

The *Saccharomyces cerevisiae* *STE14* gene encodes a methyltransferase that mediates C-terminal methylation of a-factor and RAS proteins

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Post-translational processing of a distinct group of proteins and polypeptides, including the a-factor mating pheromone and RAS proteins of *Saccharomyces cerevisiae*, results in the formation of a modified C-terminal cysteine that is S-isoprenylated and α -methyl esterified. We have shown previously that a membrane-associated enzymatic activity in yeast can mediate *in vitro* methylation of an isoprenylated peptide substrate and that this methyltransferase activity is absent in *ste14* mutants. We demonstrate here that *STE14* is the structural gene for this enzyme by expression of its product as a fusion protein in *Escherichia coli*, an organism in which this activity is lacking. We also show that a-factor, RAS1 and RAS2 are physiological methyl-accepting substrates for this enzyme by demonstrating that these proteins are not methylated in a *ste14* null mutant. It is notable that cells lacking *STE14* methyltransferase activity exhibit no detectable impairment of RAS function or cell viability. However, we did observe a kinetic delay in the rate of RAS2 maturation and a slight decrease in the amount of membrane localized RAS2. Thus, methylation does not appear to be essential for RAS2 maturation or localization, but the lack of methylation can have subtle effects on the efficiency of these processes.

Key words: a-factor mating pheromone/C-terminal farnesyl cysteine methyltransferase/methyl esterification/RAS processing/yeast *STE14* gene

Introduction

A group of eukaryotic proteins and polypeptides that undergo post-translational modification are synthesized with a C-terminal sequence, -Cys-Xaa-Xaa-Xaa (where -Xaa is any amino acid). This sequence motif, designated the CXXX motif, serves as a signal for three ordered modification events including isoprenylation of the cysteine sulfhydryl, proteolysis of the terminal three residues, and α -methyl esterification of the newly exposed carboxyl group (Clarke *et al.*, 1988; Hancock *et al.*, 1989; Rine and Kim, 1990; Stimmel *et al.*, 1990). Examples include fungal mating pheromones (Ishibashi *et al.*, 1984; Anderegg *et al.*, 1988; Akada *et al.*, 1989), RAS proteins (Gutierrez *et al.*, 1989), nuclear lamins (Farnsworth *et al.*, 1989) and the γ -subunit

of trimeric G proteins (Yamane *et al.*, 1990). All of these polypeptides are membrane associated, or pass through a membrane. While isoprenylation has been shown to play an essential role in membrane localization, the role of the methylation reaction has not been established.

Genetic analysis in the yeast *Saccharomyces cerevisiae* has provided a means for identifying cellular components that mediate these post-translational processing events. The yeast mating pheromone a-factor precursor terminates in a CXXX motif (Brake *et al.*, 1985; Powers *et al.*, 1986; Michaelis and Powers, 1988). Mature bioactive a-factor is a secreted dodecapeptide with a C-terminal cysteine that is farnesylated and methyl esterified (Anderegg *et al.*, 1988). Because defects in the synthesis of a-factor result in a readily detectable *MATa* cell-specific sterile phenotype, it has been possible to identify four mutants, *ste6* (Rine, 1979), *ste14* (Blair, 1979), *ram1* (Powers *et al.*, 1986; Wilson and Herskowitz, 1987) and *ram2* (Goodman *et al.*, 1990), that are impaired in a-factor secretion or processing. *STE6* encodes a membrane transporter responsible for the export of a-factor (McGrath and Varshavsky, 1989; Kuchler *et al.*, 1989). *RAM1*, alternatively named *DPR1*, was originally identified on the basis of its necessity for both RAS and a-factor activity (Powers *et al.*, 1986; Fujiyama *et al.*, 1987; Michaelis and Powers, 1988). In *ram1* mutants, the RAS and a-factor precursors fail to become membrane localized and remain in the cytosol (Powers *et al.*, 1986; Schafer *et al.*, 1990; P.Chen and S.Michaelis, unpublished observations). Recently, the *RAM1* gene was shown to encode a component of the farnesyltransferase enzyme responsible for isoprenylation of RAS, a-factor and the γ -subunit of a yeast G protein (Goodman *et al.*, 1988, 1990; Finegold *et al.*, 1990; Schafer *et al.*, 1990). Thus, the yeast farnesyltransferase mediates modification of multiple CXXX-terminating cellular proteins. The *RAM2* gene, like *RAM1*, appears to be critical for isoprenylation of proteins in yeast (Goodman *et al.*, 1990). Farnesyltransferase activity has also been found in mammalian systems (Reiss *et al.*, 1990; Schaber *et al.*, 1990).

We have investigated the role of the *STE14* gene product in the post-translational modification of yeast proteins containing a CXXX sequence at their C-terminus. DNA sequence analysis reveals that the *STE14* product is 239 residues in length and appears to contain multiple membrane spanning domains (Sapperstein *et al.*, 1989). We have recently demonstrated, using an *in vitro* assay, that *S.cerevisiae* contains a membrane-bound C-terminal methyltransferase activity specific for a peptide substrate terminating in S-farnesyl cysteine (Hrycyna and Clarke, 1990). This methyltransferase activity is present in both *MATa* and *MAT α* cells and is dependent upon the presence of a wild-type *STE14* gene. A mammalian counterpart of the yeast methyltransferase has been recently described that possesses similar activity (Stephenson and Clarke, 1990).

In the present report, we further examine the *in vitro* and

finding that the *STE14*-dependent C-terminal methyltransferase from yeast efficiently recognizes *N*-AcFC as a substrate suggests that peptide sequences which precede the isoprenylated cysteine residue in CXXX-terminating proteins are not required as a signal for methylation.

The wild-type and *ste14* null mutant strains we tested here are isogenic. Thus, the lack of significant levels of methyltransferase activity observed in these strains (Table I) can be attributed solely to the presence or absence of an intact *STE14* gene, and not to unrelated strain differences. Furthermore, a *ste14* mutant complemented by a high copy number *STE14* plasmid produces a higher than wild-type level of methyltransferase activity (Table I; Hrycyna and Clarke, 1990). The observation that we detect essentially

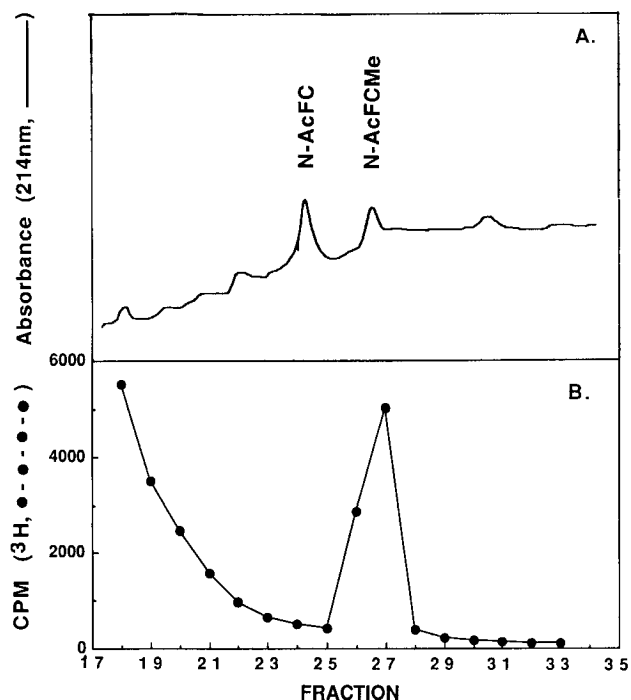


Fig. 2. Enzymatic formation of *N*-acetyl farnesyl cysteine [³H]methyl ester (*N*-AcFCMe) by the *STE14* methyltransferase. *N*-AcFC was incubated in a reaction mixture containing 30 μ l of 50 μ M [³H-methyl]AdoMet, 40 μ l of crude membranes (0.71 mg protein) from BSG24 (*pTrpE-STE14*₁₋₂₃₉) and 35 μ l 100 mM Tris-HCl (pH 7.5). After incubation for 3 h at 37°C, 200 μ l *n*-butanol was added and the sample was centrifuged at 13 000 *g* for 2 min. The *n*-butanol phase was retained and the aqueous layer was re-extracted with *n*-butanol as above. The combined *n*-butanol layers were evaporated to dryness and resuspended in 200 μ l solvent A (solvent A is 0.1% trifluoroacetic acid in water, and solvent B is 0.1% trifluoroacetic acid-90% acetonitrile-9.9% water). (A) The total sample was fractionated on a C18 reversed phase column with a non-linear elution gradient of 0-70% solvent B/100-0% solvent A over 35 min and then up to 100% solvent B at 50 min at an initial flow rate of 1 ml/min. The unreacted substrate, *N*-AcFC, and its product, *N*-AcFCMe were detected by their UV absorption at 214 nm at 24-25 min and 26-27 min respectively; the peak at 24 min corresponds to 0.04 absorbance units. (B) One-minute fractions were collected and assayed for total radioactivity by counting 250 μ l of each fraction in 10 ml of scintillation fluid. Nearly all of the radioactivity coelutes with the *N*-AcFCMe product. Aliquots (100 μ l) of fractions corresponding to the *N*-AcFC and *N*-AcFCMe peaks were evaporated to dryness. An 80 μ l aliquot of NaOH was added to each fraction and samples were directly assayed for methyl esters as described in Materials and methods. Quantitative amounts of [³H]methanol, the expected product from base hydrolysis of a methyl ester, were released from the fraction corresponding to the 26-27 min peak (data not shown).

no methyltransferase activity *in vitro* using *ste14* extracts, together with the *in vivo* results for a-factor and RAS presented below, suggest that *STE14* is the major yeast methyltransferase involved in post-translational modification of isoprenylated proteins.

