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Genes Encoding Farnesyl Cysteine Carboxyl Methyltransferase in *Schizosaccharomyces pombe* and *Xenopus laevis*

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The *mam4* mutation of *Schizosaccharomyces pombe* causes mating deficiency in h^- cells but not in h^+ cells. h^- cells defective in *mam4* do not secrete active mating pheromone M-factor. We cloned *mam4* by complementation. The *mam4* gene encodes a protein of 236 amino acids, with several potential membrane-spanning domains, which is 44% identical with farnesyl cysteine carboxyl methyltransferase encoded by *STE14* and required for the modification of a-factor in *Saccharomyces cerevisiae*. Analysis of membrane fractions revealed that *mam4* is responsible for the methyltransferase activity in *S. pombe*. Cells defective in *mam4* produced farnesylated but unmethylated cysteine and small peptides but no intact M-factor. These observations strongly suggest that the *mam4* gene product is farnesyl cysteine carboxyl methyltransferase that modifies M-factor. Furthermore, transcomplementation of *S. pombe mam4* allowed us to isolate an apparent homolog of *mam4* from *Xenopus laevis* (*Xmam4*). In addition to its sequence similarity to *S. pombe mam4*, the product of *Xmam4* was shown to have a farnesyl cysteine carboxyl methyltransferase activity in *S. pombe* cells. The isolation of a vertebrate gene encoding farnesyl cysteine carboxyl methyltransferase opens the way to in-depth studies of the role of methylation in a large body of proteins, including Ras superfamily proteins.

A number of proteins, including Ras superfamily GTP-binding proteins, are processed from precursors carrying a CAAX motif at their C termini. C, A, and X in the motif represent, respectively, cysteine, an aliphatic amino acid, and an unspecified amino acid. The precursors undergo three steps of posttranslational modifications at this motif to generate mature proteins. First, the cysteine residue is prenylated, i.e., either farnesylated or geranylgeranylated, and this is followed by removal of the C-terminal three residues, AAX. The new C-terminal residue, prenylated cysteine, is then methylesterified. These modifications are thought to be important to localize the proteins to the membrane (reviewed in references 11, 57, and 60).

Enzymes that catalyze prenylation are better characterized than ones involved in the other two steps. Genes encoding prenyltransferase have been cloned from the yeast *Saccharomyces cerevisiae* and mammals. The *S. cerevisiae RAM1*/*DPR1* and *RAM2* genes, which are essential for the modification of both Ras proteins and **a**-factor, encode the two subunits of farnesyltransferase. The product of *RAM1*/*DPR1* corresponds to the β subunit of the mammalian enzyme, whereas that of *RAM2* corresponds to the α subunit (7, 22, 25, 35, 58). The *S. cerevisiae CDC43*/*CAL1* gene encodes a subunit of type I geranylgeranyltransferase (46, 50), which is homologous to the β subunit of the corresponding enzyme in mammals (71). The *RAM2* gene product serves also as the α subunit of *S. cerevisiae* geranylgeranyltransferase (46, 71). *S. cerevisiae* and mammalian genes encoding subunits of type II geranylgeranyltransferase, which modifies proteins carrying CC or CXC at the C terminus, have also been identified (3, 40, 54).

The protease that removes the C-terminal AAX has not been conclusively identified. In *S. cerevisiae*, two types of activities were detected as candidates that were involved in physiological processing of isoprenylated protein precursors; one of these was membrane bound, and the other was cytoplasmic (27). In mammals, a microsomal endopeptidase activity that cleaves tetrapeptides containing farnesylated cysteine has been identified (42). More recently, a peptidase that carries this activity was partially purified from a microsomal fraction of pig brain, and its similarity to a thiol-dependent zinc metallopeptidase was noted (1). No cloning of the gene for this enzyme has been reported.

A membrane-associated activity that methylesterifies prenylcysteine has been detected in *S. cerevisiae* and mammals (26, 64), and a single activity appears to methylesterify both farnesyl cysteine and geranylgeranyl cysteine (28, 66). Carboxylmethylation is reversible under physiological conditions (52), raising the interesting possibility that methylation may regulate a biological activity of the target proteins. Indeed, a link between receptor-mediated signal transduction and carboxylmethylation of Ras superfamily proteins has been suggested to exist in human neutrophils (53), and carboxylmethylation of Rap1, a member of the family, has been demonstrated in regulated insulin secretion (38). Prenyl cysteine carboxyl methyltransferase is encoded by *STE14* in *S. cerevisiae* (4, 26, 28, 44, 56), but no counterpart of this gene has been identified in other organisms.

The fission yeast *Schizosaccharomyces pombe* has two mating types, termed h^+ and h^-. Heterothallic strains are fixed in either h^+ or h^-, whereas cells of homothallic strains, denoted h^{90}, change their mating type between h^+ and h^- during growth. Thus, a colony of a homothallic strain is a mixture of h^+ and h^- cells, which can perform mating followed by meiosis and sporulation within it (48a). The mating pheromones of *S.*

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TABLE 1. *S. pombe* strains used in this study

Strain	Genotype
JY1	*h*$^-$, wild type
JY334	*h*$^+$ *ade6-M216 leu1*
JY450	*h*^{90} *ade6-M216 leu1*
JY878	*h*^{90} *ade6-M216 leu1 ura4-D18*
JZ340	*h*^{90} *mam4-109 ade6-M210 leu1*
JZ653	*h*^{90} *mam4*::*ura4*$^+$ *ade6-M216 leu1 ura4-D18*
JZ745	*h*$^+$ *mam4*::*ura4*$^+$ *ade6-M216 leu1 ura4-D18*
JZ866	*h*$^-$ *mam4*::*ura4*$^+$
JX433	*h*^{90}/*h*^{90} *mam4*::*ura4*$^+$/*mam4*::*ura4*$^+$ *map2*::*ura4*$^+$/*map2*::*ura4*$^+$ *ade6-M210*/*ade6-M216 leu1*/*leu1 ura4-D18*/*ura4-D18*

