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Isolation and characterization of *Schizosaccharomyces pombe* mutants phenotypically similar to *ras1*$^-$

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Summary. We isolated mutants of *Schizosaccharomyces pombe* which have deformed cell morphology, are deficient in conjugation and poor in sporulation. This phenotype is characteristic of the *ras1* defective mutant previously identified. Tests of the mutants for allelism using cell fusion showed that they define five complementation groups, one of which is *ras1* itself. The others are named *ral1* through *ral4* (*ras* like). Mutants in *ral3* or *ral4* conjugate at a very low frequency, while the others apparently do not conjugate at all. Plasmid clones complementing *ral1*, *ral2* or *ral3*, which apparently carry the respective gene, were isolated from *S. pombe* genomic libraries. Multiple copies of either the *ral2* or the *ral3* gene could partially restore mating ability in *ral1*$^-$ strains. Multiple copies of the *ras1* gene could partially restore mating ability in *ral1*$^-$ and *ral2*$^-$ strains. These results suggest that the *ral1*, *ral2* and *ras1* genes may function in a common pathway in that order. The *ral3* gene may influence this pathway. Analysis of these gene products will aid identification of factors which interact with Ras proteins.

Key words: Fission yeast – *ras1* oncogene – Sterility – *ral* mutants – Gene cloning

Introduction

Ras is one of the best-characterized oncogenes, and is conserved in virtually all eukaryotic cells. The products of this gene (Ras proteins or p21) are localized in plasma membranes (Willingham et al. 1980; Furth et al. 1982; Sefton et al. 1982). They have guanine nucleotide binding and intrinsic GTPase activities (Scolnick et al. 1979; Sweet et al. 1984; McGrath et al. 1984; Tamanoi et al. 1984). Since these biochemical properties and their primary structures (Dhar et al. 1982; Capon et al. 1983) resemble those of G-proteins involved in transmembrane signal transduction (Hurley et al. 1984; Gilman 1984), they have been postulated to function as regulatory factors of certain effector molecules in the control of cell growth or differentiation.

In the budding yeast *Saccharomyces cerevisiae*, adenylate cyclase has been identified as an effector molecule regulated by the gene products of *RAS1* and *RAS2* (Toda et al. 1985). It does not appear, however, that Ras proteins regulate the same enzyme in the fission yeast *Schizosaccharomyces pombe* (Fukui et al. 1986a) or in vertebrates (Beckner et al. 1985; Birchmeier et al. 1985).

S. pombe apparently has only one *ras* homolog (*ras1*) (Fukui and Kaziro 1985; Nadin-Davis et al. 1986a), which has been mapped to chromosome I and is allelic to *ste5* (Lund et al. 1987). The phenotypes of *S. pombe* strains defective or hyperactive in *ras1* have been studied (Fukui et al. 1986a; Nadin-Davis et al. 1986b). It has been shown that the *ras1* gene product plays an essential role in recognition of the mating pheromone (Fukui et al 1986b), but not in cell growth (Fukui et al. 1986a; Nadin-Davis et al. 1986b). Its biochemical role, however, has not been determined.

To pursue the *ras1*-encoded function, we employed the following reasoning. If the product of the *ras1* gene functions in a cascade, say, of signal transduction, a defective mutation occurring in another element of this cascade might cause the same phenotype as *ras1*$^-$. Molecular analysis of such mutants, once obtained, should assist in identifying the biochemical role of the *ras1* gene product.

We describe in this article the isolation of *S. pombe* mutants whose phenotypes are the same as, or very close to, those of *ras1*$^-$ strains. These mutations define four genes, *ral1* through *ral4*. Functional interrelations among *ras1*, *ral1*, *ral2* and *ral3* are demonstrated using clones of these genes.

Materials and methods

Strains and media. *S. pombe* strains used in this study are listed in Table 1. They are derivatives of those originally described by Leupold (1950). Complete medium YPD (Iino and Yamamoto 1985) and synthetic media MML (Gutz et al. 1974) and SD (Iino and Yamamoto 1985) were used for growing *S. pombe* strains. SSA (Egel and Egel-Mitani 1974), MEA and SPA (Gutz et al. 1974) were the media used for induction of conjugation and sporulation.

Mutagenesis and screening of asporogenic mutants. Logarithmically growing cells of the *S. pombe* homothallic strain JY450 were collected and mutagenized with N-methyl-N′-nitro-N-nitrosoguanidine as described previously (Iino and Yamamoto 1985). All incubations were done at 30° C. Mutagenized cells were spread on SSA or MEA, on which wild-type homothallic strains can grow up to form colonies and then initiate mating and sporulation in each colony.