Recent evidence from mammalian systems suggests that the methyl esterified cysteine residue of some proteins with a C-terminal CXXX motif is not modified by a farnesyl (C₁₅) group, but rather by a geranylgeranyl (C₂₀) group (Yamane *et al.*, 1990). Although this modification has not yet been detected in yeast proteins, we examined whether the substrate *S*-geranylgeranyl LARYKC could be recognized by the *STE14* methyltransferase. We found that purified membranes from wild-type yeast catalyze the methylation of *S*-geranylgeranyl LARYKC at a rate of at least 50% that of *S*-farnesyl LARYKC, whereas membranes prepared from *ste14* mutants show no detectable activity with either substrate (data not shown). Thus it appears that the *STE14* methyltransferase can recognize a C-terminal cysteine residue modified by either a farnesyl or geranylgeranyl moiety.

STE14-mediated methylation of a-factor

The *ste14* mutation was first identified on the basis of its defect in *MATa* cell mating. To determine directly whether a-factor is methylated by the *STE14* methyltransferase, we compared *in vivo* methylation of a-factor in wild-type and isogenic *ste14* null mutant strains. To enhance our ability to detect a-factor, the strains used in these experiments contain a high copy number plasmid which over-produces a-factor. Cells were labelled with [³⁵S]cysteine and *S*-adenosyl-L-[³H-methyl]methionine ([³H-methyl]AdoMet), intracellular and extracellular fractions were separated, and immunoprecipitation was carried out using a-factor antiserum. After SDS-PAGE, [³⁵S]cysteine-labelled a-factor was visualized by autoradiography, and [³H-methyl] groups were detected using the gel slice vapor phase assay (Clarke *et al.*, 1988). This assay specifically detects ester-linked methyl groups which are liberated as [³H]methanol after base hydrolysis.

In Figure 3, the position of the [³⁵S]cysteine-labelled a-factor band detected by autoradiography is indicated by an arrow. The difference in migration of extracellular and intracellular a-factor relative to the 6.2 kd marker reflects the difference in molecular weight between mature and precursor species of a-factor. In the intracellular sample (Figure 3, panel B), a methyl ester peak is apparent in wild-type cells at a position corresponding to the a-factor precursor. In contrast, no peak is observed for the *ste14* mutant. Since both strains appear to synthesize equivalent amounts of the a-factor precursor based on autoradiography of the [³⁵S]-labelled a-factor (data not shown), the lack of a [³H]methyl peak observed for the *ste14* mutant reflects a lack of methylation, and not absence of the polypeptide *per se*. Thus, we can conclude that the *STE14* methyltransferase is responsible for methylation of a-factor.

In the extracellular fraction (Figure 3, panel A), a methyl ester peak is present at a position corresponding to mature a-factor in the wild-type strain and absent in the *ste14* mutant. We observe four-fold less [³⁵S]-labelled a-factor in the extracellular fraction from the *ste14* mutant as compared with wild-type (data not shown). Thus, the lack of methylation appears to have an adverse effect on secretion of a-factor,

in vivo activities promoted by STE14. We show that an extract from an *Escherichia coli* strain synthesizing a TrpE-STE14 hybrid protein is capable of promoting methylation *in vitro*. This result provides strong evidence that STE14 is the structural gene for the yeast methyltransferase enzyme. We also demonstrate that STE14 is required for the *in vivo* methylation of a-factor, RAS1 and RAS2. Thus, the STE14 gene product, like RAM1, is an enzyme responsible for the post-translational modifications of multiple yeast proteins. Interestingly, *ste14* null mutants are not detectably impaired in RAS activity, suggesting that methyl modification is not essential for RAS function. We do observe, however, that *ste14* mutants exhibit a significant kinetic delay in RAS2 maturation and a slight decrease in the amount of RAS2 that becomes membrane-bound.

Results

Methyltransferase activity of TrpE-STE14 hybrid proteins synthesized in *E. coli*

We recently characterized a farnesyl cysteine methyltransferase activity in membrane extracts from *S. cerevisiae* and found that the activity is absent in extracts from *ste14* mutant strains (Hrycyna and Clarke, 1990). This result raised the possibility that the STE14 gene encodes the methyltransferase enzyme. Alternatively, the STE14 product could be a rate-limiting component of this enzyme or a regulator of its synthesis. To distinguish these possibilities, we expressed STE14 as a hybrid protein in *E. coli*, an organism with no endogenous farnesyl cysteine methyltransferase activity (Figure 1). Using pATH vectors, two gene fusions were constructed, *TrpE-STE14₁₋₂₃₉* and *TrpE-STE14₁₀₂₋₂₃₉*, in which all or part of the STE14 coding sequence, respectively, was joined to the C-terminal end of the *E. coli* TrpE gene.

We assayed *in vitro* methyltransferase activity in membrane and cytosolic extracts from strains induced for synthesis of these fusions. A high level of methyltransferase activity was observed with the membrane fraction from a strain containing the *TrpE-STE14₁₋₂₃₉* fusion (Figure 1) in which the entire coding sequence of STE14 is present. This activity was dependent upon the presence of a methyl-accepting substrate and product formation was linearly dependent on time. No activity was observed in membrane fractions from the parental strain which lacks a plasmid, nor in membrane fractions from a strain with the *TrpE-STE14₁₀₂₋₂₃₉* fusion, which contains only the C-terminal portion of STE14 (Figure 1). In no case was cytosolic methyltransferase activity detected. These results indicate that the STE14 gene product is a methyltransferase and provide strong evidence that the STE14 polypeptide is the sole component of this methyltransferase. The possibility that another component required for activity might be supplied by the *E. coli* membrane fraction is unlikely, but cannot be ruled out.

Characterization of substrate recognition by the STE14 methyltransferase

In our previous work, we showed that the farnesylated peptide, *S*-farnesyl LARYKC is an efficient *in vitro* substrate for the yeast methyltransferase (Hrycyna and Clarke, 1990). To refine our understanding of the substrate requirements

for the STE14 methyltransferase and, in particular, to learn whether specific peptide sequences are required for enzyme recognition, we examined *in vitro* methylation of a compound lacking any amino acids except for a farnesylated cysteine residue. This compound, *N*-acetyl farnesyl cysteine (*N*-AcFC), has been reported to be a methylatable substrate in a mammalian cell extract (Stock et al., 1990). We compared the efficiency of methylation of *S*-farnesyl LARYKC and *N*-AcFC substrates using membrane extracts from a wild-type strain and from two isogenic *ste14* null mutants. As shown in Table I, the wild-type extract is able to methylate both substrates with comparable efficiency. The identity of the expected product, methyl esterified *N*-AcFC, was confirmed (Figure 2). No methylation of either substrate was detected in the *ste14* membrane extracts (Table I). The

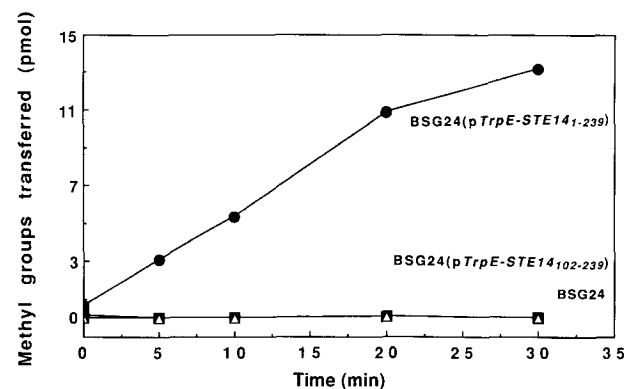


Fig. 1. Expression of the full length STE14 gene product as a TrpE fusion protein in *E. coli* generates C-terminal farnesyl cysteine methyltransferase activity. Crude membrane fractions (10 μ l) from the parent *E. coli* strain, BSG24 (Δ), and from transformants with a plasmid containing TrpE fused to the full length STE14 coding region, BSG24 (pTrpE-STE14₁₋₂₃₉) (\bullet), or to the C-terminal portion of STE14 BSG24 (pTrpE-STE14₁₀₂₋₂₃₉) (\blacksquare) were assayed with 20 μ l of 50 μ M [³H-methyl]AdoMet and 20 μ l 100 mM Tris-HCl (pH 7.5) with or without the methyl-accepting substrate *N*-acetyl farnesyl cysteine (*N*-AcFC) (1 nmol). The samples were incubated for the times indicated at 37°C. The protein concentrations of the BSG24, BSG24 (pTrpE-STE14₁₋₂₃₉) and BSG24 (pTrpE-STE14₁₀₂₋₂₃₉) samples were 15.5 mg/ml, 17.8 mg/ml and 19.5 mg/ml respectively. Methyl esters were detected as described in Materials and methods. The small amount of methyl esters formed (less than 0.1 pmol) in the absence of *N*-AcFC were subtracted to give the values presented. Similar results were obtained when *S*-farnesyl LARYKC was used as a substrate.

Table I. Methyltransferase activity in isogenic strains

Strain	Relevant genotype	Methyltransferase activity (pmol/min/mg protein) ^a
Substrate: <i>S</i>-farnesyl-LARYKC		
SM1058 (STE14 ⁺)	STE14 ⁺	0.94 (\pm 0.20)
SM1188 (<i>ste14</i>)	<i>ste14</i>	0.003 (\pm 0.008)
SM1639 (<i>ste14</i>)	<i>ste14</i>	0.0006 (\pm 0.0005)
SM1639 (pSM344)	<i>ste14</i> (2 μ STE14 LEU2)	4.42 (\pm 0.02)
Substrate: <i>N</i>-acetyl <i>S</i>-farnesyl cysteine		
SM1058 (STE14 ⁺)	STE14 ⁺	0.43 (\pm 0.01)
SM1188 (<i>ste14</i>)	<i>ste14</i>	0.011 (\pm 0.002)
SM1639 (<i>ste14</i>)	<i>ste14</i>	0.005 (\pm 0.003)
SM1639 (pSM344)	<i>ste14</i> (2 μ STE14 LEU2)	8.40 (\pm 0.05)

^a Activities from control incubations lacking farnesylated substrate have been subtracted. Each value represents duplicate incubations \pm the observed range.

