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Mutants of *Saccharomyces cerevisiae* defective in the farnesylation of Ras proteins

(C-terminal processing/"CAAX" box/farnesyltransferase)

LAURIE E. GOODMAN*, S. RENÉE JUDD*, CHRISTOPHER C. FARNSWORTH†, SCOTT POWERS‡, MICHAEL H. GELB§, JOHN A. GLOMSET†, AND FUYUHIKO TAMANOI*¶

*Department of Biochemistry and Molecular Biology, The University of Chicago, 920 East 58th Street, Chicago, IL 60637; †Howard Hughes Medical Institute Laboratory, Departments of Medicine and Biochemistry, and §Departments of Chemistry and Biochemistry, The University of Washington, Seattle, WA 98195; and ‡Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854

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ABSTRACT **Ras proteins are post-translationally modified by farnesylation. In the present investigation, we identified an activity in crude soluble extracts of yeast cells that catalyzes the transfer of a farnesyl moiety from farnesyl pyrophosphate to yeast RAS2 protein. RAS2 proteins having a C-terminal Cys-Ali-Ali-Xaa sequence (where Ali is an aliphatic amino acid and Xaa is the unspecified C-terminal amino acid) served as substrates for this reaction, whereas RAS2 proteins with an altered or deleted Cys-Ali-Ali-Xaa sequence did not. A yeast mutant, *dprl/raml*, originally isolated as a Ras-processing mutant was shown to be defective in farnesyltransferase activity. In addition, another mutant, *ram2*, also was defective in the transferase activity. These results demonstrate that at least two genes, *DPRl/RAMl* and *RAM2*, are required for the farnesyltransferase activity in yeast.**

Prenylation has been observed with a variety of proteins, including yeast mating peptides, guanine nucleotide binding proteins (such as Ras), and nuclear lamins (for review, see ref. 1). A recent addition to this list is the γ subunit of guanine nucleotide binding proteins (2–4). The polyisoprenoids detected are either all-*trans*-farnesyl or all-*trans*-geranylgeranyl groups (5, 6).

Farnesylation of Ras proteins (7–9) occurs as part of a series of C-terminal modifications needed for membrane association, including removal of three C-terminal amino acids (10, 11) and carboxyl methylation of the C-terminal cysteine (12). These modifications take place at a conserved sequence, Cys-Ali-Ali-Xaa (Ali is an aliphatic amino acid and Xaa is the unspecified C-terminal amino acid), which is termed a "CAAX" box (13). Although it appears likely that farnesylation occurs prior to amino acid removal and methylation, the order of these events has not been established.

Yeast mutants defective in the processing of Ras proteins have been isolated. The *dprl* (14) and *ram* (15) mutants were independently isolated but were shown to be allelic. (This mutant will be called *dprl/raml* hereafter). Nonmodified Ras proteins accumulate in the mutant strains (14–16), suggesting that an early step that includes farnesylation is blocked. The mutant strains are temperature-sensitive for growth and show reduced mating efficiency. The effect on mating is severe in *MAT***a** cells, since the processing of the **a**-factor mating peptide is blocked (14, 15). The processing of Ras proteins is also blocked in *hmgl hmg2* double mutants (9). These mutations abolish 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, thus affecting the production of farnesyl pyrophosphate, which is needed for the farnesylation of Ras proteins.

We have detected an activity in crude soluble extracts of yeast cells that catalyzes the farnesylation of yeast RAS2 protein. This activity is deficient in *dprl/raml* mutant strains. We further report the isolation of a mutation in a second gene, termed *ram2*, which also causes a defect in the farnesylation of Ras proteins. The availability of mutants affecting Ras processing points to the usefulness of the yeast system in analyses of protein farnesylation.

MATERIALS AND METHODS

Materials. [^{35}S]Methionine was purchased from ICN (Trans-^{35}S-label; 1199 Ci/mmol; 1 Ci = 37 GBq). [^{3}H]Farnesyl pyrophosphate (0.5 Ci/mmol) was synthesized by the method described (17) or was purchased from NEN (20 Ci/mmol). The purity of farnesyl pyrophosphate was checked by thin layer chromatography (17). Plasmids YCp19DPR1 (16), YEp51RAS2 (18), and YEp24DPR1 (19) have been described. YEpDPR1 was constructed by inserting a 4.8-kilobase *Bam*HI–*Sal* I DPR1 fragment into pYS1, which contained the 2-μm origin, *TRP1*, and pBR322. YRpDPR1 was constructed by inserting the above *DPRl* fragment into YRp7 (20).

