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On *ras* gene function in yeast

(oncogenes/glycogen/adenylate cyclase)

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ABSTRACT ***Saccharomyces cerevisiae* contains two *RAS* genes, *RAS1* and *RAS2*. An insertion mutation in *RAS2* (*ras2::LEU2*) does not affect growth on glucose based media but it does prevent growth on media with pyruvate or other noncarbohydrate carbon sources. This defect is pH sensitive and is most severe at pH 7 and above. The *ras2::LEU2* mutation also causes markedly higher levels of glycogen in the derepressed phase of growth after glucose exhaustion. Selection for restoration of growth on pyruvate yields unlinked suppressor mutations. Some of the suppressors also reduce glycogen as well as trehalose (the other reserve carbohydrate in yeast) to levels much lower than those of wild-type strains. These suppressor mutations do not suppress the lethality of *ras1 ras2* double mutants. The results indirectly accord with yeast *RAS2* governing a G protein activity of adenylate cyclase.**

Saccharomyces cerevisiae has two genes, *RAS1* and *RAS2*, homologous to the mammalian *ras* genes and encoding proteins (size *ca.* 35,000 daltons) closely resembling in the sequence of their amino terminal halves the mammalian ras proteins (1, 2). Insertional inactivation of either gene alone is without noted effect on growth but inactivation of both results in failure of spore germination (3, 4), as if *ras* gene function is essential in yeast with either one of the two cognate genes being adequate.

The present work began as an attempt to observe phenotypic alterations in a single gene *ras2* mutant. It was eventually influenced by two considerations. First, the *ras* proteins of higher cells are membrane associated and have guanine nucleotide binding (5) and GTPase (6, 7) activities. The G protein complexes of higher cells (e.g., of adenylate cyclase) have these properties and Gilman and colleagues (8, 9) have suggested on the basis of sequence homology that *ras* proteins and the α subunit of the G proteins may have analogous functions. Less is known about the structure of the yeast adenylate cyclase complex but yeast cyclase activity is activated by guanine nucleotides (10) and guanine nucleotide binding activity is associated with the cloned yeast *RAS2* product (11), a protein of similar size to known α subunits. If yeast adenylate cyclase does function like cyclase from higher cells, then loss of an α subunit would be expected to impair cyclase activity.

The second consideration concerned known aspects of cAMP metabolism in yeast. The reserve carbohydrates in yeast are glycogen and trehalose (12) and, as with glycogen in higher cells, their degradative enzymes are activated by cAMP-dependent phosphorylation [glycogen phosphorylase (13) and trehalase (14, 15), respectively]. cAMP also affects other metabolic functions in yeast (see *Discussion*). Hence, if *RAS2* governs cAMP metabolism, then a *ras2* mutant might be affected in reserve carbohydrates and perhaps in growth on certain substrates, as described below.

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MATERIALS AND METHODS

Strains. The following six strains were from M. Wigler. Strain DC5/6 is **a**/α *his3/HIS3 his4/HIS4 leu2/leu2*, and strain 2-2 (*ras2::LEU2/RAS2*) was derived from it by transplacement (4). The prototype *ras2* mutant was strain S1 (α *his3 leu2 ras2::LEU2*), a segregant from strain 2-2; it was compared with strain L2 (**a** *his4 leu2*), also a segregant from strain 2-2. *ras1* strains were T3-28D (α *his3 leu2 ras1::HIS3 trp1 ura3*) and T3-35C (**a** *can1 his3 leu2 ras1::HIS3 trp1 ura3*).