Table II. Incorporation of [³H]methyl esters and [³⁵S]cysteine in immunoprecipitated RAS1 and RAS2 in *STE14*⁺ and *ste14* strains

Strain	Plasmid	[³ H]Methyl esters in RAS1 or RAS2 (c.p.m.) ^a	[³⁵ S]Cysteine in RAS1 or RAS2 (c.p.m.) ^a	Relative extent of methylation ([³ H]c.p.m./[³⁵ S]c.p.m. × 10)
SM1058 (<i>STE14</i> ⁺)		17.3	285.8	0.61 ^b
SM1188 (<i>ste14</i>)		0.9	192.2	<0.05
SM1058 (<i>STE14</i> ⁺)	(YE <i>p-RAS2-4</i>)	148.8	1120.8	1.3
SM1188 (<i>ste14</i>)	(YE <i>p-RAS2-4</i>)	0.1	1563.8	<0.0006
SM1058 (<i>STE14</i> ⁺)	(<i>pADH-RAS2</i>)	114.8	1574.6	0.73
SM1188 (<i>ste14</i>)	(<i>pADH-RAS2</i>)	1.4	1720.5	<0.008
SM1058 (<i>STE14</i> ⁺)	(YE <i>p-RAS1</i>)	115.8	2259.2	0.51
SM1639 (<i>ste14</i>)	(YE <i>p-RAS1</i>)	0.1	1182.4	<0.0008

^aBackground radioactivity from adjacent gel slices have been subtracted.

methyl]AdoMet, and RAS proteins were examined after immunoprecipitation and SDS-PAGE. Gel slice vapor assays were performed to detect [³H-methyl]esters, and [³⁵S]cysteine incorporated into proteins was monitored by autoradiography and direct counting of gel slices. Figure 4 shows the data obtained with strains containing the *RAS2* plasmid. In the autoradiograph shown in Figure 4 (top), a 40 kd [³⁵S]cysteine-labelled band corresponding to *RAS2* is present at similar levels in both wild-type and *ste14* strains (lanes 1 and 3). In the *ste14* mutant, a doublet was consistently observed at this position (see below). The identity of the 40 kd species and doublet bands as *RAS2* was confirmed by their disappearance in control immunoprecipitations performed with a competitor peptide recognized by the Y13-259 *RAS* monoclonal antibody (Figure 4, top, lanes 2 and 4).

To determine the level of *RAS2* methylation, the dried gels shown at the top of Figure 4 were examined by gel slice vapor assays (Figure 4, lower panels A-D). In the wild-type strain (Figure 4, panel A), a methyl ester peak is apparent in gel slice number 13, which corresponds to *RAS2*, as evidenced by its disappearance with addition of the blocking peptide (Figure 4, panel B). Strikingly, no methyl esters are detected at the *RAS2* position in immunoprecipitates from the *ste14* mutant (Figure 4, panels C and D). These results indicate that *RAS2* remains unmethylated in the *ste14* mutant and demonstrate that *RAS2* is a physiological substrate for the *STE14* methyltransferase. These data are quantified in Table II, together with similar results from experiments using a different *ste14* null mutant.

We also examined methylation of *RAS1* in wild-type and *ste14* strains containing a high copy number *RAS1* plasmid. The results we obtained were analogous to those described above for *RAS2* (Table II). Taken together, the results in Figure 4 and Table II establish that *STE14* is responsible for methylation of both the *RAS1* and *RAS2* proteins. Since it is known that functional *RAS* proteins are required for viability in yeast (Powers *et al.*, 1984; Tatchell *et al.*, 1984), and since *ste14* mutants are not compromised for viability, our results point to the possibility that methylation is not essential for *RAS* function, at least under normal conditions.

Maturation of *RAS2* in strains lacking the *STE14* methyltransferase

We were interested in understanding the significance of the *RAS2* doublet detected in the *ste14* mutant, above (Figure 4, upper panel, lane 3). The upper band of the doublet has a

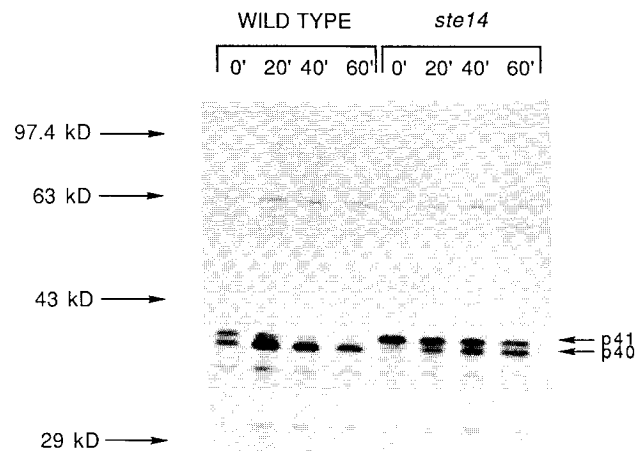


Fig. 5. Kinetics of *RAS2* processing in wild-type and *ste14* strains. Wild-type (SM1058) and *ste14* (SM1188) strains containing the plasmid *pADH-RAS2* were labelled with [³⁵S]Translabel for 2 min at 30°C, and chased for the indicated times. Total cell extracts prepared as described in Materials and methods were immunoprecipitated with anti-ras monoclonal antibody Y13-259 and immunoprecipitates were analyzed by SDS-PAGE using a 10% acrylamide gel.

mobility characteristic of the *RAS2* precursor (p41), which migrates slightly above authentic *RAS2* (p40) (Fujiyama *et al.*, 1987; Tamanoi *et al.*, 1988). Normally, maturation of the *RAS2* precursor occurs quite rapidly and p41 can only be detected in pulse-labelled wild-type strains or in mutants, such as *ram1*, in which isoprenylation is blocked (Powers *et al.*, 1986). Thus, the persistence of p41 here suggested that the rate of *RAS2* maturation might be altered in strains lacking the *STE14* methyltransferase.

To examine this possibility, we performed pulse-chase analysis of *RAS2* in wild-type and isogenic *ste14* strains. Cells were labelled briefly with a mixture of [³⁵S]cysteine and [³⁵S]methionine (³⁵S-Translabel), chased with non-isotopically labelled amino acids for varying lengths of time, and *RAS2* proteins were immunoprecipitated and analyzed by SDS-PAGE (Figure 5). Since processing in the wild-type strain is extremely rapid, we observe that even before the chase is initiated, 50% of the *RAS2* protein has already undergone processing to p40 (Figure 5). By 20 minutes, 80% of *RAS2* is converted to the p40 form, and by 40 minutes, conversion to p40 is essentially complete. In

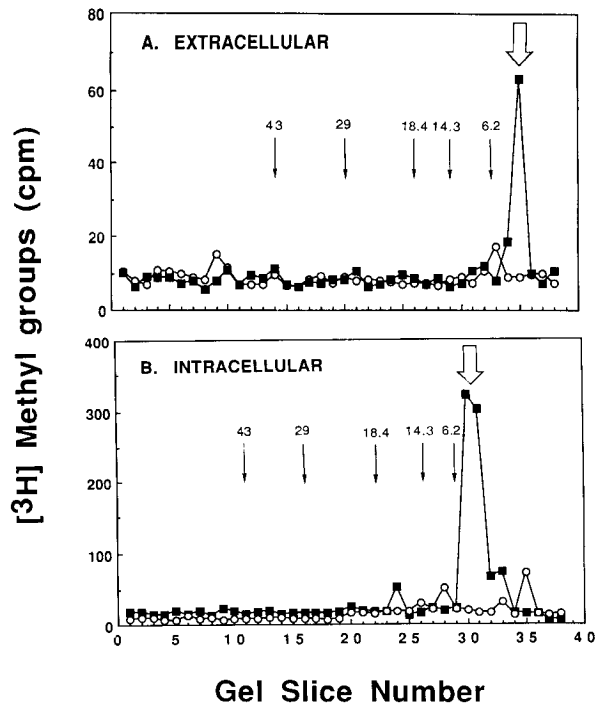


Fig. 3. Comparison of *a*-factor methylation *in vivo* in wild-type and *ste14* strains of *S. cerevisiae*. A wild-type strain, SM1058 (■) and *ste14* mutant, SM1188 (○) containing the *a*-factor plasmid pSM219 were labelled separately with [³⁵S]cysteine or [³H-methyl]AdoMet at 30°C and processed as described in Materials and methods. Extracellular and intracellular fractions were immunoprecipitated using *a*-factor antiserum. Immunoprecipitates from the extracellular (A) and intracellular (B) fractions were subjected to SDS-PAGE using a 12.5% acrylamide gel. The lanes of the dried gel containing the [³H-methyl]AdoMet samples were cut into 3 mm slices and each slice assayed for methyl esters as described in Materials and methods. The distribution of base hydrolyzed [³H]methyl groups released from extracellular (A) and intracellular (B) proteins is shown. The portion of the gel containing [³⁵S]cysteine-labelled samples was subjected to autoradiography to visualize *a*-factor. The open arrow indicates the migration position of the mature (A) and precursor (B) *a*-factor species that were detected. Positions of the pre-stained molecular weight markers are marked.

as has also been previously observed (Marr *et al.*, 1990; S.Sapperstein and S.Michaelis, in preparation). In addition, the lack of methylation greatly reduces *a*-factor biological activity; as described in Materials and methods, we determined by halo dilution assays of concentrated culture fluid that the unmethylated species of *a*-factor secreted by the *ste14* mutant appears to be at least 200-fold less active than *a*-factor produced by wild-type cells.

