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Saccharomyces cerevisiae STE6 gene product: a novel pathway for protein export in eukaryotic cells

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***Saccharomyces cerevisiae MATa* cells release a lipopeptide mating pheromone, a-factor. Radiolabeling and immunoprecipitation show that *MATa ste6* mutants produce pro-a-factor and mature a-factor intracellularly, but little or no extracellular pheromone. Normal *MATa* cells carrying a multicopy plasmid containing both *MFa1* (pro-a-factor structural gene) and the *STE6* gene secrete a-factor at least five times faster than the same cells carrying only *MFa1* in the same vector. The nucleotide sequence of the *STE6* gene predicts a 1290 residue polypeptide with multiple membrane spanning segments and two hydrophilic domains, each strikingly homologous to a set of well-characterized prokaryotic permeases (including *hlyB, oppD, hisP, malK* and *pstB*) and sharing even greater identity with mammalian *mdr* (multiple drug resistance) transporters. These results suggest that the *STE6* protein in yeast, and possibly *mdr* in animals, is a transmembrane translocator that exports polypeptides by a route independent of the classical secretory pathway.**

Key words: MATa cells/*Saccharomyces cerevisiae*/*STE6* gene/transmembrane translocator

Introduction

The yeast *Saccharomyces cerevisiae* produces and responds to secreted peptide hormones. The role of these peptide signals is to induce processes that lead to conjugation between haploid yeast cells, resulting in the formation of diploid cells (for review, see Cross *et al.*, 1988). Therefore, these secreted peptides are referred to as mating pheromones. The pheromone produced by *MATα* haploids is called α-factor. Mature biologically active α-factor (a tridecapeptide) is produced from a much larger glycosylated precursor (prepro-α-factor) by specific proteolytic processing events that occur during transit of this precursor through the yeast secretory pathway (for review, see Fuller *et al.*, 1988).

Unlike α-factor, mature biologically active **a**-factor is a post-translationally-modified peptide. The **a**-factor is 12 amino acids long (Fuller *et al.*, 1986; Betz *et al.*, 1987), but carries on the C-terminal Cys residue both a farnesyl moiety (attached via thioether linkage to the side chain) and a methyl group (attached via ester linkage to the carboxyl end) (Anderegg *et al.*, 1988; Schafer *et al.*, 1989). These same modifications also decorate both the yeast and mammalian *ras* proteins (Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; Schafer *et al.*, 1989). Also unlike α-factor, the precursors from which **a**-factor is generated (*MF***a***1* and *MF***a***2* gene products) are very short (36 and 38 amino acids respectively), lack a hydrophobic N-terminal signal sequence and are devoid of sites for attachment of Asn-linked oligosaccharides (Brake *et al.*, 1985). In further contrast to α-factor, extracellular **a**-factor is still produced when *MAT***a** cells carrying temperature-sensitive secretion-defective (*sec*) mutations (Schekman, 1985) are shifted to the restrictive temperature (Sterne, 1989; Sterne and Thorner, 1986, 1987; R.E.Sterne, L.C.Blair and J.Thorner, in preparation); and, based on protease susceptibility, pro-**a**-factor is not sequestered in a membrane-bound compartment (Sterne, 1989; R.E. Sterne, L.C. Blair and J. Thorner, in preparation). Moreover, none of the mutations that prevent proteolytic processing of prepro-α-factor block maturation of **a**-factor precursor (Julius *et al.*, 1983, 1984).

Taken together, these findings indicate that **a**-factor is processed and released from *MAT***a** cells by a route that is quite distinct from the typical secretory pathway. Several mutations have been identified that cause *MAT***a** cells (but not *MATα* cells) to be mating defective. The products of the so-called *STE* (for 'sterile') genes of this *MAT***a**-specific class should include cellular components involved in the synthesis of mature extracellular **a**-factor. Indeed, genetic and physiological studies of two such mutations, *ste6* (Rine, 1979) and *ste14* (Blair, 1979), suggested that these defects prevent the production of biologically active **a**-factor, but do not interfere with the ability of *MAT***a** cells to respond to a pheromone (α-factor) signal (Chan *et al.*, 1983). Consistent with this conclusion, transcription of the *MF***a***1* and *MF***a***2* genes is normal in *ste6* and *ste14* mutants (Brake *et al.*, 1985). A third *MAT***a**-specific mutation, *ram1* (Powers *et al.*, 1986) (also called *dpr1* and *ste16*), blocks the post-translational modification of both **a**-factor and yeast *RAS* proteins.

We have sought to clarify further the processes involved in **a**-factor biogenesis. Here we present biochemical and genetic evidence that the *STE6* gene product catalyzes the rate-limiting step in secretion of **a**-factor. By determining the complete nucleotide sequence of the *STE6* gene, we found that the *STE6* protein is likely to be a membrane-bound transporter because it is highly homologous to a family of well-studied membrane permeases from prokaryotic cells (for review, see Ames, 1986). More intriguing still, the *STE6* gene is even more closely related to the mammalian *mdr* genes which, when overexpressed or amplified, can confer multiple drug resistance (for review, see Endicott and Ling, 1989). Our findings suggest, therefore, that the true role of the *mdr* family of transporters in eukaryotic cells may be to carry out the intercompartmental translocation of peptides and proteins by a mechanism independent of the secretory pathway, as was originally speculated by Gerlach *et al.* (1986).

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Results

Export of a-factor is blocked in ste6 mutants

We developed three tools that have allowed us to examine **a**-factor biogenesis. First, we raised polyclonal anti-**a**-factor antibodies that recognize both **a**-factor precursor and mature **a**-factor, as is documented in detail elsewhere (Sterne, 1989; R.E.Sterne, L.C.Blair and J.Thorner, in preparation). Second, we prepared the 12 residue **a**-factor backbone (YIIKGVFWDPAC) by solid phase peptide synthesis for use in conjunction with the antibodies to definitively identify **a**-factor-related peptides by competition (Sterne, 1989; R.E.Sterne, L.C.Blair and J.Thorner, in preparation). Third, to raise the steady-state level of processing intermediates in *MAT***a** cells, we overproduced pro-**a**-factor by expressing the *MF***a***1* gene from a multicopy plasmid (Brake *et al.*, 1985).

When **a**-factor is produced under the direction of the genomic *MF***a***1* and *MF***a***2* genes, the level of pro-**a**-factor synthesized is just barely detectable by immunoprecipitation, even during pulse-labeling periods from 1 to 5 min with carrier-free $^{35}SO_4^{2-}$ at concentrations as high as 1 mCi/ml (data not shown). In contrast, when *MAT***a** cells carry the *MF***a***1* gene on a multicopy plasmid, intracellular pro-**a**-factor is readily observed either in a pulse label or during steady-state labeling (Figure 1). We have used this approach to examine the forms of **a**-factor present in various mutants that affect **a**-factor production.

The *ste6-1* mutation was originally identified as a lesion which causes *MAT***a** cells to be mating defective (Rine, 1979). Subsequent studies suggested that the mating defect of *ste6* mutants was due either to secretion of an insufficient amount of **a**-factor or to secretion of a non-functional **a**-factor (Chan *et al.*, 1983). The availability of anti-**a**-factor antibodies allowed us to resolve this question. As revealed by immunoprecipitation after long-term labeling, the amount of extracellular **a**-factor produced by a *ste6* mutant (*ste6ΔN*:: *TRP1) is* <3% of that secreted by an isogenic *STE6*$^+$ strain (Figure 1). Furthermore, the amount of pro-**a**-factor (and another intermediate) present intracellularly was reproducibly elevated over that found in the wild-type cells; and, mature **a**-factor was also observed, although its level was somewhat more variable from experiment to experiment. Essentially identical results were obtained when a strain (RK547-20A) carrying the original *ste6-1* allele was compared to its isogenic *STE6*$^+$ strain (REY547) (data not shown).

The decreased level of **a**-factor secreted by *ste6* mutants is not due to reduced transcription of the *MF***a***1* and *MF***a***2* genes (Brake *et al.*, 1985). Nevertheless, it was possible that *ste6* mutations reduce translation of the *MF***a***1* and *MF***a***2* mRNAs. However, pulse-labeling of cells overexpressing *MF***a***1* demonstrated that a *ste6* mutant and an isogenic wild-type cell initially synthesize nearly identical levels of pro-**a**-factor (Figure 1). The fact that *ste6* mutants release only barely detectable amounts of extracellular **a**-factor, yet synthesize normal levels of pro-**a**-factor and are capable of converting the precursor to mature **a**-factor, suggested that the *ste6* mutation prevents some aspect of **a**-factor secretion (rather than pro-**a**-factor processing). Mature **a**-factor does not accumulate intracellularly in the *ste6* mutants because it appears to be rapidly degraded (see below).

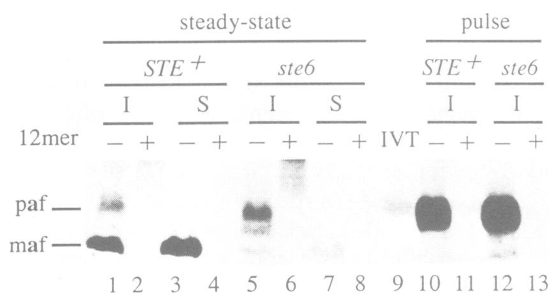


Fig. 1. *ste6* mutants are deficient in **a**-factor secretion. Expontentially-growing cultures (2 ml) at an OD_{600} = 2 of a *MAT***a** *ste6* mutant (REY21-1) and its isogenic parent (W303-1A), both carrying plasmid pAB182, were either pulse labeled at 30°C with 2 mCi $^{35}SO_4^{2-}$ for 5 min in LSM+ lacking sulfate and uracil (after a 15 min preincubation in LSM+ lacking uracil and sulfate to deplete the endogenous sulfate pool) or labeled with 2mCi $^{35}SO_4^{2-}$ in the steady-state for 1 h in LSM+ lacking uracil and containing 10 μM $(NH_4)_2SO_4$. Intracellular ('I') **a**-factor was immunoprecipitated from detergent-solubilized extracts prepared by glass bead lysis of washed whole cells (Sterne, 1989; R.E.Sterne, L.C.Blair and J.Thorner, in preparation). Secreted ('S') **a**-factor was immunoprecipitated from the combined material present in the concentrated cell-free culture medium and an organic solvent eluate of the walls of the culture flask (Sterne, 1989; R.E.Sterne, L.C.Blair and J.Thorner, in preparation). Immunoprecipitations were performed in the absence (−) or presence (+) of a 200-fold excess of synthetic **a**-factor ('12mer'). The precipitates were solubilized, fractionated on a 10−20% polyacrylamide gradient gel, and visualized by autoradiography after impregnation of the gel with 1 M salicylate (Chamberlain, 1979). Product of *in vitro* translation of synthetic *MF***a***1* mRNA, 'IVT'; pro-**a**-factor, 'paf'; and mature **a**-factor, 'maf'.