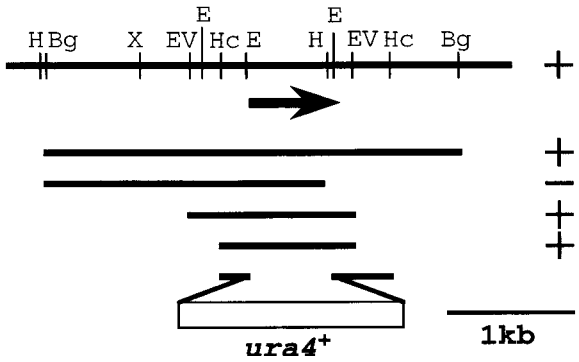

FIG. 1. Restriction map and subcloning analysis of the *mam4* gene. A restriction map of the insert of pST109-1, an original clone that could complement *mam4*, is shown at the top. The arrow represents the assigned direction and extent of the *mam4* ORF. Restriction sites: Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; and X, *Xba*I. The insert was digested with various restriction enzymes and subcloned into pDB248′. The ability of each clone to complement *mam4* is shown by + or −. The structure of a linear plasmid used for in vivo disruption of the *mam4* gene is illustrated at the bottom.

pombe, namely, P-factor secreted by *h*$^+$ cells and M-factor secreted by *h*$^-$ cells, mediate cell-cell interaction prior to mating. While P-factor is a peptide of 23 amino acids that does not appear to be modified (30), M-factor is a nonapeptide whose C-terminal cysteine residue is both farnesylated and carboxylmethylated (13) and hence resembles *S. cerevisiae* **a**-factor (2). It is likely, therefore, that *S. pombe* has farnesyltransferase and methyltransferase similar to those which modify **a**-factor in *S. cerevisiae*. Indeed, multiple farnesyltransferase activities have been detected in an *S. pombe* cell extract (12). Interestingly, the pheromone signaling is required not only for mating of haploid cells but also for sporulation of diploid cells in *S. pombe*. A number of genetic and physiological observations have contributed to establish the necessity of the pheromone signaling for *S. pombe* meiosis (15, 33, 39, 49, 65, 67), as summarized in recent reviews (48a, 70). M-factor signaling in *h*$^+$ cells and P-factor signaling in *h*$^-$ cells activate the same mitogen-activated protein kinase cascade, and either M-factor signaling or P-factor signaling alone is sufficient to promote meiosis and sporulation in diploid cells (70).

Assuming that a defect in the gene encoding either farnesyltransferase or methyltransferase may result in the production of inactive M-factor and hence cause sterility in *h*$^-$ but not in *h*$^+$ cells, we set out to analyze *h*$^-$ specific sterile mutants of *S. pombe*. So far, four *h*$^-$ specific sterility genes, named *mam1* to *mam4*, have been identified (15, 31). The *mam1* gene encodes a peptide transporter responsible for the export of M-factor (9), *mam2* encodes the P-factor receptor (33), and *mam3* codes for an agglutinin (31). The *mam4* gene was of special interest in the current analysis, as the *mam4* mutant appeared to be defective in the production of active M-factor. Because M-factor is encoded by the *mfm1*, *mfm2*, and *mfm3* genes (13, 34), the *mam4* gene product was presumed to be involved in either processing, modification, or secretion of M-factor. Here we show that the *mam4* gene encodes a farnesyl cysteine carboxyl methyltransferase similar to *S. cerevisiae* STE14. Furthermore, a cDNA clone encoding a protein highly similar to the microbial enzymes was isolated from *Xenopus laevis* by transcomplementation of the *S. pombe mam4* mutant, suggesting that farnesyl cysteine carboxyl methyltransferase is conserved from lower eukaryotes to vertebrates.

MATERIALS AND METHODS

Strains, genetic procedures, and media. The *S. pombe* strains used in this study are listed in Table 1. General genetic procedures were as described by Gutz et al. (24). Sterile mutants were crossed by protoplast fusion (61). A lithium method was used for transformation of *S. pombe* (51). The synthetic minimal media SD (59) and PM (6) and the synthetic sporulation medium SSA (16) were used.

Identification of M-factor-related peptides produced by the *mam4* mutants. Material related to M-factor was prepared from the growth media of the *mam4* mutants by methods similar to those previously described for the purification of native M-factor (13). Briefly, material was adsorbed directly from culture medium to an Amberlite XAD-2 column (Sigma, Poole, United Kingdom) and eluted with propan-1-ol. The eluate was analyzed by chromatography in methanol on Sephadex LH-60 (Sigma), and fractions equivalent to those that would be expected to contain active M-factor were further purified by reversed-phase high-pressure liquid chromatography (HPLC) on a Spherisorb ODS2 column (Phase-Sep, Deeside, Clywd, United Kingdom).

Amino acid analysis was performed on the HPLC-purified material with an Applied Biosystems model 473 A gas phase sequenator after adsorption to a Biobrene glass filter, and mass spectrometry was performed with a Kratos Kompact MALDI 3 mass spectrometer.

Cloning of the *mam4* gene. JZ340 (*h*^{90} *mam4-109*) was transformed with an *S. pombe* genomic library, which was constructed by inserting *Sau*3AI partial digests of the genomic DNA into the *Bam*HI site of the high-copy-number vector pDB248′ (5). Transformants grown on SSA plates were stained with iodine vapor, and colonies stained dark brown were selected. Microscopic inspection confirmed recovery of successful mating in these colonies. Plasmid DNA was rescued by transforming *Escherichia coli* HB101 with crude DNA preparations of *S. pombe* transformants. Plasmids that could retransform JZ340 to mating proficiency were studied further.

DNA sequencing. The DNA sequences of the *mam4* gene and its *X. laevis* homolog (*Xmam4*) were determined either by using a Sequenase kit (U.S. Biochemical Corp.) and [α-^{32}P]dCTP or by using a SequiTherm Long-Read cycle sequencing kit (Epicentre Technologies) and an automated DNA sequencer (LI-COR model 4000L). The sequences shown in Fig. 2 were determined in both orientations.

Gene disruption. A 0.7-kb *Eco*RI fragment was eliminated from the cloned *mam4* gene, and a 1.8-kb *S. pombe ura4*$^+$ cassette (23) was inserted in its place (Fig. 1). An *S. pombe* homothallic strain (JY878) was transformed with a linear *Hin*cII fragment which contained the *mam4* disruption construct. Most of the stable Ura$^+$ transformants were sterile, and the precise replacement of the *mam4* locus in them was confirmed by Southern blotting (62).

