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Mating pheromones of the fission yeast *Schizosaccharomyces pombe*: purification and structural characterization of M-factor and isolation and analysis of two genes encoding the pheromone

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Conjugation in the fission yeast *Schizosaccharomyces pombe* is controlled by the action of mating pheromones. Here I describe the isolation and characterization of M-factor, the pheromone released by M-type cells. M-factor is a nanopeptide in which the carboxy-terminal cysteine residue is carboxy-methylated and S-alkylated, probably with a farnesyl residue: Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met-Cys(*S*-farnesyl)-OCH_3. Evidence for this structure was obtained by amino acid analysis, mass spectrometry and tandem mass spectrometry of the native M-factor. Two genes encoding the M-factor were also identified and characterized. It appears that M-factor is synthesized as a larger precursor which is post-translationally cleaved and modified to yield the active pheromone. The proposed modifications are consistent with mechanisms known to exist in other yeast and higher eukaryotes.

Key words: M-factor/pheromone/*Schizosaccharomyces pombe*

Introduction

Conjugation between two haploid cells of heterothallic yeasts is generally controlled by the reciprocal action of pheromones, cells of each mating type releasing pheromones which induce mating specific changes in cells of the opposite mating type. These changes are best characterized in the budding yeast *Saccharomyces cerevisiae* (reviewed in Cross *et al.*, 1988) and include altered patterns of gene transcription (Stetler and Thorner, 1984), an increase in cell agglutinability (Fehrenbacher *et al.*, 1978), a G_1 arrest of cell growth (Hartwell, 1973) and a unidirectional elongation of the cell to form a shmoo (Lipke *et al.*, 1976). The two cell types subsequently conjugate to form a diploid zygote which can undergo meiotic division and sporulation to release ascospores to germinate to haploid cells and complete the life cycle.

Many pheromones have been characterized as short peptides, including α-factor (Stötzler *et al.*, 1976) and **a**-factor (Betz *et al.*, 1987; Anderegg *et al.*, 1988) of *S. cerevisiae*, rhodotorucine A of *Rhodosporidium toruloides* (Kamiya *et al.*, 1978, 1979), tremerogen A-I of *Tremella brasiliensis* (Ishibashi *et al.*, 1984), tremerogen A-10 and tremerogen a-13 of *T.mesenterica* (Sakagami *et al.*, 1981a,b) and the α-sk1 and α-sk2 pheromones of *Saccharomyces kluyveri* (Sakurai *et al.*, 1984). Several of the peptide pheromones are subsequently post-translationally modified by prenylation and/or carboxy methylation (see Table II), modifications which are important for their biological activity (Tsuchiya *et al.*, 1978; Fujino *et al.*, 1980; Ishibashi *et al.*, 1984; Anderegg *et al.*, 1988; Marcus *et al.*, 1991).

Given this wealth of information concerning mating pheromones in several yeast species, it is surprising that there is no information available regarding the structure of mating pheromones in the fission yeast *Schizosaccharomyces pombe*, especially since it has proved a useful species for a variety of studies (Lee and Nurse, 1987; Chappel and Warren, 1989). Many observations clearly indicated the existence of diffusible pheromones in *S.pombe* (Friedmann and Egel, 1978; Fukui *et al.*, 1986; Leupold, 1987; Leupold *et al.*, 1989) but it was only recently that such pheromones could be isolated in a cell-free preparation (Davey, 1991). This work has now extended and I describe the isolation and characterization of M-factor, a mating pheromone released by *S.pombe* cells of the cellular mating type M (minus). M-factor is a nanopeptide in which the carboxy-terminal cysteine residue is *S*-farnesylated and carboxy-methylated, modifications which appear important for full biological activity. It is encoded by two genes which contain the mature M-factor peptide sequence within short precursors, the precursors being suitable substrates for processing events that are likely to generate the mature M-factor.

Results

Purification of M-factor

A crude mixture containing active M-factor was prepared from a haploid M-type cell (h^-) as described previously (Davey, 1991). Briefly, cells were incubated in the presence of the polystyrene resin Amberlite XAD-2 and adsorbed material eluted using propan-1-ol. M-factor activity was demonstrated by monitoring changes in the volume of P-type cells; P-type (but not M-type) cells incubated in the presence of M-factor undergo a distinct change in morphology (shmoo) and a simultaneous increase in volume. This increase in cell volume is related to the amount of M-factor present during the incubation and allows quantification of M-factor activity in any preparation. Although this assay was used routinely during the purification procedures, the ability of the active fractions to induce other mating specific changes was also confirmed on a regular basis. All preparations active in the cell volume assay, for example, caused an arrest in cell growth (Davey, 1991) and induced transcription of mating specific genes (Nielsen and Egel, 1990; O.Nielsen, J.Davey and R.Egel, submitted).

Preliminary investigation into the nature of M-factor (Davey, 1991) suggested that it might be similar to the **a**-factor of *S.cerevisiae* and so techniques used to purify **a**-factor (Betz *et al.*, 1987) were applied to the purification of M-factor. M-factor was enriched by gel filtration using Sephadex LH-60 (Figure 1A) and further purified by HPLC on Spherisorb ODS2 (Figure 1B). Two species of M-factor, designated M and M$^\bullet$, could be identified and the specific biological activities were determined to be ~460 U/nmol