Yeast Strains and Preparation of Crude Soluble Extracts. The following *Saccharomyces cerevisiae* strains were used. UC100 (*MAT***a** *leu2 trpl ura3 pep4 prb*) is described (19). KMY5-2A (*MAT*α *dprl his3 leu2 ura3*) was derived from the cross of HR12 (*MAT***a** *leu2 his3 ura3 trpl ade8 canl RAS2^{val19} dprl*) (14) with wild-type strains. KMY2-3A (*MAT***a** *leu2 his3*) was the wild-type strain used for the final cross. KMY200-sgp2-No.2-1213 (*MAT***a** *ura3 his3 dprl::URA3*) was provided by K. Matsumoto (DNAX); the disruption is described by Nakayama *et al.* (21). STS39 (*MAT***a** *ras1::URA3 RAS2^{val19} ram2 his3 leu2 ade8 trpl canl gal2*) was isolated by S.P. as a suppressor of the heat-shock sensitivity of *RAS2^{val19}* cells, PT1-6 (15). STS39 was crossed with RS16-4C (*MAT*α *ura3 his3 trpl ade8 ade2 canl SUP^{84L}*) (15) and the resulting diploid was sporulated to yield RS51-3A (*MAT*α *ram2 his3 ura3 ade8 trpl canl ade2 RAS2^{val19}*) and RS51-6C (*MAT*α *ram2 his3 ura3 ade8 trpl canl ade2*). JRY1593 (*MAT***a** *ade2 met his3 lys2 ura3 hmg1::LYS2 hmg2::HIS3*) and its parent JRY1594 (*MAT***a** *ade2 met his3 lys2 ura3*) were provided by J. Rine (University of California, Berkeley). Genetic analyses were carried out according to standard methods (20). To facilitate the mating of *dprl* and *ram2* strains, the *dprl* strain KMY5-2A-N (*MAT***a** *dprl his3 leu2 ura3*; derived from KMY5-2A by *HO*-mediated mating type switch) was transformed with either YEp24 DPR1 or YCp19DPR1. Presence of the plasmid-borne wild-type *DPRl* gene complemented the **a**-specific mating defect of KMY5-2A-N, allowing efficient mating with the *ram2* strain RS51-3A and RS51-6C. Diploids

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Abbreviation: Ali, aliphatic amino acid.

¶To whom reprint requests should be addressed.

were selected by plating on medium lacking both leucine and tryptophan. Ura$^-$ isolates (which had lost the *DPR1* plasmid) were obtained and used in complementation studies and tetrad analysis.

Crude soluble extracts were prepared as follows. Cells (100-ml culture) grown in YPD medium to late logarithmic phase were resuspended in 0.2 ml of 0.1 M Mes-NaOH, pH 6.5/0.1 mM $MgCl_2$/0.1 mM EGTA/1 mM β-mercaptoethanol/2 mM phenylmethylsulfonyl fluoride and broken with glass beads. The extracts were then subjected to high-speed centrifugation at 100,000 × *g* for 1 hr in a 70 Ti rotor after removing cell debris by low-speed centrifugation. The resulting supernatant had a protein concentration of ≈10 mg/ml.

Preparation of RAS2CT Proteins. A truncated form of RAS2 protein, which is more stable than the full-length RAS2 protein, was expressed in *Escherichia coli* from plasmid pKH502-RAS2CT1. This plasmid is identical to pKH502-RAS2 (18) except that it contains a *Hin*cII–*Bal* I fragment of RAS2 (corresponding to the N-terminal 210 amino acid residues). At the *Bal* I site of the *RAS2* gene, complementary synthetic oligonucleotides were added that restored the original C-terminal 6 amino acid residues. pKH502-RAS2CT2, expressing a RAS2 protein with a cysteine to serine alteration in the CAAX box, was constructed in the same way using a pair of oligonucleotides containing the appropriate base change. Addition of a termination codon instead of the above oligonucleotides produced a construct, RAS2CTT, expressing a RAS2 protein with no CAAX box. A plasmid expressing f-RAS2CT1 was constructed by fusing the *RAS2CT1* gene with *lacZ* in pUC8. Expression of the RAS2CT1, RAS2CT2, and RAS2CTT proteins was induced by the addition of nalidixic acid. f-RAS2CT1 expression was induced by isopropyl β-D-thiogalactopyranoside. *E. coli* cells expressing these proteins were broken by sonication, and the proteins were purified to near homogeneity by DEAE-Sepharose followed by Sephadex G-75 columns in a manner similar to that described (22). To express RAS2CT1 protein in yeast, the *RAS2CT1* gene was placed under the control of the *GAL10* promoter on YEp51 (18).