Construction of a *Xenopus* oocyte cDNA library and cloning of genes that transcomplement *mam4*. *X. laevis* oocytes were treated with 10 μg of progesterone per ml for 7 h. Total RNA was extracted from them by the acid guanidinium thiocyanate-phenol-chloroform method (8). Poly(A) RNA was separated with Oligotex-dT30 ⟨super⟩ (Nippon Roche), and cDNA was synthesized with the Bethesda Research Laboratories (BRL) SuperScript lambda system, according to the manufacturer's recommendations in both cases. The *Sma*I and the *Bal*I sites of the *S. pombe* expression vector pREP3 (45) were changed to *Not*I and *Xho*I sites, respectively, by the insertion of linkers. The modified vector was digested with *Xho*I and *Not*I, and the cDNA fragments, which carried a *Sal*I site at the 5′ end and a *Not*I site at the 3′ end, were inserted into it. Plasmids were recovered into *E. coli* HB101 by electroporation. The cDNA library thus constructed contained about 720,000 independent clones, with approximate insert lengths of 0.4 to 4.0 kb. The diploid *S. pombe* strain JX433 (*h*^{90}/*h*^{90} *map2*/*map2 mam4*/*mam4*) was transformed with the *Xenopus* cDNA library, and Leu$^+$ Spo$^+$ transformants were selected as explained in Results. cDNA clones recovered from these transformants were then tested for the ability to complement the sterility of JZ653 (*h*^{90} *mam4*).

5′ RACE. The 5′ rapid amplification of cDNA ends (RACE) procedure was essentially as described by Edwards et al. (14). cDNA was synthesized from primer 5′-TGACTGTGTGATGCTCAGCAGCAAAC-3′, which corresponds to nucleotides 251 to 276 of *Xmam4* (see Fig. 2), by using SuperScript RNase H$^-$ reverse transcriptase (BRL) and 2 μg of *Xenopus* poly(A) RNA as a template.

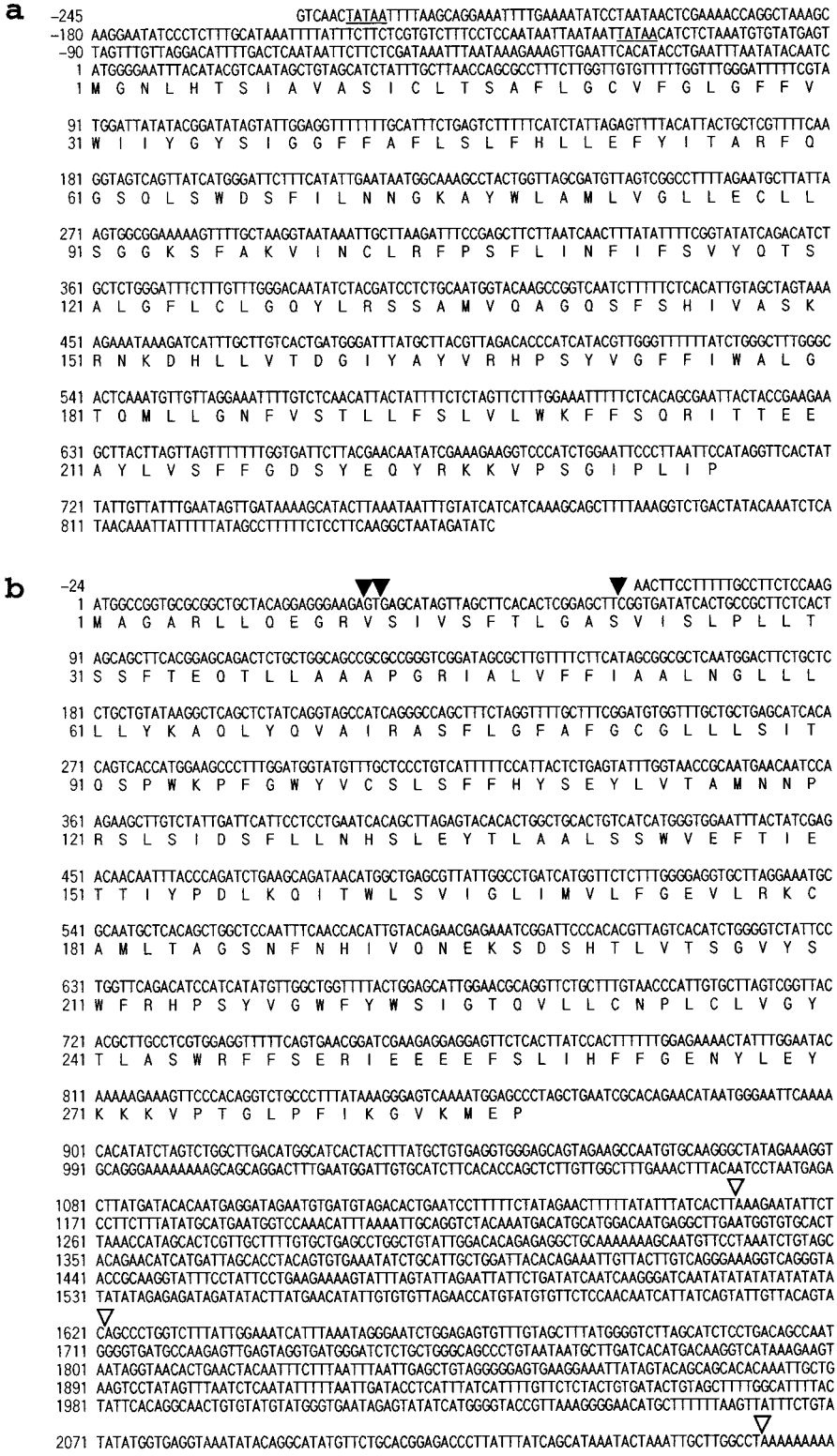

FIG. 2. Nucleotide and deduced amino acid sequences of the *mam4* gene and of the *Xmam4* cDNA. (a) DNA sequence of a 1.1-kb *Hin*cII-*Eco*RV fragment carrying the *mam4* gene together with the predicted amino acid sequence. Numbering starts at the first methionine codon of the *mam4* ORF. Possible TATA boxes are underlined. (b) Assembled nucleotide sequence of the *Xmam4* cDNA and the predicted amino acid sequence. Results obtained with the cDNA clone pXM3a and 5′ RACE analysis were combined. Numbering starts at the assumed initiation codon. The 5′ ends and poly(A) addition sites found in the cDNA clones pXM1c, pXM2a, and pXM3a are shown by closed and open arrowheads, respectively. These clones contained nucleotides 65 to 1158, 34 to 1621, and 36 to 2151, respectively.