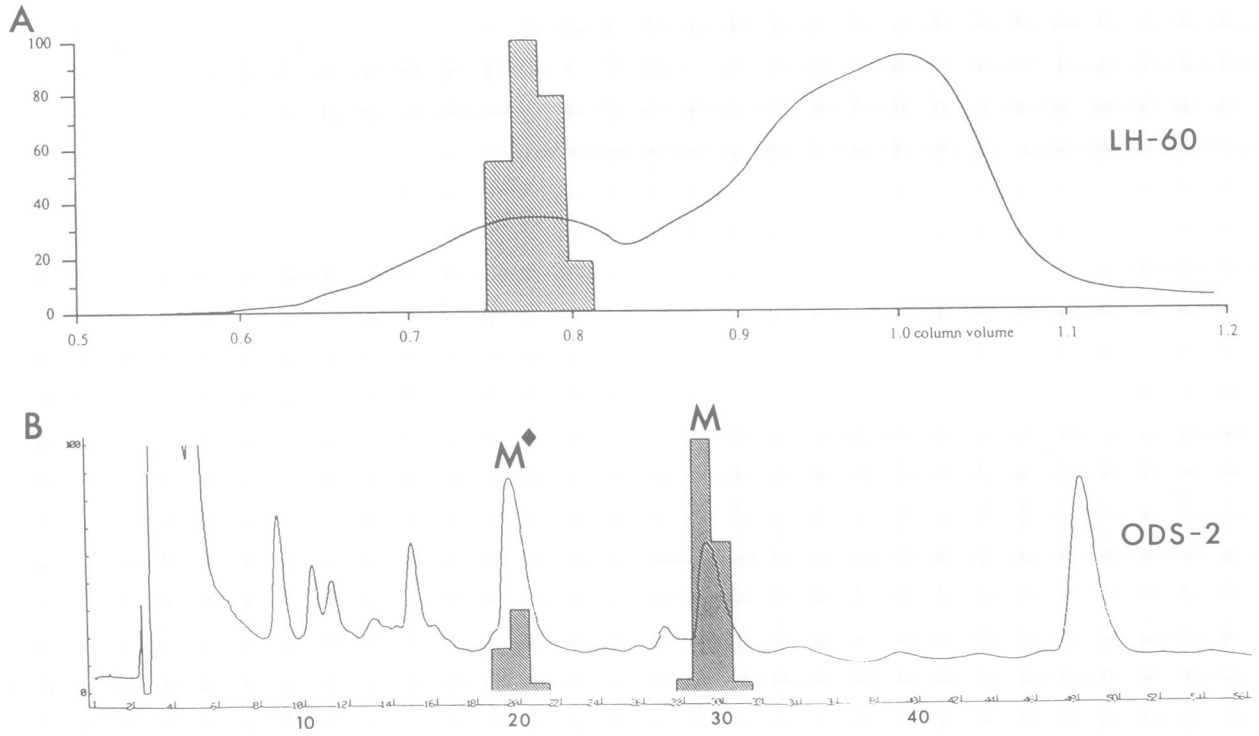

Fig. 1. Purification of M-factor. **A**: gel filtration using Sephadex LH-60. Crude M-factor was fractionated by gel filtration on Sephadex LH-60 (90 × 1.5 cm) in methanol and individual fractions were assayed for M-factor activity. The trace represents the relative absorbance at 280 nm and the hatched region indicates fractions containing M-factor. **B**: reverse phase HPLC using Spherisorb ODS2. Active fractions from (A) were further separated by reverse phase HPLC using a Spherisorb ODS2 C18 column in 0.1% trifluoroacetic acid in methanol. The trace represents the relative absorbance at 220 nm and the hatched regions indicate fractions containing M-factor. Two active species were identified.

for M and ~100 U/nmol for M◆—one unit being the amount of M-factor per ml that induces a 4% response in the cell volume change assay (Davey, 1991).

Amino acid sequencing of M-factor

Amino acid sequencing of M and M◆ revealed identical sequences: NH_2-Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met. Both peptides contain eight amino acids and no phenylthiohydantoin (PTH) derivatives were ever observed in the ninth cycle. The only difference observed between the two species of M-factor during amino acid sequencing was the faster wash-out rate of M, which is consistent with it being more hydrophobic than M◆ (Figure 1B).

The finding that M and M◆ contain identical amino acid sequences suggested that the difference between the two species might be due to modification of a common peptide backbone. This was supported by the finding that small amounts of M◆ were formed during prolonged storage of methanolic solutions of HPLC-purified M. A similar relationship exists between species of **a**-factor isolated from *S.cerevisiae* (Betz *et al.*, 1987). With **a**-factor, the **a1** species is believed to be an oxidation product of the more hydrophobic and more active **a2** species; an oxidation that occurs spontaneously in cultures during aerobic growth or which can be achieved by treatment of purified **a2** with hydrogen peroxide. Since a peptide exhibiting the chromatographic behaviour of M◆ could be obtained by hydrogen peroxide treatment of HPLC-purified M (not shown), it seems likely that M◆ arises from M due to an oxidation reaction similar to that observed with **a**-factor. Given this apparent relationship between M and M◆, all further work concentrated on the M peptide which appears to be the native M-factor.

FAB MS analysis of M-factor

FAB MS analysis of HPLC-purified M-factor gave a major protonated molecular ion peak at *m/z* 1319.6 (Figure 2A). This is some 320 mass units higher than the mass predicted for the peptide determined from amino acid sequencing (see above) and indicated either an incomplete amino acid sequence or some structural modification of the peptide, or both.

Several fungal mating factors have been shown to possess a post-translationally modified cysteine residue at their carboxy terminus (Sakagami *et al.*, 1979; Ishibashi *et al.*, 1984; Anderegg *et al.*, 1988); the cysteine residue being carboxy-methylated and *S*-farnesylated. It was striking that the presence of such a residue at the carboxy terminus of the deduced peptide sequence of M-factor could account for the difference between the observed and predicted mass values. The calculated mass of a peptide with the sequence observed earlier but with an *S*-farnesyl cysteine methyl ester as an additional residue at the carboxy terminus is 1319.7 which is in reasonable agreement with the observed value (Figure 2A). The presence of a modified cysteine residue could account for the highly hydrophobic nature of the M-factor and would provide an easily oxidizable group for the conversion of M to M◆. The fact that neither cysteine nor any other PTH derivative was detected in the amino acid sequence analysis of M-factor peptides does not preclude its presence in M-factor since a cysteine thioether appears resistant to hydrolysis (Betz *et al.*, 1987).

To investigate whether the carboxy terminus is methyl-esterified, M-factor was treated with sodium hydroxide and repurified by HPLC, a treatment that should remove the methyl group (Betz *et al.*, 1987; Anderegg *et al.*, 1988). FAB·MS of the alkali-treated M-factor gave a