Strain DFY490 (**a** *his3 leu2 ras2::LEU2*) is another segregant (2-2/1c, Tables 1 and 2) from strain 2-2, and strain DFY509 (**a** *his3 leu2*) is a segregant from strain DC5/6. Pyr^+ revertants of strain S1 were crossed with strain L2, and the following segregants identified: DFY503 (α *his3 leu2 ras2::LEU2* Rpr-1), DFY504 (α *leu2* Rpr-1), DFY505 (α *his3 leu2 ras2::LEU2* Rpr-2), DFY506 (α *his3 leu2* Rpr-2), DFY507 (α *his3 leu2 ras2::LEU2* Rpr-3), and DFY508 (α *leu2* Rpr-3).

ras1::HIS3/RAS1 diploids were D2 (S1 × T3-35C), D3 (DFY490 × T3-28D), D4 (DFY503 × T3-35C), D5 (DFY505 × T3-35C), D6 (DFY507 × T3-35C), and D8 (DFY509 × T3-28D).

Media. Enriched medium R contained 0.4% yeast extract (Difco), 1% Bacto-tryptone (Difco), and minimal salts solution 63 (16) modified in amount of KOH so as to give the desired pH (e.g., R6.2 has a pH of 6.2). Thus the medium previously referred to as rich (17) is now R7.0. Unless specified otherwise, growth employed R6.2 medium. YP medium contained 1% yeast extract (Difco) and 2% Bactopeptone (Difco). R and YP media were supplemented with 1% of a major carbon source, glucose or sodium pyruvate. Solid medium contained, in addition, 2% agar. Growth was at 30°C, aerobic. Standard genetic techniques were used (18).

Glycogen and Trehalose. The protocol to measure glycogen and trehalose (Table 3) was adapted from refs. 12 and 19. Amounts of culture of *ca.* 400 OD_{580} units (1 OD_{580} unit = 1 ml at OD_{580}) were centrifuged and washed three times with water, and the pellet was resuspended in *ca.* 0.5 ml of water. One hundred fifty OD_{580} units were made up to 1 ml 10% trichloroacetic acid (final concentration) and extracted 1 hr at room temperature. The supernatant was used for assay of trehalose as anthrone-reactive material (20). The pellets were treated with 1 ml of 0.25 M Na_2CO_3 90 min in a boiling bath and 0.2-ml portions of the suspension were brought to pH *ca.* 4.8 by addition of 0.055 ml of 3 M acetic acid and 0.2 M NaOAc (pH 4.8) to 1 ml. Five microliters of α-amylase (Boehringer 102814; 10 mg/ml) and 30 μl of amyloglucosidase (Sigma; 10 mg/ml) were added, the suspension was incubated for 2 hr at 37°C, and the released glucose was assayed by glucose oxidase (Sigma). Results are expressed as μg of trehalose or glucose, respectively, per OD_{580} unit. Dry-weight determinations on some of the cultures gave values of 129–148 μg (dry weight)/OD_{580} unit. The cultures assayed were in stationary phase of growth on R6.2/glucose medium, 1 to 3 days after glucose exhaustion. During this period glycogen and trehalose content varied little.

RESULTS

Growth of a *ras2* Mutant. Strain S1, a *ras2* mutant, was compared with strain L2 (*RAS2*) with respect to several growth parameters. Initial experiments confirmed their apparently normal and similar growth on glucose with respect to colony size, growth rate, yield, ethanol formation, and levels of enzymes of glycolysis (data not shown). However, on pyruvate and on other noncarbohydrate carbon sources, growth of the *ras2* strain was defective.

The severity of the defect in growth on pyruvate depended on the basal medium used. Thus with YP medium colonies were somewhat smaller for the *ras2* strain while with the enriched R7.0 medium often used in this laboratory, the difference was more pronounced (Table 1, part A). Two differences between the media were contributory. First, R7.0 medium includes 0.4% yeast extract and 1% tryptone while YP has 1% yeast extract and 2% peptone. Assay with glucose oxidase showed them to contain *ca.* 30 and 80 μg of glucose per ml, respectively (from the yeast extract). Supplementation of R7.0 medium with glucose at 40 μg/ml diminished the difference between growth of mutant and wild type (Table 1). Second, YP medium is unbuffered and has an initial pH of *ca.* 6.6 while R7.0 medium contains minimal salts medium 63 with 100 mM potassium phosphate at pH 7. When the R medium was composed over a range of pH from 5.5 to 7.6 (Table 1, part B) it was clear that while growth of the wild-type *RAS2* strain itself on pyruvate was quite sensitive to pH above 6.7, growth of the *ras2* mutant was affected even more and because of this the apparent difference between the strains was increased. A similar but far less pronounced trend could be discerned for growth on glucose.

