

# **APPROACHES TO THE STUDY OF PROTEIN SECRETION IN YEAST**

**Thesis Submitted for the degree  
of Doctor of Philosophy at the  
University of Leicester**

**by**

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## ABSTRACT.

### APPROACHES TO THE STUDY OF PROTEIN SECRETION IN YEAST.

**Bhupinder Gill**

Mutants of the yeast *Saccharomyces cerevisiae* that showed enhanced secretion yield of a mini-proinsulin reporter protein were isolated, following random mutagenesis, via a footprint assay screen. Three such mutants, exhibiting a 2-3 fold increase in secretion yield, were shown to contain recessive mutations in three separate chromosomal genes (*ESI1*, *ESI4* and *ESI9*). It was not possible to combine these mutations into a single strain as double heterozygous strains failed to produce viable spores. Surprisingly, all three mutants showed reduced levels of the reporter gene mRNA. Expression of a second secretory reporter, wheat  $\alpha$ -amylase, also showed altered secretory product yield and mRNA levels. However the pattern of effect was different to mini-proinsulin, indicating a specificity of action of the mutations. In contrast, cytoplasmic gene expression was unaffected, with the exception of minor codon containing heterologous genes in *esi9* cells, whose expression was blocked. Generally, the three mutants appear to enhance protein secretion by improving efficiency at the initial stage of the secretory pathway probably via the co-translation mechanism. The decreased level of mRNA may result from a direct linkage to the degradation of secretory mRNA, which occurs at an increased rate in the mutants, to its mode of translation.

A second set of mutants showing both enhanced and reduced secretion yield of the *Aspergillus niger*  $\beta$ -galactosidase secreted protein have also been isolated. Again a random mutagenesis approach was used, but this time it was coupled to visual plate assay. These mutants await further analysis.

The final part of this thesis concerns the attempted isolation of *SEC* gene homologues from *Arabidopsis thaliana*. Initially this was tried, using a functional complementation approach with *sec* mutants from *Saccharomyces cerevisiae* and two *A.thaliana* cDNA expression libraries, but no homologues were isolated. A second approach utilising the PCR and degenerate or homologous oligonucleotide primers, designed to the *SEC18* and *SEC4* genes respectively, was attempted. Use of the homologous oligonucleotides led to the isolation of possible *SEC4* homologous clones from one *A.thaliana* cDNA expression library but these clones were unable to complement a *sec4* mutant. It is possible that these represent a homologue that functions in a slightly different manner in the *A.thaliana* secretion pathway, as the gross secretion pathway is well conserved between species.

## ACKNOWLEDGEMENTS

The five years I have spent at H.M.Biocentre, although at times very frustrating, have been made very enjoyable as a result of the lads and lassies within the lab. A huge thank you therefore goes out to the following:

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'Thinking of doing a PhD?.....turn on, tune in and drop out.



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Appendix 1 BLITZ and BLAST Searches.

Appendix 2 Insulin Standard Curve.

Appendix 3 Maltose Standard Curve.

### ABBREVIATIONS

ATP	adenosine 5' triphosphate;
Amp <sup>r</sup>	ampicillin resistance;
bp	base pairs;
BSA	bovine serum albumin;
DNA	deoxyribonucleic acid;
dNTP	deoxyribonleotide triphosphate;
DTT	dithiothreitol;
ds	double stranded;
EDTA	diaminoethane tetra acetic acid;
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside;
kb	kilobase pairs;
kDa	kilodaltons;
mRNA	messenger RNA;
MOPS	3[N-morpholino]propane-sulphonic acid;
nt	nucleotide;
OD	optical density;
<i>ori</i>	origin of DNA replication;
PCR	polymerase chain reaction;
RNA	ribonucleic acid;
ss	single stranded;
SDS	sodium dodecyl sulphate;
Tris	tris (hydroxymethyl) aminomethane;
UAS	upstream activating sequence;
X-GAL	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside;

# Chapter One .

## Introduction

### 1.1 General Introduction.

The yeast *Saccharomyces cerevisiae* is a eukaryotic single celled organism that can exist in a stable haploid or diploid state, or as a polyploid or aneuploid. A haploid cell can be induced to exit cell cycle progression and undergo a series of changes to prepare it for mating. Mating occurs between two haploid cells of opposite mating types,  $\alpha$  and  $a$  to form a diploid. The mating type of the cell is determined by the *MAT* locus which has two alleles, *MAT $\alpha$*  and *MAT $a$* , specifying  $\alpha$ - and  $a$ -type cells respectively (reviewed by Herskowitz, 1981).

The *S.cerevisiae* genome is approximately 14 mega bases in length and has now been fully sequenced. Pulse-field gel electrophoresis was used to show that the genome consists of sixteen chromosomes (Carle and Olson, 1985; Carle and Olson, 1987; Link and Olson, 1991) . This value is supported by genetic data (Mortimer *et al.*, 1989), as well as by fluorescence and electron microscopy studies (Dresser and Giroux, 1988; Kuroiwa *et al.*, 1984) . The current genetic map of *S.cerevisiae* contains over 1000 markers.

Haploid *S.cerevisiae* has proved amenable to the isolation of genetic mutations, producing numerous strains that possess a number of non-lethal auxotrophic markers. The subsequent development of yeast transformation by DNA plasmids and fragments has made the organism particularly amenable to gene cloning and genetic engineering techniques. Thus mutants with identifiable phenotypes can be transformed with genomic DNA libraries to isolate the corresponding genes or suppressors which complement the defection.

*S.cerevisiae* has several properties which have established it as an important system for the expression of foreign proteins for research, industrial and medical use. It has already been utilised in brewing, baking and liquor production for many centuries, therefore establishing it as safe, and a wealth of knowledge is known abouts its biochemistry and the fermentation process. As a 'generally regarded as safe', or G.R.A.S. organism it is highly acceptable for the production of pharmaceutical proteins. In contrast *E.coli* has toxic cell wall pyrogens and mammalian cells may contain oncogenic or viral DNA, as well as prions, so that products from these organisms must be tested more extensively. Yeast can be grown rapidly on simple media and under controlled

conditions to high cell density. With its genetics being more advanced than any other eukaryote and a highly versatile DNA transformation system, it can be manipulated almost as readily as *E.coli*. As a eukaryote it has the same basic cellular processes, such as protein secretion and post translation modification activities, as higher eukaryotic organisms, thus making it a suitable host for the analysis of protein secretion and production of recombinant secreted proteins.

This Introduction covers a number of areas concerned with the yeast secretion pathway and the production of recombinant proteins. These include a general outline of the basic form of the pathway linked with a detailed account of some of the more important areas; the requirements for foreign gene expression in *S.cerevisiae*; the limitations of the secretion pathway and ways in which enhanced secreting mutants have been isolated; the selection of a reporter protein for the isolation of enhanced secretion mutants; and, finally the Aims of this Thesis.

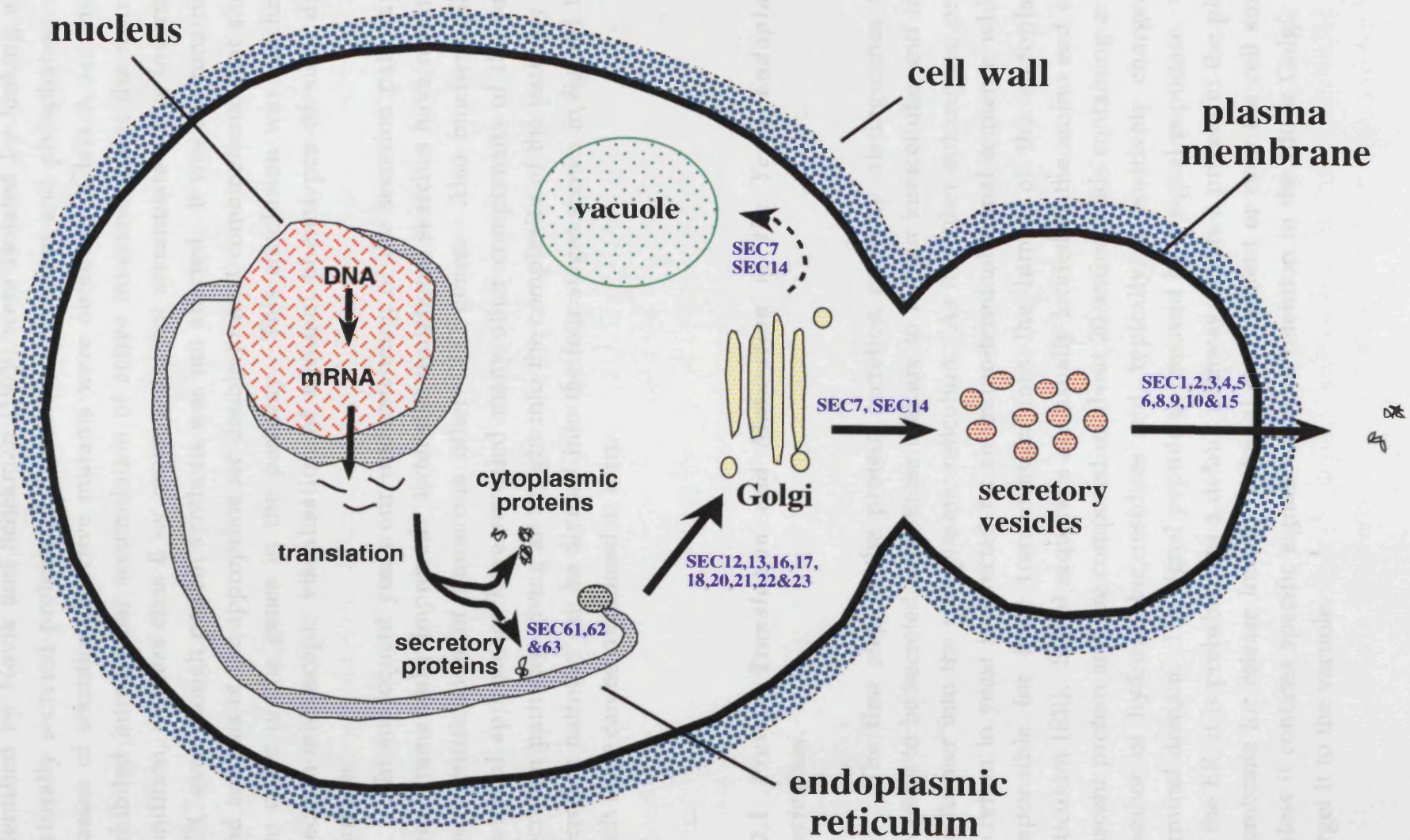
## 1.2 *The yeast secretion pathway.*

Yeast has played an important role in developing understanding of the eukaryotic protein secretion pathway at the molecular level. In particular, the micro-organism's genetic system has proved to be a particularly powerful approach to identifying genes whose products are involved in the pathway. This has been well exemplified by the isolation of the *SEC* gene mutants, encoding products essential for protein secretion (Schekman, 1985). These defined steps in the pathway and could be ordered in sequence (Novick *et al.*, 1981) All were temperature-sensitive mutants, indicating that a functional secretion pathway is essential for cell viability. Numerous mammalian, higher plant and other eukaryote homologues have since been isolated, demonstrating a high degree of conservation of pathway functions and the relevance of yeast as a model eukaryote.

The basic form of the secretory pathway and its direct analogy to that of mammalian cells, was provided by the work of Novick, Field, Ferro and Schekman in 1980, 1981, 1983 and 1985 (Novick, 1980; Novick, 1985; Novick *et al.*, 1981; Schekman *et al.*, 1983). As shown in figure1.1. secretory proteins follow the route:

**endoplasmic reticulum → Golgi body → vesicles → cell surface.**





**Figure 1.1** The *SEC* gene products originally isolated by Novick and Schekman (1981) and their ordered sequence in the secretion pathway.

Secreted proteins are exported from the cell primarily at the surface of growing buds (Novick, 1980). The original temperature sensitive *sec* mutants identified by Novik and Schekman (1979) were isolated by looking at two naturally secreted proteins of *S.cerevisiae*, invertase and phosphatase. Two classes of secretion-defective mutants were obtained. Class A *sec* mutants exhibited intracellular accumulation of active invertase under the restrictive condition, whereas class B *sec* mutants did not accumulate active invertase at 37°C even though protein synthesis was not affected. It was the construction and analysis of the appropriate *sec* double-mutant combinations, that showed the order of the genes in the pathway. This conclusion was reached by electron-microscopic visualisation of terminal phenotypes of such double mutants.

In subsequent years our understanding of the secretion pathway has been dramatically improved. Extensive mutational searches have resulted in the identification of numerous other *SEC* genes. Also biochemical and genetical approaches have revealed many other components of the protein secretion pathway, giving an insight into the complexity of the pathway. The secretion pathway can be divided into the four stages shown in figure 1.1, and each stage can be considered in turn.

### ***1.2.1 Protein Translation and Maturation in the Yeast Endoplasmic Reticulum.***

The first step in the protein secretion pathway distinguishes those proteins to be secreted from those that are to remain intracellular and inputs the former into the endoplasmic reticulum. As in higher eukaryotes, protein secretion in yeast is directed by an amino-terminal signal sequence which is responsible for their translocation into the lumen of the endoplasmic reticulum (ER). Signal sequences are usually located at the amino end of the nascent protein and are composed of about 20 amino acids containing a large number of hydrophobic residues and, typically, a positively charged N-terminal domain. The signal peptide is removed by a signal peptidase. Once in the ER, it is probable that a default pathway directs a protein to the plasma membrane for release into the external environment or onto the cell surface, unless it contains specific signals to cause retention in the ER or Golgi, or to target it to the vacuole.

Genetic selections for *S.cerevisiae* mutants exhibiting temperature-sensitive conditional defects in the targeting and translocation of secretory and membrane proteins (Deshaies and Schekman, 1989) have proved a powerful technique for identifying yeast proteins involved in these latter processes. Protein translocation in yeast can occur by both co-translation and post-translation mechanisms (Zimmerman, 1986). In vitro approaches involving chemical cross linking have provided new insights into the organisation of the ER protein-conducting channel and the interplay among its components. The figures 1.2. and 1.3. show the possible mechanisms of co-translational and post-translational targeting of secretory precursors to the endoplasmic reticulum (ER) membrane. These illustrate how various cytosolic and ER membrane-bound proteins may facilitate both targeting and translocation.

Ng *et al.*, (1996) have shown that preproteins fall into three distinct classes: Signal Recognition Particle (SRP)-dependent, SRP-independent, and those that can use both pathways. They have shown pathway specificity is conferred by the hydrophobic core of signal sequences. Analysing the hydrophobic cores of signal sequences Ng *et al.*, (1996), found that SRP-dependent substrates and those requiring both pathways carry signal sequences with significantly greater hydrophobicity than those of SRP-independent substrates. Thus, SRP-dependent substrates can use an SRP-independent pathway if SRP function is impaired or saturated. As cells are viable in the absence of SRP, every secretory pathway protein that is essential for cell growth must be able to use an SRP-independent route.

The co-and post-translational mechanisms in yeast cells differ chiefly in the steps preceding the insertion of the nascent polypeptide into the translocation apparatus. There are a number of cellular components involved in targeting for co-translation. Homologues of the mammalian 7SL RNA(scR1 RNA) and the 54KDa polypeptide subunit of the mammalian signal recognition particle (SRP54) have been identified in *S.cerevisiae* (Nunnari, 1992). The SEC65 gene, encoding the homologue of the 19KDa polypeptide subunit of the mammalian SRP((Nunnari, 1992; Sanders and Schekman, 1992) was identified using a genetic selection in *S.cerevisiae* for impaired assembly of integral membrane proteins into the ER. The *sec65<sup>ts</sup>* allele is conditionally lethal and displays a strong block of insertion into the ER of both secretory and membrane proteins, suggesting the Sec65p interacts with precursors and/or ER membrane components and at the restrictive temperature, impairs the translocation machinery.

The post-translational import mechanism is ATP-dependent and requires the cytosolic Ssa1p and Ssa2p, and members of the yeast protein hsp70

**Figure 1.2.** Model of co-translational secretory protein translocation into the yeast endoplasmic reticulum: components mediating polypeptide targeting, insertion and maturation.

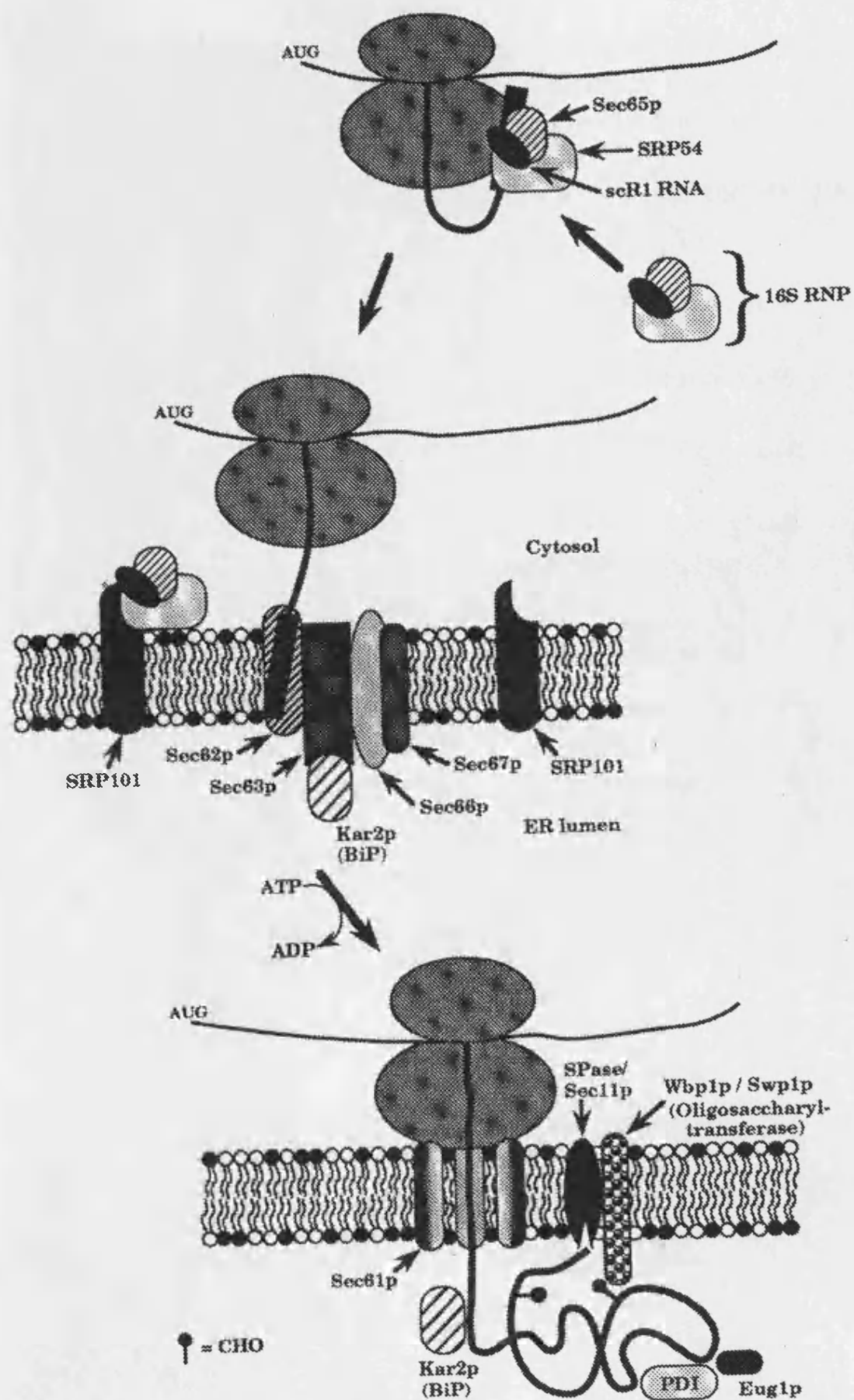
**KEY;**    **SRP54 & scRI RNA-** Homologues of the mammalian 7SL RNA and 54kDa polypeptide subunit of the mammalian signal recognition particle.

**SRP101-** Homologue of a subunit of the mammalian docking protein.

**PDI-** Protein disulphide isomerase.

**SPase-** Signal peptidase.

**Eug1p-** 65kDa protein functionally related to PDI.



**Figure 1.3.** Model of post-translational secretory protein translocation into the yeast endoplasmic reticulum: components mediating polypeptide targeting, insertion and maturation.

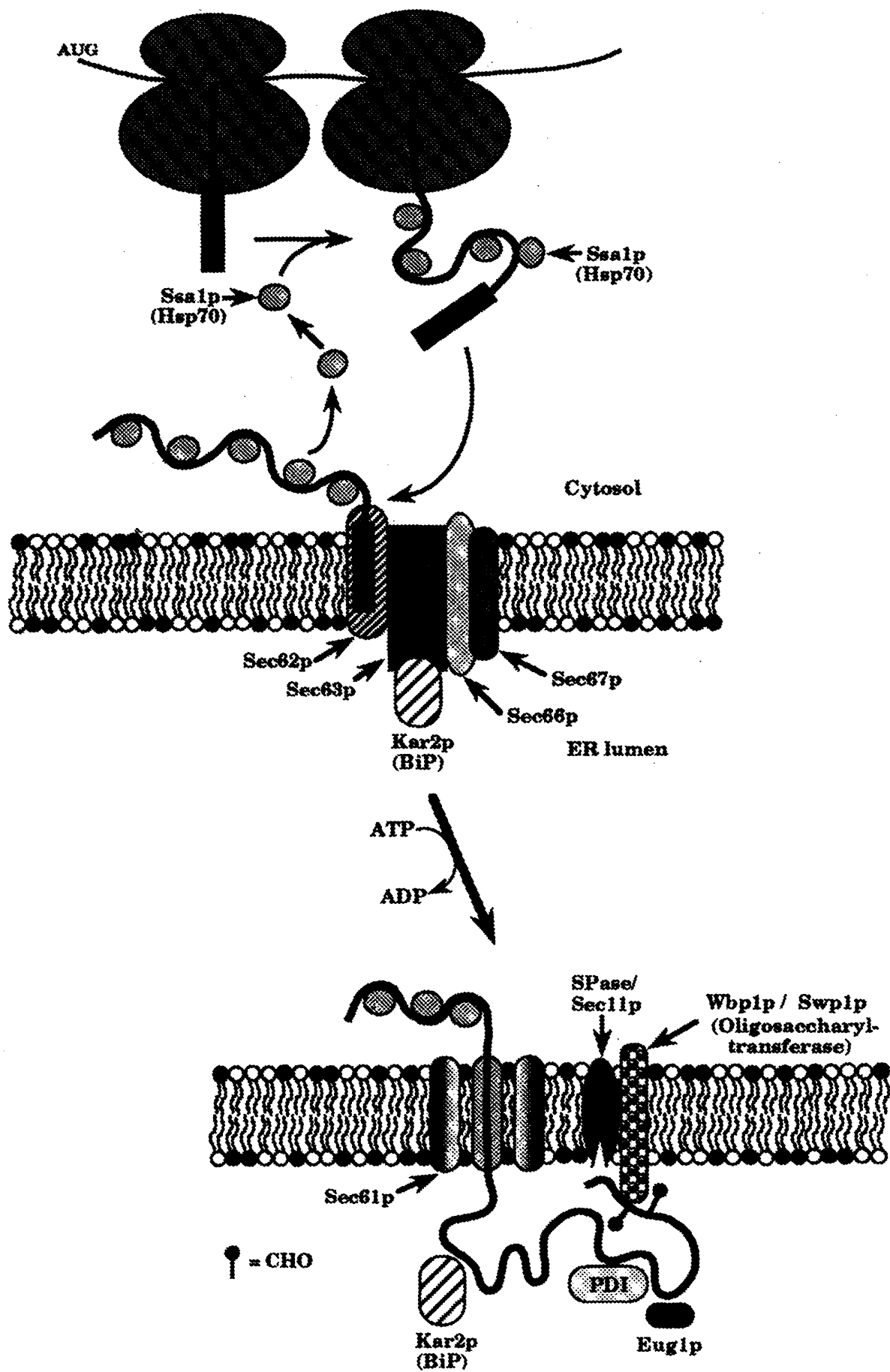
**KEY;** **SRP101**- Homologue of a subunit of the mammalian docking protein.

**PDI**- Protein disulphide isomerase.

**SPase**- Signal peptidase.

**Eug1p**- 65kDa protein functionally related to PDI.

**Ssa1p**- Member of a subfamily of the seventy kilodalton heat shock proteins (hsp70).



family (Gao *et al.*, 1991; Slater and Craig, 1989). These proteins are thought to bind nascent polypeptide chains and promote maintenance of a translocation-competent, unfolded conformation until precursor sorting and translocation into the ER are completed. This model does not preclude a role for yeast SRP and docking protein (DP) homologues in targeting to the ER, but it does suggest that other factors may allow nascent polypeptides to bypass the SRP-DP mediated pathway. Once the nascent chain has been targeted to the ER membrane the difference between the two mechanisms of translocation disappear.

Using a genetic selection strategy (Rothblatt *et al.*, 1989) that demanded cytosolic localisation of a modified histidinol dehydrogenase (the enzyme that catalyses the terminal step in histidine biosynthesis) whose normal cytosolic location had been altered by addition of a secretory signal sequence to its amino terminus, three genes coding for essential ER membrane-bound proteins were identified: *SEC61* (Stirling *et al.*, 1992), *SEC62* (Deshaies and Schekman, 1989) and *SEC63* (Stirling *et al.*, 1992). By selection for growth on minimal medium containing histidinol and screening mutants for pleiotrophic defects in secretion, it was possible to distinguish between two classes of mutants that arose as histidinol prototrophs; signal sequence mutations and mutations in the ER translocation machinery.

Evidence for interactions of Sec61p, Sec62p and Sec63p with each other, with other ER luminal (Kar2p) and membrane-bound proteins (Sec66p, Sec67p) and with translocating polypeptide intermediates, has been obtained by both genetic and biochemical analyses (Sanders and Schekman, 1992). A translocation intermediate of prepro- $\alpha$ -factor could be crosslinked to Sec61p in the presence of ATP, but this interaction was abrogated in microsomes from *sec62* or *sec63* mutant cells. These observations suggested that Sec62p and Sec63p act upstream of Sec61p for translocation across the ER membrane (Sanders and Schekman, 1992).

Kar2p, or yeast BiP (heavy chain binding protein) is required for the translocation of secretory precursors into the E.R (Normington *et al.*, 1989). Like mammalian BiP, yeast Kar2p is a resident luminal protein of the ER (Gething and Sambrook, 1992) and its function in translocation appears to be distinct and separable from that provided by cytosolic hsp70 (Brodsky and Schekman, 1993). The physical interaction of Kar2p and Sec63p has been demonstrated biochemically (Brodsky and Schekman, 1993), and is likely to occur via the luminal domain of Sec63p.

Increased synthesis of BiP is induced when abnormal proteins accumulate in the ER, and BiP preferentially associates with such proteins.



Many proteins fold poorly or aggregate when their glycosylation is inhibited, and in such cases association with BiP is frequently observed. Binding of BiP to these substrates is hydrophobic in nature, which suggests that they are recognised, at least in part, by the presence of exposed hydrophobic residues that are normally buried within the mature, properly folded protein. Although the main function of BiP is probably to promote protein assembly, it may serve to prevent export of misfolded proteins from the ER; such proteins remain associated with BiP until they either fold correctly or are degraded.

In yeast, proteolytic modification of precursors occurs concomitantly with their translocation across the ER membrane. Cleavage of N-terminal signal peptides is accomplished by the signal peptidase, which in yeast has been identified as the product of the *SEC11* gene (Sadler, 1989). Covalent addition of the N-linked carbohydrate side chains to the secreted protein, involves the assembly of dolichol-linked GlcNAc<sub>2</sub>-Man<sub>9</sub>-Glc<sub>3</sub> core oligosaccharide precursors in a series of reactions occurring on both sides of the ER membrane, followed by the transfer of the precursors from dolichol to appropriate asparagine residues on nascent polypeptides in the ER lumen (Herscovics and Orlean, 1993). A number of genes encoding enzymes involved in these processes have been identified. Amongst those required for assembly of the dolichol-linked precursors are the various *ALG* genes (Munoz *et al.*, 1994), as well as *SEC53*, (Feldman *et al.*, 1987) *SEC59* (Bernstein *et al.*, 1989) and *DPM1* (Orlean, 1992). The yeast oligosaccharyl transferase enzyme is a multi-subunit complex (Kelleher and Gilmore, 1994); one component Wbp1p, is the yeast homologue of the mammalian OST48 (Kelleher and Gilmore, 1994) transmembrane protein, a second component, Swp1p (Kelleher and Gilmore, 1994), is a 30KDa transmembrane protein that is also necessary for oligosaccharyl-transferase activity *in vivo* and *in vitro*.

Genetic and biochemical studies suggest that in addition to its role(s) in protein translocation, Kar2p/BiP is required for protein oligomerisation in the ER (Schonberger, 1991). The yeast protein disulphide isomerase (PDI) is known to catalyse disulphide bond formation in proteins entering the secretory pathway and is essential for yeast cell viability (Gunther, 1991). There are a number of other yeast homologues of mammalian ER proteins thought to be involved during protein folding that have been identified, including calnexin and the peptidyl prolyl isomerases FKB2 (Partaledis and Berlin, 1993) and cyclophilin (Frigerio and Pelham, 1993), but information as to their roles in the yeast ER is limited at present.

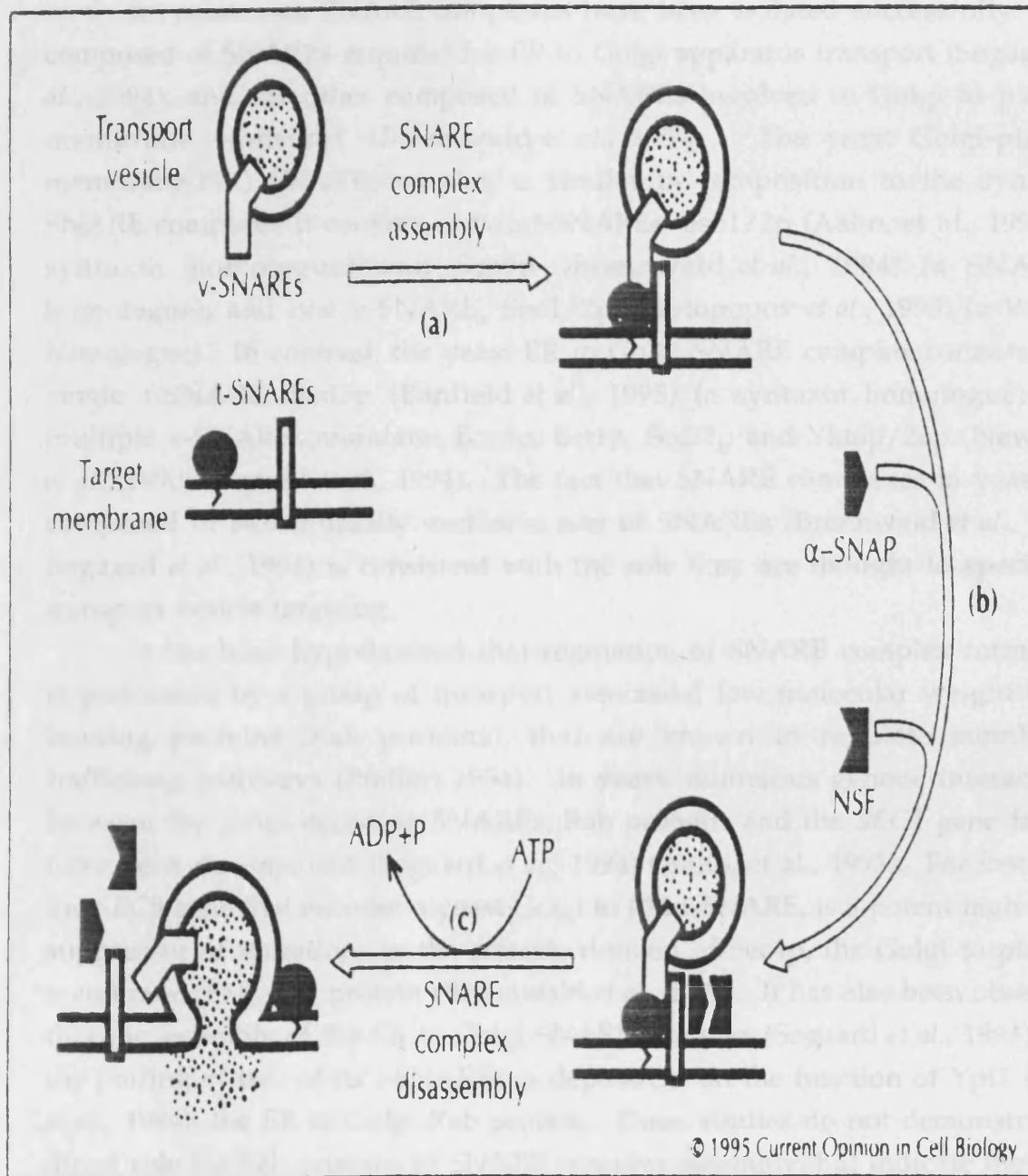
### **1.2.2 *Proteins common to transport from the ER to Golgi apparatus and through the Golgi apparatus to the cell surface.***

There are two sets of proteins involved in carrier vesicle formation (budding), targeting and fusion that are common both to the export of proteins from the ER to the *cis* Golgi network (CGN), which is the first compartment of the central Golgi apparatus, and to the transport of proteins through the Golgi apparatus to the cell surface. The first set of proteins are involved in the specificity of vesicle targeting (SNAREs) (Pelham *et al.*, 1995), these are the receptors for the soluble N-ethylmaleimide-sensitive attachment proteins (SNAPs). The second set of proteins are involved with vesicle budding and fusion (coat proteins) (Schekman and Orci, 1996).

