cellular protein was determined by measuring the trichloroacetic acid (TCA)-precipitable c.p.m. of a 2 μl aliquot of the lysate. Total protein content in the lysate was determined by the Bradford method using reagent supplied by Bio-Rad and conducted according to the vendor's instructions. Lysates were stored at −70°C.

In vitro translation

Five μg of total RNA was added to a rabbit reticulocyte lysate (Amersham) and reactions were carried out according to the supplier's directions.

Immunoprecipitation

A volume of lysate containing 10^7 TCA-precipitable c.p.m. was used per reaction. Lysates were preabsorbed for 2 h at 4°C with 100 μl of reconstituted Staph A (10% w/v; Calbiochem) previously saturated with rabbit anti-rat antibodies (Boehringer Mannheim). After removal of Staph A by centrifugation at 12 000 r.p.m. for 5 min, monoclonal antibody Y13-259 [generously provided by A.Papageorge; (Furth *et al.*, 1982)] was added to each lysate and allowed to react for at least 4 h at 4°C with constant mixing. One hundred μl of the reconstituted Staph A (saturated as above with rabbit anti-rat antibodies) were added and allowed to react at 4°C for an additional 90 min. The precipitate was collected by centrifugation, washed seven times with modified RIPA buffer and resuspended in 30 μl of Laemmli loading buffer (Laemmli, 1970). After denaturation at 90°C for 5 min, samples were electrophoresed on a 10% SDS/polyacrylamide gel. Radioactive proteins were visualized by fluorography using Kodak XAR-5 film after treatment of the gel with Enlightening (NEN).

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References

Barbacid,M. (1987) *Annu. Rev. Biochem.*, **56**, 779−827.
Botstein,D., Falco,S.C., Stewart,S.E., Brennan,M., Scherer,S., Stinchcomb, D.T., Struhl,K. and Davis,R. (1979) *Gene*, **8**, 17−24.
Bradley,O.M., Kraynak,A.R., Storer,R.D. and Gibbs,J.B. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5277−5281.
Breviario,D., Hinnebusch,A., Cannon,J., Tatchell,K. and Dhar,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4152−4156.
Buss,J.E. and Sefton,B.M. (1986) *Mol. Cell. Biol.*, **6**, 116−122.
Cannon,J.F. and Tatchell,K. (1987) *Mol. Cell. Biol.*, in press.
Cannon,J.F., Gibbs,J.B. and Tatchell,K. (1986) *Genetics*, **113**, 247−264.
Carlson,M. and Botstein,D. (1982) *Cell*, **28**, 145−154.
DeFeo-Jones,D., Scolnick,E.M., Koller,R. and Dhar,R. (1983) *Nature*, **306**, 707−709.
DeFeo-Jones,J., Tatchell,K., Robinson,L.C., Sigal,I.S., Vass,W.C., Lowy, D.R. and Scolnick,E.M. (1985) *Science*, **228**, 179−184.
De Vendittis,E., Vitelli,A., Zahn,R. and Fasano,O. (1986) *EMBO J.*, **5**, 3657−3663.
Dhar,R., Nieto,A., Koller,R., DeFeo-Jones,D. and Scolnick,E. (1984) *Nucleic Acids Res.*, **12**, 3611−3618.
Elder,R.T., Loh,E.Y. and Davis,R.W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2432−2436.
Ellis,R.W., Shih,T.Y. and Scolnick,E.M. (1982) *Adv. Viral Oncol.*, **1**, 107−126.
Fraenkel,D.G. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4740−4744.
Fujiyama,A. and Tamanoi,F. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1266−1270.
Fujiyama,A., Matsumoto,K. and Tamanoi,F. (1987) *EMBO J.*, **6**, 223−228.
Furth,M.E., Davis,L.J., Fleurdelys,B. and Scolnick,E.M. (1982) *J. Virol.*, **43**, 294−304.
Gallwitz,D., Brokel,R., Molenaar,C.M.T., Rauh,A.J.G., Schmitt,H.D. and Wagner,P. (1986) In Tanner,W. and Gallwitz,D. (eds), *Cell Cycle and Oncogenes*. Springer-Verlag, Berlin, pp. 123−130.
Gibbs,J.B., Sigal,I.S., Poe,M. and Scolnick,E.M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5704−5708.
Kataoka,T., Powers,S., McGill,C., Fasano,O., Strathern,J., Broach,J. and Wigler,M. (1984) *Cell*, **37**, 437−445.
Kataoka,T., Powers,S., Cameron,S., Fasano,O., Goldfarb,M., Broach,J. and Wigler,M. (1985a) *Cell*, **40**, 19−26.
Kataoka,T., Broek,D. and Wigler,M. (1985b) *Cell*, **43**, 493−505.
Kurtz,S. and Lindquist,S. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7232−7327.
Laemmli,U.K. (1970) *Nature*, **227**, 680−685.
Lillie,S. and Pringle,J. (1980) *J. Bacteriol.*, **143**, 1384−1394.
McGrawth,J.P., Capon,D.J., Goeddel,D.V. and Levinson,A.D. (1984) *Nature*, **310**, 644−649.
Madaule,P., Axel,R. and Meyers,M.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 779−783.
Maniatias,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499−560.
Papageorge,A., Lowy,D.R. and Scolnick,E. (1982) *J. Virol.*, **44**, 509−519.
Powers,S., Kataoka,T., Fasano,O., Goldfarb,M., Strathern,J., Broach,J. and Wigler,M. (1984) *Cell*, **36**, 607−612.
Powers,S., Michaelis,S., Broek,D., SantaAnnaA,S., Field,J., Herskowitz,I. and Wigler,M. (1986) *Cell*, **47**, 413−422.
Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237−242.
Sherman,F., Fink,G.R. and Hicks,J.B. (1985) *Methods in Yeast Genetics. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 90−93.
Tamanoi,F., Walsh,M., Kataoka,T. and Wigler,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6924−6928.
Tatchell,K. (1986) *J. Bacteriol.*, **166**, 364−367.
Tatchell,K., Chaleff,D., DeFeo-Jones,D. and Scolnick,E. (1984) *Nature*, **309**, 523−527.
Tatchell,K., Robinson,L. and Breintenbach,M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3785−3789.
Temeles,G.L., DeFeo-Jones,D., Tatchell,K., Ellinger,M.S. and Scolnick,E. (1984) *Mol. Cell. Biol.*, **4**, 2298−2305.
Temeles,G.L., Gibbs,J.B., D'Alonzo,J.S., Sigal,I.S. and Scolnick,E.M. (1985) *Nature*, **313**, 700−703.
Toda,T., Uno,I., Ishikawa,T., Powers,S., Kataoka,T., Broek,D., Cameron, S., Broach,J., Matsumoto,K. and Wigler,M. (1985) *Cell*, **40**, 27−36.
Willumsen,B.M., Christensen,A., Hubbert,N.C., Papageorge,A.G. and Lowy,D.R. (1984) *Nature*, **310**, 583−586.

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