Thus, the "Pyr$^-$" characteristic of the *ras2* strain was quite "leaky" and pH dependent. In this respect it is different from rho$^-$, which in pyruvate medium at pH 6.2 fails to show growth even when a large number of cells are deposited on a plate as a "patch." Scoring of the ras2 phenotype as patch growth was sometimes ambiguous and single-colony streaking was usually employed. In key cases (e.g., Table 1, parts C and D) plates were spread with diluted cultures.

Strains S1 and L2 were segregants from the same heterozygous diploid strain 2-2 (*ras2::LEU2/RAS2, leu2/leu2*). Of 25 asci dissected, 20 gave four germinating spores and 2:2 segregation of Leu$^+$:Leu$^-$. In 19 of these tetrads both of the Leu$^+$ segregants were Pyr$^-$ and both of the Leu$^-$ segregants were Pyr$^+$. (Growth of one such tetrad is

Table 1. Growth on plates

Part A: Strain	Carbon source, time of incubation, type of medium	YP	R7.0
L2 (*RAS2*)	Glucose (2 days)	1.5	1.5
S1 (*ras2*)	Glucose (2 days)	1.3	1.3
L2 (*RAS2*)	Pyruvate (4 days)	1.4	1.0 (1.2)*
S1 (*ras2*)	Pyruvate (4 days)	0.8	0.2 (0.5)*

Part B: Strain	Carbon source, time of incubation, type of medium	R5.5	R6.2	R6.7	R7.3	R7.6
L2 (*RAS2*)	Glucose (3.5 days)	3.3	3.4	2.7	2.6	1.8
S1 (*ras2*)	Glucose (3.5 days)	2.6	3.1	2.9	1.9	1.4
L2 (*RAS2*)	Pyruvate (3.5 days)	1.4	1.4	1.3	0.6	0.1
S1 (*ras2*)	Pyruvate (3.5 days)	0.9	0.9	0.7	0.1	NG

Part C: Strain	Carbon source, time of incubation, type of medium	Glucose (3 days)		Pyruvate (5 days)	
		R6.2	R7.2	R6.2	R7.2
2-2/1a (*ras2*)		2.5	0.8–1.6	1.4	NG
2-2/1b (*RAS2*)		2.6	0.9–2.2	2.5	1.0–1.5
2-2/1c (*ras2*)		2.4	1.2–1.8	1.2–1.5	NG
2-2/1d (*RAS2*)		2.6	1.1–1.9	1.8–2.3	0.3–0.7

Part D: Strain	Carbon source, time of incubation, type of medium	Glucose (3 days)		Pyruvate (5 days)	
		R6.2	R7.2	R6.2	R7.2
DFY503 (*ras2* Rpr-1)		2.5	2.5	1.8	0.6–1.3
DFY504 (*RAS2* Rpr-1)		2.8	2.0	2.0	0.8–1.5
DFY505 (*ras2* Rpr-2)		2.7	1.4–2.3	2.0	0.6–1.4
DFY506 (*RAS2* Rpr-2)		2.7	2.0	2.2	1.0–1.8
DFY507 (*ras2* Rpr-3)		2.6	1.4–1.8	2.0	0.6–0.9
DFY508 (*RAS2* Rpr-3)		2.6	1.4–2.5	2.0	0.9

Colony sizes are given in mm. NG, no growth. In parts A and B, cells were streaked onto plates of the indicated composition and maximum sizes of well-isolated colonies were recorded. In parts C and D, dilutions were made from liquid culture and spread to give 10–50 colony-forming units per plate. A range of colony sizes is indicated when the size distribution was broad.
*Plates were supplemented with glucose at 40 μg/ml.