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Table 1. Strains used in this study

Strain	Genotype	Origin
SG14	*h*^{90} *leu1*	H. Gutz
JY166	*h*$^-$ *arg1 lys3*	M. Yamamoto
JY167	*h*$^+$ *arg1 lys3*	M. Yamamoto
JY245	*h*$^+$ *his2 ade6-M210*	M. Yamamoto
JY260	*h*$^-$ *lys1 ura1*	M. Yamamoto
JY261	*h*$^+$ *lys1 ura1*	M. Yamamoto
JY450	*h*^{90} *ade6-M216 leu1*	M. Yamamoto
RP313	*h*$^+$ *his2 ade6-M210 ura4 ras1::URA3*$_{IK10}$	Y. Fukui
RL– – –	*h*^{90} *ade6-M216 leu1 ral*[a]	This work
RL– – –′[b]	*h*^{90} *ade6-M210 leu1 ral*[a]	This work

[a] One of *ral1*, *ral2*, *ral3*, *ral4* and *ras1*. See Table 2
[b] Genotypically identical to the strain without a prime except for the *ade6* allele

After 4 days incubation, colonies were exposed to iodine vapour, which stains spores black (Gutz et al. 1974). Colorless, hence unsporulated, colonies were picked as candidates for sterile strains.

Protoplast fusion and transformation. Protoplast fusion of *S. pombe* was performed as originally described by Sipiczki and Ferenczy (1977) and modified by C. Shimoda (personal communication). In most cases, fusants were selected by interallelic complementation of *ade6-M210* and *ade6-M216* (Gutz et al. 1974). Transformation of *S. pombe* was carried out essentially as described by Beach and Nurse (1981).

Cloning of the ral *genes.* The *ral* genes were cloned according to the procedure described by Shimoda and Uehira (1985). Representative *ral* strains, which were *leu1*$^-$, were transformed with two *S. pombe* genomic DNA libraries. The first library consisted of *Hin*dIII partial digests of *S. pombe* DNA in the vector pDB248′ (Beach et al. 1982), and was a kind gift of C. Shimoda (Shimoda and Uehira 1985). The second was constructed by inserting *Sau*3A partial digests of *S. pombe* DNA into the *Bam*HI site of the same vector. The *LEU2* gene of *S. cerevisiae* carried on pDB248′ complements the *S. pombe leu1* mutation (Beach and Nurse 1981). Leu$^+$ transformants which became sporogenic were screened by ethanol treatment and iodine staining (Gutz et al. 1974) and plasmids were recovered from them into *Escherichia coli* MC1061 (F$^-$ *araD139 Δ(araABC-leu)7679 galU galK Δ(lac)X74 hsr*$^-$ *hsm*$^+$ *rpsL thi*).

Results

Isolation of ral *mutants*

S. pombe strains defective in *ras1* show deformed cell morphology, are completely sterile regardless of their mating type and sporulate very poorly, while they grow at the same rate as *ras1*$^+$ (Fukui et al. 1986a). To isolate mutants which exhibit this phenotype, we first screened asporogenic strains derived from the homothallic parent JY450 (*h*^{90} *ade6-M216 leu1*), as described in Materials and methods. We then observed the morphology of these cells under the light microscope and chose those which were short and fat, thus being visually indistinguishable from *ras1*$^-$ cells (Fig. 1). Their sterility was confirmed by mixing them with *h*$^+$ and *h*$^-$ tester strains. Their retention of the ability to secrete the putative mating pheromone *h*$^-$-factor, which induces elongation of mating tubes in *h*$^+$ tester cells, was examined, since it is a distinctive characteristic of *ras1*$^-$ strains (Fukui et al. 1986b). Strains meeting these criteria were classified as *ral* (*ras*-like) mutants. Altogether 25 *ral* mutants (including *ras1* itself, see below) were isolated. The frequency of isolation was approximately 1 mutant out of 4000 mutagenized cells. Although their sporulation ability was not criti-