STE14-mediated methylation of RAS1 and RAS2

The RAS proteins of yeast, like *a*-factor, are farnesylated and carboxyl methyl esterified (Deschenes *et al.*, 1989; Stimmel *et al.*, 1990). Since the isoprenylation of RAS proteins and *a*-factor is carried out by a common mechanism involving the *RAM1* and *RAM2* gene products (Powers *et al.*, 1986; Goodman *et al.*, 1990) and since STE14 activity is not confined to cells of the *MATa* mating type, we were interested in determining whether RAS and *a*-factor are also methylated by a common enzyme. To test whether STE14 is responsible for methylation of RAS proteins, we compared *in vivo* methylation of RAS in wild-type and *ste14* mutant strains. Cells containing a high copy number *RAS1* or *RAS2* plasmid were double-labelled with [³⁵S]cysteine and [³H-

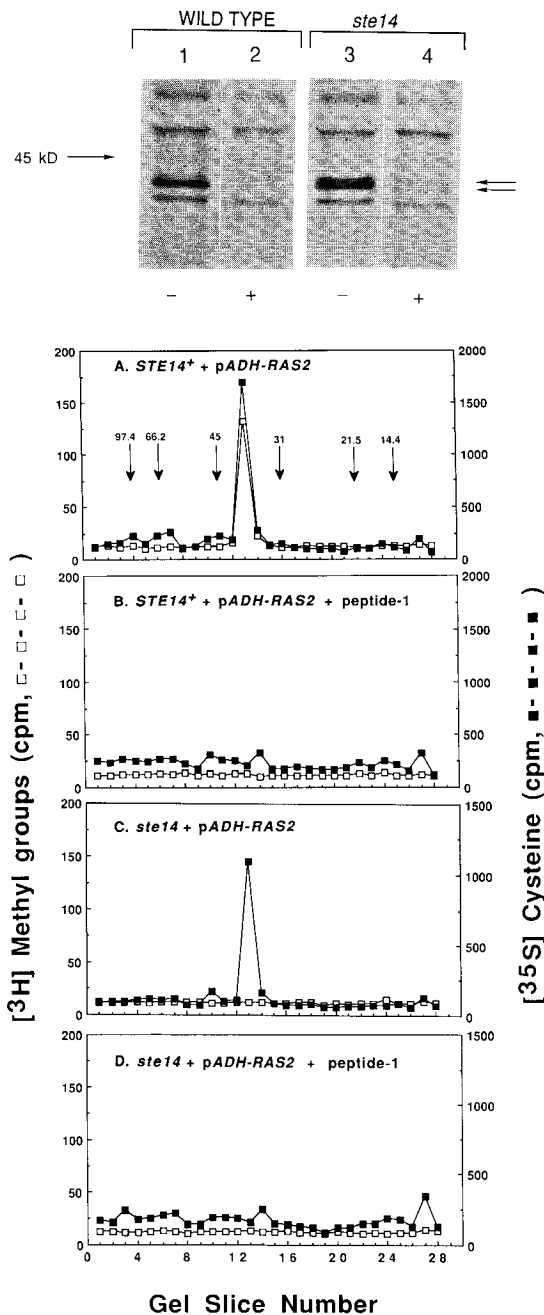


Fig. 4. Comparison of RAS2 methylation in wild-type and *ste14* strains. Wild-type (SM1058) and *ste14* (SM1188) cells containing pADH-RAS2 plasmids were double-labelled with [³⁵S]cysteine and [³H-methyl]AdoMet at 30°C and total cell extracts were prepared and immunoprecipitated with rat anti-pan-ras monoclonal antibody Y13-259 as described in Materials and methods. Immunoprecipitates were fractionated by SDS-PAGE on a 10% acrylamide gel. The dried gel was subjected to autoradiography to allow detection of [³⁵S]cysteine incorporation (top). To examine methylation, the lanes were sliced into 3 mm slices after autoradiography (lower panels A-D). The slicing was designed using the autoradiogram above as a guide, such that the band corresponding to RAS2 was fully contained in gel slice number 13 and the non-specific background band which appears immediately below RAS2 in all lanes was fully contained in gel slice number 14. These slices were assayed for methyl esters as base-volatile [³H]methanol radioactivity (panels A and C) (□). Controls with the blocking peptide are shown in panels B and D. Subsequently, the same slices were quantitatively assayed for total [³⁵S]cysteine incorporation into the RAS2 protein (■) as described in Materials and methods and shown in Table II. Migration positions of the low molecular weight standards are indicated.

independent isolates of SM1823 (*ras2^{val19} ste14*) were tested and gave results that are indistinguishable from their *ras2^{val19} STE14* parent, TK161-R2V (data not shown). Thus, the *ste14* mutation does not appear to reverse the heat shock sensitivity or lack of iodine staining of *ras2^{val19}*. Apparently, although lack of methylation can lead to subtle defects in RAS processing and localization, there are no measurable consequences for RAS activity under the conditions tested here.

Discussion

Methyl esterification appears to be the final step in formation of C-terminal farnesyl and geranylgeranyl cysteine methyl esters in peptides and proteins. The results presented here demonstrate that this methylation reaction is catalyzed by the product of the *STE14* gene in *S. cerevisiae*. We have also shown that the a-factor mating pheromone, RAS1 and RAS2 lack detectable C-terminal methylation in a *ste14* null mutant, and thus are physiological substrates of the STE14 methyltransferase. Our results strengthen the biochemical link between RAS and a-factor that was first suggested by studies of *RAM1* (Powers *et al.*, 1986), and extend the notion that a common machinery mediates the series of three modifications (isoprenylation, proteolytic cleavage, and methylation) proposed to occur on proteins that terminate with a CXXX motif, where the penultimate residue is aliphatic (Stimmel *et al.*, 1990).

Where in the cell does C-terminal methylation of isoprenylated proteins occur? Interestingly, inspection of the *STE14* DNA sequence reveals multiple potential membrane spanning domains, indicative of integral membrane proteins (Sapperstein *et al.*, 1989). Moreover, biochemical studies clearly indicate that STE14 methyltransferase enzyme activity is found in the membrane fraction (Hrycyna and Clarke, 1990). It will be interesting to determine whether methylation is carried out at the plasma membrane of yeast or on an intracellular membrane such as the cytoplasmic face of the endoplasmic reticulum. One attractive hypothesis is that the three reactions involved in maturation of CXXX-terminating proteins are carried out by a higher order complex of several polypeptides. Our observation here that a defect in methylation can impede the preceding steps of isoprenylation and proteolytic processing of RAS proteins (see below) supports the idea of a processing complex. However, it should be noted that while the STE14 methyltransferase is membrane-bound, the RAM1 component of the isoprenyltransferase is apparently soluble (Schäfer *et al.*, 1990; Goodman *et al.*, 1990) though a weak membrane association might not be easily detectable.

Methylation of a-factor by STE14

Mutants in the *STE14* gene were originally isolated on the basis of the inability of *MATa* cells to mate, due to a defect in a-factor production (Blair, 1979; Wilson and Herskowitz, 1987). We show here that methylation of a-factor fails to occur in the *ste14* mutant. The absence of methylation has dramatic consequences for a-factor. We have observed that the non-methylated a-factor produced by a *ste14* mutant is at least 200-fold less active than a-factor made by a wild-type strain. In addition, others have shown that a-factor lacking its methyl group has severely decreased biological activity (Anderegg, 1988; J. Becker, personal

communication). Thus the methyl ester on a-factor appears to be necessary either for binding of a-factor to its receptor, STE3, on the surface of MAT α cells (Nakayama *et al.*, 1985; Hagen *et al.*, 1986), or for the subsequent activation step that leads to G₁ arrest and mating. As observed here and elsewhere (Marr *et al.*, 1990; S. Sapperstein and S. Michaelis, in preparation) a second consequence of the lack of methylation is a reduced amount of a-factor secretion by *ste14* mutants. The basis for this apparent defect in a-factor export remains to be established.

Modification of RAS proteins by the STE14 methyltransferase

The profound methylation defect we observe for RAS1 and RAS2 in a *ste14* null mutant (Figure 4 and Table II) suggests that STE14 may be the major, if not the only yeast methyltransferase that can modify the C-terminus of RAS proteins. It is surprising, however, that while mutations that eliminate RAS function cause a major disruption in cell growth and survival (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984), the apparent lack of methylation in *ste14* mutants has no impact on cell viability, nor on the more subtle phenotypes of heat shock sensitivity and low starch levels conferred by a *ras2^{val19}* mutation. In comparison with *ram1* and *ram2* mutants, which are defective in farnesylation of RAS and exhibit a severe growth defect (Powers *et al.*, 1986), the *ste14* mutant grows normally under all conditions that we have examined. Although we cannot rule out the possibility that the methylation of an undetectable fraction of RAS proteins by an as yet unidentified enzyme could preserve their function, we stress that we detect < 1% of the wild-type methylation level of RAS1 and RAS2 in *ste14* mutants (Table II). In addition, methyltransferase activity is essentially undetectable in *in vitro* assays using membranes derived from *ste14* null mutants (Table I). In view of the essential nature of RAS function in yeast, the viability of the *ste14* mutant suggests that RAS proteins retain function in the absence of methylation. Alternatively, function may be somewhat impaired, but if the amount of RAS present in cells is actually far above saturating levels, then even a dramatic decrease in RAS activity would go unnoticed under normal laboratory conditions. If this were the case, it might be possible to identify a particular carbon source, temperature or ionic condition under which cell growth becomes dependent on a functional STE14 methyltransferase activity.