Table 2. Suppression of *ras2*

Diploid	No. of complete tetrads	Makeup			
		PD	NPD	T	Other
Part A					
S1 rev't 1 (Rpr-1) × L2	16	0	1	13	2
S1 rev't 2 (Rpr-2) × L2	6	1	1	4	0
S1 rev't 3 (Rpr-3) × L2	10	2	1	6	1
		Pyr^+:Pyr^-			
Part B		3:1	2:2		
DFY503 (Rpr-1) × DFY490	16	1	15		
DFY505 (Rpr-2) × DFY490	11	0	11		
DFY507 (Rpr-3) × DFY490	20	1	19		

Part A. PD, parental ditype: 2 Leu^- Pyr^+, 2 Leu^+ Pyr^+; NPD, nonparental ditype: 2 Leu^- Pyr^+, 2 Leu^+ Pyr^-; T, tetratype: 2 Leu^- Pyr^+, 1 Leu^+ Pyr^+, 1 Leu^+ Pyr^-. Part B. Strains DFY503, -505, and -507 were Leu^- Pyr^+ segregants from NPD tetrads in the three crosses in part A, respectively. Each diploid is homozygous *ras2::LEU2/ras2::LEU2*.

shown in Table 1, part C.) Thus, the Pyr^- character is closely linked with *ras2::LEU2* and seems to be a consequence of the *ras2* mutation itself.

Revertants on Pyruvate. Revertants of strain S1 were readily observed on R7.0/pyruvate plates either as discrete colonies in areas of thin confluent growth or, after prolonged incubation in liquid media from which glucose had been exhausted, as isolated colonies of normal size. To test whether these mutations were unlinked suppressors, strain S1 was outcrossed with *RAS2* strain L2 (Table 2, part A). Pyr^- (Leu^+) spores were recovered and the most frequent tetrads (tetratypes) contained three Pyr^+ spores, one of which was *ras2* (Leu^+) as if indeed a suppressor was segregating independently of *ras2*. (Although these data are limited a similar conclusion may also be drawn from the outcrosses in Table 4.) The suppressors were named Rpr-1, -2, and -3, respectively (*r*eversion on *p*yruvate of *r*as). A suppressed *ras2* segregant from each cross in Table 2 (part A) was backcrossed with a *ras2* strain (Table 2, part B). Segregation of Pyr^+:Pyr^- was 2:2 in all three cases (except for two 3:1 tetrads, which might reflect gene conversion or reversion in segregant clones) and it may be concluded that Rpr-1, -2, and -3 are almost certainly single mutations. Table 1 (part D) shows growth of six key segregants: *ras2* Rpr-1, -2, and -3 and *RAS2* Rpr-1, -2, and -3. The latter three strains were obtained from nonparental ditype tetrads (Table 2, part A).

Table 3. Glycogen and trehalose

Strain	Glycogen, $\mu g/OD_{580}$	Trehalose, $\mu g/OD_{580}$
L2 *(RAS2)*	3.3	14.4
S1 *(ras2)*	13.7	21.0
2-2/1a *(ras2)*	16.7	22.3
2-2/1b *(RAS2)*	4.1	16.9
2-2/1c *(ras2)*	12.7	28.8
2-2/1d *(RAS2)*	4.5	7.6
DFY503 *ras2* Rpr-1	0.2	<0.2
DFY504 *RAS2* Rpr-1	0.2	<0.2
DFY505 *ras2* Rpr-2	0.3	<0.2
DFY506 *RAS2* Rpr-2	0.6	<0.2
DFY507 *ras2* Rpr-3	10.4	17.6
DFY508 *RAS2* Rpr-3	2.9	7.6

Determinations were made on cultures harvested in stationary phase from R6.2 medium/1% glucose. Entries 3–6 are for the tetrad described in part C of Table 1.