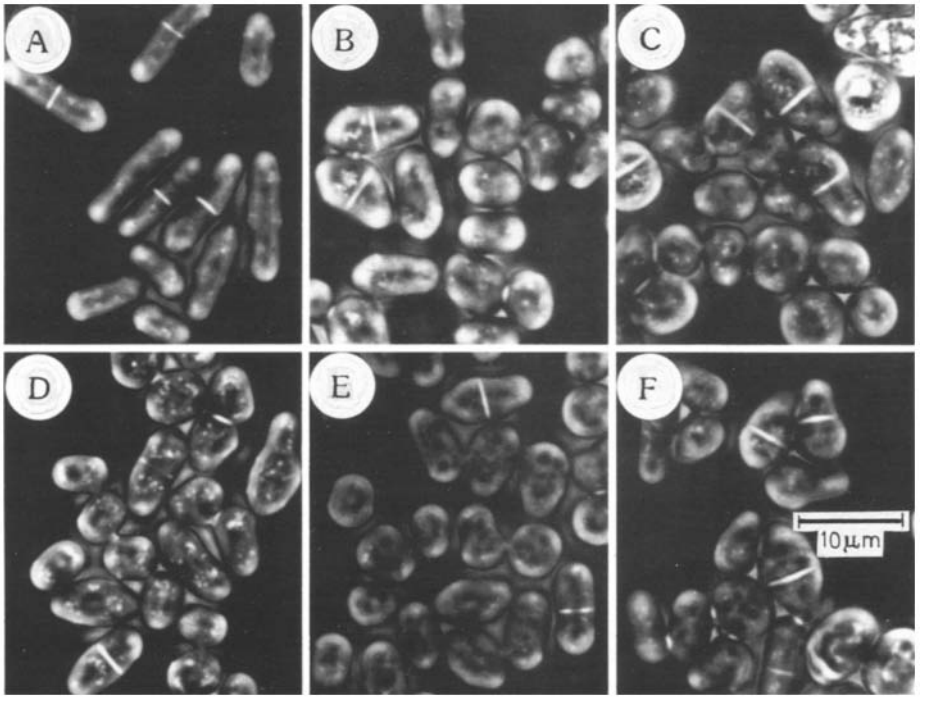

Fig. 1 A–F. Morphology of the *ral* mutants. Rapidly growing cells of representative *ral* mutants were photographed under the phase-contrast microscope. **A** Wild-type *Schizosaccharomyces pombe* strain JY7; **B** RL011 (*ras1*$^-$); **C** RL111 (*ral1*$^-$); **D** RL171 (*ral2*$^-$); **E** RL184 (*ral3*$^-$); **F** RL282 (*ral4*$^-$). Bar represents 10 μm

Table 2. Allelism tests of *ral* mutants

ade6-M210 strain	*ade6-M216* strain													
	RL353	RL011	RL101	RL111	RL183	RL241	RL171	RL809	RL212	RL184	RL018	RL211	RL282	RP313
RL353′	R	R	R	N	N	N	N	N	N	N	N	N	N	R
RL011′		R	nt	N	nt	nt	N	nt	nt	N	nt	nt	nt	R
RL101′			R	N	nt	nt	N	nt	nt	N	nt	nt	N	R
RL111′				R	R	R	N	N	N	N	N	N	N	N
RL183′					R	nt	N	nt	nt	N	nt	nt	N	N
RL171′							R	R	R	N	N	N	N	N
RL809′								R	nt	N	N	N	nt	N
RL184′										R	R	R	N	N
RL018′											R	R	N	N
RL282′													R	N

Hybrids of a *ral* mutant carrying the *ade6-M216* marker and one carrying the *ade6-M210* marker were obtained by protoplast fusion. Resultant Ade$^+$ diploids were grown on YPD plates to examine their cell morphology and were transferred to SPA to assay their sporulation activity. Fusants were clearly divided into two classes: those exhibiting wild-type cell morphology and sporulation efficiency (N), and those showing a roundish cell shape and poorly sporulating (R). The former can be taken as complementation positive and the latter as complementation negative. nt, not tested

cally tested in the above isolation procedures, these mutants turned out to be poor in sporulation, as is *ras1*$^-$, in the subsequent analyses (see below).

Allelism tests

Each of the 25 *ral* strains was fused with the wild-type strain JY245 (*h*$^+$ *his2 ade6-M210*) and Ade$^+$ hybrids were selected. All of the diploid hybrids showed normal cell shape and a high sporulation efficiency, indicating that the mutant alleles are recessive to the wild-type. Derivatives which bear *ade6−M210* instead of *ade6−M216* but are otherwise isogenic to the original *ral* strains were obtained as offspring of some of these hybrids. They are denoted by adding a prime to the original name (e.g. RL353 vs RL353′).