Maturation and membrane localization of RAS2 in a ste14 mutant

Despite the lack of an obvious effect on cell viability, we have observed several intriguing differences in RAS2 maturation between wild-type and *ste14* mutant strains. Biogenesis of RAS proteins is a complex process. In both yeast and mammalian cells, RAS is initially synthesized as a soluble precursor with a C-terminal CXXX motif. Maturation has been proposed to involve two steps (Hancock *et al.*, 1989; Fujiyama and Tamanoi, 1990): Step 1 includes isoprenylation, proteolytic cleavage and methylation, resulting in a cytosolic species whose gel mobility differs slightly from that of the precursor. Methylation is thought to occur last in this series of reactions. In Step 2, RAS becomes membrane localized and palmitoylated. In *ste14* cells we observe a striking kinetic

contrast, in the *ste14* mutant, very little of the processed p40 species of RAS2 is observed at the end of the pulse period and even after an extended chase (up to 60 min) only 50% of the RAS2 present has undergone conversion to p40. Thus, the rate of RAS2 maturation appears to be dramatically reduced in the *ste14* mutant. The results shown in Figure 5 also demonstrate that the RAS2 mobility shift does not rely on methylation, since the p41 to p40 conversion occurs despite the lack of methylation in the *ste14* mutant. Therefore, gel mobility must reflect isoprenylation or proteolytic cleavage. The finding that maturation of RAS2 is slow in the *ste14* mutant is surprising, since methylation of the cysteine carboxyl must necessarily occur after isoprenylation and proteolytic cleavage are complete. Thus, the lack of the methyltransferase would not be expected to prevent processing from p41 to p40. Possible explanations for this apparent paradox are discussed below.

Membrane localization of RAS2 in a *ste14* mutant

Farnesylation of the C-terminal cysteine residue of RAS is known to play a critical role in membrane localization. The availability of *ste14* mutants has provided us with the opportunity to assess whether methylation, like isoprenylation, plays a role in RAS2 membrane localization. To examine this possibility, total extracts from the pulse-chase experiment shown in Figure 5 were fractionated into membrane and cytosolic fractions, and RAS2 was examined after immunoprecipitation and SDS-PAGE (Figure 6). It is notable that in the *ste14* mutant, a significant amount of RAS2 reaches the membrane. On the one hand, this indicates that methylation is not essential for membrane localization. On the other hand, it is evident that the total amount of membrane-bound RAS2 is significantly less in the *ste14* mutant than in the wild-type strain. After 20 min, the *ste14* mutant contains only 20% the amount of membrane-associated RAS2 as in the wild-type strain. After 20 min, the level of RAS2 in the membrane does not increase substantially for either strain. It is interesting that with a very long chase (60 min, Figure 6) the total amount of membrane-associated RAS2 actually decreases, perhaps due to proteolytic degradation. This phenomenon is particularly evident in the wild-type case, so that the difference between the amounts of RAS2 in wild-type and *ste14* membranes is diminished at later times. We

have also examined RAS2 localization under steady state conditions by Western blotting and by steady-state isotopic labelling followed by immunoprecipitation. The results of these experiments are similar to those seen in the 40 min time point in Figure 6; the *ste14* mutant contains less membrane-bound RAS2 than wild-type and the unprocessed soluble species of RAS2 persists (data not shown).

The reduced level of membrane-associated RAS2 in the *ste14* cells may result from an altered capacity for RAS2 either to reach the membrane, or to be retained there when the C-terminal methyl ester modification is absent. Alternatively, the low amount of RAS2 in the membrane may simply reflect the p41 to p40 maturation defect discussed above, which results in a reduction of the amount of cytoplasmic p40 substrate that is available for localization to the membrane.

Physiological activity of RAS2 in a *ste14* mutant

Since cell viability is not impaired in *ste14* mutants (S.Sapperstein and S.Michaelis, in preparation), and RAS function is required for survival (Kataoka et al., 1984; Gibbs and Marshall, 1989), there appears to be no major defect in RAS activity due to a lack of methylation. However, the partial maturation and membrane localization defect described above led us to examine more subtle phenotypes associated with RAS function. A sensitive test for decreased RAS activity makes use of the hyperactive mutation *ras2^{val19}*, which activates the RAS adenylate cyclase pathway and causes cells to become heat shock sensitive and to lose the ability to accumulate glycogen stores (Kataoka et al., 1984). Mutations such as *ram1* and *ram2* which compromise RAS function result in a reversal of these phenotypes when introduced into a *ras2^{val19}* strain (Powers et al., 1986; Goodman et al., 1990). We tested whether *ste14* could also have such an effect. The *ste14::URA3* mutation was introduced into the *ras2^{val19}* strain, TK161-R2V, by gene replacement as described in Materials and methods. The resulting double mutant, designated SM1823 (*ras2^{val19} ste14*), was compared with the isogenic strains TK161-R2V (*ras2^{val19} STE14*) and SP1 (*RAS2 STE14*) for heat shock sensitivity and glycogen accumulation. As expected, the SP1 wild-type strain is heat shock resistant and exhibits strong iodine staining, whereas TK161-R2V is heat shock sensitive and stains poorly with iodine. Six

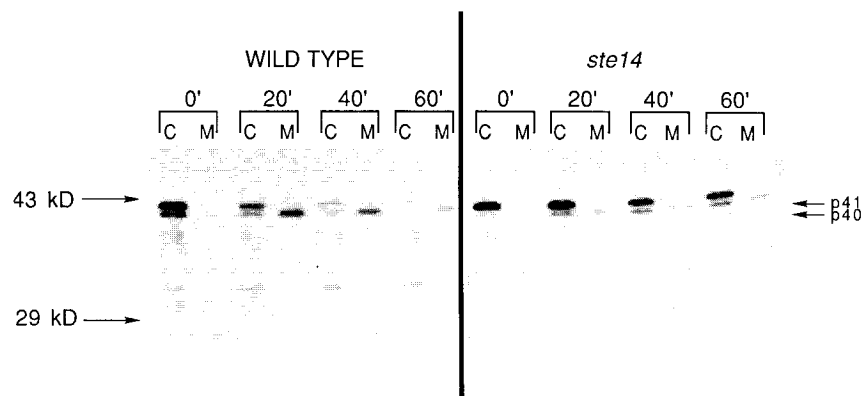


Fig. 6. Kinetics of RAS2 membrane localization in wild-type and *ste14* strains. Total cell extracts from the pulse-chase experiment shown in Figure 5 were fractionated as described in Materials and Methods. The resulting soluble cytoplasmic (C) and membrane (M) fractions were analyzed by immunoprecipitation and SDS-PAGE as described in the legend of Figure 5. Times shown indicate the length of the chase.

Table III. *Saccharomyces cerevisiae* proteins containing -Cys-Xaa-Ali-Xaa C-terminal tails and related proteins

Protein	Carboxy terminal sequence	Lipidation on conserved Cys	Methylation by <i>STE14</i>
a-factor	-Asp-Pro-Ala-Cys-Val-Ile-Ala ^b	farnesyl ^c	yes ^a
RAS1	-Gly-Gly-Cys-Cys-Ile-Ile-Cys ^d		yes ^a
RAS2	-Gly-Gly-Cys-Cys-Ile-Ile-Ser ^d	farnesyl ^c	yes ^a
RHO1	-Lys-Lys-Lys-Cys-Val-Leu-Leu ^f		?
RHO2	-Ala-Asn-Cys-Cys-Ile-Ile-Leu ^f		?
RSR1	-Ala-Ser-Thr-Cys-Thr-Ile-Leu ^g		?
STE18	-Ser-Lys-Lys-Cys-Thr-Leu-Met ^h	polyisoprene ⁱ	?
CDC42	-Ser-Lys-Lys-Cys-Thr-Ile-Leu ^j		?
YPT1	-Gly-Gly-Cys-Cys ^k		?
SEC4	-Ser-Asn-Cys-Cys ^l		?

^aThis study; ^bBrake *et al.*, 1985; ^cAnderegg *et al.*, 1988; ^dPowers *et al.*, 1984; ^eStimmel *et al.*, 1990; ^fMadaule *et al.*, 1987; ^gBender and Pringle, 1989; ^hWhiteway *et al.*, 1989; ⁱFinegold *et al.*, 1990; ^jJohnson and Pringle, 1990; ^kGallwitz *et al.*, 1983; ^lSalminen and Novick, 1987.

Table IV. *Saccharomyces cerevisiae* strains and plasmids

Name	Genotype	Source
Strains		
SM1058	<i>MATa trp1 leu2 ura3 his4 can1 STE14⁺</i>	This laboratory
SM1188	<i>ste14-Δ1::TRP1</i> , isogenic to SM1058	This laboratory
SM1639	<i>ste14-Δ2::URA3</i> , isogenic to SM1058	This laboratory
RC757	<i>MATα sst2-1 rme his6 met1 can1 cyh2</i>	Chan & Otte, 1982
SP1	<i>MATa leu2 ura3 his3 trp1 ade8 can1 gal2</i>	Toda <i>et al.</i> , 1985
TK161-R2V	<i>ras2^{val19}</i> , isogenic to SP1	Toda <i>et al.</i> , 1985
SM1823	<i>ste14-Δ2::URA3 ras2^{val19}</i> , isogenic to SP1	This laboratory
Plasmids		
pSM433	2μ <i>STE14 LEU2</i>	This laboratory
pSM219	2μ <i>MFA1 URA3</i>	This laboratory
YEp-RAS2-4	2μ <i>RAS2 LEU2</i>	Powers <i>et al.</i> , 1986
pADH-RAS2	2μ <i>RAS2 LEU2</i>	Powers <i>et al.</i> , 1986
YEp-RAS1	2μ <i>RAS1 LEU2</i>	Kataoka <i>et al.</i> , 1984

bromide (Aldrich) was added. The reaction was allowed to progress at room temperature for 30 min. After the reaction was complete, 300 μl was fractionated by HPLC using a preparative scale Econosphere C18 reversed phase column (Alltech/Applied Science, 10 mm inner diameter × 190 mm) equilibrated in solvent A at room temperature. The column was eluted using a non-linear gradient running from 0–60% solvent B and 100–30% solvent A (solvent A is 0.1% trifluoroacetic acid in water, and solvent B is 0.1% trifluoroacetic acid–90% acetonitrile–9.9% water) at an initial flow rate of 3 ml/min over 40 min, followed by a linear gradient to 100% solvent B over an additional 5 min. The *N*-AcFC product eluted at 29.4–29.6 min.