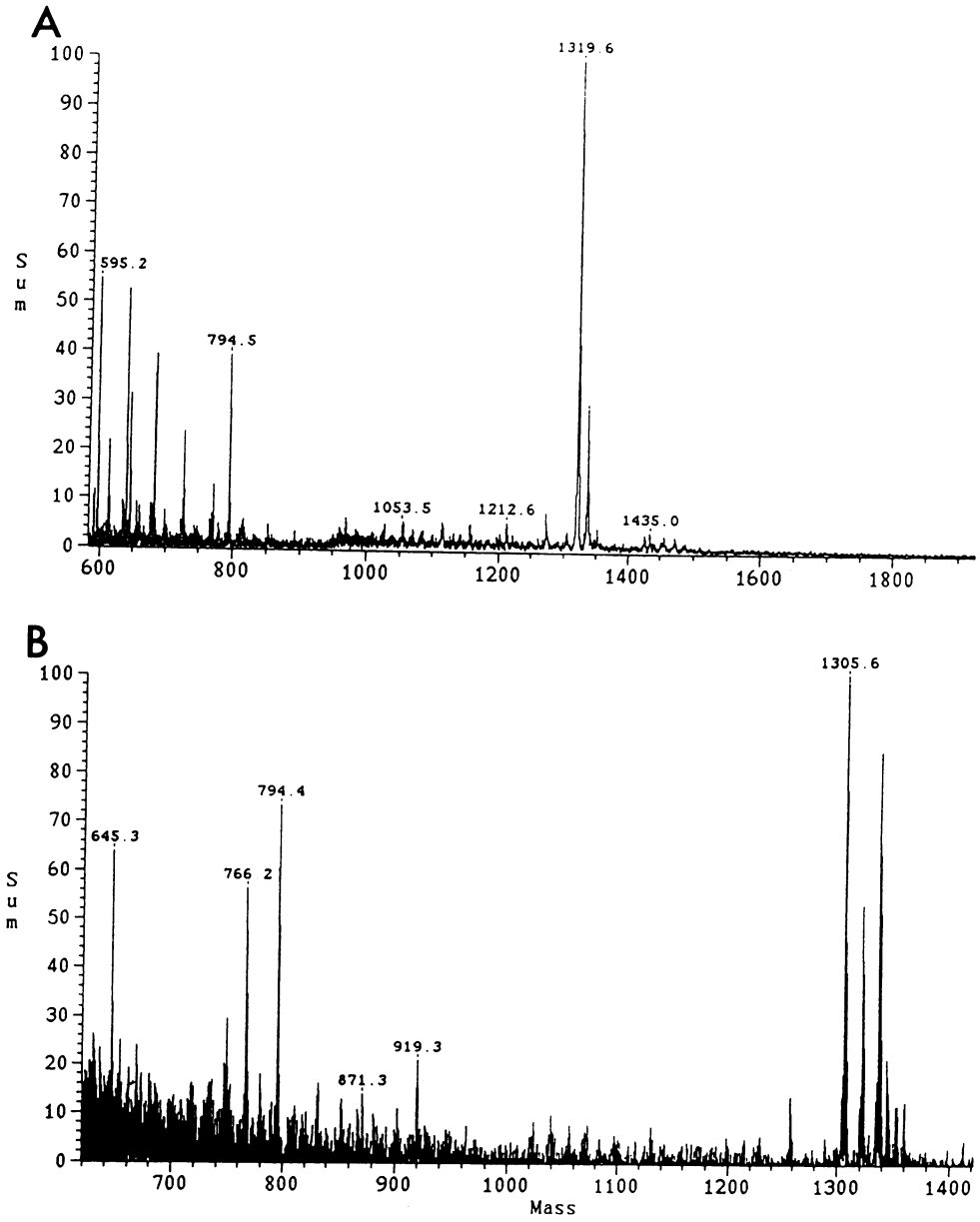

Fig. 2. FAB MS analysis of M-factor. **A**: FAB MS analysis of native M-factor. HPLC purified M-factor was subjected to FAB MS analysis (see Materials and methods) and revealed a major protonated species at 1319.6. **B**: FAB MS analysis of alkali treated M-factor. Purified native M-factor was treated with sodium hydroxide and repurified by HPLC before being subjected to FAB MS analysis. The appearance of a protonated molecular ion peak at *m/z* 1305.6 is consistent with the removal of a carboxy-terminal methyl ester (Betz *et al.*, 1987; Anderegg *et al.*, 1988).

protonated molecular ion peak at *m/z* 1305.6 (Figure 2B), a reduction of 14 mass units that is consistent with the loss of a methyl group. Following treatment with sodium hydroxide, the now presumed non-methylated M-factor was at least 100-fold less active than the non-treated factor (not shown). This apparent requirement for the carboxy-terminal methyl ester is consistent with the observations of other fungal mating factors (Fujino *et al.*, 1980; Ishibashi *et al.*, 1984; Anderegg *et al.*, 1988; Marcus *et al.*, 1991).

Tandem MS analysis of M-factor

M-factor and a synthetic peptide based upon the amino acid sequence of native M-factor were analysed by tandem MS (Figure 3). The synthetic peptide, SP1, contains the first eight amino acids of M-factor but does not contain the carboxy-terminal cysteine residue believed to be in the mature factor. Although the spectra are too weak to assign identities to all of the peaks, the similarities between the two are sufficient to confirm the peptide sequencing data presented earlier. One of the most striking features of the spectrum for M-factor is a prominent daughter ion at *m/z* 1114.5 (Figure 3A). This constitutes a loss of 205 mass units from the parent ion (*m/z* = 1319.6, Figure 2A) and could represent the removal of a $C_{15}H_{25}$ alkyl group, possibly a farnesyl residue.

Analysis of synthetic peptides

To investigate the structure of M-factor further, a variety of synthetic peptides were analysed for activity in the standard cell volume assay (Table I). Although none of the synthetic peptides induced mating specific changes in P-type cells, one of the peptides (SP2) significantly inhibited the activity of native M-factor. Although the inability of SP2 to elicit a mating specific response indicates that it is not identical to M-factor, its ability to inhibit M-factor

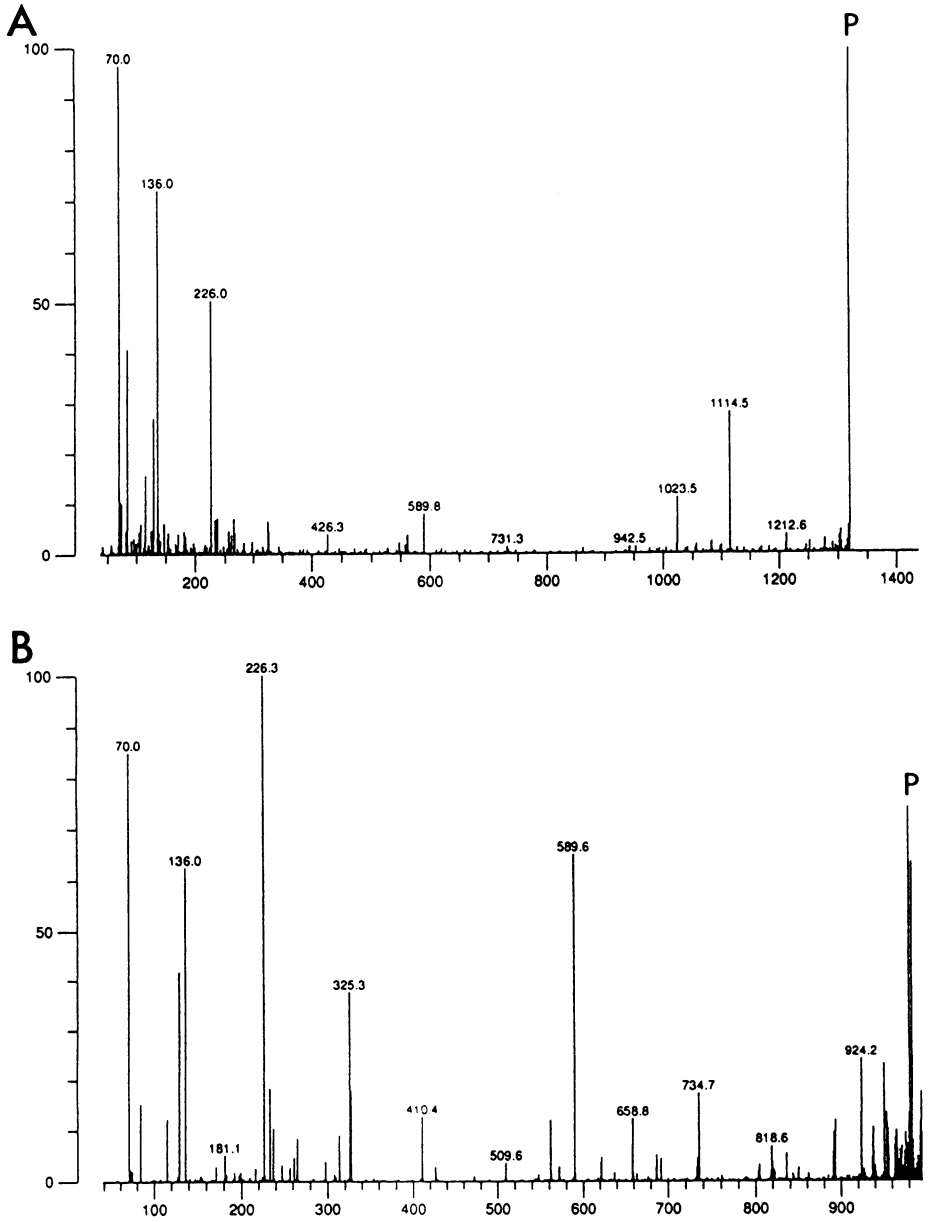

Fig. 3. Tandem mass spectrometry. **A**: tandem MS analysis of M-factor. The protonated parent ion generated by FAB MS of native M-factor (m/z = 1319.6; P; see Figure 2A) was subjected to tandem MS. **B**: tandem MS analysis of the synthetic peptide SP1. The protonated parent ion generated by FAB MS of the synthetic peptide SP1 (m/z = 998.2; P) was subjected to tandem M. SP1 represents the first eight amino acids of native M-factor but lacks the carboxy-terminal cysteine residue and associated post-translational modifications.