#### **1.2.2.1 *SNAREs and the specificity of transport vesicle targeting.***

Several molecules participating in vesicle-mediated membrane trafficking pathways have been identified and characterised in recent years. Work in three areas has led to this rapid advancement: the genetic dissection of the secretory pathway in *S.cerevisiae*; *in vitro* characterisation of the requirements for membrane trafficking in animal cells; and the biochemical characterisation of synaptic vesicle membrane trafficking in neurones (Rothman and Warren, 1994) (Bennett and Scheller, 1994). A general model of vesicle targeting and fusion has been postulated. This is based on the characterisation of the assembly and disassembly of protein complexes specifically composed of synaptic membranes and soluble membrane trafficking factors (Bennett and Scheller, 1994; Sollner *et al.*, 1993a; Sollner *et al.*, 1993b). This model is represented in figure 1.4.

The model indicates that the specificity of vesicle targeting is generated by complexes that form between membrane proteins on the transport vesicle (known as v-SNAREs) and membrane proteins on the target membrane (known as t-SNAREs). The formation of the SNARE (SNAP receptor) complex produces a scaffold for the sequential recruitment of two general membrane trafficking factors, NSF (N-ethylmaleimide-sensitive factor) (Whiteheart *et al.*, 1994) and  $\alpha$ -SNAP (Clary *et al.*, 1990). NSF is an ATPase whose catalytic activity leads to the disruption of the SNARE complex,



**Figure 1.4.** Model of the proposed role of SNAREs in transport vesicle targeting and fusion. The assembly of a SNARE complex composed of v-SNAREs and t-SNAREs (a) specifically targets the transport vesicle to its appropriate fusion partner. This is followed by the binding of the general membrane trafficking factors  $\alpha$ -SNAP and NSF (b) and the disassembly of the SNARE complex induced by ATP hydrolysis (c).

Taken from Hasson & Mooseker, *Current Opinion in Cell Biology* 1995.

an event that may lead to membrane fusion (Whiteheart *et al.*, 1994)(Figure 1.4.).

In yeast, two SNARE complexes have been isolated successfully: one composed of SNAREs required for ER to Golgi apparatus transport (Sogaard *et al.*, 1994), and the other composed of SNAREs involved in Golgi to plasma membrane transport (Brennwald *et al.*, 1994). The yeast Golgi-plasma membrane (PM) SNARE complex is similar in composition to the synaptic SNARE complex. It consists of two t-SNAREs, Sso1/2p (Aalto, *et al.*, 1993) (a syntaxin homologue) and Sec9p (Brennwald *et al.*, 1994) (a SNAP-25 homologue), and one v-SNARE, Snc1/2p (Protopopov *et al.*, 1993) (a VAMP homologue). In contrast, the yeast ER to Golgi SNARE complex consists of a single t-SNARE, Sed5p (Banfield *et al.*, 1995) (a syntaxin homologue) and multiple v-SNARE candidates Bos1p, Bet1p, Sec22p and Ykt6p/26p (Newman *et al.*, 1990; Sogaard *et al.*, 1994). The fact that SNARE complexes in yeast are composed of two mutually exclusive sets of SNAREs (Brennwald *et al.*, 1994; Sogaard *et al.*, 1994) is consistent with the role they are thought to specify in transport vesicle targeting.

It has been hypothesised that regulation of SNARE complex formation is performed by a group of transport associated low molecular weight GTP-binding proteins (Rab proteins), that are known to regulate membrane trafficking pathways (Pfeffer, 1994). In yeast, numerous genetic interactions between the genes encoding SNAREs, Rab proteins and the *SEC1* gene family have been documented (Sogaard *et al.*, 1994) (Aalto, *et al.*, 1993). For instance, the *SEC9* gene that encodes a yeast Golgi to PM t-SNARE, is a potent high copy suppressor of mutations in the effector domain of Sec4p, the Golgi to plasma membrane (PM) Rab protein (Brennwald *et al.*, 1994). It has also been observed that the assembly of the ER to Golgi SNARE complex (Sogaard *et al.*, 1994) and the pairing of two of its t-SNAREs is dependent on the function of Ypt1 (Lian *et al.*, 1994), the ER to Golgi Rab protein. These studies do not demonstrate a direct role for Rab proteins in SNARE complex assembly, but indicate they are involved.

#### **1.2.2.2 Coat proteins and their role in vesicular transport.**

Electron microscope immunocytochemistry was used to follow the progress of newly synthesised proteins and to localise the Golgi compartments in which various post-translational modifications (mainly glycosylations) take place (Dunphy and Rothman, 1985). Proteins were found to leave the ER and cross the Golgi stack together. It was only during exit from the last, *trans*

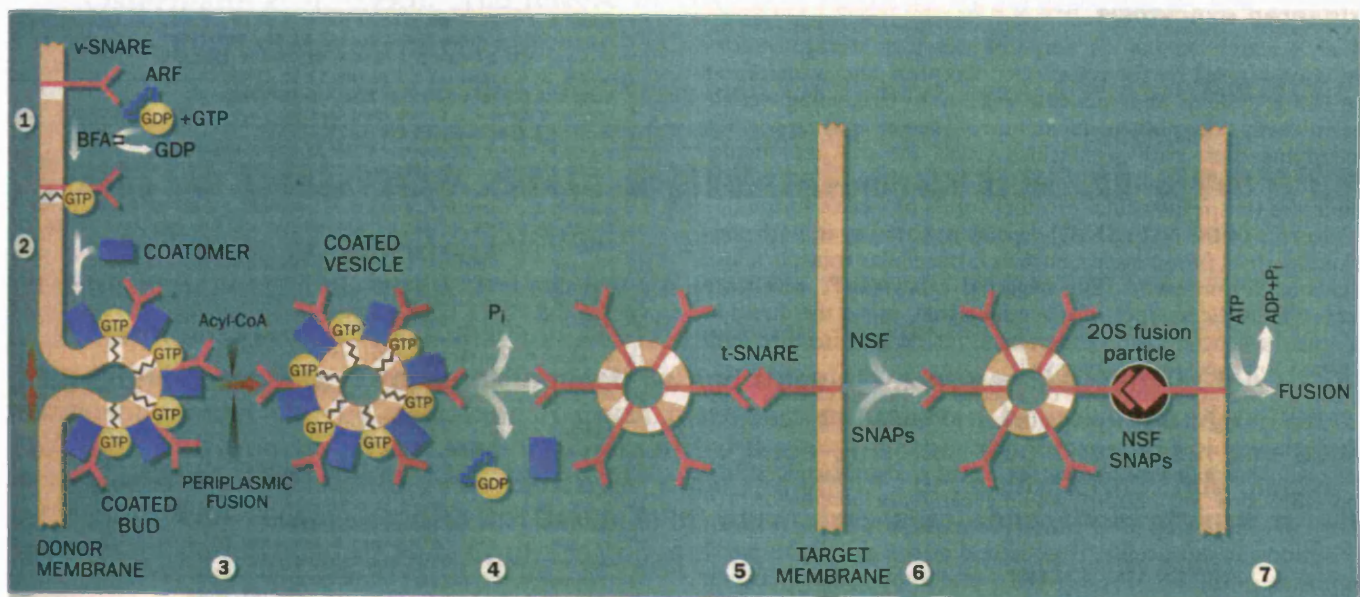
compartment that the various precursors were separated according to destination (Griffiths and Simons, 1986). Transport from the ER-to-Golgi and between each cisterna in the Golgi was found to be mediated via vesicles. Most of the fully formed vesicles and all of the vesicles still in the process of budding, had a distinct coat, which was ~18 nm thick, on their cytoplasmic surface (Orci *et al.*, 1986) and which resembled the clathrin coat of endocytic vesicles (Pearse and Robinson, 1990). The assembly of the coat from cytosolic precursors may drive budding (see figure 1.5).

Transport across the Golgi and from the ER-to-Golgi appears to employ these coated vesicles, which are termed COP (for coat protein)- coated vesicles (Orci *et al.*, 1993b). There are two sets of COP-coated vesicles that have been isolated, COPI (Bednarek *et al.*, 1995) and COPII (Barlowe *et al.*, 1994; Schekman *et al.*, 1994). The first set of proteins were originally isolated as mediating transport across the Golgi stack, although there is now considerable evidence (Lewis and Pelham, 1996) that COPI-coated vesicles can also mediate ER-to-Golgi transport. The COPII-coated vesicles have been shown to mediate ER-to-Golgi transport (Barlowe *et al.*, 1994).

The Golgi stack is specialised for protein processing and transport, and vesicles with the surface area of an entire cisterna pass between one cisterna and the next every few minutes. After coat assembly and budding the coat must be removed to allow fusion of the enclosed vesicle. The coat contains eight polypeptides; one is ADP-ribosylation factor (ARF) (Serafini *et al.*, 1991), a GTP-binding protein found mainly as a monomer in the cytosol (Kahn and Gilman, 1986). The other seven (termed COPs) are associated in an equimolar coat protomer (coatomer) complex (Waters *et al.*, 1991). Coatomer is mainly cytosolic like ARF, and therefore they co-assemble on the Golgi surface to form vesicles ; their later release constitutes uncoating (Ostermann *et al.*, 1993).

The physico-chemical properties of ARF and its presence in the coat suggested how GTP binding and hydrolysis might trigger budding and uncoating (Serafini *et al.*, 1991). ARF is N-myristylated and its GDP-bound form is water-soluble; the GTP-bound form, however, is inserted into membranes in a myristic acid-dependent manner (Kahn *et al.*, 1991). Membrane insertion appears to be modulated by the GDP-GTP conformational switch, via controlling exposure of the covalently attached fatty acid. Bud assembly is achieved by coatmer recruitment from the cytosol by the Golgi-bound ARF-GTP, with cytosolic ARF-GDP being activated for budding via GTP-GDP exchange at the Golgi surface (Serafini *et al.*, 1991). ARF is therefore required to initiate budding by providing binding sites for coatmer. Coatmer, ARF and GTP create buds on Golgi membranes that are converted to vesicles

**Figure 1.5.** Outline of steps in a round of transport.



**Step 1:** Coat assembly is initiated when a small GTP-binding protein is activated by binding GTP.

**Step 2:** The GTP-binding protein binds its receptor and recruits coat proteins, thus forming the coated bud.

**Step 3:** The bud pinches off in a distinct process involving periplasmic fusion.

**Step 4:** And the coat later disassembles following hydrolysis of the bound GTP.

**Step 5:** During budding, transport vesicles are tagged with v-SNAREs, which pair with t-SNAREs in the target membrane.

**Step 6:** The general fusion machinery then assembles on this scaffold. This consists of cytoplasmic proteins that are readily available to all cellular membranes, including the ATPase NSF and the SNAP proteins.

**Step 7:** SNAPs bind to the SNARE complex, enabling NSF to bind. ATP hydrolysis by NSF is essential for fusion.

Taken from Rothman, Nature 1994.

when palmitoyl coenzyme A is included in the incubation (Orci *et al.*, 1993a; Ostermann *et al.*, 1993). The release of ARF and coatmer by hydrolysis of the bound GTP induces uncoating.

A second set of COP-coated vesicles (COPII) have been discovered in *S.cerevisiae* (Barlowe *et al.*, 1994) which can mediate ER-to-Golgi transport *in vitro* and *in vivo*. The budding mechanism resembles that of COP-coated vesicles. Sar1p, a small GTP-binding protein similar to ARF (Nakano and Muramatsu, 1989), is recruited to the ER where a transient interaction with the cytosolically exposed domain of Sec12p converts Sar1p-GDP to the activated GTP-bound form (Yoshihisa *et al.*, 1993). Sar1p plays a role analogous to that of ARF and a complex of other polypeptides, Sec23p/Sec24p and Sec13p/p150 (150K protein) form the coat (Barlowe *et al.*, 1994). Sec23p is a GTPase-activating protein (GAP) for Sar1p (Yoshihisa *et al.*, 1993), and could ensure that uncoating follows budding since it only comes into contact with Sar1p-GTP once the coat is assembled. Once the coat is shed the targeting proteins (v-SNAREs) on the transport vesicle (Sec22p, Bos1p and Bet1p (Rexach *et al.*, 1994) ) become exposed and provide part of the binding selectivity to ensure that vesicles interact with targeting proteins, such as Sed5 (Sollner *et al.*, 1993b), on the Golgi membrane.

It is thought that the COPI may mediate the budding of Golgi -to-ER retrograde transport vesicles (Lewis and Pelham, 1996). Yeast and mammalian COPI binds to the carboxyl-terminal KKXX motif found on certain ER resident membrane proteins, allowing their return after escape to the Golgi.

### 1.2.3 ER to Golgi Transport,

Export of protein from the ER involves the sequential formation (budding), targeting and fusion of carrier vesicles to the *cis* Golgi network (CGN), which is the first compartment of the central Golgi apparatus (Dunphy and Rothman, 1985). The majority of proteins studied to date, originally identified by biochemical and genetical analysis (Pryer *et al.*, 1992; Rothman and Orci, 1992), participate in transport in the context of macromolecular protein complexes. Interactions between the proteins involved are extensive and may occur during synthesis, recycling or during the differential steps involved in carrier vesicle budding, targeting and fusion.

Using both molecular genetical analyses and biochemical assays two different sets of interacting components have been detected, which reconstitute transport between the ER and Golgi (Pryer *et al.*, 1992; Rothman



and Orci, 1992). The first set are those components required for export (budding) from the ER which lead to the formation of carrier vesicles. The second set are proteins involved in either vesicle targeting and/or fusion to the Central Golgi Network. This distinction can be confusing as it is not possible to determine between when a protein is recruited and when it actually functions; i.e. a protein may be essential for maturation of a protein "scaffold" triggering vesicle fission, as well as being involved in targeting and/or fusion.

Another set of key components involved in ER to Golgi transport are the GTP-binding proteins (Pfeffer, 1994). Maturation of protein between the ER and CGN requires at least six GTP-binding proteins. Sar1, Arf and Rab/Ypt1 are members of the Ras superfamily of small GTP binding proteins (Pfeffer, 1994). This superfamily of proteins are now recognised to be critical in regulating many aspects of membrane and cytoskeletal function (Novick and Brennwald, 1993; Schwaninger *et al.*, 1992). These proteins act as "molecular switches" whose conformations are regulated by a cycle of GDP/GTP exchange and hydrolysis. Sequential interactions between other components of the transport machinery are regulated by conformational changes in these proteins. It is anticipated that additional effector proteins, including GTPase activating (GAPs) and guanine-nucleotide exchange factors (GEFs) are integral components of the transport machinery involved in GTP-binding protein function or recycling, since purified GTP-binding proteins both hydrolyse and exchange guanine nucleotides inefficiently.

Why should so many interacting proteins be required for transport between the ER and Golgi ? The recruitment and potential concentration of protein at the export site (the first step in transport) is likely to be a critical event in initiating flow of protein through the rest of the secretory pathway. Whether export from the ER is via a "bulk" (non-selective) (Pfeffer and Rothman, 1987) or "gated" (selective)(Schwaninger *et al.*, 1992) mechanism remains to be determined. It is essential to build a "machine" that will physically segregate lipid and protein at the export site from the bulk of the ER membrane. This "fission machine" is probably linked to the quality control machinery, that ensures that only correctly folded and properly assembled proteins exit the ER, and is likely to comprise both regulatory and structural components.

Current evidence suggests that the first step(s) in vesicle budding requires the participation of the GTP-binding proteins Sar1, Arf and Rab1/Ypt1. Sar1p has strong biochemical and genetic interactions with Sec12p, Sec23p and Sec24p, where Sec23p is the Sar1p GAP (Yoshihisa *et al.*, 1993) and Sec12p a Sar1p specific GEF (Barlowe and Schekman, 1993). It is thought that



Sar1p may be involved in the selection of a vesicle budding site as it is not detected on transport vesicles. The small-GTP-binding proteins Rab1/Ypt1 and Arf on the other hand are likely to be essential for different aspects related to the recruitment and/or assembly of the coat protomer complex to the budding site. This coat-forming complex was first found in mammalian cells. The Sec21p is a subunit of a similar protein complex found in yeast cells (Stenbeck *et al.*, 1992).

When approaching the targeting/fusion end of this step in protein secretion another complex is identifiable. This consists of the ATP-binding protein NSF (Whiteheart *et al.*, 1994), which functions in the context of a biochemical complex containing the SNAPS (soluble NSF attachment protein),  $\alpha$ ,  $\beta$  and  $\gamma$  (Clary *et al.*, 1990). This complex is thought to be associated with vesicles via an integral membrane protein, the SNAP receptor (SNARE). It has been postulated that NSF is a component of a multi-subunit protein complex dictating transport vesicle formation. The yeast gene homologues to  $\alpha$ -SNAP and NSF have been identified as *SEC17* (Griff *et al.*, 1992) and *SEC18* (Eakle *et al.*, 1988) which act at a late step in transport, lending further support to the idea that NSF may be involved in targeting or fusion. There is strong genetic evidence that the yeast Ypt1 protein participates in targeting or fusion, being recruited during vesicle formation as part of a coat complex, with subsequent GTP hydrolysis serving to regulate a later event in trafficking (Bacon *et al.*, 1989).

There are a number of other gene products having homology to previously identified mammalian proteins, that also interact with complexes involved in fission and fusion. For example Bet1p (Sogaard *et al.*, 1994) has homology with synaptobrevin (a component of mature neurosecretory vesicles) indicating that this protein may participate in vesicle targeting and fusion, even though it is recruited during vesicle synthesis. The yeast homologue of one of the components of mammalian geranylgeranyl transferase is the protein Bet2, which is essential for the addition of isoprene groups to both Ypt1p and Sec4p (Rossi *et al.*, 1991). This addition is critical for both membrane association and function.

The last set of proteins isolated in the ER to Golgi flow of secretory proteins, are involved in the retention of proteins within the ER and the retrieval of proteins from the Golgi back to the ER (Pelham, 1989). The importance of retention/retrieval signals such as the "HDEL" sequence found on many yeast ER proteins, and the "KDEL" sequence found on many mammalian ER proteins, in the function of the early secretory pathway is largely known. It is becoming clear that resident ER proteins may recycle

between the ER and CGN (Dean and Pelham, 1990). There are a number of proteins that may play an important role in retrieval and/or the stabilisation of both the ER and Golgi structures. These include proteins such as Erd2, Sed4, Sed5 and Sec20 in yeast (Gimeno *et al.*, 1995; Hardwick and Pelham, 1992; Lewis and Pelham, 1996; Lewis *et al.*, 1990; Sweet and Pelham, 1992).

There are a number of proteins, isolated on the basis of their role in ER to Golgi transport, that are also required for inter-Golgi transport and in some cases, later steps. This group includes at least four proteins; Sec7,  $\beta$ -COP/Sec21, Rab1/Ypt1 and NSF/Sec18.

#### **1.2.4 Transport through the Golgi apparatus and to the cell surface.**

Three main approaches have been used to isolate components of the secretory machinery required for vesicular traffic through the Golgi apparatus and to the cell surface. One approach has been the genetic dissection of vesicular transport in yeast. This has yielded many genes whose products are required for different stages of the secretory pathway (Pryer *et al.*, 1992). A second approach from a biochemical point, involved the reconstitution of vesicular traffic. This enabled the purification of a number of proteins required for transport through the Golgi (Rothman and Orci, 1992) and for the calcium triggered fusion of secretory granules with the plasma membrane (Hay, 1992). A third approach utilises the purification of vesicular carriers and the isolation of proteins associated with them. Peripherally associated coat proteins (Duden *et al.*, 1991) and membrane spanning components of the vesicular carriers (Decamilli *et al.*, 1990) have been identified using this approach.

##### **1.2.4.1 Transport through the Golgi apparatus.**

The Golgi apparatus and the closely associated trans-Golgi network (TGN) are the site of extensive modification of transported proteins and lipids as well as a key "way-station" for intracellular membrane traffic (Farquhar, 1985). Proteins undergo covalent modifications including the addition and trimming of carbohydrate and the proteolytic processing of precursors. Many of the modifying enzymes have already been identified and localised to unique compartments of the Golgi or TGN. It is apparent that the Golgi network and TGN must contain an elaborate array of machinery as a wide range of

transported molecules are appropriately modified and correctly sorted into distinct vesicle carriers within this organelle. The Golgi apparatus has been sub-divided into three distinct compartments: cis, medial and trans Golgi and the TGN (Dunphy and Rothman, 1985; Griffiths and Simons, 1986).

As with transport through from the ER to the Golgi, transport through the Golgi apparatus requires the ATP-binding protein NSF/sec18 and its associated SNAPs, as well as the GTP binding protein ARF that is associated with coatamer (see section 1.2.2.).

Analysis of yeast secretory mutants has yielded several that are predominantly blocked in transport through the Golgi apparatus. These define two complementation groups, *SEC7* (Achstetter *et al.*, 1988) and *SEC14* (Bankaitis *et al.*, 1989). The *SEC7* gene encodes a large protein associated with carrier vesicles (Franzusoff *et al.*, 1992) that is thought to play a role in the coating of transport vesicles, similar to clathrin function in endocytosis. *SEC14* encodes a phosphatidylinositol transfer protein (Cleves *et al.* 1991) that is associated with the cytoplasmic face of Golgi membranes in vivo (Decamilli and Jahn, 1990). The current view is that Sec14p plays a direct and essential role in stimulating Golgi secretory function by maintaining an appropriate phospholipid composition in yeast Golgi membranes (Decamilli and Jahn, 1990), reflecting the importance of lipid composition to the function of the yeast Golgi.

#### **1.2.4.1.1 *KEX1*, *KEX2* and *STE13* proteases.**

The *KEX1* gene encodes a serine carboxypeptidase (kex1p) responsible for processing the carboxy terminus of secreted proteins such as  $\alpha$ -factor and K1 killer toxin (Cooper and Bussey, 1989; Dmochowska *et al.*, 1987).  $\alpha$ -factor and K1 killer toxin are produced as precursors that are endoproteolytically cleaved at dibasic sequences to release a peptide or protein with a carboxy-terminal extension of two basic residues, which are subsequently removed by Kex1p. It is now known that Kex1p (Bussey, 1988; Fuller *et al.*, 1988) is a membrane spanning protein that resides in a late Golgi compartment and is retained there by interactions that involve its cytoplasmically exposed domain (Cooper and Bussey, 1989; Cooper and Bussey, 1992).

Cleavage at the carboxyl side of Lys-Arg and Arg-Arg sequences, within polypeptide precursors of a-mating pheromone and the M1-encoded killer toxin, is performed by *KEX2* (Dmochowska *et al.*, 1987). The *kex2p* is a transmembrane,  $\text{Ca}^{2+}$  dependent serine protease of the subtilisin superfamily

and resides in a late compartment of the yeast Golgi complex (Franzusoff *et al.*, 1991; Redding *et al.*, 1991). The retention of the enzyme in this late compartment requires its cytoplasmic tail sequence and a functional clathrin heavy chain (Fuller *et al.*, 1989; Payne and Schekman, 1989).

The *STE13* protease encodes a Golgi-localised, membrane-bound dipeptidyl aminopeptidase (DPAP A) that is required for the final and rate-limiting step in the processing of the mating pheromone,  $\alpha$ -factor (Dmochowska *et al.*, 1987). DPAP A attacks the free N-terminus generated by kex2 protease cleavage and removes the -X-Ala- repeats so exposed (Julius *et al.*, 1983). Both DPAP A and Kex2p are dependant on the *CHC1* (clathrin heavy chain) and *VPS1* (vacuolar protein sorting) genes for proper localisation in the Golgi.

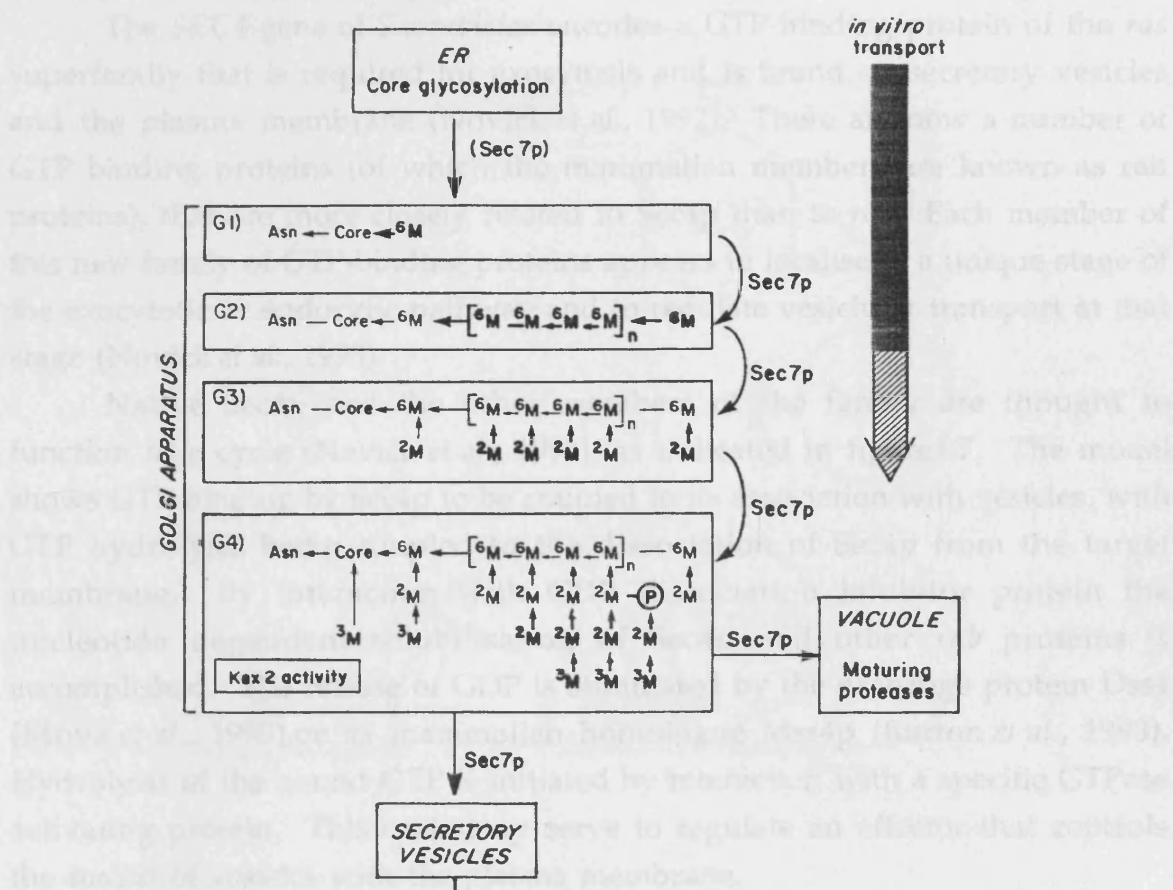
#### 1.2.4.1.2 Glycosylation in the Golgi apparatus.

It has been shown that the oligosaccharide modifications experienced by yeast glycoproteins in transit through the Golgi complex are executed in a sequential and biochemically separable manner. This supports the notion that the yeast Golgi complex possesses a functional compartmentalisation akin to that in mammalian Golgi complexes. Although core glycosylation (in the ER) in *S.cerevisiae* is identical in composition to that of mammalian cells (Herscovics and Orlean, 1993), outer chain glycosylation is considerably different. Modification of N-linked oligosaccharides proceeds in sequential fashion on proteins in transit through the secretory pathway. Outer chain carbohydrates in yeast, unlike complex oligosaccharides in mammalian cells, are composed principally of mannoses added in specific linkages by different mannosyl transferases. Mannose residues are added to core oligosaccharide units in  $\alpha$ 1-6,  $\alpha$ 1-2 and  $\alpha$ 1-3 carbon linkages, including the attachment of mannanose-phosphate in diester linkage (see Zhang *et al.*, 1982 for review).

In more recent times Franzsoff and Schekman (1989) have carried out work which suggests that the yeast Golgi apparatus does indeed consist of compartments that specialise in assembly of different aspects of the outer chain, and that Sec7p facilitates protein transport from one membrane to the next. In conjunction with their work Franzsoff and Schekman (1989) have proposed a general model for biochemical compartmentation of the yeast Golgi complex-associated mannosyltransferase activities. This model is shown in figure 1.6.

### 1.3.4.2 Transport to the cell surface.

The final stage of the secretory pathway requires the fusion of secretory vesicles with the plasma membrane. So, for yeast mutants defective in the final stage of secretion, the genes involved are thought to be products of those genes are required for the transport of the pathway, but not for earlier steps.



**Figure 1.6.** Model for the organisation of the yeast Golgi apparatus. The yeast Golgi apparatus is depicted as a series of functionally distinct compartments in which sequential aspects of the outer chain carbohydrate are assembled on glycoproteins as they move from one location to the next. One or more functional units may be contained within a single Golgi cisterna. Activities of enzymes suggested to reside in the compartments are marked by bold type. Each transfer event is mediated directly or indirectly by the Sec7 protein.

Taken from Franzusoff and Schekman 1989 in the EMBO Journal.

#### 1.2.4.2 Transport to the cell surface.

The final stage of the secretory pathway requires the fusion of secretory vesicles with the plasma membrane. So far yeast mutants, defective in the final stage of export, define ten complementation groups. The products of these genes are required for this stage of the pathway, but not for earlier steps.

The *SEC4* gene of *S.cerevisiae* encodes a GTP-binding protein of the *ras* superfamily that is required for exocytosis and is found on secretory vesicles and the plasma membrane (Novick *et al.*, 1992). There are now a number of GTP binding proteins (of which the mammalian members are known as *rab* proteins), that are more closely related to Sec4p than to *ras*. Each member of this new family of GTP-binding proteins appears to localise to a unique stage of the exocytotic or endocytic pathway and to regulate vesicular transport at that stage (Novick *et al.*, 1993).

Native Sec4p and the other members of the family are thought to function in a cycle (Novick *et al.*, 1993), as indicated in figure 1.7. The model shows GTP binding by Sec4p to be coupled to its association with vesicles, with GTP hydrolysis being coupled to the dissociation of Sec4p from the target membrane. By interaction with GDP dissociation inhibitor protein the nucleotide dependent solubilisation of Sec4p and other *rab* proteins is accomplished. The release of GDP is stimulated by the exchange protein Dss4 (Moya *et al.*, 1993), or its mammalian homologue Mss4p (Burton *et al.*, 1993). Hydrolysis of the bound GTP is initiated by interaction with a specific GTPase activating protein. This cycle may serve to regulate an effector that controls the fusion of vesicles with the plasma membrane.