C-terminal methyltransferase assay

Enzyme activity was measured in *S. cerevisiae* membrane and cytosolic fractions and in *E. coli* extracts by the method previously described by Hrycyna and Clarke (1990).

Metabolic labelling and immunoprecipitation of a-factor

To label intracellular a-factor, cells were grown in SD medium with appropriate supplements to OD₆₀₀ 0.7, harvested, and for each separate labelling 5 OD₆₀₀ units were resuspended in 0.5 ml of growth medium in a polypropylene tube. Either 150 μCi [³⁵S]cysteine (Amersham, >600 Ci/mmol) or 120 μCi *S*-adenosyl-L-[³H-methyl] methionine ([³H-methyl]AdoMet; Amersham, 15 Ci/mmol) were added to separate cultures. Labelling was carried out for only 6 min at 30°C, since intracellular a-factor is short-lived, and was terminated by addition of 0.5 ml ice-cold azide stop mix [40 mM cysteine, 40 mM methionine, 20 mM NaN₃, 500 μg/ml BSA (Miles Laboratories)]. Cells were pelleted by centrifugation at 13 600 g for 1 min, washed once with H₂O, resuspended in 500 μl immunoprecipitation buffer (1.0% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl pH 7.5, 1 mM PMSF, 0.5% Trasylol (FBA Pharmaceuticals)) and lysed in the presence of 0.5 g baked zirconium beads (0.5 mm diameter, Biospec Products) by vortexing 10 times in 1 min bursts at 4°C. The cell lysate (designated the intracellular fraction) was removed from the beads by addition of 1.3 ml immunoprecipitation buffer.

To examine extracellular a-factor, labelling was carried out as described

above, except that SD-URA media was used and labelling was allowed to proceed for 90 min. After addition of azide stop mix, cultures were moved to a new tube and centrifuged at 13 600 g for 1 min. The labelling tube was retained and a-factor adhering to the tube was recovered as described below. The cell pellet was discarded and the supernatant, which contains secreted a-factor, was retained. Extracellular proteins were concentrated from the sample by addition of an equal volume of 20% TCA (w/v), incubation at 4°C for 15 min, and centrifugation at 13 600 g for 5 min. The pellet was resuspended in 15 μl 2× Laemmli sample buffer (20% glycerol, 10% 2-mercaptoethanol, 4.3% SDS, 0.125 M Tris–HCl pH 6.8 and 0.2% bromophenol blue) and neutralized with 1 μl 1.0 M Tris base. Since a significant portion of extracellular a-factor remains bound to the polypropylene labelling tube, this tube was rinsed with H₂O and a-factor was eluted from the sides with 0.4 ml *n*-propanol. The *n*-propanol eluate was evaporated to dryness with heating. This sample was resuspended in 15 μl 1× Laemmli sample buffer and combined with the culture fluid fraction prepared as described above. This combined sample (designated the extracellular fraction) was heated at 100°C for 3 min and added to 1.3 ml immunoprecipitation buffer.

Prior to immunoprecipitation, intracellular and extracellular samples were centrifuged for 1 min at 13 600 g to remove insoluble debris, samples were transferred to a new tube and 10 μl of a-factor rabbit antiserum Ab-9–137 (S. Sapperstein and S. Michaelis, in preparation) was added and then allowed to incubate overnight at 4°C. To collect immunoprecipitates, a 45 μl aliquot of protein A–Sepharose CL-4B beads (Pharmacia) suspended in immunoprecipitation buffer (1:3 beads:total volume ratio) was added and the tubes gently agitated at 4°C for 90 min. The beads were pelleted by a 10 s spin at 13 600 g and subsequently washed four times with immunoprecipitation wash buffer A (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM EDTA, 1 mM PMSF, 0.5% Trasylol) and once with immunoprecipitation wash buffer B (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM EDTA, 1 mM PMSF, 0.5% Trasylol). After the final wash, bound immune complexes were released from the beads by the addition of 30 μl 2× Laemmli sample buffer. The samples were heated at 100°C for 3 min, clarified, and the supernatant was subjected to SDS–PAGE.

delay in Step 1 of RAS2 processing, as measured by the extremely slow conversion of p41 to p40. This result is somewhat puzzling since it suggests that a step prior to methylation (either isoprenylation or proteolytic cleavage) is affected when the *STE14* product is absent. Possible explanations are that the machinery which mediates Step 1 processing may be comprised of a complex of proteins that cannot properly form in the absence of the *STE14* product, or that a component of the machinery itself requires methylation for full activity. It could also be that the isoprenylation machinery has a sensor that responds to an insufficient amount of membrane-bound RAS by dampening further processing. Alternatively, more complicated explanations must be invoked to explain why lack of methylation affects other steps of maturation. It should be noted that the experiments described here were performed using strains overexpressing RAS2. High levels of RAS2 might cause subtle differences between wild-type and *ste14* strains to be accentuated.

Step 2 of RAS2 maturation is also in some way altered in the *ste14* mutant since we observe an apparent decrease in the total amount of RAS in the membrane. It may be that the lack of the methyl group affects the ability of RAS to reach the plasma membrane or to be retained there. Alternatively, there may be less isoprenylated substrate available for membrane attachment due to the maturation defect discussed above.

Other candidate substrates for the *STE14* methyltransferase

Characterization presented here suggests that both farnesyl (C₁₅) and geranylgeranyl (C₂₀) modified proteins might be substrates of the *STE14* methyltransferase and further predicts that residues preceding the CXXX motif do not influence methylation. Other yeast proteins that terminate in a CXXX motif, where the penultimate residue is aliphatic, and are thus candidates for methylation (Stimmel et al., 1990) are shown in Table III. We also include examples from another class of *S. cerevisiae* proteins synthesized with a C-terminal -Cys-Cys motif. Indirect evidence suggests these latter proteins may also be isoprenylated (S.Ferro-Novick, personal communication), but their state of methylation has not been evaluated.

Many of the proteins listed in Table III are known to be localized to a membrane or to be involved in membrane associated processes. In addition, all of these polypeptides, with the exception of α -factor, RHO2 and STE18, are essential for viability. Our determination here, that *STE14* appears to be the major C-terminal methyltransferase in yeast predicts that the proteins listed in Table III, if methylated, would also be substrates for this activity. Were this the case, then the viability of *ste14* mutants argues that methylation may not play an essential role for their function. However the methyl group may indeed be important for modulating processing or facilitating the interaction of these proteins with the membrane. We suggest that the critical role of methyl esterification for the biological activity of α -factor may thus be distinct from the function of this reaction for other proteins.

Materials and methods

Yeast strains, media and growth conditions

Yeast strains used in this study are listed in Table IV. SM1058 was formerly designated EG123 (Michaelis and Herskowitz, 1988). Strains SM1188 and

SM1639 were derived from SM1058 by single-step gene replacement and contain substitution mutations in which a significant portion of the *STE14* coding sequence is deleted and substituted by the selectable markers *TRP1* or *URA3*, respectively. Construction of these strains is described elsewhere (S.Sapperstein and S.Michaelis, in preparation). A double mutant containing *ste14::URA3* and *ras2^{val19}* was made by gene replacement. Briefly, a 2.5 kb *Bam*HI-*Clal* fragment containing the *ste14-Δ2::URA3* substitution mutation was used to transform TK161-R2V. Ura⁺ transformants were screened for loss of *STE14* function by the α -factor halo and mating assays (Michaelis and Herskowitz, 1988), and one of the sterile transformants was designated SM1823. Complete media (YEED), synthetic drop-out media (SD-Leu and SD-Ura), and SD minimal media were prepared as previously described (Michaelis and Herskowitz, 1988) except that drop-out media are lacking L-methionine and L-cysteine. Where necessary, SD media was supplemented with L-histidine (20 μ g/ml), L-tryptophan (20 μ g/ml), uracil (20 μ g/ml), or L-leucine (30 μ g/ml). All *in vivo* experiments were performed at 30°C.

Plasmids and manipulation of DNA

Plasmids used in this study are listed in Table IV. The *TrpE-STE14* fusion plasmids were constructed using pATH vectors (Koerner et al., 1990) and inserts from pSM191, in which a 1.6 kb *Clal*-*Bam*HI fragment containing the *STE14* gene is flanked by polylinker sites (S.Sapperstein and S.Michaelis, in preparation). Subscripted numbers designate the *STE14* codons present in each fusion. A 1.5 kb *Clal* fragment from pSM191 was cloned into the *Clal* site of pATH3 to produce p*TrpE-STE14*₁₋₂₃₉. The entire *STE14* coding sequence is present in this fusion. To construct p*TrpE-STE14*₁₀₂₋₂₃₉, a 1.1 kb *Hind*III fragment from pSM191 was cloned into the *Hind*III site of pATH3 resulting in a fusion which contains only the C-terminal half of *STE14*. Construction of plasmids pSM433 (2 μ *STE14* LEU2) and pSM219 (2 μ *MFA1 URA3*) will be described elsewhere (S.Sapperstein and S.Michaelis, in preparation). Plasmids YEp-RAS2, pADH-RAS2 and YEp-RAS1 were kindly provided by S.Powers. Transformation of plasmids into yeast and *in vitro* manipulation of DNA were performed as described previously (Michaelis and Herskowitz, 1988).