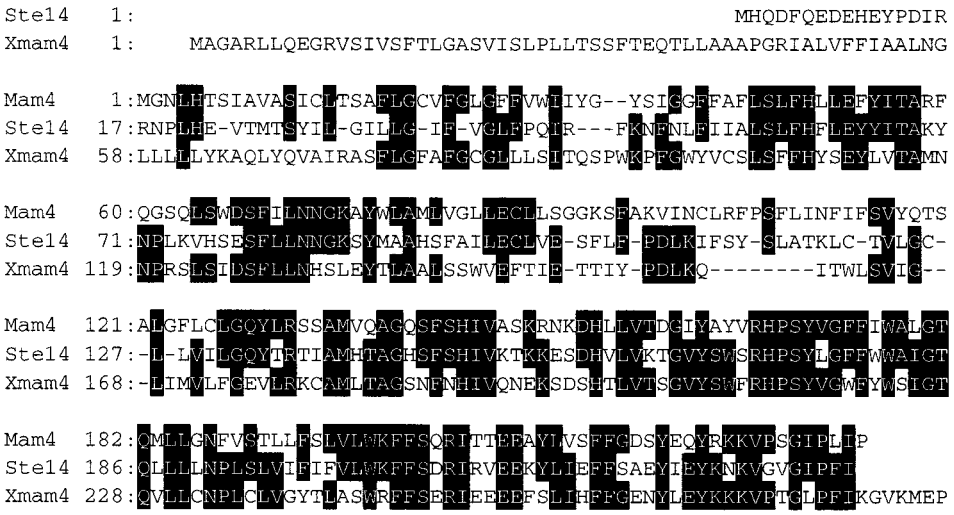

FIG. 3. Comparison of the amino acid sequences of the gene products of *S. pombe mam4*, *S. cerevisiae STE14*, and *X. laevis Xmam4*. Identical amino acids are shown in white against black.

Residual primer molecules were removed by using Tip5 (Qiagen), and RNA was hydrolyzed with 0.2 N NaOH at 65°C for 60 min. The anchor oligonucleotide 5′-pCACGAATTCACTATCGATTCTGGAACCTTCAGAGG-3′ was tailed with ddATP by using terminal deoxynucleotidyl transferase (BRL) and ligated to the cDNA with T4 RNA ligase (New England Biolabs). The products were used as the template for PCR. PCR was performed with primer 5′-CTGTCGACCA CCTCTGAAGGTTCCAGAATCGATAG-3′, which anneals with the anchor oligonucleotide, and primer 5′-GGGGATCCGATGGCTACCTGATAGAGC-3′, which anneals with nucleotides 198 to 216 of *Xmam4*, using 2.5 U of *Pfu* DNA polymerase (Stratagene) in the buffer recommended by the supplier. After 30 cycles of amplification, each of which consisted of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 45 s at 72°C, 2 μl of the product was subjected to another round of amplification (25 cycles) under the same conditions. The final PCR product was phosphorylated with T4 polynucleotide kinase (Takara Shuzo), blunt ended with Klenow fragment (Takara Shuzo), separated by 3% agarose gel electrophoresis, and cloned into the vector pBluescript KS(+).

Northern blot analysis. Six micrograms of *Xenopus* poly(A) RNA isolated as described above was electrophoresed on a 1.2% agarose gel containing 3% (vol/vol) formaldehyde. The RNA was transferred to Hybond-N (Amersham) and hybridized with ^{32}P-labelled DNA probes.

Plasmids. pART1 and pREP3 are *S. pombe* expression vectors carrying the *adh1* promoter and the *nmt1* promoter, respectively (45, 47). pART1-*mam4* and pREP3-*mam4* were constructed by inserting a 1.6-kb *Eco*RI-*Bgl*II fragment, which covers the entire *mam4* open reading frame (ORF), into the multicloning site of each vector. pREP3-*Xmam4* was constructed by inserting the entire *Xmam4* ORF (nucleotides −24 to 896 in Fig. 2) into the *Bal*I site of pREP3.

Isolation of *S. pombe* membrane fractions. *S. pombe* cells were grown to late log phase (1×10^7 to 2×10^7 cells/ml) in SD or PM medium, which was supplemented with 50 μg of adenine per ml and/or 50 μg of leucine per ml when appropriate. PM medium was used for strains carrying plasmids regulated by the *nmt1* promoter. Cells were collected by centrifugation and washed with H_2O. All of the following procedures were performed at 4°C. Three volumes of 0.3 M sorbitol–0.1 M NaCl–5 mM EDTA–5 mM Tris-HCl (pH 7.5)–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride and 4 volumes of glass beads were added to the pellet. Cells were broken by vigorous vortexing for 5 min, and extracts were briefly centrifuged in a microcentrifuge to remove cell debris. The supernatant was then centrifuged at 100,000 × *g* for 120 min, and the pellet was resuspended in 1 ml of cold glycerol buffer (20% glycerol, 10 mM Tris-HCl [pH 7.5]). Membranes were further purified by sucrose density gradient centrifugation as described previously (26), and the purified membranes were suspended in sorbitol buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride). Determination of protein concentrations was done by the procedure of Lowry et al. (41).

Synthetic peptide and C-terminal methyltransferase assay. Methyltransferase activity was assayed essentially as described by Hrycyna and Clarke (26), by using the farnesylated synthetic peptide Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys(*S-trans,trans*-farnesyl)OH described by Xue et al. (68) and *S*-adenosyl-L-[^{14}C-*methyl*]methionine.

Nucleotide sequence accession numbers. The nucleotide sequence data shown in Fig. 2 have been submitted to the GenBank/EMBL/DDBJ nucleotide sequence databases under accession numbers D87749 (*mam4*) and D87750 (*Xmam4*).

