Studies on the *STE6* encoded a-factor pump of the yeast *Saccharomyces cerevisiae*

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I dedicate this thesis to my parents Charles and Diane Brown

Declaration

This study was carried out under the supervision of Drs Alan Boyd and David Apps in the Department of Biochemistry, University of Edinburgh between October 1992 and September 1997.

The experimental work carried out in this thesis, unless otherwise stated, is my own; and this manuscript has been composed myself.

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Abstract

The *Saccharomyces cerevisiae STE6* gene product mediates the export of the peptide mating pheromone **a**-factor. The Ste6 polypeptide belong to the ABC transporter superfamily whose members include many proteins of medical significance. The Ste6p is a short lived, transmembrane protein which is produced in low levels in wild-type yeast. This thesis describes the construction, expression and partial purification of a recombinant Ste6p from *S. cerevisiae*.

In the absence of a functional assay for Ste6p, its presence was detected by Western blot analysis using polyclonal antibodies raised in this study. The antibodies were produced in rabbits in response to a recombinant Ste6p-ProteinA fusion protein. Purification studies on wild-type Ste6p were hindered due to the very low levels at which the protein was being produced. As an alternative to conventional purification techniques the Ste6p was affinity tagged at its extreme N-terminus with a hexa-histidine tail ($N(His)_6$Ste6p) so that it could be "pulled" out of a dilute solution by its high affinity to Ni-NTA (nickel-Nitrilo-Tri-Acetic-acid) resin. The chimaeric protein was expressed under the control of the *GAL* promoter in a *MAT***a**, *pep4* strain of *S cerevisiae* against a background of wild-type protein. Purification of $N(His)_6$Ste6p failed due to an apparent inability to bind the chimaeric protein to the resin. Extracts of cells expressing $N(His)_6$Ste6p were Western blotted and probed with an anti-histidine-tag monoclonal antibody. The antibody failed to detect any protein of the correct size for the Ste6p chimaera. These results suggested that the N-terminus of $N(His)_6$Ste6p had been removed during posttranslational modification of the protein.

The third approach to the purification of Ste6p involved tagging the C-terminus of the protein with an octa-histidine tag to produce $C(His)_8$Ste6p. As with the N-terminally tagged Ste6p this chimaera was expressed under the control of the *GAL* promoter however in this case the protein was expressed in a *MAT*α *pep4* strain of *S.cerevisae*. This protein could be detected by the anti-histidine tag monoclonal antibody and was able to bind to the Ni-NTA resin. The protein was partially purified and was clearly identifiable on silver stained polyacrylamide gels. It was hoped that the enigma surrounding the N-terminus of Ste6p would be resolved by sequencing the partially purified $C(His)_8$Ste6p, however only a small amount of protein was produced in this study and attempts at sequencing it were unsuccessful.

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Abbreviations used in this thesis

A_{600}	Absorbance at 600nm
ABC	ATP Binding Cassette
ATP	adenosine 5'-triphosphate
B	Bound
bp	base pair
βgal	β-galactosidase
BLAST	Basic Local Alignment Research Tool
°C	degrees centigrade
C-terminus	carboxyl terminus
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
Da	dalton
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
E. coli	*Escherichia coli*
EDTA	diaminoethanetetra-acetic acid
EGTA	1,2-di(2-aminoethoxy)ethane-N,N,N',N'-tetra acetic acid
ER	Endoplasmic Reticulum
g	relative centrifugal force
GTP	Guanosine 5'-triphosphate
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IPTG	Isopropylthiogalactoside

K	thousand
kb	kilobase
MSD	Membrane Spanning Domain
mg	milligram
min	minute
ml	millilitre
µg	microgram
µl	microlitre
MDR	Multiple Drug Resistance
MHC	Major Histocompatibility Complex
MSD	Membrane Spanning Domain
MWt	Molecular Weight
nm	nanometer
NBD	Nucleotide Binding Domain
Ni-NTA	nitrilo-tri-acetic-acid
N-terminus	amino terminus
dNTP	deoxy nucleoside triphosphate
PAGE	Polyacrylamide Gel Electrophoresis
PMSF	phenylmethylsulphonyl flouride
PCR	Polymerase Chain Reaction
RAM	Ras and **a**-factor maturation
RPM	Revolutions per Minute
S. cerevisiae	*Saccharomyces cerevisiae*
SAPU	Scottish Antibody Production unit
sec	second

SDS	Sodium Dodecyl Sulphate
STE	Sterile
TAP	Transporter Associated with Antigen Processing
Tris	2-amino-2-(hydroxymethyl) propane-1,3,-dio(tris)
UB	Unbound
V	Volts

Abbreviations for amino acids

Amino Acid	Abbreviations	
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamate	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter 1

Introduction

Introduction

The selective transport of specific molecules across biological membranes is essential for cell survival as it provides a mechanism by which a cell may acquire nutrients, excrete waste products and communicate with other cells in its environment. Selective transmembrane transport is mediated by specific transporter and channel proteins associated with cellular membranes. Molecular characterisation of these proteins has revealed that they can be grouped into discrete superfamilies. The largest of these groups is the ATP Binding Cassette (ABC); (Higgins, 1992) or Traffic ATPase (Ames *et al.*, 1992) superfamily of transport proteins which comprises of more than 100 members, with examples discovered in many organisms from simple prokaryotes to man. Family members are characterised by their highly-conserved ATP binding domain (Walker *et al.*, 1982) with which they are thought to couple the hydrolysis of ATP with the movement of substrate across biological membranes. ABC-transporters are thought to be relatively substrate-specific and transporters specific for the transport of amino-acids, sugars, inorganic ions, polysaccharides, peptides and proteins have been identified (Higgins, 1992).

The ABC transporter superfamily includes many proteins of medical significance. In humans, mutations in genes encoding ABC transporters cause genetic diseases such as cystic fibrosis (Riordan *et al.*, 1989), Zellweger syndrome (Kamijo, 1990; Gartner *et al.*, 1992), infantile hyperinsulinaemic hypoglycaemia (Aguilar-Bryan, 1995; Thomas, 1995) and adrenoleukodystrophy (Mosser *et al.*, 1994). Other ABC transporters of

medical significance include the human P-glycoprotein (Chen e*t al.*, 1986), which is involved with the development of multidrug resistance in the treatment of cancer, the Pgh1 protein of the malarial parasite *Plasmodium falciparum,* which contributes to antimalarial drug (chloroquine) resistance (reviewed in Rubio and Cowman, 1996) and the human *TAP1* and *TAP2* genes which are involved in the processing of antigen by MHC class 1 molecules (Deverson *et al.*, 1990). The significance of this family of proteins has prompted research into elucidating the mechanism by which the substrate molecules are recognised and transported.

The yeast *Saccharomyces cerevisiae* has been used as an experimental eukaryotic organism for decades and to date twenty nine ABC-transport proteins have been assigned to it (Michaelis and Berkower, 1995; Decottignies and Goffeau, 1997). The *STE6*-encoded ABC-transporter of *S. cerevisiae* transports the oligopeptide mating pheromone, **a**-factor, into the extracellular environment and is essential for the sexual life cycle (McGrath and Varshavsky, 1989; Kuchler *et al.*, 1989). The pheromonal control of the life-cycle of *S. cerevisiae* has been of interest since 1956, when Levi first reported that the mating process might be hormonally regulated. Decades of intensive study centred on the production of and response to the mating pheromones of *S. cerevisiae* have provided researchers with a model system on which to base various eukaryotic cellular processes such as peptide secretion and signal transduction. Unlike many eukaryotic ABC transporter proteins the substrate of Ste6p, **a**-factor, has been unequivocally identified and many of the cellular processes involved in the biosynthesis of **a**-factor and the mating process are understood. This,

taken in addition with the ability to manipulate yeast genetically makes Ste6p an ideal candidate for the study of eukaryotic ABC-transporter proteins.

Part 1

The ABC-transporter superfamily

1.1 The ABC-transporter superfamily of transport proteins

The ABC-transporters form one of the largest and most diverse superfamilies of proteins known to date. They are present in many organisms providing a means of transportation for a vast number of compounds ranging from inorganic ions to large polypeptides. The majority of ABC proteins are active transporters, utilising the energy of ATP hydrolysis to pump solute across biological membranes. However recent findings have shown that some ABC proteins may function in the regulation of other membrane proteins in addition to their own transport activities. P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR) are examples of mammalian ABC-transporter proteins which have been shown to regulate the activity of heterologous channels (reviewed in Higgins, 1995). The following section provides a brief introduction to structure and cellular functions associated with this family of transport proteins. This section deals mainly with the eukaryotic members of the ABC transporter superfamily; reviews concerning the structure and function of the bacterial permeases may be found in: Higgins, 1992; Ames *et al.*, 1992.

1.2 Structure

a) The four domain organisation of ABC-transporters

The ABC transporter superfamily is distinguished from other transport proteins by a highly conserved nucleotide binding domain (ATP-binding cassette) and modular structure. In general, ABC transporters are composed of homologous halves, each containing multiple (usually six) highly hydrophobic membrane spanning domains (MSD) and a nucleotide binding domain (NBD) (Figure 1.1A). The typical ABC transporter therefore contains two membrane spanning domains (MSD1, MSD2) and two nucleotide binding domains (NBD1, NBD2). The individual domains of an ABC transporter are frequently expressed as separate polypeptides, particularly in prokaryotic species (e.g. the oligopeptide transporter of *S. typhimurium*; Figure 1.1B, i). In many other ABC transporter proteins the domains are fused into larger multifunctional polypeptides, for example, the two ATP binding domains of the *E. coli* ribose transporter are fused into a double sized protein and the two membrane-spanning domains of the iron hydroxamate transporter, also from *E. coli,* are fused into a single polypeptide (Figures 1.1B, ii and iii respectively). Certain members of the ABC transporter superfamily consist of two separately encoded "half-molecules". These include the haemolysin transporter of *E. coli* (Figure 1.1B, iv) which is composed of one MSD and one NBD thought to function by existing as a homodimer and the mammalian TAP1 and TAP2 polypeptides (Figure 1.1B, v) which consist of two separately encoded "half-molecules" which are thought to associate to form a functional transporter molecule (Androlewicz *et al.*, 1994; Momburg *et al.*, 1994). Finally, many eukaryotic transporters, such as the human multidrug resistance protein

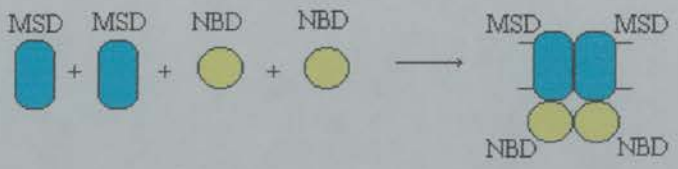
(Figure 1.1B, vi) the *S. cerevisae* Ste6p (Figure 1.1A, vii) and the cystic fibrosis gene product (Figure 1.1A, viii), have all four domains fused into a single polypeptide in the order MSD1-NBD1-MSD2-NBD2. A small subset of these "full-length" ABC transporters, for example CFTR, contain an additional domain, designated the R (regulatory) domain which lies between NBD1 and MSD2 and is the site of phosphorylation events that regulate its activity (Reviewed in Kuchler and Thorner, 1990; Ames *et al.*, 1992; Higgins, 1992; Michaelis and Berkower, 1995; Higgins 1995).

b) The Nucleotide Binding Domains

The ATP-binding domains of the ABC transporters are their most characteristic feature. Each domain is approximately 200 residues in length and contains two highly conserved motifs, the Walker A and Walker B motifs (Walker *et al.*, 1982; Hyde *et al.*, 1990) associated with many nucleotide binding proteins. The sequence identity between ABC-transporters extends over the entire nucleotide binding domain and is not restricted solely to the Walker motifs. Both ABC and non-ABC nucleotide binding proteins contain Walker A and B motifs. The ATP-binding domains are highly hydrophilic, contain no potential membrane spanning segments and are thought to be tightly associated with the cytoplasmic face of the membrane as detailed in Figure 1.1 (reviewed in Higgins, 1992). The sequence motifs found within the NBDs of the ABC-transporter superfamily are indicated in Figure 1.2. In addition to the Walker A and B motifs the NBD contains a highly diagnostic "signature" or C motif which lies

Figure 1.1 Domain organisation of typical ABC-transport proteins

(A) Structural organisation of a typical ABC transporter



(B) Domain organisation of ABC transporters

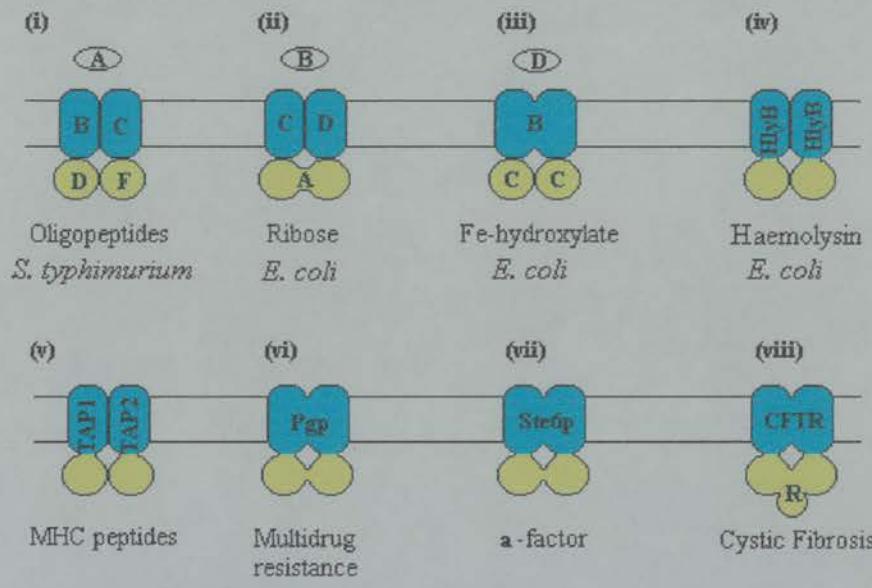
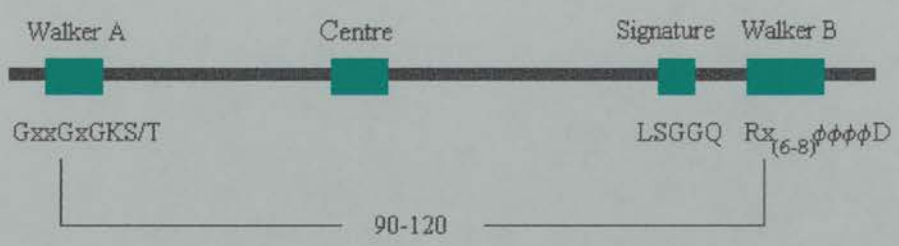


Figure 1.1A A schematic representation of the typical arrangement of the four domains that make up a ABC transporter in the membrane. The nucleotide binding domain (NBD) is shown as a yellow sphere and the membrane spanning domain (MSD), usually composed of six predicted transmembrane spans, as a blue cylinder.
Figure 1.1B A schematic representation of the domain organisation of some members of the ABC transporter superfamily. The transporter's source and organism are indicated. Each transporter consists of four domains as described in (A). Certain transporters, such as CFTR, have additional domains that are not part of the core transmembrane translocation mechanism. The regulatory, R, domain of CFTR is indicated.

Taken from: Kuchler and Thorner, 1990; Higgins, 1992; Michaelis and Berkower, 1995; Higgins 1995.

upstream of the Walker B motif and has the consensus LSGGQ. This motif generally distinguishes ABC-transporter proteins from other ATP binding proteins (Hyde *et al.*, 1990; Ames *et al.*, 1992; reviewed in Michaelis and Berkower, 1995). The final motif found within the NBD is the so called "centre region" which lies between the Walker A and B motifs and is found in many ABC-transporter proteins.

Figure 1.2 Consensus sequences within the NBD of an ABC protein



A schematised NBD (~200 residues in length) is shown with the conserved regions represented by green boxes. The Walker A, Walker B, signature and centre motifs are shown and the distance (in amino acids) between the Walker A and Walker B motifs are indicated. The consensus sequence corresponding to each region is represented below the box by the single letter amino-acid code, with ϕ denoting a hydrophobic residue. The centre region is only conserved between certain groups of ABC-transporters and as a consequence no consensus sequence is shown.

Taken from: Michaelis and Berkower, 1995.

c) The membrane spanning domains

The two MSDs of the ABC transporters are very hydrophobic and each is predicted, from its sequence, to consist of multiple α-helical segments. Hydropathy analysis

predicts that each MSD has six (or five) membrane spanning segments per domain. In general the ABC-transporter proteins do not appear to contain a high degree of sequence homology within their MSD. Certain transporters do however contain the "EEA" loop which is found in the region joining transmembrane segments IV and V of the MSDs of bacterial ABC permeases, in the yeast *PXA1* and *YKL741* gene products and in human *MDR1* (reviewed in Higgins, 1992; Michaelis and Berkower 1995; Decottignes and Goffeau, 1997).

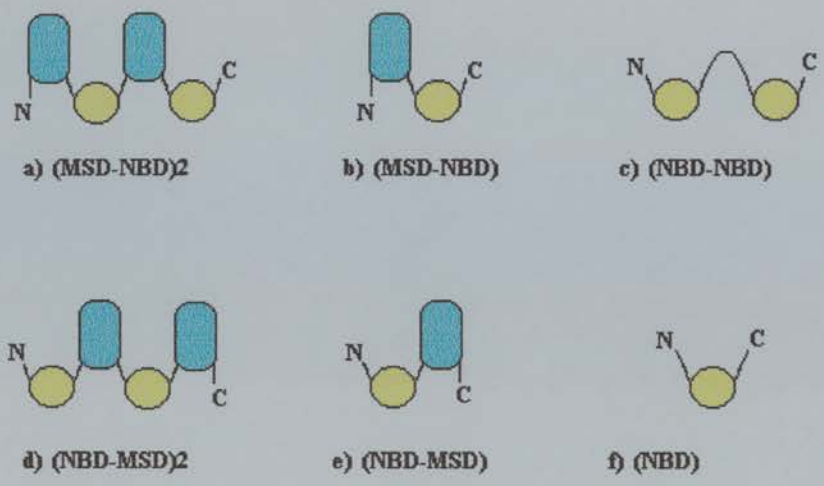
The modular design of the ABC-superfamily of transport proteins has led to the suggestion that eukaryotic members arose from the fusion of MSDs and NBDs of prokaryotic ancestors. Duplication of the fused domains followed by a further fusion event would result in the formation of a "full-size" ABC- transporter protein (reviewed in Ames *et al.*, 1992). The duplication of the initial fusion product, resulting in two MSD-NBD peptides, is consistent with the basic structure of bacterial ABC-proteins such as the haemolysin transporter of *E. coli* (Figure 1.1B, iv). The evolution of ABC-transporter proteins in vertebrates is further discussed in Hughes, 1994.

1.3 Yeast ABC transporters

Within seven years of the discovery of the first yeast ABC-transporter protein, Ste6p (McGrath and Varshavsky, 1989; Kuchler *et al.*, 1989) the entire sequence of the genome of *S. cerevisiae* was determined uncovering a further 28 family members

(Decottignes and Goffeau, 1997; Michaelis and Berkower, 1995). Sequence comparisons of yeast family members using various techniques has enabled the classification of the yeast ABC proteins into six clusters (family groups) which may in turn be broken down into ten distinguished sub-clusters of distinct predicted topology and presumed distinct function (Decottignes and Goffeau, 1997). The yeast ABC proteins range from 289-1,661 residues in length and are thought to reside in a variety of cellular locations both as membrane proteins (associated with the plasma, mitochondrial and ER membranes) and as soluble proteins (within the nucleus). Family members include both half size and full size proteins which may be categorised into one of six main topological units (Figure 1.3). Figures 1.3 a and d represent full size ABC-transporter proteins, examples of which include the **a**-factor transporter encoded by *STE6* and the protein encoded for by *PDR5* which is involved in cycloheximide and multidrug resistance respectively. Figures 1.3 b and e represent half size molecules which include the gene products encoded for by *ATM1* (involved with the maintenance of mitochondrial DNA) and *PXA1* (involved with the β-oxidation of very long chain fatty acids). Figures 1.3 c and f represent soluble members of the yeast ABC transporter superfamily. The protein encoded for by *YEF3* is expected to be a soluble protein with a NBD-NBD topology (Figure 1.3 c) and a nuclear localisation (as determined by PSORT analysis, Nakai and Kanehisa, 1992) where it is thought to be involved with the stimulation of amino-acyl-tRNA binding to the ribosome. The protein encoded by *YFL028* is predicted to contain one NBD (Figure 1.3 e) and is predicted, by PSORT analysis, to have a mitochondrial location, its function however remains to be determined (reviewed in Decottignies & Goffeau, 1997).

Figure 1.3 Predicted topology of the principal ABC proteins



Schematic representation of the domain organisation and topology of the principal yeast ABC transporters. The nucleotide binding domains (NBD) are represented by a yellow sphere. The membrane spanning domains (MSD) consist of six predicted transmembrane spans. Figures a and d represent full size transporter proteins with Figures b and e representing half-size molecules.

Taken from: Decottignies & Goffeau, 1997.

Similarities between yeast and human ABC-transporter proteins have been revealed by BLAST analysis (Basic Local Alignment Search Tool, Altschul *et al.*, 1990; Decottignes and Goffeau, 1997) which scores proteins on their degree of relatedness with a low BLAST score indicating a high degree of relatedness. All human ABC-proteins identified so far, except ABC1 and ABC2, may be included in the sub-clusters defined by the yeast ABC proteins as they have extremely low BLAST scores

with at least one yeast protein. A comparison of ABC-proteins implicated with human disease and their closest yeast homologues are shown in Table 1.1.

Examples of the sequence and functional relatedness between yeast ABC proteins and those which have an impact on human health (both human and non-human ABC proteins) are discussed below. Detailed reviews on this subject may be found in Decottignes and Goffeau, 1997; Michaelis and Berkower, (1995).

Human *MDR1* and *CDR1* and the yeast *PDR5* subgroup

Members of the yeast *PDR5* sub-cluster, which are involved in cycloheximide and multidrug resistance in yeast, are functional homologues of the human multidrug transporter *MDR1* although their modules are arranged in a "reverse" order, and the sequence identity is relatively low. Members of the *PDR5* subgroup also have a high degree of homology with the *CDR1* gene of *Candida albicans* which is responsible for drug resistant candidosis (Table 1.1; Prasad *et al.*, 1995).

Human *CFTR* and yeast *YCF1*

The yeast *YCF1* gene product confers resistance to high levels of Cd^{++} when overexpressed (Szczypka *et al.*, 1994; reviewed in Decottignes and Goffeau, 1997; Michaelis and Berkower, 1995). The Yeast *YCF1* gene product and related proteins, resemble those encoded for by mammalian *CFTR* and *MRP1* (a multidrug resistance protein distinct from that encoded by *MDR1*, Grant *et al.*, 1994). The F508 and

Table 1.1 Examples of human health problems related to ABC proteins

ABC protein	Human health impact	Closest yeast homologues	Function of yeast gene	Topology of yeast gene
CFTR[a]	cystic fibrosis	*YCF1*	Cd^{++} and multidrug resistance, glutathione S-conjugate pump	(MSD-NBD)2
ALD[a]	adrenoleukodystrophy	*PXA1*	Very long chain fatty acid β-oxidation	MSD-NBD
PMP70[a]	Zellweger syndrome	*PXA1*	Very long chain fatty acid β-oxidation	MSD-NBD
TAP1/ TAP2[a]	Behcet's disease, multiple sclerosis, bare lymphocyte syndrome type 1	*MDL1, MDL2*	not known	MSD-NBD
MDR1[a]	Cancer cells' drug resistance	*STE6*	**a**-factor export	(MSD-NBD)2
MRP[a]	Cancer cells' drug resistance	*YCF1, YLL015*	Cd^{++} and multidrug resistance, glutathione S-conjugate pump.	(MSD-NBD)2
SUR[a]	Hyperinsulinemic hypoglycaemia of infancy	*YCF1*	Cd^{++} and multidrug resistance, glutathione S-conjugate pump.	(MSD-NBD)2
ALDR[a,b]	Zellweger syndrome ?	*PXA1*	Very long chain fatty acid β-oxidation	MSD-NBD
ABC1[a,b]	not known	no close homologue		

ABC2[a,b]	not known	no close homologue		
ABC7[a,b]	not known	no close homologue		
ABC8[a,b]	not known	*ADP1*	not known	MSD-NBD-MSD
cMOAT	same phenotype as human Dubin-Johnson syndrome (mild chronic conjugated hyperbilirubinaemia)	*YCF1*	Cd^{++} and multidrug resistance, glutathione S-conjugate pump	(MSD-NBD)2
CDR1[d]	drug resistant candidosis	*PDR5, PDR10, PDR15*	cyclohexamide and multidrug resistance	(NBD-MSD)2
pfMDR2[e]	drug resistant malaria	*ATM1*	mitochondrial DNA maintenance	MSD-NBD
eEhPgp1[f]	drug resistant amoebiasis	*STE6*	**a**-factor export	(MSD-NBD)2
IdMDR[g]	drug resistant Kala azar (visceral leishmaniasis)	*STE6*	**a**-factor export	(MSD-NBD)2
SMDR2[h]	drug resistant schistosomiasis	*STE6*	**a**-factor export	(MSD-NBD)2
VgA[i]	drug resistant wound infections, pneumoniae, impetigos	*YER036*	not known	NBD-NBD

The sequence identity level between the closest yeast homologue of each ABC protein is expressed by the BLAST score of their comparison. The origin of the ABC protein is as follows: [a]human; [b]*Mus musculus*, [c]*Rattus norvegicus*; [d]*Candida albicans*; [e]*Plasmodium falciparum*; [f]*Entamoeba histolytica*; [g]*Leishmania donovani*; [h]*Schistosoma mansoni*; [i]*Staphylococcus aureus*. The function and predicted topology of the yeast ABC-transporters are also indicated. Taken from: Decottignies and Goffeau, 1997.

N1303 residues that are mutated in some cases of cystic fibrosis are conserved, as are the intracellular loops, ICL2 and ICL4, which appear to be important for CFTR function (reviewed in Decottignes and Goffeau, 1997; Michaelis and Berkower, 1995).

Human *MDR1* and *CFTR* with *S. cerevisiae STE6*

The *STE6* gene product of *S. cerevisiae* is one of the best characterised members of the ABC superfamily in yeast. Ste6p exports **a**-factor, a hydrophobic lipopeptide, into the extracellular environment providing functional similarities to MDR proteins which are thought to be involved in the expulsion of hydrophobic compounds from the cell (reviewed in Gottesman and Pastan, 1993). In addition Ste6p activity can be partially complemented by human P-glycoprotein (Mdr1p) and its murine homologue Mdr3p (Raymond *et al.*, 1992; Kuchler *et al.*, 1993) further compounding the functional relatedness between these proteins.

It has been anticipated that the study of yeast ABC-transporter proteins will increase our understanding of human ABC-protein associated disease. The yeast *S. cerevisiae* provides a convenient model for the study of ABC-proteins. The entire sequence of the yeast genome is known providing a full catalogue of all yeast ABC-proteins. The yeast is relatively easy to manipulate genetically providing a straightforward means by which protein function and location may be determined by creating a null mutation of

the gene of interest. The following sections introduce the yeast as an experimental organism and discuss the current understanding of the yeast **a**-factor export protein encoded by *STE6*.

Part 2

Yeast pheromones and control of the life cycle of *S. cerevisiae*

1.4 An overview of the life-cycle of *S. cerevisiae*

The budding yeast *S. cerevisae* is a unicellular organism which can exist as any of three distinct cell types. There are two haploid cell types, **a**-cells and α-cells, which are able to conjugate (mate) to form **a**/α diploids. All three cell types are capable of reproduction by mitotic division. When haploid cells of the opposite mating type are mixed together a series of behavioural and morphological changes occur which can be viewed through a light microscope (reviewed by Thorner 1981, Herskowitz 1988, Sprague and Thorner 1992). When haploid **a** and α cells first come in contact, they detect each others' presence by responding to the pheromone (**a** or α factor, respectively) produced by cells of the opposite mating type. **a**-type cells produce **a**-factor (**a**-pheromone) which elicits its effects through the *STE3*-encoded cell surface receptor of α-cells. In turn α-cells secrete α-factor (α-pheromone) which interacts with the *STE2*-encoded α-factor receptor of **a**-type cells. The binding of pheromone to the cell-type specific receptors activates a heterotrimeric G-protein linked protein kinase cascade mechanism which is common to both cell types. The signal

transduction cascade activates the *STE12*-encoded DNA-binding protein which acts as a transcriptional activator bringing about the changes in gene expression required for sexual conjugation. Pheromone-inducible genes include those required for cell recognition, agglutination, cell-cycle arrest, cell polarity and morphology changes, plasmogamy, karyogamy and those whose gene products are involved in the adaption and recovery processes. The pheromone-induced signal transduction pathway has been reviewed in Fields, 1990; Kurjan, 1992; Sprague and Thorner, 1992; Bardwell *et al.*, 1994 and Herskowitz, 1995.

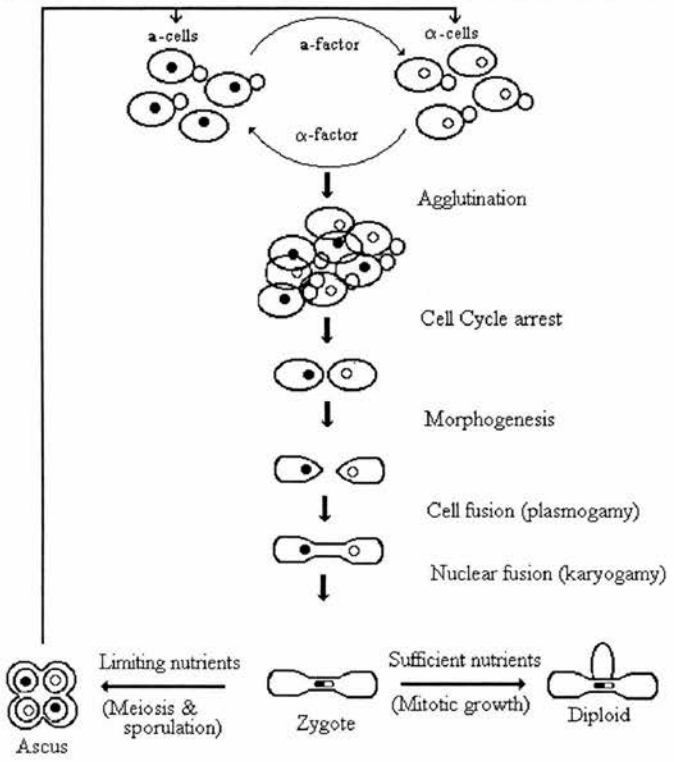
Within 30-60 min of mixing cells of the opposite mating types together they begin to aggregate into a multicellular mass. This aggregation is mediated by the production of cell-type specific sexual agglutinins which mediate cell-cell contact during the mating process (Lipke and Kurjan 1992). About 90-120 min (one doubling time) after the haploid cells first come in contact with each other they synchronise their cell-cycles by arresting growth at the G1 phase of their cell division cycle. Cultures which have gone into G1 arrest may be identified by the virtual absence of budding cells (Pringle and Hartwell 1981). Once cell-cell contact and synchronous growth have been established (about 180 min) the mating cells undergo morphological changes in which they project towards each other forming pear-shaped cells known as "Shmoos". It was the observation of such morphological changes which resulted in Levi's (1956) suggestion that the yeast mating process was under hormonal control. Once the respective projections of the mating cells have come in contact, autolytic enzymes remove, and others restructure, the cell walls and plasma membranes that originally separated the two cells resulting in cell fusion and plasmogamy, the formation of a

continuous cytoplasm. (Shimoda 1972). Zygote formation is completed when the nuclei from the mating pair fuse (karyogamy) to form a single diploid nucleus. The **a**/α diploid cell may then continue to reproduce by mitotic division but is unable to mate with either **a** or α cells. If the diploid cell is exposed to nutritional deprivation it is capable of undergoing meiosis and sporulation to produce an ascus containing four haploid meiotic progeny. A schematic representation of the life-cycle of *S. cerevisiae* is given in Figure 1.4.

1.5 An overview of the genetic control of mating

The three different cell types of *S. cerevisiae* differ in their ability to express certain cell-type specific genes: for example **a**-cells produce only **a**-factor and respond to α-factor whereas α-cells produce only α-factor and respond to **a**-factor, while **a**/α diploids neither make nor respond to either pheromone. The cell-type specific expression of genes encoding proteins required for cell-type-specific functions, such as those required in the mating process, is controlled by the composition of the genetic material contained at the *MAT* locus. Haploid cells that carry the *MAT***a** allele are **a**-type cells and haploid cells that carry the *MATα* allele are α-type cells. Diploid cells contain both the *MAT**a*** and *MATα* alleles. The *MAT* alleles encode three polypeptides, Matα1p and Matα2p are expressed by the *MATα* allele and Mat**a**1p is expressed from the *MAT**a*** allele. Together these polypeptide products

Figure 1.4 The mating process of the yeast *S. cerevisiae*



A schematic representation of the life cycle of *S. cerevisiae* reproduced from Sprague and Thorner, 1992.

form three transcriptional regulatory products, Matα1p, Matα2p and Mat**a**1p-Matα2p, which control the expression of **a,** α and **a**/α specific gene products. MATα1p activates the expression of α-specific genes in α-cells, Matα2p represses the transcription of **a**-specific genes in α-cells and Mat**a**1p-Matα2p represses the transcription of haploid specific genes in diploid cells. In an **a**-type cell the Mat**a**1p has no regulatory function: **a**-cells are such because they do not express the α-specific activator protein Matα1p, or the **a**-specific repressor protein Matα2p. The regulation of the transcription of cell-type specific genes of *S. cerevisiae* has been reviewed in Herskowitz, 1988, Dolan and Fields, 1991 and Herskowitz *et al.*, 1992.

Many genes whose products are required for the mating process have been identified by isolating mutants which were either unable to mate or unable to respond to the mating pheromones. A large number of yeast genes necessary for mating but distinct from the *MAT* locus have been identified (MacKay and Manney, 1974a; 1974b; Hartwell, 1980). These genes were termed Sterile (*STE*) genes as mutations in them conferred a sterile phenotype on affected cells. Since then other genes necessary for mating have been identified and include for example those encoding the precursors to the mating factors, which were not isolated in the original screens because they are both encoded by two genes (Kurjan and Herskowitz, 1982; Singh *et al.*, 1983; Gething, 1985) and *STE24* which is involved in an NH_2-terminal processing event in the production of mature **a**-factor (Chen *et al.*, 1997). To date some genes known to be involved in the mating process have yet to be identified and include the genes whose products are essential for **a**-factor processing and degradation (Caldwell *et al.*, 1995). The isolation of genes essential for the production and response to the mating pheromones has allowed elucidation of the mechanisms involved in these cellular events (reviewed in Sprague and Thorner, 1992).

1.6 The expression, biosynthesis and secretion of the mating pheromones

The mating pheromones of *S. cerevisiae* are oligopeptides with similar functions but which differ greatly in their structure, biosynthesis and secretion. α-factor is an unmodified tridecapeptide which is secreted through the "classic" yeast secretory pathway which involves the endoplasmic reticulum (ER), Golgi and secretory vesicles. In contrast **a**-factor is a prenylated and methylated dodecapeptide whose export from

the cell is mediated by the activity of the *STE6* gene product. For the purpose of this project a brief overview of α-factor production and secretion is given with more consideration being given to the production and export of **a**-factor. Review articles on yeast pheromone processing include Fuller *et al.*, 1988; Sprague and Thorner, 1992 and Kurjan, 1992.

a) An overview of the biosynthesis and secretion of α-factor: the secretion of a peptide by the "classic" yeast secretory pathway

Mature extracellular α-factor is an unmodified tridecapeptide which is coded for by two α-cell-type-specific, functionally redundant genes *MFα1* and *MFα2* (Kurjan and Herskowitz, 1982; Singh *et al.*,1983). These genes encode polypeptide precursors that contain tandem repeats of the α-factor peptide. The *MFα1* gene product is a 165-amino-acid polypeptide which contains four α-factor repeats whereas the *MFα2* gene product is a 120-amino-acid polypeptide which contains two α-factor repeats. Both gene products contain an amino-terminal hydrophobic signal peptide leader sequence for transit into the ER (Julius *et al.*, 1984; Waters *et al.*, 1988; reviewed in Rapoport, 1992 and Walter and Jackson, 1994); a hydrophilic domain (pro-region) which contains three consensus sites for asparagine-linked glycosylation; and a C-terminal segment containing the tandem repeats of the mature α-factor sequence. These precursor molecules are targeted to the ER by the presence of the hydrophobic signal sequence which is removed by signal peptidase (Dev and Ray,1990). The pro-region is glycosylated upon entry to the ER by the addition of three N-linked core

oligosaccharides (Julius *et al.*, 1984; Fuller *et al.*, 1992). On entry to the Golgi apparatus the core oligosaccharides are elongated and the pro-α-factor is proteolytically processed to form mature α-factor. The mature α-factor then exits the cell by exocytosis in a constitutive manner. *MFα1* and *MFα2* expression is induced when cells are exposed to **a**-factor (Achstetter, 1989; Jarvis *et al.*, 1988). The processing and secretion of α-factor is an example of the classic protein secretory pathway of yeast. Mutations in genes encoding proteins required for secretion (*SEC* genes) prevent mature α-factor from being secreted into the extracellular environment (Novick *et al.*,1980 and Novick *et al.*, 1981).

b) a**-factor**

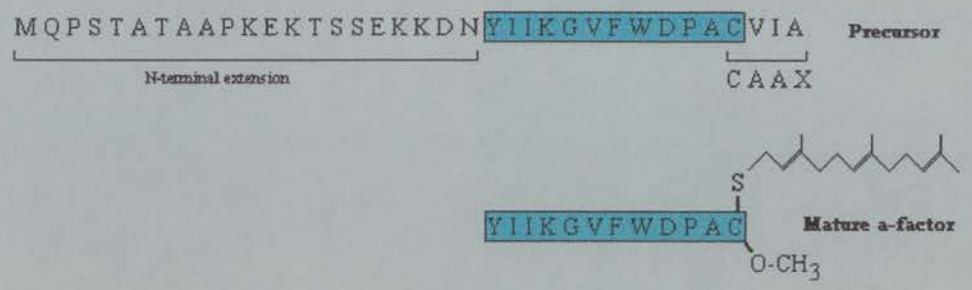
i) Introduction

Mature extracellular **a**-factor is a farnesylated and methylated dodecapepeptide which is encoded by two **a**-specific functionally redundant genes *MFa1* and *MFa2* (Brake *et al,* 1985; Michaelis and Herskowitz, 1988). The *MFa1* and *MFa2* genes encode **a**-factor precursor molecules of 36 or 38 amino-acid residues respectively which are composed of three main functional segments 1) the mature portion which is eventually secreted: b) the NH2 terminal extension: and 3) a COOH-terminal portion which contains the signal sequence for farnesylation and methyl-esterification known as the CAAX motif (Figure 1.5). Maturation of the precursor molecules includes both NH_2 and COOH terminal cleavage events and COOH-terminal modification to yield mature **a**-factor molecules YIIKG**L**FWDPAC(Farnesyl)-OCH_3 and

YIIKG**V**FWDPAC(Farnesyl)-OCH_3 from *MFa1* and *MFa2* respectively (divergent residues emboldened). Unlike the α-factor precursor molecules these polypeptides contain neither a hydrophobic signal sequence nor sites for asparagine-linked glycosylation and encode only one copy of the **a**-factor sequence. The precursor molecules are produced in equal quantities in the cell and are induced by the presence of α-factor (Dolan *et al.*, 1989; Davis *et al.*, 1992).

The differences in the structures of α and **a**-factor precursor molecules indicate that the maturation and secretion of **a**-factor may occur in a manner distinct from that occurring in the classic secretory pathway. Indeed the secretion of **a**-factor occurs when temperature-sensitive *sec* mutants are shifted to a non-permissive temperature (Sterne and Thorner, 1986; McGrath and Varshavsky, 1989). In addition mutations in genes essential for α-factor processing, for example *kex*2 and *ste*13, have no effect on the maturation of **a**-factor (Julius *et al.*, 1983, 1984) indicating that another mechanism for the secretion of the **a**-factor peptide must be present. Studies on strains of *S. cerevisiae* that were unable to mate because of a lack of **a**-factor production has led to the identification of several genes required for **a**-factor biogenesis and secretion (Chen *et al.*, 1997; reviewed in Caldwell *et al.*, 1995).