STE6 gene product is rate-limiting for a-factor transport

When normal *MAT***a** cells carry the *MF***a***1* gene on a multicopy plasmid, the intracellular precursor is overproduced at least 80-fold, as judged by radiolabeling and immunoprecipitation (Figure 2); yet, when measured either by bioassay (data not shown) or by immunoprecipitation of radiolabeled peptide (Figure 2), the amount of secreted **a**-factor is elevated only 5 to 6-fold. Therefore, it appears that some other gene product(s) are limiting the efficiency of processing and/or secretion.

Our observation that *ste6* mutants accumulate pro-**a**-factor and generate mature **a**-factor intracellularly, yet produce very little extracellular pheromone, suggested that the *STE6* gene

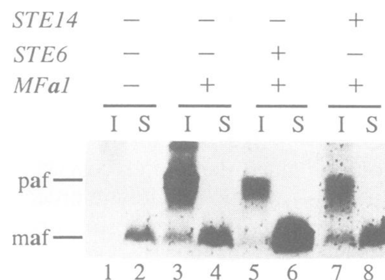


Fig. 2. Overexpression of the *STE6* gene product increases the efficiency of **a**-factor production. Exponentially growing cultures (1 ml) of a wild-type *MAT***a** strain (W303-1A) carrying multicopy plasmids with no insert (YEp352) (**lanes 1** and **2**), or with the *MF***a***1* gene alone (pKK1) (**lanes 3** and **4**), or with both the *MF***a***1* and *STE6* genes (pKK16) (**lanes 5** and **6**), or with both the *MF***a***1* and *STE14* genes (pRE 10) (**lanes 7** and **8**), were labeled for 1 h in LSM+ lacking uracil and containing 10 μM $(NH_4)_2SO_4$ and analyzed by immunoprecipitation, gel electrophoresis, and autoradiography, all as described in the legend to Figure 1.

product might be rate-limiting for **a**-factor export. To test this possibility, the *STE6* gene was inserted into the same multicopy plasmid that contained the *MF***a***1* gene. In this way, the amount of intracellular precursor and secreted **a**-factor could be measured for normal *MAT***a** cells and for the same cells overexpressing *MF***a***1* alone and overexpressing both *MF***a***1* and *STE6*. In the same way, we also examined the effect of overproducing the product of another gene that has been implicated in **a**-factor biosynthesis, *STE14* (Blair, 1979; Chan *et al.*, 1983). In comparison to *MAT***a** cells harboring the *MF***a***1* gene alone on a multicopy plasmid, simultaneous overexpression of *MF***a***1* and *STE6* resulted in a marked decrease in the level of intracellular precursor and a striking increase in the amount of mature **a**-factor released

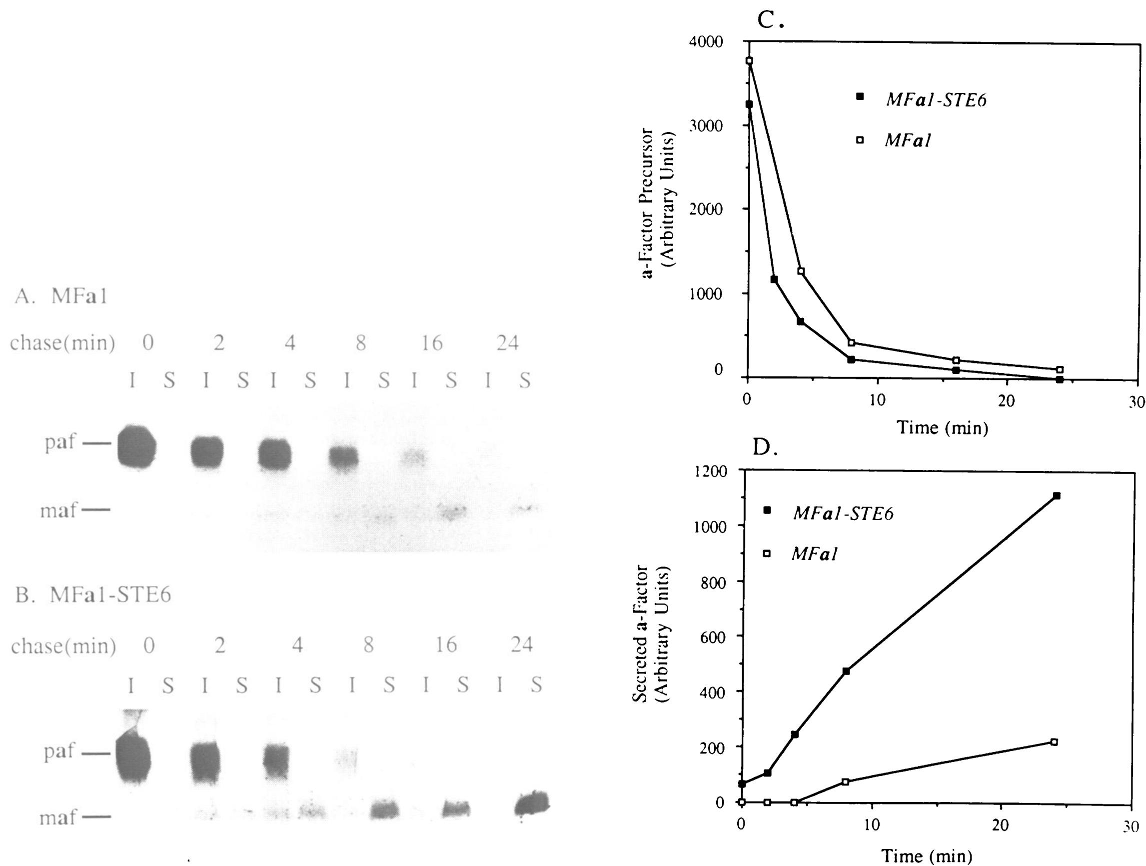


Fig. 3. The *STE6* gene product is rate limiting for **a**-factor export. Exponentially growing cultures (6 ml) of a wild-type *MAT***a** strain (W303-1A) carrying (**A**) the *MF***a***1* gene on a multicopy plasmid (pKK1), or (**B**) carrying both the *MF***a***1* and *STE6* genes on a multicopy plasmid (pKK16), were pulse-labeled with 6 mCi $^{35}SO_4^{2-}$ for 4 min in LSM+ lacking uracil and sulfate. Immediately after the pulse, a 1 ml sample was withdrawn and quenched by chilling on ice and by addition of NaN_3 to a final concentration of 10 mM. The remainder of the culture (5 ml) was removed and portions (1 ml) were transferred to five separate flasks, each containing excess methionine (20 mM), cysteine (20 mM) and sulfate (1 mM) (to yield the final concentrations given in parentheses). One of the chased samples was quenched in the same way at each of the indicated times. The **a**-factor-related peptides in all the samples were immunoprecipitated and analyzed as descibed in the legend to Figure 1. The intensity of the bands shown in the autoradiograms of (**A**) and (**B**) were quantitated by the method of Suissa (1983) and replotted as a function of time in (**C**) and (**D**) respectively.

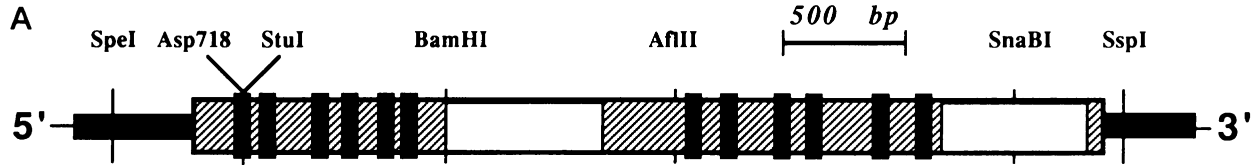
Table I. *Saccharomyces cerevisiae* strains

Strain	Genotype	Source
W303-1A	*MAT***a** *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	Rod Rothstein
W303-1B	*MAT*α *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	Rod Rothstein
W303D	diploid from crossing W303-1A with W303-1B	This study
WKK7	*ste6*Δ :: *HIS3* derivative of W303-1A	This study
REY21-1	*ste6*Δ*N* :: *TRP1* derivative of W303-1A	This study
TDY21-3D	*MAT***a** *ura3-52 leu2-3,112 trp1 his3 lys2*	Trisha Davis
CG378	*MAT***a** *ura3-52 leu2-3,112 trp1-289 ade5 can*^{r}	Craig Giroux
REY21-2	*ste6*Δ*N* :: *LEU2* derivative of CG378	This study
RK547-20A	*MAT***a** *ste6-1 kex2-1 ura3-52 leu2-3,112 ade2 ade6 arg4-17 his*$^-$	Riyo Kunisawa
REY547	*STE6* derivative of RK547-20A by DNA-mediated transformation	This study
XT1-20A	*HML*α *MAT*α *HMR*α *sst2-4*^{oc} *ura3-52 leu2-3,112 ade2 trp1 his6*	Amy Axt
RK512-5B	*MAT***a** *sst2-1 ura-52 his3*Δ*-1 ade2 cyh2*	Riyo Kunisawa
BC180	*MAT***a** *sst2*Δ*2 ura3-52 leu2-3,112 his*Δ*1 ade2-1*^{oc}	Bill Courchesne

from the cells (Figure 2). In contrast, simultaneous over-expression of *MFa1* and *STE14* did not significantly increase the amount of extracellular **a**-factor, even though the level of intracellular pro-**a**-factor was reduced somewhat.