RESULTS

The *mam4* mutant. We previously screened mutagenized *S. pombe* cells for sterile mutants (29, 65). Among mating-deficient strains obtained in this screen, three independent clones were assigned to one genetic locus, named *mam4*, by linkage analysis. The original *mam4* isolates were homothallic (h^{90}). We constructed their heterothallic derivatives and found that h^- *mam4* cells were still sterile but h^+ *mam4* cells became fully competent in mating, indicating that a defect in *mam4* causes sterility in an h^--specific manner. We then tested *mam4* mutants for their ability to secrete mating pheromones by the physiological assay previously described (19). They were judged not to secrete active M-factor (see below). Diploid strains homozygous for *mam4* sporulated only one-sixth as efficiently as the wild-type strain on synthetic sporulation medium, which was consistent with the previous observation that a diploid strain unable to produce M-factor can sporulate, but poorly (17).

Cloning and disruption of the *mam4* gene. A *mam4* mutant, JZ340 (h^{90}), was transformed with an *S. pombe* genomic library constructed in the high-copy-number vector pDB248′ (5). Six clones with overlapping inserts complemented the mating defect of JZ340, and one of them, named pST109-1, was analyzed further. This clone carried a 4.1-kb-long insert, and subcloning analysis showed that a 1.1-kb *Hin*cII-*Eco*RV fragment was sufficient to complement *mam4* (Fig. 1). DNA sequence analysis of this fragment revealed an uninterrupted ORF with a coding capacity for 236 amino acids (Fig. 2a).

One-step disruption (55) of this ORF was done by using a homothallic haploid strain as the host, as described in Materials and Methods and Fig. 1. The resultant strains showed phenotypes indistinguishable from those of the original *mam4* mutants. The disruptant was crossed with the original *mam4* mutant by protoplast fusion, and their progeny spores were analyzed. No mating-proficient segregant appeared in 14 tetrads and about 400 random spores analyzed. This tight linkage strongly suggests that the disrupted gene is indeed *mam4.*

Northern blot analysis revealed that the *mam4* gene was transcribed into three mRNAs of about 1.0, 1.2, and 1.4 kb in length in both h^+ and h^- cells, and transcription of *mam4* was not significantly affected by either nutrients or the pheromone

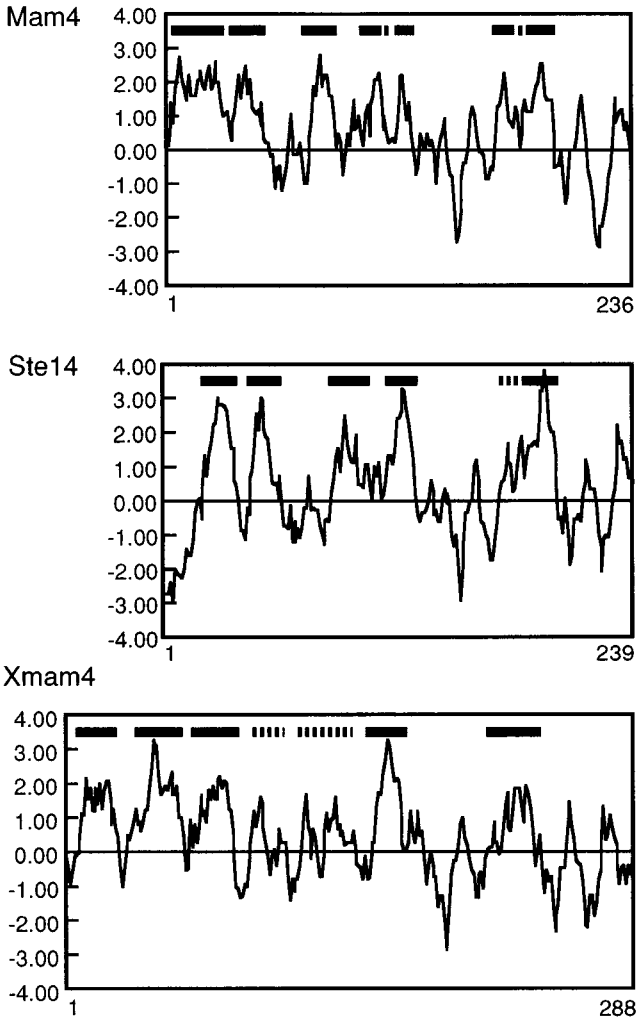

FIG. 4. Hydropathy analysis of Mam4 and Xmam4. Hydropathy was calculated for each gene product as described by Kyte and Doolittle (37), with a window of 8 amino acids. Hydrophobic regions that are likely to span the membrane are denoted by bars. Dotted bars show less hydrophobic regions, which may also span the membrane. For comparison, the hydropathy of *S. cerevisiae* Ste14 was calculated in the same manner, according to the data of Ashby et al. (4) and Sapperstein et al. (56).

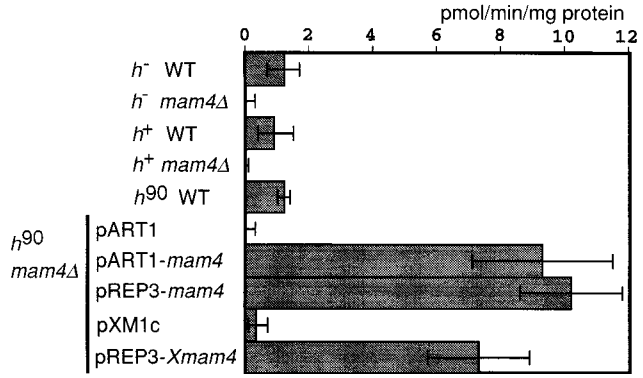

FIG. 5. Methyltransferase activity in various *S. pombe* strains carrying *mam4* or *Xmam4*. Membrane fractions were prepared from *S. pombe* cells. Each sample contained 10 μg of protein, 1 nmol of farnesylated substrate peptide, and 1 nmol of *S*-adenosyl-L-[^{14}C-*methyl*]methionine and was incubated at 37°C for 30 min. Specific activities were determined by subtracting activities determined in control experiments lacking the acceptor peptide. Averages from two to six experiments are shown with standard deviations. The *S. pombe* strains were JY1 (h^-, wild type [WT]), JZ866 (h^- *mam4*Δ), JY334 (h^+, wild type), JZ745 (h^+ *mam4*Δ), JY450 (h^{90} wild type), and JZ653 (h^{90} *mam4*Δ). pART1 is an *S. pombe* expression vector (47). pART1-*mam4* expresses the *mam4* gene under the control of the *adh1* promoter. pREP3 is another expression vector (45), and pREP3-*mam4* expresses the *mam4* gene from the *nmt1* promoter. pXM1c is also pREP3 based and expresses the truncated *Xmam4* gene, whereas pREP3-*Xmam4* expresses the full-length *Xmam4* gene.

signal (data not shown). This contrasts with the fact that transcription of many other mating-type-specific sterility genes is induced by nitrogen starvation and/or the pheromone signal (30, 33, 65, 69).