Figure 1.5 Structure of precursors and mature forms of a-factor encoded by *MFa1*



The **a**-factor precursor encoded by *MFa1* is shown with the NH_2-terminal extension, COOH-terminal CAAX motif and mature portion (shaded) indicated. The mature **a**-factor molecule derived from this precursor molecule is shown modified at the COOH-terminal with a farnesyl moiety and a carboxyl methyl group.
Taken from Chen *et al.*, 1997.

ii) The biogenesis of a-factor

The C-terminus of **a**-factor contains a CAAX motif (C is cysteine, A is aliphatic and X is one of many residues) which is CVIA in the case of **a**-factor. The CAAX motif is common to all known prenylated proteins including small GTP-binding proteins such as the ras proteins (plasma membrane-localised molecules which regulate cell differentiation and proliferation), lipopeptide pheromones, nuclear lamins and trimeric G-proteins. The CAAX motif signals a triplet of post-translational modifications which include prenylation of the cysteine residue, proteolysis of the COOH terminal AAX residues and methylation of the newly exposed cysteine carboxyl group (Clarke, 1992; Schafer and Rine, 1992; Zhang and Casey, 1996). Protein prenylation is thought to enhance membrane association and direct the

polypeptide to its correct intracellular location. Farnesylation of **a**-factor is carried out in the cytosol by the heterodimeric farnesyltransferase encoded by *RAM1* and *RAM2*. The *RAM* genes were initially identified by the discovery of mutants of *S. cerevisiae* which were deficient in both **a**-factor and Ras function suggesting that there was a maturation pathway common to both proteins. These *ram* (Ras and **a**-factor modification) mutants accumulate **a**-factor precursors intracellularly and contain ras proteins which are non-functional due their inability to localise at the plasma membrane (Fujiyama *et al.*, 1987; He *et al.*, 1991; Powers *et al.*, 1986; Schafer *et al.*, 1990; Goodman *et al.*, 1990). Following farnesylation the **a**-factor precursor polypeptide is thought to become membrane-associated by virtue of the hydrophobic interactions promoted by the lipid attachment. The **a**-factor precursor molecule then undergoes a series of membrane-associated processing events to yield mature **a**-factor. The three carboxy-terminal amino-acids distal to the farnesylated cysteine (AAX) are removed by an endoprotease whose activity has been detected and shown to be membrane-associated but whose gene(s) have yet to be identified (Ashby *et al.*, 1992; Hrycyna and Clarke, 1992). The **a**-factor precursor is then methylated by the *STE14*-encoded prenylcysteine-dependent carboxyl methyl transferase (Hrycyna and Clarke, 1990; Hrycyna *et al.*, 1991). The **a**-factor precursor is then further matured by proteolytic removal, in two stages, of the N-terminal extension by the proteins encoded by *STE24* and *AXL1* (Chen *et al.*, 1997; Michaelis, 1997) to yield mature **a**-factor.

iii) Secretion of a-factor is dependent on the *STE6* gene product

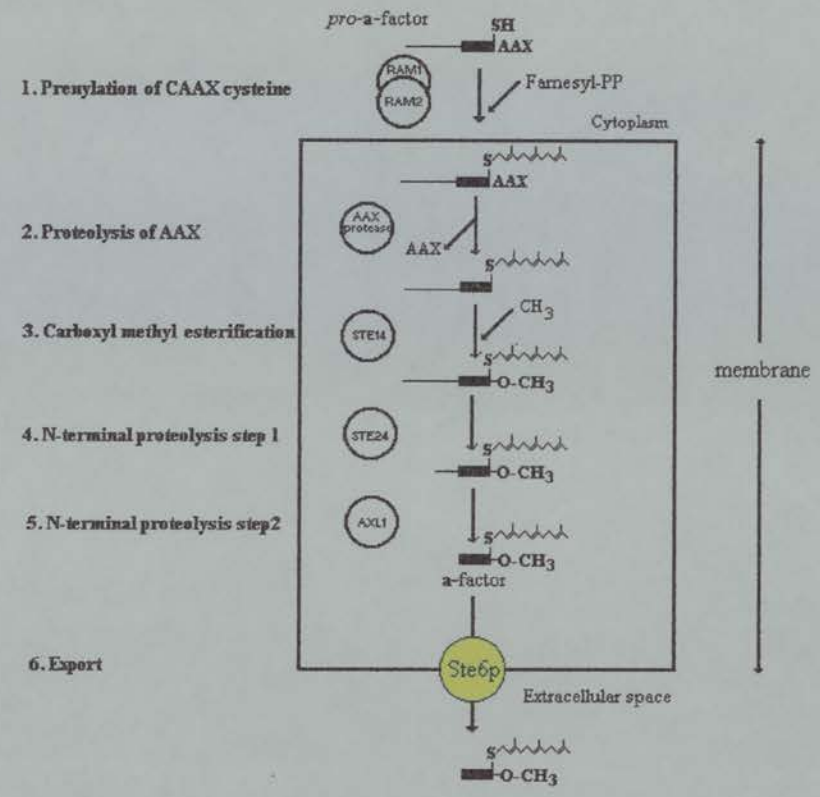
In 1989 two groups (McGrath and Varshavsky 1989; Kuchler *et al.*, 1989) reported the importance of the *STE6* gene product for the export of **a**-factor from **a**-cells. McGrath and Varshavsky discovered the previously-mapped *STE6* gene whilst working on genes involved in the yeast ubiquitin pathway. They demonstrated that deletion of the *STE6* gene in an **a**-type strain resulted in a loss of **a**-factor secretion whereas the same deletion had no effect on α-factor secretion from α-cells, further compounding the evidence that **a**-factor is secreted in a manner distinct from the classic secretory pathway. The *STE6* and the human multiple drug resistance P-glycoprotein gene, *MDR1*, were found to be 57% identical or highly conserved at the amino acid level. The involvement of the *MDR1* gene product in the energy dependent export of cytotoxic drugs out of the cell implied that the function of the *STE6* gene product was in the export of **a**-factor into the extracellular environment. Kuchler *et al.* demonstrated that the *STE6* gene product is required for, and is the rate-limiting step in, the secretion of **a**-factor. They demonstrated, by the use of antibodies directed against **a**-factor, that *ste6* mutants affected **a**-cells by preventing **a**-factor secretion. The maturation of **a**-factor is completed prior to secretion as the mature form of **a**-factor is found intracellularly in *MAT***a** *ste6* mutants. These results showed that the phenotype seen in *MAT***a** *ste6* mutants was due to the inability to secrete **a**-factor rather than to the secretion of biologically inactive **a**-factor. The transportation of the **a**-factor mating peptide by Ste6p highlighted a novel mechanism for the transport across cell membranes of peptides that lack the classic hydrophobic signal sequence.

Figure 1.6 shows a schematic representation of the biogenesis and secretion of **a**-factor.

iv) Localisation of a-factor biogenesis

In Figure 1.6 the biogenesis of **a**-factor following prenylation is shown to be localised on the plasma membrane. However although the biogenesis of **a**-factor is intimately associated with cell membranes, as determined by the localisation of all the maturation enzymes apart from the soluble farnesyl transferase encoded by *RAM1/RAM2*, the precise location of **a**-factor maturation has yet to be fully clarified. The PhD thesis of Sterne, (1989 referred to by Kuchler and Thorner, 1990; Sprague and Thorner, 1992) describes subcellular fractionation and protease accessibility studies which suggest that **a**-factor precursor molecules do not enter any intracellular membrane-bound compartment. Caldwell *et al.*, (1995) point out that enzymes involved in the maturation of prenylated proteins and peptides in animal cells have been found in microsomal membranes, for example a *STE14* functional analogue of isoprenyl cysteine methyltransferase is found to cofractionate with an endoplasmic reticulum marker in mammalian cells (Stephenson and Clarke, 1990; 1992) suggesting that such peptides and proteins pass through a microsomal membrane compartment on their way to their final destination. Microsomal membrane involvement in the maturation of **a**-factor has not been ruled out and the Michaelis group (Chen *et al*, 1997) are

Figure 1.6 Maturation and export of a-factor



Model for **a**-factor synthesis and secretion. During **a**-factor biogenesis, the unmodified **a**-factor precursor undergoes COOH-terminal processing (prenylation, proteolytic trimming of AAX, and carboxyl methylation) to yield the fully modified membrane-associated species. The N-terminal proteolytic processing events occur in two stages to generate mature **a**-factor. The CAAX processing machinery includes the Ram1p/Ram2p farnesyltransferase, the genetically unidentified AAX protease and the Ste14p methyltransferase. The N-terminal processing events are carried out by the *STE24* and *AXL1* gene products. The export of **a**-factor is mediated by ABC transporter Ste6p. The presumed localisation of **a**-factor biogenesis is as indicated. Taken from: Caldwell *et al.*, 1995; Chen *et al.*, 1997

currently conducting detailed fractionation analysis in order to determine the precise cellular location of **a**-factor intermediates. The cellular locations of the final stages of **a**-factor biogenesis are important in understanding the interaction of **a**-factor and Ste6p and are further discussed in relation to Ste6p in 1.11.

Part 3

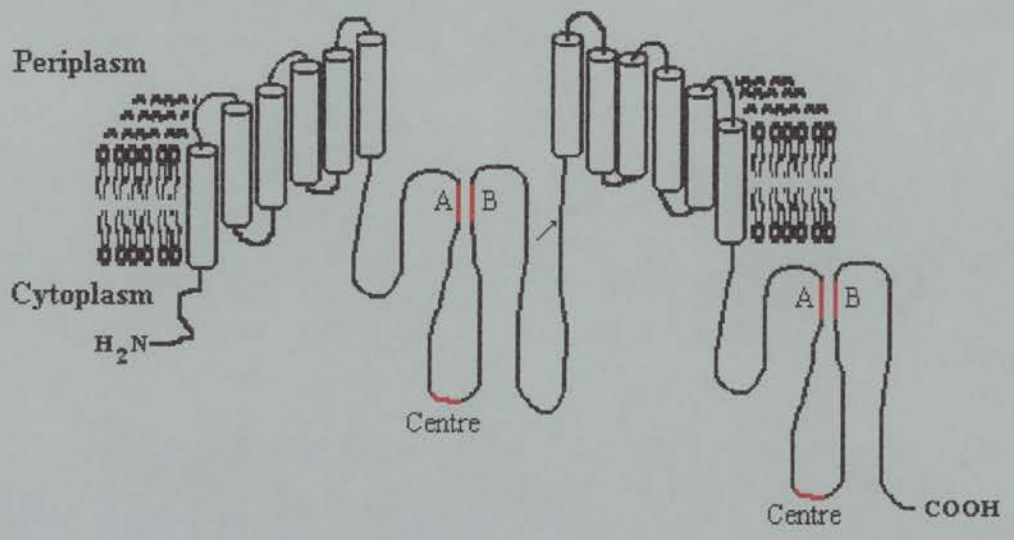
The *STE6*-encoded a-factor transporter of the yeast *S. cerevisiae*

1.7 The *STE6*-encoded a-factor transporter

The original *ste6* mutant was isolated in a screen for mutants of *S. cerevisiae* which were defective in mating (Rine, 1979 Ph.D. thesis, referred to in Wilson and Herskowitz, 1984 and subsequent papers related to *STE6*). Wilson and Herskowitz (1984) cloned the *STE6* gene and demonstrated that production of stable *STE6* mRNA is limited to *MAT***a** cells and is under negative regulation by MATa2p. The sequence of 469 nucleotides from the 5' flanking region of *STE6* was determined by Wilson and Herskowitz (1986). The *STE6* gene is located on chromosome XI (McGrath and Varshavsky, 1989) and has an open reading frame (ORF) of 3870 nucleotides which would encode a protein of 1290 amino acids with a calculated molecular weight of 144,774 (McGrath and Varshavsky, 1989; Kuchler *et al.*, 1989). Sequence analysis of *STE6* revealed that it was a member of the ABC-transporter superfamily with the topological arrangement MSD1-NBD1-MSD2-NBD2. The sequence similarities between Ste6p and Mdr1p are continuous throughout the length

of the polypeptides. In addition their hydropathy profiles are virtually superimposible suggesting that they share a common membrane topology with both the amino and carboxyl termini being located on the cytosolic face of the plasma membrane as indicated in Figure 1.7 (McGrath and Varshavsky, 1989; Kuchler *et al.*, 1989; Gottesman and Pastan, 1993).

Figure 1.7 Predicted topology of Ste6p in the plasma membrane



Schematic representation of Ste6p in the plasma membrane. The two predicted nucleotide binding domains (NBD) are shown as loops. The appropriate location of the Walker A, Walker B and Centre regions within the NBD are indicated in red. The membrane spanning domains (MSD) are represented as cylinders with each cylinder representing a membrane spanning segment. The arrow indicates the site used for the *in vivo* severing experiments discussed in section 1.10. See text for details.
Taken from: Michaelis, 1993; Browne *et al.*, 1996.

a) Ste6p is an integral membrane protein

Studies carried out on epitope-tagged versions of the *STE6* gene product have shown that Ste6p is an integral membrane protein which may be solubilised from cell membranes by the addition of the non-ionic detergent Triton X-100. In contrast treatments used for the removal of peripherally-bound proteins from cell membranes (0.1M Na_2CO_3 at pH11 and 3M urea) were unable to release detectable amounts of Ste6p (Kuchler *et al.*, 1993).

b) Ste6p is not a glycoprotein

The *STE6* sequence contains 14 consensus sites for the addition of Asn-linked carbohydrate (Kuchler *et al.*, 1989). As one of the potential glycosylation sites lies within the first putative hydrophilic loop of the protein, in an almost identical position to a single Asn-linked chain found in the *MDR1* gene product, it was reasonable to expect that Ste6p would be a glycoprotein. However the molecular weight of Ste6p has been shown to be unaffected by expression of the protein in the presence of tunicamycin (an inhibitor of N-linked glycosylation) suggesting that Ste6p does not contain any N-linked oligosaccharide. In addition Ste6p, solubilised from yeast membranes with Triton X-100, has been shown to be unable to bind to concanavalin A, a lectin that recognised both O- and N-linked sugars. Thus, native Ste6p appears not to contain any O- or N-linked oligosaccharide chains (Kuchler *et al.*,1993).

c) Ste6p is an ATP-binding protein

Ste6p is predicted, from its sequence, to contain two discrete ATP-binding domains with which it is thought to couple the hydrolysis of ATP with the movement of **a**-factor across the plasma membrane. Ste6p solubilised from membranes with Triton X-100 and partially purified by immunoprecipitation has been shown to be capable of interacting with the photoactivatable ATP analogue, 8-azido-ATP (Kuchler *et al.*, 1993; reviewed in Kuchler *et al.*, 1994). The crosslinking was shown to be specific for Ste6p by the fact that appearance of the photolabelled 145kDa product was only detectable in cells expressing Ste6p which had been exposed to the required UV irradiation. In addition the labelling was effectively competed by the presence of unlabelled 8-azido ATP.

d) Ste6p is a phosphoprotein

Several of the gene products involved in the mating signal transduction pathway including Ste2p, Ste5p and Ste12p are phosphoproteins. In each case the level of phosphorylation is stimulated by the presence of mating pheromone. Ste6p was shown to exist in a phosphorylated state by immunoprecipitation of Ste6p which had been radiolabelled with $^{32}PO_4^{3-}$ in the presence or absence of α-factor. Ste6p was shown to be phosphorylated in the absence of α-factor. On addition of α-factor the level of radioactivity incorporated into Ste6p significantly increased. However the increase in phosphate incorporation was shown to be proportional to the increase in Ste6p

concentration indicating that although Ste6p is phosphorylated the level of phosphorylatoin is unlikely to be linked to the presence of α-factor.

1.8 Structure-function analysis of Ste6p

Structure-function studies of Ste6p can be conducted by quantifying Ste6p activity either by measuring the mating efficiency of mutant cells or by quantifying **a**-factor immunoprecipitated from the culture fluid of mutated cells. The following section discusses mutagenic studies that have been carried out on Ste6p in order to determine which structural components are necessary for the protein's function.

a) Both halves of Ste6p are essential for activity

The relative importance of the two halves of the Ste6p transporter has been analysed by severing the *STE6* coding sequence (as indicated in Figure 1.7) and expressing the different halves of the molecule as two separate peptides, MSD1-NBD1 (N-half) and MSD2-NBD2 (C-half). Neither peptide, when expressed alone, was capable of complementing a *ste6* null mutation indicating that both halves of the molecule are critical for full activity and are therefore not functionally redundant. Co-expression of the different halves of Ste6p results in the formation of a functional Ste6p transporter suggesting that transport function is dependent on the formation of heterodimers (Berkower and Michaelis, 1991; Michaelis and Berkower, 1995).

The interactions between the domains of the human *MDR1* gene product, P-glycoprotein, have been examined by expressing each domain as a separate polypeptide and testing for association by coimmunoprecipitation (Loo and Clarke, 1995). It was shown that interactions between the different halves of the molecule are mediated by association between both the MSDs and the NBDs. These findings coupled with the ability of the separated halves of Ste6p to associate and reconstitute a functional transporter appear to reflect the modular architecture characteristic of this family of transport proteins. Interactions between the domains of bacterial ABC-transporters, which are composed of separate polypeptides, have been shown to be critical for their function (Kerppola *et al.*, 1991). The human TAP (transporter associated with antigen) transporter protein is an example of a eukaryotic heterodimeric ABC-transporter which is composed of the products of two separate genes, in this case *TAP1* and *TAP2* (Figure 1.1B, v).

b) The effect of mutations in the Walker A, Walker B, LSGGQ signature sequence and Centre region on Ste6p activity

The most highly conserved regions of the ABC-transport superfamily include the Walker A, Walker B, LSGGQ signature and centre regions (Figure 1.2). The high degree of conservation between these regions suggests that they may be essential for ABC-transporter function. This notion is supported by the observation that mutations in each of these four regions in the *CFTR* gene have been associated with the disease cystic fibrosis. The introduction of mutants into the conserved residues in the Walker A and LSGGQ signature regions of Ste6p have been demonstrated to cause a

dramatic loss of Ste6p function (Berkower and Michaelis, 1991; Browne *et al.*, 1996). This supports the suggestion that these residues play an vital role in ATP utilisation by Ste6p and other ABC transporters (reviewed in Michaelis and Berkower, 1995). In contrast mutations in the centre region of the NBDs of Ste6p have little effect on Ste6p activity.

i) Mutagenesis of the Walker A motif of Ste6p

Many of the mutations of *CFTR* associated with the cystic fibrosis phenotype are found within the NBDs. An alteration of the first glycine residue of the Walker A motif in either NBD of *CFTR* results in cystic fibrosis (DeVoto *et al.*, 1991). Mutagenesis of the corresponding glycine residues in Ste6p (G392 and G1087) results in a dramatic reduction in the level of **a**-factor secretion. In addition the mutagenesis of a conserved lysine residue within the Walker A motif (K398 and K1093), which leads to a loss of drug resistance when both halves of murine Mdr1p are affected, results in a reduction of **a**-factor secretion of affected Ste6p (Berkower and Michaelis, 1991).

ii) Mutagenesis of the Walker B and LSGGQ motifs of Ste6p

The LSGGQ or "signature motif" of the ABC-proteins is located just N-terminal to the Walker B motif (Figure 1.2). Several mutations of the CFTR protein associated with cystic fibrosis are clustered in or near the LSGGQ motifs. The introduction of cystic fibrosis-associated missense mutations into or near the LSGGQ motif of the

first NBD of Ste6p has been shown to result in a broad-range of **a**-factor transport defects with those mutations introduced directly within the LSGGQ motif causing the most severe defects (Browne *et al.*, 1996). Mutations of residues within the LSGGQ signature region had no effect on either the steady-state level or intracellular location of Ste6p indicating that this region of the protein may have a direct involvement in Ste6p transporter function.

iii) Mutagenesis of the Central region of Ste6p

The ABC-transporters show only limited homology over the Centre Region (Figure 1.2). Nevertheless this region is of interest as the most prevalent cystic fibrosis mutation, ΔF508, occurs in this region of the CFTR transporter. This phenylalanine residue is not conserved amongst ABC proteins however Ste6p and P-glycoprotein each contain a hydrophobic residue at an analogous position. Corresponding mutagenesis in each of the domains of Ste6p (ΔL455 in the N-terminal NBD and ΔY1150 in the C-terminal NBD) has been shown to have no effect on the activity of the Ste6p in the export of **a**-factor. This and other mutagenic alterations to the central region of Ste6p failed to exhibit any effect on the activity of the protein. This is in marked contrast to the dramatic reduction in Ste6p activity associated with mutagenesis of residues within the Walker A, Walker B and Signature regions of the protein. It would appear that the centre region of Ste6p does not play an active role in the transportation of **a**-factor (Berkower and Michaelis, 1991).

1.9 Expression of mammalian ABC transporters in *S. cerevisiae*

The successful expression of mammalian ABC-transporters in yeast provides a mechanism by which these proteins might be genetically manipulated and studied in an environment free from the inherent difficulties of working with mammalian cells. The extent of the structural and functional similarities between Ste6p and mammalian ABC-transporters has been demonstrated by functional complementation of Ste6p activity by the expression of heterologous ABC-proteins in a Δ*ste6* strain of yeast. The following section discusses some of the insights that have been obtained into the function of mammalian ABC-proteins by their expression in *S. cerevisiae*.

The extensive homology between Ste6p and mammalian P-glycoproteins has led to the suggestion that the P-glycoproteins might function to catalyse the transport of peptides or proteins under normal physiological conditions (Kuchler *et al.*, 1989; Kuchler and Thorner 1990a; 1990b). The murine homologue of human Mdr1p, Mdr3p, has been shown to partially complement a *ste6* deletion, allowing yeast to mate at low but significant frequency (Raymond *et al.*, 1992). Mdr3p behaves as a fully-functional drug transporter when expressed in yeast, conferring cellular resistance to the immunosuppressive and antifungal drug FK520, a known MDR substrate (Raymond *et al.*, 1994). A mutation in the 11th transmembrane domain of Mdr3p decreases activity and changes the substrate specificity of the protein in mammalian cells. This mutant form of Mdr3p is unable to complement a Δ*ste6* mutation in *S. cerevisiae* suggesting that the Mdr3p functions in a similar way in yeast and mammalian cells (Raymond *et al.*, 1992). Similar studies have been conducted on

the expression of human *MDR1* in *S.cerevisiae* (Kuchler and Thorner, 1992). These studies indicated that Mdr1p is capable of both complementing a Δ*ste6* mutation and conferring increased resistance toward valinomycin when expressed in *S. cerevisiae*. However other groups have been unable to repeat these expression studies and as a result doubt their authenticity (unpublished observations of S. Michaelis, reported in Michaelis and Berkower, 1995).

The *pfmdr1* gene of *Plasmodium falciparum* encodes the protein Pgh1 (P-glycoprotein homologue) which is associated with chloroquine-resistance in the parasite. Pfmdr1, like murine *MDR3*, is capable of the functional complementation of a Δ*ste6* mutation in yeast (Volkman *et al.*, 1995). The functional complementation is abolished by the presence, in Pgh1, of two naturally occurring polymorphisms which are associated with chloroquine resistance (Volkman *et al.*, 1995). The expression of wild-type Pgh1p in *S. cerevisiae* confers cellular resistance to four quinoline-containing antimalarial drugs. This drug-resistant phenotype is abolished by the introduction of Pgh1 carrying the genetic polymorphisms discussed above (Ruetz *et al* , 1996b). The heterologous expression of Pgh1 in *S. cerevisiae* has therefore not only implicated Pgh1 in anti-malarial drug resistance, but has also provided a means by which the structure-function relationships in this protein may be studied by genetic analysis in yeast.

Other heterologous ABC-proteins which have been shown to complement a Δ*ste6* mutation include the human *MRP* encoded multidrug resistance-associated protein (Ruetz *et al.*, 1996a) and the *HST6* gene product from *Candida albicans* (Raymond

et al., 1995). Ste6p-CFTR chimaeric proteins have been shown to complement a Δ*ste6* mutation and have consequently been used as tools for the study of certain properties of mammalian CFTR (Teem *et al.*, 1993).

It should be noted that not all of the mammalian ABC-transporters expressed in *S.cerevisiae* are able to complement a Δ*ste6* mutation. For example the expression of human *TAP1* and *TAP2* in *S. cerevisiae* results in the formation of a stable, ER located, TAP heterodimer which is fully functional in terms of selective peptide binding, ATP-dependent transport and specific inhibition of herpes simplex virus ICP47, but is unable to complement a Δ*ste6* mutation. (Urlinger *et al.*, 1996).

1.10 Intracellular trafficking, localisation and degradation of Ste6p

As Ste6p appears to be an integral membrane protein and functions to transport **a**-factor to the extracellular environment, it would be expected to reside primarily in the plasma membrane. This section discusses experimental evidence that indicates that Ste6p is a metabolically unstable protein that is found primarily in intracellular vesicles and is only transiently associated with the plasma membrane. It also discusses the experimental data that suggests that Ste6p is degraded by two independent pathways, one vacuolar and the other a cytosolic, ubiquitin-dependent mechanism.

a) The cellular location of Ste6p

The cellular location of Ste6p has been investigated by comparing the equilibrium distribution of Ste6p with marker proteins following fractionation of crude-cell lysates by differential centrifugation (Kuchler *et al.*, 1993; Kölling and Hollenberg 1994a). Kuchler e*t al.* (1993) found that the bulk of cellular (c-myc-epitope) tagged Ste6p co-fractionated with the plasma membrane marker protein, Pma1p (plasma membrane-associated H^+-translocating ATPase). No cytosolic marker protein (phosphoglycerate kinase) was detected in this fraction indicating that Ste6p was primarily located in the plasma membrane. Additional information on the association of Ste6p with the plasma membrane was obtained by examining the distribution of Ste6p through a sucrose density gradient. It was found that the distribution of Ste6p matched exactly that of Pma1p again indicating that Ste6p is a plasma membrane protein (Kuchler *et al.*, 1993; Kuchler *et al.*, 1994). In contrast Kölling and Hollenberg (1994a) carried out sucrose density gradient fractionation of crude cell extracts and found that only a minor portion of the total Ste6p localised with Pma1p. Instead they found that the majority of Ste6p followed a distribution pattern virtually identical to that of the Golgi marker protein dipeptidyl aminopeptidase A, indicating that Ste6p is predominantly associated with internal membranes and not the plasma membrane. The apparent discrepancy between these results might be explained by a plasma membrane enrichment step carried out by Kuchler *et al.* (1993) prior to sucrose density gradient analysis.

Further information regarding the cellular localisation of Ste6p has been obtained by the use of indirect immuno-fluorescence using overexpressed Ste6p which had been epitope-tagged either with c-myc (Kuchler *et al.*, 1993; Kölling and Hollenberg, 1994a) or with the influenza virus hemagglutanin epitope, HA1 (Berkower *et al.*, 1994). Both epitope-tagged variants of Ste6p were capable of complementing a *ste6* deletion and were detected in immunofluorescence experiments by anti-epitope antibodies. Kuchler *et al.* (1993) noted that the Ste6p had a patchy rim-like staining pattern and appeared in vesicular bodies associated with the plasma membrane. They concluded that this staining pattern was consistent for a plasma membrane protein and suggested that the vesicular bodies might represent a) newly-synthesised Ste6p within compartments of the secretory pathway *en route* to the cell surface or b) a reservoir of Ste6p-containing secretory vesicles awaiting delivery to the plasma membrane in a (perhaps) regulated manner.

The punctate staining pattern of Ste6p was observed by the other groups (Berkower *et al.*, 1994; Kölling and Hollenberg 1994a) who, however, concluded that the staining pattern was more consistent with an intracellular localisation of Ste6p. Berkower *et al.* (1994) analysed the staining pattern of the plasma membrane marker protein, Pma1p, and found that it gave a characteristic rim-staining pattern which was quite different to that seen for Ste6p. These observations were surprising considering the supposed role of Ste6p in the export of **a**-factor into the extracellular environment. The results could be interpreted in two ways; either Ste6p is an intracellular protein, which queries its role in **a**-factor export, or Ste6p is transported

to the plasma membrane but resides there for a short period of time before being internalised by endocytosis. In order to ascertain whether or not Ste6p is plasma membrane associated its distribution was examined in *end3* and *end4* strains of yeast which carry temperature-sensitive defects in genes required for endocytosis (Raths *et al.*, 1993). Any Ste6p localised in the plasma membrane of either an *end3* or an *end4* mutant would become trapped at the membrane on shifting the cells to a non-permissive temperature. Ste6p trapped at the plasma membrane would be expected to produce an intracellular distribution pattern more consistent to that observed for Pma1p. It was found that Ste6p exhibited a rim-staining pattern when expressed in both *end3* and *end4* mutant strains at the non-permissive temperature indicating that it had accumulated in the plasma membrane (Berkower *et al.*, 1994; Kölling and Hollenberg 1994a). In addition, sucrose gradient fractionation of crude cell lysates obtained from the *end4* mutant strain indicated that Ste6p fractionated with Pma1p when the cells were grown at their non-permissive temperature (Kölling and Hollenberg 1994a). Thus it would appear that when endocytosis is blocked, Ste6p appears at the plasma membrane, exposing its normally transient residency in this site. The immuno-fluorescent studies carried out on epitope-tagged Ste6p in wild-type and on *end3* and *end4* mutant strains of yeast suggest that, at any given point in time, the majority of Ste6p is found in intracellular vesicles and is either *en route* to or *en route* from the plasma membrane.

b) Trafficking and degradation of Ste6p

Pulse chase analysis of Ste6p has shown that is a metabolically unstable protein with a half-life of 13 min (Kölling and Hollenberg 1994a) or 37 min (Berkower *et al.*, 1994) in wild-type strains of *S. cerevisiae* grown on minimal medium at 30°C. The half-life of Ste6p is increased to greater than 2 hours in a *pep4* mutant strain of yeast which is deficient in several vacuolar ATPase functions (Ammerer *et al.*, 1986; Woolford *et al.*, 1986) indicating that Ste6p is degraded in the vacuole (Berkower et al. 1994 Kölling and Hollenberg 1994a). Indirect immunofluorescence studies on the intracellular localisation of epitope-tagged Ste6p in *pep4* mutants indicate Ste6p is localised in the vacuole (Berkower *et al.*, 1994).

Plasma membrane proteins are transported from the ER to plasma membrane via the Golgi apparatus and secretory vesicles. On reaching the plasma membrane the proteins may exist either as stable resident proteins such as Pma1p, which has a half life of over 5 hours (Berkower *et al.*, 1994), or as tightly regulated protein such as the pheromone receptors, Ste2p and Ste3p, which undergo rapid intracellular movement and turnover via the endocytic pathway.

Evidence of the existence of a yeast endosomal pathway was not confirmed until Riezman (1985) revealed that several yeast secretory mutants were in fact defective in endocytosis. The internalisation of the yeast pheromone receptors has been used as a model system for the study of endocytosis in yeast. Studies on the yeast **a**-factor receptor, Ste3p, have shown that the formation of a pheromone-receptor complex

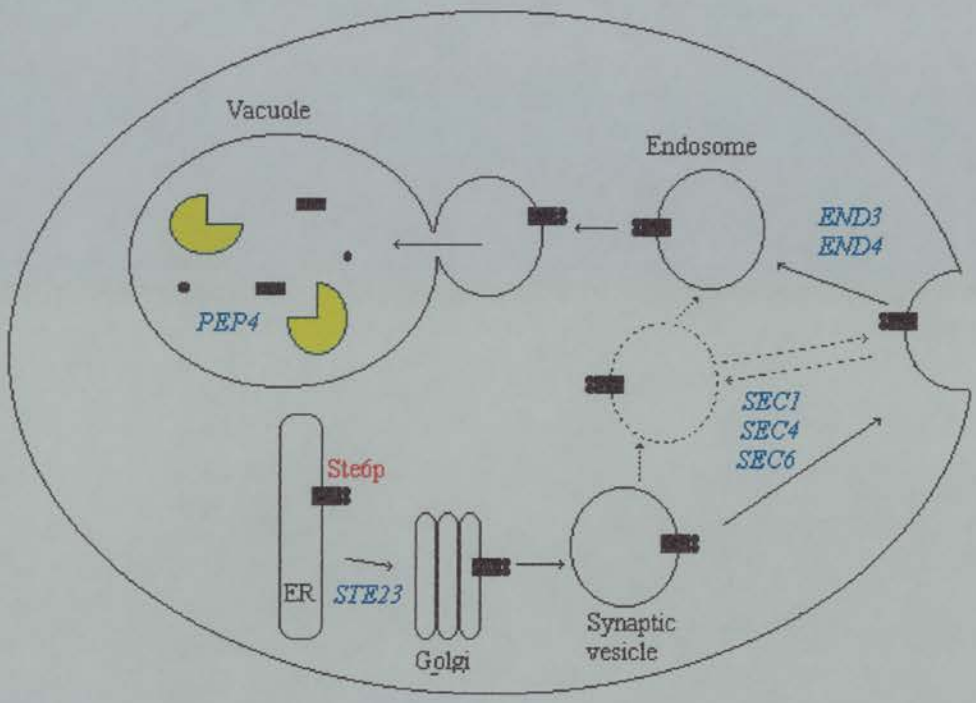
initiates endocytosis and subsequent vacuolar degradation (reviewed in Riezman, 1993). Along with pheromone-induced endocytosis, the Ste3p receptor is subject to constitutive endocytosis that occurs in the absence of pheromone (Davis *et al.*, 1993).The temperature-sensitive endocytosis mutants *end3* and *end4* were isolated through their inability to internalise α-factor or its receptor. The *end4* mutant specifically affects step(s) of the of the endocytic pathway prior to the formation of the endosomal compartment (Raths *et al.*, 1993). The ability of an *end4* mutant strain of yeast to "trap" Ste6p at the plasma membrane indicates that Ste6p is internalised via the endocytic pathway. This, together with the dramatic instability of Ste6p indicates that it is a tightly regulated protein. The following section discusses the current models relating to the intracellular trafficking and degradation of Ste6p.

i) Endocytosis and vacuolar degradation of Ste6p

A model depicting the movement of Ste6p to and from the plasma membrane is shown in Figure 1.8. Evidence in support of this model comes from cellular fractionation and immunofluorescence studies, as discussed in the previous section, combined with a series of experiments conducted both on mutants in the yeast secretory pathway and on recombinant Ste6p molecules.

Mutations in genes essential for the trafficking of Ste6p have been identified by virtue of their ability to increase the half-life of the protein by affecting its transport to the vacuole. Ste6p is stabilised when temperature sensitive mutations in genes involved in both ER to Golgi transport, (*ste23)* and in the fusion of post-Golgi secretory vesicles

Figure 1.8 Endocytosis and vacuolar degradation of Ste6p



Intracellular trafficking and vacuolar degradation of Ste6p. The model depicts the trafficking of Ste6p to the plasma membrane via the ER, Golgi and post-Golgi secretory vesicles. From the plasma membrane, Ste6p undergoes endocytosis into an endosome, which is delivered to the vacuole. In the vacuole, Ste6p is degraded by proteases (yellow "pacmen") activated by Pep4p. The products of the *SEC1*, *SEC4*, *SEC6*, *SEC23*, *END3* and *END4* genes act at the indicated steps. The dotted lines represent purely hypothetical processes: direct trafficking of Ste6p to the vacuole and a compartment for the recycling of Ste6p between the endosomes and the plasma membrane.

Taken from Berkower *et al* 1994; Kölling and Hollenberg, 1994a.

with the plasma membrane (*sec1, sec4* and *sec6*) are grown at a non-permissive temperature (Berkower *et al.*, 1994; Kölling and Hollenberg, 1994a). Likewise, in addition to "trapping" Ste6p at the plasma membrane, mutations in gene products essential for endocytosis (*end3* and *end4*) stabilise Ste6p.

Recombinant Ste6p molecules have been used in attempt to determine which physical elements of the protein are required for recognition by the cells transportation machinery. Kölling and Hollenberg (1994b) conducted studies on a chimaeric protein produced by the fusion of the first hydrophobic segment of Ste6p (amino-acids 1-78) to the secreted protein invertase which was lacking its ER signal sequence. The results of the study indicated that the first hydrophobic segment of Ste6p was capable of directing invertase to the ER membrane indicating that this region of Ste6p functions as a signal sequence. In addition a quarter molecule of Ste6p encoding the six N-terminal transmembrane domains has been shown to contain sufficient information to enable internalisation by endocytosis at a rate comparable to that of the full length molecule (Berkower *et al.*, 1994). The signal sequences required for recognition by both the endocytic machinery and the endoplasmic reticulum recognition machinery remain to be determined.

In general the results of these studies indicate that Ste6p is a highly unstable protein that is transported to the plasma membrane through the secretory pathway where it resides for a transient period before being internalised and transported to the vacuole by way of the endocytic pathway. The detection of Ste6p by indirect immunofluorescence has shown that Ste6p is only visible at the plasma membrane

when it is "trapped" there by *end 4* and *end3* mutant cells which are unable to carry out the early stages of endocytosis. This, taken in the light of the results obtained from the stability of Ste6p in various mutant strains, implies that Ste6p spends much of its short lifetime in transit to or from the plasma membrane. Kölling and Hollenberg (1994a) suggest that if there was an accumulation of Ste6p within the cell it would be likely to occur at some stage prior to reaching the plasma membrane. Berkower *et al*, (1994) suggest that the vesicular staining pattern for Ste6p could represent Ste6p in endocytic or exocytic vesicles which might function as a recycling compartment between endosomes and the plasma membrane. They suggest also that Ste6p may never reach the plasma membrane by moving directly from the secretory pathway to the vacuole. Both of the later hypotheses are represented in Figure 1.8.

It has been suggested that Ste6p may reside in a novel intracellular compartment that fuses with the plasma membrane following induction by α-factor (Kuchler *et al.*, 1993). Sucrose gradient fractionation studies on α-factor-stimulated *MAT***a** cells have indicated that the presence of α-factor does not lead to a relocalisation of Ste6p to the plasma membrane (Kölling and Hollenberg 1994a). In addition exogenous treatment of *MAT***a** cells with α-factor does not effect the half-life of Ste6p (Berkower *et al.*, 1994). Preliminary experiments therefore indicate that α-factor has no affect on the localisation and half life of Ste6p.

ii) Ste6p is multiubiquitinylated in endocytosis mutants

Ubiquitin is a small protein found in eukaryotic cells either free or covalently joined to cellular proteins. It functions as a regulatory protein and often appears to serve as a marker that targets a protein for degradation. Stable proteins are usually mono-ubiquitinylated whereas substrates targeted for degradation are often modified by the addition of a branched multiubiquitin chain. The degradation of multiubiquitinylated proteins may occur in a cytosolic ubiquitin-dependent pathway defined by the multisubunit protease yscE. yscE is structurally homologous to the so-called proteasome particle, which is a component of the 26S proteolytic complex that has been shown to specifically degrade ubiquitin-protein conjugates in reticulocyte extracts. Selective degradation of proteins by ubiquitinylation allows tight regulation and selective turn-over. Matα2p is an example of a regulatory protein which is known to exist in a multiubiquitinylated form. Matα2p activity must be limited to a single cell cycle and selective degradation would allow the concentration of the protein to be tightly monitored so that this haploid-specific protein could be removed rapidly from the cell with the onset of mating or mating type interconversion. The yeast ubiquitin system is reviewed in Finley (1992).

The dramatic stabilisation and vacuolar localisation of Ste6p in a *pep4* mutant indicates that the majority of Ste6p is degraded in the vacuole. However none of the secretory mutants examined in the previous section were capable of completely blocking the degradation of Ste6p suggesting that it can be degraded by an alternative

pathway. The accumulation of a low-mobility form of Ste6p in the plasma membrane fraction of endocytosis mutants led to the suggestion that Ste6p was being ubiquitinylated (Kölling and Hollenberg, 1994a). Studies have shown that Ste6p expressed in an *end4* mutant, grown at a non-restrictive temperature, is capable of being both immuno-precipitated and detected on Western blots by an antibody specific to ubiquitin. The appearance of this Ste6p species indicated that Ste6p most likely exists as a multiubiquitinylated protein. In addition Ste6p was found to be stabilised approximately 3-fold in a strain of yeast deficient in two ubiquitin conjugating enzymes, Ubc4p and Ubc5p. These enzymes have been implicated in the turnover of short lived and abnormal proteins (Seufert and Jentsch, 1990; Kölling and Hollenberg, 1994a). Thus it would appear that Ste6p can exist in a ubiquitinylated form and that the ubiquitinylation of Ste6p acts as a degradation signal.

It is possible that Ste6p is degraded by two independent pathways, both by the vacuole and by a cytosolic ubiquitin-dependent pathway. In this case the majority of Ste6p would be degraded in the vacuole, with the Ubc4p and Ubc5p-dependent pathway being utilised in a situation where the vacuole is overloaded or blocked. Alternatively the ubiquitinylation of Ste6p may be acting as a signal to direct Ste6p into the vacuolar degradation pathway. Recent research (Kölling, 1996) has indicated that Ste6p is stabilised by the removal of a putative ubiquitin binding domain. Removal of this domain prevented the Ubiquitinylation of Ste6p and resulted in a shift in the cellular distribution of Ste6p towards the plasma membrane. The ubiquitinylation of Ste6p may therefore play an important role in the endocytosis of Ste6p by labelling the protein for internalisation and transfer to the vacuole. Findings

from studies conducted on the ligand stimulated endocytosis of Ste2p-α-factor complexes from the plasma membrane support this notion. The ubiquitinylation of Ste2p is stimulated by the binding of α-factor to the receptor and has been shown to be required for the endocytosis of the receptor ligand complexes (Hicke and Riezman, 1996). Hicke and Riezman (1996) propose that the ubiquitinylation of Ste2p mediates the degradation of the receptor-ligand complexes, not via the proteasome, but by acting as a signal for endocytosis leading to subsequent degradation in the vacuole. A schematic representation of the ubiquitin-mediated degradation of Ste6p is given in Figure 1.9.