Table I. Activity of synthetic peptides

Peptide	Sequence	Activity (% of native M-factor)	Inhibition [activity observed (% of control) (ratio of peptide:M-factor)]			
			1	10	100	1000
SP1	Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met-COOH	NA[a]	101	105	90	75
SP2	Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met-amide	NA	93	63	32	18
SP3	Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met-Cys-COOH	NA	105	108	103	102
SP4	Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met-Cys-amide	NA	101	111	106	98

A variety of peptides and peptide amides related to M-factor were assayed for M-factor activity (as monitored by the cell volume assay) and for their ability to inhibit native M-factor. To monitor inhibition by the synthetic peptides, native M-factor was added to the samples at a concentration of 1 nM (i.e. sufficient to induce a maximum response—the 'control' of 100%).

[a]No activity detected at 10 μM (native M-factor induces a maximum response at 1 nM).

specifically further supports the amino acid sequence presented earlier. Furthermore, since SP1 (which is the same as SP2 except for the presence of a free carboxyl group) has little effect on M-factor activity, it seems likely that the difference between SP2 and native M-factor resides at the carboxy-terminal end of the peptide. Similar findings have

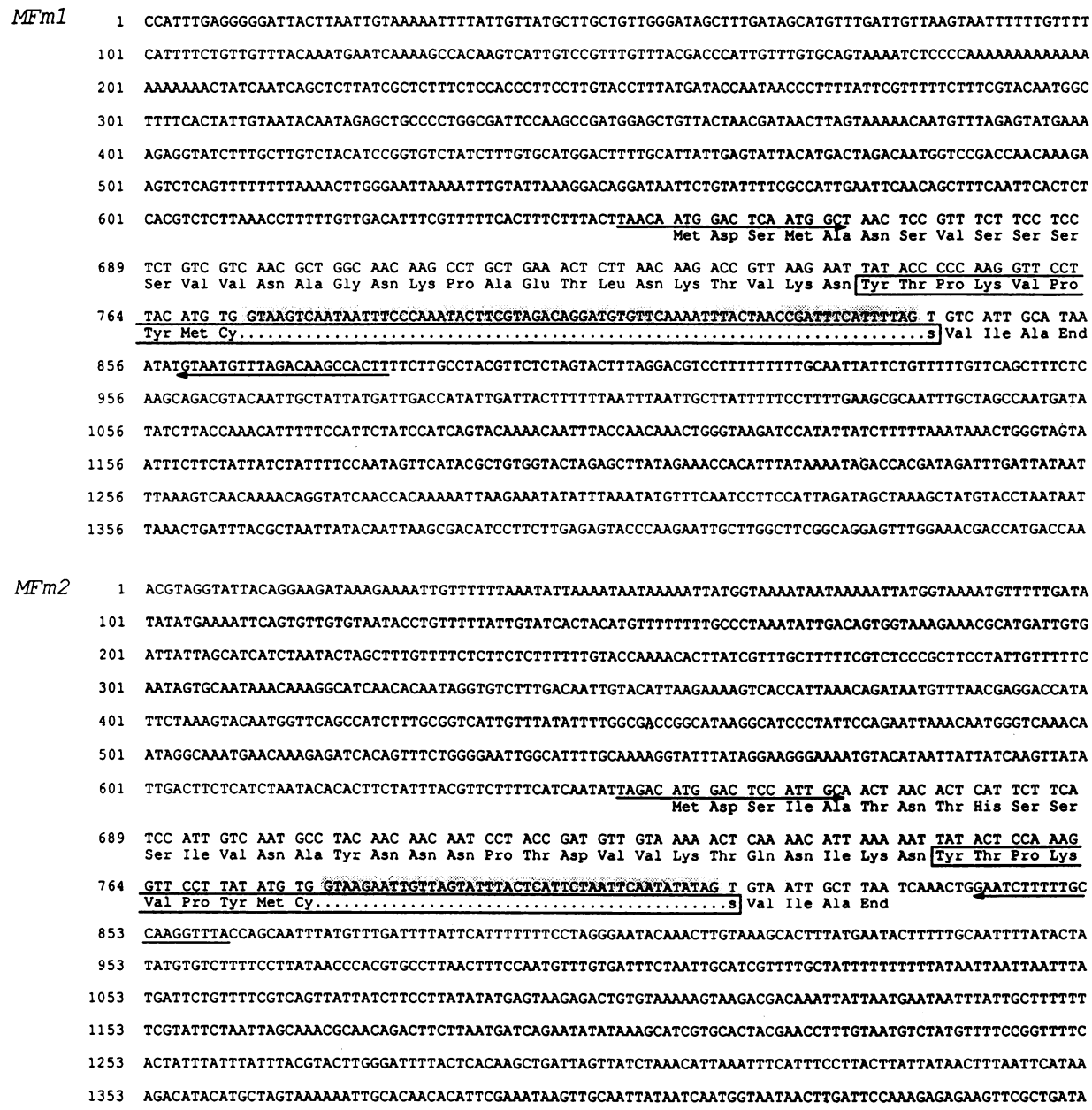

Fig. 4. Nucleotide sequences of *MFm1* and *MFm2*. The predicted translational products of *MFm1* and *MFm2* are indicated and the regions present in the mature M-factor are boxed. Initial analysis suggested that both products end with a tryptophan residue but further work (detailed in the text) demonstrated the presence of an intron in each gene (indicated by shading) which, when removed, revealed the true translation products. The sequences underlined were used to generate oligonucleotides for PCR analysis (see Figure 6).

been observed with analogues of the **a**-factor from *S. cerevisiae* (Becker *et al.*, 1987); an unmodified peptide lacking the carboxy-terminal cysteine residue was itself inactive in growth arrest assays but did block the activity of native **a**-factor. The lack of any effect with either SP3 or SP4 does not rule out the presence of a carboxy-terminal cysteine residue in M-factor since, again, an analogous peptide based on the **a**-factor of *S.cerevisiae* (i.e. with a complete peptide backbone but lacking both the farnesyl group and the methyl ester) is virtually inactive in growth arrest assays (Marcus *et al.*, 1991).

It would be desirable to confirm the structure of M-factor by completely synthesizing the modified peptide and demonstrating its activity. This has been achieved for the *S.cerevisiae* **a**-factor (Xue *et al.*, 1989) but the established methodology is not suitable for the M-factor and requires further development (F.Naider, personal communication).