#### 1.2.5 Membrane traffic to the yeast vacuole.

The two major pathways to the lysosome/vacuole are the endocytic pathway from the plasma membrane and the biosynthetic pathway from the Golgi complex (Kornfeld and Mellman, 1989). I shall only be considering the latter process. Newly synthesised vacuolar proteins are sorted from cell surface and secreted proteins in a late Golgi compartment and subsequently transported to the vacuole. A vast number of genes (over 65) required for vacuolar protein sorting and vacuolar biogenesis have been identified in genetic screens. These genes were isolated by utilising genetic screens for the identification of yeast mutants with lowered levels of vacuolar proteases (*vps*)

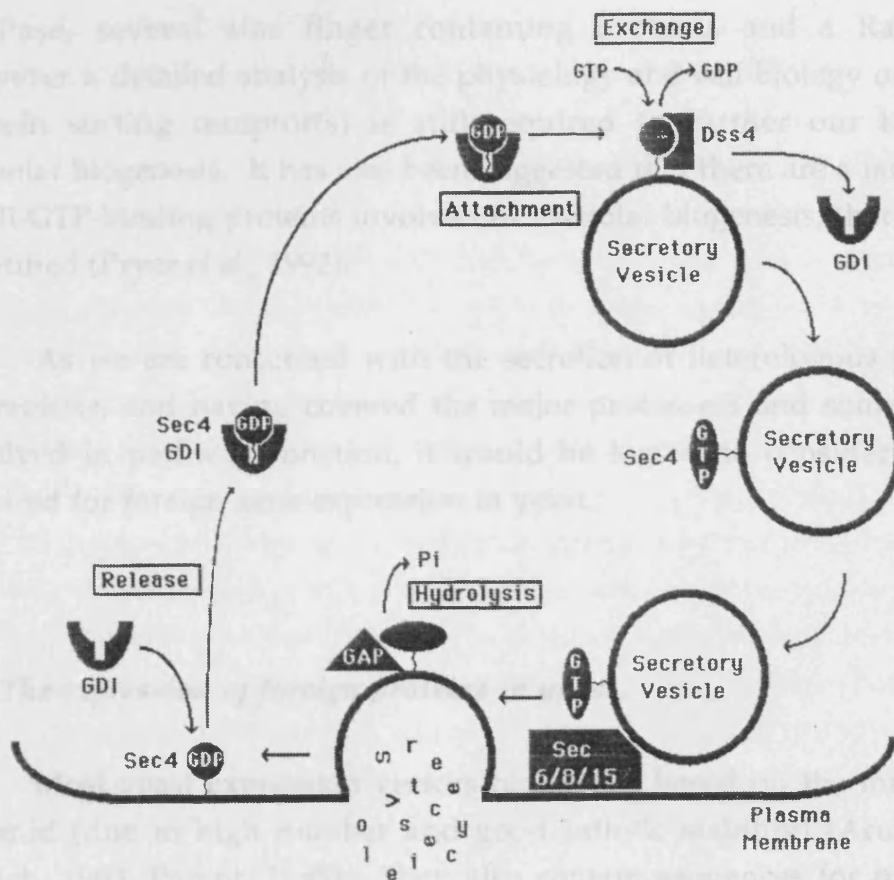


Figure 1.7. Model of the cyclical function of *ras* GTP binding protein Sec4.

KEY: GDI- GDP dissociation inhibitor  
 GAP- GTPase activating protein.  
 Pi- Inorganic phosphate.

Taken from a Guide book to the Secretory Pathway 1994.

(Raymond *et al.*, 1992), mutants with altered vacuolar morphology (*vam*) (Wada *et al.*, 1992), mutants with increased sensitivity to calcium (*cls*) (Ohya *et al.*, 1991), mutants defective for vacuolar acidification (*vma/vph*) (Preston *et al.*, 1992) and mutants defective for vacuolar inheritance (*vac*) (Shaw and Wickner, 1991).

This multitude of genes encode proteins including two kinases, a H<sup>+</sup>-ATPase, several zinc finger containing proteins and a Rab5 homolog. However a detailed analysis of the physiology and cell biology of the vacuolar protein sorting receptor(s) is still required to further our knowledge of vacuolar biogenesis. It has also been suggested that there are a large number of small GTP-binding proteins involved in vacuolar biogenesis, that are still to be identified (Pryer *et al.*, 1992).

As we are concerned with the secretion of heterologous proteins from *S.cerevisiae*, and having covered the major processes and some of the genes involved in pathway function, it would be logical to consider now what is required for foreign gene expression in yeast.

### **1.3 The expression of foreign proteins in yeast.**

Most yeast expression vectors have been based on the multi-copy 2 $\mu$ m plasmid (due to high number and good mitotic stability) (Armstrong, 1989; Broach, 1983; Parent, 1985). They also contain sequences for propagation in *E.coli* and in yeast, as well as a yeast promoter and terminator for efficient transcription of the foreign gene. Insertion of a foreign gene into an expression vector does not guarantee a high yield of the foreign protein, as gene expression is a complex multi-step process and problems can arise at numerous stages, from transcription through to protein stability. Despite increased understanding of the process of secretion, the greatest success in improving yields in recent years has been with a classical random mutagenesis approach. Also a number of other yeasts have become important host organisms for foreign gene expression because of their advantages in promoter strength, secretion efficiency, or ease of growth to high cell density. Consequently some of these will be chosen in preference to *S.cerevisiae*.



### 1.3.1 *2μm-based vectors.*

The 2μm is a 6.3kb circular double stranded plasmid present in most *S.cerevisiae* strains at about 100 copies per haploid genome (Futcher, 1988; Murray, 1987; Volkert *et al.*, 1989). The plasmid encodes four genes : *FLP* (or *A*), *REP1* (or *B*), *REP2* (or *C*) and *RAF* (or *D*.) In addition the 2μm contains an origin of replication (ORI which behaves as a typical ARS element), the *STB* locus (required *in cis* for stabilisation), and two 599 bp inverted repeat sequences. *FLP* encodes a site-specific recombinase which promotes recombination between the *FLP* recombination targets (FRT) within the inverted repeats, so that cells contain two forms of 2μm, A and B.

The 2μm is stably inherited, despite the fact that it confers no known phenotype, and indeed may be slightly disadvantageous to the host cell. Having the *STB* locus *in cis* together with the *REP1* and *REP2* gene products is required for efficient segregation. The host regulation which restricts each replication origin to one initiation per cell is overcome and appears to depend on the inverted repeat sequences and the *FLP* gene product. *FLP* promotes recombination between replicated and unreplicated inverted repeat sequences so that a double rolling circle is produced where the two replication forks can follow each other around the molecule. Such a mode of replication allows the production of multiple copies from a single initiation event (Futcher, 1988).

In 2μm<sup>+</sup> host strains, simple 2μm vectors that contain just the 2μm *ORI-STB*, a yeast selectable marker and bacterial plasmid sequences, can be used; the host strain supplies the *REP1* and *REP2* proteins in trans (Kikuchi, 1983). These *ORI-STB* expression vectors are the most convenient to use routinely in the laboratory due to their small size and ease of manipulation. The majority of plasmids used in this study are based on these simpler 2μm plasmids. Such vectors are tenfold more stable than autonomous replicating sequence (ARS) containing plasmids, and are present in 10-40 copies per cell.

### 1.3.2 *Transcriptional Promoters and Terminators.*

It has long been established that yeast promoters and terminators are essential for the efficient transcription of foreign genes. Yeast promoters consist of at least three elements which regulate the efficiency and accuracy of initiation of transcription (Struhl, 1989): upstream activation sequences (UASs), TATA elements and initiator elements. Yeast promoters may be highly complex, extending over 500bp, and containing multiple TATA elements

associated with different initiation sites (Struhl, 1989). Glycolytic promoters seem to be the most powerful *S.cerevisiae* promoters and are induced by glucose. The *ADH1* (alcohol dehydrogenase I) (Hitzeman *et al.*, 1983) and *PGK* (phosphoglycerate kinase ) (Hitzeman *et al.*, 1983; Tuite *et al.*, 1982) genes are examples of strong glycolytic promoters. As the glycolytic enzymes form approximately 50% of total cell protein, so their promoters appear to be strong.

Yeast transcription terminators are usually present in expression vectors for efficient mRNA 3' end formation. Efficient termination is probably required for maximal expression (Zaret and Sherman, 1982). It appears that yeast mRNAs follow the same pattern of termination, processing and polyadenylation of pre-mRNA as higher eukaryotes. However in yeast these processes are tightly coupled and occur within a shorter distance, near the 3' end of the gene.

### 1.3.3 RNA stability.

The half lives of yeast mRNAs range from 1 to 100 min and can therefore have a profound effect on the steady state level of a protein product (Brown, 1989). The difference between stable and unstable mRNAs may be that the latter have destabilising elements (Brown *et al.*, 1988). Insertion of premature stop codons in yeast mRNAs has been shown to cause their destabilisation, suggesting that ribosome attachment may contribute to mRNA stability (Hoekema, *et al.*, 1987)(Hoekema *et al.*, 1987; Pelsy and Lacroute, 1984). However Santiago (Santiago *et al.*, 1987) found no obvious correlation between ribosome loading and stability for a number of mRNAs. As little is known about yeast mRNA degradation mechanisms, it is impossible to predict whether a foreign mRNA will be stable. The stability of several foreign mRNAs have been tested and it is thought that poor codon usage in foreign mRNA may cause ribosome stalling (Herrick *et al* 1988) and this ribosome stalling could result in less mRNA with heterologous genes compared with homologous genes (Herrick, 1990).

### 1.3.4 Codon usage and foreign gene expression.

The degeneracy of the genetic code allows a number of different codons to code for each individual amino acid. As in most organisms, yeast shows a bias towards the use of a certain subset of possible codons. This is especially

apparent with highly expressed genes and correlates with the relative abundance of corresponding tRNAs and aminoacyl-tRNA synthetases (Bennetzen and Hall, 1982) . As codon usage varies between organisms, it is likely that a foreign gene will show a different bias to that of yeast. This does not prevent expression, however, as yeast contains the tRNAs for all 'sense' codons. The significance of codon usage becomes apparent when high-level translation is required from abundant mRNAs. When Hoekema *et al.* (1987) changed 39% of the major codons to minor ones in the highly-expressed *PGK1* gene, the yield of PGK protein decreased 10-fold. Thus, optimal codon usage is required for a high translation rate (Hadfield *et al.*, 1993) .

### 1.3.5 *Initiation and elongation of translation.*

Translational efficiency can be influenced primarily by the rate of initiation (assuming no downstream rate-limiting steps), which in turn is affected by the structure of the 5' untranslated leader of the mRNA. Initiation in eukaryotes is thought to follow a scanning mechanism whereby the 40S ribosomal subunit plus co-factors (eIF2, eIF3, eIF4C, Met-tRNA and GTP) bind the 5' cap of mRNA then migrate down the untranslated leader scanning for the first AUG codon (Kozak, 1989). However the consensus sequence around the initiating AUG in yeast is different from that in higher eukaryotes (Cigan, 1987). It should be possible to predict whether a foreign 5' leader will be deleterious by examining the sequence (Kozak, 1989). Initiation rather than elongation is usually the rate limiting step of translation, but translational elongation can become rate-limiting with very high mRNA levels. The overall rate of translation of a mRNA is not usually affected by a slower elongation rate unless ribosomes become limiting, which would affect all transcripts in the cell.

### 1.3.5 *Polypeptide folding.*

During or following translation, polypeptides must fold to adopt their functionally-active conformation. It appears that the information for correct folding is contained in the primary polypeptide structure (Gething and Sambrook, 1992). However, folding comprises rate-limiting steps during which some molecules may aggregate, particularly at high rates of synthesis and at higher temperatures (Buchner *et al.*, 1991). Certain heat-shock proteins

act as molecular chaperones in preventing the formation and accumulation of unfolded aggregates, while accelerating the folding reactions (Gething and Sambrook, 1992)(see also section 1.2.1)

### 1.3.6. *Post-translational processing.*

Amino-terminal modifications of polypeptides are the commonest processing events and occur on most cytosolic proteins (Kendall *et al.*, 1990). Two types of events normally occur; removal of the N-terminal methionine residue, catalysed by Met-amino-peptidase, and acetylation of the new N-terminal residue, catalysed by N<sup>α</sup>-acetyltransferase. Both enzymes are associated with the ribosome and act on nascent polypeptides. A variety of other post-translational modifications, e.g. phosphorylation (Perentesis *et al.*, 1988; Sambucetti *et al.*, 1986), myristylation (James and Olson, 1990) and isoprenylation (Schafer *et al.*, 1990), which are often critical for biological activity appear to be conserved between yeast and higher eukarotes.

Within the secretion pathway protein disulphide-isomerase and peptidylprolyl *cis-trans* isomerases are the two cellular enzymes, that *in vitro* catalyse the isomerisation of intramolecular covalent bonds. These enzymes catalyse the two rate-determining steps involved in protein folding. PDI catalyses thiol/disulphide interchange reactions and promotes protein disulphide bond formation, isomerisation or reduction (Freedman, 1989a; Freedman, 1989b) depending on the nature of the polypeptide substrate and the imposed redox potential. A polypeptide folding pathway is not determined by PDI, rather PDI promotes rapid reshuffling of incorrect disulphide pairings, thereby facilitating formation of the correct set of disulphide bonds.

The slow isomerisation of X-proline peptide bonds (where X is any amino-acid) are catalysed by the enzyme peptidyl-prolyl *cis-trans* isomerase. This can accelerate the refolding of proline-containing polypeptides *in vitro* and *in vivo* (Fischer and Schmid, 1990; Lang *et al.*, 1987; Schonbrunner *et al.*, 1991).

### 1.3.7 *Strategies for the secretion of foreign proteins.*

Heterologous proteins may be secreted from yeast using either a foreign signal, often derived from the protein being secreted, or a yeast signal. A

number of studies aimed at identifying the features of yeast signal peptides have been performed. Ngsee *et al.* (1989) concluded that the essential feature of a signal peptide is a hydrophobic core of 6-15 amino acids, which may be interrupted by non-hydrophobic residues. A classical signal sequence comprises a charged N-terminus, a central hydrophobic core and a consensus sequence for cleavage in the ER by signal peptidase. Some secreted proteins, such as the yeast mating pheromone,  $\alpha$ -factor, have additional *pro* sequences which may aid secretion.

An extremely wide range of heterologous proteins have been secreted from yeast and as *S.cerevisiae* secretes only 0.5% of its own proteins, a secreted protein is contaminated with few other proteins. Many foreign signal peptides function in *S.cerevisiae* and numerous foreign proteins can be secreted via their own secretion peptides (with varying degrees of success). These range from bacterial to fungal, plant and mammalian proteins, indicating that secretory leader requirements are well conserved.

Typically the expression of heterologous genes has been obtained by using signal sequences and promoters of highly expressed yeast genes. By far the most frequently used secretion leader is that of the  $\alpha$  mating factor, encoded by the gene *MF $\alpha$ 1*, composed of a *pre* and *pro* region.

#### **1.4 Apparent limitations of the secretion pathway.**

There are a number of stages in the secretory process at which problems may occur. Proteins may be retained in the Golgi due to misfolding. This is usually a consequence of overexpression as folding is a rate limiting process. The yeast proteins which assist in folding and disulphide bond formation differ from their counterparts in higher eukaryotes and this may affect folding of foreign proteins. Misfolding can result in retention in the ER and degradation.

With larger proteins especially, retention by the cell wall may be a problem, although factors other than molecular mass are known to be important. In addition to the problems of transport, other undesirable events such as aberrant processing or hyperglycosylation may take place during the secretory process. Each of these problems will be considered in turn.

Folding of secreted proteins occurs in the ER and involves accessory proteins such as BiP (Normington *et al.*, 1989), and protein disulphide isomerase (Freedman, 1989b). Nascent proteins bind to BiP co-translationally

and are released upon folding, assembly and glycosylation (if applicable); misfolded proteins bind permanently and are retained in the ER by BiP. During the secretion of foreign proteins, problems might arise either from saturation of these accessory proteins or from their inability to aid the folding of heterologous proteins, as they also can be rate limiting.

In addition to requiring successful targeting and folding in the ER, a foreign protein also has to pass through the secretory organelles in order to be released into the culture medium. Transport from the ER to the Golgi has been shown to be rate limiting in the secretion of calf prochymosin (Moir and Dumais, 1987). Also Hepatitis B virus large surface protein is retained in the ER, provoking the enlargement of this organelle.

A Golgi or post-Golgi bottleneck was postulated to represent a major obstacle in the secretion of Immunoglobulin fragment-I (Steube *et al.*, 1991); retained material may have been mal-folded. This is likely to be a common reason for intracellular accumulation of proteins within the secretory pathway.

Hyperglycosylation is also a major problem because it results in more extensive glycosylation than is found in higher eukaryotic glycoproteins. In the case of Epstein-Barr virus glycoprotein 350 (EBV gp350), secreted from *S.cerevisiae* (Schultz *et al.*, 1987), extensive glycosylation inhibited reactivity with antibodies.

The overexpression and secretion of membrane proteins can cause problems, due to their non-specific insertion into intracellular membranes, probably as a result of membrane retention signals. EBV gp350 was highly toxic due to its interaction with intracellular membranes, but could be secreted in a membrane anchor-minus form (Schultz *et al.*, 1987).

The presence of the cell wall complicates the secretion process in yeast. Permeability may be a limiting factor and it is notable that most success of secretion of foreign proteins has been with very small proteins; some proteins have been reported to be localised mainly in the cell wall when secreted from *S.cerevisiae*, e.g.  $\alpha$ -IFN (Zsebo *et al.*, 1986). A number of very large proteins such as EBV membrane glycoprotein (Schultz *et al.*, 1987) (approximately 400 kDa) and cellobiohydrolase (Penttila *et al.*, 1988) (up to 200 kDa), have been shown to be capable of passing through the cell wall and therefore this property is not related simply to the size of the molecule. It is difficult to draw conclusions from observations of permeability since many other factors, including strain, growth phase and composition of medium may have an effect on cell wall porosity (Denobel and Barnett, 1991).

Correct processing of the signal peptide or *prepro* region must take place so that the mature product is secreted. The yeast signal peptidase, *KEX2* and *STE13* gene products (Bussey, 1988) are involved in the processing of the signal peptide or *prepro* region; incomplete *STE13* or *KEX2* processing or saturation of the signal peptidase has been reported (Haguenauertsapis and Hinnen, 1984). A solution to the problem of inefficient processing is to over-express the processing enzyme genes. This approach was successfully employed in the expression of transforming growth factor  $\alpha$  (Barr *et al.*, 1987).

In addition to inefficient proteolytic processing, problems may also be caused by aberrant processing at internal sites in the protein. In the extreme case of  $\beta$ -endorphin, no complete mature protein was secreted into the medium (Bitter *et al.*, 1984), because of two trypsin-like cleavage sites at lysine residues. Use of the vacuolar protease mutant *pep 4-3* did reduce degradation, suggesting that proteolysis was taking place during passage through the normal secretory pathway.

It has been postulated (Moir *et al.*, 1985; Romanos *et al.*, 1992) that heterologous protein secretion from *S.cerevisiae* becomes saturated at a surprisingly low level, due to bottlenecks in the pathway. Despite increased understanding of the processes of secretion, the greatest success in improving yields in recent years has been with a random mutagenesis approach (see section 1.5).

### 1.5 Improving secretion yields from *S.cerevisiae*.

A number of *S.cerevisiae* strains with a "super-secreting" phenotype that overcomes an apparent blockage in the secretion pathway, have been isolated by screening for mutants with increased secretion of a particular protein product. Smith *et al.*, (Smith *et al.*, 1985) reported the isolation of three mutants (*ssc1*, *ssc2* and *SSCX*) that displayed significantly improved secretion of calf prochymosin, nearly all of which was retained previously within the secretion pathway of wild-type yeast cells. The secreted material, which was activated by the low pH of the medium, was assayed by overlaying the surface of the plate with a mixture of milk and molten agarose. Chymosin clots the milk and hence the speed of appearance of the opaque clotted regions and their size and intensity indicate the level of prochymosin secretion. One of the mutations (*ssc1*) was later identified as being in the *PMR1* gene, which encodes a  $\text{Ca}^{2+}$  ATPase (Rudolph *et al.*, 1989) (Moir *et al.* 1989). Protein

secreted by the mutant did not contain outer-chain glycosylation, suggesting that *pmr1* somehow causes a bypass of the Golgi where such glycosylation takes place.. The *pmr1* mutation significantly increased the secreted levels of prochymosin and bovine growth hormone by 5 to 50 fold (Smith *et al.*, 1985).

Working with recombinant human albumin, Sleep *et al.* (1991) developed a visual assay that relied upon antibody precipitation in solid medium, to isolate a number of super secreting strains that over-produced a range of secreted and internal proteins. However these mutations affected expression level via promoter mutations and/or an increase in plasmid copy number.

Similarly, Sakai *et al.* (1988) reported the isolation of a mutant, *ose1*, which resulted in oversecretion of mouse amylase. Like *pmr1* and *ssc2*, *ose1* also facilitated the over secretion of a different secretory protein, indicating a pleiotropic effect of the defect.

The isolation of these mutants suggested that it might be possible to use an altered secretion level phenotype as a means of identifying genes that influence the flow of secretory proteins through the pathway, rather than ones that are essential for its function, like the *SEC* genes. Although the initial screen for such mutants would be altered secretion yield, it is clear that other mutations, besides those specifically within the secretion pathway, could also give rise to such a phenotype. Thus, increases in secretion yield could be due to promoter de-repression or plasmid copy number increases (Sakai *et al.*, 1988; Sleep *et al.*, 1991), or due to factors external to the cell, such as loss of a cell-surface protease or a cell wall component (Bussey *et al.* (1983).

An indication from the isolation of these mutants is that different secretory proteins may encounter 'bottlenecks', or rate-limiting steps, at different places within the whole route from initial expression, through the secretion pathway itself, to their release into the outer environment. It is possible, therefore, that the secreted reporter protein used to isolate mutants could actually target specific steps that are limiting in its passage through the secretion pathway. This is a central tenet of the studies performed in this Thesis.

## 1.6 Selection of a secretion reporter protein.

Mini-proinsulin was chosen as a secretion reporter protein to be expressed at a high level in *S.cerevisiae*, in this initial study, for a combination



of reasons. Mini-proinsulin is a single-chain, disulphide-bonded recombinant precursor of human insulin of the structure B-LysArg-A chain, where B and A are the peptide chains of human insulin (Thim *et al.*, 1986) . When coupled to a suitable secretion leader peptide, mini-proinsulin is secreted from yeast with yields been quite high to begin with. Increasing expression to a maximum would increase pressure on any bottlenecks in its passage through the secretion pathway, whilst reducing the possibility of isolating mutants that simply result in an increase in overall expression level.

Maximum expression requires strong transcription and high translation efficiency. A strong promoter is not difficult to provide. However, a major factor influencing translation efficiency, particularly applying to the expression of foreign genes in yeast, is codon usage. The small size of mini-proinsulin therefore enabled a DNA molecule based on codons optimised for high expression in yeast, to be chemically synthesised (see figure 1.8). Being a small peptide (53 amino acids and  $M_r$  6 kDa) it would be expected to pass through the cell wall with ease, thus eliminating that stage as a potential bottleneck. Any rate limiting steps in secretion of mini-proinsulin would therefore be expected to occur during the intracellular stages.

In order to detect mutations that alter the flux of proteins through the secretory pathway, it is essential to employ a secretion reporter protein that has a reliable qualitative assay. This needed to be developed for mini-proinsulin, although preliminary studies indicated that this should be possible. In addition, yields could be accurately quantified by "ELISA" assay of liquid culture supernatants using monoclonal antibodies raised to both the A (21 amino-acid residues) and B (30 amino-acid residues) chains of human insulin. These chains are joined together by two disulphide bridges; a third disulphide bridge is formed within the A-chain (figure 1.9).

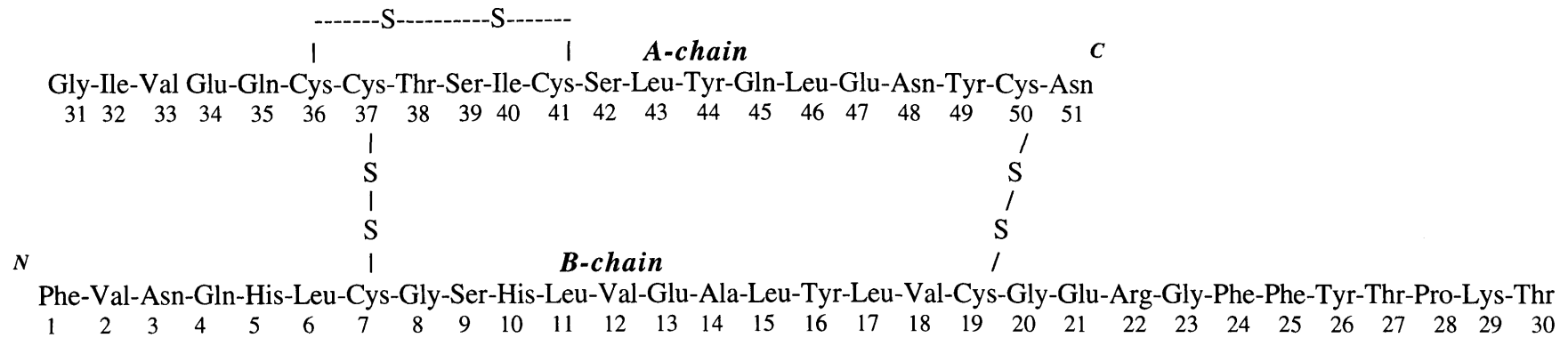
### 1.6.1 Processing and secretion of insulin in humans and yeast.

The biosynthesis of insulin occurs in the pancreatic  $\beta$ -cells via the synthesis and modification of a single-chain precursor, preproinsulin. Human preproinsulin has a prepeptide of 24 amino-acid residues, which directs the molecule (during synthesis) to the endoplasmic reticulum (ER) and into the secretory pathway, being cleaved off as it passes into the lumen of the ER. The resultant proinsulin of 86 amino-acid residues has the composition B-Arg-Arg-C-Lys-Arg-A, of which C is a peptide of 31 amino-acid residues and B and A are the B and A-chains, respectively, of mature insulin. Shortly after entry

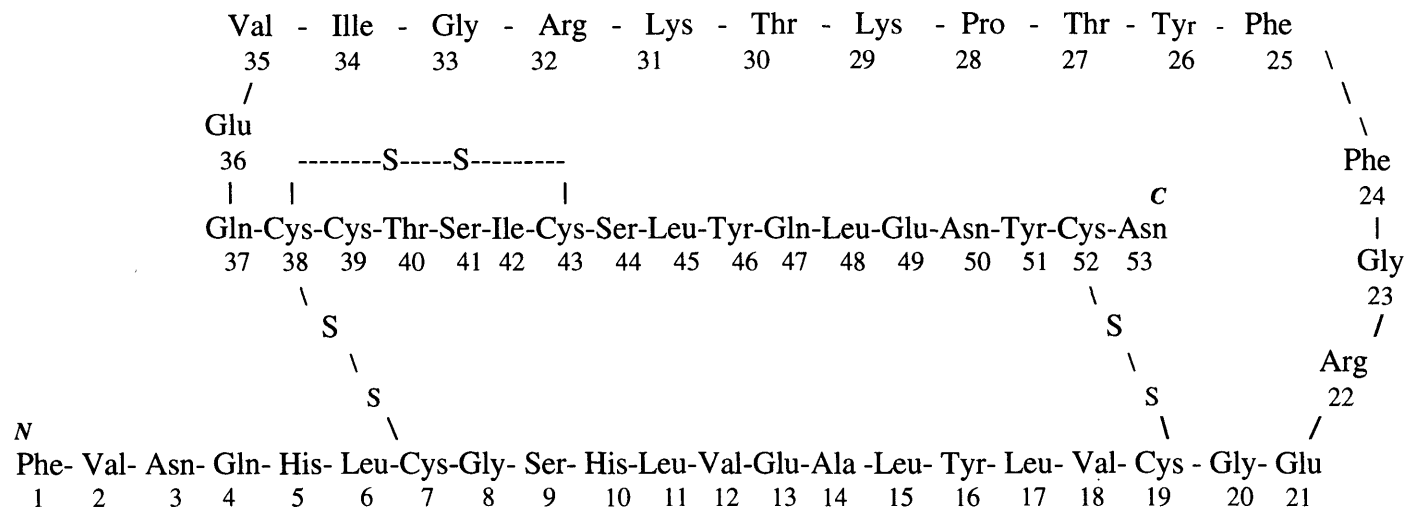


**Figure 1.9** The schematic amino acid composition of human insulin after natural biosynthesis and processing in human pancreatic  $\beta$ -cells compared to the synthetic mini-proinsulin used in this study after biosynthesis and processing in *S.cerevisiae* cells. The position of the three disulphide bridges are indicated. The only difference between the two products being the 'mini-c' peptide linking the B and A chains in the synthetic product, which is removed *in vitro* via trypsin and carboxypeptidase B digestion. Therefore before *in vitro* processing the product produced by *S.cerevisiae* cells is still a single chain precursor whereas the pancreatic  $\beta$ -cells generate a fully processed 2-chain insulin molecule.

## Human insulin



## Synthetic mini-proinsulin



into the ER, the proinsulin folds and establishes the three disulphide bridges. The proinsulin then transits to the Golgi, where it is packaged into secretory granules and wherein it is converted to mature insulin. This involves endoproteolytic cleavage at the Arg-Arg and Lys-Arg sequences to remove the C peptide. Fusion with the cell membrane to release insulin from the cell is controlled by calcium ion import, which controls membrane fusion. This in turn is regulated, so that the secretory granules accumulate in the cell until signalled for secretory release.

Yeast cells have similar processing abilities to the pancreatic  $\beta$ -cells although secretion is not regulated but constitutive, and therefore offer a similar route to the biosynthetic production. Thus, when expressed in *S.cerevisiae*, a heterologous proinsulin fused to the *MF $\alpha$ 1 prepro* secretion leader peptide was directed to the secretory pathway, where it folded correctly, formed the disulphide bonds correctly, was processed by the *KEX2* subtilisin-like activity and a mixture of mature insulin and incompletely processed forms was secreted into the medium (Thim *et al.*, 1986). However the yield of insulin was very low, whilst the C peptide was about 10-fold more abundant. As one would expect both proteins to be made in equimolar ratios, the discrepancy indicates differential degradation of insulin compared to the C peptide in yeast. This presumably reflects the differences in specificity between the yeast and  $\beta$ -cell processing enzymes. An alternative strategy was developed to circumvent this problem, involving the production of a single-chain precursor mini-proinsulin in yeast (figure 1.8), which was not endoproteolytically cleaved by *KEX2* protease and was secreted in high yields. This could subsequently be converted to insulin by an *in vitro* step (Thim *et al.*, 1986).

### 1.6.2 *Synthetic mini-proinsulin gene construction.*

The mini-proinsulin gene used by Thim *et al* (1986) was derived from modified human cDNA. Due to the problem of unfavourable codon usage limiting the level of expression in yeast it was considered appropriate to use a synthetic gene containing optimal yeast codons. The mini-proinsulin encoding sequence was therefore constructed from synthetic oligonucleotides (termed Ins1). This sequence was designed for subsequent fusion to a yeast secretion leader sequence. The strategy for the construction and design of the

mini-proinsulin gene is further described in British Patent Application No. 9513967.1.

### 1.6.3 Cloning of *Ins1* into the *pDP* series of yeast secretory expression vectors.

There were a number of reasons for choosing this series of vectors for expression of the cloned *Ins1* product. They contain the *ORI-STB* region of 2 $\mu$ m, facilitating multicopy replication (approximately 20 copies/cell) and high stability inheritance in yeast, plus the *LEU2* gene, which enables presence of the plasmid to be selected in *leu2* deficient host strains, the *MF $\alpha$ 1* promoter which provides strong transcription of heterologous genes and the *ADH1* terminator for efficient transcription termination (figure 1.10). Both vectors facilitate fusion of the *MF $\alpha$ 1 prepro* secretory leader peptide. The difference between the two plasmid vector constructs made is that pDP315 results in direct fusion at the *KEX2* endopeptidase processing site, where a *StuI* site has been engineered via *in vitro* mutagenesis, such that B-LysArg-A would be produced after digestion. The pDP314 construct results in liberation of GluAlaGluAla-B-LysArg-A after *KEX2* protease digestion and therefore requires *STE13* processing to remove the two Glu-Ala dipeptides serially and so generate the mature product.