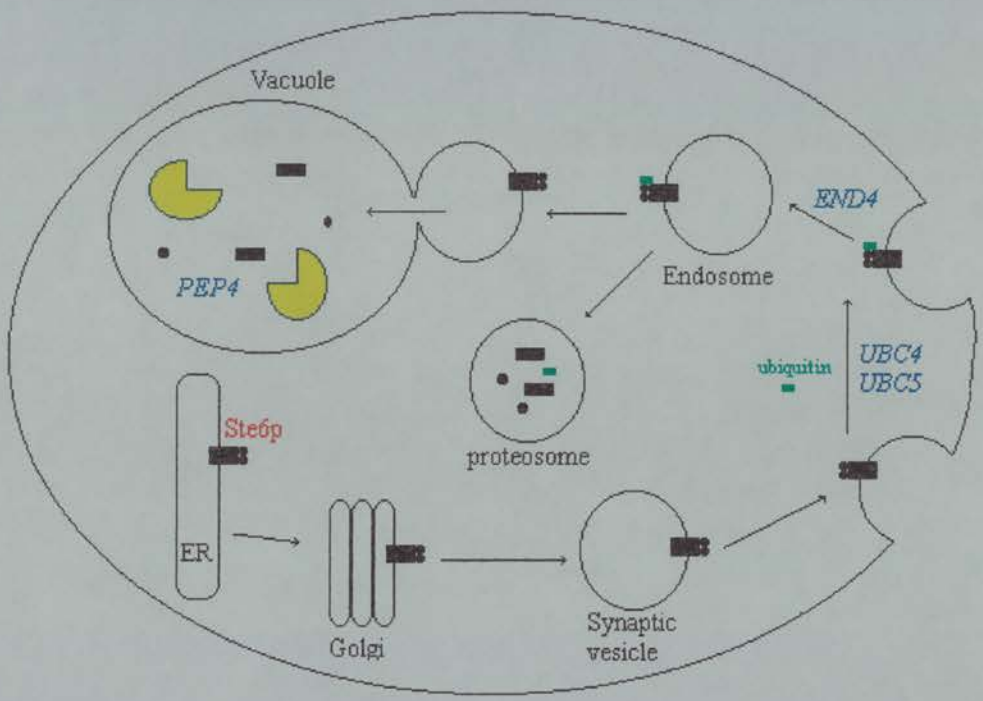
c) Possible roles for the rapid turnover of Ste6p

Since Ste6p is an **a**-type specific protein it must be capable of being rapidly cleared from the cell surface immediately following mating. In addition *S. cerevisiae* is capable of switching mating-type via interconversion of the *MAT* locus, necessitating the rapid removal of cell-type specific proteins. Thus the continuous turn-over of Ste6p provides a mechanism by which the cell can constantly monitor and modulate the protein level as required. The pheromone receptors Ste2p and Ste3p are rapidly endocytosed from the plasma membrane inferring that this might be a common feature of cell-type specific proteins involved in the mating process (Davis *et al.*, 1993).

As previously discussed the yeast endocytic pathway is thought to be responsible for the internalisation of Ste6p. Endocytosis may also play a role in the courtship phase of the mating of *S. cerevisiae* by concentrating Ste6p, and therefore **a**-factor, at the

projection tip. The role of Ste6p in the courtship behaviour of *S. cerevisiae* is further discussed in 1.12.

Figure 1.9 The Ubiquitinylation and degradation of Ste6p



Ubiquitinylation of Ste6p acts as a degradation signal. Ubiquitin is represented by the small green box. Details of the trafficking of Ste6p are as indicated in Figure 1.8. The model depicts Ste6p becoming ubiquitinylated in a *UBC4, UBC5* dependent manner. The ubiquitinylated Ste6p is internalised from the membrane in an *END4* dependent manner and enters the endocytic pathway. The ubiquitinylated Ste6p is then targeted for degradation either in the vacuole or in an ATP-dependent protease complex, the proteasome.

1.11 Ste6p and a-factor export

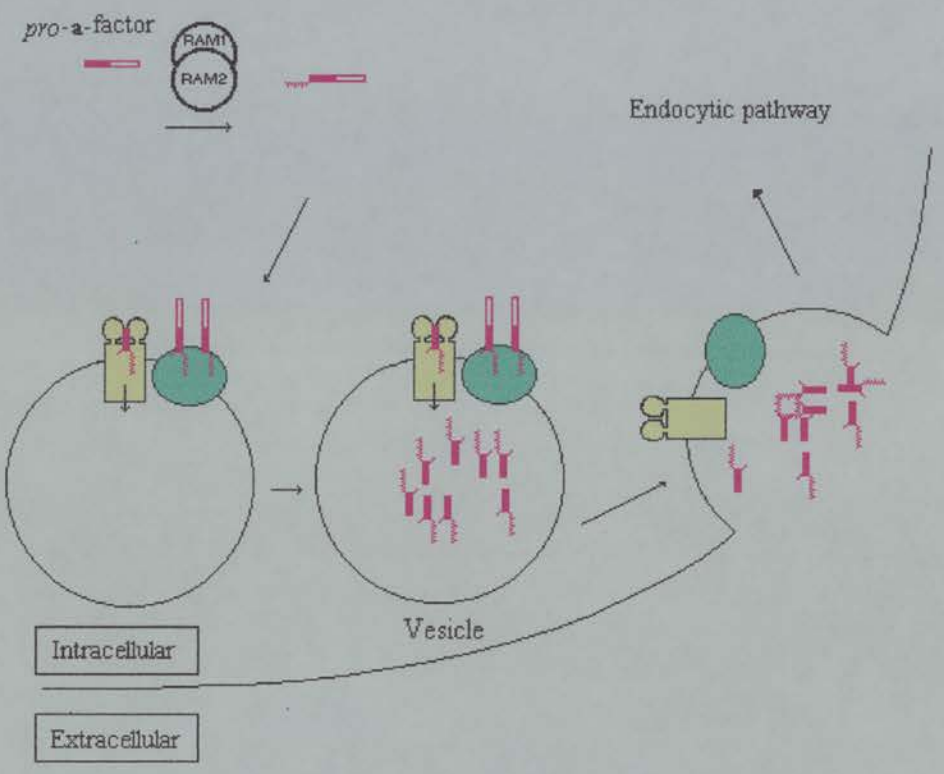
The experiments discussed in 1.10 indicate that Ste6p is located at the plasma membrane for a transient period only with the majority of Ste6p being localised in intracellular vesicles. This is surprising considering the supposed role of Ste6p in the export of **a**-factor which is generally thought to occur when **a**-factor molecules, partitioned in the plasma membrane by virtue of their hydrophobic modifications, encounter Ste6p which exports them into the extracellular environment. (McGrath and Varshavsky, 1989).

It has been suggested (Kölling and Hollenberg, 1994a) that Ste6p may function in the intracellular accumulation of **a**-factor rather than in the direct transportation of **a**-factor across the plasma membrane. In this scenario Ste6p would function to sequester mature **a**-factor into intracellular vesicles. The contents of the vesicles would be released on fusion with the plasma membrane leaving Ste6p in a position to be internalised and transported to the vacuole. This model would provide a mechanism without the requirement for Ste6p to be resident largely at the plasma membrane. It would also explain why Ste6p appears to accumulate in intracellular compartment before it becomes associated with the plasma membrane. However this model does not provide a mechanism by which the, presumably plasma membrane associated, **a**-factor molecules would be trafficked to the Ste6p vesicular compartment. In addition the export of **a**-factor is not thought to involve vesicular intermediates as its secretion occurs despite blocks imposed by various secretory

mutants (McGrath and Varshavsky, 1989). Studies have shown that pro-**a**-factor is not sequestered into vesicles as it is not protected from degradation by proteases (Kuchler *et al.*, 1989; Sprague and Thorner, 1992) however a large fraction of **a**-factor is degraded in the vacuole of *MAT***a** cells suggesting that mature **a**-factor is actually capable of entering the endosomal pathway (Kölling and Hollenberg, 1994a). Figure 1.10 proposes a model for the maturation and secretion of **a**-factor in which the **a**-factor maturation machinery and Ste6p are located within the same vesicular compartment. In this model immature **a**-factor becomes associated with the membrane located maturation machinery following COOH-terminal processing in the cytosol. On maturation the **a**-factor is transported into the vesicle by the action of Ste6p where it is accumulated until it is released by the fusion of the vesicle with the plasma membrane. The requirement for components of the yeast secretory pathway could be removed if **a**-factor is secreted by a different type of vesicular transport than normal secretory proteins. If, for example, the intracellular vesicular compartment were derived from the plasma membrane by endocytosis the homotypic fusion necessary for **a**-factor release could occur without the involvement of the yeast secretory pathway.

Both of the models described above provide a mechanism by which the requirement of Ste6p at the plasma membrane is minimal, thus providing an explanation as to how Ste6p can function effectively in the export of **a**-factor during its transient visit to this site. Further work is required to determine the precise cellular location of Ste6p and **a**-factor interaction and **a**-factor maturation. This could prove problematical due to the unstable nature and poor detectablility of both Ste6p and **a**-factor.

Figure 1.10 A proposed pathway for the intracellular accumulation of a-factor by Ste6p



The intracellular accumulation of **a**-factor by Ste6p. The trafficking and representation of Ste6p are as previously indicated. **a**-factor molecules are shown in pink. In this model immature **a**-factor associates with membrane associated maturation machinery (green sphere) following COOH-terminal processing in the cytosol. Mature **a**-factor is sequestered into intracellular vesicles by the action of Ste6p. The vesicles dock at the plasma membrane releasing **a**-factor into the extracellular environment. Ste6p is rapidly internalised from the plasma membrane via the endocytic pathway.

1.12 Ste6p, partner discrimination and cell fusion in *S. cerevisiae*

Mating between the two haploid cell types of *S. cerevisiae* is dependent upon the efficient secretion and delivery of **a** and α-pheromones to their respective target cells.

It is thought that the level of **a** and α factor secreted by *MAT***a** and *MAT*α cells, respectively, acts as the primary determinant of each haploid cell's identification as an appropriate mating partner by the target cell. This section discusses how both the pheromones and their representative receptors are thought to mediate partner choice in *S. cerevisiae.* It also discusses the evidence that indicates that Ste6p is directly involved in both partner selection and in the final stages of diploid cell formation

a) Pheromones, pheromone receptors and courtship in *S. cerevisiae*

In addition to stimulating the signal transduction pathway the pheromones and their receptors play an important role in mate selection in yeast. Cells which are unable to produce pheromone are sterile (Kurjan 1985, Michaelis 1988) as are those cells deficient in the cell-type specific receptors encoded by *STE2* and *STE3* (MacKay and Manney 1974a; 1974b; Hagen *et al.*, 1986). Immediately prior to sexual conjugation haploid cells of *S. cerevisiae* preferentially choose their sexual partners following the production and response to each others pheromone in a process termed "courtship" (Jackson and Hartwell 1990a; 1990b). Jackson and Hartwell demonstrated that, when faced with a choice of partner, both haploid cell types preferentially choose a partner which is producing the highest level of pheromone. This observation implicates the involvement of the pheromone receptors in partner discrimination and is in agreement with the observation of Bender and Sprague (1989) and Jackson *et al*, (1991). Browne *et al*, (1996) produced a series of Ste6p mutant molecules which differed from wild-type molecules only in their ability to transport **a**-factor. They found that there was a positive correlation between the level of transported **a**-factor and the

efficiency of mating. They also showed that, under conditions in which the level of **a**-factor is limiting for yeast mating, small changes in the amount of pheromone transported caused large changes in the mating efficiency. These observations are in agreement with the studies of Jackson and Hartwell (1990a; 1990b) and indicate that *MAT*α cells can efficiently discriminate between small differences in the level of **a**-factor presented by *MAT***a** cells.

b) Ste6p and mate choice in *S.cerevisiae*

The sterile phenotype of *MAT***a** cells containing null mutations in both *MFa*1 and *MFa*2 can be relieved, in part, by the addition of exogenous α-factor. In contrast the addition of exogenous **a**-factor is unable to restore mating in *MAT***a** cells which contain a *ste6* null mutation. This suggests that Ste6p can interact with the exogenous **a**-factor and that this interaction is required for recognition by *MAT*α cells (Marcus *et al*, 1991). It is plausible therefore that a Ste6p-**a**-factor interaction occurs at the cell surface and that this interaction is necessary for recognition by the *STE3*-encoded **a**-factor receptors on the surface of *MAT*α cells (Nahon *et al.*, 1995).

c) Ste6p localisation and a-factor transportation during mating of *S.cerevisiae*

Stimulation of *S. cerevisiae* by high levels of the appropriate mating pheromone results in asymmetric growth and the formation of a projection or shmoo. (1.4; Sprague and Thorner, 1992). The orientation of cell growth responds to the gradient of pheromone such that the mating projection points to the nearby partner (Jackson

and Hartwell 1990a and 1990b). The tip of the projection is the actual site of conjugation where the two haploid cells fuse to form a zygote. Indirect immunofluorescence studies on *MAT***a** cells exposed to α-factor cells has shown that the majority of cellular Ste6p becomes located at the tip of the projection (Kuchler *et al.*, 1993; reviewed in Kuchler *et al.*, 1992). The localisation of Ste6p at the projection tip may be a result of constitutive endocytosis of older Ste6p molecules, combined with a pheromone-induced increase in the synthesis of new Ste6p, all of which goes to the projection tip (Berkower *et al.*, 1994). α-factor induced expression of *STE6, MFa1* and *MFa2* coupled with the localisation of Ste6p at the projection tip would be expected to result in a dramatic increase in **a**-factor concentration at this site. The high concentration of **a**-factor at the tip of the projection may be maintained by the hydrophobicity and poor diffusibility of the molecule. As the tip of the projection is the actual site of cell fusion it is possible that the localised secretion of **a**-factor at this site is required for effective courtship and partner selection. Ste6p may be critical for the establishment and/or maintenance of the polarity in **a**-factor secretion that is required for effective courtship and partner selection. This would explain why the presence of Ste6p is essential for mating even when its role as an **a**-factor transporter has been removed by the addition of exogenous pheromone. The pheromone receptors, like Ste6p, become concentrated at the projection tip during mating (Davis *et al.*, 1993). The projection tips can therefore be considered as specialised organelles that would be highly enriched for signalling and signal reception. The close contact between two mating cells should therefore create an environment of very high concentration of pheromone and pheromone receptors. It

can be envisioned that this would create the very environment necessary for courtship and partner discrimination to occur.

d) Ste6p, a-factor and cell fusion

Studies on *MAT***a** cell specific mutants which are deficient in the ability to carry out cell fusion have shown that both high levels of **a**-factor and Ste6p are required for this final phase of mating to occur (Elia and Marsh, 1996; Brizzio *et al.*,1996). Two *MAT***a** specific cell fusion mutants, *fus5* and *fus8* were shown to produce less **a**-factor than an isogenic wild-type strain, and the mutations were found to be allelic with two genes known to be required for **a**-factor biogenesis, namely *AXL1* and *RAM1*. Several experiments have demonstrated that it is the partial defect in **a**-factor production, created by mutations in these genes, that causes the fusion defective (Fus^-) phenotype. First the overexpression of **a**-factor was found to be capable of suppressing the Fus^- phenotype. Second, a *MAT*α *sst2*Δ strain that is more sensitive to **a**-factor suppressed the Fus^- phenotype in *trans*. Finally, reduced levels of wild-type **a**-factor from a repressible promoter were shown to produce a cell fusion defect identical to that observed in the *fus5* and *fus8* mutants. It would appear that the *fus5* and *fus8* mutant strains are capable of producing enough **a**-factor to trigger the early stages of the mating process (cell cycle arrest, shmoo/ projection formation and mating pair formation) but cannot reach the levels required to elicit the cell fusion response in partner α cells. Similar experiments conducted on *MAT*α cells have shown that cells secreting lower levels of α-factor have appreciable cell fusion defects. Thus it would

appear that high levels of pheromone are required as one component of the signal for prezygotes to initiate cell fusion (Brizzio *et al.*, 1996).

Screens for mutants of *S. cerevisiae* that were defective in cell fusion (Cef) uncovered three partial-function alleles of *STE6* which elicited a substantial mating defect (Elia and Marsh, 1996). The *ste6(cef1-1)* allele was recovered, sequenced and found to contain an amber stop codon which was predicted to truncate Ste6p at amino acid residue 862. It was unclear however whether the phenotype was the result of underexpression of Ste6p, or of the expression of a mixture of truncated and functional forms of the transporter. The *ste6(cef1-1)* mutant was found to be capable of secreting **a**-factor and inducing many mating responses in a *MAT*α strain. Electron microscopy of *MAT***a** *ste6(cef1-1)* and *MAT*α wild-type mating pairs revealed that the mating process had become arrested at a late stage of conjugation in which the fusion wall remained intact. Close examination of the mating pairs revealed that many of the early mating functions remained intact, indicating that there had been adequate pheromone present to stimulate the pheromone response pathway. Mating the *ste6(cef1-1)* with a *MAT*α *sst2* strain had no effect on alleviating the defect in fusion wall degradation indicating that the *ste6(cef1-1)* defect was not due simply to a lack of pheromone secretion. In addition the overexpression of **a**-factor in the *ste6(cef1-1)* strain resulted in an increase in the level of **a**-factor secretion but did not suppress the mating fusion defect. The results of this study would suggest that Ste6p may be playing a role in cell fusion distinct from its role in **a**-factor transport. It is conceivable that Ste6p has an additional role as a regulatory protein that functions, either directly or indirectly, in the activation of the cell fusion machinery. Other members of the

ABC-transporter superfamily have been found to have additional roles in the regulation of heterologous channels, and other membrane proteins as well as carrying out their own transporter/ channel activities (reviewed in Higgins, 1995).

The findings discussed above indicate that Ste6p may be instrumental to the processes of partner selection and cell fusion in addition to its role as the **a**-factor transporter. A model providing a mechanism for the actions of Ste6p in partner discrimination is discussed in Nahon *et al.*, 1995. They propose that Ste6p and **a**-factor interact at the cell surface and that it is this **a**-factor-Ste6p complex which interacts with Ste3p receptors on the α-cell surface. This would provide direct cellular contact for courtship. They also propose a role for α-propeptide in the mating process by suggesting that exhibition of the molecule at the cell surface could act as a signal to signify cell quality during courtship. Like the **a**-factor-Ste6p conjugate, the α-propeptide would interact with Ste3p receptors on the surface of *MAT***a** cells. In both scenarios the cells would be brought into close contact by the interaction of the receptor on one cell surface binding with the pheromone attached to the opposite cell surface. This model might explain in part why the presence of Ste6p is required in the final stages of the mating process.

1.13 Structural requirements for a-factor bio-activity and recognition by Ste6p

The mature form of **a**-factor consists of three structural components, the methyl moiety, the lipid moiety and the amino-acid backbone (Figure 1.5) all of which are candidates for the recognition of **a**-factor by Ste6p and Ste3p. Studies on the

significance of the C-terminal modifications of **a**-factor have shown that both the farnesyl and methyl ester groups are required for export and biological activity (Schafer *et al.*, 1989; Schafer *et al.*, 1990; Marcus *et al* 1990). The removal of either the farnesyl or methyl group leads to a 100 times reduction in **a**-factor bioactivity, when compared to correctly modified pheromone, indicating that the methylation and farnesylation of **a**-factor are essential for its interaction with Ste3p (Marcus *et al.*, 1991). Likewise yeast defective in farnesyltransferase and methyltransferase activity, encoded by *RAM1/RAM2* and *STE14* respectively, are unable to produce extracellular **a**-factor indicating that these **a**-factor modifications are essential for the interaction with Ste6p (Schafer *et al.*, 1989; Schafer *et al.*, 1990; Sapperstein *et al* 1994). It is presumed that the lack of the farnesyl moiety prevents **a**-factor from associating with cell membranes thus impeding its interaction with either the Ste6p transporter or the Ste3p receptor. The methyl-moiety is thought to promote protein-protein interactions between **a**-factor and Ste3p/Ste6p. (Sapperstein *et al.*, 1994). The contribution made by the amino-acid backbone to the recognition of **a**-factor by Ste3p/Ste6p is thought to be minimal and is further discussed in Chapter 6.

Part 4

Outline of the project

1.16 Study of the interaction of Ste6p

The aim of this project was to study the interactions between Ste6p and **a**-factor by a combination of biochemical and genetic techniques. The biochemical approach was centred on the purification of Ste6p from an over-expressing strain of *S.cerevisiae*. In the absence of a functional assay for Ste6p a polyclonal antibody was to be raised against the C-terminal domain of Ste6p to allow detection.

Further initial aims included the reconstitution of Ste6p into phospholipid vesicles in order to allow the kinetics of its interaction with **a**-factor to be determined.

A genetic, or *in vivo*, approach to studying the interaction between Ste6p and **a**-factor was to be undertaken in which **a**-factor molecules, mutagenised at random over their amino-acid backbone, would be screened for their ability to interact with and block the Ste6p pump. The isolation of such **a**-factor molecules would allow the characterisation of residues essential for the interaction of **a**-factor and Ste6p.

Chapter 2

Materials and methods

Materials

2.1 Chemicals, antibodies and enzymes

Chemicals were generally obtained from either BDH Chemicals, Sigma Chemical Co., or Fisons Ltd. The Sequenase II DNA sequencing kit and [α^{35}S]ATP (>600Ci/mmol) were obtained from Amersham International plc. The majority of DNA manipulation enzymes e.g. restriction endonucleases, ligase, and VENT polymerase were obtained from New England Biolabs. Taq polymerase was purchased from Promega. Gel solutions for DNA sequencing were purchased from Hybaid. DNA purification kits for the preparation of high quality DNA (plasmid or M13) were purchased from Qiagen. Oligonucleotides were purchased from Oswell. The NiNTA resin for nickel affinity chromatography was also purchased from Qiagen. IgG Sepharose was from Pharmacia. Hybond C-extra for Western Blotting and the Enhanced Chemiluminescent (ECL) detection kit were supplied by Amersham. HRP antibody conjugates were gifted by the Scottish Antibody Production Unit (SAPU). Poly prep columns were from Bio Rad. Polaroid and X-ray film were obtained from Genetic Research Instrumentation. Media components were from Difco Laboratories. Protein sequence analysis was carried out by the Welmet Protein Characterisation Facility, University of Edinburgh. The Bead-beater was from Stratech Scientific LTD.

Subunit M39 of the bovine adrenal chromaffin granulae ATPase, N-terminally tagged with 10 histidine residues was gifted by D.K Apps (Edinburgh). The gene encoding this protein was originally cloned by Wang *et al.*, (1988). The histidine tagged variant was constructed by Leonora Cuifo and expressed and purified by Lorna Webster in the Biochemistry Department at the University of Edinburgh.

2.2 Bacterial and yeast strains

Strains of *E. coli* and *S. cerevisiae* used in this study are listed in Tables A1 and A2 respectively.

2.3 Plasmids.

Plasmids used in this study are listed in Table A3.

2.4 Media

In general all *E.coli* cultures were grown in Luria Broth (LB) consisting of 1% (w/v) Bacto Tryptone, 0.5% (w/v) Bacto yeast extract and 0.5% (w/v) NaCl. Bacto agar was added at a concentration of (2% (w/v) to make plates and at 0.8%(w/v) for making top agar. Antibiotics were added to a final concentration

of 15μg/ml as necessary. For blue/white colony selection the media were supplemented with 15μM IPTG and 0.005% X-gal.

For the preparation of uracil-containing single-stranded DNA the *E.coli* strain CJ236 was grown in 2xYT media which contains 1.6% (w/v) Bacto Tryptone, 1% Bacto yeast extract and 0.5% NaCl. Bacto agar was added at a concentration of 2%(w/v) to make plates and 0.8% (w/v) for top agar.

Bacterial strains NM522 and CJ236 were kept on M9 minimal plates to maintain the F' genotype. The medium was made as follows: 2%w/v Bacto agar and 0.4% glucose were autoclaved and left to cool to 40-50^{o}C. 100X additive (0.1M magnesium sulphate and 0.01M calcium chloride) and 10X M9 salts (7% (w/v) sodium phosphate (dibasic), 3% (w/v) potassium phosphate (monobasic), 0.5% (w/v) NaCl, 1% (w/v) ammonium chloride) were added to a final concentration of 1X and this was supplemented with 0.0002% thiamine. For maintenance of CJ236, chloroamphenicol was added to the medium at a concentration of 15μg/ml

Yeast cultures were grown in either rich medium (YPD; containing 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone and 2%(w/v) glucose) or minimal (selective) medium (SD; containing 0.67% Bacto yeast nitrogen base without amino acids and either 2% (w/v) glucose, 2% (w/v) mannose or 2% w/v raffinose as a carbon source. Amino acids and bases were added as required to a final concentration as follows; histidine (20μg/ml), leucine (30μg/ml),

tryptophan (20μg/ml), uracil (20μg/ml) adenine (20μg/ml). Yeast strains auxotrophic for tryptophan, due to the presence of plasmid DNA, were maintained on SD medium containing 1% casamino acids and uracil (20μg/ml). Bacto agar (2%(w/v)) was added when solid medium was required.

Methods

DNA methods

2.5 DNA manipulations

DNA manipulations such as restriction endonuclease digestion, ligation, mutagenesis using the Kunkel method, purification from host organisms and treatment with CIP were carried out as described in Sambrook *et al.*, (1989).

DNA was separated by electrophoresis through 0.4-1% (w/v) agarose gels. Fragment size was estimated by comparison with standard markers, typically digests of phage λ DNA with *Hin*dIII or *Bst*EII or the commercially available 1Kbp ladder (Gibco BRL).

2.6 Purification of DNA

Double and single-stranded DNA was purified from host bacteria according to either the methodology of Sambrook *et al.*, (1989) or by use of Qiagen kits for mini preparations, midi preparations, M13 purification and band purification of DNA from agarose gels. The production and purification of single-stranded DNA from phagemid vectors is detailed below. Yeast genomic DNA was purified either according to the method below or, by using a kit from IgI.

a) Production of single-stranded DNA from phagemid vectors using the M13KO7 helper phage

Single stranded DNA was isolated from *E. coli* transformed with a phagemid vector pVT103-U (Amp^R) containing the gene of interest (e.g. *MFa1*). 50µl of an overnight culture of transformed *E. coli* strain BW313 or CJ236 (Table A1) was inoculated into 5 ml TBG (1.2% w/v tryptone, 2.4% w/v yeast extract, 0.4% v/v glycerol, 17mM KH_2PO_4, 55mM K_2HPO_4 and 20mM glucose) supplemented with ampicillin. A 20µl aliquot of M13KO7 helper phage at 10^{11} plaque forming units (pfu)/ ml was added to the culture. The culture was then incubated, with agitation, at 37°C and 275rpm for 2 hours. Kanamycin was then added to the culture at a concentration of 75µg/ml and the culture was incubated, as before, for a further 22-24 hours. The culture supernatant was harvested by centrifugation at 14krpm for 10 min at 4°C in a microfuge. This centrifugation step was repeated and the single-stranded DNA was purified

from the supernatant either by use of a Qiagen kit or by the methodology detailed in Sambrook *et al.*, 1989; Lin *et al.*, 1992; Vernet *et al.*, (1987).

2.7 DNA Sequencing

Double-stranded plasmid DNA for sequencing was prepared from the bacterial strain DH5α using Qiagen mini or midi preparation kits. These gave very high quality sequencing-grade DNA. The Sequenase version 2.0 kit (USB) was used for all sequencing reactions. Double-stranded DNA was sequenced using either the method of Hsiao (1991) or the method noted below:

1. To 8µl (5-10ug) DNA add: 1µl primer (10ng/µl)
   1µl 1M NaOH
2. Incubate for 10mins at 68°C (during this interval it is convenient to thaw termination mixes (ddNTPs) and to prepare the labelling mix).
3. Add: 4µl TDMN
   3.2g TES (Sigma T-1375)
   0.5ml chloroform
   0.386g DTT
   4ml 1M $MgCl_2$
   2ml 5M NaCl
   dd H_20 to 50ml
   pH 1.6

4. Incubate for 10 min at room temperature. The reaction mix may be stored on ice until ready to use.

Add: 4µl labelling mix

1µl DTT (In sequencing kit)

0.4µl 5x Sequenase labelling mix

2.1µl TE (3mM Tris, pH7.5, 0.2mM EDTA)

0.5µl [α^{35}S]ATP

2µ T7 DNA polymerase (Sequenase) diluted 1:5 with enzyme dilution buffer

6. Incubate for 5 min at room temperature. It is convenient to pre-warm the termination mixes at this point (aliquot 2.5µl of each into separate microfuge tubes and incubate at 37°C).
7. Add 4µl of the labelling reaction to each termination reaction (A,C,G,T) pre-warmed for 5 min at 37°C.
8. Incubate for 10 min at 37°C.
9. Add 5µl of stop buffer to each reaction.
10. Boil reaction mixtures for 2 min before loading 3µl on to a 6% polyacrylamide gel.

M13 single stranded DNA was purified from NM522 culture supernatants using standard protocols (Sambrook *et al.*, 1989). Sequencing reactions were carried out using the instructions supplied with the Sequenase Version 2.0 kit (USB).

The products of the sequencing reactions were separated on either 0.2-0.7mm wedge gels or 4mm standard 6% polyacrylamide gels using pre-made solutions as detailed in 2.1.

2.8 Polymerase Chain reaction (PCR)

PCR extensions were carried out using a Techne thermal cycler. Both VENT and Taq polymerases were used. VENT was preferred to Taq as Taq is prone to making errors as it lacks VENT's proof-reading ability. The reactions were carried out in 100µl volumes with typically 20pmol of each primer, 1ng template DNA and 50µM of each dNTP (VENT requires 200-400 µM of each dNTP). The reactions were buffered using the buffers supplied with the enzyme. The magnesium concentration was optimised for each reaction and was usually in the region of 2-6mM. The reaction mixtures were overlaid with 50µl of mineral oil. A typical programme would be as follows:

Denaturation	95°C for 1 min
Primer Annealing	5°C below the true Tm of the amplification primers for 1 min
Primer extension	72°C extension time is calculated from the reaction proceeding at 1kb per min

In general 30 cycles were performed followed by a final extension of 10 min at 72°C.

Typically 5µl of PCR product was analysed on an agarose gel before being purified either by phenol/chloroform extraction and ethanol precipitation or by use of the appropriate Qiagen kit.

2.9 Cloning PCR products

In general PCR products were cloned into the appropriate vector following digestion with the appropriate restriction endonuclease. If this was not successful the pGEM-T vector system from Promega was employed. Essentially this utilises the fact that Taq-derived PCR products have overhangs consisting of dATP that are added by the terminal transferase activity of Taq. The pGEM-T vector has a T overhang which allows for the ligation of the A-overhang PCR product. Unfortunately VENT polymerase does not add terminal adenosine, however this can be overcome by treating a VENT-derived PCR product as follows: Phenol/chloroform extract and precipitate the DNA with two volumes of isopropanol. Resuspend in Taq buffer and add 1 unit of Taq DNA polymerase and 200µM dATP. Mix and incubate at 72°C for 20 min. The pGEM-T cloning system can now be employed. The PCR fragment can be subcloned into the appropriate vector as required.

2.10 Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis was carried out according to the Kunkel method as detailed in Sambrook *et al.*, 1989. This methodology is discussed in greater detail in Chapter 3.

2.11 Preparation of genomic DNA from yeast

Genomic DNA was prepared from BJ5465 as follows: 5ml of yeast was grown up overnight in YEPD. The following day the cells were harvested in a microfuge (20,000g, 30 sec) and resuspended in 200µl of the following: 2% Triton X100, 1% SDS, 100mM NaCl, 10mM Tris pH 8.0, 1mM EDTA. To this 100µl phenol and 100µl 24:1 chloroform: isoamyl alcohol was added along with 300mg of glass beads. The mixture was then vortexed for 2 min before centrifugation for 5 min at 20,000g. The upper aqueous layer was removed and the DNA precipitated by the addition of 2.5x volume of ethanol plus 0.1x volume 3M sodium acetate pH5.2. The precipitated DNA was harvested by centrifugation at 20,000 for 20 min and resuspended in 20µl TE. The DNA could now be used as a template for PCR amplification reactions.

In later experiments the IgI Yeast Genomic DNA kit was utilised. This enabled high quality DNA to be purified in 30 min.

2.12 Transformation of bacterial and yeast cells

Bacterial cells were made competent by treatment with $CaCl_2$ and transformed as detailed in Sambrook *et al.*, 1989. Yeast were transformed following treatment with LiOAc according to the method of Geitz *et al.*, 1992.

Protein methods

2.13 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated by electrophoresis through a 5% acrylamide stacking gel followed by a 7.5-12% acrylamide separating gels according to the basic method of Laemmli (1970). For routine analysis the Hoefer "tall mighty small" gel apparatus was used. A Bio-Rad "Protean II" apparatus was employed when larger gels were required (e.g. for band purification of proteins). The following reagents were used:

1. *Separating gel buffer*

   0.75M Tris.HCl pH8.8, 0.2% (w/v) SDS

2. *Stacking gel buffer*

   0.25M Tris.HCl pH6.8, 0.2% (w/v) SDS

3. *Acrylamide solution*

   44% (w/v) acrylamide, 0.8% (w/v) N N'-methylene-bisacrylamide.

4. *Electrophoresis Buffer*

   0.125M Tris.HCl., 0.2M glycine, 0.1% (w/v) SDS. The pH is 8.3 without adjustment.

5. *SDS sample buffer*

   0.0625M Tris.HCl pH6.8, 20% glycerol, 4% (w/v) SDS, 5% (w/v) β-mercaptoethanol.

Separated proteins were visualised by staining with either Coomassie Blue R-250 or by Silver staining as follows:

(a) *Coomassie Blue staining*

Gels were stained by soaking in a solution of 0.25% (w/v) Coomassie Brilliant Blue dissolved in 50% (v/v) methanol, 7% (v/v) acetic acid for approximately 15 min. Excess stain was removed by rinsing the gel in distilled water. The gels were then destained in a solution of 10% (v/v) methanol, 7% (v/v) acetic acid until bands could be clearly visualised.

(b) *Silver staining*

This method is based on that of Wray *et al.*, 1981. SDS-PAGE was carried out as above. Gels were fixed in a 50% methanol, 10% acetic acid solution for 20 min or overnight. The gels were then soaked in 50% methanol for 1 hour and

washed in water for 5 min. This step was repeated two more times. The following solutions were then prepared:

A) 0.4g silver nitrate dissolved in 2ml H_2O.

B) 21ml of 0.36% NaOH, 1.4ml ammonia (14.8M)

C) add solution A drop-wise to B stirring constantly. The solution should be clear. Make up to 100ml with H_2O.

D) mix 2.5ml of a 1% (w/v) citric acid solution with 0.25ml Formaldehyde (38% stock). Make to 500ml with H_2O.

E) 50% (v/v) methanol, 10% (v/v) acetic acid.

F) 3.7g NaCl, 3.7g $CuSO_4$.5H2O in 85ml H_2O. Add ammonia until solution becomes deep blue. Make up to 100ml with H_2O.

Gels were stained with solution C for 15 min and then washed in H_2O for 5 min. The gels were then soaked in solution D for 5-15 min until protein bands could be seen. The development was stopped by adding solution E. If the gel was over stained solution F was added to destain.

2.14 Transfer of proteins onto nitrocellulose

Proteins were transferred onto a nitrocellulose filter (Hybond C extra (Amersham)) following SDS-PAGE by use of a Novablot semi-dry blotter as follows:

Solutions:

Anode buffer 1:	0.3M Tris.HCl, 20% (v/v) methanol, 0.1% SDS pH10.4
Anode buffer 2:	25mM Tris.HCl, 20% (v/v) methanol, 0.1% SDS pH10.4
Cathode buffer:	25mM Tris.HCl, 20% (v/v) methanol, 0.1% SDS, 40mM 6-amino-N-hexanoic acid pH9.4.

The proteins were separated by SDS-PAGE as usual. Six pieces of Whatman 3MM filter paper and one piece of nitrocellulose membrane were cut to the same size as the gel. Two pieces of filter paper were soaked in buffer 1, one in buffer 2 and three in the cathode buffer. The gel was soaked in cathode buffer and the nitrocellulose in H_2O. The above components were then layered onto the anode plate as follows: first the two pieces of anode 1-soaked paper, then the piece of anode 2, then the nitrocellulose filter followed by the gel and finally the three pieces of cathode buffer-soaked paper. The cathode plate was placed on top and a current of 0.8mA X [area of gel]cm^2 was applied for one hour.

2.15 Detection of proteins by Western blot analysis

Proteins were transferred onto nitrocellulose as described in section 2.13. The nitrocellulose strip was then incubated for at least one hour in blocking buffer (TBST (10mM Tris.HCl pH7.4, 150mM NaCl,1% (v/v) Tween-20) plus 5% (w/v) "Marvel" (non-fat dried milk powder) to block any non-specific sites to

which antibody molecules might bind. The strip was then placed in fresh blocking buffer and the primary antibody applied at the suitable dilution in blocking buffer for the appropriate period of time (normally 20 min to overnight). The blot was then washed four times (1X 10 min followed by 3X 5 min) in TBST to remove excess antibody. The strip was then placed in fresh blocking solution with the secondary antibody. This was usually a donkey anti-rabbit HRP conjugate applied at a dilution of 1: 5,000 for 20 min. The strip was then washed as before, then developed with either the Enhanced chemiluminescence (ECL) detection system (Amersham) according to manufacturers directions, or latterly using the method detailed below which essentially follows the same principles. All washes and incubations were carried out with gentle agitation.

Stock solutions:

Luminol (Fluka) stock of 250 mM in DMSO. Stored in the dark at -20^{o}C.

p-coumaric acid (Sigma) stock of 90mM in DMSO stored as above.

Solution 1: 1 ml luminol; 0.44ml coumaric acid; 10ml of 1M Tris pH 8.5 in 100 ml distilled H_20.

Solution 2: 64µl 30% $H2O_2$; 10ml of 1M Tris pH 8.5 in 100 ml distilled H_20.

Solutions 1 and 2 were stored at 4°C. To develop a $50cm^2$ strip 2 ml of solution 1 was mixed with 2 ml of solution 2 and applied to the blot for 1 min. The blot was then removed from the solution and excess moisture removed by blotting with a tissue. The strip was then placed between two pieces of Saran Wrap (Dow) and exposed to a piece of blue sensitive X-ray film which was subsequently developed in an automatic processor.

2.16 Lysis and fractionation of bacterial cultures

Cells were harvested by centrifugation (5 min at 5000rpm in a Hareus Centrifuge), washed once in 100mM Tris.HCl (pH7.4) and resuspended in an appropriate volume (usually 5-10ml) of the same buffer. Lysozyme was then added to a concentration of $1mg.ml^{-1}$ and the cells were incubated for 20 min. Cell lysis was achieved by sonication for 4 bursts of 30 seconds interspersed with 1 min cooling intervals on ice. The lysate was centrifuged at 3000rpm for 5 min to give the P3 (inclusion) body fraction, the supernatant was then further centrifuged at 20,000rpm for 20 min to give a soluble fraction (S20) and an insoluble fraction (P20)

2.17 Glass bead lysis and preparation of membranes from *S. cerevisiae*

Cells were harvested by centrifugation for 5 min at 5000rpm (in a Hareus centrifuge) and washed once in 200mM Tris.HCl (pH8.5). The cell pellet was then weighed and resuspended in 2X volume 200mM Tris.HCl (pH8.5)

(protease inhibitors were added at this stage if required). Glass beads (0.45-0.6mm diameter) were added to the meniscus of the suspension and lysis was achieved by vigorous vortexing for three 2 min intervals interspersed with 2 min cooling on ice. The homogenate was then resuspended to 7.5X lysis volume with 200mM Tris.HCl (pH8.5) and centrifuged for 5 min at 1,000rpm (centrifuge) to remove unbroken cells and glass beads. The supernatant was recentrifuged as above as a further clearing spin. The cell membranes were then collected by centrifugation of the cleared lysate at 100,000 rpm for 20 min in a Beckman TL100 using the TLA100.3 rotor. The soluble, S100, fraction was then removed and pellet, P100, was carefully resuspended in 100-300ml 200μM Tris.HCl (pH8.5) and homogenised until a uniform solution was produced.

On occasion the breakage of large quantities of yeast cells was achieved by the use of a Bead-Beater (Stratech Scientific LTD) according to manufacturers instructions.

Preparation of protease inhibitors:

The 5X mix contained 20mM EDTA, 20mM EGTA, 20mM PMSF and 10μg.ml^{-1} each of pepstatin, leupeptin, chymostatin and antipain. The following stocks and the 5X mix were aliquoted and stored at -20^{o}C.

		per ml 5X
PMSF	200mM in isopropanol	100µl
pepstatin	1mg.ml^{-1} in methanol	10µl
antipain	1mg.ml^{-1} in water	10µl
leupeptin	1mg.ml^{-1} in 3:1 MeOH:DMSO	10µl
chymostatin	1mg.ml^{-1} in 3:1 MeOH:DMSO	10µl

2.18 Purification of a-factor on Amberlite XAD-2

The following purification method was taken from Strazdis and MacKay (1982) and Proteau *et al.*, 1990.

a) Preparation of XAD-2.