The effect of elevating the *STE6* gene product on the kinetics of **a**-factor secretion was examined in more detail.

A



B

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                 5'--cgttcacacagagagctttcaagtgccgctgaaattccactaggaaacaaagaacaagctacgtc -1
+1   ATGAACTTTTTAAGTTTTAAGACTACAAAACACTATCACATTTTCAGGTACGTGAACATACGGAATGACTACAGGCTGTTAATG 84
1    MetAsnPheLeuSerPheLysThrThrLysHisTyrHisIlePheArgTyrValAsnIleArgAsnAspTyrArgLeuLeuMet 28
85   ATAATGATAATAGGTACCGTGGCAACAGGCCTAGTGCCGGCAATTACTTCTATCCTGACGGGCAGAGTGTTCGATCTACTATCA 168
29   IleMetIleIleGlyThrValAlaThrGlyLeuValProAlaIleThrSerIleLeuThrGlyArgValPheAspLeuLeuSer 56
169  GTTTTCGTGGCTAATGGGTCACATCAAGGTTTGTATTCCCAACTAGTACAGAGGTCAATGGCAGTAATGGCACTTGGTGCGGCT 252
57   ValPheValAlaAsnGlySerHisGlnGlyLeuTyrSerGlnLeuValGlnArgSerMetAlaValMetAlaLeuGlyAlaAla 84
253  TCTGTGCCAGTAATGTGGCTTTCTCTAACAAGTTGGATGCACATCGGCGAGAGACAAGGCTTTAGAATACGGTCACAGATATTG 336
85   SerValProValMetTrpLeuSerLeuThrSerTrpMetHisIleGlyGluArgGlnGlyPheArgIleArgSerGlnIleLeu 112
337  GAGGCATATTTGGAGGAAAAGCCAATGGAATGGTACGACAATAATGAAAAATTGTTAGGAGATTTTACTCAAATCAACAGATGT 420
113  GluAlaTyrLeuGluGluLysProMetGluTrpTyrAspAsnAsnGluLysLeuLeuGlyAspPheThrGlnIleAsnArgCys 140
421  GTGGAAGAGCTAAGATCAAGCTCCGCAGAGGCATCAGCCATAACTTTCCAGAATTTAGTTGCAATATGTGCGCTTCTGGGGACG 504
141  ValGluGluLeuArgSerSerSerAlaGluAlaSerAlaIleThrPheGlnAsnLeuValAlaIleCysAlaLeuLeuGlyThr 168
505  TCATTCTACTATTCTTGGTCATTAACTTTAATTATTCTTTGCAGCTCTCCAATAATCACATTTTTTGCAGTGGTGTTTTCCAGA 588
169  SerPheTyrTyrSerTrpSerLeuThrLeuIleIleLeuCysSerSerProIleIleThrPhePheAlaValValPheSerArg 196
589  ATGATTCATGTATATTCAGAGAAGGAGAATTCTGAAACGAGTAAAGCAGCCCAATTACTTACATGGTCGATGAATGCCGCTCAA 672
197  MetIleHisValTyrSerGluLysGluAsnSerGluThrSerLysAlaAlaGlnLeuLeuThrTrpSerMetAsnAlaAlaGln 224
673  TTAGTGAGATTATATTGTACACAACGTCTAGAAAGGAAAAAATTCAAGGAAATCATACTAAATTGTAACACTTTCTTCATCAAG 756
225  LeuValArgLeuTyrCysThrGlnArgLeuGluArgLysLysPheLysGluIleIleLeuAsnCysAsnThrPhePheIleLys 252
757  AGTTGCTTTTTTGTTGCTGCAAACGCTGGGATCTTGAGATTTTTGACGTTGACTATGTTTGTACAGGGATTCTGGTTTGGTTCC 840
253  SerCysPhePheValAlaAlaAsnAlaGlyIleLeuArgPheLeuThrLeuThrMetPheValGlnGlyPheTrpPheGlySer 280
841  GCAATGATCAAAAAGGGCAAGCTGAACATTAACGATGTAATCACTTGCTTCCATTCATGCATTATGCTGGGCTCGACTTTAAAT 924
281  AlaMetIleLysLysGlyLysLeuAsnIleAsnAspValIleThrCysPheHisSerCysIleMetLeuGlySerThrLeuAsn 308
925  AATACATTACACCAAATAGTTGTTCTTCAAAAAGGCGGAGTGGCTATGGAAAAAATCATGACTCTATTAAAAGATGGATCCAAG 1008
309  AsnThrLeuHisGlnIleValValLeuGlnLysGlyGlyValAlaMetGluLysIleMetThrLeuLeuLysAspGlySerLys 336
1009 CGAAATCCTTTAAATAAAACTGTAGCCCACCAATTTCCACTAGATTATGCCACCAGTGATCTAACATTTGCTAATGTTTCGTTT 1092
337  ArgAsnProLeuAsnLysThrValAlaHisGlnPheProLeuAspTyrAlaThrSerAspLeuThrPheAlaAsnValSerPhe 364
1093 TCTTATCCAAGCAGACCTTCGGAAGCAGTTTTAAAGAACGTTAGTTTAAATTTCTCTGCAGGACAATTTACTTTCATAGTAGGA 1176
365  SerTyrProSerArgProSerGluAlaValLeuLysAsnValSerLeuAsnPheSerAlaGlyGlnPheThrPheIleValGly 392
1177 AAATCAGGCTCAGGTAAATCTACATTATCCAACTTATTATTAAGGTTCTACGATGGCTATAATGGATCGATATCTATCAATGGC 1260
393  LysSerGlySerGlyLysSerThrLeuSerAsnLeuLeuLeuArgPheTyrAspGlyTyrAsnGlySerIleSerIleAsnGly 420
1261 CACAATATCCAAACAATCGACCAAAAATTGCTAATTGAAAATATCACCGTCGTAGAACAGCGTTGTACCCTATTTAATGATACT 1344
421  HisAsnIleGlnThrIleAspGlnLysLeuLeuIleGluAsnIleThrValValGluGlnArgCysThrLeuPheAsnAspThr 448
1345 CTTAGAAAAAATATTCTTTTAGGTTCAACAGATTCGGTAAGAAATGCCGATTGCAGCACCAATGAAAATAGACATTTAATCAAA 1428
449  LeuArgLysAsnIleLeuLeuGlySerThrAspSerValArgAsnAlaAspCysSerThrAsnGluAsnArgHisLeuIleLys 476
1429 GATGCCTGTCAAATGGCTCTTCTGGATAGATTTATTCTAGACCTTCCCGATGGACTGGAAACATTAATTGGGACTGGGGGTGTC 1512
477  AspAlaCysGlnMetAlaLeuLeuAspArgPheIleLeuAspLeuProAspGlyLeuGluThrLeuIleGlyThrGlyGlyVal 504
1513 ACACTAAGTGGCGGGCAACAACAAAGAGTTGCTATAGCACGTGCATTCATCAGAGATACTCCAATATTATTCTTAGACGAAGCT 1596
505  ThrLeuSerGlyGlyGlnGlnGlnArgValAlaIleAlaArgAlaPheIleArgAspThrProIleLeuPheLeuAspGluAla 532
1597 GTATCGGCTCTAGATATTGTTCATCGCAACCTGTTGATGAAGGCAATTAGGCATTGGAGGAAAGGAAAGACTACAATCATATTG 1680
533  ValSerAlaLeuAspIleValHisArgAsnLeuLeuMetLysAlaIleArgHisTrpArgLysGlyLysThrThrIleIleLeu 560
1681 ACGCATGAGTTGAGCCAAATTGAATCTGATGACTATTTATATTTAATGAAGGAAGGTGAAGTTGTTGAAAGCGGCACCCAGTCT 1764
561  ThrHisGluLeuSerGlnIleGluSerAspAspTyrLeuTyrLeuMetLysGluGlyGluValValGluSerGlyThrGlnSer 588
1765 GAACTTCTAGCCGATCCGACCACTACATTTAGCACATGGTATCACCTACAGAATGACTACTCTGATGCGAAAACTATTGTAGAT 1848
589  GluLeuLeuAlaAspProThrThrThrPheSerThrTrpTyrHisLeuGlnAsnAspTyrSerAspAlaLysThrIleValAsp 616
1849 ACAGAGACTGAAGAAAAATCTATACACACTGTGGAAAGTTTTAACTCTCAATTGGAAACACCAAAACTTGGATCATGCTTAAGT 1932
617  ThrGluThrGluGluLysSerIleHisThrValGluSerPheAsnSerGlnLeuGluThrProLysLeuGlySerCysLeuSer 644
1933 AATCTGGGATATGATGAGACAGATCAGTTGTCCTTTTACGAAGCAATCTATCAAAAAAGATCGAACGTTAGAACAAGAAGGGTT 2016
645  AsnLeuGlyTyrAspGluThrAspGlnLeuSerPheTyrGluAlaIleTyrGlnLysArgSerAsnValArgThrArgArgVal 672
2017 AAAGTTGAAGAGGAAAATATTGGGTATGCACTAAAACAACAAAAGAACACCGAAAGTTCAACAGGGCCACAACTTCTGAGCATT 2100
673  LysValGluGluGluAsnIleGlyTyrAlaLeuLysGlnGlnLysAsnThrGluSerSerThrGlyProGlnLeuLeuSerIle 700
2101 ATTCAGATTATCAAAAGAATGATTAAAAGCATAAGATACAAAAAAATTCTAATCTTGGGACTGCTATGTTCTCTTATCGCAGGC 2184
701  IleGlnIleIleLysArgMetIleLysSerIleArgTyrLysLysIleLeuIleLeuGlyLeuLeuCysSerLeuIleAlaGly 728
2185 GCCACAAATCCCGTCTTTTCATACACATTCAGTTTCTTACTAGAAGGAATTGTCCCATCCACGGATGGAAAAACTGGCTCTTCA 2268
729  AlaThrAsnProValPheSerTyrThrPheSerPheLeuLeuGluGlyIleValProSerThrAspGlyLysThrGlySerSer 756
2269 CATTATTTGGCGAAATGGTCGCTTCTTGTTCTTGGTGTGGCTGCGGCAGATGGTATTTTCAATTTTGCTAAAGGATTCCTATTA 2352
757  HisTyrLeuAlaLysTrpSerLeuLeuValLeuGlyValAlaAlaAlaAspGlyIlePheAsnPheAlaLysGlyPheLeuLeu 784
2353 GATTGCTGCAGTGAATACTGGGTTATGGATCTTAGAAATGAAGTTATGGAAAAACTGACGAGAAAGAATATGGACTGGTTTTCT 2436
785  AspCysCysSerGluTyrTrpValMetAspLeuArgAsnGluValMetGluLysLeuThrArgLysAsnMetAspTrpPheSer 812
2437 GGTGAAAACAACAAGGCTTCTGAAATTTCTGCTCTAGTCTTGAATGATTTGCGAGATTTGAGGTCTTTGGTCTCTGAATTTTTG 2520
813  GlyGluAsnAsnLysAlaSerGluIleSerAlaLeuValLeuAsnAspLeuArgAspLeuArgSerLeuValSerGluPheLeu 840
2521 AGTGCAATGACTAGTTTCGTTACCGTATCAACGATTGGACTAATTTGGGCGTTAGTATCGGGCTGGAAGTTAAGTTTGGTTGT 2604
841  SerAlaMetThrSerPheValThrValSerThrIleGlyLeuIleTrpAlaLeuValSerGlyTrpLysLeuSerLeuValCys 868
2605 ATTTCGATGTTTCCACTCATAATTATATTTTCAGCAATATATGGAGGCATTTTACAAAAATGCGAAACAGATTATAAGACATCT 2688
869  IleSerMetPheProLeuIleIleIlePheSerAlaIleTyrGlyGlyIleLeuGlnLysCysGluThrAspTyrLysThrSer 896
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Fig. 4. Restriction map, nucleotide sequence and deduced amino acid sequence of the *STE6* gene. **A** shows a partial restriction map of the *STE6* gene and its immediate flanking sequences. The restriction endonuclease cleavage sites most relevant to this study are indicated. Hatched box indicates coding region of the gene. Solid vertical bars indicate approximate positions of putative transmembrane segments. Open boxes indicate regions of internal homology that are also homologous to prokaryotic transport proteins and to mammalian P-glycoproteins (*mdr* gene products). **B** shows nucleotide and deduced amino acid sequence of the gene. Nucleotide +1 is the A of the ATG translational start codon. Putative transmembrane segments are underlined. Consensus sites for addition of *N*-linked oligosaccharides (-Asn-X-Thr/Ser-) are indicated by the solid triangles. Boxed areas demarcate the regions of internal homology that are also homologous to bacterial permeases and mammalian *mdr* gene products.