Characterization of the structural gene(s) for M-factor

The approach used to isolate the structural gene(s) for the M-factor involved hybridization screening of an *S.pombe* genomic DNA library (an *Sau*IIIA partial digest in λ ZAP, provided by Dr Tamar Enoch, Department of Microbiology, University of Oxford) using oligonucleotides deduced from the amino acid sequence shown earlier. The oligonucleotides were designed to cover all possible codon usages in the M-factor. Screening of this library resulted in the isolation of plasmids containing two different segments of yeast chromosomal DNA and the two putative M-factor structural genes were localized to a 600 bp *Eco*RI−*Sau*IIIA fragment

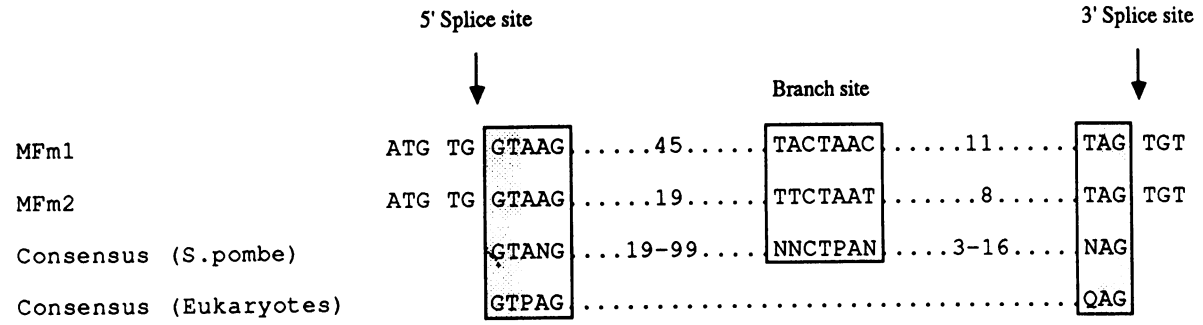

Fig. 5. Comparison of conserved splice sequences in *S.pombe* and higher eukaryotes. Consensus sequences determined by analysis of several genes from *S.pombe* and higher eukaryotes are shown and compared with the splice sites in *MFm1* and *MFm2*. The shaded regions represent nucleotides which are almost always present. N = any nucleotide; P = purine; Q = pyrimidine.

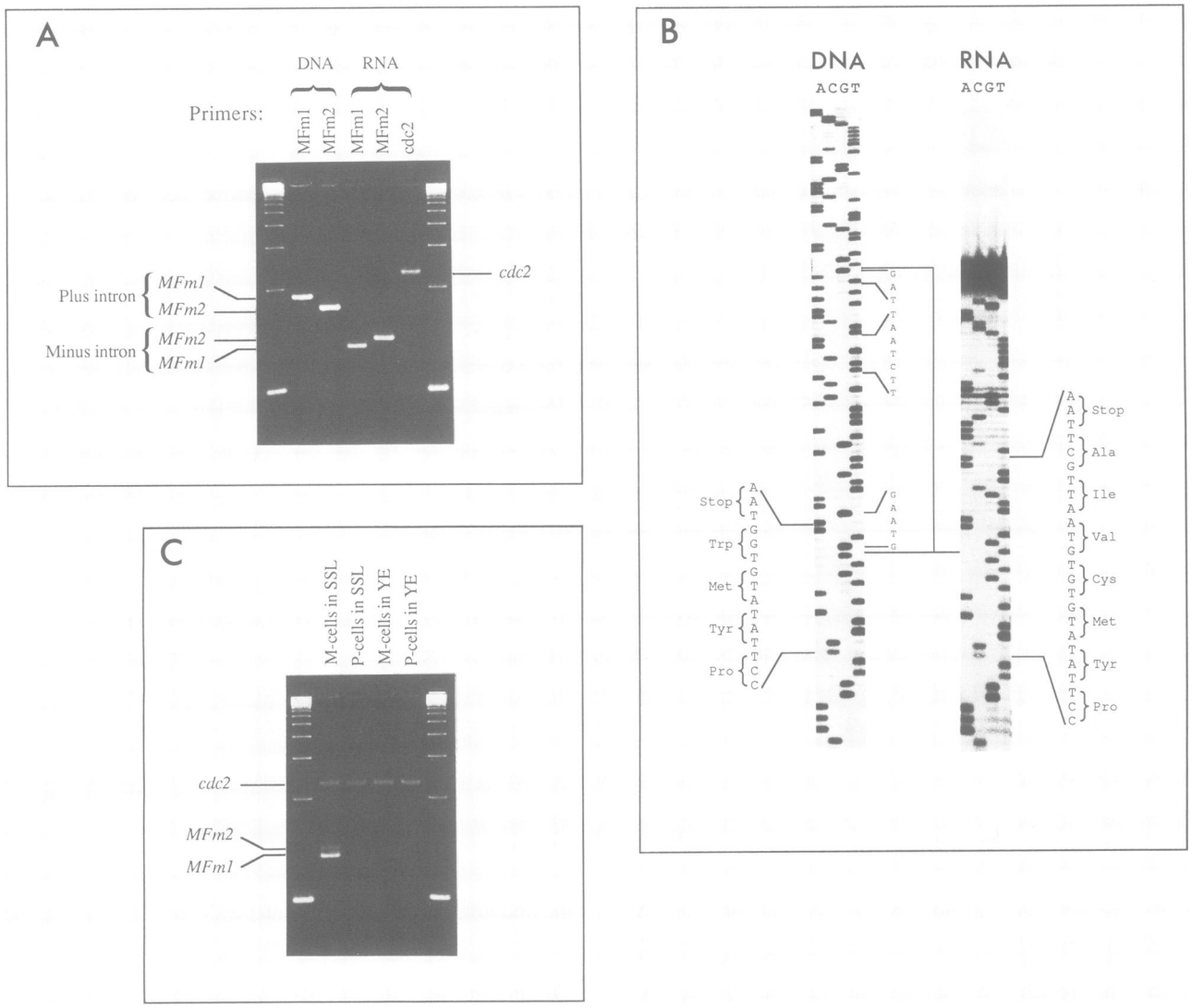

Fig. 6. PCR analysis of *MFm1* and *MFm2*. **A**: PCR products from M-type cells analysed by gel electrophoresis. Cloned genomic DNA and RNA extracted from M-type cells grown under nitrogen starvation were analysed by PCR using oligonucleotide primers described in Figure 4. The primers were designed to span the intron presumed to be present in each gene. As an internal control, the extracted RNA was also incubated with oligonucleotide primers to the *CDC2* gene of *S.pombe*—designed to generate a PCR fragment of ~280 bp. Samples were analysed by electrophoresis on a 3% agarose gel. The outside tracks are size markers consisting of a 123 bp ladder (BRL). **B**: sequence of PCR products. The fragments generated by PCR analysis in panel (A) were purified and sequenced—only the sequences related to the *MFm2* gene are shown for clarity. The translation products predicted from the sequences are indicated as is the intron and its associated consensus sequences. Sequencing of the PCR product from the RNA sample confirms the precise removal of the intron. **C**: PCR products from different cell types under different growth conditions. RNA was extracted from both M- and P-type cells grown in SSL (to induce mating response) and in YE (yeast extract; non-induced conditions) and was then analysed by PCR using the oligonucleotide primers described in (A)—primers to *MFm1*, *MFm2* and *CDC2*. After PCR incubation, the three different reactions for each RNA sample were mixed in equal volumes and analysed by electrophoresis on a 3% agarose gel.