Amberlite XAD2 resin was washed in several times in distilled H_2O. The resin was then incubated with gentle agitation for 3 hours at 40^{o}C in 6 volumes of 1.2 dichloropropane: 1-propanol (1:3). The resin was then further incubated for 3 hours with gentle agitation at 40^{o}C in 6 volumes of 1-propanol. The resin could then be stored at room temperature until needed or washed well in distilled water and autoclaved. At no point was the resin allowed to go dry.

b) Addition of XAD-2 to growth media.

An appropriate yeast culture (in this case BJ5465(pAMB8)) was inoculated into selective media and grown overnight shaking at 30°C. The following day the overnight culture was diluted into fresh medium to give an A_{600} of 0.1. At this point 100ml of autoclaved XAD-2 was added per litre of culture. The culture was then grown, shaking, at 30°C for 24 hours.

*c) Removal of **a**-factor from XAD-2.*

The resin was retrieved by decanting off the growth medium and washing the resin several times with distilled H_2O. The XAD-2 was then washed in 40% methanol at 40°C for 2 hours. The methanol was then removed by aspiration and the resin resuspended in 3 volumes of 1-propanol and incubated, shaking, for 2 hours at 40°C. The propanol containing **a**-factor was then removed by aspiration and rotary evaporated to dryness at 55-60°C. The resulting orange/ brown residue was then dissolved in a minimal quantity of DMSO, aliquoted and stored at -20°C until required. It was found that **a**-factor stored like this will maintain its activity (as detected in a "Halo" assay) for several years.

2.19 Determination of protein concentration

Protein concentration was determined by the use of the BCA kit (Pierce) following manufacturer's instructions. Bovine serum albumin (Sigma) was used as a standard in all assays conducted.

2.20 Expression of Protein A fusion proteins from the *lac* promoter.

The *E. coli* strain NM522 was transformed with either pAX12 or pAMB1 and grown in 500ml of LB plus kanamycin at 25°C until an A_{600} of 0.6 was obtained. Expression from the *lac* promoter was then induced by the addition of IPTG to a final concentration of 0.25mM. The cells were then left to grow for a further 2 hours before being harvested.

2.21 Expression of β-galactosidase fusion proteins from the P_R promoter.

The *E. coli* strain pop2136 was transformed with either pEX12 or pAMB3 and grown in 100ml LB plus ampicillin at 30°C until an A_{600} of 0.6 was obtained. Protein expression from the P_R promoter of bacteriophage λ was then induced by shifting the cells to 42°C where they were grown for a further 4 hours before being harvested.

2.22 Purification of βgal-Ste6 fusion protein from inclusion bodies

The inclusion body (P3) fraction harvested from the *E. coli* strain pop2136(pAMB3), induced as detailed in 2.21, was resuspended in 500ml 100mM Tris.HCl (pH7.4) and loaded onto a preparative 10% SDS-polyacrylamide gel. The gel was stained briefly with Coomassie Blue and the region of the gel corresponding to the .βgal-Ste6 fusion protein excised. The fusion protein was placed in dialysis tubing containing 5-10ml electrophoresis buffer and removed from the gel by electroelution (50V for 2-5 hours) in this same buffer. The buffer containing the electroeluted protein was then placed in fresh dialysis tubing and dialysed against distilled H_2O overnight. The protein was then concentrated by lyophilisation.

2.23 Immunisation of rabbits for antibody production and collection of serum

Two New Zealand white rabbits were subcutaneously injected with 150μg of purified PrtA-Ste6p chimera which had been mixed with an equal volume of Freund's complete adjuvant to form an emulsion. A second injection was administered six weeks later, however in this, and all subsequent injections, Freund's incomplete adjuvant was used in the emulsion. A 5 ml test bleed was taken a week after the third injection. The blood was left at room temperature for 10 min before being placed at 4°C overnight. The following day the serum

was collected by centrifugation (3,000rpm for 15 min in a MSE bench top centrifuge) and filtered through a 0.22μm ultrafilter (Acrodisc). After the addition of sodium azide to a final concentration of 0.1%, the serum was stored at -20°C in aliquots. The rabbits were boosted and bled at regular intervals until they were finally sacrificed and the serum stored at -20°C.

2.24 Affinity purification of antibodies using antigen immobilised on nitrocellulose

A sample of antigen, in this case βgal-Ste6 fusion protein (2.22), was subjected to 10% SDS-PAGE then blotted onto nitrocellulose (2.14). The nitrocellulose was then stained with 0.2% Ponceau S in 3% TCA for 5 min and then washed in water to remove background staining. The band corresponding to the βgal-Ste6p was excised from the blot with a scalpel and marked to indicate which side had protein bound. The Ponceau S was then removed by washing the strip of nitrocellulose in TBST several times. The strip was then incubated in 5% Marvel in TBST for at least 60 min to block any non-specific sites on the nitrocellulose. The strip was then washed in TBST and placed, antigen side up, on a piece of Nescofilm. As much serum that could be held in place by surface tension (typically 0.5-1ml) was then placed on top of the affinity strip. A lid was placed over the strip to avoid evaporation and the strip was incubated like this at room temperature for 2 hours. The excess serum was then removed from the affinity strip and the strip was washed 4 times, for 5 min each wash, in TBST. The antibody was eluted from the strip by incubating it for 20 min in the

presence of as much 0.2M glycine (pH2.8) as could be held by surface tension. The eluted antibody was removed from the strip and added to an equal volume of 0.1M Tris.HCl (pH8.5), sodium azide was then added to a concentration of 0.1%. The affinity-purified antibodies were aliquoted and stored at -20°C until required. The strip could be re-used by washing it in a copious amount of TBST then storing it in the presence of 0.1% sodium azide.

2.25 Protein sequencing from Immobilon P

a) Preparation of polyacrylamide gels for separation of proteins prior to protein sequence analysis.

The following method utilises a buffer exchange procedure to provide enhanced resolution of polyacrylamide gels pre-run for protein sequencing in the presence of a free radical scavenger, to prevent blockage of the N-terminus of the protein and it is taken from Dunbar and Wilson (1993).

All solutions used in this procedure were made up fresh and filtered immediately before use. The electrophoresis was carried out using either the Hoefer Tall Mighty Small or Bio-Rad Protean II apparatus. The polyacrylamide gels were produced according to 2.13 however in this case both the separating and stacking gels were made using the separating gel buffer. The gel was loaded with SDS-PAGE sample buffer containing tracker dye (2.13) and was pre-run at 20mA in the buffers detailed below until the dye had reached the bottom.

Top reservoir	Stacking buffer (diluted 2X) containing 5μM reduced glutathione.
Bottom reservoir	Separating buffer (diluted 2X)

The sample, which had been resuspended in SDS-PAGE sample buffer and heated at 37°C for 10 min, was then electrophoresed through the gel at 20mA in the presence of elecrophoresis buffer (2.13) to which 100μM thioglycollate had been added.

b) Electrotransfer of protein onto Immobilon P.

The following method for the electotransfer of material from a polyacrylamide gel to Immobilon P is taken from Matsudaira, (1987). Following electrophoresis the gel was soaked in transfer buffer (10mM CAPS, 10% methanol, pH11.0) for 5 min. During this time a piece of Immobilon P was cut to the same size as the gel, soaked in 100% methanol and stored in the transfer buffer. The gel, sandwiched between a sheet Immobilon P and several sheets of 3MM paper, was assembled into a wet blotting apparatus and electroeluted for 16 hours at 0.2 amps in transfer buffer.

c) *Staining Immobilon P with Coomassie Blue R-250.*

The staining method was taken from Matsudaira, (1987). The Immobilon P membrane onto which protein had been transferred was washed in deionised water for 5 min. The proteins were detected by staining the membrane with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min then destaining in 50% methanol, 10% acetic acid for 10 min at room temperature. The membrane was then washed in deionised water. The band corresponding to the protein of interest ($C(His)_8$Ste6p in this case) was then excised and retained for protein sequence analysis.

d) Direct transfer of protein onto Immobilon P.

The following method was obtained from D.K. Apps (University of Edinburgh). The $C(His)_8$Ste6p was separated from contaminating proteins by polyacrylamide gel electrophoresis as detailed in 2.25a. The gel was stained with filtered Coomassie Blue staining solution (2.13a). The band corresponding to $C(His)_8$Ste6p was excised, weighed, crushed and added to 2X volume per weight 100mM NH_4HCO_3. SDS was added to a concentration of 0.1% and the mixture was incubated for 24 hours at room temperature. The mixture was diluted 2X with distilled water and a $100mm^2$ piece of Immobilon, which had been wetted with methanol, was added to the mixture. The protein solution and Immobilon membrane were incubated for several hours to allow binding to take

place. The membrane was washed with water and dried and used for N-terminal protein sequence analysis.

Miscellaneous methods

2.26 Detection of Ste6p activity, the "halo assay"

The **a**-factor supersensitive strain, RC757, was grown in YEPD until an A_{600} of 0.6 was obtained. A 1 ml aliquot of the cells was then added to 4ml of top agar (YPD or YPG plus 0.8% Bacto agar which had been maintained at 45°C) and poured immediately onto the required agar plate (YPD or YPG) to create a lawn of sensitive cells. The plates were allowed to set and were dried by placing them in a 30°C incubator for 30 min. Overnight cultures of the strains to be tested for **a**-factor secretion were harvested by brief centrifugation (6,000 rpm for 3 min in a bench top microfuge) and 3µl of the cells, or purified **a**-factor, were spotted onto the lawn. The plates were then incubated for 2-3 days at 30°C until the RC757 lawn had grown and halos of growth inhibition due to the presence of **a**-factor could be seen. Halo size is indicative of how much **a**-factor is present, the greater the halo the more **a**-factor present.

Chapter 3

Production and affinity purification of rabbit antibodies specific for the C-terminal region of Ste6p

3.1 Introduction

In the absence of a functional assay for Ste6p it was necessary to raise antibodies against Ste6p to allow detection. In order to produce (Ste6p) specific antibodies in rabbits an appropriate antigen had to be produced. One way of producing antigen from a cloned gene is to create a fusion protein which can be overexpressed in *E.coli* and affinity purified from bacterial extracts. A variety of suitable expression vectors are available including one which permits fusing the gene of interest to the N-terminal portion of protein A, consisting of two and a half IgG binding domains, by use of the pAX vector system (Zueco and Boyd, 1992). Protein A, a polypeptide from the cell wall of *S. aureus*, is an ideal candidate for fusion protein production as it has a high affinity for the F_c domain of immunoglobulins. This enables the fusion protein to be readily affinity purified on commercially available IgG Sepharose. The IgG binding capacity of protein A also allows for detection of chimaeric proteins with commercially available rabbit anti-bovine IgG HRP conjugate (SAPU) following Western Blot analysis. It is thought also that the repetitive structure of protein A enhances the immunological response to the fusion protein (Lowenadler *et al.*, 1986).

Antigen-specific antibodies may be purified from serum by immunoaffinity absorption. This may be achieved by the production in *E. coli* of a second fusion protein by means of the pEX vector system (Kusters *et al.*, 1989). Here the gene is expressed from the P_R promoter as a cro-lacZ (β-galactosidase) fusion protein under control of the temperature-sensitive cI875 repressor protein giving a very high and consistent level

of gene expression. The fusion protein is purified from inclusion bodies by preparative SDS-PAGE and attached to a solid support as described in Chapter 2.

This Chapter describes the production and purification of a bacterially-expressed Ste6p-proteinA fusion protein to use as an antigen for the production of anti Ste6p antibodies. I also describe the production of a β-galactosidase-Ste6p fusion protein and its use in the affinity-purification of Ste6p specific antibodies.

3.2 Construction of a protein A-tagged Ste6p chimaeric protein

Ste6p is thought to have 12 membrane-spanning domains (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989) making it a fairly hydrophobic molecule. For this reason it was decided to raise antibodies against the extreme C-terminal portion of the protein. This region is thought to be exposed on the cytosolic face of the plasma membrane. The solubility of the fusion protein was an important consideration as the production of insoluble aggregates (inclusion bodies) would make purification of the fusion protein on IgG Sepharose difficult. Another important consideration when choosing the region of Ste6p to use in antibody production was the degree of sequence similarity between domains of Ste6p and other members of the ABC transporter family which may be present in yeast. The other major extramembrane domains of the protein are thought to be mainly located on the cytosolic face of the cell membrane and are thought to encode the conserved nucleotide binding-domains associated with the ABC transporter family. At this time little was known about other *S. cerevisiae* ABC transporter proteins. It was hoped that the C-terminal region of

Ste6p would share little sequence homology with other family members so as to avoid immunological cross reactivity which could render a polyclonal antibody useless.

A *Sal*I fragment of approximately 6.6kb containing the entire *STE6* gene and 600bp of upstream sequence cloned into pUC7 was obtained from Benjamin Glick, University of Basel (originally supplied by Alexander Varshavsky MIT). A 1.2kbp fragment corresponding to the extreme 3' end of the *STE6* open reading frame was amplified by PCR using as primers the oligonucleotides;

1) 5'-CGG AAT TCA GAT ACC CGA TAT AAG TAG AGG-3'

2) 5'-CGG GAT CCT TTC TTA TGG CGT TTC TCT TTA TGC CTC-3'.

These two oligonucleotides contain the restriction sites *Eco*RI and *Bam*HI, respectively which were used for sub-cloning. The PCR product was cloned into the *Bam*H1 and *Eco*R1 sites of pAX12 (Zueco and Boyd, 1992) to create an in-frame fusion with IgG binding domains of protein A under control of the *lac* promoter. Expression from the *E. coli lac* promoter can be induced by the presence of IPTG (Chapter 2). This construct was called pAMB1 and was predicted to encode a protein of 56kDa (16.9kDa from the protein A and 39kDa from the Ste6p). This fusion protein was called PrtA-Ste6p.

3.3 Expression of PrtA-Ste6p in *E. coli*

The *E.coli* strain NM522 was transformed with either pAX12 or pAMB1 to make strains NM522(pAX12) and NM522(pAMB1) respectively. Expression of PrtA-Ste6p in the presence of IPTG was compared with IPTG-induced protein expression from untransformed NM522 and NM522(pAX12) (Figure 3.1a). The blot indicates that no protein was expressed from untransformed NM522 cells which was capable of interacting with rabbit IgG (lane 1). In lane 2 a band of around 17kDa corresponding to the protein A moiety from pAX12 was detected. Induced NM522(pAMB1) cells produced a protein of approximately 56kDa (lane 3). This corresponds to the expected molecular weight of the PrtA-Ste6p chimaera demonstrating that the fusion protein is being expressed correctly. To examine further the expression of PrA-Ste6p in NM522, cell lysates of IPTG induced NM522(pAMB1) were subjected to differential centrifugation and Western Blot analysis. The results shown in Figure 3.1b indicate that the majority of the chimaera is found in the insoluble, P20, fraction in cells grown at 37°C.

3.4 Expression of the PrtA-Ste6 fusion protein in cells grown at 25°C

For ease of purification it was important that the PrtA-Ste6p chimaera could be isolated from a soluble extract of the cells. Early expression studies carried out at 37°C showed that the fusion protein was found predominantly in the insoluble P20 fraction (Figure 3.1b). In an attempt to prevent the formation of insoluble protein

aggregates (inclusion bodies) cells were grown and expressed at 25°C. Figure 3.2 indicates that the majority of the fusion protein is found in the soluble S20 fraction in NM522(pAMB1) grown at 25°C. In comparison, when grown at 37°C, nearly all of the fusion protein is in the insoluble P20 fraction. There was a considerable amount of protein degradation apparent in cells grown at 25°C. Subsequent experiments on the PrtA-Ste6p were conducted at 25°C with an induction time of 2 hours.

3.5 RIPA buffer enhances binding of the PrtA-Ste6 fusion protein to IgG Sepharose

Affinity purification of protein A fusion proteins is normally achieved by binding soluble cell extract onto IgG Sepharose under suitable conditions (low salt concentration) washing the Sepharose to remove non-specifically bound protein and then eluting the fusion protein either by increasing the salt concentration or by decreasing pH. Initial purification studies on the PrtA-Ste6 fusion protein indicated that it failed to bind efficiently to the IgG Sepharose. In an attempt to increase binding efficiency the S20 fraction of the cell lysate was treated with a complex detergent mixture called RIPA buffer (added to give a final concentration of 150mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate and 0.1% SDS). The results obtained using RIPA-treated S20 extracts compared with those in a low salt solution are shown in Figure 3.3. It can be seen that a far greater proportion of the fusion protein is bound to IgG Sepharose when RIPA buffer is added to the S20 fraction prior to addition to the IgG. Figure 3.3 also demonstrates that the temperature at which binding is carried

out is unimportant. Subsequent purifications were carried out at 4°C in the presence of RIPA buffer.

3.6 Purification of the PrtA-Ste6 fusion protein and its use as an antigen for production of Ste6p specific antibodies

The PrtA-Ste6p fusion protein was bound onto IgG Sepharose in the presence of RIPA buffer as detailed in section 3.5. Contaminating proteins were removed by washing the IgG Sepharose in wash buffer (150mM NaCl, 50mM Tris-HCl and 0.05% Tween 20 pH 8.0) until the wash buffer gave an A_{280} of less than 0.1. At this point several attempts were made to elute the chimera from the IgG Sepharose. It was found that neither high salt (1M NaCl, 50mM Tris.HCl, 0.05% Tween 20 pH8.0) nor 0.5M acetic acid were able to remove the majority of the chimera from the IgG Sepharose (data not shown). To overcome this problem the fusion protein was electroeluted from a preparative polyacrylamide gel loaded with IgG-Sepharose-bound PrtA-Ste6p which had been dissociated in gel sample buffer. The eluate was dialysed overnight against distilled water and concentrated by lyophilization. Sufficient pure PrtA-Ste6p was obtained to use as antigen for antibody production in two New Zealand White rabbits (as detailed in Chapter 2). At a later date it was discovered that the fusion protein could be dissociated from the IgG Sepharose by incubation in the presence of 0.1M Glycine pH 3, 1% CHAPS for 20 min (Figure 3.4). This method gave greater yields of fusion protein and was less time demanding than elution from a preparative gel. Protein purified by this later method was used for all subsequent booster inoculation of the rabbits.

3.7 Construction and expression of a βgal-ste6 chimaeric protein for the affinity purification of anti Ste6p antibodies

Antibodies specific to Ste6p protein were affinity-purified by the use of a second fusion protein. This time a βgal-Ste6p fusion protein was produced by sub-cloning the 1.2kbp *Eco*RI/ *Bam*HI fragment of pAMB1 corresponding to the extreme 3' end of *STE6* into pEX12 (Kusters *et al.*, 1989) to create an in-frame fusion with β-galactosidase. The resultant plasmid, pAMB3, was transformed in to the *E. coli* strain pop2136. Growth and expression from the P_R promoter was carried out as detailed in Chapter 2 and was compared with pop2136 cells containing either no vector or the parent pEX12 vector. The results are shown in Figure 3.5. The results indicate that the βgal-Ste6p fusion protein is being correctly expressed in pop2136.

3.8 Detection of Ste6p in yeast with the affinity-purified anti-Ste6p antibody

Polyclonal antibodies specific for Ste6p were affinity purified from rabbit serum by the method detailed in 2.24. The affinity purified antibodies were tested for their ability to bind specifically to Ste6p expressed in yeast by use of the *S. cerevisiae* stains BJ5464 and WKK7 (Table A2). The BJ5464 strain has a wild-type *STE6* gene whereas WKK7 is a *STE6*-deleted strain. As shown in Figure 3.6a the antibodies recognise a protein of approximately 145kDa (the expected molecular weight of Ste6p) in a membrane extract of BJ5464 cells whereas no protein was detected in a membrane fraction of WKK7. To further demonstrate that the antibody was specific for Ste6p, WKK7 was transformed with pAMB14 (Table A3) which contains the *STE6* gene

under the control of the powerful *GAL* promoter. When WKK7(pAMB14) was grown on mannose no immunoreactive protein was detectable in the membrane fraction, however when grown in the presence of galactose a protein of 145kDa was detected (Figure 3.6b). Thus the affinity-purified antibody was specific for Ste6p and was capable of detecting Ste6p in yeast cell membranes by Western blot analysis.

3.9 Discussion

In this Chapter the successful production, expression and purification of an affinity purified anti-Ste6p antibody was described. The antibody was capable of detecting Ste6p found in membrane fractions of glass bead-lysed yeast cells by Western Blot analysis. The affinity-purified antibody was found to be quite unstable and tended to be non-functional after 1-2 months even when stored in aliquots at -20^{o}C. The addition of 1-2% BSA appeared to help stabilise the antibody; however, it was still necessary to re-purify antibody from rabbit serum at regular intervals.

Figure 3.1 Expression of PrtA-Ste6p in *E. coli*

A. The *E. coli* strain NM522 was transformed with either pAX12 or pAMB1 and grown shaking at 37°C in LB plus ampicillin until an A_{600} of 0.6 was obtained. The cultures were then induced by the addition of.0.25mM IPTG and grown at 37°C for a further 4 hours. Cell pellets were weighed and resupended in an equal volume of 2x SDS-PAGE sample buffer. The samples were then boiled for 10 min before analysis by 12% SDS-PAGE. The proteins were visualised by Western blot analysis using a rabbit anti-bovine IgG HRP conjugate at a 1:10,000 dilution for 20 min. The blot was developed by use of enhanced chemiluminescence (ECL).

B. A culture of NM522(pAMB1) was grown and induced as in 3.1a. The cells were harvested then subjected to glass bead lysis and differential centrifugation as described in Chapter 2. The cell fractions (P3, S20 and P20) were made up to an equal volume and combined with 2x SDS-PAGE sample buffer. The samples were then subjected to Western blot analysis as detailed in Figure 3.1a.

A.

PrtA-Ste6p
(56KDa)

PrtA
(17kDa)

Lane 1 NM522

Lane2 NM522(pAX12)

Lane3 NM522(pAMB1)

B. **NM522(pAMB1)**

PrtA-Ste6p
(56KDa)

Lane 1 P3

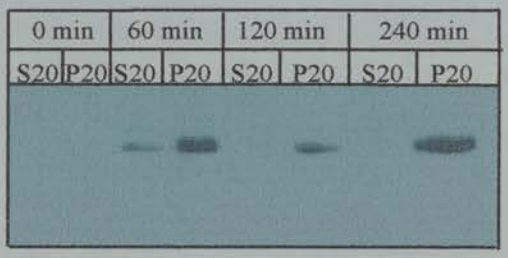
Lane 2 S20

Lane 3 P20

Figure 3.2 The PrtA-Ste6p fusion protein is soluble in cells grown at 25°C

NM522(pAMB1) cells were grown at either 37°C or 25°C until an A_{600} of 0.6 was reached. They were then induced by the addition of 0.25mM IPTG and grown for a further 4 hours. Samples were taken at hourly intervals. The cells were lysed and then subjected to differential centrifugation to give S20 and P20 fractions as described in materials and methods. The samples were then run on 12% SDS-PAGE and the presence of the fusion protein detected by Western blot analysis using a rabbit anti-bovine IgG HRP conjugate at a 1:10,000 dilution for 20 min. The blot was developed by use of ECL.

a) NM522(pAMB1) grown at 37°C



b) NM522(pAMB1) grown at 25°C

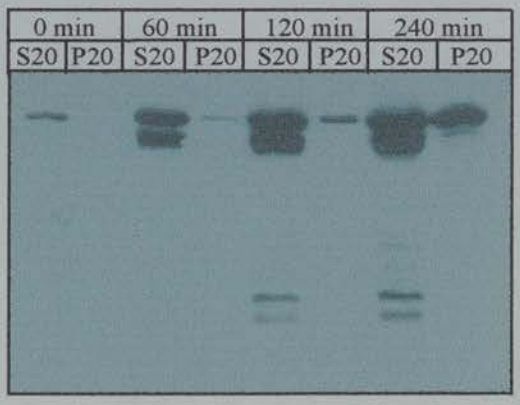


Figure 3.3 Solubilisation of the PrtA-Ste6p chimera with RIPA buffer aids binding to IgG Sepharose

Overnight cultures of NM522(pAMB1) were inoculated into LB with ampicillin and grown shaking at 25°C until an A_{600} of 0.6 was obtained then induced by the addition of 0.25mM IPTG and grown for a further 2 hours. The cells were lysed and the S20 fraction collected as detailed in Chapter 2. The S20 fraction was then either left untreated or treated with RIPA buffer (final concentration 150mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate and 0.1% SDS) and applied to 1ml of IgG Sepharose previously equilibrated with wash buffer (150mM NaCl, 50mM Tris-HCl and 0.05% Tween 20 pH8.0). Binding of the fusion protein occurred during gentle rotation at either room temperature or at 4°C for 1 hour. The IgG Sepharose was then pelleted by centrifugation (30 seconds at 3,000g) and the supernatant fraction ("unbound") collected for analysis by SDS-PAGE. The IgG Sepharose was then washed with wash buffer until an A_{280} of less than 0.01 was obtained, then resuspended in 2X SDS-PAGE sample buffer (Chapter 2). Samples of unbound and bound chimaeric protein were analysed by 12% SDS-PAGE and Western blot analysis as described in Figure 3.1. In the following blot B signifies Chimaeric protein Bound to the IgG Sepharose with UB indicating material which remained UnBound following incubation with IgG Sepharose.

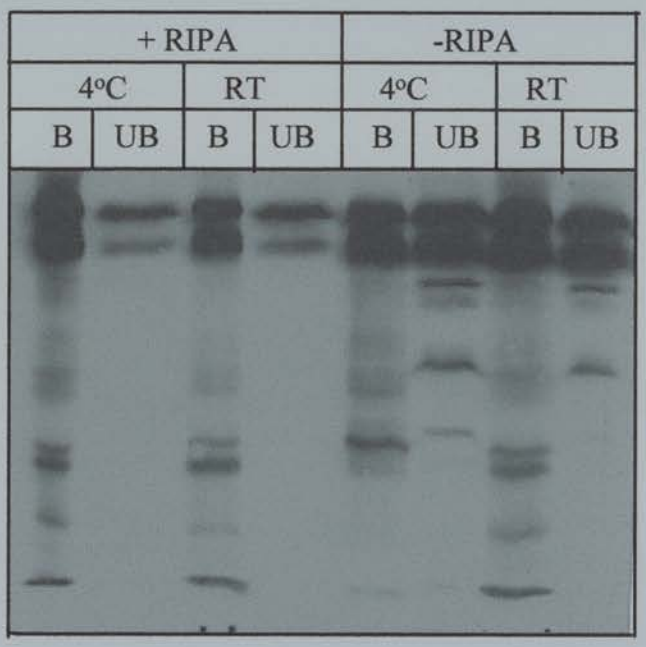

+ RIPA
-RIPA
4°C
RT
4°C
RT
B
UB
B
UB
B
UB
B
UB

Figure 3.4 Purification of the PrtA-Ste6p fusion protein on IgG Sepharose

The S20 fraction from a 500ml culture of NM522(pAMB1), grown and expressed as detailed earlier in this Chapter, was treated with RIPA buffer before being applied to 1ml of IgG Sepharose previously equilibrated in wash buffer. Binding was carried out for 1 hour at 4°C with constant gentle agitation. The IgG Sepharose was then washed with wash buffer until an A_{280} of less than 0.01 was obtained. The chimaera was removed from the IgG Sepharose by incubation in the presence of 0.1M glycine pH3, 1% CHAPS for 20 min with gentle agitation. The eluate was then dialysed and concentrated by lyophilization. The purified fusion protein was analysed by 12% SDS-PAGE and either stained with Coomassie Blue or transferred onto nitrocellulose and visualised with ECL following probing with rabbit anti-bovine IgG HRP conjugate at a 1:10,000 dilution for 20 min.

A) SDS-PAGE: a 12% polyacrylamide gel stained with Coomassie Blue.

Lane 1 Molecular Weight markers

Lane 2 PrtA-Ste6p

B) Western blot of the stained gel shown in A.

Lane 1 PrtA-Ste6p

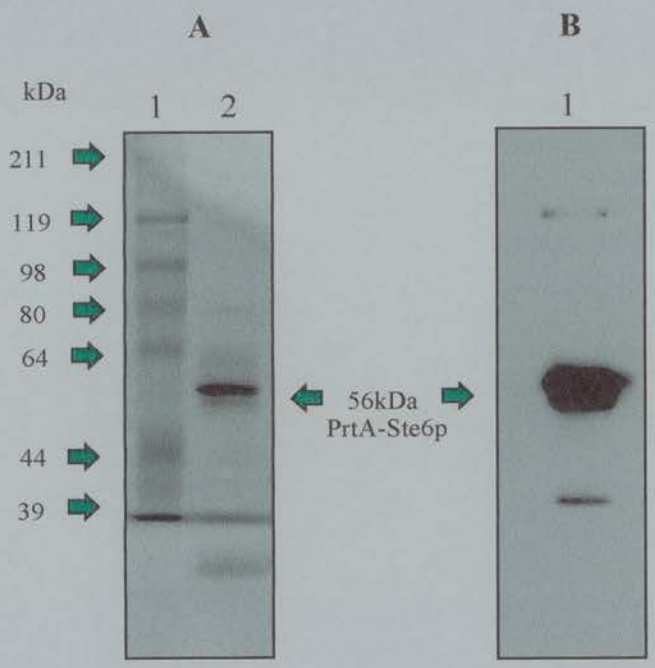

A
B
kDa
1
2
1
211
119
98
80
64
44
39
56kDa
PrtA-Ste6p

Figure 3.5 Expression of a βgal-Ste6 fusion protein in pop2136 cells

Untransformed pop2136, pop2136(pEX12) and pop2136(pAMB3) cells were grown, shaking, in 100ml LB plus ampicillin at 30°C until an A_{600} of 0.6 was obtained. Half the culture from each was then shifted to 42°C, the remainder staying at 30°C, and grown for a further 2 hours. The cells were then lysed and the P3 (inclusion body containing) fraction collected as detailed in Chapter 2. The inclusion body fraction from each culture was resupended in 5ml lysis buffer, mixed with 2X sample buffer and analysed by SDS-PAGE (Chapter 2).

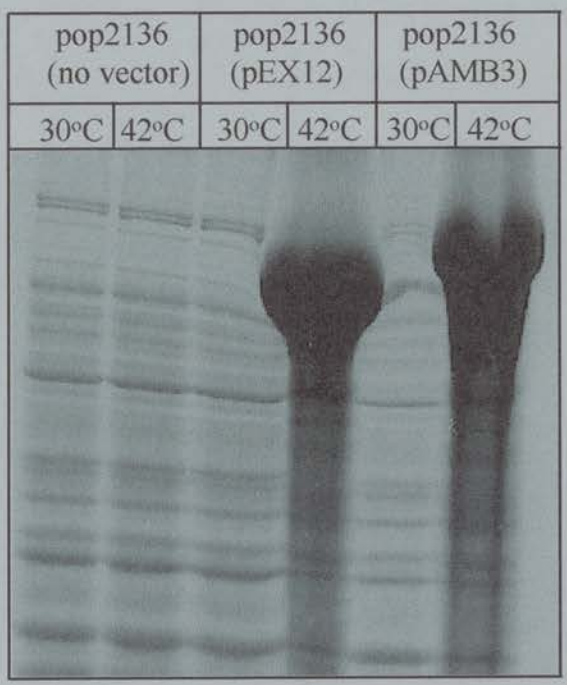

pop2136
(no vector)
pop2136
(pEX12)
pop2136
(pAMB3)
30°C
42°C
30°C
42°C
30°C
42°C

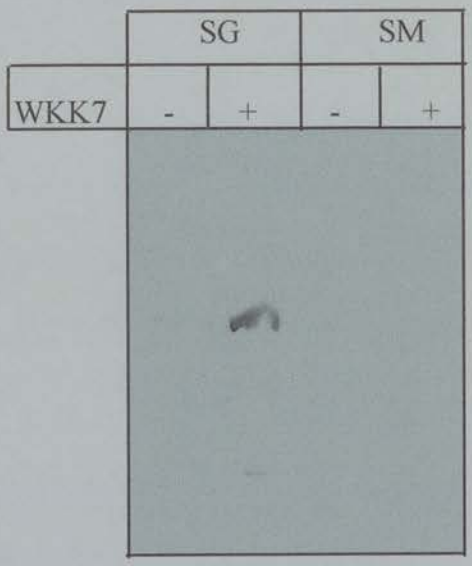
Figure 3.6 Detection of yeast Ste6p with affinity purified antibodies

Figure 3.6a single colonies of yeast strains BJ5464 and WKK7 were grown overnight in selective media containing glucose as a carbon source (SD). The following day the cultures were inoculated into 50ml of SD and grown until an A_{600} of 0.6 was obtained. The cells were harvested then subjected to glass bead lysis and differential centrifugation to yield a membrane, P100, fraction as described in Chapter 2. The P100 fractions were then subjected to SDS-PAGE and Western blot analysis. The blot was probed with affinity-purified anti-Ste6p antibody at a 1:100 dilution for 16 hours followed by a 1: 1,000 dilution of Donkey anti-rabbit HRP conjugate for 20 min. The blot was then developed by use of ECL.

Figure 3.6b single colonies of yeast strains WKK7 and WKK7(pAMB14) were grown in selective medium containing mannose as a carbon source (SM). The following day the cultures were inoculated into 50ml of either SM or SG (selective media containing galactose) and grown until an A_{600} of 0.6 was obtained. The cells were then lysed and subjected to Western blot analysis under identical conditions to those used for 3.6a.

A.

B.



- Wild-type WKK7
+ WKK7(pABK2)

Chapter 4

The attempted purification of a recombinant Ste6p tagged at the amino terminus with six histidine residues

4.1 Introduction

In order to carry out biochemical analysis of the Ste6p and **a**-factor substrate it was essential that the Ste6p could be purified and reconstituted into phospholipid vesicles. It was intended that the wild-type Ste6p would be isolated from a membrane enriched fraction of lysed yeast cells that were overexpressing the protein. The first stage of the purification would be to determine which detergents were capable of solubilising the Ste6p. Once solubilised it was hoped that the protein could be purified using standard techniques such as ion-exchange chromatography and exclusion chromatography.

As an alternative to traditional purification techniques there are various methods available that enable a protein of interest to be tagged to allow rapid affinity purification. One example of this is tagging a protein with a domain of Protein A to allow purification on IgG Sepharose as detailed in Chapter 3. Another, now widely used technique exploits metal chelate chromatography. For this the protein of interest is tagged at either the amino or carboxyl terminus with six or more consecutive histidine residues. These histidine residues have a high affinity for the metal nickel that may in turn be bound to a solid matrix. The commercially available Ni-NTA (nitrilo-tri-acetic-acid) resin (Qiagen) is capable of binding hexa-histidine tags with a $Kd=10^{-13}$ M at pH8. The high binding efficiency permits the removal of contaminating protein either by competition with imidazole or by lowering the pH and theoretically leads to a one-step purification procedure. The tag is very small and is therefore unlikely to interfere with protein structure. Another advantage of using this type of affinity tag is that the resin does not require any functional protein structure and can therefore bind

tagged protein in the presence of 8M Urea, 10mM β-mercaptoethanol, up to 1% non-ionic detergent and salt concentrations of 1M. All of the above considerations are important in the purification of a large membrane protein which has a low level of expression and a relatively short half life.

Initial purification studies were centred around the constitutive expression of wild-type protein from a yeast episomal vector. The protein was found to be easily solubilised with a wide variety of detergents and was detectable by Western blot analysis. However, the level of protein expression, even when expressed in a strain in which the vacuolar ATPase encoded by the *PEP4* gene had been deleted, was poor and it was envisioned that it would be virtually impossible to purify by conventional means.

As an alternative to conventional purification procedures the amino terminus of Ste6p was tagged with a hexa-histidine tail. It was hoped that resultant recombinant protein ($N(His)_6$Ste6p) could be easily purified from the membranes of yeast expressing the protein. Several different purification procedures were attempted using both native and denaturing conditions, however, none appeared to enable binding of $N(His)_6$Ste6p to the Ni-NTA resin. It was proposed that the $N(His)_6$Ste6p failed to bind to the Ni-NTA resin because the hexa-histidine tail had been removed during posttranslational processing of the protein.

This Chapter describes the attempts made to purify from *S. cerevisiae* both wild-type Ste6p and an N-terminally hexa-histidine tagged variant.

4.2 Production of a Ste6p-overexpressing strain of *S. cerevisiae*

For the purification of wild-type Ste6p to be successful it was essential that its expression in *S. cerevisiae* could be optimised to give high protein yields. As discussed in Chapter 2 Ste6p is a metabolically unstable protein which is stabilised by yeast containing a defect in the vacuolar protease A gene *PEP4* (Kölling *et al.*, 1994; Berkower *et al.*, 1994). With this in mind it was decided that BJ5465 a *pep4 MAT***a** strain of *S. cerevisiae* (Table A2) would be used for the expression studies.

An expression vector for wild-type Ste6p was produced by cloning the *Sal*I fragment of pSTE6, which contains the entire *STE6* gene, into the *Sal*1 site of the yeast episomal vector YEplac181 (Geitz and Sugind., 1988) to make pAMB7. This plasmid was transformed into the yeast BJ5465 to create BJ5465(pAMB7). Expression of Ste6p from BJ5465 and BJ5465(pAMB7) was compared by Western blot analysis of crude cell lysates as detailed in Figure 4.1. As can be seen from Figure 4.1 there was a large increase in the amount of Ste6p expression from BJ5465(pAMB7) when compared to wild-type BJ5465. All subsequent experiments on the purification of wild-type Ste6p utilised the BJ5465(pAMB7) strain. A control experiment was carried out in which pAMB7 was transformed into the *MAT*α strain JRY188. Expression of Ste6p from JRY188 and JRY188(pAMB7) was carried out as detailed in Figure 4.1. As expected no Ste6p expression was detected in either strain (data not shown) in agreement with Wilson and Herskowitz (1984) who observed that *STE6* expression is cell type specific and negatively regulated by the *MAT*α2 product.

The Ste6p detected by Western blot analysis appears as a diffuse band. The appearance of the band would correspond with that of a glycoprotein; however Ste6p is not thought to be glycosylated (Kuchler *et al.*, 1993). Attempts were made at improving the resolution of the band by altering the conditions of protein treatment prior to and during analysis by SDS-PAGE. The most favourable conditions found are those detailed in Figure 4.1.

4.3 Localisation of Ste6p by differential centrifugation

In order to optimise the purification conditions it was first necessary to determine which cell fraction contained the greatest quantity of Ste6p. Crude cell lysates of growing BJ5465(pAMB7) were subjected to differential centrifugation and analysed by SDS-PAGE and Western blot analysis (Figure 4.2). As can be seen from Figure 4.2b the greatest quantity of Ste6p was found in the membrane fraction (P100). A large quantity of Ste6p was also found in the P3 and P12 fractions. The most likely cause of this is incomplete cell lysis. Figure 4.2a indicates that there are a great many contaminating proteins in the P100 fraction. It also shows that Ste6p is not a major protein and is not identifiable as a distinct protein in a crude cell lysate such as this. All further experiments on the isolation of Ste6p were conducted on the P100 fraction from BJ5465(pAMB7) cells lysed with glass beads.

4.4 Ste6p is solubilised by a wide range of detergents

When determining which detergent to use for the purification it was necessary to take into account its critical micelle concentration (CMC). A detergent with a high CMC is preferable for the reconstitution of protein into proteoliposomes and a CMC of 1mM or greater is preferable for detergent removal by dialysis (Neugebauer, 1990). Preliminary experiments conducted on the solubilisation of Ste6p with a range of non-ionic detergents indicated that Ste6p was readily solubilised by 1% Thesit, CHAPS, Triton X-100 and Zwittergents 3-8 to 3-14. Of the detergents tested only LDAO and MEGA-8 were inefficient at solubilising Ste6p. The Zwittergent 3-10 was picked for the solubilisation of Ste6p, due to its favourable CMC and its relative cost efficiency.

At this point it became clear that it was going to be very difficult to purify wild-type Ste6p from *S. cerevisiae*. The main problem envisioned was the very low level at which the protein was being produced. This could have been overcome if large culture volumes had been utilised, however this was deemed impractical due to the constraints of handling large cell quantities. The expression of the protein might also have been improved had it been put under the inducible control of the powerful *GAL* promoter. Another alternative was to affinity-tag the protein with a oligo-histidine tail so that it could be "pulled" out of a dilute solution by its high affinity to Ni-NTA resin (Qiagen). It was decided that the Ste6p should be tagged with six consecutive histidine residues at its amino terminus and be put under control of the *GAL* promoter to produce $N(His)_6$Ste6p. The amino terminus was chosen for several

reasons. First the anti-ste6p antibody is directed against the C-terminus of the Ste6p and there were worries that a tag here would interfere with some antibody recognition. The C-terminus is thought to contain a large non-membrane associated domain on the cytosolic face whereas the N-terminus is thought to be part of a short, non-membrane associated domain on the cytosolic face of the plasma membrane. Thus it was reasoned that the larger C-terminal domain was more likely to have an integral role in the function of the Ste6p than the rather shorter N terminal region. As a consequence it was decided not to place the oligo-histidine tag on the C-terminus in case it affected the activity of the protein.