Farnesyltransferase Reaction and Product Analyses. Farnesyltransferase activity was assayed by incubating RAS2 proteins with [^{3}H]farnesyl pyrophosphate. A typical reaction mixture of 30 μl contained 50 mM potassium phosphate (pH 7.4), 5 mM dithiothreitol, 10 mM $MgCl_2$, 0.8 μM [^{3}H]farnesyl pyrophosphate (20 Ci/mmol was used if not indicated), 30 μg of RAS2CT1 protein, and 50 μg of crude soluble extracts. The reaction mixture was incubated at 30°C. To assay for the incorporation of radioactivity into acid-insoluble products, the reaction mixture was spotted onto Whatman no. 3 paper, immersed into 10% (wt/vol) trichloroacetic acid twice, and washed twice each with ethanol and acetone. The filters were dried and radioactivity was measured by using Liquifluor (Amersham) as the counting solution. Analyses by SDS/polyacrylamide gel and immunoprecipitation using Y13-259 were carried out as described (18). To determine the structure of the transferred polyisoprenoid, the radioactive band on a SDS/polyacrylamide gel was cut out and the radioactivity was released by incubation with trypsin (100 μg/ml) in 50 mM ammonium bicarbonate. After lyophilization, the sample was solubilized in 400 μl of formic acid/ethanol, 1:4 (vol:vol), and 50 mg of Raney nickel and 1 ml of pentane were added. The sample was incubated for 15 hr at 100°C in a tightly sealed screw cap tube. After cooling at −20°C for 1 hr, 400 μl of H_2O was added. Then, the sample was vortex mixed and then centrifuged for 1 min at 1500 × *g*, and the pentane phase was removed. The concentrated sample was analyzed by radiometric GC both before and after hydrogenation over Pt (23).

RESULTS

Detection of Yeast Farnesyltransferase Activity. To detect farnesylation of yeast RAS proteins, we used a RAS2 protein that had an internal deletion in the C-terminal hypervariable domain but retained the C-terminal 6 amino acid residues Gly-Cys-Cys-Ile-Ile-Ser. This truncated RAS2 protein, termed RAS2CT1, is functional in yeast, since its expression complements the growth defect of *ras2*$^-$ cells (24) on nonfermentable carbon sources (data not shown). RAS2CT1 was expressed in *E. coli* and purified.

Incubation of RAS2CT1 protein and [^{3}H]farnesyl pyrophosphate in the presence of crude extracts of yeast cells resulted in the incorporation of radioactivity into acid-insoluble products (Fig. 1 and also Table 1). Little incorporation occurred when no protein was added to the extracts. This was presumably because (*i*) the amount of endogenous protein was much less than that of the exogenously added protein and/or (*ii*) the endogenous proteins were already farnesylated. Similarly, little incorporation of ^{3}H was observed when RAS2CT2, which has an amino acid alteration from cysteine to serine within the CAAX box, was used. A RAS2 protein lacking the CAAX box (RAS2CTT) also did not support farnesylation (data not shown). These results strongly suggest that the farnesyltransferase activity recognizes the CAAX box.

Radioactivity Is Incorporated into RAS2 Protein. Fig. 2 demonstrates that the farnesyl radioactivity was incorporated into RAS2 protein. A radioactive band having an apparent molecular mass of 30 kDa, corresponding to the molecular mass of the RAS2CT1 protein, was detected on a SDS/polyacrylamide gel (lane 2). This band was not detected with the RAS2CT2 reaction (lane 3). When a fusion RAS2 protein, f-RAS2CT1, having an additional 20 amino acids at the N terminus of the CT1 protein was used instead of RAS2CT1, a radioactive band on a SDS/polyacrylamide gel appeared at a position slightly higher than that seen with RAS2CT1, reflecting the molecular mass increase (lane 5). In addition, the radioactivity incorporated could be immunoprecipitated with a monoclonal antibody (Y13-259) against Ras proteins (data not shown).