(*MFm1*) and to a 900 bp *Eco*RI–*Sau*IIIA fragment (*MFm2*).

DNA sequence analysis of both fragments revealed the presence of regions encoding the M-factor peptide sequence within what appear to be short precursors (Figure 4). Each precursor contains several amino acids at the amino terminus and, at first, it seemed that both contain a tryptophan residue at the carboxy terminus. The presence of a terminal tryptophan residue was surprising since it did not agree with earlier findings; there is, for example, no known post-translational modification of a tryptophan which could account for the mass of the M-factor as revealed by FAB MS. A possible explanation for this discrepancy was suggested by the homology with eukaryotic introns (Mount, 1982), including those found in *S.pombe* (Russell, 1989; Figure 5). Both M-factor genes contain sequences homologous to 5′ and 3′ splice sites and the intervening sequences contain a characteristic branch site, a pyrimidine-rich region lacking the dinucleotide AG. Furthermore, the putative introns are quite short (71 nt in *MFm1* and 42 nt in *MFm2*), consistent with known introns in *S.pombe* (Russell, 1989).

Removal of the introns during the formation of mRNA would generate M-factor precursors terminating with the sequence . . . Cys-Val-Ile-Ala. Such precursors would be consistent with post-translational modifications resulting in the formation of an *S*-farnesyl cysteine methyl ester as the carboxy-terminal residue of the mature M-factor (for review, see Glomset *et al.*, 1990); isoprenylation of the cysteine residue being followed by proteolytic removal of the three carboxy-terminal amino acids and carboxyl methylation of the resulting carboxy-terminal isoprenylated cysteine residue.

The removal of both introns was confirmed by DNA sequencing of products generated by PCR amplification of RNA transcripts extracted from M-type cells (Figure 6). Haploid M-type cells were grown to mid-exponential phase in SSL medium and then transferred to SSL medium lacking a nitrogen source, conditions expected to induce the transcription of mating specific genes (Kelly *et al.*, 1988; Nielsen and Egel, 1990). Six hours after transfer, RNA was extracted from these cells and subjected to PCR analysis using oligonucleotide primers designed to span the proposed intron (see Figure 4). The same primers were also used for PCR analysis of the cloned genomic DNA.

Gel electrophoresis of the various products from PCR analysis showed bands of the sizes predicted: 231 bp for the *MFm1* gene and 160 bp for the transcript of the *MFm1* gene, and 211 bp for the *MFm2* gene and 169 bp for the transcript of the *MFm2* gene (Figure 6A). DNA sequence analysis of the PCR amplified products from the M-type cells confirmed the precise splice sites in the transcripts (Figure 6B).

PCR analysis also provided insights into transcription of the M-factor genes (Figure 6C). No M-factor-related transcripts could be detected in RNA extracted from P-type cells cultured under mating conditions or in RNA extracted from either cell type cultured in yeast extract medium, suggesting that transcription from the M-factor genes is cell type-specific and induced by nitrogen starvation.

Discussion

Structure of M-factor

This paper describes the results of a study into the nature of M-factor, a mating pheromone released by M-type cells of the fission yeast *S.pombe*. M-factor is shown to be a nanopeptide in which the carboxy-terminal cysteine residue is carboxy-methylated and S-alkylated, probably with a farnesyl residue: Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met-Cys-(*S*-farnesyl)-OCH_3. Evidence for this structure was obtained from three different approaches.

Amino acid analysis. The first eight residues were identified by amino acid sequencing of purified M-factor and although no PTH derivative was ever observed in the ninth cycle, this does not preclude the presence of a cysteine thioether which is likely to be resistant to hydrolysis (Betz *et al.*, 1987).

Mass spectrometry. FAB·MS of purified M-factor revealed a mass of 1319.6 [Figure 2A; high resolution analysis has recently provided a mass of 1319.71965 (not shown)] which is consistent with the proposed structure (calculated mass = 1319.72). Mass spectrometry of alkali-treated M-factor showed a reduction of 14 mass units (Figure 2B), presumably due to the removal of the carboxy-terminal methyl ester (Anderegg *et al.*, 1988). Although tandem MS of M-factor was unable to confirm the complete amino acid sequence, it did reveal sufficient similarities to that of a synthetic peptide based on the above sequence to suggest the sequencing was correct (Figure 3). In addition, native M-factor generated a daughter ion at 1114.5 mass units which probably results from the removal of the farnesyl residue.

DNA sequence analysis. The M-factor amino acid sequence was contained within precursors encoded by two structural genes. The two precursors terminate with the sequence . . . Cys-Val-Ile-Ala and would be suitable substrates for post-translational modifications (described below) leading to the formation of an *S*-farnesyl cysteine methyl ester as the carboxy-terminal residue of the mature M-factor (Glomset *et al.*, 1990). The observation that these genes are only transcribed in M-type cells under conditions which cause the release of M-factor (Figure 6C) strongly supports their proposal as the structural genes for the M-factor.

Further, indirect support for the proposed structure of

Table II. Structures of mating pheromones isolated from a variety of species

Species	Pheromone	Structure	Reference
S.pombe	M-factor	Tyr-Thr-Pro-Lys-Val-Pro-Tyr-Met-Cys(*S*-farnesyl)-OCH_3	This study
S.cerevisiae	**a**-factor	Tyr-Ile-Ile-Lys-Gly-(Val/Leu)-Phe-Trp-Asp-Pro-Ala-Cys(*S*-farnesyl)-OCH_3	Anderegg *et al.* (1988)
R.toruloides	rhodotorucine A	Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg-Asn-Gly-Cys(*S*-farnesyl)-OH	Kamiya *et al.* (1979)
T.mesenterica	tremerogen A-10	Glu-His-Asp-Pro-Ser-Ala-Pro-Gly-Asn-Gly-Tyr-Cys(*S*-farnesyl)-OCH_3	Sakagami *et al.* (1979)
T.mesenterica	tremerogen a-13	Glu-Gly-Gly-Gly-Asn-Arg-Gly-Asp-Pro-Ser-Gly-Val-Cys(*S*-farnesyl)-OH	Sakagami *et al.* (1981)
T.brasiliensis	tremerogen A-I	Asp-Ser-Gly-Ser-Ser-Arg-Asp-Pro-Gly-Ala-Ser-Ser-Gly-Gly-Cys(*S*-farnesyl)-OCH_3	Ishibashi *et al.* (1984)