The fusions were made as described by Hadfield., *et al* (1997) and verified by sequencing. Both constructs provide in-frame fusions to the *MF $\alpha$ 1* secretion leader peptide, as shown in figure 1.11.

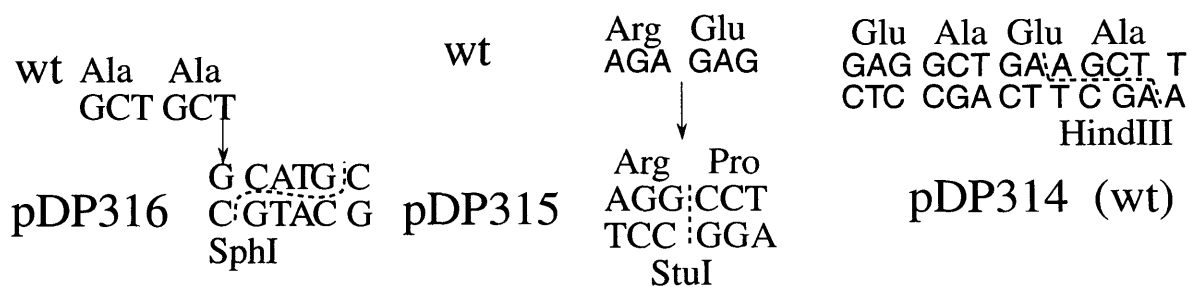
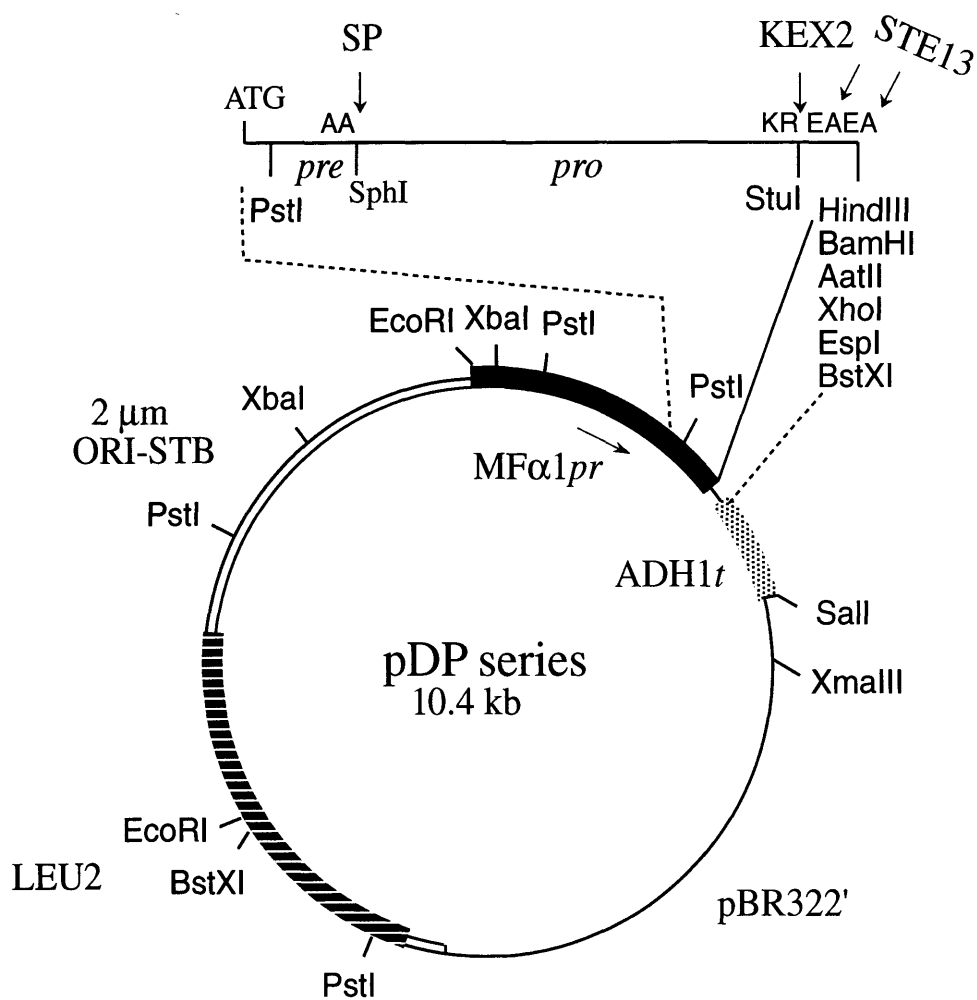
## 1.7. Aims

The overall aim of this project was to identify rate limiting steps in the yeast secretion pathway. To achieve this new secretion mutants were to be generated, using a number of secretion reporters and plate assays in conjunction with random mutagenesis. Prior to the start of this project, the vectors containing the synthetic mini-proinsulin gene had already been constructed and transformed into a number of yeast strains. Initial studies had been performed with these strains and an appropriate antibody based plate assay was being developed.

The aims of this study can be divided into two distinct sections;

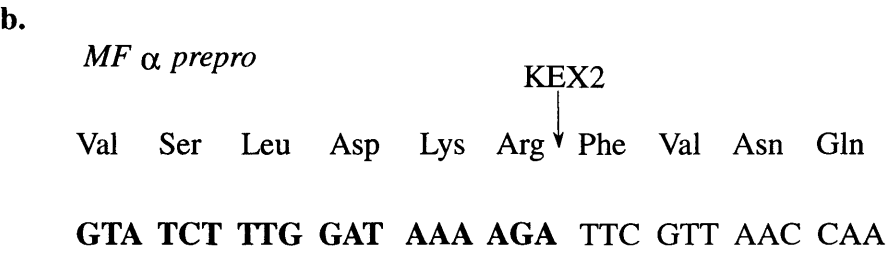
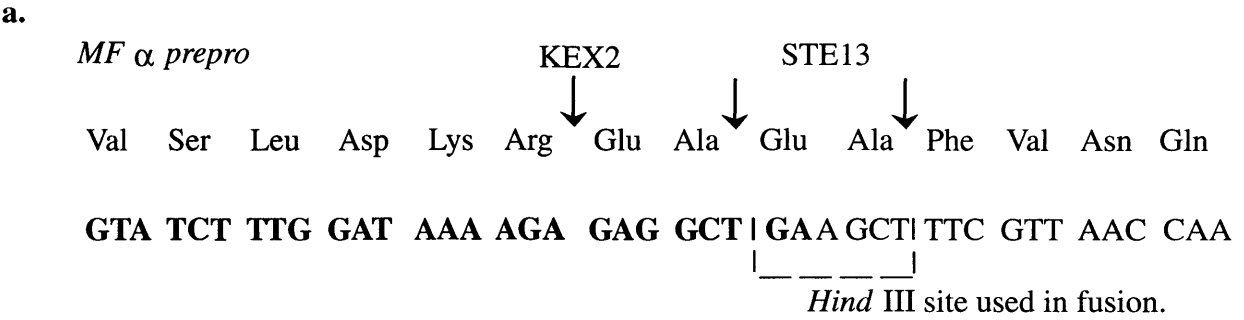
(i) the work carried out with the mini-proinsulin reporter; and

**Figure 1.10.** The pDPseries of expression vectors. This series allows the construction of three different vector constructs; pDP314 which utilises the natural *Hind*III site in the *MFα1* leader for its translational fusion: pDP315 which provides a engineered *Stu*I site for direct fusion to the KEX2 cleavage site and pDP316 where a *Sph*I site has been introduced to allow fusion directly to the *pre* peptide. KEX2, STE13 and signal peptidase (SP) sites are indicated by arrows. Only the first two constructs were utilised.





**Figure 1.11** In-frame fusions of Ins1 in vectors pDP314 (a) and pDP315 (b). The vector sequence is in bold print and the *KEX2* and *STE13* (pDP314 only) processing sites have been indicated, as well as the *Hind*III site used in the fusion in pDP314.



(ii) the selection and utilisation of another secretion reporter protein.

I shall consider each of these areas in turn.

#### The mini-proinsulin reporter protein.

Once the antibody-based plate assay had been fine tuned, the next stage of the project was to select a yeast strain for mutagenesis. The mutagenised cells would then be screened and strains showing enhanced secretion of mini-proinsulin isolated, via the plate assay. These mutant strains would be analysed to confirm that their mutations were chromosomal in origin and did not lead to an elevation in transcript levels of the reporter protein.

After these preliminary analyses the mutant strains would be further analysed on a genetic and molecular basis. Also other secreted and cytoplasmic, reporter proteins could now be transformed into these mutant strains, to see if the mutation was specific for secreted proteins. The final part of the analysis would attempt the isolation of the genes resulting in the enhanced secretion phenotype.

#### The selection and utilisation of another secretion reporter protein.

A different secretion reporter was used to attempt the isolation of other enhanced secretion mutants. The  $\beta$ -galactosidase protein of *Aspergillus niger* was selected. This protein is much larger in size than the mini-proinsulin reporter and glycosylated. Therefore this might target different areas of the secretion pathway that were rate limiting for the secretion of this protein.

There were a number of criteria used for the selection of this secretion reporter protein;

(1) The secretion reporter protein must not have been utilised in a previous study.

(2) The size of the protein; large and small proteins may encounter different bottlenecks.

(3) The extent of glycosylation.

(4) The availability of a suitable assay to allow rapid screening of yeast colonies for potential mutants. This could be based on staining or antibody precipitation of substrate or product.

(5) Availability of a reliable culture assay.

Once these criteria had been met the procedure would be similar to the one followed for the isolation and characterisation of the mini-proinsulin mutant strains.

## Chapter Two

### DEVELOPMENT OF A MINI-PROINSULIN REPORTER FOOTPRINT ASSAY SYSTEM FOR THE ISOLATION OF ENHANCED SECRETION MUTANTS OF *SACCHAROMYCES CEREVISIAE*.

#### 2.1 Results

##### 2.1.1 *Detection and quantification of mini-proinsulin in the culture medium by ELISA assay.*

Once vectors for the expression of mini-proinsulin from *S.cerevisiae* had been constructed and checked via sequencing, the next stage was to select a number of yeast strains to be transformed with these vectors. Additionally a reliable assay system was required, to detect and quantitate any mini-proinsulin secreted into the liquid medium. Early methods used to detect insulin secreted from yeast cells into the medium, extracted from *E.coli* inclusion bodies or isolated from human patients, involved radioimmunoassays utilising I<sup>125</sup>. Working with highly penetrative radioactivity is however undesirable (though very sensitive) and more recently non-radioactive methods have been developed (Boehringer 1990), based upon enzyme linked immuno-sorbant assay (ELISA) of insulin for quantification. It was this method that was selected for the assay of mini-proinsulin in liquid medium.

##### 2.1.2 *Strain selection and variation.*

A number of haploid laboratory strains were available for transformation with pDP314-Ins. In order to assess whether host strain genotype had any effect on the level of insulin produced, a number of strains were chosen based on the following criteria:

- 1) all strains were mating-type  $\alpha$  to enable the *MF $\alpha$*  promoter on the plasmid to function.

2) each had to possess a number of auxotrophic markers that would allow selection of plasmids transformants and facilitate the construction of diploids for genetic analysis.

3) strains which had been previously used for the successful expression of other foreign proteins were utilised (e.g. JHRY188) .

4) protease deficient strains (BJ2168 and TGY47.1) and non-protease deficient strains were compared, since secreted products can occasionally suffer from protease degradation (Jones, 1991; Wingfield and Dickinson, 1993).

Using this rationale the following yeast strains, BF307-10, DBY746, JRY188, BJ1991 and TGY47.1 were transformed by the lithium acetate procedure with the pDP-Ins1 plasmid and transformants able to grow in minimal medium lacking leucine isolated. Transformant clones of each strain were grown on minimal medium and then inoculated into 10 mls of YPD and incubated. The secretion of mini-proinsulin into the medium was measured via ELISA (table 2.1). These assays were also performed on cell extracts of each strain in order to determine whether any insulin remained intracellular . All transformants containing a pDP-Ins1 plasmid secreted ELISA reactive material into the culture medium, indicating that the pDP-Ins1 constructions gave rise to immunologically identifiable mini-proinsulin. There was no reactive material present in the control culture medium or within the cell extracts. This indicated that all of the insulin produced was secreted. The assay results showed that there was significant strain variation for the production of mini-proinsulin. Strain DBY746 secreted approximately half the amount of mini-proinsulin of strain BF307-10, and the two protease deficient strains BJ1991 and TGY47.1 did not secrete increased levels of mini-proinsulin; indeed they gave levels similar to those obtained with strain JRY188 which had previously been used for the secretion of human EGF (Brake *et al.*, 1984). All three of these strains gave secretions levels lower than those obtained with BF307-10 but slightly higher than those obtained with DBY746.

The objective of the project was to put the secretion pathway in *S.cerevisiae* under pressure. This was achieved by synthesising the mini-proinsulin gene with optimal codons for expression in yeast, with expression itself driven from a strong promoter on a multi-copy plasmid. Thus, secretion of mini-proinsulin was optimised allowing us to isolate mutants that further improved the efficiency of secretion, rather than mutants that affected essential genes (e.g. the *sec* mutants). Strain BF307-10 was chosen for future work as it secreted the greatest levels of quantifiable mini-proinsulin and would therefore be putting the pathway under the greatest "stress".

**Table 2.1.** An example of the levels of insulin secretion from different strains of *S.cerevisiae*.

Strain	Insulin mU/L*	
	Secreted	Intracellular
BF307-10	144(±13)	0.5
DBY746	83(±8)	0.5
BJ1991	90(±10)	0.6
TGY47.1	98(±4)	0.5
JRY188	101(±6)	0.6

\*By ELISA (see Methods) using human insulin as the standard; mean values for 3 separate assays.

All strains were cultured to OD<sub>650</sub> =1 before harvesting.

The experiment was repeated on at least three other occasions and the results were similar to those shown above.

### **2.1.3 Stability of insulin plasmids in yeast.**

It is possible that heterologous protein expression might be disadvantageous and reduce the growth rate of yeast cells expressing the protein. Hence plasmid-free cells will out grow plasmid containing ones leading to a reduction in measurable insulin levels. It has also been shown that 2 $\mu$ m based vectors (as utilised in this study) are lost from cells during vegetative growth because of asymmetric distribution during mitosis (Murray and Szostak, 1985). This rate of loss can vary, depending on the laboratory strain of *S.cerevisiae* being utilised (Dr.K.Duffy. personal communication). Therefore it was necessary to establish the stability of the insulin expressing plasmids in *S.cerevisiae*. Transformant yeast colonies of strain BF307-10 were inoculated into 10 mls of minimal medium lacking leucine to maintain the selection for the plasmid, and incubated at 30°C overnight. Cells from the resultant culture were used to inoculate both non-selective YPD medium and selective minimal medium. After growth for at least 50 generations, cells were plated onto YPD and incubated at 30°C for 2-3 days. The colonies were then replica-plated onto minimal medium to determine the proportion of plasmid-containing (Leu<sup>+</sup>) cells in the original populations. In minimal medium 97% of 2000 colonies were Leu<sup>+</sup>, indicating that the propagated cells had retained the plasmid; 92% of the cells still carried the plasmid after culturing in non-selective YPD medium. This demonstrated a high degree of mitotic stability indicating that the expression of the insulin precursor products did not affect plasmid stability. As a control plasmid stability was also monitored with BF307-10 cells transformed with vector pDP314 only. The results were nearly identical to those obtained with recombinant pDP314-Ins1 indicating that the expression of the insulin precursor did not affect plasmid stability.

Additionally the growth rate of strain BF307-10 containing pDP314-Ins was compared against strain BF307-10 without plasmid. It was found that the growth rate of both strains was almost identical, indicating that the expression of the insulin precursor products did not cause toxicity.

### **2.1.4 Standardisation of insulin production in shake flask cultures.**

Transformant yeast colonies containing pDP314-Ins1 were inoculated into 10 ml of minimal medium lacking leucine and incubated at 30°C overnight. 100 $\mu$ l aliquots of cells at a concentration of 10<sup>8</sup>cells/ml were used

to inoculate 100 mls -YPD medium in 200 ml conical flasks. Culture growth was followed using a spectrophotometer to measure cell density and the ELISA technique to measure insulin levels. The results (Table 2.2 and figure 2.1.) showed that insulin production increased gradually with culture density until a peak level of production was reached at the end of exponential growth, approximately thirty hours after culture inoculation. If the culture was allowed to continue growing eventually insulin levels fell, until none was detectable as the cells entered stationary phase. This may be due either to an unknown protease activity released into the culture medium, the drop in pH of the medium with time, or to aggregation of the mini-proinsulin proteins to produce fewer separate discernible molecules. For subsequent assays a time point around the twenty hour (O.D<sub>650nm</sub> 1.8-2.0 approximately  $6 \times 10^7$  cells/ml) period was used, with samples diluted if need be, to allow measured insulin levels to fall within the standard curve limits.

#### ***2.1.5 Development of a plate footprint assay for mini-proinsulin producing colonies.***

The plate assay devised for detection of mini-proinsulin secreted by transformed yeast colonies was dependant upon the general property of proteins to bind to nitro-cellulose filters. When a filter was placed upon the surface of nutrient agar, its porosity enabled water and nutrients to pass through, so that yeast cells inoculated on the filter could grow and form colonies. Cells secreting mini-proinsulin deposited the peptide on the surface of the filter and the material became bound to the filter in a quantitative manner. Mini-proinsulin bound to the filters was detected immunologically using goat anti-insulin primary antibody and rabbit anti goat secondary antibody, conjugated to the enzyme alkaline phosphatase to give a chromogenic reaction. This appeared as a coloured deposit. Thus the colony produced an immuno reactive 'footprint' where the intensity reflected the amount of mini-proinsulin secreted, and could be used as a basis for detecting changes in the amount secreted.

As a preliminary test of the system, solutions of human insulin (1pg-10ng in a volume of 5 ml H<sub>2</sub>O, adjusted to pH 4.0 by HCl) were spotted onto nitro-cellulose membrane filters with pore sizes of 0.45 µm, 0.2 µm, 0.15 µm and 0.1 µm placed in double thickness upon agar plates and left overnight at

**Table 2.2.** An example of insulin production from BF307-10 *S.cerevisiae* cells in shake flask cultures.

Time (Hrs)	O.D. <sub>650nm</sub>	Cell Number	Insulin mU/L*
0	0.06		1
6	0.096	3 x 10 <sup>6</sup> /ml	9
8	0.221		43
10	0.49	1.3 x 10 <sup>7</sup> /ml	68
12	1.00	2.8 x 10 <sup>7</sup> /ml	160
16	1.80		242
23	2.01	6.2 x 10 <sup>7</sup> /ml	440
30	2.50		473
96	2.89		176
110	3.00	2.2 X 10 <sup>8</sup> /ml	87

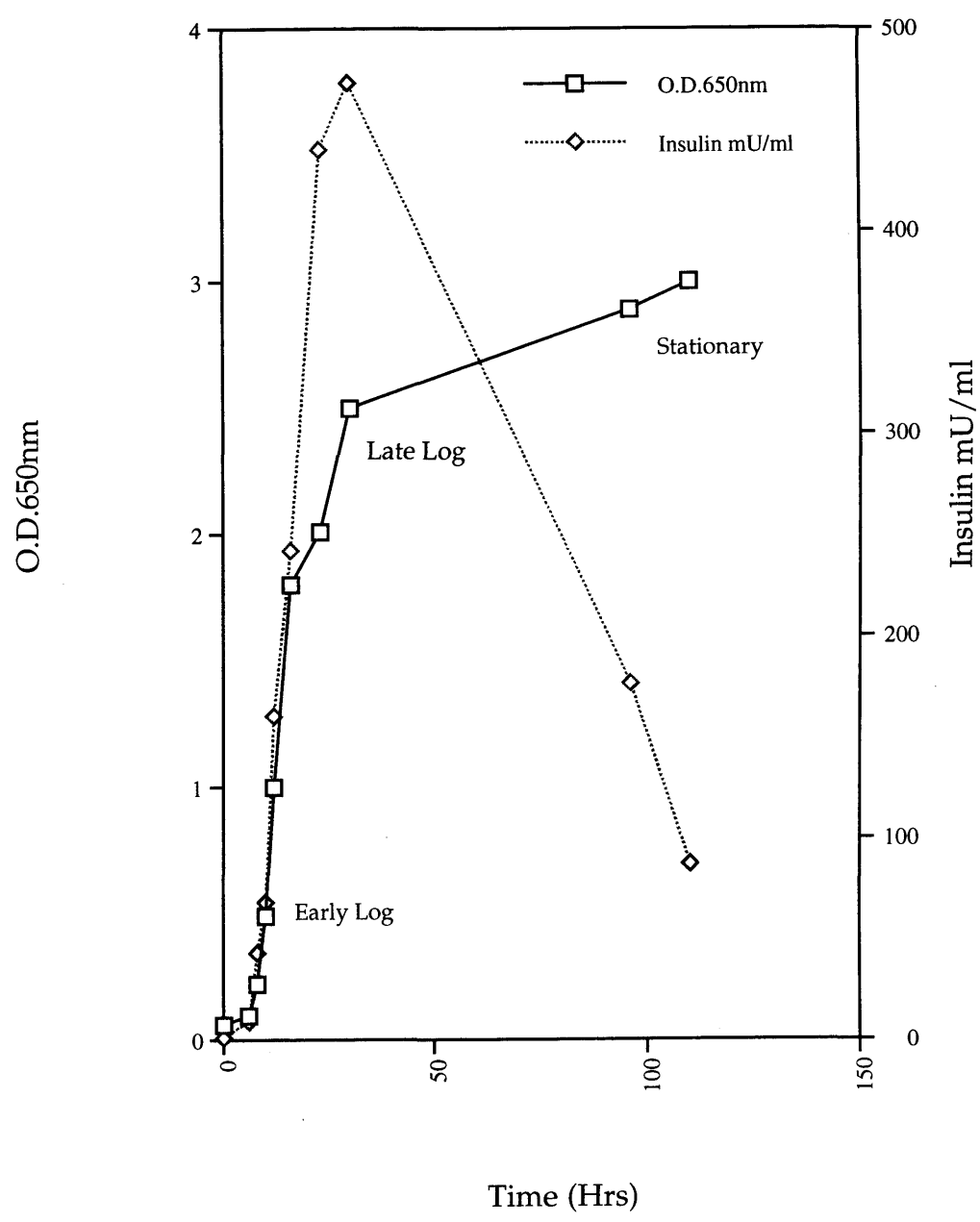
\*By ELISA (see Methods); mean values for 3 separate assays.

The production of insulin was followed over a period of 110hrs in BF307-10 *S.cerevisiae* cells to determine the optimum time point (optical density) for insulin assay.

The experiment was repeated on at least three other occasions and the results were similar to those shown above.



**Figure 2.1.** The production of mini-proinsulin in shake flask culture with respect to time and cell density.



room temperature to allow the insulin to bind. The filters and subsequent footprints were then developed.

The results showed that insulin would bind to the nitro-cellulose filters under conditions suitable for colony growth. The best results were obtained with 0.2  $\mu\text{m}$  pore size, which bound most of the insulin, with only about 5% passing through to a second filter underneath. With 0.45  $\mu\text{m}$  pore size, about 25% passed through to the second filter. Both of these pore sizes showed spot intensities (as estimated by eye) that reflected the relative quantity of insulin applied to the filter. In contrast, the 0.15  $\mu\text{m}$  and 0.1  $\mu\text{m}$  pore sizes were too poorly wettable to be used for effective footprint production.

Since the mini-proinsulin secreted by transformed yeast cells was a single chain precursor (Thim *et al.*, 1984) there was no guarantee that it would bind to nitro-cellulose with the same capability as normal insulin. To test this and complete development of the footprint plate assay, a number of different *MAT $\alpha$*  yeast strains were transformed with the pDP-Ins1 plasmids and the amounts of mini-proinsulin produced in liquid culture medium quantified by ELISA (Table 2.1). Cells from strains BF307-10 and DBY746 cultures were also washed in sterile water, resuspended in water at  $10^8$  cells per ml and then 5  $\mu\text{l}$  spotted onto 0.2  $\mu\text{m}$  nitro-cellulose filters placed upon the surface of SD-minimal selective or YPD agar plates. This test was performed to see whether the two-fold difference in levels of insulin secretion as analysed by ELISA could be visualised on colony footprints. The plates were then incubated at 30°C for 2-4 days to enable colony growth. To minimise the chance of bacterial or fungal contaminant growth, the filters were pre-sterilised by exposure to ultraviolet light and 15  $\mu\text{g/ml}$  tetracycline was incorporated into the growth medium.

After colony growth, the filters were placed upside down in a beaker of SB buffer and the liquid gently swirled to wash off the yeast cells. The filters were given two further washes in SB buffer and then incubated in SB-BSA prior to antibody detection-staining. Footprints were detected where the mini-proinsulin secreting colonies had been located and they varied in intensity relative to the amount assayed in liquid culture medium. Figure 2.2a shows results for two different yeast strain hosts. Transformed strain DBY746 secreted half the amount of mini-proinsulin as BF307-10 (Table 2.1) and their colony footprints showed a corresponding difference in intensity.

A 2-fold difference in footprint intensity visible on filters was sufficiently sensitive for detecting enhanced secretion mutants. In the case of control colonies that did not secrete mini-proinsulin, a negative footprint was observed due to other proteins secreted by the yeast cells blocking the filter to

**Figure 2.2** Colony footprint assay for mini-proinsulin secretion.

(a) Colony footprints for two different non-mutant yeast stains, BF307-10 and DBY746 both containing plasmid pDP314-Ins1. A negative colony footprint is seen for wild-type BF307-10 and DBY746 cells that do not contain plasmid pDP314-Ins1 (C<sup>-</sup>). It is possible to distinguish the two fold difference in footprint intensity between BF307-10 and DBY746.

(b) Detection of an enhanced secretion mutant (arrowed) from original mutagenesis of strain BF307-10.

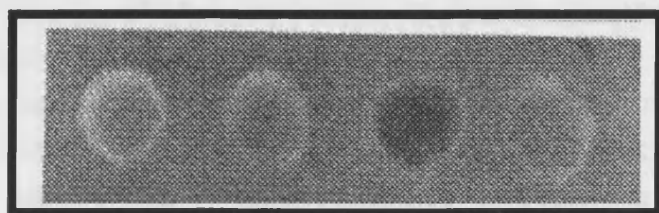
(a) C<sup>-</sup> +



} BF 307-10

} DBY 746

(b)



background staining more effectively than BSA. This does not block the mini-proinsulin secreted by transformed cells from binding to the membrane, however. This would appear to be because yeast naturally secretes few proteins at low levels, into its growth medium. Additional mini-proinsulin is therefore able to bind without being blocked.

### 2.2.5 *Mutagenesis and isolation of enhanced secretion mutants.*

The colony footprint assay was used to isolate mutant cells showing enhanced levels of secretion of mini-proinsulin. BF307-10 (pDP314-Ins1) cells were chemically mutagenized with either ethyl methyl sulphonate (EMS) or nitrosoguanidine (NTG) at concentrations determined to give 60% killing (Sleep *et al.*, 1991). Preliminary experiments were performed to determine the percentage of surviving cells after mutagenesis and the appropriate concentration of mutagen selected. Surviving cells were plated onto minimal selective agar. The resultant colonies were then either replica-plated or toothpicked onto sterile 0.2  $\mu\text{m}$  pore size nitro-cellulose filters placed upon YPD-tetracycline agar plates, incubated at 30°C for 3-6 days, and the mini-proinsulin footprints stained as before. Prospective mutants having footprints of greater intensity were selected (25 out of 80 000 mutagenized colonies) and rescreened to test how reproducible the footprint assay was. It was noted on the control plate of unmutagenised cells that the variation in footprint intensities within the strain BF307-10 was minimal. Also it was noted that footprint intensity was not dependant on colony size or age. An example of an enhanced secreting colony can be seen in figure 2.2b.

For final confirmation of enhanced secretion, the colonies were inoculated into liquid medium and yields of secreted mini-proinsulin accurately assayed by ELISA. Of the rescreened mutagenized colonies, 10 consistently showed increased secretion yields of mini-proinsulin ranging from 1.3- to 3-fold (Table 2.3). Thus these appeared to be candidate mutants showing enhanced efficiency of the protein secretion pathway and were denoted as *esi* mutants, for the enhanced secretion of mini-proinsulin.

**Table 2.3.** Insulin assays on ten prospective *esi* mutants isolated by footprinting screening.

Strain	Insulin mU/L*\$	Relative Yield
+ untransformed	0	0
+	58( $\pm$ 4)	1.0x
<i>esi1</i>	<b>127(<math>\pm</math>8)</b>	<b>2.1x</b>
<i>esi2</i>	78( $\pm$ 3)	1.3x
<i>esi3</i>	91( $\pm$ 6)	1.6x
<i>esi4</i>	<b>103(<math>\pm</math>5)</b>	<b>1.8x</b>
<i>esi5</i>	81( $\pm$ 2)	1.4x
<i>esi6</i>	89( $\pm$ 5)	1.5x
<i>esi7</i>	83( $\pm$ 4)	1.4x
<i>esi8</i>	81( $\pm$ 9)	1.4x
<i>esi9</i>	<b>109(<math>\pm</math>7)</b>	<b>1.9x</b>
<i>esi10</i>	83( $\pm$ 6)	1.4x

\* Measured by ELISA technique, using human insulin as the standard. All strains were cultured to an OD<sub>650</sub>=0.8 before harvesting. Strains highlighted were those with the most enhanced secretion.

+ = BF307-10 non-mutant parent strain.

\$ = Levels of insulin secretion are lower than those in table 2.1 as cultures were harvested at a lower cell density.

The experiment was repeated on at least three other occasions and the results were similar to those shown above.

## 2.3 Discussion.

A general strategy has been developed and employed to enable the isolation of mutations affecting the efficiency of the secretion pathway. Such mutants might alter the rate of flow of secretory proteins through the pathway, rather than eliminate pathway function altogether. Using a synthetic mini-proinsulin as a secretion reporter, and a footprint assay for detecting the amount of protein secreted by yeast cells we isolated a number of mutants that showed an increased secretion phenotype. The mutation frequency (at approximately 1 in  $10^4$  mutagenised cells) was very similar to that observed by Sleep *et al* (1991) who used another immunological method to recover mutants showing enhanced secretion of human serum albumin.

Levels of mini-proinsulin in shake flask cultures reached a maximum of approximately 19mg/L in YPD medium. When minimal media was used, a two fold reduction in levels was seen. These levels of mini-proinsulin production are roughly equivalent to those quoted by Thim *et al* (1986)-10mg/L in minimal media, using mini-proinsulin expressed from modified human cDNA (B-chain-Lys-Arg-A-chain). However the mini-proinsulin gene used in their study is based on the human cDNA and the codons were far from optimal for expression in yeast, unlike the synthetic mini-proinsulin used in this study. Insulin levels in the supernatant have been determined by radioimmune assay rather than ELISA, and one would expect the radioimmune assay to be as sensitive as the ELISA as both have been calibrated against the international standard for insulin. It is difficult however to compare expression levels accurately as different plasmid selection systems were used as well as different host strains and culture conditions.

From the initial screen and subsequent rescreening ten *esi* mutant strains were isolated that secreted greater amounts of mini-proinsulin than non mutagenised cells. These strains were cultured and assays revealed that three mutants showed secretion levels 2-3 fold greater than wild-type, whilst the other seven showed enhancement of between 1.3-1.5 fold. No mutants were isolated that secreted lower levels of mini-proinsulin than wild-type (which could have been identified as colonies with white footprints) nor were any mutants recovered that secreted mini-proinsulin at levels more than three times greater than wild-type. The rationale behind the design of this mutant screen was to isolate a different subset of genes involved in the secretion pathway, to those essential genes isolated as temperature sensitive mutations by Novik and Schekman (1980). As the rationale for the screen relied on a situation with maximum possible flow through, but still within

the capacity of the secretion pathway to try to isolate new genes. It is possible that mutants may have been isolated that are defective in non-essential genes, (including essential genes that are part of a gene family) or carry non-lethal mutations in essential genes.