4.5 Production of a recombinant Ste6p by attaching six consecutive histidine residues on the amino terminus to produce N(His)$_6$Ste6p

The addition of six consecutive histidine residues to the extreme N-terminus of Ste6p was carried out by the use of PCR using the following primers:

1) 5' primer

Start 5' end of *STE6*

5'-CGCC AAG CTT ATG **CAT CAC CAT CAC CAT CAC** AAC TTT TTA AGT TTT AAG TA C-3'

*Hin*dIII 6 consecutive histidine residues

2) 3' primer:

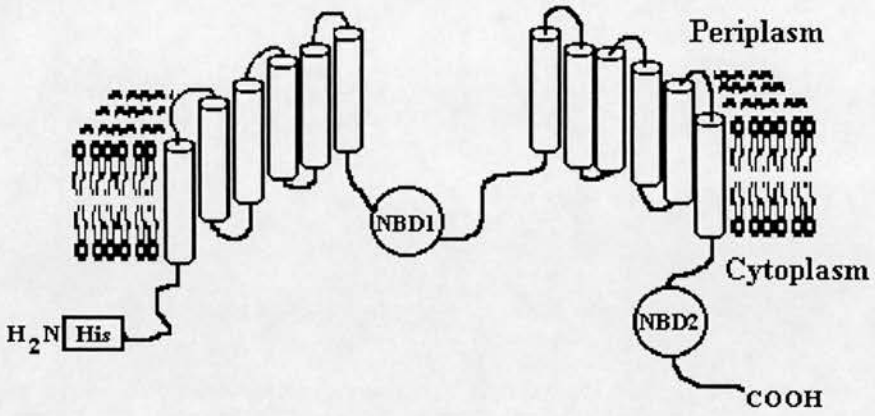
3' end of *STE6*

5'-CGC CAA GCT TTT ACC ATT CCA TCT ATG AGT AAC-3'

*Hin*dIII

The PCR reaction was carried out using VENT polymerase with pSTE6 as the template as detailed in Chapter 2. The 4.3kb product was digested with *Hind*III and cloned into the *Hind*III site of pK19, a standard laboratory general purpose vector, to produce pAMB10. The *Hin*dIII fragment containing the tagged *STE6* gene was then subcloned into the *Hin*dIII site of the yeast episomal vector YEpGAL thus placing the tagged *STE6* gene under the control of the inducible *GAL* promoter. The resultant vector was called pAMB11. It was possible that the PCR process could introduce an error into the *STE6* gene that would render it useless. It was decided therefore to sequence to the first *Eco*R1 site (1kb down stream of the extreme 5'-end of the tagged *STE6* gene) so that, if necessary, the tagged region of the gene could be sub-cloned into the wild type sequence using a simple *Hin*dIII/*Eco*R1 digest of both pAMB11 and pSTE6 to produce a "wild-type" gene. A high quality sample of pAMB10 was produced by the Qiagen midi-preparation kit from the *E .coli* strain DH5α. The DNA was then subjected to double stranded DNA sequencing (method 1) as detailed in Chapter 2 with the M13 forward sequencing primer and primers N1384-5'-AGC GGC ATT CAT CGA CCA-3' and N2710-5'-CAT CAA GGT TTG TAT TCC-3'. This enabled the first 1 kb of DNA corresponding to the extreme 5' end of the histidine tagged *STE6* gene to the first *Eco*R1 site be read. The sequence was checked by two non-biased readers and was found to contain no mistakes.

A cartoon of N(His)$_6$Ste6p spanning the plasma membrane is shown below. The boxed "His" motif at the N-terminus indicates the position of the six consecutive histidine residues and NBD stands for Nucleotide Binding Domain.



4.6 N(His)$_6$Ste6p complements a *ste6* strain of *S. cerevisiae*

It was essential that N(His)$_6$Ste6p could act as a functional **a**-factor transporter. In order to test this an **a**-factor supersensitive strain of *S. cerevisiae*, RC757 (*MAT*α *sst2* Table A2), was utilised. Mutations in *sst2* cause both **a** and α cells to display both hypersensitivity and greatly prolonged responses (especially to division arrest) to pheromone (Chan and Otte, 1982a,b; reviewed in Sprague and Thorner, 1992). As the *sst2* mutation in RC757 renders it hypersensitive to **a**-factor, the secretion of **a**-factor can be semi-quantitatively monitored by the production of an area of growth inhibition, or "halo", around an **a**-factor producing patch of cells on a lawn of RC757 cells. To test whether N(His)$_6$Ste6p was capable of producing active Ste6p, pAMB11 was transformed into the *S. cerevisiae* strain WKK7 which has a *MAT***a** Δ*ste6*::*HIS3* genotype (Table A2). The expression of N(His)$_6$Ste6p and its ability to complement the WKK7 Δ*ste6*::*HIS3* genotype is detailed in Figure 4.3. Wild-type WKK7 was unable to secrete **a**-factor in the presence of either glucose or galactose. As expected

the control strains, BJ5465 and WKK7(pAMB7), were capable of secreting **a**-factor on both carbon sources. No halo was produced from WKK7(pAMB11) grown on glucose however **a**-factor secretion was apparent when the cells were grown on galactose. The $N(His)_6$Ste6p was thus capable of complementing a *ste6* strain of yeast and was produced in the presence of galactose and not glucose.

4.7 Expression of $N(His)_6$Ste6p in BJ5465

$N(His)_6$Ste6p was expressed in BJ5465 in order to stabilise the protein by virtue of the strain's *pep4* genotype. As a consequence the $N(His)_6$Ste6p would be expressed in a *STE6* wild-type background producing a protein mix that would be indistinguishable by Western blot analysis unless the addition of the histidine residues greatly altered the molecular weight of $N(His)_6$Ste6p in comparison to Ste6p. This mixture of anti-Ste6p antibody reactive proteins will be referred to simply as Ste6p immunoreactive material.

The expression of $N(His)_6$Ste6p was checked by comparing the amount of Ste6p produced in BJ5465 cells with anti-Ste6p antibody immunoreactive protein produced by BJ5465(pAMB11) cells. The cells were grown and induced with galactose as detailed in Figure 4.4. As can be seen from Figure 4.4 the membrane fraction of BJ5465(pAMB11) cells appeared to contain more Ste6p immunoreactive material than wild-type BJ5465 membranes isolated under identical conditions. The protein detected by the anti-Ste6p antibody appears as a distorted, or smudgy, band. This

banding pattern had been observed previously and did not appear to be due to the $N(His)_6$Ste6p.

The DNA sequence data, halo assay and Western blot of $N(His)_6$Ste6p expression gave the clear impression that an active, galactose induced N-terminal hexa-histidine tagged variant of Ste6p was being produced from the pAMB11 vector. From these data we decided to continue the purification studies in the knowledge that the protein was active and correctly tagged at the N-terminus. In addition the solubilisation of $N(His)_6$Ste6p protein with the range of detergents detailed in section 4.4 gave comparable results to the those obtained from the wild-type protein (data not shown).

4.8 Attempted purification of $N(His)_6$Ste6p solubilised from BJ5465(pAMB11) membranes

It was hoped that the $N(His)_6$Ste6p could be purified from the membranes of galactose-induced BJ5465(pAMB11) cells under relatively mild conditions. Initial purification studies were centred on solubilising the protein from a glass bead lysate using the Zwittergent 3-10. The results shown in Figure 4.5 indicate that a large amount of Ste6p immunoreactive material was present in the cell lysate suggesting successful induction of $N(His)_6$Ste6p expression. Comparison of the amounts of protein in the S20 and P20 fractions indicates that the protein was at best only 50% soluble. The disappointing solubilisation of anti-Ste6p antibody reactive protein may have been due partially to incomplete cell lysis. The lysis could have been monitored by measuring the absorbance due to the release of DNA from lysed cells at 260nm

until it reached a constant level. The blot also indicates that there was a large amount of Ste6p immunoreactive material in the unbound fraction suggesting that very little, if any, soluble $N(His)_6$Ste6p applied to the Ni-NTA resin actually bound to it. There was no Ste6p immunoreactive protein found in either of the washes nor the eluate. The results shown in Figure 4.5 indicate that the $N(His)_6$Ste6p was unable to bind to the Ni-NTA resin under these conditions.

An identical experiment to that described in Figure 4.5 was carried out using Triton X-100 instead of Zwittergent 3-10. The Triton X-100 was marginally better at releasing anti-Ste6p antibody reactive protein from membranes of induced BJ5465(pAMB11) cells and was an economical detergent to use while initial protein binding experiments were being conducted. Virtually identical results were obtained when Triton X-100 was used (data not shown) indicating that no anti-Ste6p antibody reactive protein was being bound to the Ni-NTA resin. Further experiments in which the concentration of TX-100 for both protein solubilisation and binding were varied were also carried out. No Ste6p immunoreactive material was found to have been bound to or eluted from the Ni-NTA resin under any of the detergent concentrations attempted (data not shown).

It was possible that the protein was being solubilised with Zwittergent 3-10 and Triton X-100 but had somehow folded and as a result the histidine "tail" was inaccessible to the Ni-NTA resin. In an attempt to overcome this problem both the solubilisation and binding of the protein to the Ni-NTA resin were carried out in the presence of 8M Urea (Figure 4.6). As demonstrated in Figure 4.6 there was no

binding of Ste6p immunoreactive material to the Ni-NTA resin in the presence of 8M urea and 1% Triton X-100.

4.9 Discussion

This Chapter described preliminary experiments relating to the purification of wild-type Ste6p and the production and expression of a N-terminally-hexa-histidine tagged variant of the protein, $N(His)_6$Ste6p. $N(His)_6$Ste6p was expressed as an active protein as shown by its ability to complement a *ste6* null mutation in the yeast strain WKK7 and was detectable as an increase in Ste6p immunoreactive material when expressed against a wild-type background in BJ5465.

Attempts made at purifying $N(His)_6$Ste6p on Ni-NTA resin were unsuccessful due to the inability to detect any $N(His)_6$Ste6p binding to the Ni-NTA resin. This may have been due to a low concentration of $N(His)_6$Ste6p leading to poor visualisation on Western blots. The blots themselves might have been misleading as the Ste6p immunoreactive material visualised on the blots might have consisted mainly of the wild-type Ste6p. Ideally the $N(His)_6$Ste6p would have been expressed in a *pep4* strain in which the *STE6* gene had been deleted to remove contaminating wild-type protein. This was attempted following the protocol detailed in the appendix to this Chapter. Numerous attempts at making this *ste6::URA3* strain failed due to inability to ligate the blunt ended, filled-in *URA3* fragment into the parent vector.

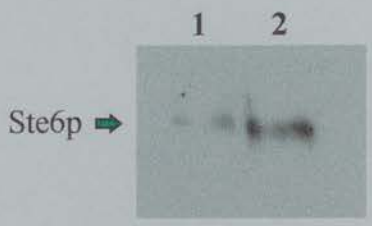
It was possible that the binding of $N(His)_6$Ste6p to the Ni-NTA resin failed because the tagged N-terminus was in some way unavailable for binding to the resin. As there was no binding evident in the presence of 8M urea is would seem unlikely that this was due to any residual protein structure which may have "hidden" the oligo-histidine "tail". Alternatively it was conceivable that the N-terminus of the $N(His)_6$Ste6p was being cleaved during a posttranslational event and was therefore not available to bind to the Ni-NTA resin. At this time Kölling and Hollenberg (1994b) conducted studies on a chimaeric protein produced by the fusion of the 1st hydrophobic segment of Ste6p (amino-acids 1-78) to the gene of the secreted protein invertase (*SUC2*) which was lacking its ER signal sequence. The results of the study showed that the first hydrophobic segment of Ste6p was capable of directing invertase to the ER membrane indicating that this region of Ste6p functions as a signal sequence. During the course of their experiments they observed that, when deglycosylated, the chimaeric protein had the same mobility on SDS-gels as wild-type invertase. This was unexpected as the chimaeric protein had a calculated molecular weight of 8.8 kDa larger than wild-type invertase. It was concluded that this discrepancy was due to the removal of the N-terminal Ste6p sequences by signal peptidase upon transition into the lumen of the ER. Analysis of the Ste6p sequence by the rules set up by von Heijne (1986) predicted that there would be a potential signal cleavage site at Gly62↓Ser63.

In order to determine whether or not the extreme N-terminus was being removed during some posttranslational event it was decided that the C-terminus of Ste6p should be tagged with a oligo-histidine tail and tested for its ability to bind to Ni-NTA resin. At this time monoclonal antibodies specific for an oligo-histidine sequence were

becoming commercially available. It was hoped that such antibodies could be used to determine whether or not the extreme N-terminus of Ste6p was present in the mature protein. The results on the study of a C-terminally-oligo-histidine tagged variant of Ste6p are detailed in Chapter 5.

Figure 4.1 Overexpression of Ste6p from BJ5465(pAMB7)

Overnight cultures of BJ5465 and BJ5465(pAMB7) were inoculated into 50ml of glucose-containing selective medium (SD) supplemented with the necessary amino acids and uracil. The cultures were grown, shaking, at 30°C until an A_{600} of 1.0 was obtained. The cells were then harvested, lysed with glass beads as detailed in Chapter 2. The crude cell lysate was diluted in 2X SDS-PAGE sample buffer and subjected to electrophoresis on a 7.5% polyacrylamide gel. The electrophoresed protein was transferred onto nitrocellulose. The blot was probed with a 1:100 dilution of anti-Ste6p serum for 2 hours followed by a 1:5,000 dilution of donkey anti-rabbit HRP conjugate for 20 min. The blot was then developed by ECL.



Lane 1.BJ5465
Lane 2 BJ5465(pAMB7)

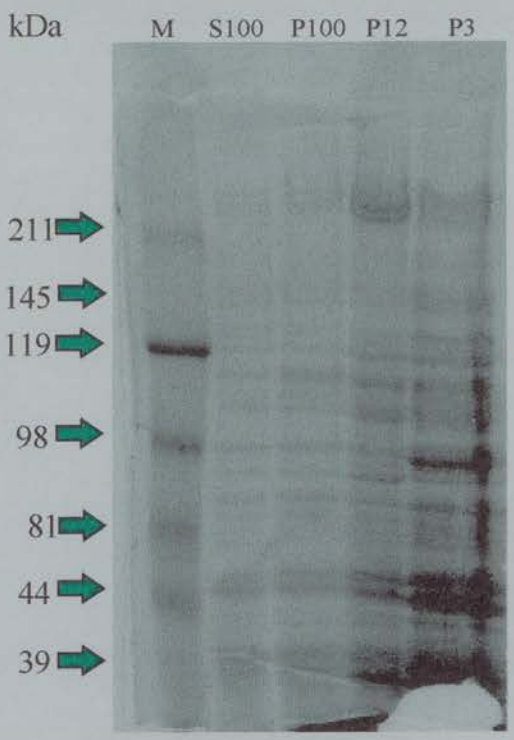
Figure 4.2 Localisation of Ste6p by differential centrifugation of BJ5465(pAMB7) cell lysate

An overnight culture of BJ5465(pAMB7) was inoculated into 50ml of selective medium with glucose as a carbon source. The culture was grown shaking at 30°C until an A_{600} of 1.0 was obtained. The cells were then harvested and subjected to glass bead lysis and differential centrifugation as detailed in Chapter 2. The cell fractions were resuspended to an equal volume in lysis buffer and diluted in an equal volume of 2X SDS-PAGE sample buffer (Chapter 2). The samples were then heated at 37°C for 10 min and subjected to electrophoresis on a 7.5% polyacrylamide gel. Gels were either stained with Coomassie Blue or transferred onto nitrocellulose. The Western blot was probed with a 1:100 dilution of anti-Ste6p serum for 2 hours and a 1:5,000 dilution of Donkey anti-rabbit conjugate for 20 min. The blot was developed with ECL.

A. SDS-PAGE: a 7.5% polyacrylamide gel stained with Coomassie Blue.

B. Western blot of the stained gel shown in A.

A.


kDa
M
S100
P100
P12
P3
211
145
119
98
81
44
39

B.

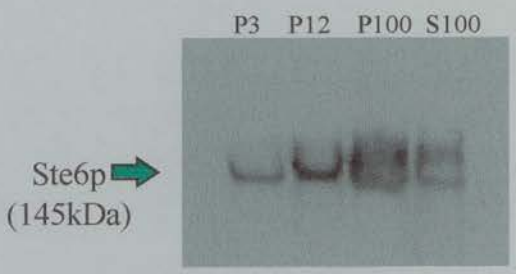

P3
P12
P100
S100
Ste6p
(145kDa)

Figure 4.3 Expression of N(His)6Ste6p in WKK7; the Halo Assay

Agar plates containing either glucose or galactose as a carbon source were seeded with RC757 cells as detailed in Chapter 2. Single colonies of WKK7, WKK7(pAMB7), WKK7(pAMB11) and BJ5465 were inoculated into 5 ml of selective medium containing mannose as a carbon source and supplemented with necessary amino-acids and uracil (SM). The cultures were grown for 16 hours until they appeared turbid to the eye. The cells from a 1.5 ml aliquot of each culture were harvested by centrifugation (3g for 5 min). The pellets were gently resuspended by pipetting until a uniform cell solution was obtained. A 3µl aliquot of each cell suspension was then carefully pipetted on to the lawn of RC757 cells. The plates were incubated at 25°C in a static incubator until the halos were visible (2-3 days).

A. Glucose

B. Galactose

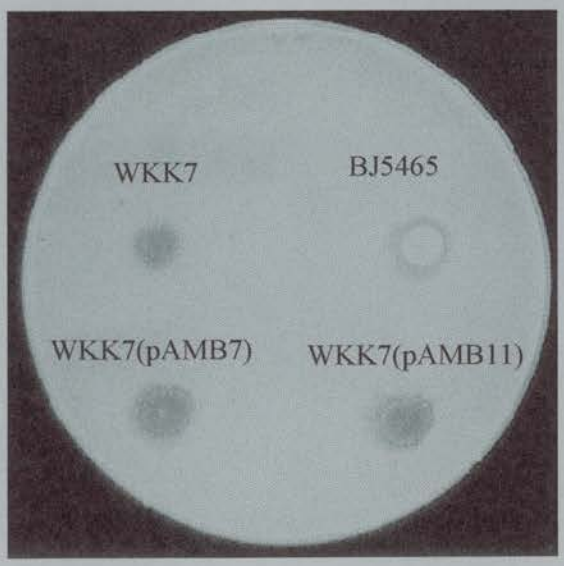


Figure 4.4 Expression of N(His)6Ste6p in BJ5465

Overnight cultures of BJ5465 and BJ5465(pAMB11) were inoculated into 50ml of selective medium containing mannose as a carbon source and supplemented with the necessary amino-acids and uracil. The cultures were grown, shaking, at 30°C until an A_{600} of 0.6 was obtained. The growth medium was then supplemented by the addition of galactose to a concentration of 2%. The cultures were then grown, shaking, at 30° C for a further 12 hours. The cells were then harvested and subjected to glass bead lysis and differential centrifugation to yield S100 and P100 fractions as detailed in Chapter 2. The cell fractions were made up to an equal volume in cell lysis buffer and diluted in 2X SDS-PAGE sample buffer. The samples were then heated at 37°C before being separated by electrophoresis through a 7.5% polyacrylamide gel. The gel was then blotted onto nitrocellulose and probed for the presence of immunoreactive Ste6p with a 1:100 dilution of anti-Ste6p serum for 2 hours and a 1:5,000 dilution of Donkey anti-rabbit HRP conjugate for 20 min. The blot was developed by ECL.

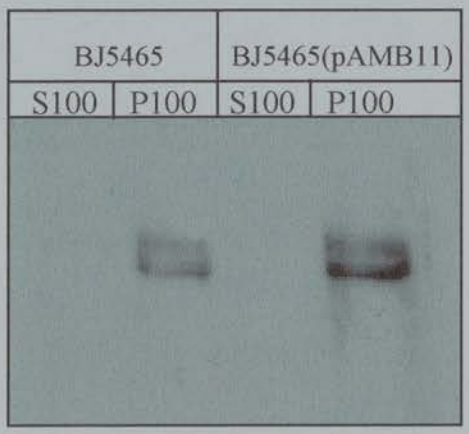

BJ5465
BJ5465(pAMB11)
S100
P100
S100
P100

Figure 4.5 Attempted purification of N(His)$_6$Ste6p solubilised from BJ5465(pAMB11) membranes with Zwittergent 3-10

Overnight cultures of BJ5465(pAMB11) were inoculated into 1 litre of selective medium containing 2% mannose as a carbon source and supplemented with the necessary amino acids and uracil (SM). The cultures were grown, shaking, at 30°C until an A_{600} of 0.6 was obtained. The cells were then harvested and resuspended in 1 litre of SG (as SM but using galactose as a carbon source). The cultures were incubated as before for a further 16 hours. The cells were harvested and washed in 50mM Tris.HCl pH 7.5, then weighed and resuspended in an equal volume of lysis buffer (50mM Tris.HCl, 300mM NaCl plus protease inhibitors). The cell suspension was placed in the small chamber of the Bead Beater (Chapter 2), which had been half filled with glass beads. The cells were lysed by 4 bursts of 1 min on the bead beater interspersed with 1 min cooling intervals on ice. The cell lysate was separated from the beads by centrifugation at 6,000g for 5 min (centrifuge) and solubilised by the addition of Zwittergent 3-10 to a concentration of 1%. The lysate was incubated with gentle agitation for 1 hour at 4°C then centrifuged to yield an S20 fraction (Chapter 2) which was added to 1ml of Ni-NTA resin that had been previously equilibrated in Solubilisation buffer (lysis buffer plus protease inhibitors and 1% Zwittergent 3-10). The Ni-NTA resin/cell lysate mixture was incubated, with gentle agitation, for 1 hour at 4°C. The unbound material was removed from the resin by centrifugation (1,200g for 3 min). The resin was washed in 10 volumes of solubilisation buffer (Wash 1) and then in 10 volumes of wash buffer 2 (solubilisation buffer plus 10% glycerol pH6.0) until the A_{280} of the wash buffer was less than 0.01. Oligo-histidine containing proteins were competed off the resin by the addition of a high concentration of imidazole in the final elutant wash (wash buffer 2 plus 500mM imidazole). Samples of the cell lysate, unbound material, washes 1 and 2 and eluate were diluted with 2x sample buffer and subjected to 7.5% SDS-PAGE and Western blot analysis. The blot was probed with a 1:100 dilution of anti-Ste6p serum for 2 hours and a 1:5,000 dilution of Donkey anti-rabbit HRP conjugate for 20 min. The blot was developed by ECL.

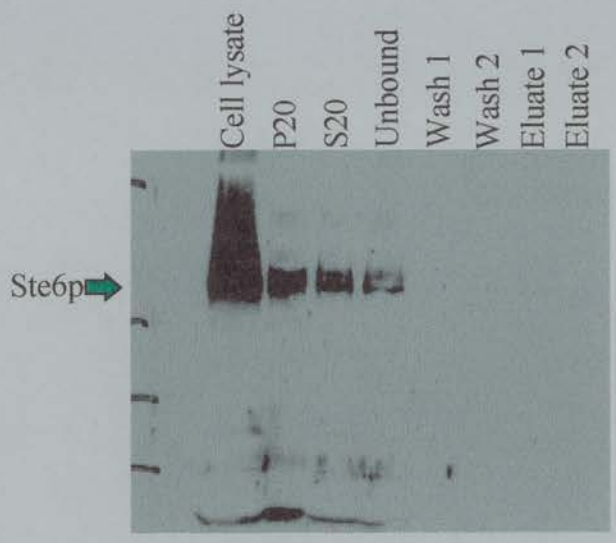

Ste6p
Cell lysate
P20
S20
Unbound
Wash 1
Wash 2
Eluate 1
Eluate 2

Figure 4.6 Attempted purification of N(His)$_6$Ste6p solubilised from BJ5465(pAMB11) membranes with Triton X100 and 8M urea

Cells from 1 litre of BJ5465(pAMB11) cells, cultured and galactose induced as described in Figure 4.5, were resuspended in buffer A (50mM NaPO4 pH 8.0, 500mM NaCl) supplemented with protease inhibitors and lysed with the Bead-Beater as before (Figure 4.5 and Chapter 2). The cell lysate was separated from the beads by centrifugation at 6,000g for 5 min and solubilised by the addition of Triton X-100 to a concentration of 1%. The lysate was incubated with gentle agitation for 1 hour at 4°C then centrifuged to yield an S20 fraction (Chapter 2) which was added to 1ml of Ni-NTA resin that had been previously equilibrated in Buffer A plus protease inhibitors and 1% Triton X-100. The resin was incubated on a rotating wheel for 60 min at 4°C. The unbound material was removed from the resin by centrifugation (2,000 X *g* for 1 min) and the resin was washed in buffer B (8M urea, 0.1M NaPO4, 0.01M Tris. HCl, pH 8.0. and 0.1% Triton X-100) until the A_{280} of the wash buffer was less than 0.01. The N(His)$_6$Ste6p was eluted by incubation with 100µl elution buffer (10mM Tris pH 7.5, 500mM NaCl, 0.1% Triton X-100, 300mM imidazole) for 5 min and centrifugation at 2,000 X G for 2 min. The elution step was carried out twice. Samples taken from each point in the purification procedure subjected to polyacrylamide gel electrophoresis and Western blot analysis as detailed in Figure 4.5.

Cell lysate
Unbound
Eluate 1
Eluate 2
NiNTA resin
Ste6p

Appendix to Chapter 4

Protocol for the production of a *ste6*::*URA3* strain of BJ5465

The *STE6* gene disruption strategy was based on the one-step gene replacement method described in Kuchler *et al.*, 1989.

1) The plasmid pAMB4 (Table A3) would be digested with *Stu*1 and *Sna*B1 (both of which generate blunt ends) to remove a 3.4 kb fragment representing 87% of the *STE6* coding region.
2) The deleted fragment would be replaced with the *URA3* containing *Bam*H1 fragment of YDp-U (Berben *et al.*, 1991) which had had its sticky ends filled in by the action of the Klenow fragment of DNA polymerase 1 prior to ligation.
3) The resultant plasmid would be digested with *Sal*I and *Sac*1 to release the *STE6* disruption cassette which would be transformed into BJ5465 (Table A2).
4) Transformants would be screened for by their ability to grow on media deficient in uracil.
5) Integration of the deletion construct into the genome would be confirmed by DNA hybridisation and tetrad analysis.

Chapter 5

The partial purification of a recombinant Ste6p tagged at the Carboxyl terminus with eight histidine residues

5.1 Introduction

In Chapter 4 the production, expression and partial purification of an N-terminally hexa-histidine tagged Ste6p was discussed. The failed attempts at purification of both the recombinant and the wild-type protein left us with an interesting dilemma. It remained of utmost importance to try and purify Ste6p from *S. cerevisiae* yet an interesting question regarding the possible cleavage of the N-terminus of Ste6p had also been posed. Although the state of the N-terminus of Ste6p could be determined by protein sequence analysis, for this a large amount of purified protein would be required.

In an attempt to purify Ste6p from *S.cerevisiae,* and in turn provide material for protein sequence analysis, it was decided that a recombinant Ste6p would be produced in which the C-terminus had been tagged with eight consecutive histidine residues. This chapter describes the production, expression and partial purification of a C-terminally octa-histidine tagged Ste6p variant. It also describes attempts made at protein sequence analysis and how the use of a monoclonal antibody, specific for a sequence of six or more histidine residues, was used to further substantiate the evidence that the N-terminus of Ste6p had been removed by post-translational modification of the protein.

5.2 Production of a recombinant Ste6p by attaching eight consecutive histidine residues to the carboxyl terminus to produce C(His)$_8$Ste6p

As discussed in Chapter 4 the inability to detect any binding of N(His)$_6$Ste6p to Ni-NTA resin may have been due to several reasons. Although the possibility of the N-terminus being removed from mature Ste6p was of interest it led to doubt whether conditions would ever be found which would enable N(His)$_6$Ste6p to be purified by metal chelate chromatography. It was decided that a C-terminally octa-histidine tagged Ste6p should be produced as an alternative to the studies on N(His)$_6$Ste6p. Tagging the C-terminus of Ste6p contradicted the strategy of tagging the N-terminus of the protein however it appeared to be the obvious step to take. It was expected that a C-terminally tagged protein would be purifiable on Ni-NTA resin and would enable the analysis of the nature of the N-terminus of Ste6p.

The addition of eight consecutive histidine residues to the extreme C-terminus of Ste6p was carried out by the use of PCR using the following primers:

1) 5' primer:

Start 5' end of *STE6*

5'-GGC CAA GCT TAT GAA CTT TTT AAG TTT TAA GAC TAC A-3'

*Hin*dIII

2) 3' primer:

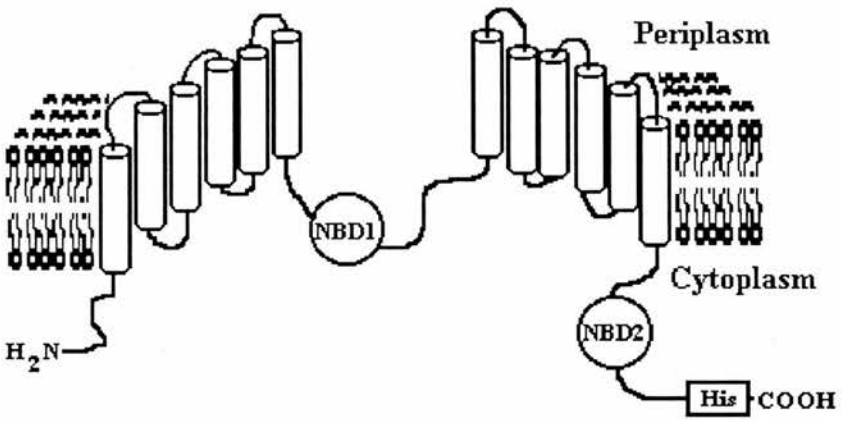
8 consecutive histidine residues

5'-G CGG AAG CTT TTA **GTG ATG GTG ATG GTG ATG GTG ATG** ACT GCT TTG GTT GGA AAC AAT TTG-3'

*Hin*dIII ←3'end of *STE6*

The PCR reaction was carried out using VENT polymerase with pSTE6 as the template as detailed in Chapter 2. The PCR product was digested with *Hin*dIII and cloned into the *Hin*dIII site of YEpGAL thus placing the tagged *STE6* gene under the control of the inducible *GAL* promoter. The resultant plasmid was called pAMB14. In order for DNA sequence analysis to be carried out the *Hin*dIII fragment of pAMB14 containing the C-terminally histidine tagged *STE6* gene was subcloned into the *Hin*dIII site of pK19 to produce pAMB15. A high quality sample of pAMB15 was produced by the Qiagen midi-preparation kit from the *E. coli* strain DH5α. The DNA was then subjected to double-stranded sequencing (method 2) with the following primer: 5'-AGC GGA TAA CAA TTT CAC ACA GGA-3'. This enabled the DNA corresponding to the extreme 3' end of the histidine-tagged *STE6* encompassing the octa-histidine tag to be read. The sequence was checked by two non-biased readers and was found to contain no mistakes, so it was concluded that the DNA sequence encoding the octa-histidine tag was correctly placed.

A cartoon of C(His)$_8$Ste6p spanning the plasma membrane is shown below. The boxed "His" motif at the C terminus indicates the position of the eight consecutive histidine residues and NBD stands for Nucleotide Binding Domain.



5.3 C(His)$_8$Ste6p complements a *ste6* strain of *S. cerevisiae*

It was essential that C(His)$_8$Ste6p, like N(His)$_6$Ste6p, could act as a functional **a**-factor transporter. The **a**-factor bioassay was carried out essentially as described in 4.6. This time the yeast strain WKK7 was transformed with pAMB14 to produce WKK7(pAMB14). Samples of WKK7, WKK7(pAMB14), BJ5465 and purified **a**-factor (Chapter 2) were tested for their ability to produce a halo on a lawn of RC757 cells grown using either glucose or galactose as a carbon source (Figure 5.1). As expected BJ5465 and purified **a**-factor were capable of producing a halo effect when grown on either carbon source. Wild-type WKK7 was unable to secrete **a**-factor in the presence of either glucose or galactose and no halo was produced from WKK7(pAMB14) grown on glucose; however **a**-factor secretion was apparent when the cells were grown on galactose. The C(His)$_8$Ste6p was thus capable of complementing a *ste6* defect and was produced in the presence of galactose and not glucose.

The expression of N(His)$_6$Ste6p and C(His)$_8$Ste6pN from WKK7(pAMB11) and WKK7(pAMB14) respectively were examined as detailed in Figure 5.2. It was found that, when grown on galactose, both recombinant proteins were equally good at complementing the *ste6* mutation of WKK7. Interestingly when cultures of both strains were grown on galactose and then placed on an RC757 lawn grown containing glucose as a carbon source halo production was also evident. This was an unexpected observation when one considers that the half-life of wild-type Ste6p is supposedly only 13-30 min (Kölling and Hollenberg, 1994; Berkower *et al.*, 1994). If this were the case and if the glucose present in the RC757 lawn was sufficient to repress the expression of the recombinant proteins, it is surprising that such an obvious halo was produced. However it is conceivable that residual **a**-factor had remained associated with the cell wall of the cells grown in the presence of glucose with subsequent leaching resulting in the pheromone response of the RC757 tester lawn.

5.4 The expression of C(His)$_8$Ste6p in a *MAT*α strain of *S. cerevisiae*

The efficient expression of recombinant Ste6p required that it should be produced in a *ste6* strain of *S cerevisiae* which was preferably deficient in the vacuolar hydrolase activities attributable to the *PEP4* gene product (Ammerer *et al.*, 1986; Woolford *et al.*, 1986). A strategy for the production of a WKK7 *pep4* knockout strain is detailed in the appendix to this Chapter. Suspected mutant cells were produced; however they failed to grow on either liquid or solid media and were therefore deemed useless for the expression of recombinant Ste6p.

As discussed in Chapters 2 and 4, expression of *STE6* in *S. cerevisiae* is **a**-cell type specific, that is, it is not normally expressed in α-type cells. As it was expected that expression of C(His)$_8$Ste6p would be as inefficient as that observed with N(His)$_6$Ste6p, it was decided to try and express the protein in the *MATα pep4* strain BJ5464 under the control of the *GAL* promoter. Expression of the recombinant protein under the control of the *GAL* promoter was expected to provide a high level of protein expression which would not be subjected to transcriptional repression by the Matα2p. If this was successful the expression of C(His)$_8$Ste6p would be stabilised by the absence of a functional *pep4* gene and detection of the recombinant protein could be achieved without the interference from the wild-type protein. In addition the studies of Berkower *et al.*, (1994) indicated that degradation of wild-type Ste6p occurs with identical kinetics in *MAT***a** and *MATα* strains of *S. cerevisiae*.

The expression of C(His)$_8$Ste6p in a *MATα ste6* strain of *S. cerevisiae* (BJ5464) is shown in Figure 5.3. The expression of wild-type Ste6p in the presence of glucose and galactose is shown in lanes 1 and 2 respectively. The *MATα* strain, BJ5464, gave no banding pattern for Ste6p when grown either in the presence of glucose or galactose as shown in lanes 3 and 4 respectively. The expression of C(His)$_8$Ste6p from BJ5465(pAMB14) grown in the presence of galactose (lane 5) gave a banding pattern comparable to that of wild-type Ste6p (lane 1) and appeared similar to that observed from the expression of N(His)$_6$Ste6p in BJ5465. No anti-Ste6p immunoreactive material of the correct molecular weight for C(His)$_8$Ste6p was

detected in the membrane fraction of BJ5464(pAMB14) cells grown on glucose (lane 6). When BJ5464(pAMB14) cells were grown in the presence of galactose (lane 7) a heavy band of approximately 145kDa corresponding to C(His)$_8$Ste6p was detected. The results of the Western blot indicated that the expression of C(His)$_8$Ste6p in BJ5464 had been successful. All future experiments involving purification studies on C(His)$_8$Ste6p were conducted on protein obtained from galactose-induced BJ5464(pAMB14).

5.5 An anti-oligo-histidine monoclonal antibody is immunoreactive with C(His)$_8$Ste6p but not with N(His)$_6$Ste6p

The practice of purifying oligo-histidine-tagged recombinant proteins by metal chelate chromatography has stimulated the development of two different means by which the poly-histidine "tail" can be detected. The first uses a Ni-NTA conjugate (Qiagen) consisting of Ni-NTA coupled to either calf intestinal alkaline phosphatase (AP) or horseradish peroxidase (HRP). These conjugates can be used for chromogenic or chemiluminescent detection of any recombinant protein containing an accessible oligo-histidine tag. The second method of detection involves the use of antibodies specific for a oligo-histidine tag. Commercially-available monoclonal antibodies are available, however they were usually directed against an oligo-His tag plus its flanking sequence, for example the Qiagen His antibody is directed against the sequence Arg-Gly-Ser-His-His-His-His which is the sequence contained in recombinant proteins expressed from their commercially available expression system. Dianova GmbH (Germany) produced the first monoclonal antibody (13/45/31) against the oligo-

histidine tag. It was claimed to be effective in detecting oligo-histidine tagged proteins when used in a variety of different situations including immunoblot and immunoprecipitation analysis. Unfortunately the expense of this product prevented its use.

A mouse monoclonal antibody directed against an oligo-Histidine residue epitope was made available by Dr Dieter Engelcamp at the Western General Hospital, Edinburgh. This antibody offered a means by which the presence or absence of an oligo-histidine tag could be determined, so as to indirectly identify the N-terminus of $N(His)_6$Ste6p. If the antibody were immunoreactive with a protein of the correct size for $N(His)_6$Ste6p it would suggest that the failure to purify the protein was due to either the experimental conditions or the inability to detect minute quantities of the protein. On the other hand if the protein was not immunoreactive with the anti-His tag antibody it would further confirm the idea that the N-terminus of Ste6p was cleaved during posttranslational modifications of the protein. $C(His)_8$Ste6p would act as a control protein being expressed under identical conditions in the same strains of *S. cerevisiae*. If the $C(His)_8$Ste6p were immunoreactive with the antibody it should be able to be purified on Ni-NTA resin.

Figure 5.4a shows a Western blot of a bacterially expressed N-terminally-deca-Histidine-tagged protein, probed with the anti-His-tag monoclonal antibody. It confirms that this antibody is capable of immunoreacting with a protein containing a poly-Histidine motif attached to the N-terminus. Western blot analysis of the membrane fractions of BJ5464(pAMB11) and BJ5464(pAMB14) probed with anti-

Ste6p antibody and anti-His-tag antibody are shown in Figure 5.4b. The Western blot probed with the anti-Ste6p antibody demonstrates that Ste6p is being produced from BJ5464(pAMB14) and BJ5464(pAMB11) cells respectively in the presence of galactose. The Western blot probed with the anti-His-tag antibody indicates that a protein of identical size to that detected with the anti-Ste6p antibody could be detected in the membranes of cells expressing $C(His)_8$Ste6p, in contrast, this was not observed in cells expressing $N(His)_6$Ste6p. To eliminate the possibility that this was the consequence of expressing the protein in an α-cell type the experiment was repeated using the WKK7 strain of *S. cerevisiae* (Figure 5.4c). The results shown in Figure 5.4c are identical to those shown in Figure 5.4b, the high background on the blot being due to a longer exposure time (necessitated by the lower protein concentrations resulting from the expression of protein in a *PEP4* strain of *S. cerevisiae*).

The results shown in Figure 5.4 demonstrate that the anti-His-tag monoclonal antibody was immunoreactive with a bacterially-expressed N-terminally-deca-Histidine tagged protein and $C(His)_8$Ste6p. This antibody was not immunoreactive with $N(His)_6$Ste6p suggesting that there was no histidine tag accessible to the antibody. The comparative Western blots probed with anti-Ste6p antibody indicate that this was not due to poor expression of the protein. It is possible that this lack of detection was due to the inability of the antibody to detect the hexa-histidine tag on $N(His)_6$Ste6p as the proteins that were successfully detected in this study had eight and ten histidine residues. However this explanation would seem unlikely as the antibody had been tested against at least ten different histidine tagged proteins which

were tagged with six to ten residues at either the N or C terminus and expressed from different plasmid expression systems in a variety of different organisms. In each case the protein of interest was positively identified by the antibody (D. Engelcamp personal communication). The antibody has now been marketed commercially.

The results of the experiments carried out with the anti-His-tag antibody demonstrate that the histidine tag of C(His)$_8$Ste6p was accessible and as a consequence it was expected that it would be able to bind to and therefore be purifiable on Ni-NTA resin.

5.6 Studies on the binding of C(His)$_8$Ste6 solubilised with Triton X-100 from the membranes of galactose-induced BJ5464(pAMB14) cells

The successful purification of a histidine-tagged variant of human P-glycoprotein by its affinity to Ni-NTA was reported by Loo and Clarke (1995). This purification of another eukaryotic ABC-transporter by nickel chelate chromatography suggested that C(His)$_8$Ste6p could also be purified using this technique, and provided a set of conditions on which to base the purification of C(His)$_8$Ste6p. The P-glycoprotein purification was based on the transient expression of a C-terminally deca-histidine tagged P-glycoprotein variant in the mammalian cell line HEK 293. Material solubilised by the non-ionic detergent *n*-dodecyl-β-D-maltoside was bound onto a Ni-NTA spin column (Qiagen) in the presence of 50mM imidazole. Contaminating proteins were removed by washing the resin with 80mM imidazole. The protein was eluted from the resin with 300mM imidazole. This protocol yielded 6-12mg of purified P-glycoprotein which had ATPase activity in the presence of lipid.