In a 4 min pulse, *MAT***a** cells carrying the *MF***a***1* plasmid and the *MF***a***1*–*STE6* composite plasmid produced virtually identical amounts of intracellular pro-**a**-factor (Figure 3A and B). In the cells overexpressing *MF***a***1* alone, however, the amount of precursor steadily declined during the chase, but was not accompanied by a commensurate increase in the amount of either intracellular processed **a**-factor or extracellular mature **a**-factor (Figure 3A). We have observed this inefficient processing and secretion in several different wild-type *MAT***a** strains derived from different origins, including W303-1A, CG378 and TDY21-3D (Sterne, 1989; R.E.Sterne, L.C.Blair and J.Thorner, in preparation) (Table I). When expressed as a fraction of the pro-**a**-factor initially synthesized, the amount of mature **a**-factor secreted is only 3–5% of the radioactivity incorporated into the precursor (Figure 3C and D). Theoretically, if all of the precursor were processed and secreted, then half of the ^{35}S incorporated into pro-**a**-factor should appear in mature **a**-factor (because the pheromone has only one of the two sulfur atoms present in the precursor). These observations suggest that, in the absence of efficient export, intracellular precursor and/or its processed forms are subject to non-specific degradation. This proteolysis may occur in the yeast vacuole because *MAT***a** *pep4* mutants which are deficient in the major vacuolar proteases (Jones, 1984) convert a significantly larger fraction of pro-**a**-factor into the secreted pheromone (Sterne, 1989).

In the cells overexpressing both *MF***a***1* and *STE6*, the amount of precursor also declined steadily during the chase; but, in contrast to cells overexpressing *MF***a***1* alone, the disappearance of pro-**a**-factor was accompanied by a concomitant increase in extracellular **a**-factor (Figure 3B). The increase in extracellular pheromone over that seen in cells overexpressing *MF***a***1* alone was nearly an order of magnitude, and approached about half the theoretical limit expected for complete conversion of the precursor initially synthesized (Figure 3C and D). Even more strikingly, when both *MF***a***1* and *STE6* were co-expressed from a multicopy plasmid, **a**-factor began to appear extracellularly during the pulse period, whereas in cells overexpressing *MF***a***1* alone, extracellular **a**-factor could only be detected 8 min after the chase (Figure 3A and B). When plotted as a function of time (Figure 3C and D), these data indicate that overexpression of *STE6* increased the rate of **a**-factor export nearly 5-fold. Hence, elevation of the level of the *STE6* gene product increased both the rate and the extent of **a**-factor secretion.

Complete nucleotide sequence of the STE6 gene

To gain additional insight into the possible function of the *STE6* gene product, a total of 4500 nucleotides was sequenced from the genomic DNA containing the *STE6* gene, from 102 bp upstream of the *Spe*I site to 115 bp downstream of the *Ssp*I site (Figure 4), and has been deposited in GenBank (accession no. M26376). Within this region there is an open reading frame of 3870 nucleotides (Figure 4), which could encode a protein of 1290 amino acids (calculated mol. wt 144 774). The N-terminal sequence obtained is in complete agreement with the first 41 amino acids of the *STE6* coding region which were reported previously by Wilson and Herskowitz (1986) when they determined the nucleotide sequence of 469 nucleotides from the 5′ flanking region of this gene. The size of the *STE6* coding region is also consistent with the length of the *STE6* transcript (4 kb)

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Ste6a  325 E K I M T L L K D G S K - R N P L N K T V A H Q F P L D Y A T S D L T F A N
Ste6b 1021 E K H N T L E V E N N N A R - T V G - I A G H T Y H G K E K K P I V S I Q N

Ste6a  362 V S F S Y P S R P S E A V L K N V S L N - F S A G Q F T F I V G K S G S G K
Ste6b 1057 L T F A Y P S A P T A F V Y K N M N F D M F - C G Q T L G I I G E S G T G K

Ste6a  399 S T L S N L L L R F Y D G Y N G S I S I N G H N I Q T I D Q K L L I - E N I
Ste6b 1094 S T L V L L L T K L Y N C E V G K I K I D G T D V N D W N L T S L R K E - I

Ste6a  436 T V V E Q R C T L F N D T L R K N I L L G S T D S V R N A D C S T N E N R H
Ste6b 1131 S V V E Q K P L L F N G T I R D N L T Y G L Q D E I L E I E M Y D A L K Y V

Ste6a  474 L I K D A C Q M A L L D R F I L D L P D G L E T L I G T G G V T L S G G Q Q
Ste6b 1169 G I H D F V I - - - - - - S S P Q G L D T R I D T T L - - - - - - - L S G G Q A

Ste6a  512 Q R V A I A R A F I R D T P I L F L D E A V S A L D I V H R N L L M K A I R
Ste6b 1196 Q R L C I A R A L L R K S K I L I L D E C T S A L D S V S S S I I N E I V K

Ste6a  550 H W R K G K T T I I L T H E L S - Q I E - S D D Y L Y L M K E G E V V E S G
Ste6b 1234 K G P P A L L T M V I T H - - S E Q M M R S C N S I A V L K D G K V V E R G