The incorporated radioactivity consisted exclusively of a C_{15} farnesyl moiety. This was shown by releasing the incorporated radioactivity by treatment with Raney nickel and subjecting it to GC analyses. As shown in Fig. 3, the radioactivity recovered in the nonhydrogenated sample coeluted with authentic all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene, the expected product of Raney nickel cleavage of a thioether-linked farnesyl group. After hydrogenation, the radioactivity coeluted with farnesane.

The farnesyltransferase activity was not detected in the presence of EDTA, suggesting that Mg^{2+} or other divalent

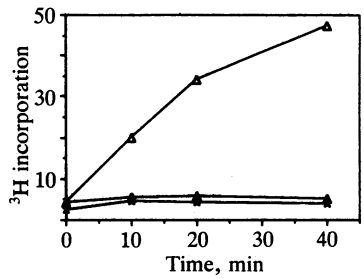

FIG. 1. Transfer of a farnesyl group from [^{3}H]farnesyl pyrophosphate to RAS2CT1 protein. Farnesyltransferase reactions (90 μl) were carried out using crude soluble extracts of UC100 cells with RAS2CT1 (△), RAS2CT2 (▲), or with no protein (×). After incubation at 30°C, 20 μl were taken out at various time points and the incorporation of radioactivity into acid-insoluble products was determined by a filter binding assay and presented as cpm × 10^{-3}.

Table 1. Mutant extracts do not inhibit farnesyltransferase

Exp.	Extract(s)	[^{3}H]Farnesyl incorporated, cpm
1	WT (17 μg)	44,345
	WT (34 μg)	66,164
	dprl (17 μg)	7,576
	WT (17 μg) + *dprl* (17 μg)	37,396
	No protein control	3,550
2	WT (8 μg)	17,379
	ram2 (15 μg)	1,700
	WT (8 μg) + *ram2* (8 μg)	15,306
	No protein control	1,262

In experiment 1, UC100 and KMY5-2A cells were used for wild-type (WT) and *dprl* cells, respectively. Farnesyltransferase reactions were carried out with RAS2CT1 protein at 30°C for 30 min. In experiment 2, RS16-4C and RS51-3A cells were used for wild-type (WT) and *ram2* cells, respectively, with incubations at 30°C for 20 min. For both experiments, the total amount of extract protein per 10 μl is indicated in parentheses.

cations are required. Dithiothreitol stimulated the activity ≈2-fold. The transferase activity was detected in a pH range of pH 5.5 to 8 with an optimum of pH 6.5 in Mes buffer. Incubation temperatures between 25°C and 45°C supported the activity, but little activity was detected at 15°C.

Yeast Mutants Defective in Farnesyltransferase. To gain insight into the genes involved in the farnesylation of Ras proteins, we sought to find mutants defective in farnesyltransferase. One candidate was *dprl/raml*, known to be defective in the processing of Ras proteins (14, 15). This mutation is within the *DPR1/RAM1* gene, which encodes a hydrophilic protein of 431 amino acids (16). In addition, another mutant, termed *ram2*, has been isolated. The mutant was obtained as a second site suppressor of heat-shock sensitive *RAS2^{val19}* yeast cells. (Detailed characterization of *ram2* will be published elsewhere.) The *ram2* mutation is recessive and causes temperature-sensitive growth. Processing of the RAS2 protein is defective in the mutant and a-factor production is greatly reduced (unpublished results).