Induction of *TrpE-STE14* fusions and preparation of *E. coli* extracts

Fusion plasmids were transformed into *E. coli* strain BSG24, also designated FZ-392, which is a mutagenized derivative of LE392 (Murray et al., 1977) obtained from Abbott laboratories via S.Gerring. Media used for propagation and induction of fusion strains, 'modified M9' and 'modified M9-Trp', were prepared essentially as described (Koerner et al., 1990), except that vitamin free casamino acids (Difco no. 0288 Vitamin Assay Quality) were used and ampicillin was replaced by carbenicillin (50 μ g/ml). For induction of *TrpE* fusion proteins, cells were grown in 50 ml 'modified M9' to an OD₆₀₀ of 0.7–1.2, harvested at 11 000 g for 10 min at 4°C, washed once with 30 ml 'modified M9-Trp', and resuspended in 500 ml of this media. After incubation for 1.5 h at 30°C with constant shaking, indoleacrylic acid (5 μ g/ml final concentration) was added to initiate induction. The cultures were allowed to grow for an additional 6 h and harvested as before. The cell pellet was weighed and resuspended at a concentration of 0.5 g/ml of *E. coli* lysis buffer A [5 mM sodium phosphate, 5 mM EDTA, 10% glycerol (v/v), 25 μ M PMSF and 15 mM 2-mercaptoethanol, pH 7.0; the last two components added just prior to use]. The cells were subjected once to French press treatment at 1000 lb/in². Unbroken cells and large debris were removed by centrifugation at 11 000 g for 10 min at 4°C. This extract was spun at 100 000 g for 1 h at 4°C. The supernatant (cytoplasmic fraction) was stored at -20°C and the pellet (crude membrane fraction and insoluble proteins) was resuspended in 1.5 ml cold *E. coli* lysis buffer A and stored at -20°C.

Preparation and purification of *S. cerevisiae* membrane and cytosolic fractions.

Cells were grown in SD medium containing required nutrients to an OD₆₀₀ of 0.7–1.2. Crude membranes and cytosolic fractions were prepared and the membranes were subsequently purified by sucrose gradient centrifugation as previously described by Hrycyna and Clarke (1990).

Synthetic substrates

L-Leu-L-Ala-L-Arg-L-Try-L-Lys-*S-trans,trans*-farnesyl-L-Cys (*S*-farnesyl LARYKC) and L-Leu-L-Ala-L-Arg-L-Tyr-L-Lys-*S-all trans*-geranylgeranyl-L-Cys (*S*-geranylgeranyl LARYKC) were provided by Robert Stephenson (UCLA) and are described elsewhere (Stephenson and Clarke, 1990). *N*-acetyl farnesyl cysteine (*N*-AcFC) was synthesized by a modification of the method of Stephenson and Clarke (1990). *N*-acetyl cysteine (450 nmol) was dissolved in 0.35 ml of dimethyl formamide, H₂O, 0.5 M KHCO₃ (5:1:1, v/v/v) and then two equivalents (200 nmol) of *trans,trans*-farnesyl

- Biology*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 103–108.
- Broek,D., Toda,T., Michaeli,T., Levin,L., Birchmeier,C., Zoller,M., Powers,S. and Wigler,M. (1987) *Cell*, **48**, 789–799.
- Chan,R.K. and Otte,C.A. (1982) *Mol. Cell. Biol.*, **2**, 11–20.
- Clarke,S., Vogel,J.P., Deschenes,R.J. and Stock,J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4643–4647.
- Deschenes,R.J., Stimmel,J.B., Clarke,S., Stock,J. and Broach,J. (1989) *J. Biol. Chem.*, **264**, 11865–11873.
- Farnsworth,C.C., Wolda,S.L., Gelb,M.H. and Glomset,J.A. (1989) *J. Biol. Chem.*, **264**, 20422–20429.
- Finegold,A.A., Schafer,W.R., Rine,J., Whiteway,M. and Tamanoi,F. (1990) *Science*, **249**, 165–169.
- Fujiyama,A. and Tamanoi,F. (1990) *J. Biol. Chem.*, **265**, 3362–3368.
- Fujiyama,A., Matsumoto,K. and Tamanoi,F. (1987) *EMBO J.*, **6**, 223–228.
- Gallwitz,D., Donath,C. and Sander,C. (1983) *Nature*, **306**, 704–707.
- Gibbs,J.B. and Marshall,M.S. (1989) *Microbiol. Rev.*, **53**, 171–185.
- Goodman,L.E., Perou,C.M., Fujiyama,A. and Tamanoi,F. (1988) *Yeast*, **4**, 271–281.
- Goodman,L.E., Judd,S.R., Farnsworth,C.C., Powers,S., Gelb,M.H., Glomset,J.A. and Tamanoi,F. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9665–9669.
- Gutierrez,L., Magee,A.I., Marshall,C.J. and Hancock,J.F. (1989) *EMBO J.*, **8**, 1093–1098.
- Hagen,D.C., McCaffrey,G. and Sprague,G.F., Jr (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1418–1422.
- Hancock,J.F., Magee,A.I., Childs,J.E. and Marshall,C.J. (1989) *Cell*, **57**, 1167–1177.
- Hrycyna,C.A. and Clarke,S. (1990) *Mol. Cell. Biol.*, **10**, 5071–5076.
- Ishibashi,Y., Sakagami,Y., Isogai,A. and Suzuki,A. (1984) *Biochemistry*, **23**, 1399–1404.
- Johnson,D.I. and Pringle,J.R. (1990) *J. Cell Biol.*, **111**, 143–152.
- Kataoka,T., Powers,S., McGill,C., Fasano,O., Strathern,J., Broach,J. and Wigler,M. (1984) *Cell*, **37**, 437–445.
- Koerner,T.J., Hill,J.E., Myers,A.M. and Tzagoloff,A. (1990) In Guthrie,C. and Fink,G.R. (eds), *Guide to Yeast Genetics and Molecular Biology*, Academic Press, Vol. 194, pp. 477–490.
- Kuchler,K., Sterne,R.E. and Thorner,J. (1989) *EMBO J.*, **8**, 3973–3984.
- Madaule,P., Axel,R. and Meyers,A.M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 779–783.
- Marr,R.S., Blair,L.C. and Thorner,J. (1990) *J. Biol. Chem.*, **265**, 20057–20060.
- McGrath,J.P. and Varshavsky,A. (1989) *Nature*, **340**, 400–404.
- Michaelis,S. and Herskowitz,I. (1988) *Mol. Cell. Biol.*, **8**, 1309–1318.
- Michaelis,S. and Powers,S. (1988) In Bradshaw,R.A., McAlister-Henn,L. and Douglas,M.G. *Molecular Biology of Intracellular Protein Sorting and Organelle Assembly*, Alan R.Liss, Inc., New York, NY, pp. 193–202.
- Murray,N.E., Brammar,W.J. and Murray,K. (1977) *Mol. Gen. Genet.*, **150**, 53–60.
- Nakayama,N., Miyajima,A. and Arai,K. (1985) *EMBO J.*, **4**, 2643–2648.
- 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., Santa-Anna,A.S., Field,J., Herskowitz,I., and Wigler,M. (1986) *Cell*, **47**, 413–422.
- Reiss,Y., Goldstein,J.L., Seabra,M.C., Casey,P.J. and Brown,M.S. (1990) *Cell*, **62**, 81–88.
- Rine,J.D. (1979) Regulation and Transposition of Cryptic Mating Type Genes in *Saccharomyces cerevisiae*. Ph.D. Thesis, University of Oregon, Eugene, OR.
- Rine,J. and Kim,S.-H. (1990) *New Biol.*, **2**, 219–226.
- Salminen,A. and Novick,P.J. (1987) *Cell*, **49**, 527–538.
- Sapperstein,S., Berkower,C. and Michaelis,S. (1989) Abstracts Cold Spring Harbor Yeast Meeting, Cold Spring Harbor, NY, p. 139.
- Schafer,W.R., O'Hara,M.B., Garsky,V.M., Mosser,S.D., Bergstrom,J.D., Moores,S.L., Marshall,M.S., Friedman,P.A., Dixon,R.A.F. and Gibbs,J.B. (1990) *J. Biol. Chem.*, **265**, 14701–14704.
- Schafer,W.R., Trueblood,C.E., Yang,C.-C., Mayer,M.P., Rosenberg,S., Poulter,C.D., Kim,S.-H. and Rine,J. (1990) *Science*, **249**, 1133–1139.
- Stephenson,R.C. and Clarke,S. (1990) *J. Biol. Chem.*, **265**, 16248–16254.
- Stimmel,J.B., Deschenes,R.J., Volker,C., Stock,J. and Clarke,S. (1990) *Biochemistry*, **29**, 9651–9659.
- Stock,J., Miller,R. and Volker,C. (1990) Abstract, Cold Spring Harbor RAS Meeting, Cold Spring Harbor, NY, p. 28.
- Strazdis,J.R. and MacKay,V.L. (1983) *Nature*, **305**, 543–545.
- Tamanoi,F., Hseuh,E.C., Goodman,L.E., Cobitz,A.R., Detrick,R.J., Brown,W.R. and Fujiyama,A. (1988) *J. Cell. Biochem.*, **36**, 261–273.
- Tatchell,K., Chaleff,D., DeFeo-Jones,D. and Scolnick,E.M. (1984) *Nature*, **309**, 523–527.
- 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.
- Whiteway,M., Hougan,L., Dignard,D., Thomas,D.Y., Bell,L., Saari,G.C., Grant,F.J., O'Hara,P. and MacKay,V.L. (1989) *Cell*, **56**, 467–477.
- Wilson,K.L. and Herskowitz,I. (1987) *Genetics*, **155**, 441–449.
- Yamane,H.K., Farnsworth,C.C., Xie,H., Howald,W., Fung,B.K.-K., Clarke,S., Gelb,M.H. and Glomset,J.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5868–5872.