Ste6a  586 T Q S E L L A D P T T T F S T W Y H L Q N D . . .
Ste6b 1270 N F D T L Y N N R G E L F Q I V S N - Q S S.
```

Fig. 5. Extensive internal homology in the *STE6* gene product. The two hydrophilic domains (Ste6a and Ste6b) indicated (positions correspond to the boxed residues shown in Figure 4) have been aligned to maximize the number of identities (boxed). The dash symbols (−) indicate single residue gaps inserted to optimize the alignment.

identified by hybridization of fragments from the coding region to poly(A)$^+$ RNA isolated from *MAT***a** cells (data not shown), and is in agreement with the transcript length reported previously by Wilson and Herskowitz (1984).

Hydropathy analysis of the amino acid sequence using the parameters of either Hopp and Woods (1981) or Kyte and Doolittle (1982) revealed 12 potential membrane-spanning domains (Figure 4). Further, multiple matches (14 total) to the consensus site for addition of Asn-linked oligosaccharides are found in the sequence. These features are frequently observed in polytopic membrane glycoproteins.

The STE6 gene product is a transport protein

The predicted *STE6* protein contains two domains (residues 325−607 and 1021−1290) that share 33% identity with each other (Figure 5). The homology between these two domains is increased to >50% if standard conservative amino acid substitutions are considered. When compared with the protein sequences available in various databases, each of these domains of internal homology in *STE6* was found to have significant similarity over its entire length to two different classes of known membrane-associated transport proteins (Figure 6A and B).

First, both domains of the *STE6* gene product share 21−31% identity with component proteins of well-characterized, periplasmic binding protein-dependent permeases from prokaryotic cells, including *pstB* (phosphate transport), *malK* (maltose transport), *rbsA* (ribose transport), *hisP* (histidine transport) and *opp*D and *opp*F (oligopeptide transport) (Ames, 1986). In general, however, the homology of *STE6* is greatest (34−37% identity) with those members of this family of bacterial transport proteins that are involved in the export of polypeptides (as opposed to those involved in the uptake of ions, sugars, amino acids or other small molecules). Components of bacterial transport systems of this type include gene products involved in the secretion of: a 3300 mol. wt peptide antibiotic, microcin B17, by *Escherichia coli (mcbF)* (del Carmen Garrido *et al.*, 1988); a 45 000 mol. wt calmodulin-stimulated adenylate cyclase by the whooping cough pathogen, *Bordetella pertussis (cyaB)* (Glaser *et al.*, 1988); and, a 110 000 mol. wt hemolysin from pathogenic strains of *E.coli (hlyB)* (Felmlee *et al.*, 1985). Alignments of the domains of *STE6* to some of these prokaryotic gene products are shown in Figure 6A and B.

The role of these particular bacterial proteins in membrane transport appears to be as peripheral membrane components that intimately associate on the cytosolic side of the plasma membrane with separate, much more hydrophobic, integral membrane proteins (see, e.g. Gallagher *et al.*, 1989). Strong evidence indicates that the function of these peripheral proteins is to provide energy coupling for the transport process via binding and hydrolysis of ATP. Indeed, these proteins all possess sequences highly homologous to the residues known to comprise a primary ATP-binding site in the β subunits of proton-translocating membrane ATPases (Futai *et al.*, 1989); and, in some cases, these proteins can be covalently labeled by various reactive ATP derivatives (Hobson *et al.*, 1984; Higgins *et al.*, 1985) or transport in an *in viro* system can be stimulated by ATP (Bishop *et al.*, 1989; Dean *et al.*, 1989). The putative ATP binding regions of the prokaryotic proteins are completely conserved in both domains of the *STE6* gene product (G x SG x GKS beginning at residues 392 and 1087, and L xx DE xx SALD beginning at residues 527 and 1211).

Remarkably, the hydrophilic domains of *STE6* gene product are most closely related (40% identity) to the hydrophilic domains of a second class of membrane transporters, the mammalian multiple drug-resistant (*mdr*) gene products (Figure 6A and B). The similarity between *STE6* and the *mdr* proteins in these regions rises to >60% homology if conservative amino acid substitutions are allowed. Like the bacterial transport proteins mentioned above, the *mdr* gene products are ATP-binding proteins (Cornwell *et al.*, 1987) and possess ATPase activity (Hamada and Tsuruo, 1988); however, like *STE6*, *mdr* proteins have two ATP-binding domains. Unlike the ATP-binding components of the prokaryotic permeases, but like the *STE6* gene product, the *mdr* proteins also contain 12 potential transmembrane segments. The *mdr* gene products have been shown to be integral membrane glycoproteins, the so-called 'P-glycoproteins' (Ueda *et al.*, 1986). No significant homology could be found by computer between the hydrophobic domains of *STE6* and the integral membrane proteins that are components of the bacterial permeases. In contrast, the region of the *STE6* gene product that contains the first six transmembrane segments (Ste6M1 domain) shares significant homology (22% identity) over its entire length with the corresponding membrane-spanning segments of human and mouse *mdr1* proteins; similarly, the region of *STE6* containing the second set of six transmembrane helices (Ste6M2 domain) shares 20% identity with the corresponding region of human *mdr1* (Figure 7). Thus, the yeast *STE6* gene product and the *mdr* proteins from mammalian cells possess significant homology throughout their entire primary sequence, are nearly identical in length, contain two ATP-binding regions, and are presumably anchored in the plasma membrane via two sets of six membrane-spanning helices.

Phenotype of cells with ste6 null mutations

The *STE6* gene is normally expressed only in *MAT***a** cells (Wilson and Herskowitz, 1984), suggesting that *MAT*α cells do not require this protein for viability. To determine if the *STE6* locus is an essential gene, a deletion mutation (*ste6*Δ :: *HIS3*), in which 87% of the coding sequence was removed

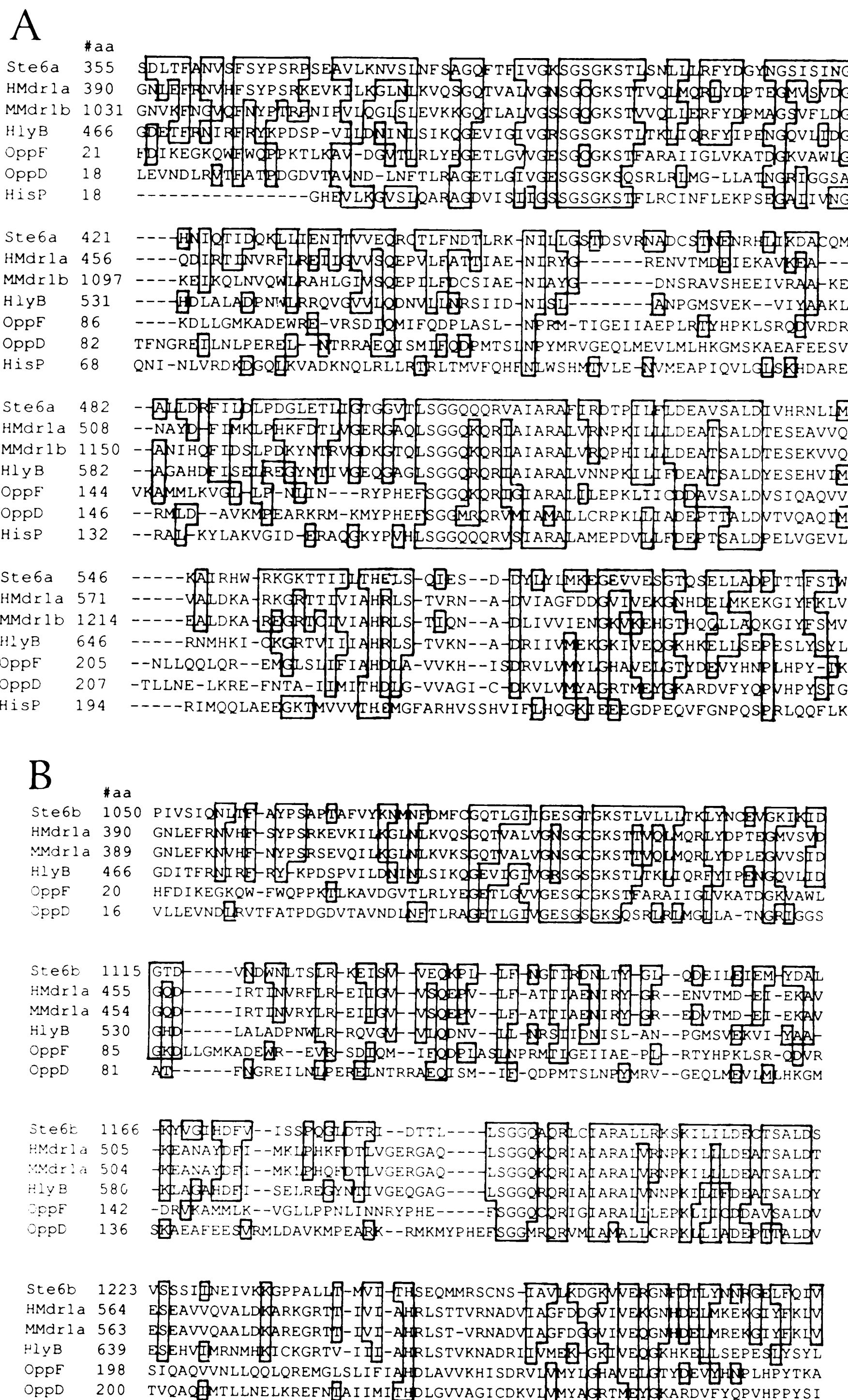

Fig. 6. The *STE6* gene product is homologous to both prokaryotic and eukaryotic transport proteins. Each of the domains of internal homology (Ste6a and Ste6b) are compared separately (**panels A** and **B** respectively) to a number of known membrane bound transporters. Alignments were generated by the FASTP algorithm of Lipman and Pearson (1985). Identities are boxed. Sequences for the bacterial permeases can be found in the following citations: *hisP*, histidine transport (Higgins *et al.*, 1982); *oppD*, oligopeptide transport (Higgins *et al.*, 1985); *oppF*, oligopeptide transport (Hiles *et al.*, 1987); and *hlyB*, hemolysin export (Felmlee *et al.*, 1985). Sequences for the mammalian multiple drug resistance translocators can be found in the following citations: human *mdr1* (Chen *et al.*, 1986) and mouse *mdr1* (Gros *et al.*, 1986).

(from the *Stu*I site to the *Sna*BI site, Figure 4) and replaced by the *HIS3* gene, was constructed and used to transform a diploid recipient. Appropriate restriction endonuclease digestion and hybridization analysis of DNA from candidate transformants confirmed that the transformed cells possessed one homolog carrying the intact *STE6* gene and the other carrying the disrupted locus (see Materials and methods). Upon sporulation and tetrad dissection, both *MAT***a** *ste6Δ* :: *HIS3* and *MATα ste6Δ* :: *HIS3* spore clones were readily recovered and were obtained in equal numbers. Cells carrying the *ste6Δ* :: *HIS3* mutation displayed no obvious growth defect in either liquid or solid medium (data not shown). Thus, a functional *STE6* gene is not required for either germination or viability of haploid cells.

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          #aa
Ste6M1    26  MIMII-GTVATGLV-PAITSILTGRVFDLLSVFVANGSHQGLYSQLVQRSMAVMALGAASVPVM
MMdr1M1   66  LLMLVFGNMTDSFT-KAEASILPSITNQSGPNSTLIISNSSLEEEMAIYAYYYTGIGAGVLIVA
HMdr1M1   67  LMMLVFGEMTDIFANAGNLEDLMSNITN-RSDINDTGFFMNLEEDMTRYAYYYSGIGAGVLVAA

Ste6M1    90  WLSLTSWMHIGERQGFRIRSQILEAYLEEKPMEWYDNNEKLLGDF-IQINRCVEELRSSSAEAS
MMdr1M1  129  YIQVSLWCLAAGRQIHKIRQKFFHAIMNQE-IGWFDVHD--VGELNIRLTDDVSKINDGIGDKI