The initial studies on the interaction of C(His)$_8$Ste6p with Ni-NTA resin were concerned with finding conditions which would enable efficient binding and elution of the protein. In order to keep the cost of these initial binding studies to a minimum the detergent Triton X-100 was used for the solubilisation of C(His)$_8$Ste6p from the membranes of galactose-induced BJ5464(pAMB14) cells.

Initial attempts at binding C(His)$_8$Ste6p onto Ni-NTA resin used the protocol of Loo and Clarke (1995), who loaded the recombinant P-glycoprotein onto the Ni-NTA resin spin column in the presence of 50mM imidazole in order to reduce non-specific protein binding. An attempt at binding the C(His)$_8$Ste6p to Ni-NTA resin in the presence of 50mM imidazole (Figure 5.5) resulted in no C(His)$_8$Ste6p binding to the resin under these conditions. The experiment was then repeated with the omission of imidazole from the binding buffer (Figure 5.6) and C(His)$_8$Ste6p was found to bind to the Ni-NTA resin under these conditions. The protein was eluted from the resin by the addition of 300mM imidazole.

Although Triton X-100 appeared to be adequate for the solubilisation of C(His)$_8$Ste6p from the membranes of BJ5464(pAMB14) Figures 5.5 and 5.6 indicate that a large amount of the protein was not solubilised under these conditions. Figure 5.6 also demonstrates that although some C(His)$_8$Ste6p was bound to the Ni-NTA resin a large proportion of the protein remained unbound under these conditions. To overcome these problems a strong denaturant, 8M urea was used. As can be seen from Figure 5.7 the addition of 8M urea resulted in the total solubilisation of

C(His)$_8$Ste6p from the membranes of BJ5464(pAMB14). Unfortunately the addition of urea did not decrease the proportion of C(His)$_8$Ste6p remaining unbound. Figure 5.7, also shows that the protein remains bound to Ni-NTA when the pH was lowered to pH6.0. This is an important consideration as contaminating proteins are usually selectively eluted from Ni-NTA resin by the addition of a low concentration of imidazole (c.f. Loo and Clarke, 1995). C(His)$_8$Ste6p did not bind to Ni-NTA resin in the presence of 50mM imidazole indicating that a lower imidazole concentration, which would allow C(His)$_8$Ste6p to bind to the resin, would have to be determined in order that imidazole could be used as a means of removing contaminating protein from the resin. A reduction in pH, resulting in the protonation of histidine residues and their dissociation from the resin might provide an alternative mechanism by which contaminating proteins could be removed. Proteins containing histidine monomers are usually eluted at around pH 6.0 whereas proteins containing 6 or more histidine residues are eluted at around pH 4.5 (Qiagen). The data obtained from Figure 5.7 would indicate that C(His)$_8$Ste6p remains bound to Ni-NTA when the pH is lowered to pH 6.0. In addition the supplementation of the solubilisation buffer with 8M urea appears to greatly increase the amount of C(His)$_8$Ste6p solubilised from membranes of BJ5464(pAMB14) with Triton X-100.

In order to determine the imidazole concentration required to elute C(His)$_8$Ste6p from Ni-NTA a discontinuous imidazole gradient was applied to C(His)$_8$Ste6p which had been solubilised and bound to Ni-NTA in the presence of 1% Triton X-100 and 8M urea (Figure 5.8). As can be seen from Figure 5.8 C(His)$_8$Ste6p remained bound

to the Ni-NTA resin in the presence of buffer at pH 6.0 and in the presence of 20mM imidazole, but was eluted by imidazole concentrations of 40mM and greater.

Although Triton X-100 solubilised C(His)$_8$Ste6p could be purified on Ni-NTA in the presence of 8M urea the use of the detergent *n*-dodecyl-*β*-D-maltoside was also explored. This was prompted by the success of Loo and Clarke (1995) who used it in their purification of deca-histidine tagged P-glycoprotein. They found that this detergent solubilised around 80% of the available P-glycoprotein from mammalian cell line membranes. In addition their purification procedure was successful without the use of strong denaturants, e.g. 8M urea, and resulted in the recovery of P-glycoprotein ATPase activity.

5.7 The partial purification of C(His)$_8$Ste6p solubilised with *n*-dodecyl-*β*-D-maltoside

The studies conducted on Triton X-100-solubilised C(His)$_8$Ste6p indicated that the protein remained bound to the Ni-NTA resin in the presence of 20mM imidazole. Figure 5.9 shows an experiment in which C(His)$_8$Ste6p solubilised from the membranes of BJ5464(pAMB14) with *n*-dodecyl-*β*-D-maltoside was bound to Ni-NTA resin in the presence of 20mM imidazole. The protein was then subjected to elution by a discontinuous imidazole gradient using concentrations of 30mM-300mM imidazole. As can be seen from Figure 5.9A, C(His)$_8$Ste6p bound to the resin in the presence of 20mM imidazole and was eluted from the resin with imidazole concentrations in excess of 40mM. There is a slight band corresponding to

C(His)$_8$Ste6p in the 30mM imidazole wash, however, examination of the silver stained gel (Figure 5.9B) indicates that a large amount of contaminating protein is being eluted in the 30mM imidazole wash. A faint band corresponding to the expected molecular weight is detectable in the silver stained gel. The inability to clearly detect C(His)$_8$Ste6p on silver stained gel was disappointing especially when the amount of contaminating proteins is taken into account. It was decided that subsequent attempts at the purification of C(His)$_8$Ste6p would be carried out with the addition of 20mM imidazole to the binding buffer. In addition a 30mM imidazole wash would also be included. This may result in the loss of a small amount of C(His)$_8$Ste6p however this loss would appear to be insignificant when one takes into account the benefits obtained by removing such a large amount of contaminating protein. A duplicate experiment was conducted in which the 20mM imidazole was omitted from the binding buffer. The omission of imidazole from the binding buffer was found to have no effect on the binding/elution pattern of C(His)$_8$Ste6p, which was found to be identical to that indicated in Figure 5.9 (results not shown) indicating that the protein behaves in an similar manner whether in the presence or absence of 20mM imidazole.

In an attempt to reduce the amount of contaminating proteins present in the final eluate the resin was washed with a low pH buffer. Figure 5.10 shows an experiment in which the Ni-NTA resin with C(His)$_8$Ste6p bound was washed with buffers of pH 6.3 and pH 5.7 before washing with buffer of pH 8.0 containing 30mM imidazole. The Western blot (Figure 5.10A) shows that no C(His)$_8$Ste6p is lost from the resin in any of the washing steps. It also shows that most of the C(His)$_8$Ste6p is efficiently eluted from the resin by 300mM imidazole. The silver-stained gel (Figure 5.10B) shows that

contaminating protein is eluted from the resin in each of the washes. A candidate band for C(His)$_8$Ste6p is also evident on the silver-stained gel but there was still a considerable amount of contaminating protein present in the eluate fraction; however the majority of these proteins appeared to be at a much lower molecular weight than C(His)$_8$Ste6p.

As the C(His)$_8$Ste6p appeared to be a distinct, albeit faint band on the silver stained gel shown in 5.10B the purification was scaled up in an attempt to obtain enough protein for protein analysis. The partial purification of C(His)$_8$Ste6p from a 1 litre culture of BJ5464(pAMB14) is detailed in Figure 5.11. The purification was carried out by binding the C(His)$_8$Ste6p onto the resin in the presence of 20mM imidazole and contaminating proteins were removed by washing the resin with 30mM imidazole at pH 8.0 and then at pH 6.3. The silver stained gel shows C(His)$_8$Ste6p as a clearly identifiable band in the eluate fraction, distinct from the lower molecular weight contaminating proteins. In addition the Western blot shows that the majority of C(His)$_8$Ste6p was eluted from the Ni-NTA resin by of 300mM imidazole. The eluted protein was retained for amino-acid sequence analysis as discussed in 5.8.

5.8 Attempted sequencing of C(His)$_8$Ste6p immobilised on Polyvinylidene Difluoride (Immobilon P) membranes

Until recently sequencing the N-terminus of a protein has required large (>nmolar) quantities of purified protein. In recent years improvements in protein sequencing techniques (Elizinga, 1982; Matsudaira, 1987) have resulted in the ability to directly sequence pmol quantities of protein after spotting or electrotransfer onto Polvinylidene Difluoride (PVDF) membranes. PVDF membranes, which are marketed as Immobilon (Millipore), provide a mechanically strong solid phase support with a capacity for protein adsorption similar to that of nitrocellulose (Matsudaira, 1987). In practice it is possible to transfer proteins separated by SDS-PAGE onto Immobilon P membranes either directly or by electrotransfer. The protein may then be detected by staining with Coomassie Blue and the portion of Immobilon P containing the protein excised and sequenced directly.

In an attempt to sequence the N-terminus of C(His)$_8$Ste6p, the eluate fractions from Figure 5.11 were concentrated and separated by SDS-PAGE according to the method of Dunbar and Wilson, (1993) (Chapter 2) and as detailed in Figure 5.12. The gel was electrotransferred onto Immobilon P by wet blotting and stained with Coomassie blue as detailed in Chapter 2. The region of Immobilon corresponding to C(His)$_8$Ste6p was cut out and sent for sequence analysis by Dr Andrew Cronshaw at the Welmet protein sequencing facility at Edinburgh University. The material was subjected to 6 cycles of sequence analysis by the method of Hays *et al.*, (1989) however the

sequence analysis was unsuccessful and no information on the amino-acid sequence at the N-terminus was obtained.

As it was possible that the blotting procedure may have blocked the N-terminus another method for binding C(His)$_8$Ste6p to Immobilon P was attempted. The concentrated partially purified protein from Figure 5.11 was separated by SDS-PAGE as before and as detailed in Chapter 2 and Figure 5.12. On this occasion gel was stained with filtered Coomassie Blue stain (Chapter 2) and the region of the gel corresponding to C(His)$_8$Ste6p excised (Figure 5.12). The C(His)$_8$Ste6p was eluted from the gel and bound to the Immobilon P membrane by the method of Apps as described in Chapter 2. The Immobilon membrane was then subjected to 6 rounds of sequence analysis as described above. The sequence analysis was unsuccessful and no information regarding the N-terminus of C(His)$_8$Ste6p was obtained. The failure may have been due to insufficient quantities of protein or to the N-terminus being blocked and therefore inaccessible to sequence analysis.

5. 9 Discussion

In this Chapter the production, expression and partial purification of a C-terminally octa-histidine tagged Ste6p variant have been discussed. The protein (C(His)$_8$Ste6p) was correctly expressed as an active protein as shown by its ability to complement a *ste6* null mutation.

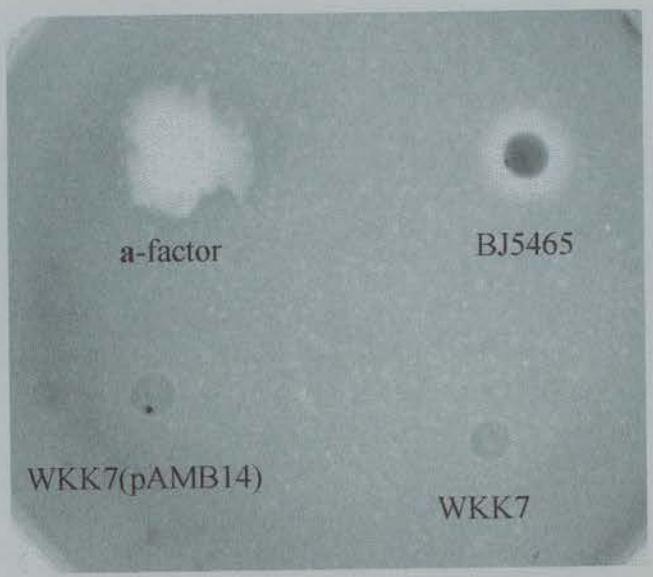
This protein (C(His)$_8$Ste6p) was found to be immunoreactive with a mouse monoclonal antibody specific for a sequence of several histidine residues. In contrast N(His)$_6$Ste6p showed no immunoreactivity with this monoclonal antibody suggesting that the hexa-histidine tag was either inaccessible to the antibody or had been removed during posttranslational modifications to the protein. In an attempt to determine the amino acid sequence at the N-terminus of mature protein the partially-purified C(His)$_8$Ste6p was subjected to protein sequence analysis. Unfortunately the protein sequence analysis was unsuccessful perhaps due to insufficient quantities of protein or to the N-terminus being blocked and therefore inaccessible to sequence analysis.

The studies conducted on the N(His)$_6$Ste6p, as detailed in Chapters 4 and 5, indicate that the protein was correctly expressed as an active protein but was unable to interact with Ni-NTA resin or a monoclonal antibody specific for an oligo-histidine tag. These findings coupled with the recombinant Ste6p studies conducted by Kölling and Hollenberg (1994b) would suggest that the N-terminus of Ste6p was removed during posttranslational processing of the protein.

Figure 5.1 Expression of C(His)$_8$Ste6p in WKK7; the Halo Assay

Agar plates containing either glucose or galactose as a carbon source were seeded with RC757 cells as detailed in Chapter 2. Single colonies of BJ5465, WWK7 and WKK7(pAMB14) were inoculated into 5 ml of selective medium containing mannose as a carbon source and supplemented with the necessary amino-acids and uracil (SM). The cultures were grown for 16 hours until they appeared turbid. The cells from a 1.5 ml aliquot of each culture were gently resuspended by pipetting until a uniform cell suspension was obtained. A 3µl aliquot of each cell suspension and of purified **a**-factor (Chapter 2) were then pipetted on to the lawn of RC757 cells. The plates were incubated at 25^{o}C until the haloes were visible (2-3 days).

A. Glucose



B. Galactose

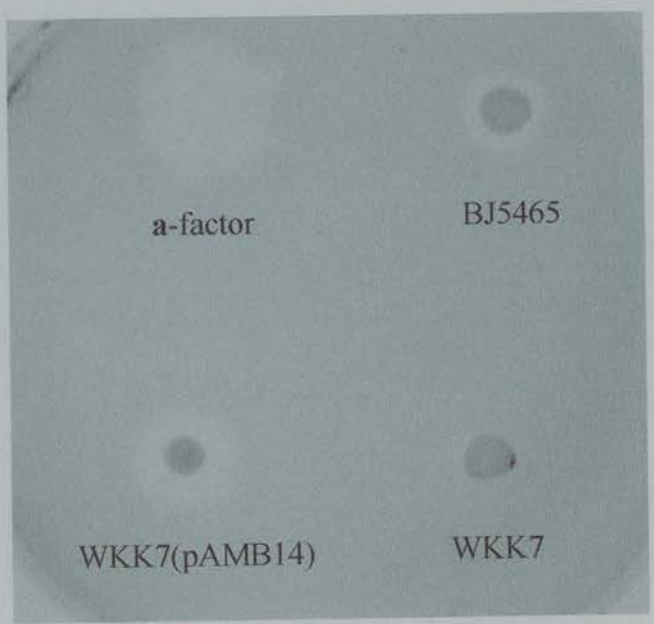


Figure 5.2 Comparison of Halos produced from WKK7(pAMB11) and WKK7(pAMB14) cells grown on either glucose or galactose as a carbon source

Agar plates containing either glucose or galactose as a carbon source were seeded with RC757 as detailed in Chapter 2. Single colonies of WKK7, WKK7(pAMB11) and WKK7(pAMB14) were inoculated into 5 ml of selective medium containing either glucose (D) or galactose (G) as a carbon source and supplemented with necessary amino-acids and uracil. The cultures were grown and harvested and applied to the RC757 lawn as detailed in Figure 5.1. The plates were incubated at 25^{o}C until the halos were visible (2-3 days).

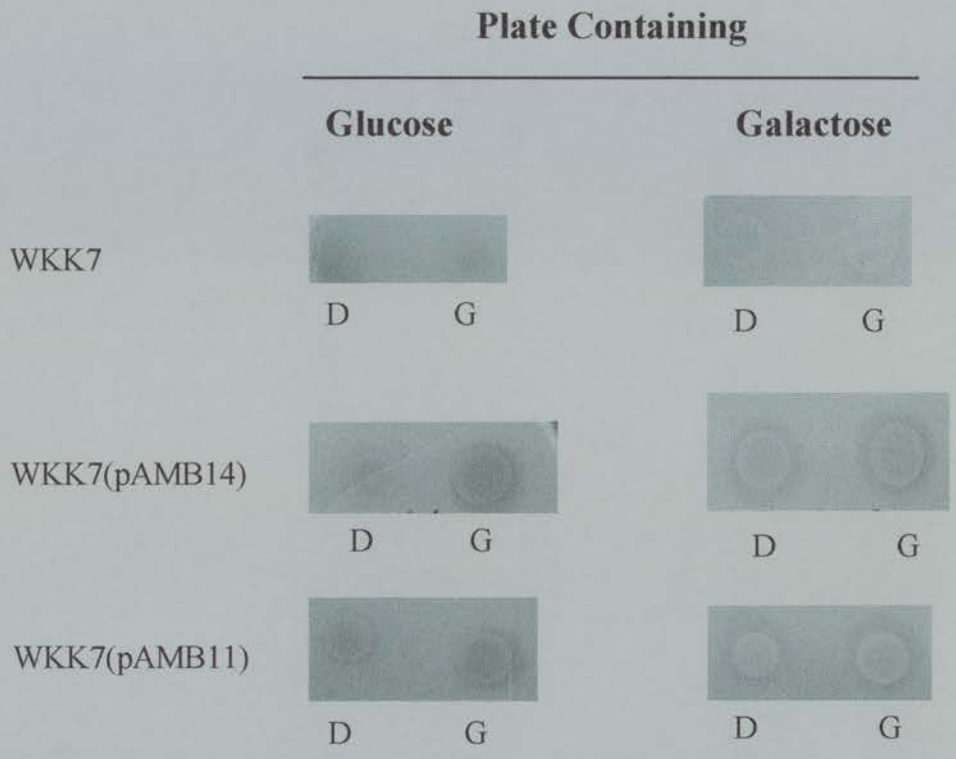


Plate Containing
Glucose
Galactose
WKK7
D
G
D
G
WKK7(pAMB14)
D
G
D
G
WKK7(pAMB11)
D
G
D
G

Figure 5.3 Expression of C(His)$_8$Ste6p in *S. cerevisiae*

Overnight cultures of BJ5464, BJ5465, BJ5464(pAMB14) and BJ5465(pAMB14) were inoculated into 100ml selective medium containing 2% mannose as a carbon source supplemented with 1% casamino acids (plus tryptophan for BJ5464 and BJ5465) and uracil (SM). The cells were then grown shaking at 30°C until an A_{600} of 0.6 had been obtained. The cells were harvested, split in half and inoculated into 50ml fresh selective medium containing either mannose (SM) or galactose (SG) as a carbon source. The cells were incubated, shaking for 16 hours at 30°C before being harvested, weighed and subjected to glass bead lysis and differential centrifugation to yield S100 and P100 fractions as detailed in Chapter 2. The membrane (P100) fractions were resuspended in 100µl of lysis buffer, diluted in an equal volume of 2X SDS-PAGE sample buffer, heated at 37°C for 10 min, and loaded onto a 7.5% polyacrylamide gel. The gel was blotted onto nitrocellulose and probed with a 1:100 dilution of affinity purified anti-Ste6p antibody for 16 hours. The blot was then probed with a 1:5,000 dilution of donkey anti-rabbit HRP conjugate for 20 min and developed by the use of ECL.

Lane 1. BJ5465, Glucose
Lane 2. BJ5465, Galactose
Lane 3. BJ5464, Glucose
Lane 4. BJ5464, Galactose
Lane 5. BJ5465(pAMB14), Galactose
Lane 6. BJ5464(pAMB14), Glucose
Lane 7. BJ5464(pAMB14), Galactose

Figure 5.4 Detection of C and N-terminally oligo-histidine-tagged-Ste6p chimaeras with an anti-histidine tag monoclonal antibody

A. A 39kDa deca-histidine N-terminally tagged bacterially-expressed protein (Chapter 2) was diluted in 2X SDS-PAGE sample buffer and subjected to electrophoresis on a 12% SDS polyacryalmide gel. The electrophoresed protein was Western blotted onto nitrocellulose and probed with a 1:50 dilution of anti-His-tag antibody for 16 hours. The blot was then probed with a 1:1,000 dilution of anti-mouse IgG HRP conjugate for 20 min and developed by the use of ECL.

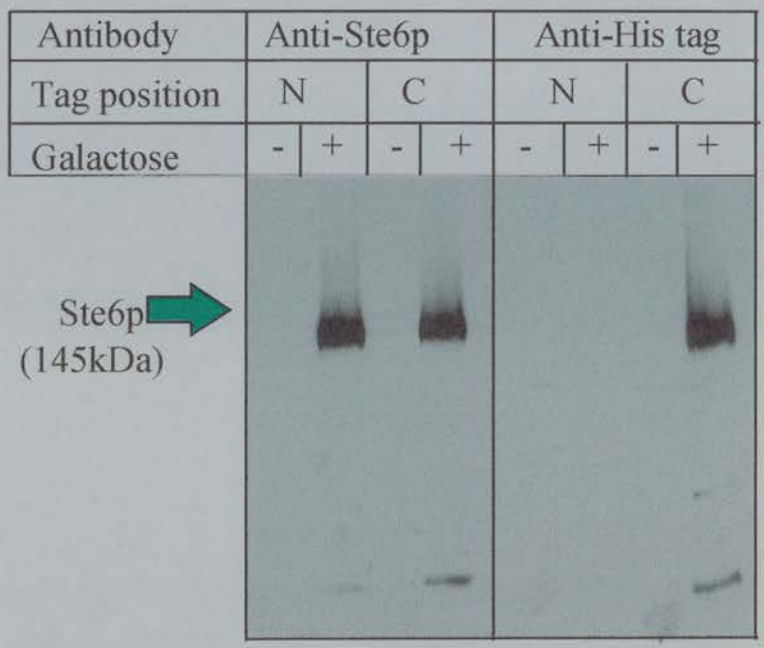
B. Overnight cultures of BJ5464(pAMB11) and BJ5464(pAMB14) were inoculated into 100ml of selective medium containing either 2% glucose or galactose as a carbon source and supplemented with 1% casamino acids and uracil. The cells were then grown, harvested and subjected to glass bead lysis and differential centrifugation as detailed in the legend to Figure 5.3. The membrane (P100) fractions were resuspended in lysis buffer and diluted with an equal volume of 2X SDS-PAGE sample buffer, heated for 10 min at 37°C and loaded in duplicate onto a 7.5% polyacrylamide gel. The proteins were then separated by electrophoresis and blotted onto nitrocellulose. The blot was then cut in half, half of the blot was probed with a 1:50 dilution of the anti-His-tag monoclonal antibody, the other half was probed with a 1:100 dilution of affinity-purified anti-Ste6p antibody. The blots were incubated with the primary antibody for 16 hours. Secondary antibodies were then applied as follows; donkey anti-rabbit HRP conjugate was applied to the anti-Ste6p antibody blot at a 1:5,000 dilution for 20 min and the anti-mouse IgG HRP conjugate was applied at a 1:1,000 dilution to the anti-His-tag antibody for 20 min. The blots were developed by the use of ECL.

C. WKK7 cells were transformed with pAMB11 and pAMB14 to produce WKK7(pAMB11) and WKK7(pAMB14) respectively. These cell lines were grown and subjected to Western blot analysis in an identical manner to that described in Figure 5.4B.

A.


39kDa

B.


Antibody
Anti-Ste6p
Anti-His tag
Tag position
N
C
N
C
Galactose
- + - + - + - +
Ste6p
(145kDa)

C.


Antibody
Anti-Ste6p
Anti-His tag
Tag position
C
N
C
N
Galactose
- + - + - + - +
Ste6p
(145kDa)

Figure 5.5 Binding of C(His)$_8$Ste6p onto Ni-NTA resin in the presence of 50mM imidazole

BJ5464(pAMB14) cells were grown and galactose induced as described in Figure 5.3. The cells were harvested and subjected to glass bead lysis in 50mM $NaPO_4$ pH8.0 plus protease inhibitors and subjected to differential centrifugation to yield a membrane (P100) fraction as detailed in Chapter 2. The cells were resuspended in buffer A (50mM NaPO4 pH 8.0, 500mM NaCl, 50mM imidazole and 20% v/v glycerol) supplemented with 1% Triton X-100, thoroughly homogenised and incubated with gentle agitation at 4^{o}C for 1 hour. Insoluble material (P16) was removed by centrifugation at 16,000 X *g* for 15 min. The soluble material (S16) was mixed with 150µl packed Ni-NTA resin, which had been equilibrated in buffer B (buffer A plus 0.1% Triton X-100), and incubated with gentle agitation for 60 min at 4^{o}C. The unbound material was removed from the resin by centrifugation (2,000 X *g* for 1 min).The resin was washed in 10 bed volumes of buffer B, then incubated in 100µl of buffer C (buffer B plus 80mM imidazole) for 2 min then centrifuged at 2,000 X *g* for 1 min. The supernatant (wash C) was retained for Western blot and the resin was further washed in 10 bed volumes of buffer C. The resin was then incubated in 100µl of elution buffer (buffer B plus 300mM imidazole) and centrifuged as before. The supernatant was retained for Western blot analysis and the elution step was repeated. Samples taken from each point in the purification procedure were subjected to gel electrophoresis and Western blot analysis with affinity purified anti-Ste6p antibody as described in Figure 5.3.


S16
P16
Unbound
300mM Imidazole
300mM Imidazole
Ni-NTA
C(His)8Ste6p

Figure 5.6 Binding of C(His)$_8$Ste6p, extracted from the membranes of BJ5464(pAMB14) with Triton X-100, onto Ni-NTA resin

The membranes from galactose-induced BJ5464(pAMB14) were collected as detailed in Figure 5.5, resuspended in 1.5 ml buffer A (50mM $NaPO_4$ pH 8.0, 500mM NaCl and 20% v/v glycerol) supplemented with 1% Triton X-100, thoroughly homogenised and incubated with gentle agitation at 4^{o}C for 30 min. Insoluble material (P16) was removed by centrifugation at 16,000 X *g* for 15 min. The soluble material (S16) was then mixed with 150µl packed of Ni-NTA resin which had been equilibrated in buffer B (buffer A plus 0.1% Triton X-100). The resin was incubated on a rotating wheel for 60 min at 4^{o}C. The unbound material was removed from the resin by centrifugation (2,000 X *g* for 1 min) and the resin was washed in 20 bed volumes of buffer B. C(His)$_8$Ste6p was eluted by incubation with 100µl 10mM Tris pH 7.5, 500mM NaCl, 0.1% Triton X-100, 20% v/v glycerol, 300mM imidazole for 5 min. The elution step was carried out twice. Samples taken from each point in the purification procedure subjected to Western blot analysis as detailed in Figure 5.3.

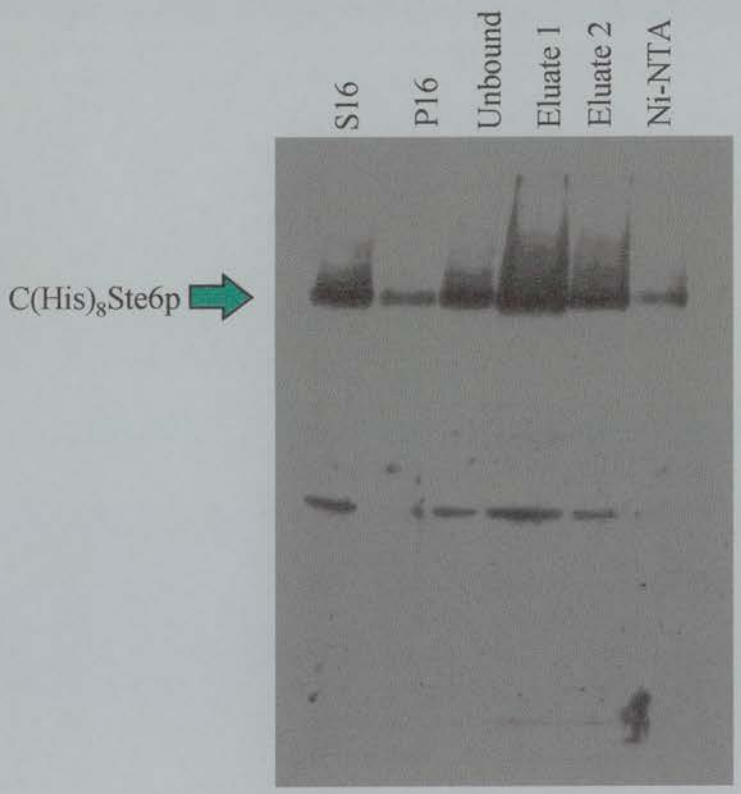

S16
P16
Unbound
Eluate 1
Eluate 2
Ni-NTA
C(His)8Ste6p

Figure 5.7 Binding of C(His)$_8$Ste6p, extracted from the membranes of BJ5464(pAMB14) with 1% Triton X-100 and 8M urea, onto Ni-NTA resin

The membranes from galactose-induced BJ5464(pAMB14) were collected as detailed in Figure 5.5, resuspended in buffer A (8M Urea, 0.1M NaPO4, 0.01M Tris. HCl, and 20%v/v glycerol pH 8.0) with the addition of 1% Triton X-100 and incubated with gentle agitation for 1 hour at 4^{o}C. Soluble material (S16) was separated from insoluble material (P16) gathered by centrifugation at 16,000 X *g* for 15 min. The S16 fraction mixed with 150µl packed of Ni-NTA resin which had been equilibrated in buffer A plus 0.1% Triton X-100 and incubated with gentle agitation at 4^{o}C for 1 hour. Unbound material was removed by centrifugation at 2,000 X *g* for 1 min. The resin was washed with 2 X 2ml buffer A, then incubated in 100µl of buffer B (8M Urea, 0.1M NaPO4, 0.01M Tris. HCl, 0.1% Triton X-100 and 20%v/v glycerol pH 6.0) for 2 min. The resin was then centrifuged at 2,000 X *g* for 1 min. The supernatant (Wash B) was retained for Western blot analysis. The resin was then washed in 10 bed volumes of buffer B. This washing process was repeated with buffer C (buffer B plus 80mM imidazole) and buffer D (buffer B plus 300mM imidazole). Samples taken from each point in the purification procedure were analysed by Western blot analysis as detailed in Figure 5.3.

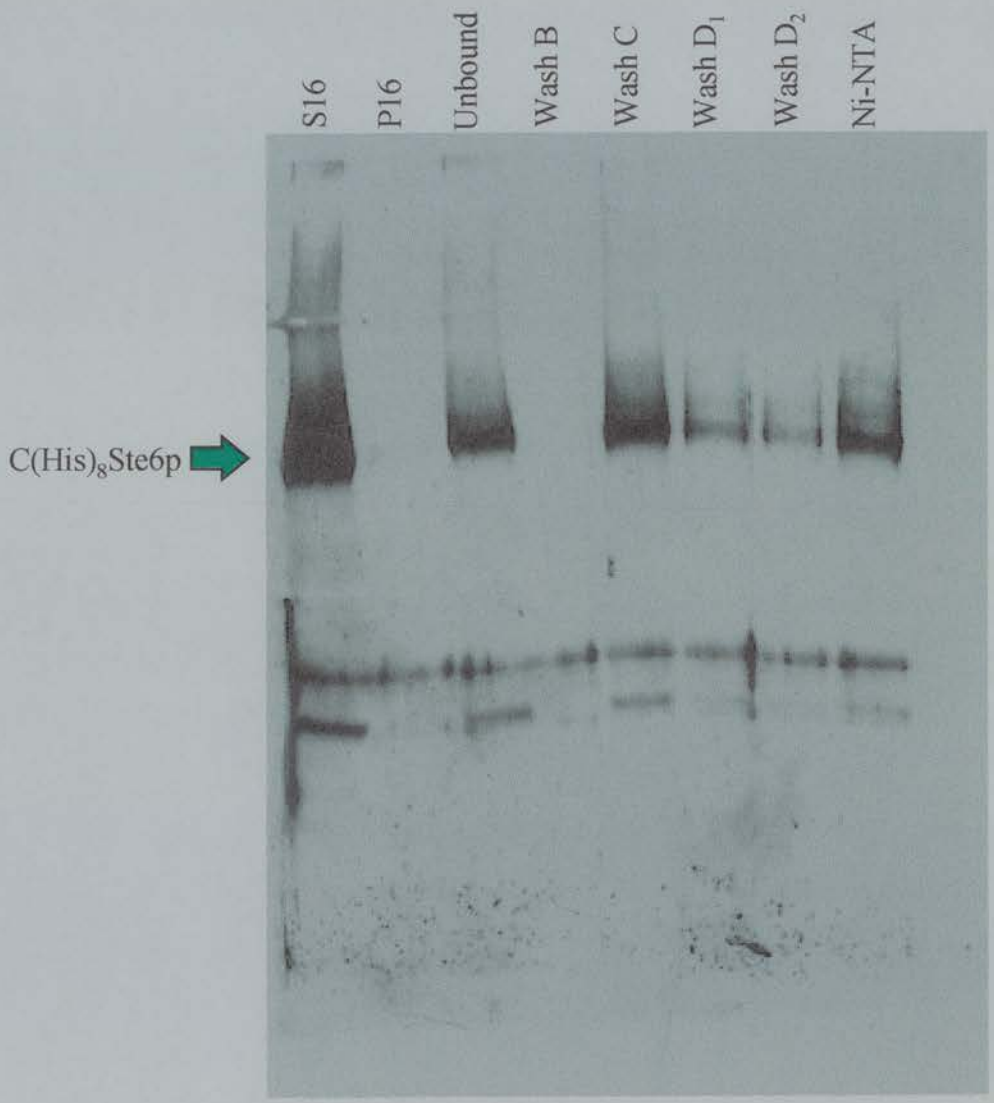

S16
P16
Unbound
Wash B
Wash C
Wash D_1
Wash D_2
Ni-NTA
C(His)$_8$Ste6p

Figure 5.8 Elution of C(His)8Ste6p from Ni-NTA resin with a discontinuous imidazole gradient

$C(His)_8$Ste6p was solubilised from cell membranes from galactose induced BJ5464(pAMB14) and bound to Ni-NTA as detailed in Figure 5.7. Protein was eluted from the resin in a stepwise fashion using a discontinuous imidazole gradient. The resin was washed with 2 X 2ml buffer A, then incubated in 100µl of buffer B (8M Urea, 0.1M NaPO4, 0.01M Tris. HCl, 0.1% Triton X-100, 20%v/v glycerol pH 6.0) for 2 min, and centrifuged at 2,000 X *g* for 1 min. The supernatant (Wash B) was retained for Western blot analysis. The resin was then washed in 10 bed volumes of buffer B. The elution/ washing steps were carried with buffer B containing 20mM, 40mM, 60mM, 80mM and 300mM imidazole. Samples taken from each point in the purification procedure were subjected to Western blot analysis as detailed in Figure 5.3.

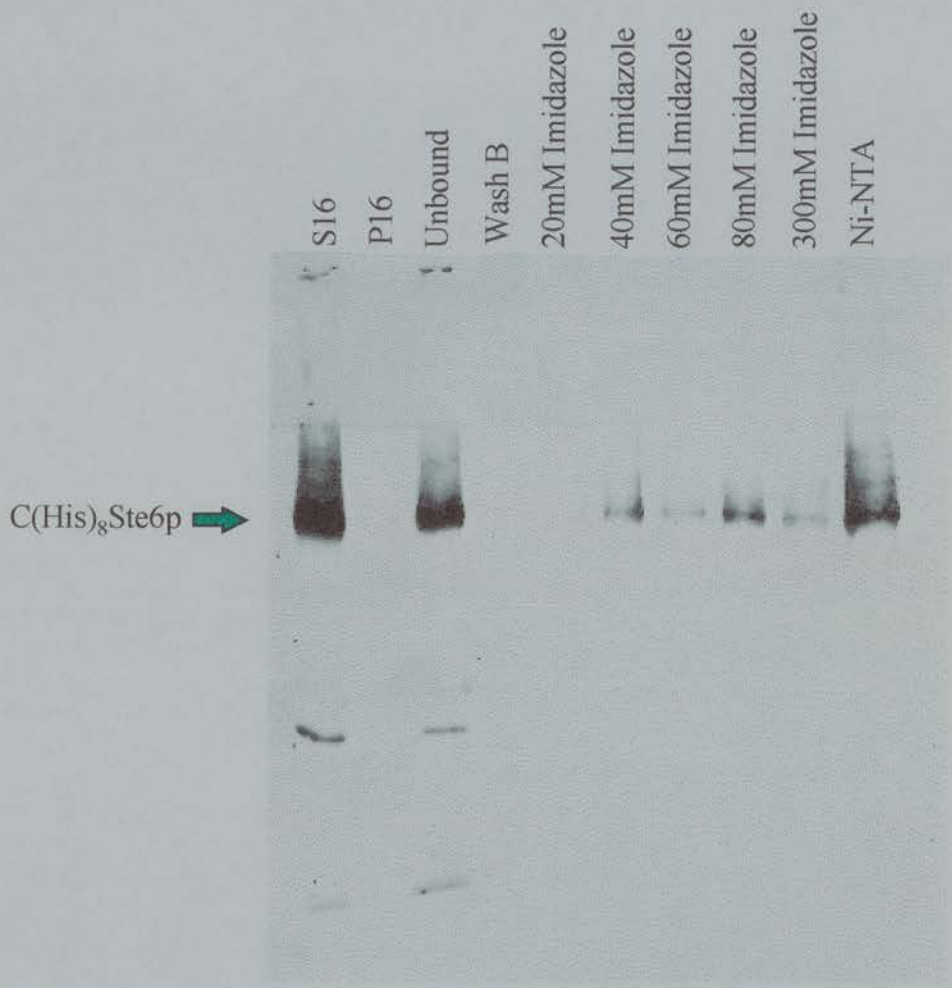
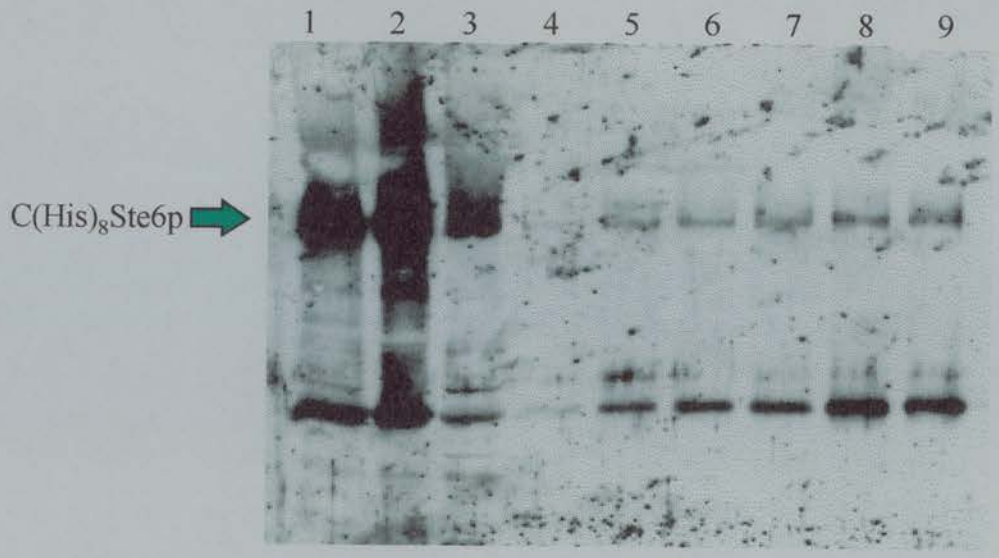

C(His)8Ste6p
S16
P16
Unbound
Wash B
20mM Imidazole
40mM Imidazole
60mM Imidazole
80mM Imidazole
300mM Imidazole
Ni-NTA

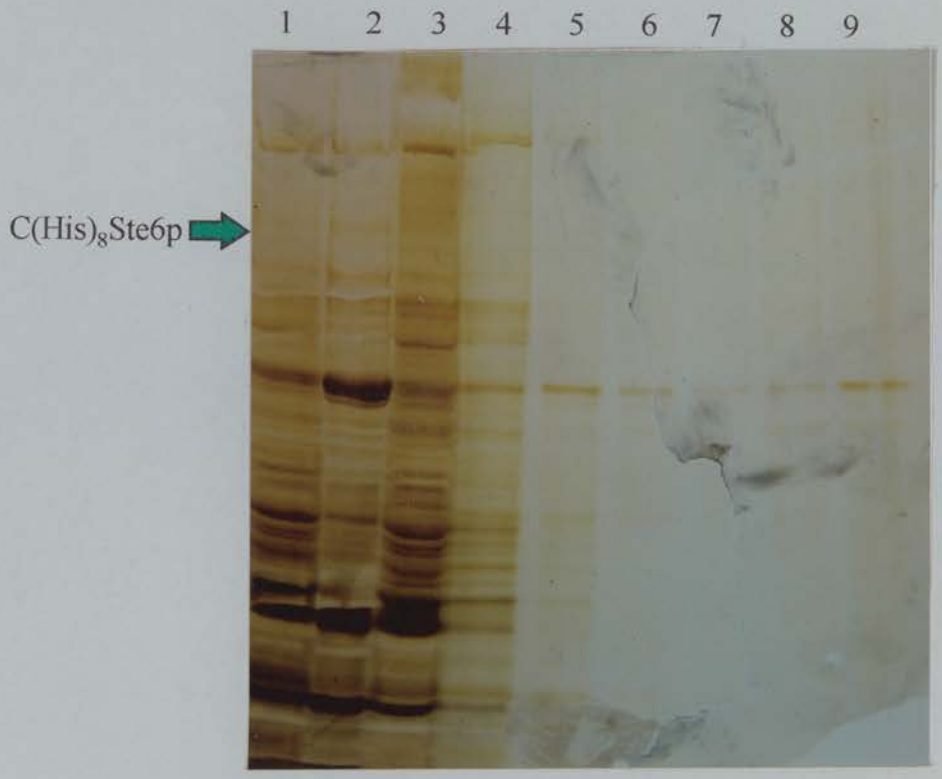
Figure 5.9 Binding of n-dodecyl-β-D-maltoside solubilised C(His)$_8$Ste6p to Ni-NTA resin in the presence of 20mM imidazole

The membranes from a 100ml galactose induced BJ5464(pAMB14) culture were collected as detailed in Figure 5.5, resuspended in 400µl buffer A (50mM NaPO4 pH 8.0, 500mM NaCl, 20mM Imidazole and 20% v/v glycerol) and homogenised until a uniform solution was obtained. The homogenate was then diluted in 1ml of buffer A which had been supplemented with 1% n-dodecyl-β-D-maltoside (DDM) and incubated with gentle agitation at 4^{o}C for 1 hour. Insoluble material (P100) was removed by centrifugation at 100,000 rpm for 20 min in a Beckman TL100 centrifuge. The soluble fraction (S100) was mixed with a 150µl packed Ni-NTA resin which had been equilibrated in buffer A supplemented with 0.1% DDM. The resin was incubated with gentle agitation for 1 hour at 4^{o}C. Unbound material was separated from the resin by centrifugation at 2000 X *g* for 1 min. The resin was then washed in 10 bed volumes of buffer A supplemented with 0.1% DDM. Stepwise elutions were then carried out in buffer B (10mM Tris. HCl pH 7.5, 500mM NaCl, 20% v/v glycerol and 0.1% DDM) which had been supplemented with 30mM, 50mM, 60mM, 80mM and 300mM imidazole. The elution and washing steps were carried out by resuspending the resin in 100µl of buffer, incubating for 2 min on ice, then separating the resin from the eluate by centrifugation for 1 min at 2000 X *g*. The resin was then washed in 10 bed volumes of the same buffer. Samples taken from each point in the purification procedure were subjected to polyacrylamide gel electrophoresis as detailed in Figure 5.3. The gels were either silver stained according to the method detailed in Chapter 2, or subjected to Western blot analysis with the affinity purified anti-Ste6p antibody as detailed in Figure 5.3. The region of the silver stained gel in which the 145kDa C(His)$_8$Ste6p protein would be expected to appear is as indicated.