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Labelling and immunoprecipitation of RAS proteins

Cells were grown to mid-logarithmic phase (OD_{600} 0.4–0.6) in SD medium supplemented with appropriate nutrients. For the double labelling experiment shown in Figure 4, 5 OD_{600} units of cells were harvested, washed once with 5 ml H_2O and then incubated in a total vol of 1.0 ml containing 150 μCi [3H -methyl]AdoMet, 150 μCi [^{35}S]cysteine (Amersham, >600 Ci/mmol), and 835 μl fresh growth media for 15 min at 30°C with constant shaking. Labelled cells were harvested by centrifugation at 13 000 g for 1 min, washed once with H_2O and resuspended in 50 μl of 1% SDS, 1 mM PMSF. Zirconium beads (0.2 g) were added and samples were heated at 100°C for 2 min. Cells were lysed by vortexing for 10 min at 4°C in 1 min bursts followed by heating at 100°C for 3 min. This cell extract was removed from the lysis beads and added to 45 μl 2 \times Laemmli sample buffer.

For immunoprecipitation, 25 μl of cell extract in sample buffer and 15 μl (1.5 μg) rat anti-pan-ras (Ab-1) monoclonal antibody Y13–259 (Oncogene Sciences Inc., Manhasset, NY) were added to 1.3 ml immunoprecipitation buffer. In control incubations, the monoclonal antibody was neutralized prior to addition of the antibody with a ten-fold excess (w/w) of a blocking peptide, peptide 1 (Oncogene Sciences Inc.), that corresponds to the binding region of the Y13–259. Immunoprecipitation was carried out as described for a-factor, except that the protein A–Sepharose CL-4B beads were pre-coated with rabbit antibodies against IgG since rat antibodies do not bind well to protein A. To coat the beads, an 800 μl aliquot of dry protein A–Sepharose CL-4B beads (Sigma) was swelled in 1.5 ml cold H_2O for 2 h, 160 μl rabbit anti-rat IgG heavy and light chain antibodies (Cappel Laboratories) were added and the beads were placed on a rotator at room temperature for 5 h. Beads were washed twice with 1.3 ml of immunoprecipitation buffer and resuspended in 1 ml of this buffer. An aliquot of the coated bead suspension (80 μl) was added to each immunoprecipitation reaction.

Pulse–chase labelling, fractionation and immunoprecipitation of RAS2

To analyze the kinetics of maturation and membrane localization of RAS2, cells were grown in SD medium containing necessary supplements to an OD_{600} of 0.4–0.6. Twenty OD_{600} units of cells were harvested and resuspended in 2.0 ml of the same medium. Pulse labelling was carried out by incubation of cells with 600 μCi [^{35}S]Translabel (ICN, >1000 Ci/mmol) for 2 min. The chase was initiated by addition of 40 μl chase mix (1.0 M L-cysteine, 1.0 M L-methionine) and was terminated after various lengths of time by removal of an 0.5 ml aliquot of cells into 0.5 ml ice cold azide stop mix. Cells were pelleted, resuspended in lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1 mM PMSF, 0.5% Trasylol) and disrupted at 4°C by vortexing 6 times in 1 min bursts in the presence of 0.25 g zirconium beads. This cell extract was adjusted to 1 ml with lysis buffer, removed from the lysis beads, and centrifuged for 10 min at 500 g to remove cellular debris. A 0.5 ml portion of this sample (designated the whole cell extract) was directly subjected to immunoprecipitation after addition of Triton X-100 and sodium deoxycholate to a final concentration of 1% each. The remaining 0.5 ml portion was separated into membrane and cytoplasmic fractions prior to immunoprecipitation.

For fractionation, lysed cellular material was centrifuged at 100 000 g for 1 h. The supernatant (cytoplasmic fraction) was transferred to a new tube and adjusted to a final concentration of 1% Triton X-100. The pellet (membrane fraction) was washed with 500 μl lysis buffer, resuspended in 1 ml of extraction buffer (1.0% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1 mM PMSF, 0.5% Trasylol) and sonicated for 15 s.

The total cell extract, membrane fraction and cytoplasmic fraction prepared as described above were subjected to immunoprecipitation. Prior to addition of antibody, insoluble material was removed by centrifugation at 13 600 g for 5 min, and the clarified sample transferred to a new tube. Ten microlitres of anti-pan-ras monoclonal antibody 259 (kindly provided by J.Gibbs) was added and samples were incubated at 4°C overnight. Immunoprecipitates were collected using coated beads as described above and resuspended in 30 μl 2 \times Laemmli sample buffer.

SDS–PAGE analysis, gel slice vapor phase equilibrium assay for methyl esters, and gel slice assay for total ^{35}S incorporation.

SDS–PAGE was performed using the buffer system described by Laemmli (1970). Electrophoresis of a-factor immunoprecipitates was carried out on 12.5% gels with pre-stained low molecular weight markers (Bethesda Research Laboratories) as standards. RAS samples were analyzed in 10% gels using low molecular weight standards (Biorad). Gels were fixed in 10% acetic acid for 15 min, rinsed in H_2O for 5 min, and soaked in 0.7 M sodium salicylate pH 7.0 for 15 min. Gels were dried under vacuum at

80°C and subjected to autoradiography at –80°C. For the experiment shown in Figure 4, gels were stained in 50% methanol, 10% acetic acid (wt/v), 0.1% Coomassie brilliant blue for 15 min and destained in 10% acetic acid, 5% methanol for 3 h, prior to drying and autoradiography.

Radiolabelled methyl esters were assayed by a modification of the method of Clarke et al. (1988). The lanes on the dried gels were cut into 0.3 cm slices and mixed with 200 μl 1 M NaOH in a polypropylene microcentrifuge tube. The tube was placed in a 20 ml scintillation vial containing 5 ml of scintillation fluid (ASCI, Amersham) and capped. After 24 h at 37°C, the microcentrifuge tube was removed and the radioactivity that had been transferred by diffusion to the scintillation fluid as [3H]methanol was assayed in a liquid scintillation counter. Total ^{35}S radioactivity incorporation was assayed by adding 1 ml PROTOSOL (New England Nuclear), a tissue solubilizer, to each of the microcentrifuge tubes containing the gel slices and allowing them to incubate for 6 h at 55°C. The tubes were opened and put directly into 10 ml of organic counting scintillation fluid (OCS, Amersham) with 100 μl of glacial acetic acid and subsequently counted in a liquid scintillation counter.

Physiological assays

Bioassay of a-factor: To compare the activity of extracellular a-factor produced by wild-type and *ste14* cells, a-factor was collected from the culture fluid of strains SM1058 (pSM219) and SM1188 (pSM219) using polystyrene beads (Strazdis and MacKay, 1983) and assayed by the spot halo method described previously (Michaelis and Herskowitz, 1988). Briefly, cells were grown to saturation in one liter of appropriately supplemented SD medium containing 50 g of Amberlite XAD-2 polystyrene beads (Sigma) to which a-factor quantitatively adsorbs. After discarding the culture, beads were washed several times with water and a-factor was eluted from the beads with n-propanol and reduced to 5 ml. This concentrated a-factor was serially diluted into 200 $\mu g/ml$ bovine serum albumin and assayed by spotting 3 μl aliquots onto a lawn of the supersensitive strain RC757. For the wild-type strain, a zone of clearing was seen up to the 1000-fold dilution. By contrast, no a-factor activity was observed for the *ste14* culture fluid, even using the undiluted material. Since the culture fluid from this *ste14* mutant contains only 20% as much a-factor as the wild-type culture fluid as determined by immunoprecipitation and densitometry, the non-methylated a-factor secreted by the *ste14* mutant appears to be at least 200-fold less active than authentic methylated a-factor.

Heat shock sensitivity assay: Heat shock sensitivity was performed essentially as described (Broek et al., 1987). Cells were patched onto YEPD plates and grown 36 h at 30°C. Patch plates were replica plated onto a YEPD plate that was immediately set into a shallow water bath at 53°C. After incubation for 30 min, plates were transferred to a 30°C incubator. After one day, wild-type RAS2 cells formed a confluent patch whereas no growth was observed for *ras2^{val19}* derivatives. By 2 days, 2–15 papillae per patch were observed for the latter strains.

Starch accumulation assay: To test starch accumulation, cell patches on YEPD were grown for 2 days. Plates were inverted over iodine crystals in a closed container for 10 min and color was observed. The RAS2 wild-type strain stained brown, whereas *ras2^{val19}* mutants did not stain brown, but turned a bright yellow color.

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References

- Akada,R., Minomi,K., Kai,J., Yamashita,I., Miyakawa,T. and Fukui,S. (1989) *Mol. Cell. Biol.*, **9**, 3491–3498.
- Anderegg,R.J., Betz,R., Carr,S.A., Crabb,J.W. and Duntze,W. (1988) *J. Biol. Chem.*, **263**, 18236–18240.
- Bender,A. and Pringle,J.R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9976–9980.
- Blair,L.C. (1979) Genetic Analysis of Mating Type Switching in Yeast. Ph.D. Thesis, University of Oregon, Eugene, OR.
- Brake,A.J., Brenner,C., Najarian,R., Laybourn,P. and Merryweather,J. (1985) In Gething,M.-J. (ed.), *Current Communications in Molecular*