HMdr1M1  130  YIQVSFWCLAAGRQIHKIRKQFFHAIMRQE-IGWFDVHD--VGELNIRLTDDVSKINEVIGDKI

Ste6M1   153  AITFQNLVAICALLGTSFYYSWSLTLIIILCSSPIITFFAVMFSRMIHVYSEKENSETSKAAQLL
MMdr1M1  190  GMFFQSITTFLAGFIIGFISGWKLTLVILAVSPLIGLSSALWAKVLTSFTNKELQAYAKAGAVA
HMdr1M1  191  GMFFQSMATFFTGFIVGFTRGWKLTLVILAISPVLGLSAAVWAKILSSFTDKELLAYAKAGAVA

Ste6M1   217  TWSMNAAQLVRLYCTQRLERKKFKEIILNCNTFFIKSCFFVAANAGILRFLTLTMFVQGFWFGS
MMdr1M1  254  EEVLAAIRTVIAFGGQQKELERYNKNLEEAKNVGIKKAITASISIGIAYLLVYASYALAFWYGT
HMdr1M1  255  EEVLAAIRTVIAFGGQKKELERYNKNLEEAKRIGIKKAITANISIGAAFLLIYASYALAFWYGT


Ste6M2   792  VMDLRNEVMEKLTRKNMDWFSGENNKASEISALVLNDLRDLRSLVSEFLSAMISFVIVSTIGLI
MMdr1M1  143  IHKIRQKFFHAIMNQEIGWF--DVHDVGELNTRLTDDVSKINDGIGDKIGMFFQSIITFLAGFI
HMdr1M2  785  TKRLRYMVFRSMLRQDVSWFDDPKNTTGALTTRLANDAAQVKGAIGSRLAVIIQNIANLGTGII

Ste6M2   856  WALVSGWKLSLVCISMFPLIIIFSAIYGGIIQKCETDYKTSVAQLENCLYQIVTNIKTIKCLQA
MMdr1M1  205  IGFISGWKLTLVILAVSPLIGLSSALWAKVLTSFTNKELQAYAKAGAVAEEVLAAIRTVIAFGG
HMdr1M2  849  ISFIYGWQLTLLLLAIVPIIAIAGVVEMKMLSGQALKDKKELEGAGKIATEAIENFRTVVSLTQ

Ste6M2   920  EFHFQLTYHDLKIKMQQIASKRAIATGFGISMTNMIVMCIQAIIYYYGLKLVMIHEYTSKEMFT
MMdr1M1  269  QQKELERYNKNLEEAKNVGIKKAITASISIGIAYLLVYASYALAFWYGTSLVLSNEYSIGEVIT
HMdr1M2  913  EQKFEHMYAQSLQVPYRNSLRKAHI--FGITFSFTQAMMYFSYAGCFRFGAYLVAHKLMSFEDV

Ste6M2   984  --TFTLILFTIMSCTSLVSQIPDISRGQRAASWIYRILD
MMdr1M1  333  --VFFSILLGTFSIGHLAPNIEAFANARGAAFEIFKIID
HMdr1M2  975  LLVFSAVVFGAMAVGQVSSFAPDYAKAKISAAHIIMLIE
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Fig. 7. The transmembrane domains of yeast *STE6* gene product and mammalian *mdr* proteins are homologous. Identities between the two hydrophobic domains of *STE6*, Ste6M1 (residues 26–317) and Ste6M2 (residues 716–1002), and the corresponding domains of mammalian *mdr* gene products are boxed. See text for further explanation.

However, the *ste6*Δ :: *HIS3* mutation did have two striking phenotypic consequences in *MAT***a** cells. First, this null allele prevented *MAT***a** haploids from releasing any extracellular **a**-factor (Figure 8A). Two other deletion–insertion mutations we constructed (*ste6*Δ*N* :: *TRP1* and *ste6*Δ*N* :: *LEU2*) also greatly reduced the amount of **a**-factor secreted (Figures 1 and 8A). Unlike the *ste6*Δ :: *HIS3* null mutation which eliminates any detectable *STE6* mRNA, these latter two alleles produce copious amounts of a 3 kb transcript corresponding to the C-terminal two-thirds of the coding region (perhaps driven by promoters fortuitously created by the insertion constructions) (data not shown). Similarly, a *ste6–lacZ* fusion (in which *lacZ* has been inserted in-frame to a *Pst*I site corresponding to codon 384 of the *STE6* sequence) also greatly reduces, but does not eliminate completely, **a**-factor secretion (Wilson and Herskowitz, 1984); however, this construction also expresses several transcripts complementary to the C-terminal two-thirds of the *STE6* gene (Wilson and Herskowitz, 1984). Given the internal homology in the *STE6* sequence (Figures 4 and 5), the simplest explanation for the low level of pheromone released by *MAT***a** cells carrying the *ste6*Δ*N* :: *TRP1* and *ste6*Δ*N* :: *LEU2* alleles (and the *ste6–lacZ* fusion) is that there is residual *STE6* activity resulting from partial function of aberrant forms of the protein that include the C-terminal two-thirds of the molecule but are truncated at their N-terminal end.

Because of the similarity of the yeast *STE6* gene product to the mammalian *mdr* proteins, we also determined whether *ste6* null mutants displayed any increased sensitivity to various growth inhibitory agents. Unfortunately, the drugs typically used to select *mdr* variants in animals cells (including vinblastine, adriamycin and colchicine) have little or no effect on the growth of yeast cells, even at the limits of solubility of these compounds. On the other hand, we found that *MAT***a** *STE6* cells are reproducibly less sensitive than isogenic *MAT***a** *STE6*Δ :: *HIS3* cells to the growth inhibitory action of a peptide antibiotic, valinomycin, that is an effective inhibitor of yeast growth when present at the millimolar level (Figure 8B). If this effect is due to the absence of *STE6* function, then cells not expressing the *STE6* gene for other reasons should also be more sensitive to valinomycin. Indeed, *MAT*α *STE6* cells displayed the same sensitivity to valinomycin as the *MAT***a** *ste6*Δ :: *HIS3* mutant (Figure 8B). Conversely, *MAT***a** cells carrying the *STE6* gene on a multicopy plasmid displayed even greater resistance to valinomycin than *MAT***a** cells expressing *STE6* from its normal chromosomal locus (data not shown).

In contrast, for drugs of other chemical classes that also block yeast growth (including cycloheximide and tunicamycin), there were small or no effects on sensitivity caused by either deletion or overexpression of the *STE6* gene (Figure 8B).

Discussion

We have shown here that the *STE6* gene product is required for, and is rate-limiting, in the release of a peptide mating pheromone, **a**-factor, by *MAT***a** cells of the yeast *S.cerevisiae*. Given that pro-**a**-factor appears to be synthesized and processed in the cytosol (Sterne, 1989; R.E.Sterne, L.C.Blair and J.Thorner, in preparation) and given the striking homology between the *STE6* gene product and known membrane-associated transport proteins from both pro-

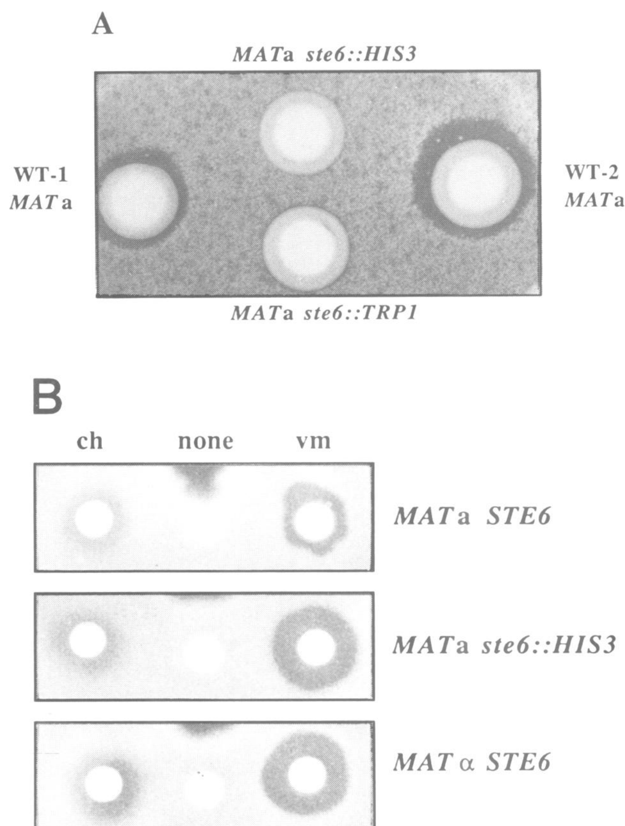

Fig. 8. Phenotypic consequences of a *ste6* null mutation. (**A**) Bioassay of **a**-factor production. Equal numbers of cells of the indicated strains were spotted on sterile filter disks and placed on the surface of a YPD plate that had been covered with top agar seeded with $\sim 10^5$ cells of a *MATα sst2* tester strain and then incubated at 30°C for 2 days (see Materials and methods for details). (**B**) Bioassay of drug sensitivity. YPD plates were covered with top agar seeded with $\sim 10^5$ cells of the strains to be tested. Sterile filter disks containing different concentrations of various inhibitors dissolved in ethanol were then placed on the surface and the plates incubated at 30°C for 2 days (see Materials and methods). ch, 5 μl of 100 μM cycloheximide; vm, 5 μl of 5 mM valinomycin; none, 5 μl of ethanol.

karyotic and mammalian cells, it seems reasonable to propose that the function of the *STE6* protein in **a**-factor production is to serve as an ATP-driven transporter that actually translocates the peptide through the plasma membrane.

If this is indeed the case, then the *STE6* gene product should be an **a**-factor-binding protein. Yeast cells possess at least one other known **a**-factor-binding protein, the **a**-factor receptor (*STE3* gene product) expressed on the surface of *MATα* cells (Hagen *et al.*, 1986). In fact, two segments of the *STE6* sequence, each over 40 amino acids long (residues 663–708 and 1002–1042), possess significant similarity to regions of the *STE3* protein (Figure 9A). Assuming that **a**-factor approaches its receptor from the external face of the plasma membrane, and given the proposed seven transmembrane helices structure of the receptor (Hagen *et al.*, 1986), only the segment of *STE3* following the sixth putative transmembrane segment (Figure 9A) will be on the exocellular side of the membrane and thus available for **a**-factor recognition. Conversely, because *STE6* is required for **a**-factor export, it seems likely that it should recognize **a**-factor on the cytoplasmic side of the plasma membrane. It is also likely that the two putative ATP-binding domains are also disposed on the cytosolic face of the membrane. Furthermore, because of the high net positive charge (+5) of the first 25 residues of the *STE6* sequence, the N terminus of the protein probably also resides on the cytoplasmic side of the membrane (Hartmann *et al.*, 1989). These considerations, and analogy with the mammalian *mdr* gene products which it resembles most closely, suggest a model for the topology of the *STE6* protein within the membrane (Figure 9B). This model has two additional appealing features. First, it places the only consensus site for addition of *N*-linked carbohydrate that exists in the N-terminal region (Figure 4) in a hydrophilic loop on the extracellular face of the plasma membrane. This site is in a position identical to the single glycosylation site found in the mammalian P-glycoproteins (Chen *et al.*, 1986; Endicott and Ling, 1989). All the other Asn-x-Thr/Ser sites in *STE6* would reside on the cytosolic side of the membrane and, therefore, would presumably remain unglycosylated. Secondly, both of the segments of similarity between *STE6* protein and the **a**-factor receptor (Figure 9A) would lie on the cytoplasmic face of the membrane—the first just preceding the seventh transmembrane domain, and the second just following the last transmembrane domain (Figure 9B). It is not hard to envisage that tertiary folding of the *STE6* protein might bring these segments into juxtaposition.