Both the *dprl/raml* and *ram2* mutants were defective in farnesyltransferase activity. As shown in Fig. 4 *A* and *B*, virtually no incorporation of farnesyl radioactivity into the RAS2 protein was detected in either the *dprl/raml* extracts

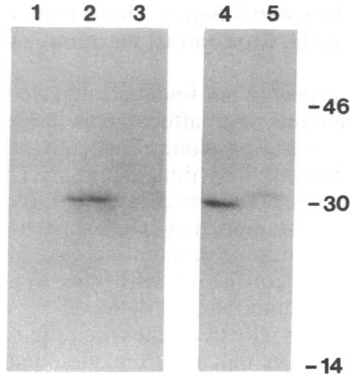

FIG. 2. SDS/polyacrylamide gel electrophoresis of the products of farnesyltransferase reaction. Farnesyltransferase reactions were carried out at 30°C with [^{3}H]farnesyl pyrophosphate (0.5 Ci/mmol). The entire sample from each reaction mixture (30 μl) was then analyzed on a 12.5% polyacrylamide gel containing SDS. Lanes: 1, UC100 extracts (50 μg) alone; 2, UC100 extracts (50 μg) + RAS2CT1 (7 μg); 3, UC100 extracts (50 μg) + RAS2CT2 (7 μg); 4, KMY2-3A extracts (121 μg) + RAS2CT1 (7 μg); 5, KMY2-3A extracts (121 μg) + f-RAS2CT1 (7 μg). Molecular mass markers indicated are ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14 kDa).

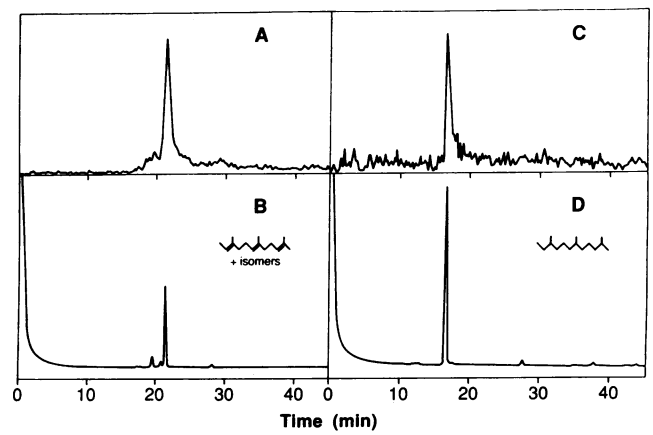

FIG. 3. Identification of the isoprenoid transferred to RAS2CT1. The farnesyltransferase reaction was catalyzed by UC100 extracts and RAS2CT1 protein. The radioactivity incorporated into RAS2CT1 protein was identified on a 12.5% polyacrylamide gel containing SDS. The radioactivity was eluted from the gel and was analyzed by GC. (*A*) Nonhydrogenated ^{3}H-labeled material released from RAS2CT1. (*B*) Authentic all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene with trace amounts of other cis/trans isomers (23). (*C*) ^{3}H-labeled material released from RAS2CT1, which had been hydrogenated over platinum. (*D*) Authentic farnesane. No C_{20} isoprenoid was observed (all-*trans*-2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene, t_R = 43.9 min; phytane, t_R = 38.3 min).

or the *ram2* extracts. Similar results were obtained with other *dprl* alleles including a gene disruption and a complete deletion (data not shown). Lack of detectable farnesyltransferase activity was not due to the presence of any endogenous inhibitors, since mixing the mutant and wild-type extracts did not significantly reduce wild-type activity (Table 1). At-

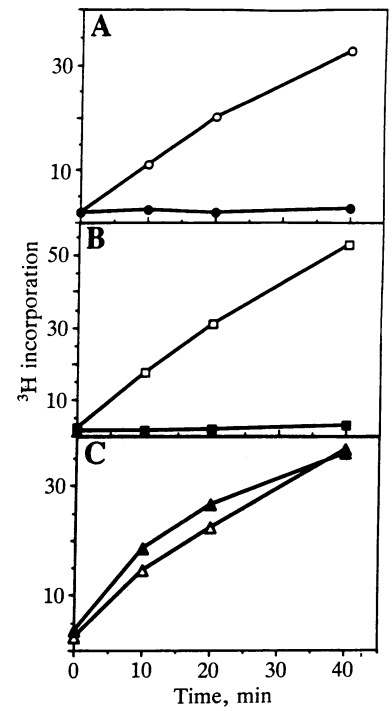

FIG. 4. Farnesyltransferase activity in yeast mutants. The farnesyltransferase reaction was carried out at 30°C using RAS2CT1 protein. Cell extracts were used as indicated below. After various incubation periods, samples were spotted onto filter papers and acid-insoluble radioactivity was determined. (*A*) KMY5-2A (*dprl*) (●) and KMY2-3A (○). (*B*) RS51-3A (*ram2*) (■) and RS16-4C (□). (*C*) JRY1593 (*hmg1 hmg2*) (▲) and JRY1594 (△). Data for ^{3}H incorporation are presented as cpm × 10^{-3}.

tempts to recover farnesyltransferase activity by mixing the *dpr1/ram1* and *ram2* extracts have been unsuccessful (see *Discussion*).