To test for allelism, *ral* strains with the *ade6-M210* marker were fused with a series of *ral* isolates carrying *ade6-M216*. All homozygous *ral/ral* fusants tested exhibited deformed cell morphology and poor sporulation. Therefore recovery of a normal diploid cell shape in a fusant, which was always associated with a high sporulation efficiency, was taken as an indication of mutual complementation. Results of complementation tests which include 13 *ral* strains are shown in Table 2. These strains were divided into 5 complementation groups. One group is obviously *ras1* itself, because a standard *ras1* disruptant, RP313, does not complement within this group (Table 2). The other 4 complementation groups were named *ral1* through *ral4*. The remaining 12 *ral* strains not shown in Table 2 were subsequently shown to belong to one or other of these 5 complementation groups, as summarized in Table 3 (data not shown). Out of 25 *ral* isolates, 10 turned out to be *ras1*$^-$. This assignment was confirmed by the observation that their defects were fully complemented by introduction of plasmid pDB248SPRAS, which carries the *ras1* gene (Fukui et al. 1986a).

Characterization of ral *mutants*

After establishing the genetic classification of *ral* strains, the characteristics of the mutants of each group were re-

Table 3. Classification of the mutants

Assigned complementation group	Number of strains	Strains
ras1	10	RL005, RL011, RL101, RL141, RL161, RL181, RL221, RL262, RL353, RL409
ral1	3	RL111, RL183, RL241
ral2	5	RL171, RL212, RL253, RL261, RL809
ral3	6	RL018, RL184, RL211, RL231, RL242, RL281
ral4	1	RL282

examined carefully. Although mutants in *ral1, ral2, ral3* and *ral4* show a phenotype quite similar to that of *ras1*$^-$ mutants, small differences are apparent among them.

The cell morphologies of representatives of *ral1* through *ral4* are shown in Fig. 1. Compared with *ras1*$^-$, *ral1*$^-$ and *ral4*$^-$ cells are more roundish, while *ral2*$^-$ and *ral3*$^-$ cells are hardly distinguishable from *ras1*$^-$.

To assess the degree of their sterility, *ral* mutants were mixed on sporulation agar with *h*$^+$ (JY167 and JY261) or *h*$^-$ (JY166 and JY260) tester strains carrying appropriate auxotrophic markers. After 4 days at 30° C, the mixture was replated on SD plates to score prototrophic cells generated by mating. Starting with roughly 10^7 cells, *ral3* or *ral4* strains generated some 10^2 prototrophic cells, while *ras1*, *ral1* and *ral2* generated no prototrophs at all. Thus *ral3* and *ral4* strains are leaky in conjugation; their mating efficiency is $\sim 10^{-4}-10^{-5}$. The leakiness of *ral3* mutants appears to be intrinsic to the gene function, since preliminary results indicate that even strains whose *ral3* gene is completely disrupted conjugate leakily (data not shown).

The sporulation efficiency of *ral/ral* homozygous diploids, constructed by protoplast fusion, was measured. As summarized in Table 4, all *ral* strains sporulate as poorly as *ras1*$^-$ except for *ral4* which sporulates more efficiently.

Table 4. Sporulation efficiency of *ral* mutants

Diploid genotype	Sporulation efficiency (%)
ral1/ral1	3.7
ral2/ral2	3.2
ral3/ral3	5.6
ral4/ral4	13.4
ras1/ras1	4.5
ral^+/ral^+ (JY362)	56.3

Diploids homozygous for each *ral* gene were obtained as described in Table 2. Sporulation efficiency was determined after 24 h incubation on SPA at 25° C and is expressed as no. of asci/no. of total cells. The value given is an average of a few measurements done with two independent diploid constructs except for *ral4/ral4* and JY362

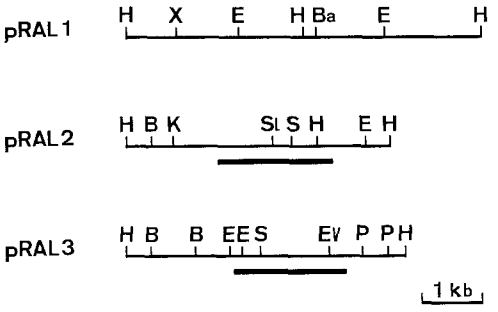

Fig. 2. Restriction maps of the inserts in pRAL1, pRAL2 and pRAL3. These inserts are carried at the *Hin*dIII site of the high copy vector pDB248′ (Beach and Nurse 1981), whose size is 12.7 kb. The approximate size of the insert in pRAL1 is 5.7 kb in length, in pRAL2, 4.2 kb and in pRAL3, 4.5 kb. Restriction endonucleases are abbreviated as follows: B, *Bgl*II; Ba, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; S, *Sac*I; Sl, *Sal*I; X, *Xho*I. The location of the *ral2* or the *ral3* gene, assigned preliminarily, is indicated by a *bar* under the respective restriction map