Mam4 is a hydrophobic protein homologous to *S. cerevisiae* STE14. The predicted *mam4* gene product is homologous to the *S. cerevisiae STE14* gene product (44.0% amino acid identity) (Fig. 3), which is a farnesyl cysteine carboxyl methyltransferase responsible for the modification of Ras proteins and the mating pheromone **a**-factor (4, 26, 28, 44, 56). The *STE14* gene product is believed to contain five or six membrane-spanning domains (4, 56), and the predicted *mam4* gene product is also highly hydrophobic and appears to contain five to seven putative membrane-spanning domains (Fig. 4).

Mam4-dependent membrane-associated methyltransferase activity. Membrane fractions were prepared from various *S. pombe* strains, and their carboxyl methyltransferase activities were assayed by using a synthetic peptide carrying C-terminal farnesyl cysteine as the substrate. The substrate was identical to *S. cerevisiae* mature **a**-factor except that it was not methylesterified. The membrane fractions prepared from *S. pombe* wild-type strains contained a methyltransferase activity, the level of which was relatively constant irrespective of the mating type (Fig. 5). The membrane fractions prepared from *mam4*-disrupted strains showed no detectable methyltransferase activity, whereas the fraction prepared from a *mam4*-overexpressing strain showed an enzymatic activity about eight times higher than that of the wild type (Fig. 5). These observations strongly suggest that the *mam4* gene encodes farnesyl cysteine methyltransferase that modifies M-factor in *S. pombe.*

M-factor-related products secreted by the *mam4* mutant. We tried to identify M-factor-related peptides produced by the *mam4* mutant. The *S. cerevisiae ste14* mutant generates a farnesylated but unmethylated heptapeptide that corresponds to the C terminus of **a**-factor (44, 56). Peptides secreted into the medium by the *mam4* mutant were recovered by essentially the same method as used previously to isolate the intact M-factor (13). They were analyzed by mass spectroscopy and amino acid sequencing. The major product detected by mass spectroscopy had a mass of 326.2 Da, which corresponds to farnesylated but nonmethylated cysteine. Amino acid sequencing of a fraction separated by HPLC revealed a Tyr-Met peptide with an unresolvable C-terminal residue. We assume that this peptide is most likely to be the unmethylated tripeptide Tyr–Met–Cys–S-farnesyl, which corresponds to the C terminus of M-factor, because farnesylated cysteine is not resolved in the conventional sequence analysis. A minor peak that apparently corresponds to this tripeptide (mass of about 650 Da) was detected by mass spectroscopy (data not shown). We could not detect any larger M-factor-related peptide in either the culture medium or the cell extract.

Cloning of *Xenopus* cDNAs that transcomplement *S. pombe mam4.* Although farnesyl cysteine methyltransferase activity has been demonstrated in higher eukaryotes, genes coding for these enzymes have not been identified. We thus set out to screen for *Xenopus* genes that could functionally complement

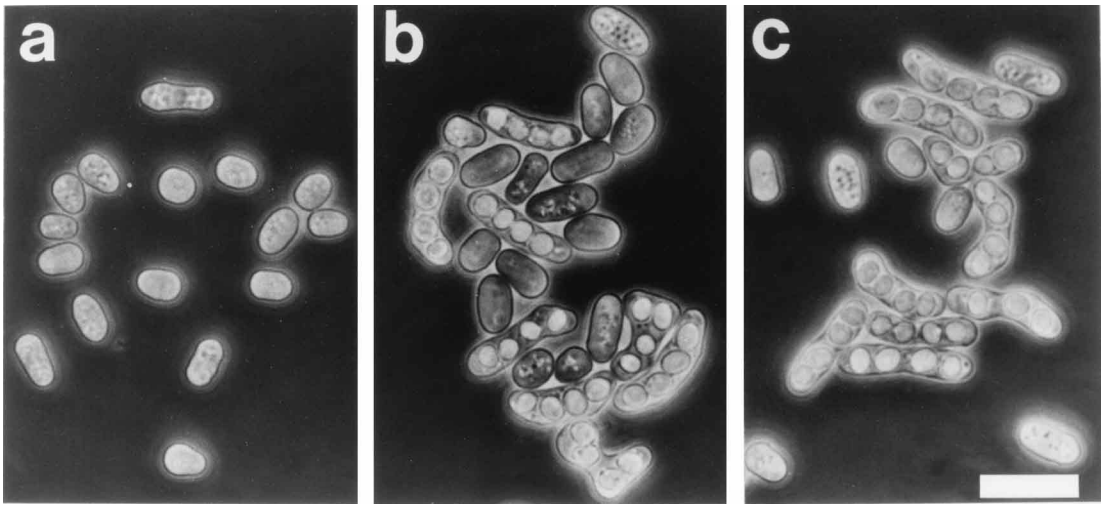

FIG. 6. Complementation of the *mam4* mutation by the *mam4* or *Xmam4* gene. *mam4* (JZ653) cells were transformed with the vector pREP3 (a); pREP3-*mam4*, which expresses the *mam4* gene (b); or pXM1c, which expresses the truncated *Xmam4* gene (c). The transformants were grown on the synthetic sporulation medium SSA for 3 days and photographed under phase-contrast microscopy. Bar, 10 μm.

the *mam4* deficiency in *S. pombe*. Our screen exploited the observations that the pheromone signal not only is required for haploid cells to mate but also is required for diploid cells to undergo sporulation (15, 33, 39, 49, 65). This is because the pheromone signal is required for transcription of *mat1-Pi(m)* and *mat1-Mi(m)*, coexpression of which then enables expression of *mei3*, whose gene product is a critical factor for the initiation of meiosis (48a, 67, 70). Signaling induced by either P-factor or M-factor alone is sufficient to activate meiosis in diploid cells (15, 33, 39, 49, 65). The host we used for the screen was a diploid strain homozygous for both *mam4*$^-$ and *map2*$^-$ (JX433), and the *map2* gene encodes P-factor (30). This strain cannot sporulate because it produces neither M-factor nor P-factor, due to the *mam4* and the *map2* mutations. We transformed JX433 with a *Xenopus* oocyte cDNA library and isolated plasmid clones that apparently complemented its sporulation deficiency. These *Xenopus* clones were expected either (i) to complement *mam4* or *map2* functionally and allow the strain to produce M-factor or P-factor or (ii) to activate the pheromone signaling pathway of *S. pombe* fortuitously. To further select *Xenopus* counterparts of *mam4*, we examined these clones for their ability to convert a homothallic haploid strain defective in *mam4* (JZ653) to mating proficiency. Of 100,000 JX433 cells originally transformed, we eventually recovered three cDNA clones that were able to complement the sterility of JZ653 and named them pXM1c, pXM2a, and pXM3a (Fig. 6).