## Chapter Three.

### GENE EXPRESSION ANALYSIS OF THE *ESI* MUTANTS.

#### 3.1 Introduction.

It was decided that it would be probably too difficult to work with mutants showing less than 2-fold enhanced levels of secretion, thus only the three mutants *esi1*, *esi4* and *esi9*, showing 2- to 3-fold enhanced secretion (Table 3.1a) were selected for further characterisation. Initially it was important to ascertain whether each mutant's phenotype was independent of the "secretion-reporter" plasmid used for the isolation. Secondly we decided to express the synthetic mini-proinsulin from a different promoter to test the possibility that the enhanced secretion phenotypes seen were due to enhanced transcription and were promoter specific. Also the effects of the mutations on secretion reporter transcription would have to be investigated. This was to determine whether the enhanced secretion phenotype was a general property for all secreted proteins or specific to the mini-proinsulin reporter. The final part of this analysis involved studying the effects of the mutations on cytoplasmic reporter expression, as this would indicate whether the mutations specifically affected proteins to be secreted or generally affect both cytoplasmic and secreted proteins.

**Table 3.1.** Amount of mini-proinsulin secreted into the yeast cell culture medium by *esi* mutants. \* All strains were cultured initially in selective medium before inoculation into non-selective YPD. Cultures were harvested after cells had reached OD<sub>650</sub> of 2.

**a. Cells containing pDP314-Ins1**

Strain	Insulin mU/L*	Relative Yield
+	194 (± 3)	1.0x
<i>esi1</i>	501(± 4)	2.6x
<i>esi4</i>	340 (± 5)	1.8x
<i>esi9</i>	346 (± 5)	1.8x

**b. Cells containing pBG1 (see section 3.2.3)**

Strain	Insulin mU/L*	Relative Yield
+	100 (± 10)	1.0x
<i>esi 1</i>	274 (± 15)	2.7x
<i>esi 4</i>	207 (± 11)	2.1x
<i>esi 9</i>	220 (± 6)	2.2x

\* Measured by ELISA, using human insulin as the standard.

## 3.2 Results.

### 3.2.1 *Effect of assay point.*

As a first step, insulin levels were determined for higher cell densities. Insulin levels were again determined by ELISA but this time when the cells had reached an OD<sub>650</sub> of 2 (approximately  $6 \times 10^7$  cells/ml). This is closer to the point of peak insulin production as previously determined in chapter two (Table 2.2. and Figure 2.1) and explains why the levels of insulin produced are higher than those recorded in table 2.1 and 2.3, where cells were harvested when they had reached OD<sub>650</sub> of one (approximately  $2.8 \times 10^7$ /ml).

### 3.2.2 *Plasmid based or chromosomal mutations?*

The first step in analysis was to ascertain whether the mutant phenotypes of *esi* 1, 4 and 9 were plasmid derived or chromosomal in origin. Thus, plasmid free segregants were recovered as leucine-auxotrophs by growing the mutant strains for 30 -40 generations in non-selective YPD medium . These strains were then retransformed with fresh pDP314-Ins1 and transformants selected on minimal medium plates lacking leucine. Leucine-prototrophic transformants were grown overnight in minimal medium, inoculated into 10 mls of YPD and the amount of mini-proinsulin secreted into the culture medium determined by ELISA. The results showed the retransformed *esi* mutants to have the same secretion pattern as the original isolates, with levels of secreted insulin being 2-3 fold greater in the *esi* mutants than the wild type BF307-10. Thus the *esi* phenotypes were chromosomal in origin rather than plasmid based.

### 3.2.3 *Construction of pBG1.*

A new yeast expression vector was constructed for two reasons: firstly to allow the expression of the synthetic insulin from a different yeast promoter, in order to exclude the possibility of promoter bias on insulin expression and mutant production: secondly, as the *MF $\alpha$*  promoter used in the vector pDP314-Ins1 is not active in diploid cells a different yeast promoter was

required to allow diploid strains to be constructed for genetic analysis of the mutants.

As the *MF $\alpha$*  secretion leader peptide was still required to direct the insulin produced into the secretion pathway, and there were no available restriction sites to release the leader peptide encoding region from the *MF $\alpha$*  promoter in vector pDP314-Ins1, the region encoding the *pre-pro MF $\alpha$*  leader/insulin cassette was extracted from pDP314-Ins1 using the PCR technique. For this purpose oligonucleotides were designed to be homologous to the *MF $\alpha$*  *pre-pro* leader sequence and to the end of the synthetic Ins1 gene (see experimental procedures). The PCR conditions were set up in accordance with the guidelines provided by the manufacturers of the Deep Vent Polymerase used in the reaction, and the calculated melting temperatures of the oligonucleotide sequences. The Deep Vent Polymerase used has a proof reading element in the 3' to 5' direction. Therefore the single stranded exonuclease activity only removes misincorporated bases from the 3' end. These conditions were modified until only the required product was isolated. The final reaction conditions are set out below;

Step 1:	95°C	5 mins (DNA denaturation)
Step 2:	94°C	30 secs (To prevent DNA reannealing)
Step 3:	50°C	1 min (Annealing of oligonucleotides)
Step 4:	72°C	30 secs (DNA synthesis)
Step 5:	15°C	Hold temperature.

Thirty cycles were completed from step 2 to step 4.

The synthetic oligonucleotides were designed to incorporate cleavage sites for the *Bam*HI restriction endonuclease both before the start of the *pre* open reading frame and at the end of the synthetic insulin gene. The products of the PCR were checked by agarose gel electrophoresis and the correct sized product of 470 bp isolated from the gel by electroelution. The DNA fragment isolated was cleaved with restriction endonuclease *Bam*HI to produce cohesive ends and ligated to DNA of plasmid vector pUC19, which had also been digested with restriction endonuclease *Bam*HI. The PCR product cloned in pUC19 was then sequenced to check there was no misincorporation of bases during the PCR procedure. An autoradiograph of the corresponding sequence can be seen in figures 3.1 and 3.2. The sequence generated was compared with the known sequence and it was noted that a one base substitution had occurred during the PCR procedure. The base substitution involved the replacement of a cytosine residue with a guanine residue at codon A13 of the insulin sequence, which results in the amino acid change, leucine to phenylalanine.

**Figure 3.2.** Sequence of Ins1 of the PCR product MF $\alpha$ /Ins1, cloned into the vector pUC19 to facilitate sequencing.

(a) A representative autoradiograph of the MF $\alpha$  *pre-pro* sequence. The reverse -48 primer (see experimental procedures) was used for sequencing from pUC19, which yields the top coding strand of the MF $\alpha$  *pre-pro*. The MF $\alpha$  *pre-pro* DNA sequence read from the bottom to the top of the autoradiograph (shown below) was checked against the known MF $\alpha$  *pre-pro* sequence on the data base and found to have no errors.

5' CGA TTT TAT TCG CAG CAT CCT CCG CAT TAG CTG CTC CAG TCA

ACA CTA CAA CAG AAG ATG AAA CGC CAC AAA TTC CGG CTG AAG

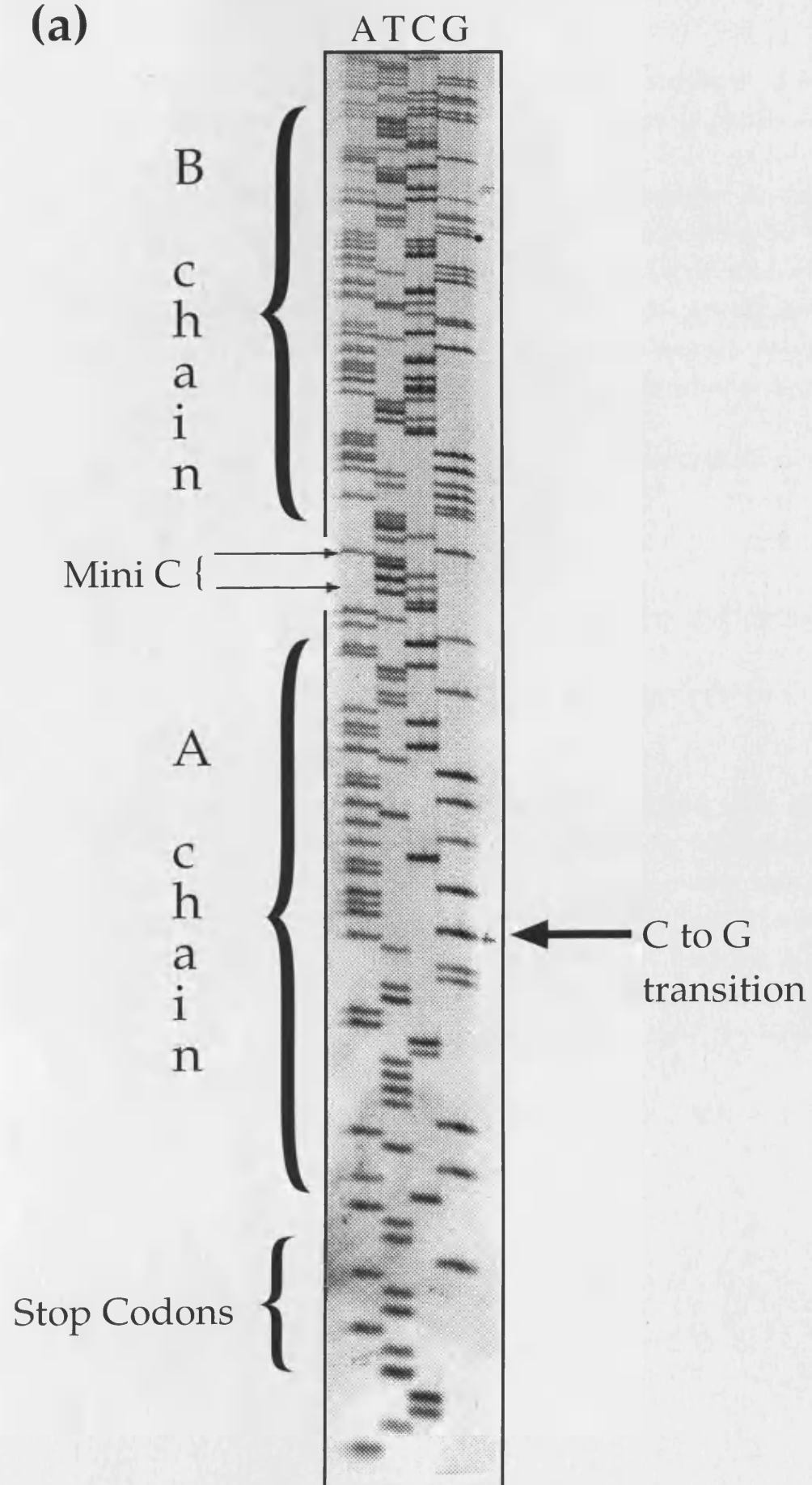
CTG TCA TCG GTT ACT CAG ATT TAG AAG GGG ATT TCG ATG TTG

CTG TTT TGC CAT TTT 3'

(b) The Insulin B-chain/MF $\alpha$ *pre-pro* junction. The universal -20 primer (see experimental procedures) was used for sequencing from pUC19, which yields the bottom non-coding strand of mini-proinsulin sequence in the reverse orientation. The junction of the MF $\alpha$ *pre-pro* leader peptide and the B-chain peptide is arrowed. Given below is the extrapolated DNA sense sequence of the junction. Where the Insulin B-chain peptide DNA is given in normal print and the MF $\alpha$ *pre-pro* leader peptide DNA in bold print.

5' CAA AAC GTT TTC **AAG TAA GGA** 3'

(a)



**Figure 3.2.** Sequence of Ins1 of the PCR product MF $\alpha$ /Ins1, cloned into the vector pUC19 to facilitate sequencing.

(a) A representative autoradiograph of the MF $\alpha$  *pre-pro* sequence. The reverse -48 primer (see experimental procedures) was used for sequencing from pUC19, which yields the top coding strand of the MF $\alpha$  *pre-pro*. The MF $\alpha$  *pre-pro* DNA sequence read from the bottom to the top of the autoradiograph (shown below) was checked against the known MF $\alpha$  *pre-pro* sequence on the data base and found to have no errors.

5' CGA TTT TAT TCG CAG CAT CCT CCG CAT TAG CTG CTC CAG TCA

ACA CTA CAA CAG AAG ATG AAA CGC CAC AAA TTC CGG CTG AAG

CTG TCA TCG GTT ACT CAG ATT TAG AAG GGG ATT TCG ATG TTG

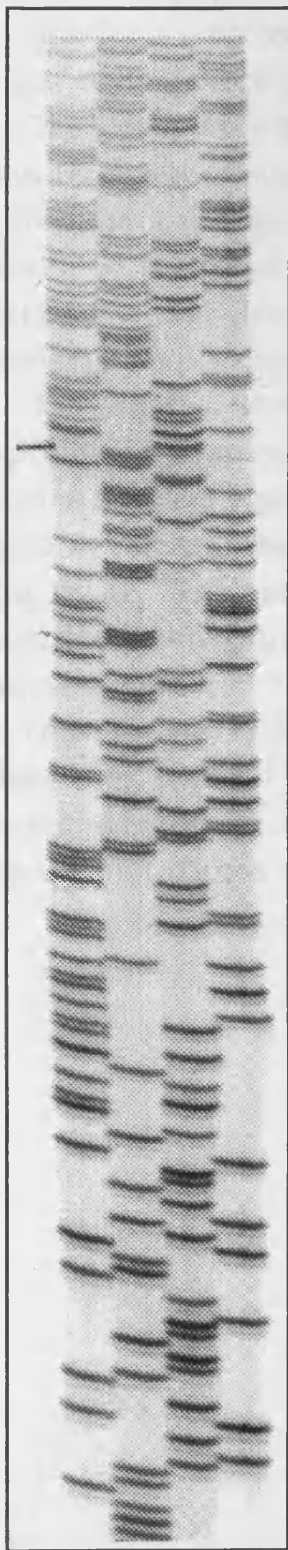
CTG TTT TGC CAT TTT 3'

(b) The Insulin B-chain/MF $\alpha$  *pre-pro* junction. The universal -20 primer (see experimental procedures) was used for sequencing from pUC19, which yields the bottom non-coding strand of mini-proinsulin sequence in the reverse orientation. The junction of the MF $\alpha$  *pre-pro* leader peptide and the B-chain peptide is arrowed. Given below is the extrapolated DNA sense sequence of the junction. Where the Insulin B-chain peptide DNA is given in normal print and the MF $\alpha$  *pre-pro* leader peptide DNA in bold print.

5' CAA AAC GTT TTC **AAG TAA GGA** 3'

(a)

A T C G



(b)

A T C G



B-chain/MFα  
junction

B-chain

*MFα pre-pro*



A number of clones were sequenced and all showed the same base substitution indicating an early PCR error. However this is not at a residue considered to be critical in folding and receptor recognition (Hua *et al.*, 1991) and is therefore acceptable for a reporter protein. It was not necessary therefore to report the PCR and cloning procedure to obtain a none-defective clone.

The 470 bp *MFα* leader/insulin cassette was isolated from pUC19-*MFα*/insulin by digestion with *Bam*HI, electrophoresis and the DNA electroeluted from the appropriate gel slice. This fragment was then cloned into the unique *Bgl*II site of the yeast expression vector pBEJ15 (figure 3.3a); as the two restriction enzymes cleave DNA to produce compatible cohesive ends, to form the recombinant plasmid pBG1 (figure 3.3b.).

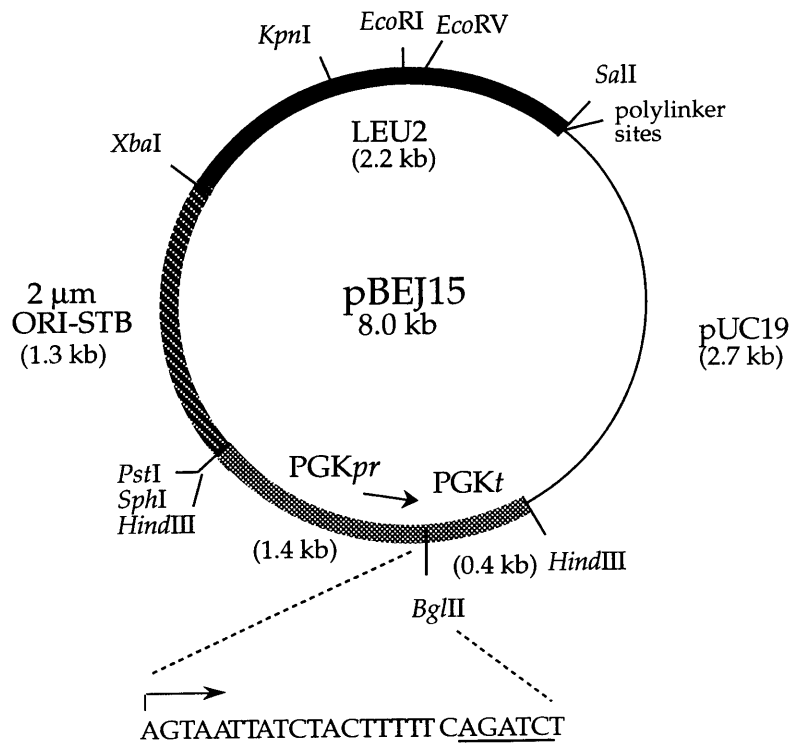
The three *esi* strains were transformed with pBG1, which carries the mini-proinsulin coupled to the *PGK1* promoter. Transformants were isolated on leucine deficient agar plates, as the vector pBG1 contains the *LEU2* marker, cultured in minimal medium and then inoculated into YPD for the assay of insulin levels. The amount of mini-proinsulin secreted by the strains was measured after the cultures had reached O.D<sub>650</sub> of 2. This again showed that the mutants produced 2- to 3-fold more secreted insulin than wild-type (Table 3.1b). Thus, the elevated yields were independent of promoter type. Generally the levels of secreted insulin, when expression is driven by the *PGK1* promoter, were lower than those seen when expression was driven by the *MFα* promoter. This is consistent with observations made by Jordan (1993).

**Figure 3.3.** Construction of the expression vector pBG1.

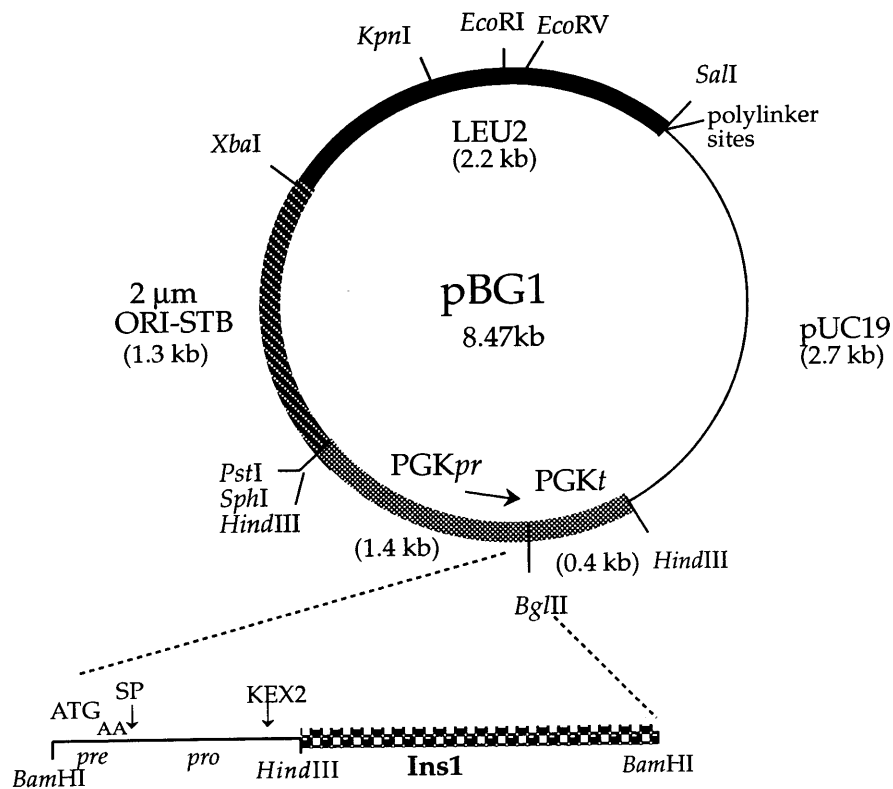
(a) Expression vector pBEJ15 ((Jordan, B. 1993) that contains a unique *Bgl*III cloning/expression site generated by *Bal*31 deletion initiated from within the natural coding sequence of the gene, deleting the ATG translation start codon, but retaining the transcription start-point. A linker was then added to insert the restriction site. The arrow on the DNA sequence marks the transcription start-point.

(b) Expression vector pBG1, with the *MF $\alpha$ pre-pro* mini-proinsulin sequence inserted at the unique *Bgl*III site.

(a)



(b)



### 3.2.4 Transcriptional analysis of *esi* mutants.

In order to assess whether the phenotype of the mutants was due to a gain in efficiency of the secretion pathway or simply due to elevated expression levels, transcript analysis was carried out. The strains were grown overnight to an OD<sub>650</sub> of approximately 2 and total RNA isolated from BF307-10 (wild-type) and *esi1*, 4 and 9 strains containing pDP314-Ins and also from a wild-type control strain containing no plasmid. The level of insulin transcript present in the total RNA prepared was measured by Northern blot hybridisation (figure 3.4a). Hybridisation with an actin probe (figure 3.4a) was included as a control for variations in sample preparation and the levels of hybridisation to the actin and insulin probes quantified by phosphorimager (figure 3.4b). As shown in figure 3.4, none of the mutants contained more mini-proinsulin mRNA than the wild-type control. Thus the increased secretion of mini-proinsulin in the mutants was not due to elevated transcription, resulting from elevated promoter activity or elevated gene dosage due to raised plasmid copy number.

The ratio of mini-proinsulin to actin mRNA levels from *esi1* was slightly reduced compared to wild-type, but in the cases of *esi4* and *esi9* the amount of mini-proinsulin mRNA was significantly reduced (by approximately 5-fold and 30-fold, respectively). As reduced *denovo* transcription levels typically result in reduced product yields it is not immediately obvious how such decreases in mini-proinsulin mRNA can be linked with the enhanced secretion phenotype. One possibility these results suggest, is that the mini-proinsulin message may be being used and turned over at a faster rate to facilitate the enhanced secretion yield. However, there is no direct correspondence between the extent of product yield increase and message levels, as *esi1* showed the greatest mini-proinsulin secretion, but the smallest decrease in mRNA. The *esi4* and *esi9* mutants both gave a two-fold increase in mini-proinsulin secretion, but different reduced levels of mRNA.

**Figure. 3.4.** Mini-proinsulin transcripts in *esi* mutants. The filter was hybridised separately with the insulin and actin probes, before being exposed to X-ray film.

**(a)** Northern blot of total yeast RNA (10µg total RNA loaded per track) from wild-type BF307-10 and mutant strains carrying plasmid pDP314-Ins1 hybridized with mini-proinsulin (Insulin) and actin (Actin) probes. This is a composite picture of the two X-ray films.

**Lane1:** *esi1* pDP314-Ins1

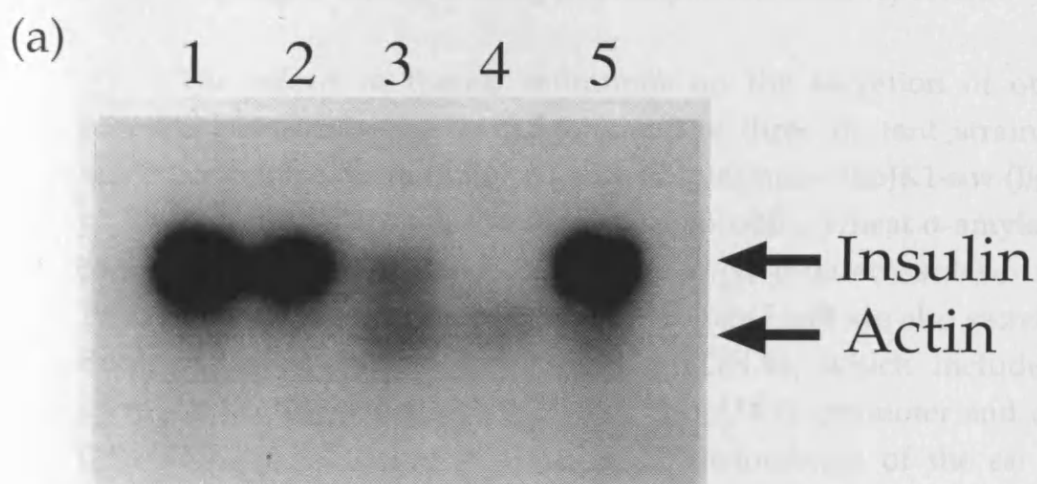
**Lane2:** *esi4* pDP314-Ins1

**Lane3:** *esi9* pDP314-Ins1

**Lane4:** BF 307-10 negative control (no plasmid present)

**Lane5:** BF 307-10 pDP314-Ins1 (positive control)

**(b)** Phosphorimager quantification of mRNA levels.



(b)

Strain	mRNA abundance		Relative mRNA abundance
	Insulin	Actin	insulin/actin
+	126277	3997	31.6 (1.0x)
esi1	118941	4574	26.0 (0.8x)
esi4	53416	8889	6.0 (0.2x)
esi9	12979	9020	1.4 (0.04x)

† Phosphorimager counting units (mean of duplicates)

### 3.2.5 Phenotypic analysis using alternative secretion reporters

The effects of the *esi* mutations on the secretion of other secretory reporter proteins were investigated. The three mutant strains, along with wild-type parent, were transformed with plasmids YEpJK1- $\alpha$ w (figure 3.5a) and pVK1.1 (figure 3.5b), which carry genes encoding wheat  $\alpha$ -amylase (Kirkham., 1989; Rothstein, 1984) and *Aspergillus niger*  $\beta$ -galactosidase (Kumar *et al.*, 1992). These two proteins are naturally secreted and are also secretable by yeast. Both plasmids contain the encoding cDNAs, which include the natural secretion leader sequence, coupled to the *ADH1* promoter and carry the 2 $\mu$ m *ORI-STB* for multicopy replication. Transformants of the *esi* mutants and wild-type BF307-10, were cultured in selective liquid medium lacking tryptophan for the transformants containing YEpJK1- $\alpha$ w and lacking uracil for transformants containing pVK1.1. All strains were cultured overnight in minimal media and then inoculated into YPD medium, and harvested when they had reached an OD<sub>650</sub> of 1.5 (approximately  $4 \times 10^7$  cells/ml). Assays on cell extracts and secreted products were performed. The DNS assay (see Chapter 8) was used to ascertain wheat  $\alpha$ -amylase activity as the formation of maltose from soluble starch, and  $\beta$ -galactosidase activity was measured by the rate of ONPG hydrolysis (see Chapter 8). Both reactions could be monitored spectrophotometrically.

Wheat  $\alpha$ -amylase is non-glycosylated like mini-proinsulin, but at approximately 30 kDa it is about 5-times as large. As shown in Table 3.2a., it is secreted with approximately 99% efficiency from wild-type BF307-10 yeast cells. The *esi1* mutant showed the same secretion pattern and yield as wild-type BF307-10, in contrast to secreting 3-fold more mini-proinsulin than wild-type BF307-10. However, the *esi4* mutant secreted about 3-times more  $\alpha$ -amylase than wild-type, which, in combination with the amount of product found in the cells, constituted almost a 3-fold increase in total yield. This represents a greater enhancement of product yield than observed with mini-proinsulin (approximately 2-fold increase). In contrast again, the *esi9* mutant failed to secrete any  $\alpha$ -amylase, whilst the amount found in the cell showed an almost 8-fold increase in yield over wild-type, but with an overall ten-fold decrease in total amylase yield. This failure to secrete differs from the phenotype with mini-proinsulin, in that secretion is restored following meiosis (see chapter 4). Figure 3.6. shows the phenotypes as seen on starch plates. Thus, the mutant phenotypes were different for the different secretory product.

Analysis of the amylase transcripts produced in the mutants (figure 3.7) also showed differences with mini-proinsulin. Thus, *esi1* cells contained

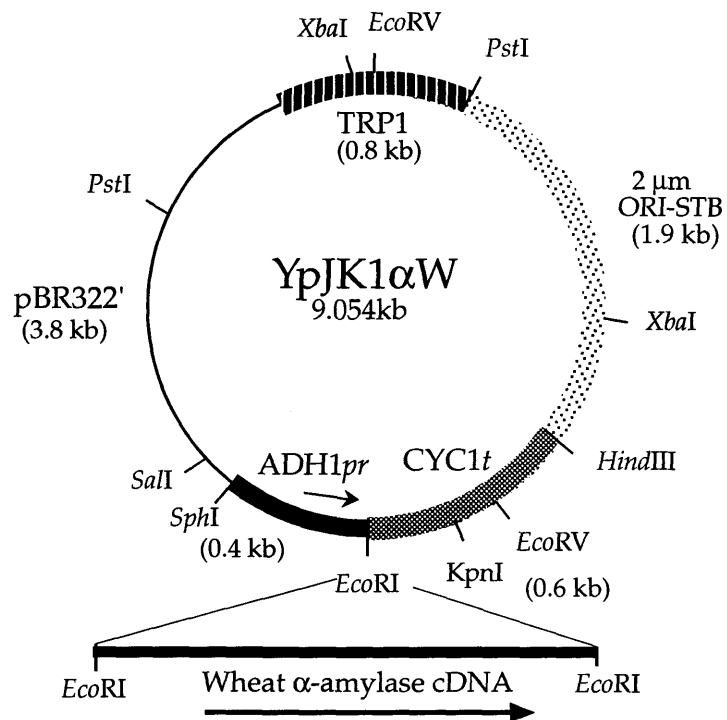
**Figure 3.5.** Vectors for the expression of wheat  $\alpha$ -amylase and *A.niger*  $\beta$ -galactosidase cDNAs.

**(a)** YpJK1 $\alpha$ w (Kirkham, J. 1989). Shows the wheat  $\alpha$ -amylase cDNA, cloned into the unique EcoRI site, (again generated by *Bal*31 deletion and linker insertion) with the *ADH1* promoter utilised to drive expression.

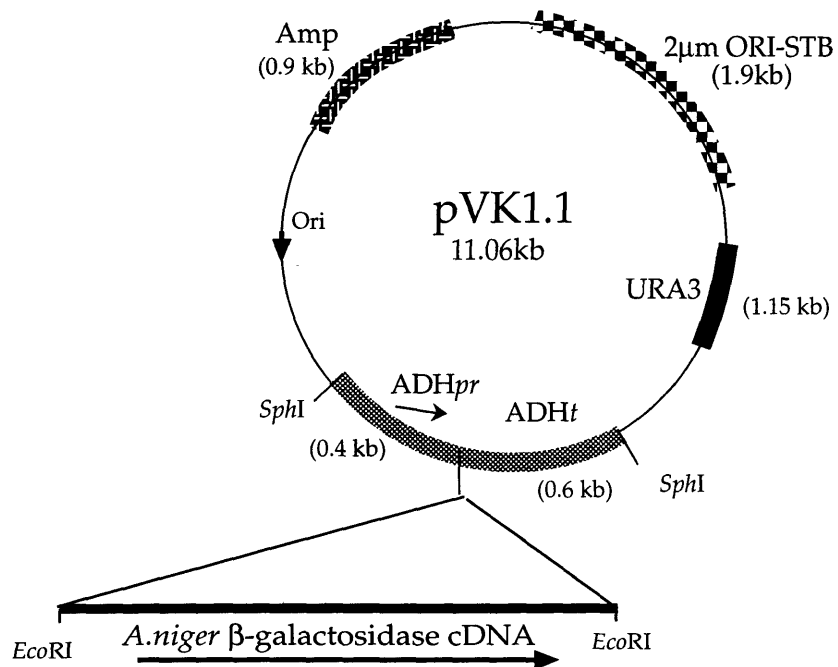
**(b)** pVK1.1 (Kumar *et al.*, 1992). Shows the *A.niger*  $\beta$ -galactosidase cDNA cloned into the unique EcoRI site, with the *ADH1* promoter being utilised to drive expression.