Isolation of the ral *genes*

Cloning of the *ral* genes was performed as described in Materials and methods. RL111 (*ral1*), RL171′ (*ral2*) and RL184 (*ral3*) were transformed with the two *S. pombe* gene libraries. Plasmids complementing either the *ral2* or *ral3* defects could be recovered from the *Hin*dIII library and were named pRAL2 and pRAL3 respectively. A plasmid complementing *ral1* was recovered from the *Sau*3A library and two adjacent *Hin*dIII fragments were subcloned as a single fragment in the vector pDB248′. This subclone, which complemented *ral1*, was named pRAL1. Figure 2 shows restriction maps of the inserts of pRAL1, pRAL2 and pRAL3. Probable locations of the *ral2* and *ral3* genes assigned by subcloning and preliminary sequencing analysis are also indicated. The *ral1* gene apparently spans the central *Hin*dIII site in the insert, but no further delimitation of this gene has been done. So far no hint of homology has been detected among these three genes nor between them and the *ras1* gene (data not shown).

The following observations strongly suggest that the genes carried on these plasmids are the authentic *ral1*, *ral2* and *ral3* genes and not some sort of suppressors. (1) Gene disruptants constructed using each plasmid were all found to exhibit the Ral phenotype and to be allelic to the original *ral* isolates (data not shown). (2) Introduction of these plasmids into the corresponding mutants restores mating and sporulation proficiency to the wild-type level.

Table 5. Recovery of mating activity by multicopy plasmids[a]

Mutant gene	Plasmid[b]					
	pRAS (Gly)	pRAS (Val)	pRAL1	pRAL2	pRAL3	pDB248′
ras1	+++	+	−	−	−	−
ral1	+	+	+++	+	+	−
ral2	+,−[c]	+	−	+++	−	−

[a] Mutant strains (RL011, RL353, RL171, RL809, RL111 and RL183) were transformed with each plasmid listed and crossed with testers for mating. Mating activities could be classified into three categories: +++, conjugation occurred at the wild-type level; +, conjugation at very low efficiency (~0.01%) could be detected by complementation of auxotrophic markers; −, no conjugation could be detected

[b] pRAS(Gly) represents pDB248SPRAS (Fukui et al. 1986a), which carries the wild-type *ras1* allele, while pRAS(Val) is a plasmid carrying an activated *ras1* allele, in which the glycine at residue 17 is substituted by valine, as was described previously (Fukui et al. 1986a; Nadin-Davis et al. 1986b). pDB248′ is a vector plasmid used in construction of the others

[c] Results differ depending on the mutant allele

Dose effects of the ral *genes*

Each *ral* mutant was transformed with pRAL1, pRAL2 and pRAL3, as well as with plasmids carrying either the wild-type (Gly-17) or an activated (Val-17) *ras1* allele. As summarized in Table 5, weak but significant suppression of sterility was observed in some cases. Defects in *ral1* were apparently suppressed by multiple copies of *ras1*, *ral2* or *ral3*, and defects in *ral2* by multiple copies of *ras1*. Suppression was seen in no other combination of recipients and plasmids, although recipients defective in *ral3* or *ral4* gave no conclusive results because they were leaky and allowed mating at around 10^{-4} by themselves. The nonreciprocal nature of suppression observed here may suggest that products of these genes interact in a regulatory cascase rather than share the same function (see Discussion).

Discussion

This study demonstrates that *S. pombe* has at least a few genes whose deficiency causes almost the same effects on cell physiology as a defect in the *ras1* gene. Such genes are genetically distinct from *ras1* and comprise four complementation groups, *ral1–ral4* (see below). The initial screen for these *ral* mutants was the isolation of sterile (mating-deficient) strains. Then sterile strains which had deformed cell morphology were screened. As we have pointed out previously (Lund et al. 1987), only *ste5* mutants have deformed cell morphology among the sterile mutants of *S. pombe* reported in the literature (*ste1–ste8*) (Thuriaux et al. 1980; Girgsdies 1982; Michael and Gutz 1987). We have furthermore shown that *ste5* is allelic to *ras1* (Lund et al.