Three lines of evidence support the hypothesis that *dpr1/ram1* and *ram2*, although having similar phenotypes, are two different mutations. (*i*) *dpr1* and *ram2* belong to different complementation groups. This was demonstrated by crossing *dpr1* and *ram2* strains. Seven diploids resulting from the crosses were screened for temperature sensitivity; all grew at the nonpermissive temperature (37°C), even though the individual mutants were temperature-sensitive for growth. Extracts prepared from the diploids exhibited farnesyltransferase activity significantly above the mutant level (data not shown). (*ii*) These diploids were sporulated and tetrads were dissected. Due to poor sporulation efficiency and poor spore viability (56% overall), only six complete tetrads were analyzed. (The reasons for the low spore viability are unknown; however, multiple factors are known to affect sporulation and spore viability. It is possible that temperature-sensitive spores have decreased viability even at the permissive temperature.) The ability to grow at 37°C segregated $0^+:4^-$ in one tetrad, $1^+:3^-$ in three tetrads, and $2^+:2^-$ in two tetrads, consistent with the pattern expected for mutations in two different genes. (If the two mutations were in the same gene, the expected segregation pattern would be all $0^+:4^-$.) The third line of evidence indicating that *dpr1/ram1* and *ram2* are nonallelic is shown in Fig. 5. The temperature-sensitive growth of the *dpr1* mutant can be complemented by either a single-copy DPR1 plasmid (YCpDPR1) or a multicopy DPR1 plasmid (YRpDPR1). In marked contrast to this, the temperature-sensitive growth of the *ram2* mutant was complemented neither by a single-copy DPR1 plasmid (YCpDPR1) nor by multicopy DPR1 plasmids (YEpDPR1 or YRpDPR1).

Farnesylation of Ras Proteins in *hmg1 hmg2* Double Mutants Is Blocked *in Vivo* but Not *in Vitro*. The *hmg1* and *hmg2* mutations affect mevalonate production, thus limiting the supply of farnesyl pyrophosphate (9). Extracts prepared from the *hmg1 hmg2* double mutant, however, were active in the farnesylation of Ras proteins *in vitro* (Fig. 4*C*). The mutant extracts were prepared from cells grown in the presence of mevalonic acid (5 mg/ml) and then starved for mevalonic acid for 3 hr before collection. Such starvation blocks the processing of Ras proteins *in vivo* (9).

DISCUSSION

Crude soluble extracts of yeast cells catalyze the addition of a farnesyl moiety to the RAS2 protein. The C-terminal sequence of the RAS2 protein is required for the activity as evidenced by the inability of RAS2 proteins having a mutated CAAX box (alteration of cysteine to serine within the CAAX box or deletion of the CAAX box) to serve as substrates. The 6 C-terminal amino acids are sufficient for recognition of the substrate, as indicated by our use of a truncated RAS2 protein retaining only the last 6 amino acids of the C-terminal domain. These observations are in line with the proposal that the CAAX box is important for the modification of Ras proteins.