Ever since the molecular characterization of the mammalian *mdr* gene products, it has been appreciated that they bear considerable resemblance, especially in the ATP-binding domains, to constituent proteins of prokaryotic permeases of the periplasmic binding protein-dependent class of transport systems (Gerlach *et al.*, 1986; Gros *et al.*, 1986). This homology immediately suggested that the mechanism of multiple drug resistance conferred by the mammalian P-glycoproteins was due to their ability to pump the drugs out of the cell, thereby keeping the intracellular concentration of these agents below the threshold for their toxic or growth inhibitory effects. All *mdr* strains carry either amplified or overexpressed versions of a cellular *mdr* gene and produce the cognate P-glycoprotein at a higher than normal level, suggesting that elevation of activity is required to achieve pumping of these xenobiotic compounds, which are presumably not the natural cellular substrates for the *mdr* gene products. Additional support for this view of *mdr* function comes from the fact that resistance to the anti-malarial drug, chloroquine, in a unicellular eukaryote, the malarial parasite *Plasmodium falciparum*, is attributable to amplification of an *mdr*-like gene (Wilson *et al.*, 1989).

Despite this somewhat satisfying picture, the endogenous substrates for the *mdr* class of eukaryotic transporters have remained elusive. Because the yeast *STE6* gene product is required for export of an oligopetide normally produced by the cells, our finding that the *STE6* gene is also clearly a member of the *mdr* class of transporters may provide the missing link in interpreting the normal physiological function of the *mdr* proteins in eukaryotic cells. Our results raise the possibility that the true role of the *mdr* gene products is to serve as transmembrane translocators of proteins via a route that is independent of the standard secretory pathway. Indeed, there are quite a number of eukaryotic proteins that are released from animals cells which have no obvious signal sequence or other strikingly hydrophobic domain and whose mechanism of secretion does not appear to involve the secretory pathway, including interleukin-1α and -1β (March *et al.*, 1985) and both acidic and basic fibroblast growth factors (Burgess and Maciag, 1989). For lack of a better explanation, appearance of such proteins in the extracellular

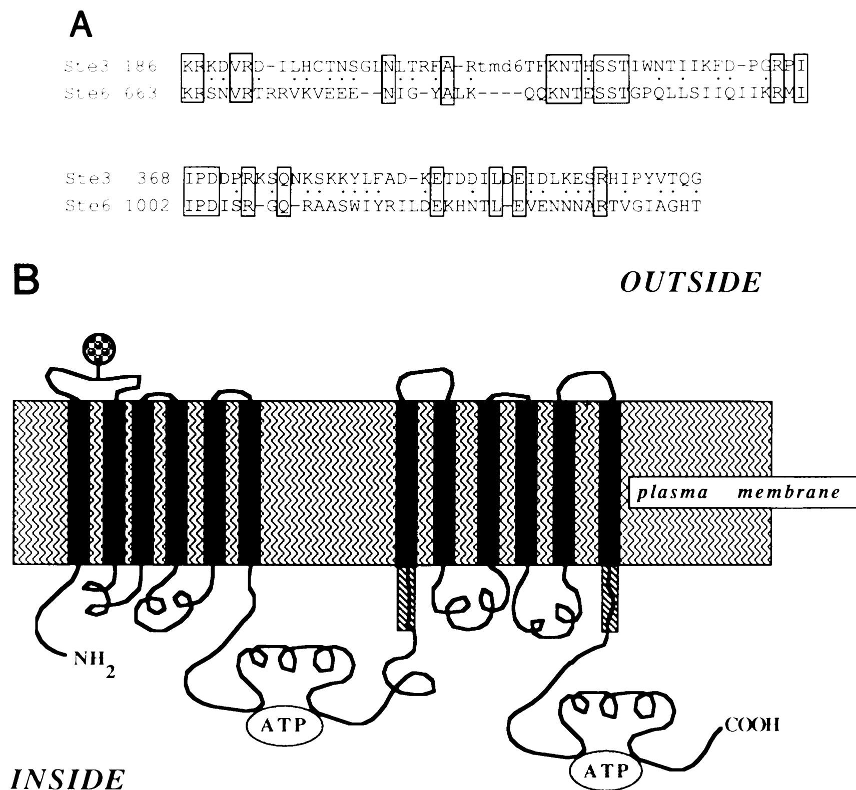

Fig. 9. Model for the *STE6* gene product in the yeast plasma membrane. (**A**) Homology between *STE6* and the **a**-factor receptor (*STE3* gene product). Identities are boxed and conservative amino acid substitutions, derived from the PAM matrix (Lipman and Pearson, 1985), are indicated by a dot. (**B**) Schematic representation for the disposition of the *STE6* protein in the plasma membrane. Solid line represents polypeptide chain. Solid vertical bars indicate transmembrane helices. Dotted ball represents a single Asn-linked carbohydrate chain. Hatched bars just preceding the seventh membrane-spanning segment and just following the last transmembrane segment indicate the regions of homology between *STE6* and the **a**-factor receptor (see **A**). The two hydrophilic ATP-binding domains are marked by the ovals.

fluid has been attributed to damage and death of the producing cells; however, we propose that these types of proteins may exit the cell via the agency of the *mdr* transporters. This hypothesis should be readily testable.

There appears to be a small, but ever growing, family of *mdr*-related genes that can be identified, even within the genome of a single organism (Van der Blick *et al.*, 1987; Endicott and Ling, 1989). Thus, there may be different classes of plasma membrane-associated P-glycoproteins for specific types of proteins. Alternatively, *mdr* transporters may be differentially regulated, spatially or temporally or in a tissue-specific manner, during development. A third, even more intriguing possibility is that there may be more distantly related *mdr*-like proteins resident in cellular membranes other than the plasma membrane. These molecules could be responsible for the translocation of polypeptides between all intracellular compartments in a eukaryotic cell. Indeed, the 'machinery' responsible for transposing any polypeptide across any cellular membrane has remained, until now, uncharacterized. For example, it is clear that signal recognition particle (SRP) and SRP receptor target a secretory protein to the endoplasmic reticulum membrane (Römisch *et al.*, 1989), and that ATP-dependent protein unfolding enzymes of the *hsp70* class are required to put the secretory molecule in a transport-competent state (Deshaies *et al.*, 1988); but, the actual mechanism by which the polypeptide traverses the membrane has remained obscure. We propose, therefore, that members of the *mdr* gene family may serve this function.

If this scenario is correct, there are many potential implications. For example, *mdr*-like activities could explain how soluble cytosolic proteins are imported into the lysosome-like vacuole of yeast to initiate their proteolytic degradation. In animal cells, perhaps there is an *mdr*-like protein in the lysosomal membrane that is specific for recognizing ubiquitinated proteins. It may even be possible that the secretory pathway itself may have such proteins, for example to import processed peptide antigens into the secretory system so that productive complexes with the MHC locus receptors can be formed and displayed on the surface of antigen-presenting cells.

Yeast **a**-factor is a lipopeptide, and the drugs carried by the *mdr* transporters are, in general, large, planar molecules with aromatic rings. Furthermore, P-glycoproteins are abundant in tissues that produce steroids and related compounds. Thus, certain *mdr* transporters may require that their substrates possess some hydrophobic character or, perhaps, that their substrates be conjugated (either transiently or permanently) to a lipophilic substituent to achieve efficient export.

Materials and methods

Yeast strains, growth conditions and DNA-mediated transformation

S.cerevisiae strains used in this study are listed in Table I. Synthetic medium (SD), supplemented with nutrients appropriate for maintenance of plasmids, or rich medium (YPD), were prepared as described (Sherman *et al.*, 1986). Low sulfate minimal medium (LSM+) was prepared as described previously

(Julius *et al.*, 1983), but was supplemented with all L-amino acids [at the levels recommended by Sherman *et al.* (1986)] except cysteine and methionine. Top agar contained 0.75% agar (Difco) in YPD. DNA-mediated transformation of yeast cells was performed by the lithium acetate procedure (Ito *et al.*, 1983). Sonicated calf thymus DNA (20–50 μg per transformation) was used as a carrier to enhance transformation efficiency. Strain construction and tetrad analysis was performed by standard procedures (Sherman *et al.*, 1986).

Plasmids and bacterial strains

pAB182 (provided by A.Brake) consists of a 1.6 kb genomic *Eco*RI–*Xba*I fragment containing the *MF***a***1* gene and its promoter (Brake *et al.*, 1985) inserted, after addition of *Bam*HI linkers, into the *Bam*HI site of a 2 μm DNA-based multicopy yeast vector (pAB24) which also has the *URA3* gene and the *LEU2*^{d} allele (Beggs *et al.*, 1981) as selectable markers. pRE4 was constructed by ligating a 6 kb genomic *Hin*dIII–*Sal*I fragment containing the *STE6* gene and its promoter (Wilson and Herskowitz, 1984), excised from YIp5-STE6 (provided by K.Wilson and I.Herskowitz), into the polylinker of pGEM1^{TM} (Promega) that had been digested with *Hin*dIII and *Sal*I. pKK1 consists of the 1.6 kb *Bam*HI fragment containing the *MF***a***1* gene inserted into the *Bam*HI site of YEp352 (Hill *et al.*, 1986). pKK16 was constructed by inserting the 6 kb *Hin*dIII–*Sal*I fragment containing the *STE6* gene into pKK1 that had been digested with *Hin*dIII and *Sal*I. pRE10 consists of a 4 kb genome *Bam*HI fragment containing the *STE14* gene (L.Blair and R.E.Sterne, unpublished results) inserted into pAB182 at a unique *Bgl*II site that resides within the *MF***a***1*-containing insert ~420 bp upstream from the TATA box for *MF***a***1* transcription (Dolan *et al.*, 1989).