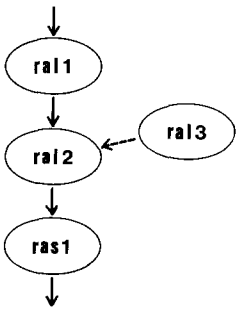

Fig. 3. A schema for the possible interaction of the gene functions

1987). Thus it seems that *ral1–ral4* mutants had eluded previous screens. Although *ras1* mutants emerged most abundantly among deformed sterile mutants, they occupy only 40% of this kind (see Results). This may mean that isolation of sterile mutants of *S. pombe* is as yet unsaturated. Indeed, a new sterile mutation of *S. pombe* (*steX*), which could be related to a regulatory cascade involving cAMP, has recently been identified in our laboratory (Watanabe et al. 1988). Clarification of the mechanisms of mating now appears to be essential for understanding the role of the Ras protein in *S. pombe*. Analysis of sterile mutants, deformed or nondeformed, will become increasingly important in this regard.

A possible flow chart of gene functions was deduced from the suppression pattern shown in Table 5 and is shown in Fig. 3. We assumed that each defect suppressed is a null type and that if a high dosage of gene A can suppress a defect in gene B, A should function downstream of B. The *ral4* gene has been excluded from the schema since there is no evidence to demonstrate an interaction between this gene and *ras1* or other genes. Following the above logic, *ral1* is positioned furthest upstream of those genes under consideration. It is similarly logical that *ral2* functions upstream to *ras1*. We suspect an intimate relationship between these two genes, because *ral2* mutants mimic *ras1*$^-$ perfectly in cell morphology as well as in the tightness of sterility under our experimental conditions. It remains as an interesting possibility that the *ral2* gene product directly interacts with the Ras protein.

As supporting evidence for the above schema, we have seen that *ral1, ral2* and *ras1* mutations have very similar effects on gene expression in *S. pombe* (Y. Watanabe and M. Yamamoto, in preparation). The *S. cerevisiae CDC25* gene product has been shown to function upstream of the *RAS* genes and to regulate them (Martegani et al. 1986; Camonis et al. 1986; Robinson et al. 1987; Broek et al. 1987). Our preliminary comparison, however, has revealed no sequence homology betwen the *S. pombe ral1* or *ral2* genes and the *S. cerevisiae CDC25* gene (data not shown).

The *ral3* gene cannot be positioned on the linear pathway through *ral1, ral2* and *ras1* (Fig. 3). At present its function can best be explained by the assumption that *ral3* encodes a modifier which boosts the activity of *ral2*. This assumption is even more attractive in that it could explain the intrinsic leakiness of *ral3* mutations. The *S. cerevisiae RAM* or *DPR* gene product is known to modify the *ras* function (Powers et al. 1986; Fujiyama et al. 1987), but preliminary experiments indicate that *ral3* and *DPR* do not cross-hybridize (S. Miyake, M. Yamamoto and F. Tamanoi, unpublished results). Thus, *S. pombe* and *S. cerevisiae* may have differences not only in the physiological roles of the Ras proteins but also in the regulatory cascades involving these proteins.

Although complementation analysis suggested that *ral4* could be the fifth member of the group of *S. pombe* sterile mutations which cause deformation in cell morphology, there remain some uncertainties. (1) Only one mutant allele has been assigned to this group. (2) This mutant sporulates more efficiently than other *ral* strains. (3) Cloning of the *ral4* gene has not been successful. (4) No functional link has been established between *ral4* and *ras1*. Obviously further extensive genetic analysis of this group is necessary to exclude a trivial possibility such as, for instance, that the phenotype is caused by a combination of a few silent mutations.

The present genetic data are not sufficient to allow us to speculate on the biochemical nature of the gene interactions shown schematically in Fig. 3. Preliminary DNA sequence analysis indicates that the putative Ral2 and Ral3 proteins have no strong homology with known proteins. Recently, a cytoplasmic protein named GAP (GTPase activating protein) present in mammalian cells has been shown to interact physically with Ras proteins (Trahey and McCormick 1987; Cales et al. 1988; Adari et al. 1988). It is the first protein demonstrated to have an interaction with mammalian Ras. While it has been reported that *S. pombe* cytosol does not reveal GAP-like activity (when assayed with mammalian Ras proteins) (Adari et al. 1988), we can still speculate that the *ral2* product may be a counterpart of GAP in this yeast. Further characterization of the *ral2* product as well as GAP is necessary to see if these two proteins have any characteristics in common.

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