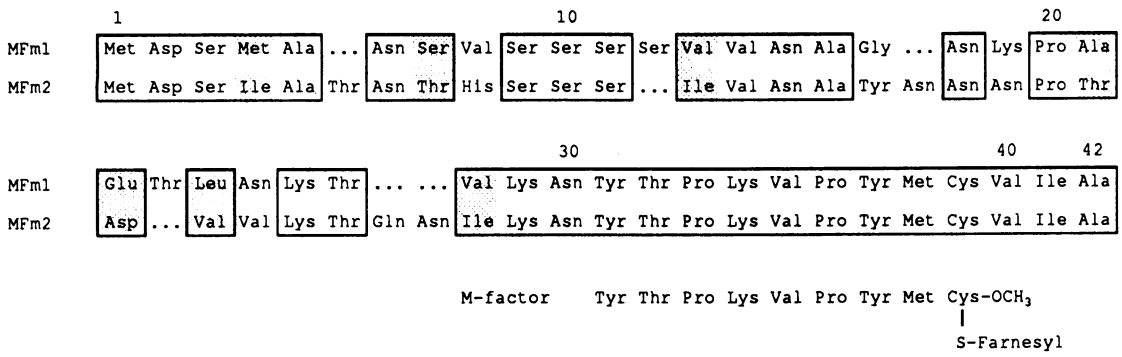

Fig. 7. Comparison of proposed M-factor precursor sequences from *MFm1* and *MFm2*. The proposed precursors from *MFm1*and *MFm2* are aligned for maximal homology and homologous regions are boxed. Conserved amino acid changes are indicated by shading.

M-factor is provided by its similarity to the structures of mating factors released by other species (Table II). When considered together, the available evidence strongly supports the structure that I have proposed for M-factor. Of course, there are other investigations that could be performed to show unequivocally that the structure is correct. For example, one could use proton NMR and gas chromatography to confirm the modifications to the cysteine residue, while the total synthesis of an active M-factor analogue would remove any possible doubt about the structure.

Analysis of the structural genes for M-factor

M-factor is encoded by two similar, but not identical genes which I have called *MFm1* and *MFm2*. This terminology is based on that of *S.cerevisiae* and should also prevent confusion in assigning names to the structural gene(s) for the P-factor, when they are identified. Each gene contains one intron and both introns contain features which are characteristic of introns observed in other genes from *S.pombe* (Russell, 1989) and higher eukaryotes (Mount, 1982); the introns are small (71 nt in *MFm1* and 42 nt in *MFm2*) and possess highly conserved sequences at the exon−intron boundaries and at a putative branch site within the intron. The position of the introns within the coding regions for the M-factor could be significant. Each intron occurs between the codons for the methionine and valine residues towards the carboxy terminus of the precursors, with the cysteine codon being generated at the junction following splicing. Therefore, splicing adds the cysteine residue and the last three amino acids and generates a precursor which would be a suitable substrate for prenylation. It is possible that the carboxy-terminal exon developed independently as a general mechanism for producing precursors suitable for prenylation. Clearly, the genomic structure of other prenylated proteins will need to be determined to test this suggestion.

The presence of two structural genes, each encoding a single copy of the mature M-factor, is similar to the arrangement of the structural genes of the **a**-factor in *S. cerevisiae* (Brake *et al.*, 1985). The α-factor of *S.cerevisiae* is also encoded by two discrete genes but each encodes a precursor containing multiple copies of the mature factor (Kurjan and Herskowitz, 1982; Singh *et al.*, 1983), *MFα1* encodes four copies of α-factor and two copies of *MFα2*. The structural genes for mating factors from other species are not yet characterized.

Processing of M-factor precursors

Comparison of the M-factor precursors encoded by *MFm1* and *MFm2* reveals a high degree of homology (Figure 7). Both have identical, short carboxy-terminal extensions (. . . Val-Ile-Ala) and longer amino-terminal extensions (30 residues in *MFm1* and 32 residues in *MFm2*) which show some similarity. The mechanism by which these precursors are processed to generate the mature, extracellular M-factor is currently under investigation but, using available information, one can propose a reasonable scenario.

Several proteins are now known to be post-translationally modified by S-prenylation of carboxy-terminal cysteine residues, the substrate for prenylation being a precursor in which the cysteine is followed by three additional amino acids, the first two of which are often aliphatic residues (for review, see Glomset *et al.*, 1990). Addition of the prenyl group, via thioether linkage, is followed by proteolysis of the carboxy-terminal tripeptide and, usually, methyl esterification of the exposed carboxyl group. Considerable progress is being made towards identifying the enzymes responsible for these modifications. Biochemical studies suggest that the *DPR1* gene product of *S.cerevisiae* is a component of the prenyltransferase (Finegold *et al.*, 1990; Goodman *et al.*, 1990; Schafer *et al.*, 1990) and the *STE14* gene product a component of the methyltransferase (Hrycyna and Clarke, 1990; Marr *et al.*, 1990; Hrycyna *et al.*, 1991). Mammalian counterparts of the yeast prenyltransferase (Reiss *et al.*, 1990; Schaber *et al.*, 1990) and methyltransferase (Stephenson and Clarke, 1990) have also been reported. Characterization of the peptidase responsible for the removal of the carboxy-terminal tripeptide has not yet been reported.

Processing of the amino-terminal extension of the M-factor precursor is even more speculative but could again reflect events with the **a**-factor of *S.cerevisiae*. The cleavage site between the amino-terminal extension and the mature factor sequence for both **a**-factor and M-factor has the conserved sequence . . . Lys-Asn-Tyr . . . with cleavage occurring between the asparagine and tyrosine residues. It is tempting to speculate that cleavage is performed by endopeptidase that is similar in the two species.

Whether the processing of the amino-terminal extension is a single proteolytic event or involves sequential proteolysis is unclear, but two minor species of M-factor, which co-purified with the M$^{\blacklozenge}$ species (see Figure 1B), were detected and found to possess the amino acid sequence of the mature factor extended by three residues at the amino

terminus (not shown). These minor species, which were present at <1% of the mature peptide, began with the sequences Val-Lys-Asn . . . and Ile-Lys-Asn . . . and could represent M-factor that had been released from the cell before complete processing had occurred (the two amino acid sequences represent minor, conserved changes between the two M-factor genes). It is not possible to conclude whether these are genuine intermediates in the processing pathway or are the result of a 'non-specific' proteolytic event that is not essential for the release of mature M-factor. However, given the homology of the cleavage sites in M-factor and **a**-factor, the lack of homology just previous to these cleavage sites and the possibility that the premature cleavage of the M-factor could be the result of a less specific action of the same endopeptidase responsible for generating the mature M-factor (both cleavage sites occur between a hydrophobic residue and a neutral polar residue), it is perhaps more likely to represent a non-essential cleavage event.

In those instances where it has been demonstrated, enzymes analogous to those believed to be involved in the modification of the M-factor are either cytosolic (Goodman *et al.*, 1990; Schafer *et al.*, 1990) or exposed on the cytoplasmic face of cellular membranes (Hrycyna and Clarke, 1990) and would generate a cytosolic form of the M-factor requiring export into the medium. This cytosolic scenario is supported by the lack of a suitable hydrophobic signal sequence in the amino-terminal extensions of the M-factor precursors and it seems likely that M-factor is released from cells by a mechanism similar to that used by **a**-factor. Modified **a**-factor is exported into the medium by a membrane bound transporter encoded by the *STE6* gene (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). The *STE6* peptide transporter is one of a family of ATP-driven transporters that are found in a range of species and are believed to control the transmembrane movement of a variety of molecules (Kuchler and Thorner, 1990). The proposed structure of M-factor would suggest that a transporter related to this family is present in *S.pombe*.