(a)



(b)



**Table 3.2.****(a)** The effect of *esi* mutations on the secretion of wheat  $\alpha$ -amylase. <sup>†</sup>

Strain	$\alpha$ -amylase activity.*		% Secreted	Relative Yield.	
	Cell	Supernatant		Secreted	Total
+	0.55	40.5	98.7	1.0x	1.0x
<i>esi</i> 1	0.71	50.5	98.6	1.2x	1.2x
<i>esi</i> 4	1.57	124.7	98.7	3.1x	3.1x
<i>esi</i> 9	4.3	0	0	0	0.1x

<sup>†</sup> BF307-10 parent and mutant cells containing YEpJK1- $\alpha$ w. Strains were cultured in minimal medium, inoculated into non-selective YPD medium and cells harvested when they had reached an O.D.<sub>650</sub> of 1.5

\* Maltose produced in mg/ml/of supernatant or cells as determined spectrophotometrically by DNS assay (see Experimental procedures). The figures are the mean of duplicate assays.

**(b)** The effect of *esi* mutations on the secretion of *A.niger*  $\beta$ -galactosidase. <sup>†</sup>

Strain	$\beta$ -galactosidase activity.*		% Secreted	Relative Yield.	
	Cell	Supernatant		Secreted	Total
+	40	8.3	17	1.0x	1.0x
<i>esi</i> 1	47.7	12.8	21	1.5x	1.2x
<i>esi</i> 4	45.5	10.2	18	1.2x	1.2x
<i>esi</i> 9	8.7	0.67	7	0.08x	0.2x

<sup>†</sup> BF307-10 parent and mutant cells containing pVK1.1.

\* nM of ONPG converted/minute (see Experimental procedures). The figures are the mean of duplicate assays.

**Figure 3.6.** *esi* mutant phenotypes after transformation with YpJK1 $\alpha$ w. Single colonies have been streaked onto minimal medium containing 2% starch and lacking tryptophan. The diploid strain *esi9* X BJ2168 has been included to show that amylase secretion is restored after mating. The areas of starch hydrolysis are easily distinguishable as zones of clearing. The plates are marked as to which strain they contain.

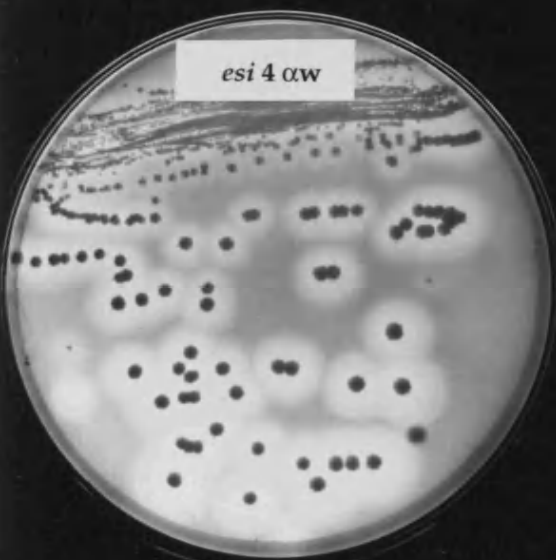
BF 307-10  $\alpha$ w (WT)



*esi* 1  $\alpha$ w



*esi* 4  $\alpha$ w



*esi* 9  $\alpha$ w



*esi* 9 X BJ2168



**Figure 3. 7.** Wheat  $\alpha$ -amylase transcripts in *esi* mutants. The filter was hybridised separately with the wheat  $\alpha$ -amylase and actin probes, before being exposed to X-ray film.

**(a)** Northern blot of total yeast RNA (10 $\mu$ g total RNA loaded per track) from wild-type BF307-10 and mutant strains carrying plasmid YEpJK1- $\alpha$ w hybridized with wheat  $\alpha$ -amylase (Amylase) and actin (Actin) probes. This is a composite picture of the two X-ray films.

**Lane1:** BF 307-10 negative control (contains no plasmid).

**Lane2:** BF 307-10 YEpJK1- $\alpha$ w positive control.

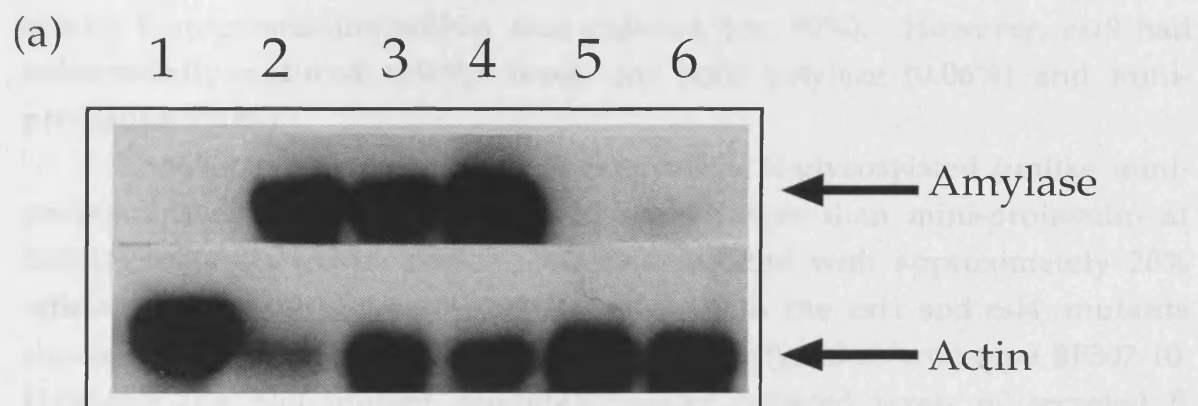
**Lane3:** *esi1* YEpJK1- $\alpha$ w.

**Lane4:** *esi4* YEpJK1- $\alpha$ w.

**Lane5:** *esi9* YEpJK1- $\alpha$ w.

**Lane6:** *esi9* YEpJK1- $\alpha$ w X BJ2168 diploid.

**(b)** Phosphorimager quantification of mRNA levels.



(b)

Strain	mRNA abundance		Relative mRNA abundance	
	Amylase	Actin	Amylase/Actin	
+	60566	3905	15.0	(1.0x)
esi1	5852	11254	5.2	(0.3x)
esi4	117113	7284	16.0	(1.0x)
esi9	<100	12833	<0.008	(<0.0005x)
esi9/+	5109	10322	0.5	

† Phosphorimager counting units (mean of duplicates)

reduced levels (ca. 40%) of amylase mRNA, compared with levels similar to the non-mutant parent for mini-proinsulin (fig3.4). Mutant *esi4* showed the converse, with amylase mRNA levels similar to the non-mutant parent, whilst mini-proinsulin mRNA was reduced (ca. 40%). However, *esi9* had substantially reduced mRNA levels for both amylase (0.06%) and mini-proinsulin (10%).

The *A.niger*  $\beta$ -galactosidase is extensively N-glycosylated (unlike mini-proinsulin) and is approximately 20 times larger than mini-proinsulin at 110kDa. As shown in Table 3.2b., it is secreted with approximately 20% efficiency from wild-type BF307-10 cells. Both the *esi1* and *esi4* mutants showed essentially the same secretion pattern and yield as wild-type BF307-10. However the *esi9* mutant provided greatly reduced levels of secreted  $\beta$ -galactosidase (0.08 times that of wild-type) as well as a reduced level within the cell (0.2 times that of wild-type) and therefore a reduced overall total yield (0.2 times that of wild-type). This pattern is very similar to the observed with the wheat  $\alpha$ -amylase secretion reporter. The overall pattern indicated by these results suggests that the enhanced secretion phenotype seen for mini-proinsulin is specific for that reporter, and not a general effect seen with all secreted proteins. No transcript analysis were therefore performed in the mutants containing the  $\beta$ -galactosidase reporter plasmids.

The reduced mRNA levels in the mutants may have resulted from either a reduced transcription rate or more rapid turnover. This question was addressed by examining the abundance of a mRNA encoding a cytoplasmic protein expressed from the *ADH1* promoter. We already had data for the expression of wheat  $\alpha$ -amylase from the ADH1 promoter.

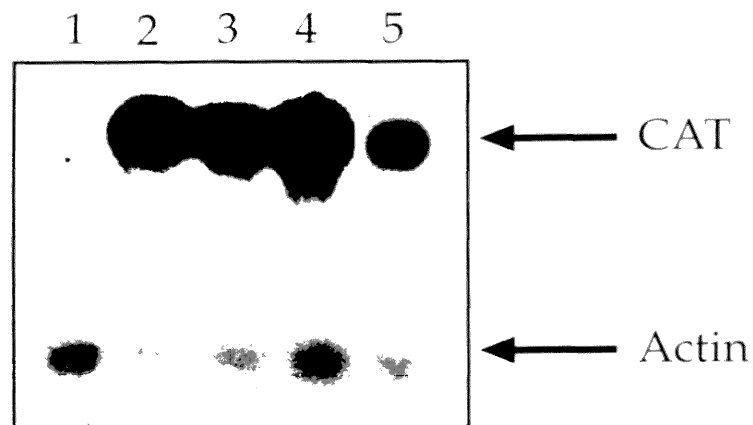
### 3.2.6 Effect of mutations on cytoplasmic reporter expression.

By considering the effects of the *esi* mutations on cytoplasmic reporter expression, we were asking the question whether the mutations were specific for secretory reporter expression or general affecting cytoplasmic protein genes as well. Thus the *esi* mutants and non-mutant parental strains were transformed with pCH100 (Hadfield *et al.*, 1986), a 2 $\mu$ m-based plasmid encoding chloramphenicol acetyltransferase (CAT), a cytoplasmically expressed protein. This plasmid was chosen for two reasons; firstly expression of CAT was from the *ADH1* promoter and secondly it is cytoplasmic in nature. This second feature would enable us to determine whether the reductions seen in mRNA levels for the *esi* mutations are specific for genes encoding proteins destined for the secretion pathway. Transformed yeast strains were cultured overnight in selective YPGE/chloramphenicol selective medium and harvested when cells had reached a concentration of  $1 \times 10^7$  cells/ml. CAT enzyme assays of total soluble cell protein extracts showed that expression levels in *esi1* and *esi4* cells were the same as in the non-mutant parent (figure 3.8b). This was associated with similar levels of mRNA (figure 3.8a). Thus, the decreases in mRNA level and altered secretion yields associated with these two mutants appear to be due to defects specifically within the secretion pathway.

In the case of *esi9*, the level of CAT mRNA was reduced two fold but no product was detected (Figure 3.8a and b). This indicates a possible defect at the level of translation. This was examined further by transforming the *esi9* mutant and wild-type control strains with plasmid pLG669-Z, which expresses cytoplasmic  $\beta$ -galactosidase from the *CYC1* promoter (Guarente and Ptashne, 1981). In the wild-type BF307-10 strain, an average (from 4 different transformants) of 260  $\beta$ -galactosidase activity units (nmol ONPG converted per minute per OD<sub>650nm</sub> culture) was found in the cells, compared with only 0.9 units in *esi9* cells. This substantiated the occurrence of a blocking effect of the *esi9* mutation on the translation of the cytoplasmic reporter genes. A universal blockage of cytoplasmic expression would prove greatly debilitating - if not lethal - to the cells, however, the *esi9* cells showed no difference in growth rate to non-mutant cells. Nor were there any other signs of toxicity that would be expected with such an effect. This suggested that it could be atypical, possibly reflecting the fact that both the CAT and  $\beta$ -galactosidase genes are heterologous and contain highly unfavourable codons for translation in yeast.



(a)



(b)

Strain	mRNA abundance †		Relative mRNA abundance CAT / actin	CAT units per mg cell protein	ADH units per mg cell protein
	CAT	Actin			
+	444321	8728	50.9 (1.0x)	63.5	0.27
esi1	326498	7477	43.7 (0.9x)	63.5	0.32
esi4	453410	8224	55.1 (1.1x)	78.7	0.28
esi9	74760	3629	20.6 (0.4x)	none	0.30

† Phosphorimager counting units (mean of duplicates)

**Figure 3.8** Expression of chloramphenicol acetyltransferase in *esi* mutants containing pCH100.

**(a)** Northern blot of total yeast RNA (10µg total RNA loaded per track) from wild-type BF307-10 and mutant strains carrying plasmid pCH100 hybridized with chloramphenicol acetyltransferase (CAT) and actin (Actin) probes.

**Lane1:** BF 307-10 negative control (contains no plasmid).

**Lane2:** BF 307-10 pCH100 positive control.

**Lane3:** *esi1* pCH100.

**Lane4:** *esi4* pCH100.

**Lane5:** *esi9* pCH100.

**(b)** Quantification of mRNA levels (by phosphorimager) and CAT and ADH protein product levels (by enzyme assay).

To investigate this possibility, intracellular levels of alcohol dehydrogenase (ADH) were assayed in mutant and wild-type cells. The ADH in glucose-grown mid-log phase cells is mainly encoded by *ADH1*, with *ADH2* and *mADH* expression being repressed (Ciriacy, 1975). Importantly for this investigation, *ADH1* has close to optimal codon usage (Sharp, 1986). It was found that non-mutant control cells, *esi9*, and also the other two *esi* mutants, all contained approximately 0.3 ADH units per mg cell protein (see figure 3.8). Thus, the *esi9* mutation had no effect on expression of the highly expressed, major codon containing *ADH1* gene. Poor codon adaptation, as in the CAT and  $\beta$ -galactosidase genes, is thought to cause translational stalling (Herrick, 1990), which may act in association with the mutant *esi9* phenotype to inhibit cytoplasmic expression. This would appear to confirm that the blockage of cytoplasmic expression in *esi9* is atypical affecting only those genes that contain unfavourable codons for translation in yeast.

### 3.3 Discussion.

Although mutant *esi1* showed elevated levels of secreted mini-proinsulin with unaltered mRNA levels, in the cases of *esi4* and *esi9* the phenotype was associated with reduced mRNA levels. The latter is most surprising, as it would appear unlikely that reduced primary transcription would result in increased secretion yield. In fact, it would be expected to have the reverse effect. This might arise if the product was toxic to the cells - which it does not appear to be, as both growth rates and plasmid stability are unaffected (see Hadfield *et al*, 1993, for review of effects of toxic products on yeast cells). Another possibility is that by expressing a heterologous protein at a high level a 'log-jam' effect at a rate-limiting step may be caused, which is reduced by reducing expression. Alternatively, primary transcription may be unchanged in the mutants, but mRNA turnover rate increased in *esi4* and *esi9* for some reason.

Additionally, when wheat  $\alpha$ -amylase was expressed in the same cells using the *ADH1* promoter rather than *MF $\alpha$ 1*, thereby eliminating promoter-specific effects, its mRNA level was reduced in *esi1* and *esi9*, but not in *esi4*. Furthermore, the secretion phenotypes for the amylase reporter differ to those for mini-proinsulin: *esi1* was no different to non-mutant levels; *esi4* showing 5-fold more secreted product and a 3-fold increase in total yield; and *esi9* showing no secretion, but a 3-fold elevation of intra-cellular amylase.

Gains in product yield without associated transcriptional increases suggest gains in translation efficiency. Analysis of cytoplasmic CAT expression showed no differences in product or mRNA yields for *esi1* and *esi4*, indicating that reduced mRNAs and elevated products were specific for secretory proteins. In the case of *esi9* CAT, expression was reduced but not the mRNA level, indicating that expression of CAT was being impeded in the mutant.

The effects at the level of mRNA and translation associated with the three *esi* mutants suggest that all act at an early stage of the secretion pathway and translocation into the endoplasmic reticulum. This would support the view that the main barrier to secretion of mini-proinsulin - the reporter with which they were isolated - is at this level. Reduced mRNA half-life has been associated with translational pausing. The signal recognition particle (SRP) is thought to interact with the secretion leader peptide directly upon synthesis by the ribosome and cause a translational pausing (Siegel, 1988; Wolin, 1989). Its action in initiating protein secretion can therefore be linked with the phenotypes of enhanced secretion yield and reduced steady-state mRNA level.

The observations made in this study lead us to a different view than that proposed by Siegel (1988) or Wolin(1989).

Differences in phenotype of amylase secretion may come about because the leader peptide is different, causing altered efficiency of interaction with the SRP, or because the amylase is transcribed and translated at a lower rate and is also larger. This may also be the same for  $\beta$ -galactosidase secretion.

Indications from gene expression analysis of the *esi* mutants is that they do not affect the same function within the yeast cell. Therefore they may be mutations in three different genes all acting at an early stage of the secretion pathway.

## Chapter Four.

### GENETIC ANALYSIS OF ESI MUTANTS.

#### 4.1 Introduction.

The next stage in the analysis of the *esi* mutants utilised a genetic approach. Here a number of questions would need to be addressed concerning the mutations.

(1) Were they dominant or recessive?

(2) Are the phenotypes due to mutations in single genes or as can happen with random chemical mutagenesis, mutations in a number of different genes.

(3) Were the three *esi* mutations in separate genes.

As the mutants were isolated in a haploid background it is possible to answer the above questions by mating the *esi* mutants initially with a wild-type haploid *MATa* strain (as the original strain used for mutagenesis was *MATα*), and then against each other.

## 4.2 Results.

### 4.2.1 Dominance tests.

The first part of the genetic analysis was to determine whether the *esi* mutations were dominant or recessive. To achieve this heterozygous diploids were made by mating each of the *esi* mutant strains and the wild-type parent (BF307-10) control, all of which were *MAT $\alpha$*  and contained plasmid pBG1, with a suitable *MATa* strain. The initial *MATa* strain selected was 74/2. However this cross failed to produce viable spores even with the wild-type control, after sporulation of the diploid and tetrad dissection. This incompatibility between yeast strains of opposite mating types forming inviable spores, is occasionally witnessed (general laboratory observation). Another *MATa* strain was used (JHRY20-2C) and the cross set up as detailed below;

BF307-10 *esi*<sup>+</sup>-(pBG1)*MAT $\alpha$*  X JHRY20-2C *MATa*

Matings were carried out as described in the experimental procedures and diploids selected on plates lacking the amino acids leucine, tryptophan, arginine, adenine and methionine (therefore not allowing haploids to grow). The diploids were then grown in selective liquid minimal medium and the amounts of mini-proinsulin secreted into the medium assayed. The results (Table 4.1a) showed that heterozygous diploids containing an *esi* mutation gave rise to a similar amount of secreted mini-proinsulin as that made with the wild-type parent control. This indicated that each of the *esi* mutations is recessive in nature.

### 4.2.2 Meiotic Segregation Analysis: are the *esi* mutations in single genes ?

The diploids of each cross including the wild-type parental control were sporulated after being cultured in enriched YPD presporulation medium. This was achieved by plating the diploids under conditions of nitrogen starvation (see experimental procedures) which allows cells to commit to the meiotic cell cycle, so creating four haploid spores. These four haploid spores, known as a tetrad, are encased in an ascus (which can be digested away to allow dissection) and are the product of a single meiotic event. Dissection of these tetrads and

**Table 4.1.** Amount of mini-proinsulin secreted into the culture medium by *esi* diploids containing pBG1.

**a.** *esi*/wild type diploids.

Diploid <sup>a</sup>	Insulin mU/L
+/+	97 (± 11)
<i>esi</i> 1/+	75 (±13)
<i>esi</i> 4/+	106 (± 15)
<i>esi</i> 9/+	77 (± 13)

<sup>a</sup> BF307-10 MAT $\alpha$  *esi*<sup>±</sup> (pBG1) / JHRY20-2C MAT $\alpha$  = +/+

**b.** *esi*/*esi* diploids (complementation tests).

Diploid <sup>b</sup>	Insulin mU/L
+/+	100 (± 15)
<i>esi</i> 1/ <i>esi</i> 4	83 (± 15)
<i>esi</i> 4/ <i>esi</i> 9	82 (± 17)
<i>esi</i> 1/ <i>esi</i> 9	94 (± 28)

<sup>b</sup> BF307-10 MAT $\alpha$  *esi*<sup>-</sup> (pBG1) / BF307-10 MAT $\alpha$  *esi*<sup>-</sup> URA3<sup>+</sup> = +/+



germination of each of the four spores individually allows study of the products of a single meiosis. Each of the four haploid progeny from complete tetrads (ones where all four spores germinated) from the diploid strains were cultured in selective liquid medium, inoculated into non-selective YPD liquid medium and grown to an  $OD_{650}=2$ , before analysis by ELISA.

With the BF307-10 control strain all four spores from complete tetrads showed the same (wild-type) levels of insulin secretion. In the case of the *esi4*/+ heterozygous diploid, a 2:2 ratio of inheritance of the enhanced secretion phenotype to wild-type was obtained for ten complete tetrads analysed. This indicated that the *esi4* mutation occurred in a single chromosomal gene. A selection of analysed tetrads are represented in Table 4.2. A number of other chromosomal mutations, such as those for adenine (*ade4*, *ade6*), methionine (*met4*) and tryptophan (*trp1*) auxotrophies were also shown to display allelic segregation from their wild type alleles.

The *esi1*/+ heterozygous diploid showed 2:2 segregation for the presence or absence of the plasmid pBG1. This showed the possibility of a second chromosomal mutation affecting meiotic cell division inheritance of the 2 $\mu$ m based plasmids. To test whether this defect was specific to meiosis, the mitotic stability of plasmid pBG1 was analysed in *esi1* mutant strains over a period of approximately 60 cell doublings. The results indicated no difference between plasmid stability in the *esi1* haploid strain compared with its non-mutant (*ESI1*+) BF307-10 parent strain, indicating that the defect was specific to meiotic division.

This meiosis specific mutation was further investigated. Mutant strain *esi1* containing plasmid YpJK1 $\alpha$ w, was mated with wild-type MATa strain BJ2168 to form diploids. The diploids were sporulated under conditions of nitrogen starvation and the resulting tetrads dissected. The spores were germinated on non-selective YPD solid medium and then replica plated onto selective solid media, to screen for maintenance of the plasmid. Again a 2:2 segregation for presence or absence of the plasmid YpJK1 $\alpha$ w was noted indicating that the mutation giving plasmid instability occurred at a single single locus.

Of the twenty complete tetrads that showed 2:2 segregation for plasmid pBG1, ten were selected for further analysis. In the ten complete tetrads those spores that failed to inherit the plasmid were cultured in non-selective YPD medium and then transformed with the plasmid pBG1. Each set of meiotic segregants were cultured in selective liquid medium and then inoculated into non-selective YPD liquid medium and grown to an  $OD_{650}=2$ . Analysis of secreted mini-proinsulin levels by ELISA showed a 2:2 segregation of the *esi1*

**Table 4.2.** Meiotic segregation analysis of *esi4*/*ESI4* heterozygotes of the enhanced secretion of insulin following the sporulation of *esi 4* pBG1/JHRY20-2CMATa *ESI*<sup>+</sup> diploids.

Sporulated diploid Asci <sup>a</sup>	Yield of secreted mini-proinsulin (μU/ml) from germinated spores <sup>b</sup> ,				Ratio + : <i>esi</i>
	A	B	C	D	
+ / + (control) asci	99	82	89	80	4 : 0
<i>esi4</i> / + asci	100	98	200	178	2 : 2
1	76	81	224	241	2 : 2
2	228	232	491	441	2 : 2
3	103	90	241	274	2 : 2
4					

<sup>a</sup> BF307-10 MATα *ESI*<sup>+</sup> (pBG1)/JHRY20-2C MATa *ESI*<sup>+</sup> = + / + diploid.

*esi4* MATα *esi*<sup>-</sup> /JHRY20-2C MATa *ESI*<sup>+</sup>

<sup>b</sup> By ELISA assay (see Methods); mean values from 3 separate assays.

single chromosomal gene (Table 4.3 shows a selection of complete tetrads that were analysed). A number of other chromosomal mutations, such as adenine (*ade4,6*), methionine (*met4*) and tryptophan (*trp1*) were shown to be inherited in a 2:2 ratio. The results indicated no linkage between the meiotic plasmid loss phenotype and the *esi* phenotype as meiotic products showed all four possible combinations;  $2\mu^- esi1^+$ ,  $2\mu^- esi1^-$ ,  $2\mu^+ esi1^+$  and  $2\mu^+ esi1^-$ . When *MAT $\alpha$  esi1* derivatives isolated from the initial *esi1* (pBG1) cross with JHRY20-2C, were backcrossed with strain JHRY20-2C, the 2:2 meiosis segregation phenotype was observed again. This backcross was performed in an attempt to try to separate the *esi* mutation from the lesion causing plasmid instability. These data seem to contradict the results recorded in Table 4.3. As only a small number of *MAT $\alpha$  esi1* derivatives (three) showing the enhanced secretion phenotype were backcrossed, no firm conclusion can be made about linkage between the two genes. Subsequently a further backcross between a *MAT $\alpha$  esi1* derivative (isolated from the first backcross) and a *ESI*<sup>+</sup> strain showed a 2:2 segregation of wild-type and meiosis specific plasmid instability phenotypes.

Heterozygous diploids formed from the cross of *MAT $\alpha$  esi9*pBG1 with *MAT $\alpha$*  strain JHRY20-2C produced tetrads on sporulation medium. After digestion and dissection onto non-selective YPD solid medium very few of the spores germinated, making it impossible to complete the meiotic segregation analysis. However one advantage of the Singer MSM dissection machine, is the facility to utilise the machines' automated grid pattern to re-examine the dissected spores after incubation of the plate. Therefore it is possible to see at what stage of germination or development individual spores had arrested. As shown in figure 4.1, the spores arrested at different stages of germination and colony development; some were unable to germinate and remained as single cells (approx. 50%), whilst others had been able to go through one cell division or even a number to form a multicellular microcolony structure before arresting (approx. 40%).

A number of other *MAT $\alpha$*  strains were tested in matings with the *esi9* mutant (*MAT $\alpha$* ) to try to obtain a diploid that would sporulate to give asci with four viable spores. In all cases this was unsuccessful. In addition, it was also noticed that the *esi9* haploid strain eventually stopped secreting mini-proinsulin, or as an ultimate consequence of the mutant phenotype. This acquired secretion defect was maintained when the *esi9* mutant was transformed with a plasmid carrying a wheat  $\alpha$ -amylase gene, as described in chapter 3. The *esi9* YpJK $\alpha$ w1 (see chapter three) strain was mated against *MAT $\alpha$*  strain BJ2168 resulting in a diploid that was able to secrete  $\alpha$ -amylase. This showed the defect to be recessive as initially observed with mini-

**Table 4.3.** Meiotic segregation analysis of *esi1/ESI1* heterozygotes of the enhanced secretion of insulin phenotype following the sporulation of *esi1* pBG1/JHRY20-2C MATa *ESI*<sup>+</sup> diploids.

Sporulated diploid Asci <sup>a</sup>	Yield of secreted mini-proinsulin (μU/ml) from germinated spores <sup>b</sup> ,				Ratio + : <i>esi</i>
	A	B	C	D	
+ / + (control) asci	90	88	82	94	4 : 0
<i>esi1</i> / + asci <sup>c</sup>	12 <sup>+</sup>	20 <sup>+</sup>	319 <sup>-</sup>	231 <sup>-</sup>	2 : 2
	10 <sup>+</sup>	11 <sup>-</sup>	303 <sup>-</sup>	267 <sup>+</sup>	2 : 2
	10 <sup>-</sup>	9 <sup>+</sup>	269 <sup>+</sup>	265 <sup>-</sup>	2 : 2
	24 <sup>+</sup>	16 <sup>+</sup>	160 <sup>-</sup>	155 <sup>-</sup>	2 : 2

<sup>a</sup> BF307-10 MATα *ESI*<sup>+</sup> (pBG1)/JHRY20-2C MATa *ESI*<sup>+</sup> = + / + diploid.

*esi1* MATα *esi*<sup>-</sup> /JHRY20-2C MATa *ESI*<sup>+</sup> = *esi1* / + diploid.

<sup>b</sup> By ELISA assay (see Methods); mean values from 3 separate assays

<sup>c</sup> + and - respectively indicate presence or absence of inherited plasmid pBG1.

Those spores that did not contain the plasmid were transformed prior to assay.

**Figure 4.1** Individual spores dissected out from four spore asci onto YPD medium from the *esi9*pBG1/JHRY20-2C diploids after sporulation. Letters (a)-(d) show a selection of these germinated spore cultures from the same meiosis, that have arrested at different stages of development. The figure shows the aberrant morphology of these germinated spores.

(a)



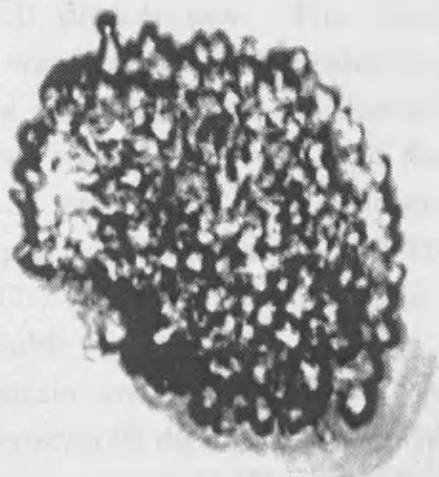
(b)



(c)



(d)



proinsulin as the secretion reporter protein. Sporulation of this diploid yielded 4 viable spores per ascus indicating that the defect in spore viability may be associated with the presence of secretory mini-proinsulin. Germination of the tetraspores and assay of  $\alpha$ -amylase secretion levels showed a 2:2 segregation of the enhanced secretion phenotypes versus wild-type, indicating that it was encoded by a single chromosomal gene (Table 4.4).

#### 4.2.4 Complementation analysis between *esi* mutants.

Complementation analysis was undertaken to investigate whether the *esi* mutations were in separate genes. Before the complementation analysis could be performed *MATa esi* haploids were recovered from tetrad dissections of *esi1*pBG1 x JHRY20-2C and *esi4*pBG1 x JHRY20-2C diploids. The phenotype of the spores were determined and are listed below;

*esi1 MATa* Leu<sup>+</sup> Trp<sup>+</sup> Met<sup>-</sup> Ura<sup>-</sup> His<sup>-</sup> Ade<sup>+</sup> (pBG1)

*esi4 MATa* Leu<sup>+</sup> Trp<sup>-</sup> Met<sup>+</sup> Ura<sup>-</sup> His<sup>-</sup> Ade<sup>-</sup> (pBG1)

As both of the *MATa* enhanced secreting spores were uracil auxotrophs, the *esi* (pBG1) mutant strains and wild-type strain BF307-10 were transformed with the *URA3* gene marker to make them uracil prototrophs. The yeast integrating vector YIp31 (Hadfield et al., 1987) was digested with restriction endonuclease *HindIII* and the *URA3* gene isolated as a 1.1kb fragment after gel electrophoresis. After purification the fragment was transformed into the strains allowing integration of the *URA3* gene into the genome. Transformants were isolated as uracil prototrophs. The *MATa esi4* (pBG1) strain was mated against the BF307-10 Ura<sup>+</sup> (pBG1) wild-type control and *esi1* or *esi9* Ura<sup>+</sup> *MATa* (pBG1) strains to create double heterozygous diploids, *esi1/esi4*, and *esi4/esi9*. The *MATa esi1* (pBG1) strain was mated with the *esi9* Ura<sup>+</sup> *MATa* (pBG1) strain to create a double heterozygous diploid carrying the *esi1/esi9* mutations, as well as with wild-type control strain *MATa* BF307-10 Ura<sup>+</sup> (pBG1) to check the effect of the integrated *URA3* gene on sporulation and spore viability. These diploids were then grown in selective liquid medium lacking leucine, methionine and uracil and the amounts of mini-proinsulin secreted into the medium assayed. In all cases, the levels were

**Table 4.4.** Secreted wheat  $\alpha$ -amylase from meiotic segregants of *esi9*/ESI9 heterozygotes following the sporulation of *esi 9* (pJK1- $\alpha$ w) / BJ2168 MATa ESI<sup>+</sup> diploids.