A. Western Blot of gel shown in B.



B. Silver stained 7.5% polyacrylamide gel

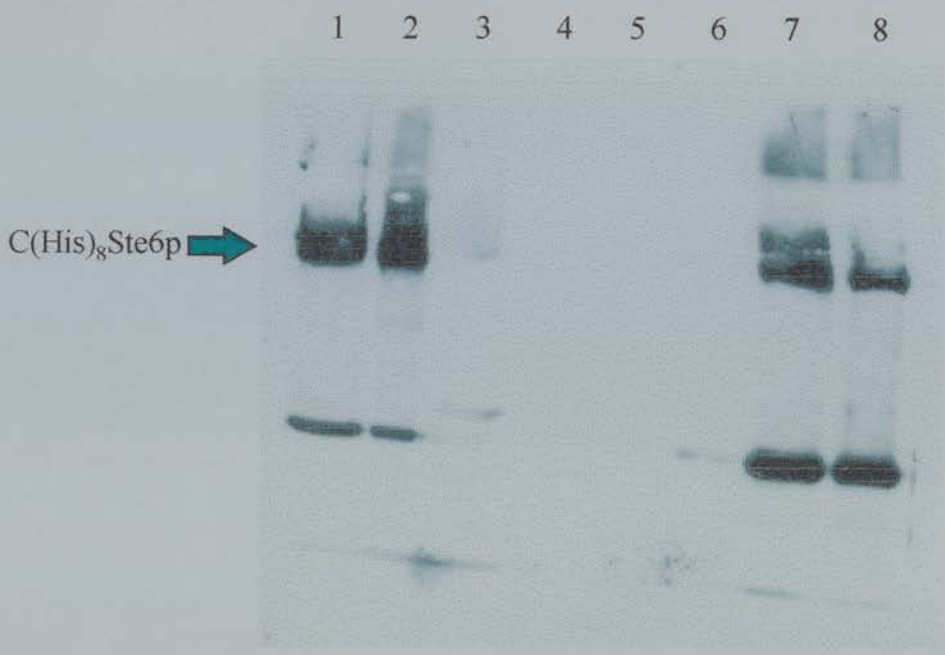


Lane 1. S100
Lane 2. P100
Lane 3. Unbound
Lane 4. 30mM Imidazole
Lane 5. 40mM Imidazole
Lane 6. 50mM Imidazole
Lane 7. 80mM Imidazole
Lane 8. 300mM Imidazole
Lane 9. Ni-NTA

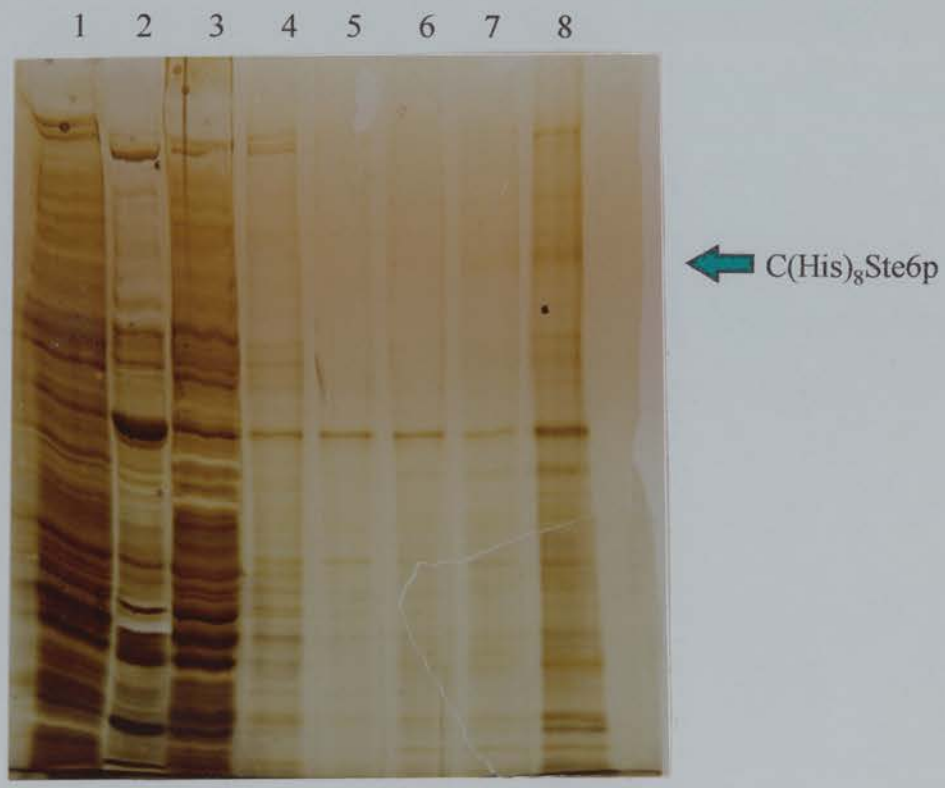
Figure 5.10 The effect of lowering the pH on the removal of contaminating proteins from Ni-NTA

$C(His)_8$Ste6p was solubilised from the membranes of galactose-induced BJ5464(pAMB14) cells and bound on to Ni-NTA resin in the presence of 20mM imidazole as detailed in Figure 5.9. The resin was washed in 10 bed volumes of buffer A (50mM NaPO4 pH 8.0, 500mM NaCl, 20mM Imidazole and 20% v/v glycerol) which had been supplemented with 0.1% DDM. The resin was then subjected to the elution and washing steps (as described in Figure 5.9) with citrate-phosphate buffer (50mM citrate-phosphate, 500mM NaCl, 20%v/v glycerol and 0.1% DDM) which was at either pH 6.3 or pH 5.7. The resin was then subjected to elution and washing steps with buffer A which had been supplemented with either 50mM or 300mM imidazole. Samples taken from each point in the purification procedure were subjected to polyacrylamide gel electrophoresis as detailed in Figure 5.3. The gels were either silver stained according to the method detailed in Chapter 2, or subjected to Western blot analysis with the affinity purified anti-Ste6p antibody as detailed in Figure 5.3.

A. Western Blot of gel shown in B.



B. Silver stained 7.5% polyacylamide gel

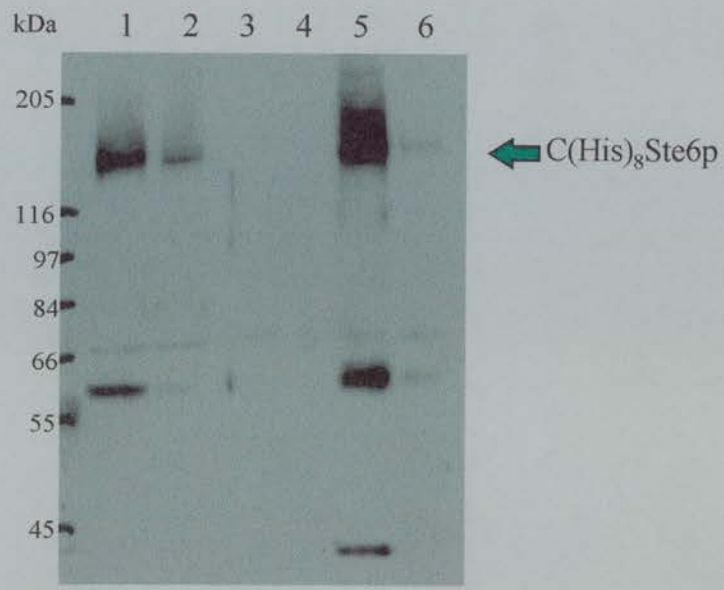


Lane 1. S100
Lane 2. P100
Lane 3. Unbound
Lane 4. pH6.3 wash
Lane 5. pH5.7 wash
Lane 6. 50mM Imidazole
Lane 7. 300mM Imidazole
Lane 8. Ni-NTA

Figure 5.11 Partial purification of C(His)$_8$Ste6p solubilised from the membranes of BJ5464(pAMB14) cells with DDM on Ni-NTA resin

The cells from a 1 litre culture of galactose-induced BJ5464(pAMB14) were subjected to glass bead lysis and differential centrifugation, and solubilisation with 1% DDM as detailed in Figure 5.9. The DDM-solubilised material (S100) was collected following centrifugation at 100,000 rpm for 20 min in a Beckman TL-100 centrifuge, applied to a 350µl packed Ni-NTA which had been equilibrated in buffer A (50mM NaPO4 pH 8.0, 500mM NaCl and 20% v/v glycerol) supplemented with 0.1% DDM. The resin was incubated with gentle agitation at 4°C for 16 hours then washed in 20 bed volumes of 0.1% DDM supplemented buffer A. The resin was then subjected to elution and washing cycles (as described in Figure 5.10) with the following buffers; buffer B (buffer A plus 30mM imidazole) and buffer C (50mM citrate-phosphate, 500mM NaCl, 20%v/v glycerol and 0.1% DDM). C(His)$_8$Ste6p was eluted from the resin by incubation with 1ml elution buffer (buffer A plus 300mM imidazole and 0.1% DDM) for 15 min. The eluate was harvested by centrifugation for 1 min at 2,000 X *g*. An aliquot of the eluted material was retained for further analysis and the elution procedure was repeated with a further 9ml of elution buffer. Samples taken from each point in the purification procedure were subjected to polyacrylamide gel electrophoresis as detailed in Figure 5.3. The gels were either silver stained according to the method detailed in Chapter 2, or subjected to Western blot analysis with the affinity purified anti-Ste6p antibody as detailed in Figure 5.3.

A. Western blot of gel shown in B

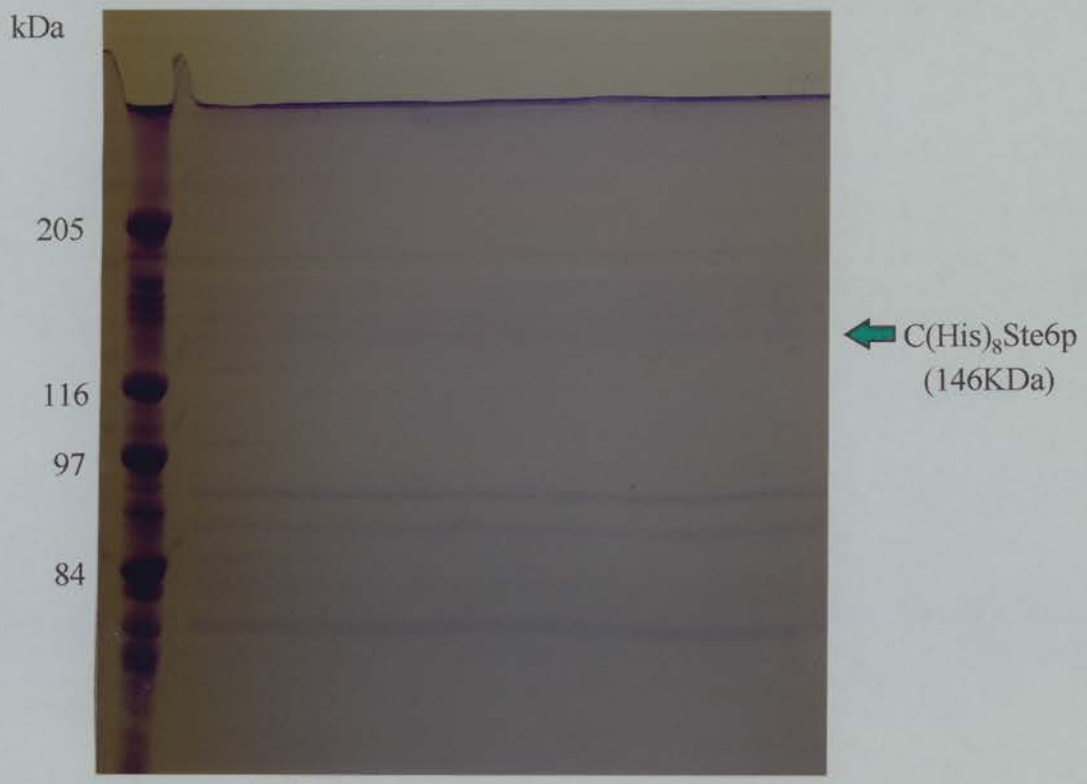


B. Silver stained 7.5% polyacrylamide gel

Lane 1. S100
Lane 2. Unbound
Lane 3. 30mM Imidazole wash.
Lane 4. pH6.3 wash
Lane 5. 300mM Imidazole elution
Lane 6 . Ni-NTA.

Figure 5.12 Separation of partially purified C(His)$_8$Ste6p by SDS-PAGE prior to attachment to Immobilon P membrane and sequence analysis

The eluate from the purification experiment detailed in 5.8 was dialysed against distilled water and concentrated by lyophilisation. The material was resuspended in 300µl 1X SDS-sample buffer and subjected to gel electrophoresis according to the method detailed in 2.25a. The gel was lightly stained with Coomassie Brilliant Blue as detailed in 2.13a. The band corresponding to C(His)8Ste6p (as indicated) was excised from the gel and prepared for protein sequencing as detailed in 2.25d.

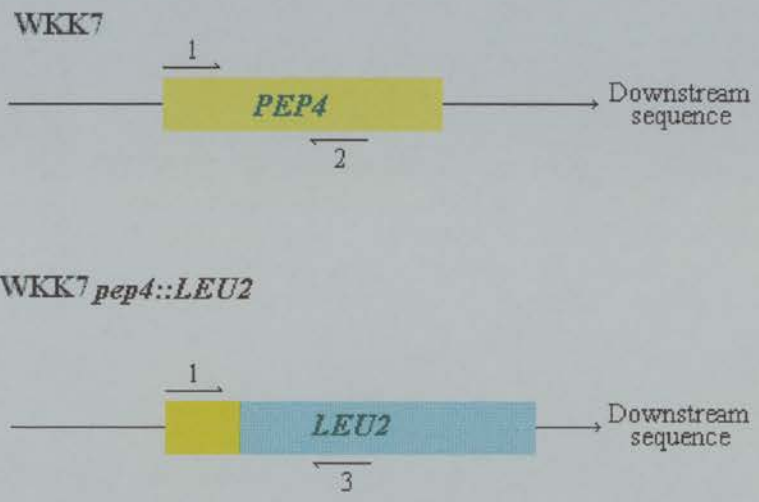

kDa
205
116
97
84
C(His)8Ste6p
(146KDa)

Appendix to Chapter 5

Protocol for the production of a *pep4*::*LEU2* strain of WKK7

A vector containing the *PEP4* gene, in which the majority of the open reading frame had been replaced by the *LEU2* marker gene, was made available by Carol Woolford (Carnegie-Mellon University). This vector, known as pTS17 or BJ5783, was used in the creation of a *pep4*::*LEU2* strain of WKK7. pTS17 was created by replacing a *Hin*dIII fragment of the *PEP4* open reading frame and down stream sequence with the *LEU2* marker. The digestion of pTS17 with *Bam*HI released the 8kb disruption cassette which was purified by agarose gel electrophoresis and transformed into the *S. cerevisiae* strain WKK7 (Table A2). WKK7 transformants were selected for their ability to grow on media deficient in leucine. A PCR strategy was designed to determine whether or not the correct recombination event had occurred between WKK7 and the pTS17 disruption cassette and is detailed in Figure 5.13. The genomic DNA from colonies of WKK7 transformed with the *LEU2* disruption cassette, which were capable of growing on leucine deficient medium, was extracted and used as a template for PCR analysis. The analysis was carried out using the template DNA with either primers 1 and 2 or primers 1 and 3 together. Successful integration of the *LEU2* disruption cassette into the WKK7 genome resulted in the production of a PCR product only when primers 1 and 3 were used. The production of a PCR product when primers 1 and 2 were used together indicated that correct integration had not occurred and that the *PEP4* gene of WKK7 remained intact. The genomic DNA from untransformed WKK7 was used as template for control PCR reactions.

Figure 5.13 PCR strategy to analyse the integration of a *LEU2* disruption cassette into the *PEP4* gene of WKK7



The region of the WKK7 genome incorporating the *PEP4* gene with and without the integration of the *LEU2* disruption cassette is shown. The *PEP4* gene is indicated in yellow and the *LEU2* gene in blue. The PCR primers 1, 2 and 3 are also indicated. Primer 1, sequence 5'-ATGTTCAGCTTGAAAGCATT-3', was directed against the 5' end of the *PEP4* gene which would be untouched if a successful disruption occurred. Primer 2, sequence 5'-TATCGATGGCGGCACC-3', was directed against a region in the centre of the *PEP4* gene which is present in wild-type WKK7 but would be absent if the successful disruption of *PEP4* by the *LEU2* disruption cassette occurred. Primer 3, sequence 5'-AGCCACCA TTGCCTATT-3', was directed against the centre of the *LEU2* gene.

Chapter 6

A genetic approach to studying the interaction between Ste6p and a-factor

6.1 Introduction

In addition to the biochemical approach to studying the interaction between **a**-factor and Ste6p a genetic study involving the random mutagenesis of the *MFa1* gene was attempted. It was hoped that random mutagenesis of the *MFa1* gene would result in the production of mutant **a**-factor molecules which, when expressed in a wild-type *MAT***a** strain, were capable of interacting with and blocking the Ste6p pump. This mutagenic strategy was expected to provide some insight as to which residues of the mature **a**-factor molecule were involved in the interaction with Ste6p.

The mature form of **a**-factor consists of three parts (Figure 1.5), the C-terminal farnesyl and methyl groups and the N-terminal amino-acid backbone. The structural requirements of **a**-factor that are required for transport by Ste6p and detection by Ste3p are not well defined. Previous studies (Marcus *et al.*, 1990; He *et al.*, 1991) have demonstrated that both the farnesylation and methyl-esterification of **a**-factor are essential for its export and biological activity. For the purpose of this project we were interested in determining which residues of the amino-acid backbone of the mature molecule play an essential role in mediating the interaction between Ste6p and **a**-factor.

Region-directed oligonucleotide mutagenesis is a technique which allows random mutagenesis of a defined region of DNA. Valls *et al.*, (1990) successfully employed oligonucelotide-directed mutagenesis to determine which amino-acids were essential

for targeting yeast carboxypeptidase Y to the vacuole. It was hoped that this technique could be used as a means of producing **a**-factor molecules which were able to interact with and block the Ste6p pump. It was proposed that a library of mutant *MFa1* genes could be produced following mutagenesis with primers directed against all residues of the mature **a**-factor molecule with the exception of the C-terminal sequence, CVIA. This sequence is known to be required for **a**-factor modifications (farnesylation and methylation) which are essential for **a**-factor secretion and bioactivity (Marcus *et al.*, 1990; He *et al.*, 1991). Mutant **a**-factor molecules which were able to interact with and block the Ste6p pump could, in theory, be detected by transforming the mutated **a**-factor libraries carried at one copy per cell on a yeast centromere-based plasmid, into a *MAT***a** strain of yeast containing intact *MFa1* and *MFa2* genes. Transformants would then be screened for their ability to secrete **a**-factor using a growth inhibition (halo) assay in which colonies of **a**-type cells secreting **a**-factor can be detected by the ability of **a**-factor to inhibit the growth of α-type cells plated in a lawn. As the mutated **a**-factor molecules would be expressed in the presence of wild-type **a**-factor it could be rationalised that only those mutant **a**-factor molecules capable of interacting with and blocking the pump would prevent the secretion of **a**-factor. Such **a**-factor mutants would exert a dominant negative effect and could be described as dominant negative mutations (DNM) of the **a**-factor gene. The plasmid DNA from transformed cells displaying such a phenotype could be rescued and retransformed into yeast to ensure that any observed inhibition of **a**-factor secretion was due to the presence of the mutated **a**-factor. The plasmids of interest could then be subjected to DNA sequence analysis to detect which mutations in the *MFa1* gene product conferred the dominant trait.

It is conceivable that such **a**-factor mutants could exert their effects by blocking some part of the **a**-factor maturation machinery for example those enzymes involved in C-terminal modification or N-terminal proteolysis. To eliminate this doubt it was intended that extracts of cells synthesising a confirmed DNM would be assayed for their ability to modify a chemically synthesised **a**-factor precursor molecule (Marcus *et al.*, 1990).

It was hoped that this genetic screen would allow the isolation of **a**-factor DNMs which were capable of interacting with and blocking the Ste6p-**a**-factor pump. Such mutants would then be chemically synthesised and tested for their ability to block Ste6p pump which had been purified and reconstituted into phospholipid vesicles. It was hoped that kinetic studies on the interaction of mutant **a**-factor and Ste6p along with cross-linking studies would help identify which amino-acid residues, if any, were essential for **a**-factor-Ste6p interaction.

The following Chapter describes the attempts made to produce a library of mutant **a**-factor genes. It also discusses the results of others' recent studies conducted on the N-terminal region of **a**-factor.

6.2 Cloning *MFa1*

The nucleotide sequences of the genes encoding the **a**-factor precursor molecules, *MFa1* and *MFa2*, were originally reported by Brake *et al.*, (1985). For the purpose of

this study it was decided that the *MFa1* gene would be cloned and subjected to mutagenesis. A fragment of DNA encoding the entire *MFa1* structural gene was amplified by PCR using VENT DNA polymerase, genomic DNA isolated from the *S. cerevisiae* strain BJ5465 (as detailed in Chapter 2) and the following primers:

1) 5' primer (E127)

5'-GCC CCA AGC TTC TTT GTT CTT GTT ACA AAC GAG TGT GT-3'

*Hin*dIII

2) 3' primer (E126)

5'-CGG GAT CCG TGC ATG GAT GTA CAA CGA TAA CC-3'

*Bam*HI

The 560bp PCR product was digested with *Hin*dIII and *Bam*HI and cloned into the *Hin*dIII/*Bam*HI site of the yeast phagemid vector pVT103-U (Vernet *et al.*, 1987) to produce pAMB8. The cloned material was subjected to double stranded DNA sequencing (method 1) as detailed in Chapter 2 using primer "53" (Vernet *et al.*, 1987) as detailed below:

Primer "53"

5'-CTG CAC AAT ATT TCA AGC-3'

The region of the cloned material containing the **a**-factor structural gene was found to contain no mistakes.

6.3 Design and production of oligonucleotide primers for the random mutagenesis of mutagenesis of *MFa1*

The cloned *MFa1* gene was used as a template for oligonucleotide-targeted mutagenesis by the method of Kunkel (1987) and in Sambrook *et al.*, (1989). The mutagenic primers were designed so that a library of mutant **a**-factor molecules that contained amino acid substitutions at any position in the mature **a**-factor, with the exception of the C-terminal CVIA, would be produced as discussed above. Mutagenic oligonucleotides directed against the remaining 11 amino-acid residues of the mature **a**-factor were produced according to the strategy detailed in Figure 6.1. Two oligonucleotides were produced: a 30-mer (G1192) corresponding to the region encoding [DN]YIIKGV[FW] and a 27-mer (G1191) corresponding to [GV]FWDPA[CV]. For each oligonucleotide the first and last six residues (corresponding to the bracketed amino-acids) were synthesised according to the *MFa*1 sequence. The intervening 18 (or 15) residues were "doped" (contaminated) during synthesis with 0.22% of each of the incorrect nucleotides as described in Valls *et al.*, (1990). This strategy was calculated to yield single mutational frequencies of 20% (that is one in five oligonucleotides within the synthesised population will have a single base change) and 4% and 0.8% for double and triple mutations respectively.

6.4 Attempted mutagenesis of *MFa1* with random "doped" primers.

To increase the efficiency of oligonucleotide directed mutagenesis the Kunkel method (Sambrook *et al.*, (1989) taken from Kunkel, 1987) was used, in which the single-stranded template DNA is produced in a strain of *E. coli* which has a *dut⁻ ung⁻* genotype. The *dut⁻ ung⁻* genotype results in the production of uracil-containing single-stranded template DNA due to a deficiency in the dUTPase and uracil-N-glycosylase activities respectively. Oligonucleotide directed-mutagenesis relies on the semiconservative nature of DNA replication resulting in half of the progeny carrying the desired mutation. The Kunkel method increases the chances of the progeny carrying a mutation to 80% due to the instability of the parent, uracil-containing, strand of DNA.

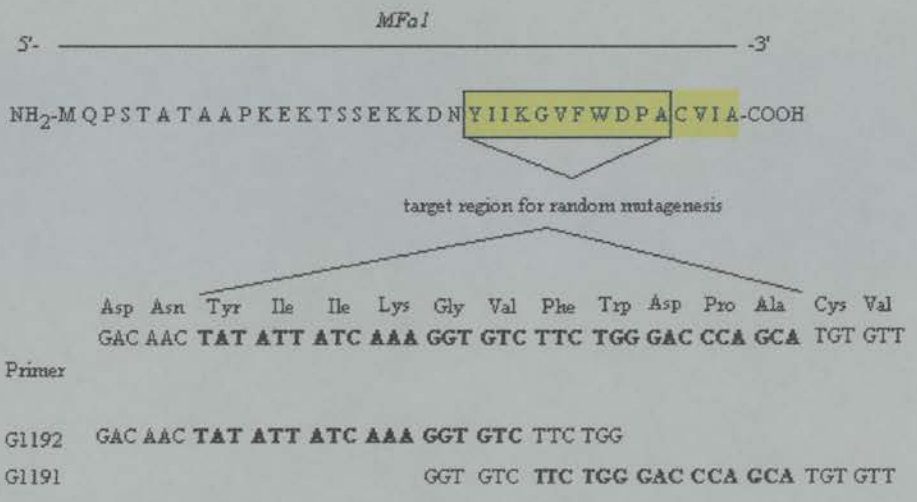
Uracil-containing single-stranded template DNA was produced in the *E. coli* strain BW313 transformed with pAMB8 as described in Chapter 2. The single-stranded DNA was purified from the culture supernatant with the Qiagen M13 kit and used as a template for oligonucleotide-directed mutagenesis with either primer G1191 or G1192 as described in Sambrook *et al.*, (1989). The extension reactions were carried out using Sequenase 2 (Amersham). The resultant DNA was transformed into the *E. coli* strain NM522.

Initial attempts at mutagenesis failed due to the small number (<100) of transformants per reaction carried out. Transformation efficiency was increased by the use of

commercial competent *E. coli* strain DH5α (GIBCO BRL). Transformants were picked and subjected to DNA sequence analysis (Chapter 2) following extraction of plasmid DNA with Qiagen spin-column mini-preparation kits. In the first instance six transformants, from a mutagenesis reaction carried out with the G1191 primer, were characterised and none contained mutations. Colonies picked from subsequent mutagenesis reactions were screened by DNA sequence analysis and in all cases failed to show any sequence alteration. It was thought that this apparent lack of mutagenesis might be due to the quality of the single stranded DNA; in addition it was possible that the BW313 strain was failing to produce uracil-containing DNA.

Figure 6.1

The design of oligonucleotide primers for the random mutagenesis of *MFa1*



Representation of the primary gene product of the **a**-factor gene, *MFa*1. The yellow region represents the portion of the precursor molecule present in mature **a**-factor with the boxed area representing the target region for random mutagenesis. The mutagenic oligonucleotides, G1191 and G1192, are shown with the emboldened residues representing those which were doped during synthesis.

In order to rule out any doubt regarding the uracil content of the template DNA another *dut⁻ ung⁻* strain of *E. coli*, CJ236 (Table A1), was employed. Single-stranded DNA produced from CJ236 harbouring pAMB8 and purified using the Qiagen kit was used in a mutagenesis reaction with G1191 as the mutagenic primer. Transformants were characterised by DNA sequence analysis and once again failed to show any sequence alteration over the region supposedly mutagenised by G1191.

In all, approximately 50 transformants, recovered from mutagenesis with G1191, were sequenced. The Kunkel method of mutagenesis is thought to be about 80% efficient (Sambrook *et al.*, 1982) therefore out of the 50 colonies screened some 40 would be expected to carry the mutagenised strand of DNA. The oligonucleotides were doped to the extent that 20% of oligonucleotides would carry a single base change. If this were the case then 8 out of the 40 supposedly mutagenised transformants would be expected to carry a single base change. If the *E. coli* strains BW313 and CJ236 had been efficient at producing uracil-containing single-stranded template DNA then 5 transformants would have been expected to carry a single base change.

In an attempt to determine why the mutagenesis was unsuccessful the decision was taken to clone and sequence the primers to determine the exact level of doping. The primer G1191 was cloned following the PCR reaction detailed in Figure 6.2 where pAMB8 was used as a template of *MFa1* with primers E126 (*MFa1* 3' primer) and G1191 (mutagenic primer). The PCR reaction was carried out in duplicate using Taq

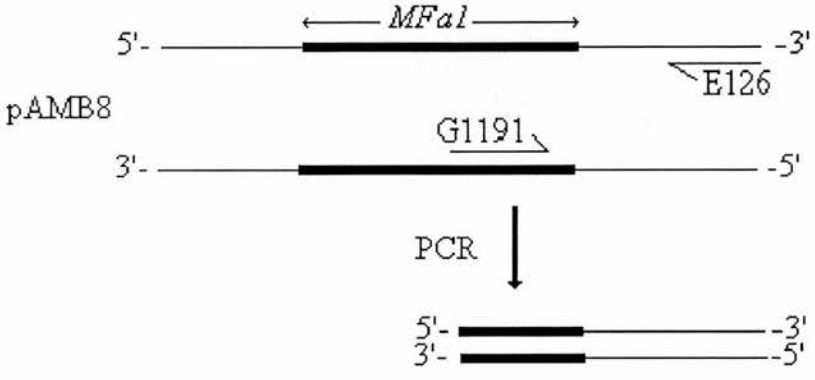
polymerase and the pooled product was cloned using the p-GEMT vector (Chapter 2).

The PCR reaction was carried out under low stringency conditions to ensure that the reaction was non-biased against G1191 oligonucleotides which contained mutations. Transformants were screened for the presence of PCR product by restriction digestion and sequence analysis. 15 transformants were sequenced, but none were found to contain any sequence alteration. If 20% of the oligonucleotide primers produced had had a single base change then we would have expected to see 3 mutant forms of the *MFa1* gene over the G1191 priming region. This estimate assumes that the PCR reaction was un-biased and accurate.

In all, approximately 50 transformants, recovered from mutagenesis with G1191, were sequenced. This taken with the results of the studies on the cloned primer G1191 would suggest that the primers were not doped to a sufficiently high level to allow efficient screening of mutant forms of the *MFa1* gene.

Figure 6.2

The use of PCR to produce an *MFa1* containing product incorporating the mutagenic primer G1191.



The template, pAMB8, is shown with the region of DNA corresponding to *MFa1* highlighted. The PCR product produced by reactions carried out with primers E126 and G1191 is also shown.

Information obtained in discussions at the Yeast Genetics and Molecular Biology Meeting (Seattle, 1994) indicated that analysis of the amino-acid determinants for **a**-factor-Ste6p interaction had already been attempted with little success. This information taken with the apparent difficulty we were having with the creation of a library of mutant **a**-factor molecules prompted us to drop this side of the project. The decision was taken to concentrate on the biochemical approach to studying **a**-factor-Ste6p interaction by focusing on the purification and reconstitution of Ste6p.

6.5 Discussion

In recent years several studies have concentrated on the molecular determinants of **a**-factor bioactivity and Ste6p interaction. Caldwell *et al.*, (1994) examined the role of the N-terminal amino-acid residues on the bioactivity of **a**-factor. Their analysis of truncated **a**-factor molecules showed that sequential removal of the N-terminal amino-acids resulted in a progressive loss of potency. To further examine the importance of N-terminal residues for the bioactivity of **a**-factor they used doped oligonucleotide mutagenesis to create a library of mutagenised **a**-factor molecules. As their study was concerned with the bioactivity of mutant **a**-factor molecules they expressed the mutagenic **a**-factor library in an *mfa1 mfa2* null mutant and screened for colonies which secreted biologically inactive **a**-factor. The identification of mutant **a**-factor molecules which were biologically inactive (or less active than wild-type) but secreted could act as a reverse screen for amino-acids involved in **a**-factor secretion. Their analysis showed that the mutant **a**-factor molecules were present at a lesser concentration than wild-type molecules and they suggested that this was due to their inability to immunoprecipitate mutant variants as efficiently as wild-type molecules. This could also have been due to less efficient secretion by the Ste6p pump. The data presented in this paper is too limited to draw any precise conclusions regarding which residues might be important for bioactivity and secretion. In general they showed that only extreme changes in the peptidyl structure affect the biological activity suggesting that Ste6p may be a promiscuous peptide transporter. This is in marked contrast to the effects of removal of either the methyl or farnesyl moieties.

Studies on the importance the carboxyl methyl esterification of **a**-factor by the *STE14*-encoded protein (Sapperstein *et al.*, 1994) suggest that is essential for the export of **a**-factor by the Ste6p pump. These studies (of Sapperstein *et al.*, 1994) indicate that although **a**-factor is correctly synthesised and membrane associated in a *ste14* null mutant, the (*ste14* null) mutant exhibits a block in **a**-factor export which is as severe as that seen in a *ste6* mutant strain. They suggest that the carboxyl methyl esterification of **a**-factor is likely to be an essential determinant in the recognition of the peptide by Ste6p. In addition they refer to unpublished data (Kistler, *et al.*) that states that most amino-acid alterations in mature **a**-factor do not cause an **a**-factor export defect.

To date the precise structural requirements for the recognition of **a**-factor by Ste6p remain unknown. The studies by Sapperstein *et al.*, (1994) suggest that C-terminal methyl esterification of **a**-factor is necessary for its interaction with Ste6p. In addition attempts made at elucidating which N-terminal amino-acid residues are important for the recognition of **a**-factor both by Ste6p and Ste3p have shown that neither protein is able to discriminate against a variety of amino-acid changes within the N-terminal portion of the peptide (Caldwell *et al.*, 1994, Kistler *et al.*, unpublished data).

Recent studies (Quinby and Deschenes, 1997) have shown that processing of the CVIA sequence is not sufficient for the generation of **a**-factor competent for export. Experiments conducted on truncated **a**-factor precursor molecules and GST-(pro)-**a**-factor fusion molecules implicated the amino terminal prosequence in **a**-factor

biogenesis. Deletion of the **a**-factor prosequence was found to lead to a dramatic decrease in the level of intracellular **a**-factor resulting in sterility. They proposed that the amino terminal prosequence may play a role in directing the **a**-factor precursor through a transport pathway that culminated in Ste6p.

In order to ascertain which components of the **a**-factor molecule are involved with the interaction of Ste6p it will be necessary carry out kinetic studies on purified, reconstituted Ste6p and a variety of chemically synthesised mutant **a**-factor molecules.

Chapter 7

Discussion

Discussion

The *STE6* gene product of the yeast *Saccharomyces cerevisiae* encodes a transmembrane ATPase which is essential for the export of the **a**-factor mating pheromone. Ste6p is a member of the ATP binding cassette superfamily of transport proteins which includes many human proteins of medical significance, as discussed in Chapter 1. The identification of the Ste6p substrate, **a**-factor, coupled with the power of yeast genetics made this an ideal candidate protein with which to study this family of transport proteins.

During the course of this project I set out to purify Ste6p from the membranes of yeast cells which were overexpressing the protein. The Ste6p was detected by use of a rabbit polyclonal antibody directed against the C-terminal 400 amino-acids of Ste6p. The antibody was raised and affinity-purified from the rabbit serum by use of two bacterially-expressed recombinant Ste6p proteins which contained the C-terminal 400 amino acids of Ste6p fused to Protein A or β-galactosidase respectively. The affinity-purified antibody was shown to be specific for Ste6p and was capable of detecting the protein in yeast cell membranes by Western blot analysis (Chapter 3).

Initial purification studies were centred on the purification of wild-type Ste6p from the membranes of yeast which were constitutively overexpressing the protein from a yeast episomal vector (Chapter 4). Although conditions were found in which the protein could be solubilised from the membranes, it was soon realised that the

purification of wild-type Ste6p, by conventional means, could be extremely difficult, the main reason for this being the poor level of Ste6p expression. This could possibly have been overcome by greatly increasing the culture volume, or by placing the *STE6* gene under the control of a powerful inducible promoter such as *GAL*. However the decision was taken to tag the extreme N-terminus of Ste6p with six consecutive histidine residues in order to allow its affinity purification on Ni-NTA resin (Chapter 4). The N-terminally histidine tagged version of Ste6p ($N(His)_6$Ste6p) was fully functional, as shown by its ability to complement a Δ*ste6* mutation and its expression under the control of the *GAL* promoter was detectable when expressed in BJ5465. However attempts made at purifying the $N(His)_6$Ste6p by its supposed affinity for Ni-NTA resin failed. It was proposed that the N-terminus was being proteolytically removed during the post translational processing of the protein thus preventing it from binding to the Ni-NTA resin. This proposal was supported by the observations of Kölling and Hollenberg (1994b) who suggested that the N-terminus of Ste6p is removed by signal peptidases on transit to the ER (as discussed in Chapter 4).

A functional octa-histidine-C-terminally tagged variant of Ste6p ($C(His)_8$Ste6p) was then produced and conditions were found by which it could be partially purified from yeast cell membranes (Chapter 5). A monoclonal antibody specific for a poly histidine tag failed to detect the $N(His)_6$Ste6p in Western blots analysis whereas the $C(His)_8$Ste6p was readily detected under identical conditions. These observations also supported the suggestion that the N-terminus of Ste6p was indeed being removed during maturation of the protein.

Recent studies in which the metabolic stability of Ste6p tagged with an HA tag at either the N-terminus (between amino acids 7 and 8) or at its C-terminus have indicated that the N-terminus of Ste6p is intact in the mature protein (Geller *et al.*, 1996). In this study the metabolic stability of the N and C-tagged variants of Ste6p were compared by pulse-chase analysis and detection by SDS-PAGE following immunoprecipitation with anti-HA antibodies. Geller *et al.*, (1996) reasoned that a cleavage event involving the N-terminus of Ste6p would occur very shortly if not immediately after synthesis. Therefore, if the N-terminus were being removed, the pulse-chase experiment would show a more rapid disappearance of the radioactive label from the N-terminally tagged molecule that from Ste6p tagged at the C-terminus. Their results indicated that there was no difference in the stability of the N and C-terminally tagged Ste6p suggesting that no cleavage event was taking place.

The findings of Geller *et al.*, (1996) conflict with those detailed in this thesis. The discrepancy in the findings might be explained by differences in tagging strategies. Their N-terminally tagged Ste6p variant was labelled between amino acids 7 and 8 whereas the hexa-histidine tag was added to the extreme N-terminus of $N(His)_6$Ste6p. It is conceivable therefore that the addition of the HA tag at this position in some way disrupted the proteolysis of the N-terminus. The definitive answer regarding the nature of the N-terminus of Ste6p will require protein sequence analysis to be carried out. Attempts made at sequencing the N-terminus of the partially purified $C(His)_8$Ste6p proved unsuccessful probably as a result of the small quantity and low purity of the material used (Chapter 5).