Standard methods were used for the propagation and isolation of plasmid DNA (Maniatis *et al.*, 1982). *E.coli* K-12 strains HB101, DH1, DG98, or XL-1 Blue (Stratagene) were used for manipulation and amplification of plasmids, where appropriate.

Radiolabeling, cell lysis and immunoprecipitation

The procedures used for labeling of cells with $^{35}SO_4^{2-}$, preparation of cell lysates, extraction of **a**-factor from culture medium and from the walls of the culture vessel, immunoprecipitation with anti-**a**-factor antibodies, electrophoresis of **a**-factor-related peptides in polyacrylamide gradient gels, *in vitro* translation of synthetic *MF***a***1* mRNA generated by transcription of the *MF***a***1* gene under control of a *Salmonella typhimurium* bacteriophage SP6 promoter, and analysis of the gels by autoradiography and densitometry are described in detail elsewhere (Sterne, 1989; R.E.Sterne, L.C.Blair and J.Thorner, in preparation).

Recombinant DNA methods and nucleotide sequence analysis

Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs, Pharmacia or Boehringer Mannheim. All radiochemicals were purchased from Amersham. Cycloheximide, valinomycin, and ampicillin were from Sigma; hydrazine, dimethylsulfate, and formic acid were from Aldrich; and piperidine was obtained from Serva.

Yeast genomic DNA was isolated as described (Sherman *et al.*, 1986). After digestion with various restriction enzymes, DNA was fractionated in 0.7% agarose gels, transferred to nitrocellulose filters (0.45 μm pore size, Schleicher & Schuell) and hybridized to radioactively labeled DNA fragments (Southern, 1975). Total RNA and poly(A)$^+$ RNA was isolated according to Sherman *et al.* (1986). For hybridization analysis of RNA, poly(A)$^+$ RNA (2–5 μg) was fractionated in a 1.2% agarose gel containing 2.2 M formaldehdye (Williams *et al.*, 1985). RNA was transferred to Hybond-C^{TM} membranes (Amersham) and hybridized to radioactively labeled restriction fragments of *STE6*. Restriction fragments of *STE6* used for hybridization analysis were purified on agarose gels (Maniatis *et al.*, 1982), denatured, and labeled with [^{32}P]dCTP using Klenow polymerase and random primers (Amersham). Unincorporated nucleotides were removed from reaction mixtures using commercially available Nick^{TM} columns (Pharmacia).

For sequence analysis of *STE6*, various restriction fragments were subcloned into the polylinker of Bluescript phagemids (Stratagene) and nested deletions were generated using an *Exo*III/mung bean nuclease kit and conditions recommended by the supplier (Stratagene). Enzymatic sequencing of DNA (Sanger *et al.*, 1980) was carried out on double-stranded plasmid DNA using a Sequenase^{TM} kit (United States Biochemicals). The second strand was sequenced by the chemical degradation method (Maxam and Gilbert, 1980). For chemical sequencing, DNA of various subclones was end-labeled at the 3′ termini of various restriction sites using reverse transcriptase and [^{32}P]dNTPs.

Bioassays of a-factor production and drug sensitivity

a-Factor secretion by different strains was measured by replica-plating patches of the *MAT***a** cells to be tested onto indicator lawns which were prepared by spreading a suspension of *MAT*α *sst2* cells on the surface of a YPD plate, or by spotting an identical number of freshly grown *MAT***a** cells onto a sterile filter disk which was then laid on the indicator lawn. **a**-Factor causes G_1 arrest in cells of opposite mating type. Because *MAT*α *sst2* cells are ~100 times more sensitive to pheromone than wild-type cells (Chan *et al.*, 1983) and cannot recover from pheromone-induced G_1 arrest (Chan and Otte, 1982), diffusion of **a**-factor in the top agar results in a clear zone ('halo') in the lawn surrounding the **a**-factor producers. The size of the halo, therefore, is a meausre of the amount of pheromone secreted. Indicator lawns were prepared by adding 3 ml of top agar in YPD, equilibrated at 45°C, to 0.4 ml of a suspension (OD_{600} = 0.5) of *MAT*α *sst2* cells and pouring this mixture on prewarmed (37°C) YPD plates.

Drug sensitivity of cells was assessed using a modified halo assay as follows. A lawn of the cells to be tested was prepared as decribed above and plates were left to sit on the bench until the top agar solidified. Sterile filter disks were placed on the lawn and 5–15 μl of solutions of various drugs dissolved in ethanol were spotted on the filter disk. Plates were incubated at 30°C for 24–36 h. Clear zones surrounding the filter disks were caused by growth inhibition and the halo size reflects the apparent sensitivity of cells to different drugs (Cooper, 1963).

Gene disruptions

STE6 was deleted from its corresponding chromosomal locus by one-step gene replacement (Rothstein, 1983) as follows. pRE4 was digested with *Stu*I and *Sna*BI (which both generate blunt ends) to remove a 3.4 kb fragment representing 87% of the *STE6* coding region (Figure 4). The deleted segment was replaced with a 1.8 kb *Bam*HI fragment containing the *HIS3* gene, excised from pWJ79 (provided by K.Struhl) and converted to flush ends by incubation with the Klenow fragment of *E.coli* DNA polymerase I, by blunt-end ligation, to generate plasmid pKK06. A linear 2.6 kb *Spe*I/*Ssp*I restriction fragment from pKK06, bearing the *ste6* :: *HIS3* construct, flanked by 480 bp and 415 bp of *STE6* sequence at its 5′ and 3′ ends, respectively (Figure 4), was purified on an agarose gel and used to transform a *his3*/*his3* homozygous diploid strain (W303D). Integration of this deletion construct into the genome was confirmed by both DNA hybridization and tetrad analysis. Segregation of *MAT*α mating type in spores was followed by halo assay, using an *MAT***a** *sst2* indicator strain (BC180 or RK512-5B).

Two other deletion constructions of *STE6* were made by removing a portion of the promoter region and part of the N-terminal coding sequence of *STE6*. To do so, YIp5-STE6 was linearized with Asp718 (Figure 4) and subsequently digested with *Bal*31 exonuclease for various times. The products so generated were converted to flush ends by incubation with the Klenow fragment of *E.coli* DNA polymerase I and then *Bam*HI adaptors were added by blunt-end ligation. After digestion with *Bam*HI to remove excess linkers, and also to remove the DNA fragment extending to the *Bam*HI site within the *STE6* gene (Figure 4), the deletion plasmids were recircularized by intramolecular ligation and recovered by transformation of *E.coli*. The plasmid isolated from one transformant, termed pRESTE6Δ22, contained a *Sal*I–*Bam*HI fragment that was 175 bp shorter than the *Sal*I–Asp718 fragment in the original YIp5-STE6. Given the total number of nucleotides removed and the fact that the Asp718 site resides 100 nucleotides downstream from the translational start site (where +1 is the A of the ATG initiator codon), the nucleotides corresponding to the wild-type mRNA start sites at −25 and −50 (Wilson and Herskowitz, 1986) were deleted as well as ~1 kb of N-terminal coding sequence. An *Xho*I/*Sal*I fragment from YEp13 containing the *LEU2* gene was converted to flush ends with Klenow enzyme and cloned into the *Bam*HI site of pRESTE6Δ22, using *Bgl*II linkers, to yield plasmid pREste6-ΔN :: LEU2. A *Bgl*II fragment carrying the *TRP1* gene was inserted into the *Bam*HI site of pRESTE6Δ22, resulting in plasmid pREste6-ΔN :: TRP1. The *LEU2* and *TRP1* genes were inserted in the *anti* orientation, such that transcription of the marker genes was opposite to the direction of *STE6* transcription. To generate linear fragments for one-step gene replacement by DNA-mediated transformation of appropriate recipients, the *ste6ΔN* :: *TRP1* construct was excised by digestion with *Sal*I and *Cla*I (at nucleotide 1712 in the *STE6* coding sequence) and the *ste6ΔN* :: *LEU2* construct was excised by complete digestion wth *Hin*dIII and partial digestion with *Sal*I. The structures of the resulting alleles in yeast transformants were confirmed by hybridization analysis.

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