There is considerable structural similarity between the M-factor and the **a**-factor from *S.cerevisiae*. This could reflect similarities in their biosynthesis and it would be interesting to study the synthesis of M-factor in *S.cerevisiae* and of **a**-factor in *S.pombe*. Such work would identify common elements in the biosynthetic pathways and might reveal insights into the function of certain components; for example, it could explain how the membrane transporters recognize the intracellular form of the pheromones.

Structure–function relationships

Although a detailed investigation into the biological significance of different aspects of the M-factor has only recently begun, some structure–function relationships are evident from the work presented here and preliminary observations seem to agree with conclusions from studies of mating pheromones from other yeasts. Since the non-modified peptide is unable to induce mating specific changes (Table I), it is clear that some modification is required for biological activity. However, it is less clear which modification is required. Removal of the carboxy-terminal methyl ester by treatment of native M-factor with sodium hydroxide reduced activity by ~100-fold and would suggest that this modification is almost essential for biological activity. This is consistent with other mating factors where methylation has been shown to be important for full biological activity (Fujino *et al.*, 1980; Ishibashi *et al.*, 1984; Anderegg *et al.*, 1988; Marcus *et al.*, 1991). Although I have no evidence concerning the role of prenylation in the activity of M-factor, it should perhaps be noted that this modification is required for the export of **a**-factor (Powers *et al.*, 1986; Schafer *et al.*, 1990) and for full biological activity of several mating factors (Tsuchiya *et al.*, 1978; Fujino *et al.*, 1980; Anderegg *et al.*, 1988; Marcus *et al.*, 1991).

Conclusion

This paper describes the isolation and characterization of a pheromone involved in the mating reaction of *S.pombe*. There is currently much interest in understanding how eukaryotic cells communicate with their neighbours and the interaction observed during the mating process could provide a valuable system for investigating such communication. The characterization of M-factor is an important step in these studies and the availability of the pure pheromone will now allow further investigation of the interaction.

Materials and methods

Yeast strains and culture conditions

All of the work described here used the wild-type haploid heterothallic strains of *S.pombe*, M-types cells were h^- (L972) and P-type cells were h^+ (L975). Culture conditions were as described previously (Davey, 1991). Unless specified otherwise, all chemicals were reagent grade and were purchased from standard suppliers.

Purification of M-factor

A crude mixture containing active M-factor was prepared from M-type cells as described previously (Davey, 1991). M-factor was adsorbed directly from culture medium to the polystyrene resin Amberlite XAD-2 (Sigma, Poole, UK) and eluted with propan-1-ol. The eluted material was taken to dryness by rotary evaporation and resuspended in a small volume of methanol. Insoluble material was removed by centrifugation and the sample loaded onto a column (90 × 1.5 cm) of Sephadex LH-60 (Sigma) in methanol and eluted, in methanol, at a rate of 10 ml/h. Elution was monitored by absorbance at 280 nm and individual fractions were taken to dryness and assayed for M-factor activity as described below. Active fractions were pooled, concentrated by evaporation and further purified by reverse phase HPLC on a Spherisorb ODS2 column (Phase-Sep, Clywd, UK) using 0.1% trifluoroacetic acid in methanol at a flow rate of 1 ml/min. Elution was monitored by absorbance at 220 nm and individual fractions were taken to dryness and assayed for M-factor activity.

Determination of M-factor activity

M-factor activity was assayed by monitoring the volume of cells following their exposure to the factor as described previously (Davey, 1991) except that a Coulter Channelyser C256 (Coulter Electronics, Luton, UK) was used to monitor cell volumes. One unit of activity is defined as the amount of M-factor per ml that induces a response in P-type cells of 4% in the cell volume assay (Davey, 1991).

Oxidation of M-factor

Approximately 3 nmol of HPLC-purified M-factor (in 100 μl of methanol) was mixed with 10 μl of hydrogen peroxide and incubated at room temperature for 30 min before being analysed by HPLC as described above.

Amino acid analysis

Automated Edman degradation was performed on the HPLC-purified M-factor (1–2 nmol) with an Applied Biosystems model 473 A gas phase sequenator after adsorption to a Biobrene glass filter. PTHs were identified by HPLC as recommended by the manufacturers. This work was performed by Alta Bioscience, University of Birmingham.

Mass spectrometry

FAB·MS and tandem MS were performed by Dr Jonathan Curtis (Department of Chemistry, University of Warwick) on a Kratos Analytical Concept IIHH four-sector mass spectrometer equipped with an Ion Tech fast atom gun producing a beam of 8 keV Xe atoms. Samples were dissolved in a 10% trifluoroacetic acid solution and added to a matrix of either a 1:1 mixture of glycerol and monothioglycerol or *meta*-nitrobenzyl alcohol (mNBA). An accelerating voltage of 8 kV was used throughout. All spectra were acquired as raw data onto the Kratos DS90 data system and processed using the Kratos Mach3 processing software. Mass spectra were recorded using a scan speed of 10 s per decade with the resolution of MS1 set to 1000. Tandem MS spectra were recorded with the collision cell electrically floated to 4 keV, a scan speed of 30 s per decade and with the resolution of MS2 set to 1000. The collision gas used was helium at a pressure giving a beam attenuation of 70%.

Demethylation of M-factor

HPLC-purified M-factor was treated with 0.1 M sodium hydroxide for 2.5 h at 45°C and repurified by HPLC as described above.

Synthesis of M-factor related peptides

A number of peptides and peptide amides based on the native M-factor (see Table I) were synthesized by solid phase methodology using FMOC protection chemistry on a Biotech Instruments BT7300 Multiple Peptide Synthesizer using the materials and conditions recommended by the manufacturer. Peptides were deprotected and cleaved with TFA/anisole/water/2-mercaptoethanol (95:2:2:1). This work was performed by Alta Bioscience, University of Birmingham.

Oligonucleotide synthesis

Oligonucleotides were synthesized on a Biotech Instruments BT8510 Automatic Synthesizer using materials and conditions recommended by the manufacturer. This work was performed by Alta Bioscience, University of Birmingham.

Nucleic acid methodology

Standard nucleic acid methodology was performed as described by Sambrook *et al.* (1989). Structural genes encoding the M-factor were identified by hybridization screening of an *S.pombe* genomic DNA library with oligonucleotides deduced from the amino acid sequence of native M-factor. The library was kindly provided by Dr Tamar Enoch (Department of Microbiology, University of Oxford) and consists of a *Sau*IIIA partial digest of genomic DNA in λ-ZAP. DNA sequencing was by the dideoxynucleotide method (Sanger *et al.*, 1977) using [^{35}S]dATP (Amersham, Aylesbury, UK) and double stranded DNA. Isolation of nucleic acids from *S.pombe* cells was by standard techniques (Moreno *et al.*, 1991). Polymerase chain reactions were performed using a DNA thermal cycler (Perkin Elmer Cetus, USA) with material and conditions recommended by the manufacturer. The sequences of the *MFm1* and *MFm2* genes have been deposited in the EMBL Data Library under the accession numbers X63627 and X63628, respectively.

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