Glycogen and Trehalose. Examination of colonies by iodine staining (for glycogen) indicated that *ras2* mutant strains indeed might contain more glycogen and two of the suppressors of Pyr^-, Rpr-1 and Rpr-2, seemed to reduce glycogen content. Thus in all 26 of the 2 Pyr^+:2 Pyr^- tetrads from Rpr-1/+ and Rpr-2/+ *ras2/ras2* diploids (Table 2, part B, lines 1 and 2), the Pyr^+ segregants stained pale and the Pyr^- ones stained dark, the latter indicating higher than normal glycogen levels. In the Rpr-3/+ cross (line 3), however, all the segregants stained dark like the *ras2* mutant itself.

Assay data on glycogen and trehalose are presented in Table 3. The top entries show that the original *ras2::LEU2* strain, S1, contains considerably more glycogen than the *RAS2* control strain L2 and the differences between *ras2* and *RAS2* strains were likewise observed in the tetrad. For trehalose the results were somewhat less clear, the three *ras2* strains containing on the average a 50% higher level than the *RAS2* strains.

Levels in the strains containing the suppressors are also shown in Table 3. Both Rpr-1 and Rpr-2 reduced glycogen and trehalose to barely detectable levels and the mutations acted this way in both the *ras2* and *RAS2* backgrounds. Rpr-3, on the other hand, was without marked effect on glycogen and trehalose.

Thus as predicted the *ras2* mutation affected the levels of reserve carbohydrate and two of the suppressor mutations selected for their restoration of growth on pyruvate had a striking and opposite effect from *ras2* in this respect.

Suppression of *ras1 ras2*. To determine whether the mutations suppressing the Pyr^- characteristic of *ras2* strains would also allow growth of the *ras1 ras2* combination, *ras2* strains carrying the three suppressors (the same strains described in Tables 1–3) were crossed with a *ras1::HIS3* mutant strain (diploids D4–D6). In control crosses, *ras1* strains were crossed with a *RAS2* strain (diploid D8) as well as with *ras2* strains unsuppressed for Pyr^- (diploids D2 and D3). All diploids were homozygous for *his3* and *leu2* so that scoring of His and Leu would allow assignment of the *ras* alleles (Table 4).

For diploids D4 and D5 containing Rpr-1 and Rpr-2, respectively, the results were clear: no His^+ Leu^+ segregants were obtained although scoring for Pyr showed the expected segregation of a suppressor. Thus, these two suppressors definitely did not allow growth of *ras1 ras2* segregants.

For diploid D6 containing Rpr-3, His^+ Leu^+ segregants were obtained but about half were Pyr^+ and half were Pyr^-, the same pattern observed for the His^- Leu^+ segregants. It is likely, therefore, that whatever the reason for recovery of the *ras1 ras2* segregants, it was not their inheritance of Rpr-3.

One of the control diploids, D3 (*ras1/RAS1 ras2/RAS2*), gave as expected (almost) no *ras1 ras2* segregants (3, 4). However, the other diploid of this composition, D2, unexpectedly gave an appreciable number of such segregants. Unlike other crosses, diploid D8, heterozygous only for *ras1/RAS1*, usually produced asci with four germinating spores and the His^+ (*ras1*) segregants had no clear abnormality on pyruvate.

These results show that the three suppressors in question probably do not allow growth of *ras1 ras2* segregants. The apparent appearance of such segregants in two of the crosses will be discussed.