Further aims:

This thesis details the expression and partial purification of $C(His)_8$Ste6p from yeast cell membranes. The initial aims of the project were concerned with the isolation and reconstitution of Ste6p into phospholipid vesicles to allow kinetic analysis to be carried out. The reconstitution of $C(His)_8$Ste6p into phospholipid vesicles will first require that improvements be made to the purification procedure in order that large quantities of purified protein can be obtained. In addition protein sequence analysis of $C(His)_8$Ste6p is more likely to be successful if large amounts of pure protein were used. The $C(His)_8$Ste6p might be further purified by eluting it from the resin in the presence of a continuous imidazole gradient. However this may prove difficult as $C(His)_8$Ste6p is competed off the Ni-NTA resin by relatively low concentrations of imidazole. Alternatively it may be possible to further enrich the crude cell membrane extract for $C(His)_8$Ste6p prior to its incubation with Ni-NTA. This could be achieved, for example, by passing crude cell extract through a lectin column to remove contaminating glycoproteins. The quantity of crude material will most likely have to be greatly increased to overcome losses encountered by the introduction of an extra purification step.

The successful reconstitution of $C(His)_8$Ste6p into phospholipid vesicles and the development of a suitable assay with which to measure the transportation of **a**-factor, will provide a mechanism in which the kinetics of Ste6p-**a**-factor interaction may be studied (as discussed in Chapter 6). Ste6p-substrate specificity could be studied by

measuring the kinetics of the interaction between $C(His)_8$Ste6p and **a**-factor molecules lacking, for example, the methyl moiety, the farnesyl moiety or which have been truncated at the N-terminus.

The studies discussed in 1.10 suggest that Ste6p may function in the accumulation of **a**-factor into intracellular vesicles prior to reaching the plasma membrane. It would be interesting to determine whether Ste6p and proteins involved in the modification of **a**-factor were co-localised within any intracellular compartment. Indirect immunofluorescence and cellular fractionation may be used to determine if this were the case. An alternative approach might lie in the isolation of the Ste6p containing intracellular membranes. This might be achieved by the use of techniques developed in this laboratory for the immunoisolation of the Kex2-containing compartment of yeast Golgi (Bryant, 1992). The isolation of the hypothetical Ste6p containing compartment would enable the identification of other compartmental components, for example the **a**-factor maturation machinery. It can be envisioned that, if such a compartment were isolated, that it could be used in the development of a cell free assay system for the maturation and export of **a**-factor.

Appendix

Table A1 *Escherichia coli* strains used in this study

Escherichia coli strain	Genotype	Remarks	Source
NM522	*supE thiΔ(lac-proAB) hsd*5 *F'[proAB*$^+$ *lacI*^{q} *lacZΔ*M15*]*.	General lab strain for the propagation of plasmids. Used for the expression of fusion proteins.	Gough and Murray, 1993.
pop2136	*F*$^-$*supE44 hsdR17 mcrA*$^+$ *mcrB*$^+$ r_k^- m_k^+ *thi*-1 *aroB mal-l* contains chromosomally integrated copy of λcI857 (ts).	Used for the expression of β-galactosidase fusion proteins from the P_R promoter of pEX vectors.	Kusters *et al.*, 1989.
DH5α	*supE*44 *Δλac*U169 *(Φ80 lacZΔ*M15*) hisR*17 *recA*1 *endA*1 *gyrA*96 *thi-1 relA*1.	General lab strain for the propagation of plasmids. The *endA*1 mutation improves plasmid yield. This strain makes good DNA for sequencing.	Gibco, BRL.
BW313	*F' dut*$^-$ *ung*$^-$.	A *dut*$^-$*ung*$^-$ strain used to make uracil-containing DNA for Kunkel mutagenesis.	Gifted by Phil Meaden (Heriot Watt University).

CJ236	*dut*1*ung*1*thi*-1*rel*A1 (*cam*^{r} F' on plasmid JC105)	A *dut*$^{-}$*ung*$^{-}$ strain used to make uracil-containing DNA for Kunkel mutagenesis. The F' is carried on the plasmid JC105.	Kunkel (1987) this was gifted by J. Beags (Edinburgh University)

Table A2 *Saccharomyces cerevisiae* strains used in this study.

***S. cerevisiae* strain**	**Genotype**	**Remarks**	**Source**
BJ5464	*MATα ura3-52 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*	protease deficient lab strain.	Yeast Genetic Stock Centre, University of California, Berkley.
BJ5465	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*	protease deficient lab strain.	Yeast Genetic Stock Centre, University of California, Berkley.
WKK7	*MAT***a** *ura3-52 leu2-3, 112 ΔSte6::HIS3 trp1-1 ade2-1 can1-100.*	*Ste6* null mutant for use with complementation studies.	Karl Kuchler (Vienna).
RC757	*MATα sst2-1 met1 his6 can1 cyh2 rme1.*	**a**-factor tester strain.	R. Chan (University of Oregon).
JRY188	*MATα leu2-3, 112 ura3-52 trp1 his4 sir3-8 rme GAL.*	General lab strain.	Brake *et al.*, 1994.

Table A3 Plasmids used in this study.

PLASMID	CONSTRUCTION/ COMMENTS	SOURCE
pAX12	Used in the production of protein A fusion protein. Discussed in Chapter 3.	Zueco and Boyd (1992).
pEX12	Used in the production of β-galactosidase fusion proteins. Discussed in Chapter 3.	Kusters *et al.*, (1989).
pk19	Small, high copy number *E. coli* plasmid cloning vector.	NEB.
pVT-103U	Yeast/*E. coli* shuttle phagemid vector used for the production of single-stranded DNA in mutagenesis studies.	Vernet *et al.*, (1987)
YDp-U	Yeast integrating vector carrying a *URA3* disruption cassette.	Berben *et al.*, (1991).
Yeplac181	Yeast episomal vector with *LEU2* selective marker.	Gietz and Sugind (1988).
YEpGAL	*GAL* promoter cloned into the *Eco*R1/*Bam*H1 site of Yeplac112.	Boyd lab.
pSTE6	6.6kb *Sal*1 fragment of genomic DNA containing entire *STE6* gene and 600bp upstream sequence cloned into the *Sal*1 site of pUC7.	Benjamin Glick (University of Basel), originated from A. Varshavsky (MIT).
BJ5783 (p17)	pUC4 vector containing the *PEP4* gene disrupted by *LEU2* for the creation of *pep4* knockout strains of yeast. Discussed in the appendix to Chapter 5.	Gifted by Beth Jones.

pAMB1	PCR amplified 1.2Kbp fragment corresponding to the extreme 3' end of the *STE6* open reading frame cloned into the *Eco*RI/*Bam*H1 sites of pAX12.	This study.
pAMB3	The *Eco*RI/*Bam*H1 fragment of pAMB1 corresponding to the extreme 3' end of the *STE6* open reading frame subcloned into the *Eco*RI/*Bam*H1 site of pEX12.	This study.
pAMB4	The *Sal*1 *Sac* 1 fragment of pSTE6 containing the entire *STE6* gene cloned into the *Sal*1 *Sac*1 Sites of pk18.	This study.
pAMB7	The *Sal*1 fragment of p*STE6* containing the entire *STE6* gene subcloned into the *Sal*1 site of Yeplac181.	This study.
pAMB8	PCR amplified *MFa1* cloned into the *Bam*HI/*Hin*dIII site of pVT-103U.	This study.
pAMB11	PCR amplified N-terminally hexa-histidine tagged *STE6* cloned into *Hin*dIII site of YEpGAL.	This study.
pAMB14	PCR amplified C-terminally octa-histidine tagged *STE6* cloned into *Hin*dIII site of YEpGAL	This study.
pAMB15	*STE6* containing *Hin*dIII fragment of pAMB14 subcloned into *Hin*dIII site of pk19.	This study.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic Local alignment search tool. *J. Mol. Biol.* **215**:403-410.

Achstetter, T. 1989. Regulation of α-factor production in *Saccharomyces cerevisiae*: **a**-factor pheromone-induced expression of *MFα1* and *STE13* genes. *Mol. Cell. Biol.* **9**:4507

Aguilar-Bryan, L., Nichols, C.G., Eechsler, S.W., Celment, J.P., Boyd, A.E., Gonzalez, G., Herrera-Sosa, H., Nguy,K., Bryan, J. and Nelson, D.A. 1995. Cloning of the beta-cell high-affinity sulphonurea receptor a regulator of insulin secretion *Science*. **268**:423-426.

Ames, G. F-L. 1986. Bacterial periplasmic transport systems: structure, mechanism and evolution. *Ann. Rev. Biochem*. **55**:397-425.

Ames, G.F., Mimura, C.S., Holbrook, S.R. and Shyamala, V. 1992. Traffic ATPases: A superfamily of transport proteins operating from *Escherichia coli* to humans. 1992. *Advances in Enzymology*. **65**:1:47.

Ammerer, G., Hunter, C.P., Rothman, J.H., Saari, G.C., Valls, V.A. and Stevens, T.H. 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. *Mol. Cell. Biol*. **6**. 2490-2499.

Androlewicz, M.J., Ortmann, B., van Endert, P.M., Spies, T. and Cresswell, P. 1994. Characteristics of peptide and major histocompatibility complex class I/β2-microglobulin binding to the transporters associated with antigen processing (TAP1 and TAP2). *Proc. Natl. Acad. Sci*. **91**:12716

Ashby, M.N., King, D.S. and Rine, R. 1992. Endoproteolytic processing of a farnesylated peptide in vitro. *Proc. Natl. Acad. Sci.* **89**:4613

Bardwell, L., Cook, J. G., Inouye, C.J. and Thorner, J. 1994. Signal propagation and regulation in the mating response pathway of the yeast *S. cerevisiae*. *Developmental Biology.* **166**:363-379.

Bender, A. and Sprague, G.F.Jr. 1989 Pheromones and pheromone receptors are the primary determinants of mating specificity in the yeast *Saccharomyces cerevisiae*. *Genetics.* **121**:463-176.

Berben, G., Dumont, J., Gilliquet, V., Bolle, P. and Hilger, F. 1991. The YDp plasmids: a uniform set of vectors bearing versatile gene disruption cassettes for *Saccharomyces cerevisiae*. *Yeast.* 7:475-477.

Berkower, C. and Michaelis, S. 1991. Mutational analysis of the yeast **a**-factor transporter STE6, a member of the ATP binding cassette (ABC) protein superfamily. *EMBO J.* **12**: 77-3785.

Berkower, C., Loayza, D. and Michaelis, S. 1994. Metabolic instability and constitutive endocytosis of Ste6, the **a**-factor transporter of *Saccharomyces cerevisiae. Mol. Biol. Cell.* **5**:1185-98.

Brake, A.J., Merryweather, J.P., Coit, D.G., Heberlein, U.A., Masiarz, F.R., Mullenach, G,T., Urdea, M.S., Valenzuela, P. and Barr, P.J. 1984. Alpha factor directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **81**. 4642-4646.

Brake, A.J., Brenner, R., Najarian, R., Laybourn, P. and Merryweather, J. 1985. Structure of genes encoding precursors of the yeast peptide mating pheromone **a**-factor. In: *Protein Transport and Secretion*, ed. M.J. Gething. Cold Spring Harbour, NY: Cold Spring Harbour Lab.

Brizzio, V., Gammie, A.E., Nijbroek, G., Michaelis, S. and Rose, M. 1996. Cell fusion during yeast mating requires high levels of **a**-factor mating pheromone. *J. Cell. Biol.* **135**:1727-1739.

Browne, B.L., McClendon, V. and Bedwell, D.M. 1996. Mutations in the first LSGGQ motif of Ste6p cause defects in **a**-factor transport and mating in *Saccharomyces cerevisiae*. *J. Bacteriology.* **178**:1712-1719.

Bryant, N. 1992. PhD Thesis. Immunoisolation of a Golgi subcompartment of *Saccharomyces cerevisiae*.

Caldwell, G.A., Wang, S., Naider, F. and Becker, J.M. 1994a. Consequences of altered isoprenylation targets on **a**-factor export and bioactivity. *Proc. Natl. Acad. Sci. USA.* **91**:1275-1279.

Caldwell, G.A., Wang, S., Xue, C. Jiang, Y., Lu, H., Naider, F. and Becker, J.M. 1994b. Molecular determinants of bioactivity of the *Saccharomyces cerevisiae* lipopeptide mating pheromone. *J. Biol. Chem.* **269**:19817-19826.

Caldwell, G.A., Naider, F. and Becker, J.M. 1995. Fungal lipopeptide mating pheromones: a model system for the study of protein prenylation. *Microbiological Reviews.* **59**:406-422.

Chan, R.A. and Otte, C.A. 1982a. Isolation and genetic analysis *of Saccharomyces cerevisiae* mutants supersensitive to G_1 arrest by **a** factor and a factor pheromones. *Mol. Cell. Biol.* **2**:11-20.

Chan, R.A. and Otte, C.A. 1982b. Physiological characterisation of *Saccharomyces cerevisiae* mutants supersensitive to G_1 arrest by **a** factor and a factor pheromones. *Mol. Cell. Biol.* **2**:21-29.

Chen, C., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesmann, M.M. and Roninson, I.B. 1986. Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multi-drug resistant human cells. *Cell.* **47**:381-389.

Chen, P., Sapperstein, S.K., Choi, J.D. and Michaelis, S. 1997. Biogenesis of the *Saccharomyces cerevisiae* mating pheromone **a**-factor. *J. Cell. Biol.* **136**:251-269.

Clarke. S. 1992. Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu. Rev. Biochem.* **61**:355-386.

Davis, J.L., Kunisawa, R, and Thorner, J. 1992. A presumptive helicase (MOT1 gene product) affects gene expression and is required for viability in the yeast *Saccharomyces cerevisiae. Mol. Cell. Biol.* **12**:1879.

Davis, J.L., Horecka, N.G,and Sprague, G.F, Jr. 1993. *Cis*-and *Trans*-acting functions required for endocytosis of the yeast pheromone receptors. *J. Cell. Biol.* **122**:53-65.

Decottignies, A. and Goffeau, A. 1997. Complete inventory of the yeast ABC proteins. *Nature Genetics.* **15**:137-145.

Deutscher, M.P. (ed.) Guide to protein purification. *Methods in Enzymology*. **182**:162-164.

Dev, I.K. and Ray, P.H. 1990. Signal peptidases and signal peptide hydrolases . *J. Bioenerg. Biomembr*. **22**:271-290.

Deverson, E.V., Gow, I.R., Coadwell, W.J., Monaco, G.W. and Howard, J.C. 1990. MHC class II region encoding proteins related to the multidrug resistance family of transmembrane proteins. *Nature*. **348**:738-741.

DeVoto, M., Ronchetto, P., Faran, P., Orriols, J.J.T., Romeo, G., Goossens, M., Ferrari,M., Magnani, C., Seia, M. and Cremonesi, L. 1991. Screening for non-Delta F508 mutants in 5 exons of the cystic-fibrosis regulator (CFTR) gene in Italy. *Am. J. Hum. Genet.*, **48**:1127-1132.

Dolan, J.W., Kirkman, C. and Fields, S. 1989. The yeast *STE12* protein binds to the DNA sequence mediating pheromone induction. *Proc. Natl. Accad. Sci.* **86**:5703

Dolan, J.W. and Fields, S. 1991. Cell-type-specific transcrition in yeast. *Biochim. et Biophysicia. Acta.* **1088**:155-169.

Dunbar, B. and Wilson, S.B. 1993. A buffer exchange procedure giving enhanced resolution to polyacrylamide gels pre-run for protein sequencing. *Analytical Biochemistry*. **216**:227-228.

Elia, L. and Marsh, L. 1996. Role of the ABC transporter Ste6 in cell fusion during yeast conjugation. *J. Cell. Biol.* **135**: 741-751.

Elizinga, M (ed.) 1982. *Methods in Protein Sequence* Humana Press. Clifton, NJ

Fields, S. 1990. Pheromone response in yeast. *TIBS.* **15**:270-273.

Finlay, D. 1992. The yeast ubiquitin system. In *The molecular and cellular biology of the yeast* Saccharomyces*: Gene expression* (ed Jones, E.W., Pringle, P.R. and Broach, J.R.) p539-582. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Fuller, R.S., Sterne, R.E. and Thorner, J. 1988. Enzymes required for yeast pheromone processing. *Ann Rev Physiol.* **50**:345-62.

Fujiyama, A., Matsumoto, K. and Tamanoi, F. 1987. A novel yeast mutant defective in the processing of ras proteins: assessment of the effect of the maturation of processing steps. *EMBO. J.* **6**:223-228.

Gartner, J., Moser, H. and Valle, D. 1992. Mutations in the 70 kDa peroxisomal membrane protein gene in Zellweger syndrome. *Nature Genet.* **1**:16-23.

Geitz, D. and Sugino,M. 1988. New yeast *Escherichia coli* shuttle vectors constructed with in vitro mutagenised yeast genes lacking six base pair restriction sites. *GENE.* **74**:515-522.

Geitz, D., St. Jean. A., Woods, R.A. and Schiestl, R.H. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nuc. Acids. Res.* **20**:1425

Geller, D., Taglicht, D., Edgar, R., Tam, A., Pines, O., Michaelis, S. and Bibi, E. 1996. Comparative topology studies on *Saccharomyces cerevisiae* and in *E. coli*. The N-terminal half of the yeast ABC transporter Ste6. *J. Biol. Chem.* **271**:13746-13753.

Gething, M.-J. ed. 1985. *Current communications in molecular biology. Protein transport and secretion.* Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Goodman. L.E., Judd. .R., Farnsworth. C.C., Powers. S., Gelb. M.H., Glomset. J.A. and Tamanoi. 1990 Mutants of *Saccharomyces cerevisiae* defective in farnesylation of Ras proteins. *Proc. Natl. Acad. Sci.* **87**:9665-

Gough, J.A. and Murray, N.E. 1983. Sequence diversity among related genes for recognition of specific target molecules. *J. Mol. Biol.* **166**:1-19.

Gottesman, M.M. and Pastan, I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62**:385-427.

Grant, C.E., Valdimarsson, G., Hipfner, D.R., Almquist, K.C., Cole, S.P. and Deeley, R. 1994. Overexpression of multidrug resistance protein (MRP) increases resistance to natural product drugs. *Cancer. Res.* **54**:357.

Hagen, D.C., McCaffrey, G. and Spargue, G.F. Jnr. 1986. Evidence the yeast *STE3* gene encodes a receptor for the peptide pheromone **a**-factor: gene sequence and implications for the structure of the presumed receptor. *Proc. Natl. Acad. Sci. USA.* **83**:1418-1422.

Harding, T.M., Morano, K.A., Scott, S.V. and Klionsky, D.J. 1995. Isolation and characterisation of yeast mutants in the cytoplasm to vacuole targeting pathway. *J. Cell. Biol.* **131**:591-602.

Hartwell 1980. Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by polypeptide mating hormones. *J. Cell. Biol.* **85**:811-822.

He, B., Chen, P., Chen. S.Y., Vancure, K.L., Michaelis, S. and Powers, S. 1991 *RAM2,* an essential gene of yeast, and *RAM1* encode two polypeptide components of the farnesyltransferase that prenylates **a**-factor and Ras proteins. *Proc. Natl. Acad. Sci. USA*. **88**:11373-11377.

Herskowitz, I. 1988. Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52**:536-553.

Herskowitz, I., Rine, J. and Strathern, J. 1992. Mating-type determination and mating-type interconversion in *Saccharomyces cerevisiae*. In *The molecular and cellular biology of the yeast* Saccharomyces*: Gene expression* (ed Jones, E.W., Pringle, P.R. and Broach, J.R.) p 657-744. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Herskowitz, I. 1995. MAP kinase pathways in yeast: for mating and more. *Cell.* **80**:187-197.

Hicke, L. and Riezman, H. 1996. Ubiquitination of a yeast Plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell.* **84**:277-287.

Higgins, C.F. 1992. ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* **8**:67-113.

Higgins, C.F.1995. The ABC of channel regulation. *Cell.* **82**:693-696.

Higgins, C.F., Hiles, I.D., Salmond, G.P.C., Gill, D.R., Downie, J.L., Evans, I.J., Holland, I.B., Gray, L., Buckel, S.D., Bell, A.W. and Hermodson, M.A. 1986. A family of ATP-binding subunits coupled to many distinct biological processed in bacteria. *Nature*. **323**: 448-450.

Hrycyna. C.A. and Clarke. S. 1990. Farnesyl cysteine C-terminal methyltransferase activity is dependent on the *STE14* gene product in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**:5071-5076.

Hrycyna. C.A., Sapperstein. S.K., Clarke. S. and Michaelis. S. 1991. The *Saccharomyces cerevisiae STE14* gene encodes a methyltransferase that mediates C-terminal methylation of **a**-factor and RAS proteins. *EMBO.J.* **10**:1699-1709.

Hrycyna. C.A. and Clarke. S. 1992. Maturation of isoprenyalted proteins in *Saccharomyces cerevisiae*. Multiple activities catalyses the cleavage of three carboxyl-terminal amino acids from farnesylated substrates in vitro. *J. Biol. Chem*. **267**:10457-10464.

Hughes, A. 1994. Evolution of the ATP-Binding-Cassette Transporters of Vertebrates. *Mol Biol. Evol*. **11**:899-910.

Hyde, S.C., Emsley,P., Hartshorn, M.J., Mimmack, M.M., Gilaedi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E. and Higgins, C.F. 1990. Structural model of ATP-binding proteins assocuated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature*. **346**:363-365.

Hsiao, Ku-chan. 1991.A fast and simple procedure for sequencing double stranded DNA with Sequenase. *Nucleic Acids Research*. **19**:2787.

Jackson, C.L. and Hartwell, L.H. 1990a. Courtship in *S. cerevisiae*: an early cell-cell interaction during mating. *Mol. Cell. Biol.* **10**:2202-2213.

Jackson, C.L. and Hartwell, L.H. 1990b. Courtship in *S.cerevisiae*: Both cell types choose mating partners by responding to the strongest pheromone signal. *Cell.* **63**:1039-1051.

Jackson, C.L., Konopka, J.B. and Hartwell, L.H. 1991. *S. cerevisiae* a pheromone receptors activate a novel signal transduction pathway for mating partner discrimination. *Cell.* **67**:389-402.

Jarvis, E.E., Hagen, D.C, and Sprague, G.F. Jnr. 1988. Identification of DNA segment that is necessary and sufficient for α-specific gene control in *Saccharomyces cerevisiae*: Implications for regulation of α-specific and **a**-specific genes. *Mol. Cell. Biol.* **8**:309.

Julius, D., Blair, L., Brake, A., Sprague, G. and Thorner, J. 1983. Yeast α-factor is processed from a larger precursor polypeptide: The essential role of a membrane-bound dipeptidyl aminopeptidase. *Cell.* **37**:309

Julius, D., Scheckman, R. and Thorner, J. 1984. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. *Cell.* **39**:309-318.

Kamijo, K., Taketani, S., Yokata, D., Osumi, T. and Hashimoto, T. 1990. The 70-kDa peroxisomal membrane protein is a member of the Mdr (P-glycoprotein)-related ATP-binding protein superfamily. *J. Biol. Chem.* **265**:4534-4540.

Kerppola, R.E., Shyamala, V.K., Klebba, P. and Ames, G. 1991. The membrane-bound proteins of periplasmic permeases form a complex- identification of the histidine permease HISQMP complex. *J. Biol. Chem.* **266**:9857-9865.

Kistler M., Mainer, K. and Eckardtschupp, F. 1990. Genetic and Biochemical-analysis of glutathione-deficient mutants of *S. cerevisiae. Mutagenesis.* **1**:39-44.

Kölling, R. 1996. An acidic domain mediates ubiquitination and fast turnover of the BC-transporter Ste6. Meeting Abstract. Molecular Biology, Madison, Wisconsin.

Kölling, R., Bottner, B. and Hollenberg, C.P. 1993. Synthesis and farnesylation of **a**-factor fusion proteins in *Saccharomyces cerevisiae*. *FEBS Letters*. **336**:129-132.

Kölling, R. and Hollenberg, C.P. 1994a. The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquinated form in endocytosis mutants. *EMBO J.* **13**:3261-3271.

Kölling, R. and Hollenberg, C.P. 1994b. The first hydrophobic segment of the ABC-transporter, Ste6, functions as a signal sequence. *FEBS Letters*. **351**:155-158.

Kuchler, K., Sterne, R.E. and Thorner, J. 1989. *Saccharomyces cerevisiae STE6* gene product: a novel pathway of protein export in eukaryotic cells. *EMBO J.* **8**:3973-3984.

Kuchler, K. and Thorner, J. 1990a. Membrane translocation of protein without hydrophobic signal sequences. *Curr Op Cell Biol.* **2**:617-624.

Kuchler, K. and Thorner, J. 1990b. Secretion of peptides and proteins lacking hydrophobic signal sequences: The role of adenosine triphosphate-driven membrane translocators. *Endocr. Rev.* **13**:499-514.

Kuchler, K. and Thorner, J. 1992. Functional expression of human mdr1 in the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **89**:2302

Kuchler, K., Göransson, H.M., Viswanathan, M.N. and Thorner, J. 1992. Dedicated transporters for peptide transport and incompartmental traffic in the yeast *Saccharomyces cerevisiae*. *Cold Spring Harbour Symp. Quant. Biol.* **57**:579-592.

Kuchler, K., Dohlman, H.G. and Thorner, J. 1993. The **a**-factor transporter (*STE6* gene product) and cell polarity in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **120**:1203-1215.

Kuchler, K., Swartzman, E.E. and Thorner.J. 1994. A novel mechanism for transmembrane translocation of peptides: the *Saccharomyces cerevisiae STE6* transporter and export of the mating pheromone **a**-factor. *Current Topics in Membranes.* **41**:19-42.

Kunkel, T.A., Roberts, R.J. and Zakour, R.A. 1987 *Methods in Enzymology*. **154**: 367-382.

Kurjan, J. 1985. α-factor structural gene mutations in yeast: effect on α-factor production and mating. *Mol. Cell. Biol.* **5**:787-796.

Kurjan, J. 1992. Pheromone response in yeast. *Annu. Rev. Biochem.* **61**:1097-1129.

Kurjan, J. and Herskowitz, I. 1982. Structure of the a yeast pheromone gene (*MFa*): a putative a-factor precursor contains four tandem copies of mature a-factor. *Cell.* **30**: 933-943.

Kusters, J.D., Jager, E.J. and Vanderzeijst, B.A.M. 1989. Improvement in the cloning linker of the bacterial expression vector pEX. *Nuc. Acids. Res.* **17**:8007.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**:680-685.

Leighton, J. and Schatz, G. 1995. An ABC transporter in the mitochondrial membrane is required for the growth of yeast. *EMBO J.* **14**:188-195.

Levi, J.D. 1956. Mating reaction in yeast. *Nature*. **177**:753-754.

Lin, J., Smith, M., Jesse, J. and Bloom, F. 1992. DH11S: An *Escherichia coli* strain for preparation of single-stranded DNA from phagemid vectors. *Biotechniques* **12**:718-721.

Lipke, P.N. and Kurjan, J. 1992 Sexual agglutination in budding yeasts: Structure, function and regulation of adhesion glycoproteins. *Microbiol. Rev.* **56**:180-

Loo, T.W. and Clarke, D.M. 1995 Rapid purification of human P-glycoprotein mutants expressed transiently in HEK 293 cells by Nickel-chelate chromatography and characterisation of their drug stimulated ATPase activities. *J. Biol. Chem.* **270**: 21449-21452.

MacKay, V. and Manney, T.B. 1974a. Mutations affecting the conjugation and related processes in *Saccharomyces cerevisiae*. I. Isolation and phenotypic characterisation of non-mating mutants. *Genetics* **76**:255-273

MacKay,V and Manney, T.B. 1974b. Mutations affecting the conjugation and related processes in *Saccharomyces cerevisiae*. II. Genetic analysis of non-mating mutants. *Genetics* **76**:273-288

McGrath, J.P. and Varshavsky, A. 1989. The yeast *STE6* gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature* **340**:400-404.

Marcus, S., Caldwell, G.A., Xue, C.-B., Naider, F. and Becker, J.M. 1990. Total *in vitro* maturation of the *Saccharomyces cerevisiae* a-factor lipopeptide mating pheromone. *Biochem. Biophys. Res. Comm.* **172**:1310-1316.

Marcus, S. Caldwell, G.A., Miller, D., Xue, C.B., Naider, F. and Becker, J.M. 1991. Significance of C terminal modifications to the biological activity of

Saccharomyces cerevisiae **a**-factor mating pheromone, *Mol. Cell. Biol.* **11**:3603-3612.

Marr. R.S., Blair. L.C. and Thorner. J. 1990. *Saccharomyces cerevisiae STE14* gene is required for COOH-terminal methylation of **a**-factor mating pheromone. *J. Biol. Chem.* **265**:20057-20060.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene diflouride membranes. *J. Biol. Chem.* **262**:10035-10038.

Michaelis, S. 1993. STE6, the yeast **a**-factor transporter. *Sem. Cell. Biol.* **4**:17-27.

Michaelis, S. and Herskowitz, I. 1988. The **a**-factor pheromone *of Saccharomyces cerevisiae* is essential for mating. *Mol. Cell. Biol.* **8**:1309-1318.

Michaelis, S. and Berkower, C. 1995. Sequence comparison of yeast ATP-binding cassette proteins. *Cold Spring Harbour Symp. Quant. Biol.* **LX**: 291-307.

Momburg, F., Neefjes, J.J. and Hämmerling, G.J. 1994. Peptide selection by MHC-encoded TAP transporters. *Curr. Opin. Immunol.* **6**:32.

Mosser, J., Lutz, Y., Stoeckel, M.E., Sarde, S.O., Kretz, C., Douar, A.M., Lopez, J., Aubourg, P. and Mandel, J.L. 1994. The gene responsible for adrenoleukodystrophy encode a peroxisomal membrane protein. *Human Mol. Gen.* **3**:265-271.

Nahon, E., Atzmony. D., Zahavi, A. and Granot, D. 1995 Mate selection in yeast: a reconsideration of the signals and the message encoded by them. *J. Theor. Biol.* **172**:315-322.

Nakai, K. and Kanehisa, M.A. 1992. A knowledge base for predicting protein localisation sites in eukaryotic cells. *Genomics*. **14**:897-977

Novick, P. and Scheckman, R. 1979. *Proc. Natl. Acad. Sci.* **76**:1858-1862.

Novick, P., Field. C. and Schekman, R. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell*. **21**:205-215.

Novick, P. Ferro, S. and Schekman, R. 1981. Order of events in the yeast secretory pathway. *Cell*. **25**:461-469.

Neugebauer, J. ed. 1990. A guide to the properties and uses of detergents in biology and biochemistry. Calibochem Biochemicals.

Powers. S., Michaelis. S., Broek, D. Santa. A.S., Field. J., Herskowitz, I. and Wigler, M. 1986. *RAM* a gene of yeast required for functional modification of RAS proteins and for production of the mating pheromone *a*-factor. *Cell*. **47**:413-422.

Prasad, R., De Wegifosse, P., Goffeau, A. and Balzi. 1995. Molecular cloning and characterisation of a novel gene of *Candida albicans, CDR1*, conferring multiple resistance to drugs and antifungals. *Curr. Genet*. **27**:320-329.

Pringle and Hartwell 1981, Mol Biol of the yeast Saccharomyces: life cycle and inheritance p97 ed. J.N Strathern.

Proteau, G., Gelanznikas, R. and Merante, T. 1990. The isolation of biologically active mating pheromone, **a**-factor, from the yeast, *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Comm*. **170**:182-186.

Quinby, G.E. and Deschenes, R.J. 1997. An amino terminal prosequence is required for efficient synthesis of *S. cerevisiae* **a**-factor. *Biochim. Biophys. Acta.* **1356**:23-24.

Rapoport, T.A. 1992. Transport of molecules across the endoplasmic reticulum membrane. *Science*. **258**:931-936.

Raths. S., Rohrer, J., Crausaz, F. and Riezman, H. (1993) *end3* and *end4* mutants: two mutants defective in receptor mediated and fluid phase endocytosis in *Saccharomyces cerevisiae. J. Cell. Biol.* **120**:55-65.

Raymond, M., Gros,P., Whiteway, M. and Thomas, D.Y. (1992). Functional complementation of yeast *ste6* by a mammalian multidrug resistance *mdr* gene. *Science*. **256**:232-234.

Raymond, M., Ruetz, S., Thomas, D.Y. and Gros, P. 1994. Functional expression of P-glycoprotein in *Saccharomyces cerevisiae* confers cellular resistance to the immunosuppressive and antifungal agent FK520. *Mol. Cell. Biol.* **14**:277

Raymond, M., Alarco, A.M., Dignard, D., Magee, B.B. and Thomas, D.Y. 1995. Structural and functional analysis of *HST6*, an ABC transporter from *Candida albicans* that complements the mating defect of *Saccharomyces cerevisiae ste6Δ* mutants. *Yeast*. **11**:S85.

Reizman, H.1985. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell*. **40**:1001-1009.

Reizman, H. 1993. Yeast endocytosis. *Trends Cell Biol.* **3**:273-277.

Rine, J. 1979. Ph.D thesis, University of Oregon, Eugene, USA.

Riordan, J.R., Rommens, J.M., Kerem, B., Alon,N., Rozmahel, R., Grzelczak, Z., Zielinski, J., Lok, S.,Palvsil, N., Chou, J.-L., Drumm, M.L., Oannuzzi, M.C., Collins, F.s. and Tsui, L.-C. 1989. Identification of the cystic fibrosis gene: cloning and characterisation of complementary DNA. *Science*. **245**:1066-1073.

Rubio, J.P. and Cowman, A.F. 1996. The ATP-binding cassette (ABC) gene family of *Plasmodium falciparum. Parasitology Today*. **12**:135-140.

Ruetz, S., Brault, M., Kast, C., Hemenway, C., Heitman, J., Grant, C.E., Cole, S.P., Deeley, R.G. and Gros, P. 1996a. Functional expression of the multidrug Resistance-associated protein in the yeast *Saccharomyces cerevisiae. J. Biol. Chem*. **271**:4154-4160.

Ruetz, S., Delling, U., Brault, M., Schurr, E. and Gros. P. 1996b. The *pfmdr1* gene of *Plasmodium falciparum* confers cellular resistance to anti-malarial drugs in yeast cells. *Proc. Natl. Acad. Sci*. **93**:9942-9947.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: A laboratory manual. 2nd edition. Cold Spring Harbour Laboratory Press, New York.

Sapperstein, S., Berkower, C. and Michaelis, S. 1994. Nucleotide sequence of the yeast *STE14* gene, which encodes farensylcysteine carboxyl methylatransferase, and demonstration of its essential role in **a**-factor transport. *Mol. Cell. Biol.* **14**:1438-1449.

Schafer, W.R., Kim, R., Sterne, R., Thorner, J., Kim, S-H. and Rine, J. 1989. Genetic and pharmacological supression of oncogenic mutations in *RAS* genes of yeast and humans. *Science*. **245**:379-385.

Schafer, E.R., Trueblood,C.E., Yang, C-C., Mayer, M.P., Rosenberg, S., Poulter, C.D., Kim, S-H. and Rine, J. 1990. Enzymic coupling of choloesterol intermediates to a mating pheromone precursor and to the Ras protein. *Science*. **249**:1133-1139.

Schafer W.R. and Rine, J. 1992. Protein prenylation: genes, enzymes, targets and functions. *Ann. Rev. Genet.* **30**:219-37.

Seufert, W. and Jentsch, S. 1990. Ubiquitin-conjugating enzymes *UBC4* and *UBC5* mediate the selective degradation of short-lived and abnormal proteins. *EMBO J.* **9**:543-550.

Shimoda 1972. Mating reaction in *S. cerevisiae* changes in autolytic activities. *Arch. Microbiol.* **85**:310

Singh, A., Chen, J.M., Lugovoy, J.M., Chang, J.M., Hitzeman, R.A. and Seeburgh, P.H. 1983. *Saccharomyces cerevisiae* contains two discrete genes coding for the a-factor pheromone. *Nuc. Acids. Res.* **11**:4049-

Sprague, G.F. and Thorner, J.W. 1992. Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae.* In: *The molecular and cellular biology of the yeast* Saccharomyces*: Gene expression* (ed. Jones, E.W., Pringle, P.R. and Broach, J.R.) p 657-744. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Stephenson, R.C. and Clarke, S. 199. Identification of a C-terminal protein carboxyl methyltransferase in rat-liver membranes utilising synthetic farnesyl cysteine-containing peptide substrate. *J. Biol. Chem.* **265**:16248-16254.

Stephenson, R.C. and Clarke, S. 1992. Characterisation of rat-liver carboxyl methyltransferase involved in the maturation of proteins with the -CXXX C-terminal sequence motif. *J. Biol. Chem.* **267**:13314-13319.

Sterne, R.E. and Thorner, J. 1986. Processing and secretion of a yeast peptide hormone by a novel pathway. *J. Cell. Biol.* **103**:189a.

Strazdis, J. and MacKay, V. 1982. Reproducible and rapid methods for the isolation and assay of **a**-factor, a yeast mating hormone. *J. Bacteriology.* **151**:1153-1161.

Szczypka, M.D., Wemmie, J.A., Moye-Rowley, W.S. and Thiele, D.J., 1994. A yeast metal resistance protein similar to human cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance-associated protein. *J. Biol. Chem.* **269**:22853-22857.

Teem, J.L., Berger, H.A., Ostedgaard, L.S., Rich, D.P., Tsui, L-C. Welsh, M.J. 1993. Identification of revertants for the cystic fibrosis ΔF508 mutation using STE6-CFTR Chimeras in yeast. *Cell.* **73**:335-346.

Thomas, P.M., Cot, G.J., Wohlik, N., Haddad, B., Mathew, P.M., Rabl, W., Aguilar-Bryan, L., Gagel, R.F. and Bryan, J. 1995. Mutations in the Sulphonurea receptor in familial persistent hyperinsulinemic hypoglycemia of infancy. *Science.* **268**:426-429.

Thorner, J. 1981. Pheromonal regulation of development in *Saccharomyces cerevisiae*. In *The molecular biology of the yeast* Saccharomyces: *Life cycle and inheritance* (ed. J.N. Strathern *et al.*), p. 143. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Urlinger, S., Kuchler, K., Meyer, T.H., Uebel, S. and Tampė, R. 1996. Intracellular location, complex formation, and function of the transporter associated with antigen processing in yeast. *Eur. J. Biochem.* **245**:266-272.

Valls, L.A., Nither, J.R. and Stephens, T.H. 1990. Yeast carboxypeptidase-Y vacuolar targetting signal is defined by 4 pro-peptide amino-acids. *J. Cell. Biol.* **111**:361-368.

Vernet, T., Dignard, D. and Thomas, D.Y. 1987. A family of yeast expression vectors containing the phage f1 intergenic region. *GENE.* **52**:225-233.

Vida, T.A., Huyer, G. and Emr, S.D. 1993. Yeast vacuolar proenzymes are sorted in the late Golgi complex and transported to the vacuole via prevacuolar endosome-like compartments. *J. Cell. Biol.* **121**:1245-1256.

Volkman,S.K., Cowman, A.F. and Wirth, D.F. 1995. Functional complementation of the *ste6* gene of *Saccharomyces cerevisiae* with the pfmdr1 gene of plasmodium falciparum. *Proc. Natl. Acad. Sci.* **92**:8927-8925.

Von Heijne, G. (1986) *Nucleic Acids Research.* **14**:4683-4690.

Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. 1982. Distantly related sequences in the a- and b-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO.J.* **1**:945-951.

Walter, P. and Jackson, A.E. 1994. Signal sequence recognition and protein targeting to the Endoplasmic Reticulum. *Annu. Rev. Cell. Biol.* **10**:87-119.

Wang, S-Y., Mariyama, Y., Mandel, M., Hulmes, J.D., Pan, Y-C.E., Danho, W. and Nelson, N. 1988. Cloning of cDNA encoding a 32kDa protein. An accessory

polypeptide of the H^+-ATPase from chromaffin granules. *J. Biol. Chem.* **263**:17683-17642

Waters, M.G., Evans, E.A. and Blobel, G. 1988. Prepro-α-factor has a cleavable signal sequence. *J. Biol. Chem.* **263**:6209-6214.

Wilson, K.L. and Herskowitz, I. 1984. Negative regulation of *STE6* gene expression by the a2 product of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**:2420-2427.

Wilson, K.L. and Herkowitz, I. 1986. Sequences upstream of the *STE6* gene required for its expression and regulation by the mating type locus in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*. **83**:2536-2540.

Woolford, C.A., Daniels, L.B., Park, F.J., Jones, E.W., Van Arsdell, J.N. and Innis, M.A. 1986. The *PEP4* gene encodes an aspartyl protease implicated in the posttranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. *Mol. Cell. Biol.* **6**:2500-2510.

Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. 1981 Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197-203.

Zhang, L. and Casey, P.J. 1996. Protein prenylation: molecular mechanisms and functional consequences. *Ann. Rev. Biochem.* **65**:241-269.

Zueco, J. and Boyd, A. 1992. Protein-A fusion vectors for use in combination with pEX vectors in the production of affinity purification of specific antibodies. *GENE*. **121**:181-182.