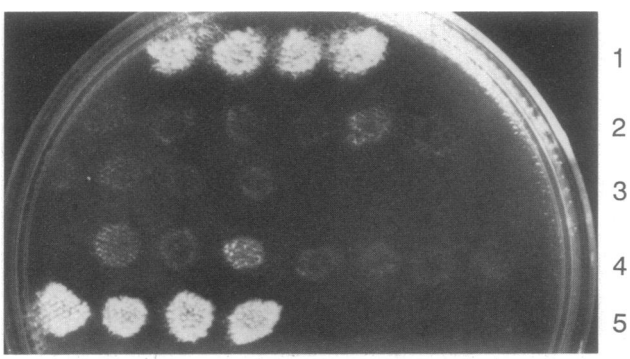

FIG. 5. Lack of complementation of the temperature-sensitive growth of *ram2* mutant with cloned *DPR1* gene. KMY200-sgp2-No.2-1213 (*dpr1*) or STS39 (*ram2*) cells were transformed with the DPR1 plasmids as follows. Rows: 1, YCpDPR1/dpr1; 2, YCpDPR1/ram2; 3, YEpDPR1/ram2; 4, YRpDPR1/ram2; 5, YRpDPR1/dpr1. After growing the transformants under nutritional selection, the cells were replica plated onto YPD and incubated at 37°C.

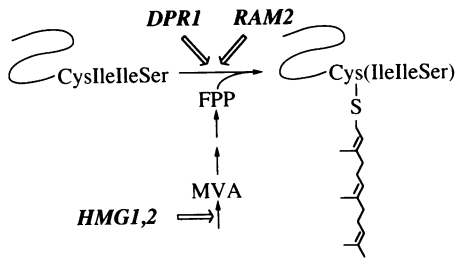

FIG. 6. Schematic representations of three yeast genes involved in farnesyltransferase. *DPR1* and *RAM2* genes appear to affect the farnesyltransferase directly, rather than altering the supply of farnesyl pyrophosphate (FPP) as for *HMG1,2* genes. Whether they encode subunits of the enzyme has not yet been elucidated. MVA, mevalonic acid.

A similar activity in rat brain has been purified to near homogeneity (25). Properties of this rat enzyme including the requirement for Mg^{2+} and the CAAX box are very similar to those of the yeast enzyme. In addition, we have shown that extracts prepared from *Schizosaccharomyces pombe* cells exhibit a similar activity (J. Brown and F.T., unpublished data). Thus, farnesyltransferase appears to be a ubiquitous eukaryotic enzyme.

Yeast provides an ideal system for the genetic analysis of Ras farnesylation. Three genotypes, *dpr1/ram1*, *ram2*, and *hmg1 hmg2*, are known to affect processing of Ras proteins *in vivo* (9, 14, 15). The *hmg1 hmg2* mutations limit the supply of farnesyl pyrophosphate but do not affect farnesyltransferase activity, since addition of farnesyl pyrophosphate to the *in vitro* assay allows full farnesyltransferase activity. In marked contrast to this, the *dpr1/ram1* and *ram2* mutants are still defective in the *in vitro* farnesyltransferase assay‖; these mutations affect a different step in the farnesylation of Ras proteins than does *hmg1 hmg2*. These points are presented schematically in Fig. 6 to contrast the various steps that these genes affect.

One striking aspect of our finding is that we have identified two distinct mutants that affect yeast farnesyltransferase activity. Either mutation alone drastically reduces this activity, rather than partially diminishing it. Thus, it appears that at least two genes are required for farnesyltransferase activity. Our observation that overexpression of only one gene, *DPR1*, does not result in an increase of farnesyltransferase activity is consistent with the hypothesis above (L.E.G. and F.T., unpublished data).

A possible reason why two genes are required for the yeast farnesyltransferase is that they encode subunits of the enzyme. The farnesyltransferase purified from rat brain has an apparent molecular mass of 70–100 kDa and contains equimolar amounts of two polypeptides of ≈50 kDa (25). The yeast enzyme might have a similar structure. However, we have not been successful in recovering farnesyltransferase activity by mixing the *dpr1* and *ram2* extracts. This might be because we have not found the right conditions for reconsti-

‖While this paper was under review, a report describing defective farnesyltransferase activity in extracts prepared from *dpr1/ram1* mutant appeared (26).

tution or because the subunits are stable only when both are present. Although we favor the hypothesis that the *DPR1* and *RAM2* encode subunits of farnesyltransferase, it is also possible that one or both of these genes affect the expression or activity of the transferase. Purification of the yeast enzyme should provide clues to these questions.

Farnesyltransferase presumably contains two functional domains; one recognizing one or more elements of the CAAX box and another recognizing farnesyl pyrophosphate. Yeast mutants defective in farnesyltransferase activity described here as well as other mutants that can be generated should provide valuable tools for structural and mechanistic studies of this type of enzyme.

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