DISCUSSION

These results show that a yeast *ras2* mutant is impaired in growth on noncarbohydrate carbon sources and accumulates excessive glycogen. Unlinked suppressor mutations restore growth and some of them also reduce glycogen as well as

Table 4. Crosses with *ras1::HIS3*

Diploid*				No. of asci	Viable spores and His/Leu segregation†					Suppression of Pyr^-	Viable His^+Leu^+ spores
	RAS1	*RAS2*	*Sup*		*4*	*3*	*2*	*1*	*0*	(Pyr^+/Leu^+)‡	
D2	+/−	+/−	+/+	43	9 (1/0/5)	24 (1/2/17)	11	0	0	0/21	13§
D3	+/−	+/−	+/+	93	10 (9/0/0)	60 (3/0/54)	16	4	3	0/20	1
D4	+/−	+/−	+/Rpr-1	18	1 (1/0/0)	11 (1/0/10)	4	1	1	11/18	0
D5	+/−	+/−	+/Rpr-2	22	2 (2/0/0)	10 (1/0/9)	8	1	1	7/19	0
D6	+/−	+/−	+/Rpr-3	47	5 (2/0/2)	31 (0/9/20)	6	2	3	12/34‖	13¶
D8	+/−	+/+	+/+	24	20	4	0	0	0	**	**

*+, wild-type allele; − or Sup, mutant allele (*ras1::HIS3*, *ras2::LEU2*, Rpr-1, Rpr-2, and Rpr-3). All strains are homozygous *his3/his3* and *leu2/leu2*.

†Number of tetrads giving 4, 3, 2, 1, and 0 viable spores. For 3 and 4, constitution of tetrads [parental ditype/nonparental ditype/tetratype (PD/NPD/T)] with respect to His and Leu is presented (unassigned tetrads were presumed gene conversions). For 3, the nongerminating spore was assigned on basis of the other 3.

‡Scoring of some His^- Leu^+ (i.e., *ras2::LEU2*) spores for growth on pyruvate.

§Three His^+ Leu^+ were from NPD, nine were from T, and five were from complete asci.

‖Twelve Pyr^+/21 His^-, 5 Pyr^+/13 His^+.

¶Nine His^+ Leu^+ were from NPD, four were from T and two were from complete asci.

**All spores Leu^-, none Pyr^-.

trehalose to levels much lower than wild type. Considering the cited similarities between yeast *ras* proteins and the α subunit of G proteins of adenylate cyclase in higher cells, as well as the lethality of *ras1* and *ras2* together, the present results would fit with yeast *RAS2* governing a G protein of yeast cyclase and perhaps *RAS1* governing another one. *RAS2* inactivation would be expected to impair cyclase activity and consequently cause higher levels of glycogen (i.e., less degradation), as well as defects in growth. The suppressors that also reduce glycogen and trehalose in a wild-type background recall the *bcy1* and *glc1* mutations of yeast that cause cAMP independence of a protein kinase activity, activation of trehalase, and low levels of the reserve carbohydrates (15, 21).

These results complement the direct evidence now reported by Wigler and co-workers (22) for the yeast *ras* proteins indeed being involved in adenylate cyclase. The remainder of this discussion, therefore, focuses on other questions: the nature of the Pyr^- characteristic of *ras2* strains, the involvement of cAMP in yeast reserve carbohydrate metabolism, the suppressors reported here, and the apparent *ras1 ras2* segregants.

The defective growth on pyruvate (and on glycerol, lactate, and ethanol, data not shown) might involve problems in gluconeogenesis or respiration but assay of gluconeogenic enzymes (malate dehydrogenase, phospho*enol*pyruvate carboxykinase, and fructose bisphosphatase) did not reveal major differences in the mutant (data not shown) and respiration is also likely to be adequate because yields were fairly normal on glucose. However, the leakiness of the defect and the fact of pH dependence make interpretation difficult. Possible roles of cAMP in carbon catabolite repression in yeast have been reviewed (23) but recent evidence does not favor its involvement (24, 25). At present the known roles of cAMP-dependent phosphorylation in inactivation of fructose bisphosphatase (26, 27) and activation of fructose 6-phosphate kinase 2 (28, 29) are not clearly related to the described *ras2* phenotype either. Thus, the growth phenotype of *ras2* mutants is not understood. It is interesting, nonetheless, that in *Neurospora crassa* the *cr-1* mutant is defective in adenylate cyclase and grows normally submerged on glucose (30) but not on some respiratory substrates (31), provocative similarities to the yeast *ras2* phenotype. However, *cr-1* is likely affected in the catalytic component of its cyclase (32).