Sporulated diploid Asci <sup>a</sup>		Yield of secretory $\alpha$ -amylase from germinated spores <sup>b, c</sup>				Ratio + : <i>esi</i>
		A	B	C	D	
+/+ (control) asci	1	40.4 <sub>0.6</sub>	41.0 <sub>0.6</sub>	40.2 <sub>0.5</sub>	39.9 <sub>0.6</sub>	4 : 0
	2	29.0 <sub>0.6</sub>	28.0 <sub>0.6</sub>	25.7 <sub>0.5</sub>	28.0 <sub>0.6</sub>	4 : 0
	3	30.0 <sub>0.6</sub>	31.0 <sub>0.5</sub>	28.0 <sub>0.5</sub>	32.0 <sub>0.5</sub>	4 : 0
<i>esi9</i> /+ asci	1	18 <sub>0.6</sub>	20 <sub>0.3</sub>	41 <sub>2.0</sub>	37 <sub>0.3</sub>	2 : 2
	2	15 <sub>0.3</sub>	18 <sub>0.4</sub>	31 <sub>1.4</sub>	31 <sub>0.7</sub>	2 : 2
	3	13 <sub>0.5</sub>	15 <sub>0.3</sub>	35 <sub>1.0</sub>	31 <sub>0.6</sub>	2 : 2
	4	43 <sub>0.6</sub>	47 <sub>0.6</sub>	65 <sub>1.6</sub>	73 <sub>1.4</sub>	2 : 2
	5	43 <sub>0.5</sub>	42 <sub>1.2</sub>	68 <sub>1.1</sub>	64 <sub>0.5</sub>	2 : 2
	6	36 <sub>0.3</sub>	47 <sub>1.0</sub>	68 <sub>1.0</sub>	67 <sub>0.5</sub>	2 : 2

<sup>a</sup> BF307-10 MAT $\alpha$  / BJ2168 MATa = +/+ diploid.

*esi9* (pJK1- $\alpha$ w) / BJ2168 MATa ESI<sup>+</sup> diploid.

<sup>b</sup> mg of maltose equivalents liberated from soluble starch per ml of culture  
(see Methods); mean values from 3 separate assays.

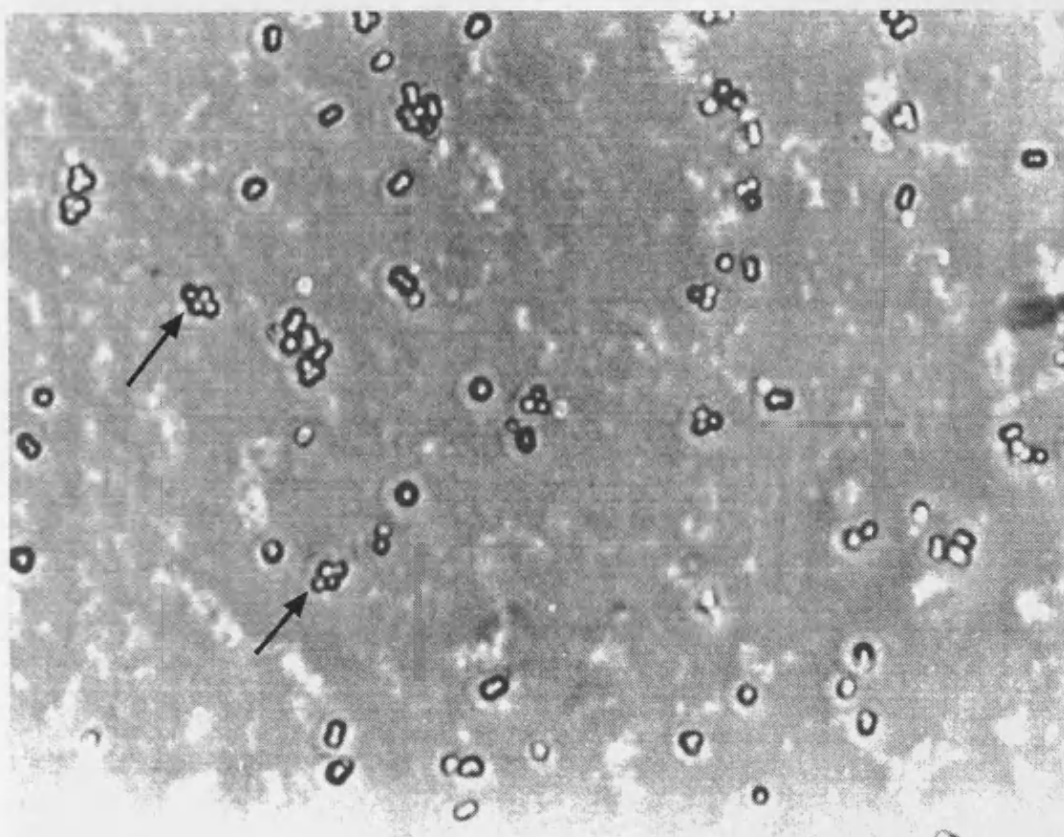
<sup>c</sup> Subscript value is for residual activity within the cell.

similar to that for the wild-type diploid (Table 4.1.b), indicating that the mutations were in different genes.

The double heterozygous diploids carrying the *esi* mutations and the wild-type controls were sporulated under conditions of nitrogen starvation; all produced four spore asci on sporulation medium (see figure 4.2) and the two control heterozygous diploids made from *MAT $\alpha$*  BF307-10 Ura<sup>+</sup>X*MATaesi1* (pBG1) and *MATa esi4* (pBG1) produced viable spores after dissection and germination. After tetrad digestion and dissection of the *esi* double heterozygotes on to non-selective YPD solid medium very few spores germinated, making it impossible to perform any further analysis. It was noted however that the walls of the asci from double *esi* heterozygotes seemed very fragile, requiring little or no digestion with  $\beta$ -glucuronidase before they were dissected. Again it was possible to monitor individual spores using the Singer MSM dissection machine, and spores were seen arrested at a range of different stages of development. Examples from the double *esi* heterozygote dissections can be seen in figure 4.3.

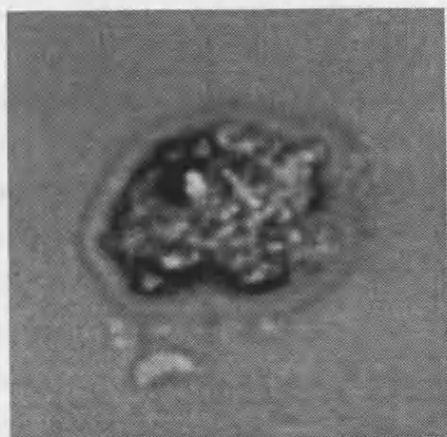


**Figure 4.2.** Tetrads produced after sporulation of *esi* heterozygous diploids. Two four spore asci are arrowed.



**Figure 4.3.** Examples of arrested spores from the double mutant dissections *esi1/esi9,esi4/esi9* and *esi1/esi4*. Letters (a)-(f) show a selection of spores that have arrested at different stages of development.

(a)



(b)



(c)



(d)



(e)



(f)



### 4.3 Discussion.

Genetic analysis has shown all three *esi* mutations to be recessive. Although the *esi1* and *esi9* strains had secondary chromosomal mutations, it was still possible to deduce that all three *esi* defects, leading to the enhanced secretion of mini-proinsulin phenotype are mutations in single genes. Complementation analysis indicated that the mutations are in three different chromosomal genes. Another reason for constructing the diploid strains containing two of the *esi* mutations was to try to isolate a haploid derivative with both mutations and then to test the levels of insulin secretion in this haploid strain. Combining two such mutations within a haploid cell could possibly result in greater increases in yield. It was not possible to isolate such a derivative as heterozygous diploids expressing mini-proinsulin, had very low levels of spore germination after sporulation and dissection. The tetrads of such diploids were quite fragile, suggesting the possibility that high mini-proinsulin secretion may impede secretion of proteins necessary for spore viability or result in their mislocalisation.

Cells enter meiosis through the meiotic prophase, which includes a round of DNA synthesis and the events associated with recombination. This is where chromosomes condense and transient double-stranded chromosome breaks occur, allowing gene convertants and recombinants appear. Cells then go through meiosis I (reductional) and meiosis II (equational) divisions. Finally, spore walls form through deposition of spore coat materials within a membrane outgrowth near the spindle poles ((Briza *et al.*, 1990). Meiotic genes have been divided into three classes - early, middle and late - based on their time of expression (Clancy *et al.*, 1983; Wang *et al.*, 1987). It is the late genes that may be affected in the *esi* mutants as their products are involved in spore packaging and possibly require secretory processes. Therefore the low level of spore viability seen in the double heterozygous diploids could be a result of aberrant deposition of spore coat materials. This would allow spores and tetrads to be formed and would only become a problem after dissection and attempted germination.

Another consideration to be addressed is that of tetrad fragility seen in the *esi* double heterozygotes. The tetrads of the double heterozygotes required little or no  $\beta$ -glucuronidase enzyme to digest the ascus wall surrounding the four spores. This would imply that combining the *esi* mutations impairs the cells ability to secrete all the proteins required for effective ascus wall formation.

The *esi1* mutant possesses a secondary mutation that results in a meiosis specific plasmid segregation defect. The results show 2:2 segregation of two 2 $\mu$ m based plasmids (pBG1 and YpJK $\alpha$ w1) after sporulation and tetrad dissection, indicating a chromosomal mutation in a single gene. This would imply that a host cell function is involved in the movement of 2 $\mu$ m based plasmids into the spores during sporulation, confirming the present view that 2 $\mu$ m based plasmids either passively diffuse into the spores or use a host cell function for segregation into the spores.

Like other extrachromosomal elements there is a 4:0 segregation of the 2 $\mu$ m in the spores produced by meiosis. It was reported that 82% of the 2 $\mu$ m plasmid pool present in a diploid cell, is segregated to the spores. Whereas, only 55% of the mitochondrial DNA is segregated into the spores (Brewer and Fangman, 1980). This is probably indicative of the cellular location of the two extrachromosomal elements. The 2 $\mu$ m plasmid is present within the nucleus, whilst the mitochondrial DNA is present within the cytoplasm, only a fraction of which is enveloped into the spores. There is little information about 2 $\mu$ m maintenance during the host cell's alternative developmental pathways. For example, it has not been determined whether the partitioning mechanism is required for the inheritance of the plasmid during sporulation but there is strong evidence to imply that it is required during diploid vegetative growth. It has been shown however that 2 $\mu$ m based plasmids are significantly more stable in diploids, than in haploids (Mead *et al.*, 1986).

Little is known about 2 $\mu$ m plasmid maintenance during meiosis. Meiotic nuclear division differs from mitotic division in that there is a high rate of homologous recombination during pre-meiotic S phase. The investigation of plasmid inheritance during meiosis could improve the understanding of the processes involved in the segregation of nuclear material during sporulation. It would be interesting to determine whether the meiosis specific segregation defect seen in mutant *esi1* is a general defect affecting all nuclear plasmids or only 2 $\mu$ m.

Of the three mutants *esi4* was the easiest to analyse from a genetic point of view as it had no secondary mutations that affected spore viability or plasmid segregation. Therefore it should be possible to use the initial insulin colony footprint assay, after the mutant strain containing pBG1 had been transformed with the YCp50 yeast genomic library (Rose *et al.*, 1987), to isolate the wild-type *ESI4* gene. A number of attempts were made to isolate the gene in this way, but none were successful as the primary antibody had deteriorated. As it was not possible to obtain more of the primary antibody, mutant *esi4* was transformed with the Ins2 reporter plasmid, which contained a 9E10 epitope

tag at the end of the gene. Using the appropriate primary and secondary antibodies footprints were detected after mutant *esi4* Ins2 had been transformed with the YCp50-based genomic library (Rose *et al.*, 1987). It was not possible however to use this new reporter to isolate the wild-type gene as the putative positive clones identified were shown to be false positives after ELISA assay.

The *esi9* mutant was the most difficult to analyse genetically due to secondary chromosomal mutations that drastically reduced spore viability. This reduction in spore viability could however have resulted from a single chromosomal mutation that affected the secretion of proteins required for spore formation (considered earlier) as well as resulting in higher levels of mini-proinsulin secretion.

## Chapter Five.

### THE ISOLATION OF ALTERED SECRETION MUTANTS USING THE *ASPERGILLUS NIGER* $\beta$ -GALACTOSIDASE.

#### 5.1 Introduction.

With the *esi* mutants already isolated and their genetic and phenotypic characterisation underway, a different secretion reporter was used to attempt the isolation of other enhanced secretion mutants. The industrially important secreted  $\beta$ -galactosidase protein of *Aspergillus niger* was selected. This protein is much larger in size than the mini-proinsulin reporter and glycosylated. Therefore this might target different areas of the secretion pathway that were rate limiting for the secretion of this protein.

The annual world production of whey, a major by-product of cheese and caesin manufacture, exceeds over  $10^{11}$ kg. The protein contained in whey has significant value in the food industry, and is separated and concentrated for these purposes by ultrafiltration (Irvine and Hall, 1985). The lactose and salts (whey permeate) however have lower value and are frequently discarded which can represent a major source of water pollution without expensive sewage treatment. Therefore alternative uses need to be found for whey permeate. As *S.cerevisiae* lacks both a lactose uptake system and a  $\beta$ -galactosidase ( $\beta$ -D-galactohydrolase E.C.3.2.1.23.), it cannot utilise lactose directly (Sreekrishna and Dickson, 1985). Therefore the expression and secretion of *A.niger*  $\beta$ -galactosidase from *S.cerevisiae* would be of biotechnological importance as it would allow the fermentation of whey, solving the disposal problem and producing useful alcohol and biomass for yeast extracts.

As discussed in chapter 3 the *Aspergillus niger*  $\beta$ -galactosidase is a large protein approximately 130kDa in size, with 12 potential sites of N-linked glycosylation. As the protein is much larger than mini-proinsulin and heavily glycosylated, it was thought a different type of enhanced secretion mutant could be generated utilising this reporter. It has been observed that other *A.niger* genes utilise non-preferred *S.cerevisiae* codons which reduce overall expression, for example the glucoamylase (Boel *et al.*, 1984) and pectin lyase D (Gysler *et al.*, 1990) genes. The  $\beta$ -galactosidase protein shows little codon bias towards *A.niger* codons and is expressed using its own signal peptide in *S.cerevisiae*. A plasmid construction, pVK1.1, containing the *Aspergillus*

*niger*  $\beta$ -galactosidase gene (Kumar *et al.*, 1992) was kindly supplied by Dr.W.Lancashire from Whitbread. The plasmid contained the encoding *lacA* cDNA, which includes the natural secretion leader sequence, coupled to the *ADH1* promoter and carries the 2 $\mu$ m *ORI-STB* for multicopy replication.

## 5.2 Results.

### 5.2.1 Strain selection and variation.

As described in chapter two a number of haploid laboratory strains were available for transformation with recombinant plasmids. Following the same criteria as before, in order to assess whether host strain genotype had any effect on  $\beta$ -galactosidase production, the yeast strains BF307-10, DBY746, JRY188, TGY47.1 and BJ2168 were each transformed by the lithium acetate procedure, with the pVK1.1 plasmid (see figure 3.5b). Transformants able to grow in minimal medium lacking uracil were isolated. Transformant clones of each strain were grown in minimal medium and inoculated into 10 mls of YPD and incubated. The secretion of  $\beta$ -galactosidase into the medium was measured via ONPG hydrolysis (table 5.1). Assays were also performed on cell extracts of each strain in order to determine the levels of intracellular  $\beta$ -galactosidase. As we can see from table 5.1. only approximately 10% of the  $\beta$ -galactosidase produced is actually secreted, with the other 90% therefore remaining within the cell. The results also showed that there was strain variation for the production of  $\beta$ -galactosidase; the two protease deficient strains BJ2168 and TGY47.1 secreted approximately half the amount of  $\beta$ -galactosidase of strain BF307-10 with intracellular levels being lower as well. There was little difference between the other three strains tested, however strain BF307-10 secreted the greatest amount of  $\beta$ -galactosidase and had the greatest accumulation of intracellular  $\beta$ -galactosidase. Hence the secretion pathway is probably being overwhelmed in this strain. Because of this strain BF307-10 was chosen for future work, as the passage through the secretion pathway seemed limiting for protein production.



**Table 5.1.** Levels of  $\beta$ -galactosidase secretion from different strains of *S.cerevisiae*.

Strain	$\beta$ -galactosidase activity.*	
	Cell	Supernatant
BF307-10	158	15.2
DBY746	132	12.11
BJ2168	98	8.3
TGY47.1	100	7.9
JRY188	146	13.01

\* nM of ONPG converted/minute (see Experimental procedures). The figures are the mean of duplicate assays.

All strains were cultured to OD<sub>650</sub>=2 before harvesting.

### 5.2.2 Stability of pVK1.1 plasmid in yeast.

In some cases heterologous protein expression can have deleterious effects upon the host yeast cells resulting in reduced growth and unusually high incidence of plasmid-free cells, which out-grow plasmid-containing ones. Therefore, as an indication of possible product toxicity, it was necessary to establish the stability of the  $\beta$ -galactosidase expressing plasmids in *S.cerevisiae*. Transformant yeast colonies of strain BF307-10 were inoculated into 10 mls of minimal medium lacking uracil to maintain the selection for the plasmid, and incubated at 30°C overnight. Cells from the resultant culture were used to inoculate both non-selective YPD medium and selective minimal medium. After growth for at least 50 cell doublings, cells were plated onto YPD agar and incubated at 30°C for 2-3 days. The resultant colonies were then replica-plated onto minimal medium to determine the proportion of plasmid-containing (Ura<sup>+</sup>) cells in the original populations. On minimal medium out of 1500 colonies scored, 92% were derived from cells that had retained the plasmid; whilst 87% of the cells still carried the plasmid after culturing in non-selective YPD medium. As a control the experiment was also undertaken at same time with BF307-10 cells transformed with vector only. The results were nearly identical to those obtained with recombinant pVK1.1 indicating that the expression of the  $\beta$ -galactosidase reporter protein did not affect plasmid stability. This demonstrated a reasonable degree of mitotic stability indicating that the expression of the  $\beta$ -galactosidase reporter did not affect plasmid stability and is consistent with the observations made by Kumar *et al.*, (1992).

For a second assay of potential toxicity by the accumulated intracellular  $\beta$ -galactosidase, the growth rate of strain BF307-10 containing pVK1.1 was compared against strain BF307-10 without plasmid in non-selective YPD medium. It was found that the growth rate of both strains was almost identical, indicating that the accumulation of the  $\beta$ -galactosidase reporter protein did not cause toxicity. Thus, the inefficient secretion of the  $\beta$ -galactosidase reporter protein, which might be expected to overwhelm the secretion pathway, at some stage- did not appear to have any detectable toxic effects upon the host cell.

### 5.2.3 *Standardisation of $\beta$ -galactosidase production in shake flask cultures.*

Transformant yeast colonies containing pVK1.1 were inoculated into 10 ml of minimal medium lacking uracil and incubated at 30°C overnight. 100 $\mu$ l aliquots of culture at a concentration of 10<sup>8</sup>cells/ml were used to inoculate 100 mls -YPD medium in 200 ml conical flasks. Culture growth and secreted  $\beta$ -galactosidase levels were then followed (table 5.2. and figure 5.1). For subsequent assays a time point around the twenty four hour (OD<sub>650nm</sub> 1.8-2.0) period was used to measure secreted  $\beta$ -galactosidase activity, as this was towards the end of log phase and before stationary phase. It was noted that unlike mini-proinsulin the levels of  $\beta$ -galactosidase did not fall drastically after approximately 36 hours.

### 5.2.4 *Plate assay for the detection of $\beta$ -galactosidase producing colonies.*

There was a plate assay available for the detection of *S.cerevisiae* colonies secreting  $\beta$ -galactosidase, which removed the need to develop one. This assay relied on the chemical X-gal being incorporated into solid minimal media. As transformant yeast colonies grow and secrete  $\beta$ -galactosidase they breakdown the X-gal in the media, which results in a blue halo appearing around the colony. The size of the halo produced around the colony would be expected to be indicative of the level of  $\beta$ -galactosidase being secreted by the colony.

When cells of strain BF307-10 containing plasmid pVK1.1. were grown overnight in selective minimal media and then inoculated into non-selective YPD medium, and plated onto solid minimal medium containing X-gal, the halo size was highly reproducible, with little difference between colonies. Therefore it seemed possible to use this visual halo assay for the isolation of enhanced secreting colonies after random mutagenesis.

### 5.2.5 *Mutagenesis and isolation of enhanced secretion mutants.*

The plate assay was used to isolate mutant cells showing enhanced and reduced levels of secretion of  $\beta$ -galactosidase. BF307-10 (pVK1.1) cells were

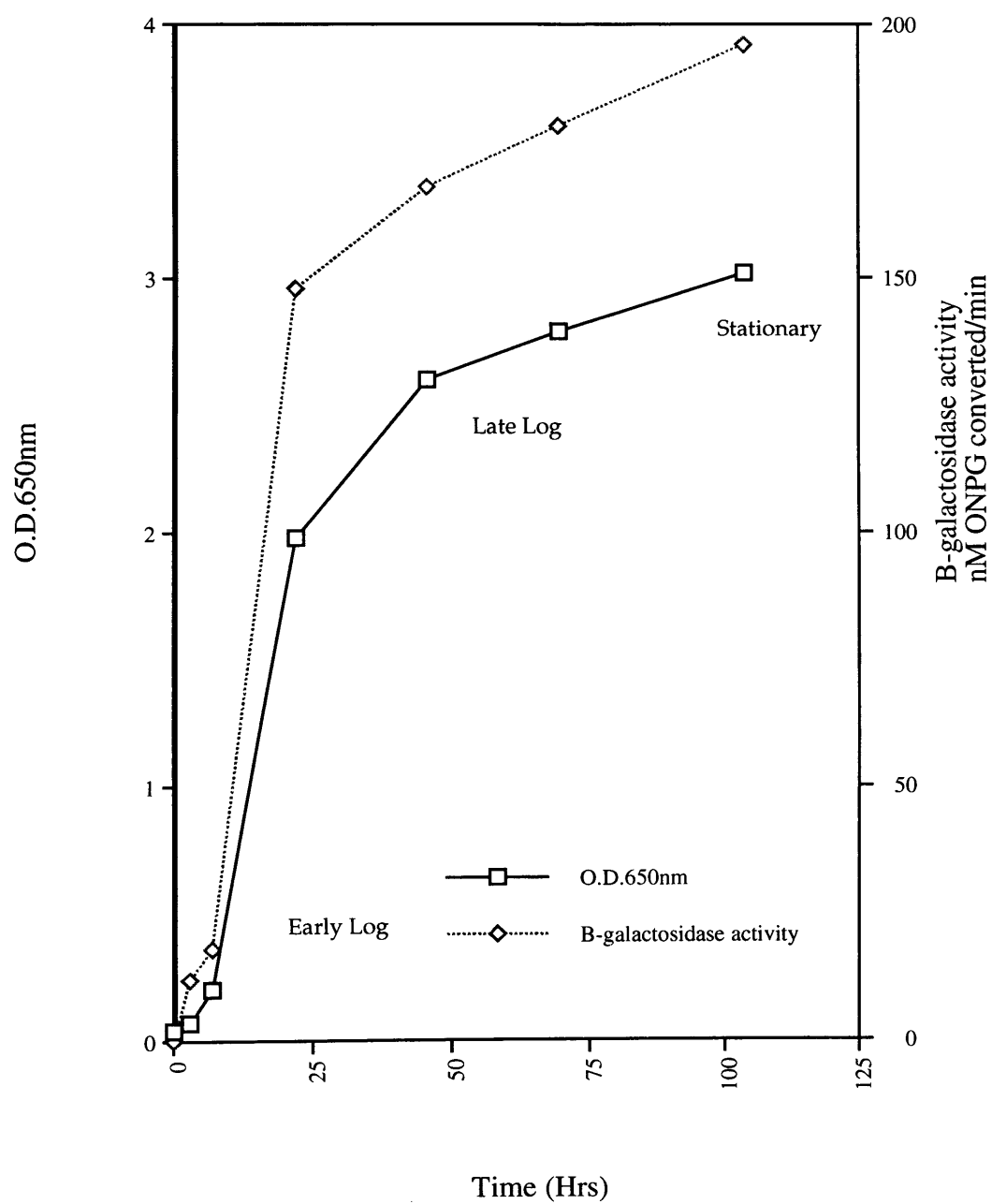
**Table 5.2.**  $\beta$ -galactosidase production from BF307-10 *S.cerevisiae* cells in shake flask cultures.

Time (hrs)	Culture O.D. 600 <sub>n m</sub>	$\beta$ -galactosidase activity* (secreted)	Cell number
0	0.04	0	-
3	0.07	12	$2.2 \times 10^6$ /ml
7	0.201	18	$8.1 \times 10^6$ /ml
22	1.98	148	$6.0 \times 10^7$ /ml
46	2.60	168	-
70	2.79	180	$1.5 \times 10^8$ /ml
104	3.02	196	$2.2 \times 10^8$ /ml

\* nM of ONPG converted/minute (see Experimental procedures). The figures are the mean of duplicate assays.

The production of  $\beta$ -galactosidase was followed over a period of 104hrs in BF307-10 *S.cerevisiae* cells.

**Figure 5.1.**  $\beta$ -galactosidase activity in shake flask culture with respect to time and cell density



chemically mutagenized with ethylmethyl sulphonate (EMS) at a concentration determined to give 60% killing. Surviving cells were plated onto minimal selective agar and incubated at 30°C for 3-6 days. Prospective mutants having larger or smaller blue halos were selected (approximately 38 out of 40 000 mutagenised colonies ) and rescreened to test how reproducible the halo assay was. An example of an "enhanced secretion" colony can be seen in figure 5.2.

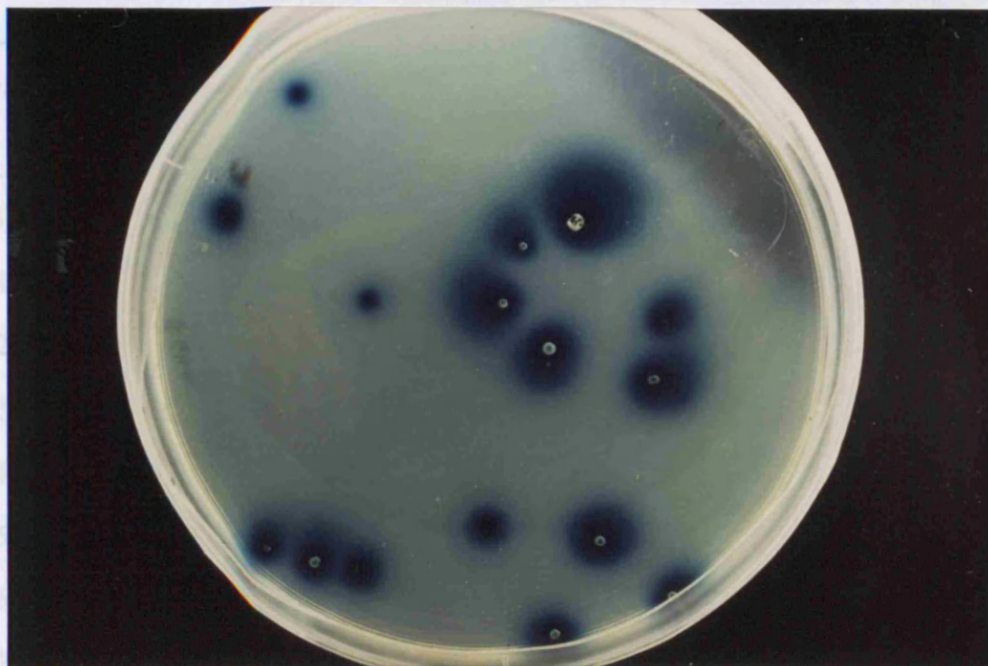
For quantitative confirmation of enhanced secretion, the colonies were cultured in liquid medium and yields of secreted  $\beta$ -galactosidase accurately assayed by ONPG hydrolysis. Of the rescreened mutagenized colonies, only 4 consistently showed increased secretion yields of  $\beta$ -galactosidase and were termed *esg* (enhanced secretion of  $\beta$ -galactosidase), approximately 4-fold greater than wild-type (Table 5.3.). Thus these appeared to be candidate mutants showing enhanced efficiency of the protein secretion pathway. There were four other mutagenised colonies that showed decreased yields of secreted  $\beta$ -galactosidase and were termed *dsg* (decreased secretion of  $\beta$ -galactosidase), approximately 3-fold lower than wild-type (Table 5.3.) The failure of the majority of mutants to maintain the enhanced secretion phenotype of initial isolation, suggested a possible instability in the secretion phenotype, that made analysis of potential mutants difficult.

#### 5.2.6 Is the *A.niger* $\beta$ -galactosidase toxic to *S.cerevisiae* cells?

It was noted that colonies of *S.cerevisiae* expressing and secreting the  $\beta$ -galactosidase reporter gene began to lose their blue colour when left on solid media for periods of time longer than five-ten days. After fifteen-twenty days the colonies were dead. Thus the secreted product did after all appear to have a cryptic toxic effect on the host cells. There are a number of instances where the product may affect the cell. One may see lethality upon induction of expression, if the gene has been introduced into the cell under repressing conditions, an example of this been the expression of Diphtheria toxin (Perentesis *et al.*, 1988). However in less severe cases, the recombinant product may not be lethal, but reduce growth or viability such as that seen with the expression of polyoma virus middle-T antigen (Belsham *et al.*, 1986).

None of these adverse affects were witnessed on the initial introduction and expression of the *A.niger*  $\beta$ -galactosidase within *S.cerevisiae* cells and it was possible to isolate prospective enhanced secretion mutants. However, it

**Figure 5.2.** *S.cerevisiae* BF307-10 cells plated out onto X-gal plates after random mutagenesis. To the top and right of the photograph, an example of a colony showing enhanced secretion of  $\beta$ -galactosidase can be seen.



**Table 5.3.**  $\beta$ -galactosidase assays on nine prospective mutants isolated by halo size.

Strain	$\beta$ -galactosidase activity.*		% Secreted	Relative Yield.	
	Cell	Supernatant		Secreted	Total
BF	168	14.53	8	1.0x	1.0x
<b>1</b>	117	70.9	27	<b>4.9x</b>	1.0x
<b>2</b>	148	6	4	<b>0.4x</b>	0.84x
<b>3</b>	145	6	4	<b>0.4x</b>	0.83x
<b>4</b>	190	56	23	<b>3.9x</b>	1.35x
<b>5</b>	179	59	25	<b>4.0x</b>	1.30x
<b>6</b>	148	2	1	<b>0.14x</b>	0.82x
<b>7</b>	149	6.8	4	<b>0.47x</b>	0.85x
8	112	13.5	11	0.93x	0.69x
<b>9</b>	155	50	25	<b>3.44x</b>	1.12x

\* nM of ONPG converted/minute (see Experimental procedures). The figures are the mean of duplicate assays.

Strains highlighted were those with altered secretion.



would appear that a build up of  $\beta$ -galactosidase inside the cells might affect colony size, secretion yield and eventually result in cell death. This provided a major barrier to successful mutant isolation and characterisation.

#### 5.2.7 *The androgen inducible expression vector system.*

To try to overcome this problem of cell toxicity caused by constitutive expression of the  $\beta$ -galactosidase reporter gene, an inducible expression vector system was selected, enabling expression to be turned off until required for assay. In this way toxic effects would be kept to an absolute minimum. Although a number of inducible promoter systems are available, for example *GAL1, 10* (Johnston, 1987), *PHO5* (Meyhack *et al.*, 1982) and *CUP1* (Butt *et al.*, 1984), the changes in the growth media used to induce transcription can cause profound effects on cellular metabolism, which would not be desirable. A inducible promoter system was therefore chosen that would avoid such problems. This utilised a hormone-inducible promoter (Purvis *et al.*, 1991).

The human androgen receptor can be expressed in *S.cerevisiae* in a form capable of activating transcription from a yeast promoter carrying androgen-responsive elements, in an androgen-dependent manner. This system requires two plasmid constructs to function in *S.cerevisiae*; one expresses the androgen receptor and the other contains a promoter under the control of an androgen response element, so that expression of a downstream coding sequence is only activated when an androgen hormone (usually dihydrotestosterone (DHT) is added to the medium. The DHT would be taken up from the medium by the yeast cells via the androgen receptors, which are produced when the cells are grown on glucose as expression of the *hAR* gene is driven by the *PGK* promoter.

This system was constructed for inducible expression of secretory  $\beta$ -galactosidase as follows. The first plasmid pPGK-hARI (figure 5.3a), containing the complete encoding sequence of the human androgen receptor gene, expressed from the *PGK* promoter, was integrated into the *trp1* locus of the prospective *esg* mutants and wild-type BF307-10.