The insert in pXM1c was 1.1 kb in length, that in pXM2a was 1.6 kb, and that in pXM3a was 2.1 kb. DNA sequence analysis suggested that these three *Xenopus* cDNA clones derived from a single gene, with their poly(A) addition sites being different from each other (Fig. 2b and 7a). Although these clones apparently gave rise to functional proteins in *S. pombe*, their nucleotide sequences all appeared to lack an authentic initiation codon (Fig. 2b). We therefore performed 5′ RACE to obtain the missing 5′ region, as detailed in Materials and Methods. A DNA fragment obtained by this method carried an ATG that was in frame with the predicted ORF and had a flanking sequence (AAGATGG) that was consistent with it functioning as an initiation codon (36). Furthermore, the

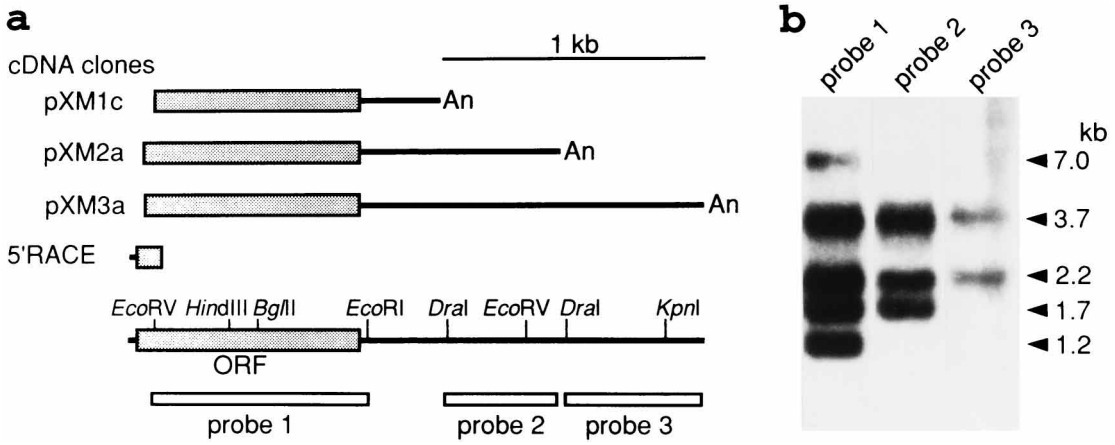

FIG. 7. Structures of *Xmam4* cDNA clones and Northern blot analysis. (a) The structures of cDNA inserts in the clones pXM1c, pXM2a, and pXM3a and the cDNA obtained by 5′ RACE are shown with a restriction map. Stippled boxes represent the *Xmam4* ORF. (b) Northern blot analysis. Six micrograms of poly(A) RNA prepared from *Xenopus* oocytes was separated on a formaldehyde agarose gel and hybridized with the probes shown in panel a.

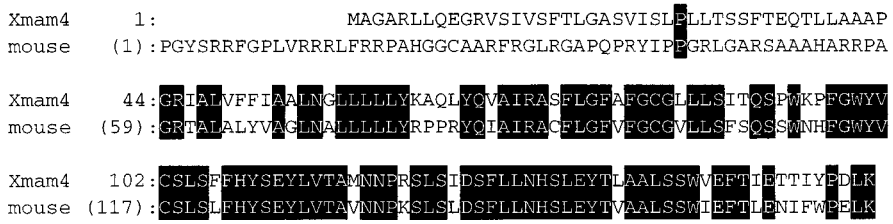

FIG. 8. Comparison of the amino acid sequences of Xmam4 and a mouse cDNA clone. Amino acids 1 to 158 of Xmam4 and the possible translation product of a mouse cDNA identified in the Expressed Sequence Tags project (accession number AA022288) are aligned. Identical amino acids are shown in white against black.

summed length of each cDNA clone appeared to match the length of the corresponding mRNA species detected in Northern analysis (see below). Based on these observations, we conclude that the ATG codon identified in the 5′ RACE is likely to be the initiation site for translation, although we have been unable to find a stop codon upstream of it (Fig. 2b). The assembled cDNA sequence (Fig. 2b) indicates that this gene, which we named *Xmam4*, could encode a protein of 288 amino acids (Fig. 2b). A plasmid carrying the assembled *Xmam4* cDNA sequence could complement the *S. pombe mam4*-disrupted strain efficiently (data not shown). The deduced *Xmam4* product is 35.9% identical with Mam4 and 41.9% identical with Ste14 (Fig. 3). It is larger than Mam4 and Ste14, mainly due to its long N terminus, and appears to contain at least five putative membrane-spanning domains (Fig. 4). There may be extra membrane-spanning domains in the N terminus, compared to those in its yeast homologs (Fig. 4).

Expression of *Xmam4* in *Xenopus* oocytes. Northern blot analysis with most of the *Xmam4* ORF as a probe (probe 1) detected five transcripts of 7.0, 3.7, 2.2, 1.7, and 1.2 kb in length, in the extract of *Xenopus* oocytes (Fig. 7). A probe corresponding to the most 3′ region of pXM2a (probe 2) hybridized with 3.7-, 2.2-, and 1.7-kb transcripts, whereas one corresponding to the most 3′ region of pXM3a (probe 3) hybridized with 3.7- and 2.2-kb transcripts (Fig. 7). From these results we presume that cDNA clones pXM1c, pXM2a, and pXM3a, the sizes of which increase in this order, are likely to have derived, respectively, from 1.2-, 1.7-, and 2.2-kb transcripts. The identities of the 7.0- and 3.7-kb transcripts remain unclear, although an isogene of *Xmam4* was identified during the 5′ RACE experiment (data not shown), and the transcripts may derive from this or another isogene.