Roles of cAMP in reserve carbohydrate metabolism in yeast are far less understood than in higher cells. So, although the effects of the various mutations on glycogen and trehalose seem to fit with the governance of cAMP by *RAS2*, there are interesting complications. Both reserve materials accumulate under a variety of starvation conditions (12) and trehalase activation is not always correlated with cAMP increases (33). There are at least two trehalase activities in yeast (34) and a role of phosphorylation in transport of trehalase into vacuoles has been suggested (35). Glycogen phosphorylase in yeast, which has two forms of different activity (36, 37), is activated by both cAMP dependent and independent protein kinases (13) but apparently not by the cascade mechanism found in higher cells. Yeast spores also have glycogen hydrolase activities (38). And glycogen synthetase in yeast is found in two interconvertible forms (39) but cAMP-dependent phosphorylation has not been directly implicated.

As to the suppressor mutations of Pyr^-, there are several possibilities. If *ras2* impairs cyclase activity, then a variety of mutations known to suppress cAMP-dependent yeast mutants might also suppress *ras2* effects. These include *bcy1* (cAMP-independent protein kinase, ref. 40), as mentioned, *pde* (decreased phosphodiesterase activity, ref. 41) and *IAC* (increase in adenylate cyclase, ref. 42). One could also imagine alterations at *cyr1* (the catalytic subunit, ref. 43) or at *RAS1*. Suppressors of cAMP dependence might or might not be able to suppress the lethality of a *ras1 ras2* double mutant and there also might be specific suppressors of the Pyr^- phenotype. Regarding the present suppressors, one can only say they are unlikely to be *bcy1* (for *bcy1* suppresses the double *ras* mutant, ref. 22) or to be at *RAS1* (for they segregated independently from it, Table 4). Also, with respect to suppression of Pyr^- (and, for Rpr-1 and Rpr-2, the effect on reserve carbohydrates), Rpr-1 and Rpr-2 seem to be dominant and Rpr-3 recessive (data to be reported).

None of the above considerations provide compelling evidence against a *ras2*/cAMP linkage but they do serve to emphasize how much there is to learn about cAMP in yeast. Furthermore, an *essential* role for cAMP in the cell cycle would not necessarily relate to pyruvate or reserve carbohydrate metabolism (44, 45).

Finally, there is the question of the apparent *ras1 ras2* segregants arising in some of the crosses. One possibility might be that His^+ Leu^+ spores reflect gene conversion of a resident *his3* or *leu2* allele, but this seems unlikely in view of 2:2 segregation of both His and Leu even in complete tetrads with a His^+ Leu^+ spore. Perhaps it is more likely that an unrecognized suppressor of *ras1 ras2* was present in two of the immediate parental strains or arose in the diploids.

There is also another possibility. The reported nonviability of *ras1 ras2* spores was for *ras1::URA3 ras2::LEU2* (3, 4), while the present crosses involved *ras1::HIS3 ras2::LEU2*. *ras1::URA3* is a deletion of amino acid residues 62–149 replaced by *URA3* while *ras1::HIS3* is merely an insertion of *HIS3* at amino acid residue 162, so *ras1::HIS3* might have residual expressed function somehow influencing recovery of *ras1 ras2* segregants, which *ras1::URA3* does not have. Fitting this idea is the observation that, although the *ras1::URA3 ras2::LEU2* spores did not bud (3, 4), in the present experiments even in the crosses where no His^+ Leu^+ segregants were recovered the putative His^+ Leu^+ spores generally gave a microcolony on the germination plate.

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