Methyltransferase activity of the *Xmam4* gene product. The *mam4*-disrupted *S. pombe* strain JZ653 was transformed with pXM1c, which carries the shortest truncated *Xmam4* gene on the vector pREP3. A membrane fraction prepared from the transformed cells had a low but detectable carboxyl methyltransferase activity (Fig. 5). JZ653 transformed with plasmid pREP3-*Xmam4*, which could express the assembled *Xmam4* gene, was also examined for the methyltransferase activity. As shown in Fig. 5, this transformant exhibited a much higher level of the activity than the pXM1c transformant (nearly as high as that of the pREP3-*mam4* transformant), suggesting that the N-terminal region identified by the 5′ RACE technique is important for the carboxyl methyltransferase activity.

DISCUSSION

Protein methylesterification is a reversible reaction that could play an important role in controlling protein function. For example, methylation at the side chains of glutamate residues in bacterial chemoreceptors plays a key role in regulating the chemotactic response (reviewed in references 10 and 63). In contrast, little is known about the role of methylesterification in eukaryotes, despite the fact that many proteins, including several that possess a prenylated C-terminal cysteine residue, undergo this modification. Cloning of the genes coding for the eukaryotic methyltransferases will provide an important first step towards understanding the biological significance of methylation in these cells. We now report the isolation of the *mam4* gene from *S. pombe* and demonstrate that it is likely to encode a farnesyl cysteine carboxyl methyltransferase. Furthermore, we report the identification of a *Xenopus* homolog of Mam4, named Xmam4, which is the first example of a methyltransferase from a eukaryote higher than yeast.

We have cloned the *mam4* gene of *S. pombe* by complementation of its inability to secrete active M-factor and have presented evidence to suggest that this gene encodes a methyltransferase. The cloned gene has the potential to encode a protein of 236 amino acids that is highly similar to the STE14 farnesyl cysteine methyltransferase of *S. cerevisiae*. Furthermore, cells lacking *mam4* lack the methyltransferase activity normally associated with membranes prepared from wild-type cells, while cells overexpressing this gene have increased activity. These results strongly suggest that *mam4* encodes a farnesyl cysteine carboxyl methyltransferase that is responsible for methylation of the M-factor mating pheromone. The precise role of methylation in the biological activity of M-factor is not known, although it is clearly necessary, as removal of this group by base hydrolysis of the native M-factor inactivates the pheromone (13). Carboxyl methylation is also required for the full activity of the **a**-factor pheromone of *S. cerevisiae* (2, 43) and the tremerogens of *Tremella brasiliensis* (32) and *Tremella mesenterica* (18). The *mam4* mutant does not produce active M-factor; however, the product generated by the mutant was not simply unmethylated M-factor but was a mixture containing a farnesylated but unmethylated tripeptide corresponding to the C terminus of M-factor, farnesylated cysteine, and small amounts of a variety of other molecules of low molecular weight that are most likely to have derived from M-factor or its precursor. This suggests that carboxylmethylation of M-factor is required either to prevent degradation of the pheromone or to allow proper N-terminal processing of the precursor. Our results with the *mam4* mutant are similar to those observed with the *ste14* mutant of *S. cerevisiae*, where a number of **a**-factor-related products are released into the medium. Processing of the **a**-factor precursor is unaffected in this mutant, and the released products are produced as a consequence of the reduced stability of the intracellular pheromone in the mutant (56). By analogy, therefore, it appears that the M-factor derivatives released by the *mam4* mutant result from the instability of the nonmethylated precursor.

The enzyme encoded by the *S. cerevisiae STE14* gene is also responsible for methylation of the Ras proteins (28), although this does not appear to be essential for the activity of these proteins. The loss of *ras* function in *S. cerevisiae* would nor-

mally cause growth arrest (reviewed in reference 21), while the *ste14* mutation has no affect on cell growth. A similar argument appears to hold true with the *S. pombe* Ras protein encoded by *ras1*, the single *ras* homolog in these cells. Although Ras1 is not required for cell growth in *S. pombe*, it is indispensable for mating in both h^+ and h^- cells (20, 48), and yet the *mam4* mutation has no effect on the ability of h^+ cells to mate. It will be interesting to determine whether the Ras1 protein is totally devoid of methylation in the *mam4* mutant.

By exploiting the *S. pombe mam4* mutant, we have been able to isolate a homolog from *X. laevis*. The *Xmam4* gene product is the first example of a methyltransferase from a higher eukaryote, and our results indicate that a family of methyltransferases is conserved from unicellular organisms to higher animals. This view has been further supported by our recent computer search: a partial mouse cDNA sequence with a deduced product that is highly similar to Xmam4 (Fig. 8) has been deposited in the DNA database (accession number AA022288). This cDNA was isolated in the mouse Expressed Sequence Tags project; it has no known function and has even been suspected of being a reversed clone. However, the high degree of similarity between the mouse cDNA product and Xmam4 strongly suggests that mammals also possess a member of the methyltransferase family. The deduced mouse protein is much more similar to Xmam4 than to yeast enzymes.

The methyltransferases of budding yeast, fission yeast, and frog are most highly conserved in the C-terminal region, suggesting that the catalytic domains of the enzymes may lie near the C terminus. This might explain why the three *Xenopus* cDNA clones that we isolated could restore methyltransferase activity and complement the sterility of the *mam4* mutant despite the fact that they lack the 5′ region of the gene. The first methionine residue present in these truncated clones normally appears at position 117 in the complete protein (Fig. 2b), and the ability of the truncated proteins to complement the *mam4* mutation suggests that the polypeptide containing residues 117 to 288 possesses some catalytic activity. The greater activity observed in cells expressing the full-length *Xmam4* gene would suggest that the N-terminal region either enhances the activity of the enzyme or increases its stability. It is also possible that the full-length *Xmam4* gene is better expressed in *S. pombe* than the truncated gene.

Loss of either *STE14* function in *S. cerevisiae* or *mam4* function in *S. pombe* results in the inactivation of a mating pheromone but in no other obvious defects even though farnesyl cysteine methyltransferase seems likely to be involved in the modification of a number of additional proteins. Either these other proteins are sufficiently active without methylesterification or there is another, as-yet-unidentified, methyltransferase in yeast. The physiological role of the conserved methyltransferase in the higher eukaryotes remains to be discovered.

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