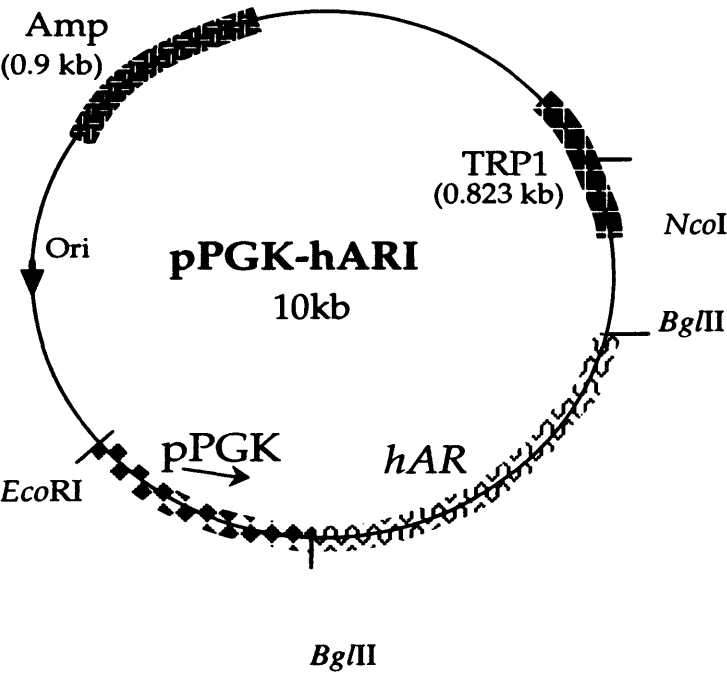
The second plasmid YEpPGKareBRSNX (figure 5.3.b) contained a modified *PGK* promoter, lacking the normal upstream activation sequence (UAS) , but with a triple arrangement of androgen response elements (ARE) positioned at the normal UAS site. It carried the 2 $\mu$ m *ORI-STB* for multicopy replication, the *LEU2* marker gene to maintain selection and it contained a

**Figure 5.3.** Vectors required for the androgen inducible system.

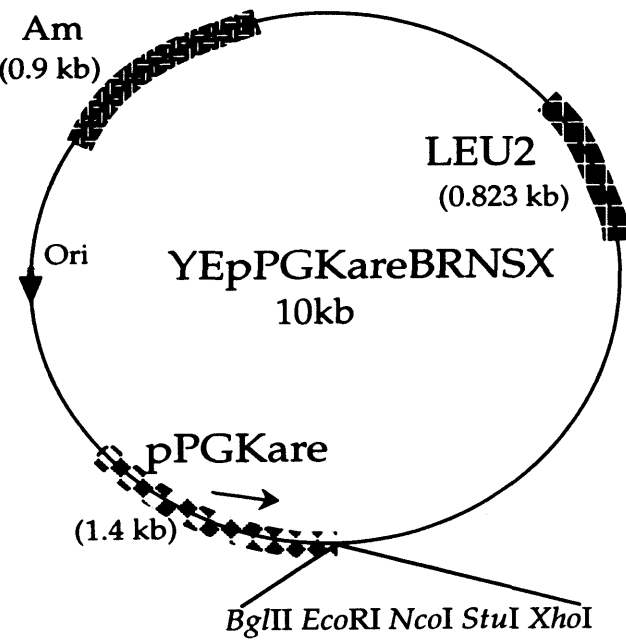
(a) pPGK-hARI integrative plasmid contains the complete encoding sequence of the human androgen receptor gene (*hAR*) expressed from the PGK promoter. Integration is achieved after restriction endonuclease digestion with *NcoI* in the middle of the *TRP1* marker and transformation.

(b) YE<sub>p</sub>PGKareBRNSX plasmid that contains a modified PGK promoter with a triple arrangement of androgen response elements (ARE). The plasmid has a multi-cloning site at the end of the ARE elements that incorporates an ATG site, if required at the *NcoI* restriction site.

(a)



(b)



multiple cloning site downstream of the heterologous promoter, to enable insertion of the heterologous coding sequence to be expressed.

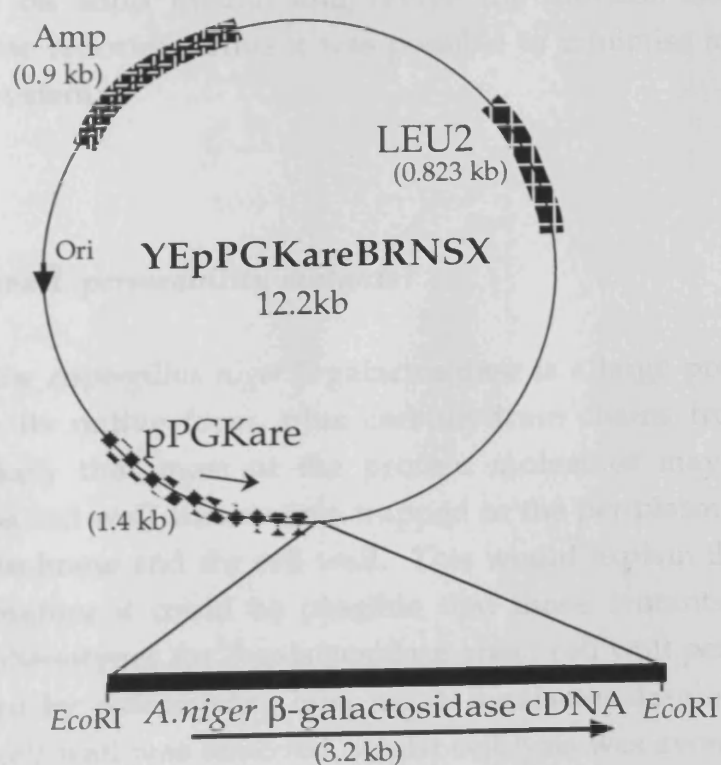
#### 5.2.8 Construction of pBG2- $\beta$ -gal.

The complete cDNA sequence encoding the full length secreted  $\beta$ -galactosidase was isolated from the expression vector pVK1.1 by partial digestion with the restriction endonuclease *EcoRI* and gel electrophoresis; a time course of the digestion was performed to calculate the time required to yield the correct 3.2kb fragment containing the whole of the  $\beta$ -galactosidase gene. The final reaction conditions required one unit of restriction endonuclease *EcoRI* with the digestion being stopped after nine minutes. After gel electrophoresis the 3.2kb fragment was purified using the BIO101 Gene Clean Kit and ligated into vector YEpPGKareBRSNX, that had also been digested with restriction endonuclease *EcoRI*, to form vector pBG2- $\beta$ -gal (figure 5.4.).

It was not possible to check the fragments orientation by endonuclease restriction analysis. Therefore in order to identify a plasmid isolate that had the *A.niger*  $\beta$ -galactosidase gene in the correct orientation for expression, strain BF307-10 containing an integrated copy of the *hAR* gene was transformed with a number of the pBG2- $\beta$ -gal isolates. Transformants were isolated on leucine and tryptophan deficient agar plates, as the integrated vector pPGK-*hAR* contained the *TRP1* marker and the pBG2- $\beta$ -gal isolates contained the *LEU2* marker. These transformants were then replica plated onto leucine and tryptophan plates that contained the androgenic ligand (DHT) at a concentration of 100nM plus X-gal. This DHT concentration was high enough to induce expression (Purvis *et al.*, 1991) of the  $\beta$ -galactosidase gene via the ARE's in the vector. Yeast colonies transformed with plasmid pBG2- $\beta$ -gal with the  $\beta$ -galactosidase gene in the correct orientation, would be blue and have blue halos around them as they would be able to breakdown the X-gal. Several such colonies were obtained, at a frequency of approximately 50% as would be expected for random insertion in either orientation.

Once a plasmid with the  $\beta$ -galactosidase gene in the correct orientation had been isolated it was transformed into the *esg* mutants (which had been previously cured of pVK1.1) containing the integrated *hAR* gene. Transformants were isolated on leucine and tryptophan deficient agar plates containing glucose. These transformants were cultured in minimal medium,

Figure 5.4 Expression vector pBG2- $\beta$ -gal that has had the *A.niger*  $\beta$ -galactosidase inserted at the unique *EcoRI* site of YE<sub>p</sub>PGKareBRNSX



inoculated into YPD containing DHT and  $\beta$ -galactosidase activities assayed (table 5.4). The results obtained with this androgen inducible system were similar to those obtained with plasmid pVK1.1, although the levels of  $\beta$ -galactosidase activity within the cell and secreted were generally lower than those seen with the pVK1.1 plasmid. However it was now possible to store (especially on solid media) and revive the mutants isolated using the  $\beta$ -galactosidase reporter. Thus it was possible to minimise toxicity by using this inducible system.

### 5.2.9 Cell wall permeability mutants?

As the *Aspergillus niger*  $\beta$ -galactosidase is a large protein, approximately 130kDa in its native form, plus carbohydrate chains from glycosylation, it seemed likely that most of the protein molecules may be unable to pass through the cell wall and become trapped in the periplasmic space between the plasma membrane and the cell wall. This would explain the blue colour of the cells. Therefore it could be possible that those mutants showing enhanced secretion phenotypes for  $\beta$ -galactosidase affect cell wall permeability. This was investigated by determining how much  $\beta$ -galactosidase activity was released when the cell wall was removed, whilst cell lysis was avoided.

The four enhanced secretion mutants and the wild-type control, were grown overnight in minimal media and then inoculated into 10mls of YPD media containing DHT. Cultures were harvested when they had reached an OD<sub>650nm</sub> of 2. The cell walls were removed by zymolase treatment in osmotically stabilising medium (see experimental procedures) and cells were checked under the microscopic until approximately 50% spheroplasting had occurred (this level was chosen so that spheroplasting did not go too far and produce large amounts of cell debris. The resultant spheroplasts were harvested by centrifugation and the supernatant, containing liberated periplasmic proteins, assayed for  $\beta$ -galactosidase activity. This determined how much  $\beta$ -galactosidase activity was released when the cell wall was removed, whilst avoiding cell lysis. The spheroplasts were then lysed and assayed for intracellular  $\beta$ -galactosidase activity. The results (table 5.5) indicated that two of the enhanced secretion mutants, *esg1* and *esg9*, do have altered cell wall permeability, as they had similar levels of intracellular  $\beta$ -galactosidase activity to wild-type but had reduced levels of activity present in

**Table 5.4.**  $\beta$ -galactosidase assays on nine prospective mutants isolated by halo size using the new androgen inducible vector system.

Strain	$\beta$ -galactosidase activity.*		% Secreted	Relative Yield.	
	Cell	Supernatant		Secreted	Total
<b>BF</b>	108	10.53	9	1.0x	1.0x
<b>1</b>	77	40.9	34	<b>3.9x</b>	1.0x
<b>2</b>	108	3.5	3	<b>0.33x</b>	0.94x
<b>3</b>	85	4.1	4.6	<b>0.4x</b>	0.76x
<b>4</b>	130	35	21	<b>3.3x</b>	1.40x
<b>5</b>	119	38	24	<b>3.61x</b>	1.33x
<b>6</b>	88	1.1	1.2	<b>0.11x</b>	0.82x
<b>7</b>	99	4.6	4	<b>0.47x</b>	0.76x
<b>8</b>	62	8.23	11.7	0.78x	0.60x
<b>9</b>	95	28	23	<b>2.66x</b>	1.04x

\* nM of ONPG converted/minute (see Experimental procedures). The figures are the mean of duplicate assays.

Strains highlighted were those with altered secretion.

Originally proteins assays were performed on cell extracts and supernatants using the Biorad assay kit. However it was found that proteins were nearly identical for each mutant and the wild type control. Subsequently proteins assays were not performed.

**Table 5.5.** Intracellular, periplasmic and supernatant  $\beta$ -galactosidase assays on *esg* mutants.

Strain <sup>a</sup>	$\beta$ -galactosidase activity.*		
	Intracellular	Periplasmic	Supernatant
+	63 (63)	27 (27)	10 (10)
<i>esig1</i>	65 (56)	12 (11)	39 (33)
<i>esig4</i>	89 (55)	40 (25)	34 (21)
<i>esig5</i>	73 (52)	31 (22)	36 (26)
<i>esg 9</i>	70 (61)	18 (16)	27 (24)

\* nM of ONPG converted/minute (see Experimental procedures). The figures are the mean of duplicate assays and those enclosed in parenthesis indicate percentages.



the periplasmic space. The other two mutants, *esg4* and *esg5*, had very similar levels of both intracellular  $\beta$ -galactosidase activity and periplasmic to wild-type, but more  $\beta$ -galactosidase was secreted into the medium. This suggests they affect secretion in some other way.

### 5.3 Discussion.

Using a strategy based on the *A.niger*  $\beta$ -galactosidase gene reporter protein I have been able to isolate another set of altered secretion mutants. Again these seem to be mutations that affect the efficiency of the secretion pathway; such mutants might alter the rate of flow of secretory proteins through the pathway or carry cell wall permeability mutations, rather than eliminate pathway function altogether. The mutation frequency (at approximately 1 in  $5 \times 10^3$  mutagenised cells) was greater than that observed by Sleep *et al.* (1991), and that seen when isolating the *esi* mutants (see chapter 2).

From the initial screen and subsequent rescreening eight mutant strains that showed an altered secretion pattern of  $\beta$ -galactosidase compared to non mutagenised cells were isolated. These were cultured and assays revealed that four mutants showed secretion levels 3 fold greater than wild-type, whilst the other four showed reduced secretion levels approximately 4-fold lower than wild-type. As with the mini-proinsulin reporter no mutants were recovered that secreted  $\beta$ -galactosidase at levels more than three times greater than wild-type. Further analysis has shown that two of *esg* mutants could be cell wall mutants.

As previously discussed, the rationale behind the design of this mutant screen was to isolate a different subset of secretion pathway genes, to those essential genes identified by temperature sensitive mutations by Novik and Schekman (1980). However the situation with the  $\beta$ -galactosidase reporter was very different to that seen with the mini-proinsulin reporter, in that nearly 90% of the  $\beta$ -galactosidase activity remained within the cell, whereas all of the mini-proinsulin was secreted into the medium. Therefore it is probable that the mutations affect different components of the secretion pathway, which are limiting for the secretion of  $\beta$ -galactosidase, to those identified using the mini-proinsulin reporter. This is clearly the case for those affecting cell wall permeability and is a justification for using different reporter systems to isolate different subsets of mutants. As with the *esi* mutants it is possible that the *esg/rsg* mutants may be defective in non-essential genes, or carry non-lethal mutations in essential genes.

These mutants need to be further analysed in a number of ways: firstly to determine whether or not altered secretion phenotype seen is due to altered  $\beta$ -galactosidase transcription: secondly the effects of the mutations on secretion reporter transcription need to be investigated and genetic studies undertaken to determine if the mutations are in separate genes: the final part of this analysis would involve studying the effects of the mutations on

cytoplasmic reporter expression, as this would indicate whether the mutations specifically affected proteins destined to be secreted or generally affect both cytoplasmic and secreted proteins.

## Chapter Six.

### General Discussion.

The rationale for this work was the employment of two secretory reporter systems that should exert pressure upon the yeast protein secretion pathway, in order to create bottlenecks in the passage of the reporter protein through the pathway. Mutants that relieved or caused such bottlenecks, recognisable by a phenotype of enhanced or decreased secretion yield, would then enable the identification of gene products that acted at the rate limiting steps. Such mutants would alter the rate of flow of secretory proteins through the pathway, rather than eliminating pathway function altogether.

#### 6.1 *The mini-proinsulin reporter expression system.*

In this study a footprint assay has been developed that allows the detection of small increases in mini-proinsulin secretion. Using this expression system a number of mutations giving rise to enhanced secretion were isolated. All gave fairly small increases in mini-proinsulin yield, to a maximum of 3-fold, with the majority giving less than 2-fold. Thus, inefficiencies in the pathway could be genetically identified, but they were not unduly large.

Three of the mutations analysed by genetic methods were shown to be in separate genes, designated *ESI1*, *ESI4* and *ESI9* and enhanced mini-proinsulin secretion without increasing expression levels of cytoplasmic proteins. The evidence provided from studies of levels of messenger RNAs encoding secreted and cytoplasmic reporter proteins, suggested that the mutations act at the very beginning of the secretion pathway and enhance secretion by stimulating the introduction of more products into the pathway, with an associated degradation of the mRNA. Furthermore as all three genes act at this initial stage, it suggests that this is the major rate-limiting step for the secretion of mini-proinsulin in *S.cerevisiae*.

Interestingly, in both non-mutant and mutant cells no mini-proinsulin could be detected within the cells, suggesting that once entered into the secretion pathway the polypeptide is transported through it, and secreted, with high efficiency. Alternatively, invisible losses may occur within the pathway,

such as rapid proteolytic degradation of a proportion of the molecules, perhaps as a result of mis-folding or erroneous routing.

Two mechanisms are known to operate in yeast for directing proteins into the secretion pathway, co-translational and post-translational (Hann and Walter 1991; Rothblatt *et al.*, 1994) (see Introduction). The co-translational mechanism involves recognition and binding of the nascent secretion leader peptide by a signal recognition particle (SRP) as the secretory protein mRNA is being translated by the ribosome. Binding of the SRP causes a pausing of translation until the SRP binds with its receptor in the ER membrane. The leader peptide can then direct secretion and translation resumes with concurrent passage of the newly translated protein through the ER membrane and simultaneous removal of the signal peptide. In the post-translational mechanism SRP binding is not involved. Instead SSA proteins bind to the newly translated polypeptide to prevent it from folding. Subsequent interaction of the signal peptide with the ER membrane then facilitates entry into the secretion pathway. Having been kept in an unfolded state, the polypeptide is able to pass through the ER membrane.

Of these two alternative mechanisms, it would seem more likely that the co-translational mechanism is enhanced in all three of the mutants. This is suggested by the different specificity effect noted between mini-proinsulin and amylase secretion, which is understandable if SRP recognition of two different leaders (*MF $\alpha$  pre-pro* and wheat  $\alpha$ -amylase) is involved. In a recent review a kinetic partitioning model was proposed to explain the affinity of a signal sequence for SRP (Zheng and Gierasch 1996). Zheng and Gierasch (1996) have collated evidence from the work of Hann and Walter (1991) and Ng *et al.*, (1996), to show that it is possible to classify signal sequences by their translocation pathway preference in yeast. They have qualitatively ranked a number of signal sequences in terms of their dependence on the yeast SRP:

DPAP-B (dipeptidyl aminopeptidase B), Pho8 (repressible alkaline phosphatase) > Kar2, Och1 ( $\alpha$ -1,6-mannosyltransferase) > invertase, prepro- $\alpha$ -factor, Gas1 (glycophospholipid-anchored surface protein), PDI > prepro CPY (carboxypeptidase Y).

Ng *et al* (1996) argue that the hydrophobicity of signal sequences correlates with preference for translocation pathway. Signal sequences for proteins that follow the SRP independent were found to be relatively less hydrophobic than those utilizing both. Therefore under normal conditions in yeast the *MF $\alpha$*  signal peptide has a low affinity for SRP. The *esi* mutations could act to

enhance the binding affinity of the SRP for the *MF $\alpha$*  signal peptide, whilst sequence differences with the amylase signal peptide could result in the mutations having quite different effects on binding affinity for the amylase leader, as observed. A second factor is the association of reduced mRNA levels for secreted proteins with the *esi* mutations. This could occur if mRNAs for secreted proteins were being selected more efficiently for translation and this was associated with degradation. As the co-translational mechanism involves pausing of translation, this could have the consequence of initiating message degradation, analogous to the situation in which the presence of rare codons appears to cause reduced message levels by stalling translation (Herrick *et al* 1990).

The odd action of the *esi9* mutant in inhibiting expression of minor codon-containing genes encoding reporter proteins, but not expression of major codon containing genes, suggests that the *esi9* mutation reduces mRNA half life and hence protein yield. However, any mRNAs coupled to SRP via signal peptides are stabilised and product is yield unaffected or even increased.

In contrast, it is difficult to explain the action of the mutants via a modification of the post-translational mechanism. Thus, although an increase in SSA protein production could improve secretion yield, it might be expected to have a general effect on other secretory proteins, which was not the case. Furthermore, the post-translational mechanism cannot be linked with the decreased levels of mRNAs encoding secretion proteins that are associated with the mutations. This explanation would promote the notion that the co-translational mechanism may be potentially more efficient than the post-translational one, which is less sophisticated and absent in higher eukaryotes.

Further clarification of the molecular effects of the *esi* mutations on the mechanism of protein secretion must await the cloning of the genes and molecular analysis of the products they encode. At the present time this has been attempted but not achieved, so corresponding with a general picture that has emerged over the past ten years. Although a number of enhanced or reduced secretion mutants have been isolated over the years (Bussey *et al.*, 1983; Sakai *et al.*, 1988; Sleep *et al.*, 1991; Smith *et al.*, 1985) only in one case has a gene been identified that is clearly responsible for the enhanced secretion phenotype- namely *PMR1* (Rudolph *et al.*, 1989).

It is interesting to reflect that, despite the great complexity of the secretion pathway, the basic approach of looking for functions acting at rate-limiting steps in the secretion pathway has been successful. Although the

mutants analysed in this study only seem to affect the beginning of the pathway, this approach is by no means exhausted. Other mutants that act elsewhere in the pathway, may be obtained. Screening for altered levels of a secreted reporter protein (elevated or lowered) enables the isolation of mutations that are not lethal. This is either because they partially alter the activity of the gene product or because it is not essential to viability.

The *esi* mutants generated and analysed in this study have potential usefulness in the production of biotechnologically important proteins from yeast at elevated levels. The general procedure used in this study is still employed in industry to improve secretion yields of biotechnologically important proteins. The yeast strains used by Delta Biotechnology Ltd for the secretion of recombinant human albumin, have been developed over a number of years using this approach.(Sleep *et al.*, 1991). Strains isolated initially with enhanced secretion of recombinant human albumin, were subjected to further mutagenesis and screened for an even greater level of secreted product. This process was repeated again and again until no visible increase in secreted product was seen. Another approach would be to characterise genetically the mutant strains and combine two or more mutations into one strain, to see the effect on secreted product.

Although this approach has been very good at generating mutants with altered secretion levels, further clarification of the molecular effects of the mutations on the mechanism of protein secretion and gene isolation has not been very forthcoming. Consequently this approach may be reaching the end of its shelf-life. However, it may be possible to carryout high density colony screens using automation/robotics, to look for colonies that have reverted back to their wild-type levels of secretion after transformation with a yeast genomic DNA library. It is this reversion back to wild-type levels of secretion, that has been the stumbling block in this project.

## **6.2 The $\beta$ -galactosidase reporter.**

Using a strategy based on the *A.niger*  $\beta$ -galactosidase gene reporter protein I have been able to isolate another set of altered secretion mutants. Again these seem to be mutations that affect the efficiency of the secretion pathway; such mutants might alter the rate of flow of secretory proteins through the pathway, or carry cell wall permeability mutations rather than

eliminate pathway function altogether. Eight mutant strains have been isolated, four that showed secretion levels 3 fold greater (*esg*) than wild-type, whilst the other four showed reduced secretion levels (*rsg*) approximately 4-fold lower than wild-type. Further analysis has shown that two of the *esg* mutants could be cell wall mutants.

These mutants need to be further analysed in a number of ways: firstly to determine whether or not altered secretion phenotype seen is due to altered  $\beta$ -galactosidase transcription: secondly the effects of the mutations on secretion reporter transcription need to be investigated and genetic studies undertaken to determine if the mutations are in separate genes: the final part of this analysis would involve studying the effects of the mutations on cytoplasmic reporter expression, as this would indicate whether the mutations specifically affected proteins destined to be secreted or generally affect both cytoplasmic and secreted proteins.



## Chapter Seven.

### CAN SEC GENE HOMOLOGUES BE ISOLATED FROM *ARABIDOPSIS THALIANA*?

#### 7.1 Introduction.

The last ten years has seen an increase in the expression and secretion of antibodies and enzymes in plants. This has subsequently led to a greater interest in the plant secretory pathway, the mechanism by which these foreign proteins are produced. The plant cell signalling initiative by the EPSRC has also provided a valuable insight into inter-cellular signalling within plants. A number of methods have been used to try to isolate genes that are involved in the secretion pathway of *Arabidopsis thaliana*. The reasons for choosing this organism are apparent. It has a manageable genome with regards to size and is therefore not as complex on the evolutionary scale as higher plants such as tobacco. This introduction gives a very general outline of the plant secretion pathway as viewed today and considers the isolation of gene homologues using a complementation approach.

##### 7.1.1 *The plant secretion pathway.*

Protein secretion is ubiquitous in nature but was until recently a poorly understood process in plants. As with animal and yeast cells (see Chapter 1) secreted proteins are synthesised on the membranes of the rough endoplasmic reticulum and transported to the cell surface by secretory vesicles formed at the Golgi apparatus. Understanding the process of secretion in plants has been slow, in part because of their autotrophic mode of nutrition, that negates the need for specialised digestive glands. However, plant cells are surrounded by a cell wall that can contain as much as 20% structural proteins by weight (Lamport, 1981), and therefore the synthesis and secretion of these proteins has been extensively studied (reviewed by (Cassab and Varner, 1988). It is now apparent that constitutive secretion of proteins occurs via a default pathway as in animals and yeast (Burgess and Kelly, 1987; Denecke *et al.*, 1992).

The proteins that are secreted from plant cells can be separated into two distinct groups based on differences in their structure and biological functions.

Secreted glycoproteins, with sugars attached to the protein through an O-glycosidic linkage, are thought to play principally structural, non-enzymic roles (Fincher, 1983) (Cassab and Varner, 1988)). On the other hand, enzymic proteins are generally N-glycosylated (Akazawa and Hara-Nishimura, 1985), although it has been reported that some secreted enzymes are not post-translationally modified by oligosaccharide addition (Jacobsen, 1988).

#### **7.1.1.2 *Transport into the ER.***

As with animal and yeast, plant secretable proteins contain amino terminal signal sequences that direct them into the lumen of the ER (Dorrel *et al* 1988). An examination of the signal peptides from different secreted plant proteins shows little sequence homology even though there can be a high degree of sequence conservation among related proteins (Vonheijne, 1985). It is now clear that the molecular components required for the translocation of proteins into the ER share many common features in animal, yeast and plant cells. Indeed the *A.thaliana* gene for the 54-kDa subunit of the signal recognition particle has been isolated and characterised (Denfert *et al.*, 1992; Sze *et al.*, 1992). Subsequent work has shown that *A.thaliana* actually expresses three distinctly divergent SRP genes (Schnepf, 1993).

In plants, but not in animals, the hydroxyproline residues of O-glycosidic proteins are replaced by either galactose residues or arabinose oligosaccharides (Robinson, 1985). The biochemical and molecular details of protein N-glycosylation in plant cells are well understood, and are similar to those in animal cells (Elbein, 1979; Elbein, 1987; Kornfeld and Kornfeld, 1985). Thus these proteins are co- or post-translationally glycosylated in the ER (see Chapter 1) and transferred to the Golgi stacks in vesicles (Morre *et al* 1989). The initial step of N-glycosylation occurs in the lumen of the ER (Faye *et al* 1992).

#### **7.1.1.3 *Transport to and through the Golgi apparatus.***

It is thought that following their synthesis and cotranslation in the ER, plant secretory proteins exit the ER in transition vesicles using processes similar to those described in Chapter One. Indeed functional homologues of the yeast *ERD2*, *SEC12* and *SAR1* genes have been isolated from *A.thaliana* (Denfert *et al.*, 1992; Lee *et al.*, 1993). The work of Kinal *et al.*, (1995) has also shown evidence for a Kex2p-type processing pathway in plants. These ER

vesicles enter the *cis*-face cisternae of the Golgi , and proteins move through the Golgi stack and exit through the *trans*-Golgi network, where vacuolar proteins are sorted from proteins destined for the plasma membrane and the cell wall. The major synthetic products of the Golgi apparatus of most plant cells, however, are not glycoproteins but complex polysaccharides that are components of the cell wall (Moore and Staehelin, 1988; Robinson and Kristen, 1982).

The Golgi apparatus of plant cells differs in several important aspects from its animal counterpart (Rambourg and Clermont, 1990). For example, onion root tip cells at interphase typically contain about 400 individual Golgi stacks (Garciaherdugo *et al.*, 1988) which are usually dispersed throughout the cytoplasm; most animal cells contain only ten stacks clustered in a juxtanuclear position (Rambourg and Clermont, 1990).

An *Arabidopsis* ARF (ADP-ribosylation factor) gene has been isolated that encodes a GTPase probably involved in Golgi vesicle trafficking (Regad *et al.*, 1993). Many other small GTP-binding proteins, that are important for GTPase/GTP-binding activities have been isolated from *A.thaliana* (Anai, 1991; Anuntalabhchai, 1991; Ma, 1994; Reynolds *et al.*, 1993; Yi and Gueriot, 1994) ; some of these have been classified as structurally and functionally similar to the *ypt* gene family of yeast (Palme *et al.*, 1993). This would indicate that there is a family of small GTP-binding proteins in plants, carrying out a similar function to those isolated in mammals and yeast , involved in the secretion pathway.

#### 7.1.1.4 Targeting to the plasma membrane and vacuole.

Little is known about the proteins involved in the transport of secretory proteins to the plasma membrane in plants. The flow of proteins to the plant vacuole has received much greater attention however and numerous genes have been isolated that are involved in vacuolar protein sorting/targeting (Dallmann *et al.*, 1992; Lee and Wickner, 1992; Sze *et al.*, 1992). The signal sequences required for targeting have also been studied (Gal and Raikhel, 1994; Neuhaus *et al.*, 1991; Nieto *et al.*, 1993). The whole process of vacuolar targeting has been reviewed ((Pryer *et al.*, 1992; Vitale and Chrispeels, 1992).

As mentioned in this section numerous gene homologues have been isolated using functional complementation of yeast mutants, and it is this technique that I shall consider next.

### 7.1.2 *The isolation of gene homologues using the complementation approach.*

Cloning by complementation (this is not complementation in the true sense, but more phenotypic rescue) is a powerful method for gene isolation in prokaryotic and lower eukaryotic systems. This technique has not been used extensively in higher eukaryotes because of the difficulty of introducing large numbers of clones into a mutant organism- e.g. humans, mice, fruit flies and plants- and the general lack of mutations in the systems where complementation might be possible (e.g. mammalian tissue culture). This problem is overcome by the expression of genes from higher eukaryotes in organisms more amenable to single gene introduction, using selections and screens to identify the genes of interest. Interspecies complementation has been used previously to isolate genes (Haubruck *et al.*, 1990; Jones and Robinson, 1989). The first gene from yeast was isolated by complementation of an amino acid biosynthetic mutation in *E.coli* (Struhl, 1977). Since the cloning of a human cDNA by direct complementation of the fission yeast *Schizosaccharomyces pombe* (Moore *et al.*, 1991), many other genes have been obtained by this approach (Bowser *et al.*, 1992; Mollenhauer *et al.*, 1991; Neuhaus *et al.*, 1991; Vitale and Chrispeels, 1992).

For complementation of a particular mutation in the host organism, a library of the genes expressed (cDNA) from the organism of interest must be cloned into a suitable expression vector for expression in the host organism.

#### 7.1.2.1 *Arabidopsis thaliana* cDNA expression libraries.

There were two *A.thaliana* cDNA expression libraries constructed in vectors able to be propagated in *E.coli* and yeast, available for the purpose of this study. The first constructed in the bacteriophage vector  $\lambda$  YES-R was kindly supplied by Dr.S.J.Elledge , and the second constructed in plasmid vector pFL61 by Dr.F.Lacroute . I shall consider each of these libraries in turn.

##### 7.1.2.1.1 *The $\lambda$ YES -R cDNA expression library.*

The  $\lambda$ YES, a multifunctional phage  $\lambda$  vector system is designed to facilitate gene isolation from eukaryotes by complementation of *E.coli* and *Saccharomyces cerevisiae* mutations (Elledge *et al.*, 1991). The cDNA library in  $\lambda$ YES-R can be converted into a *S.cerevisiae*/*E.coli* shuttle vector form by *cre*-

*lox* excision following infection of the *E.coli* strain BNN132 (Elledge *et al.*, 1991). BNN132 is a lysogen resistant to  $\lambda$  infection, which carries the *cre* gene from P1, encoding a protein that catalyses the site specific recombination of *lox* sites. Two *lox* sites are present in  $\lambda$  YES-R, flanking a linearised plasmid sequence to yield a circular plasmid (figure 7.1), conferring ampicillin resistance on the host bacterium.

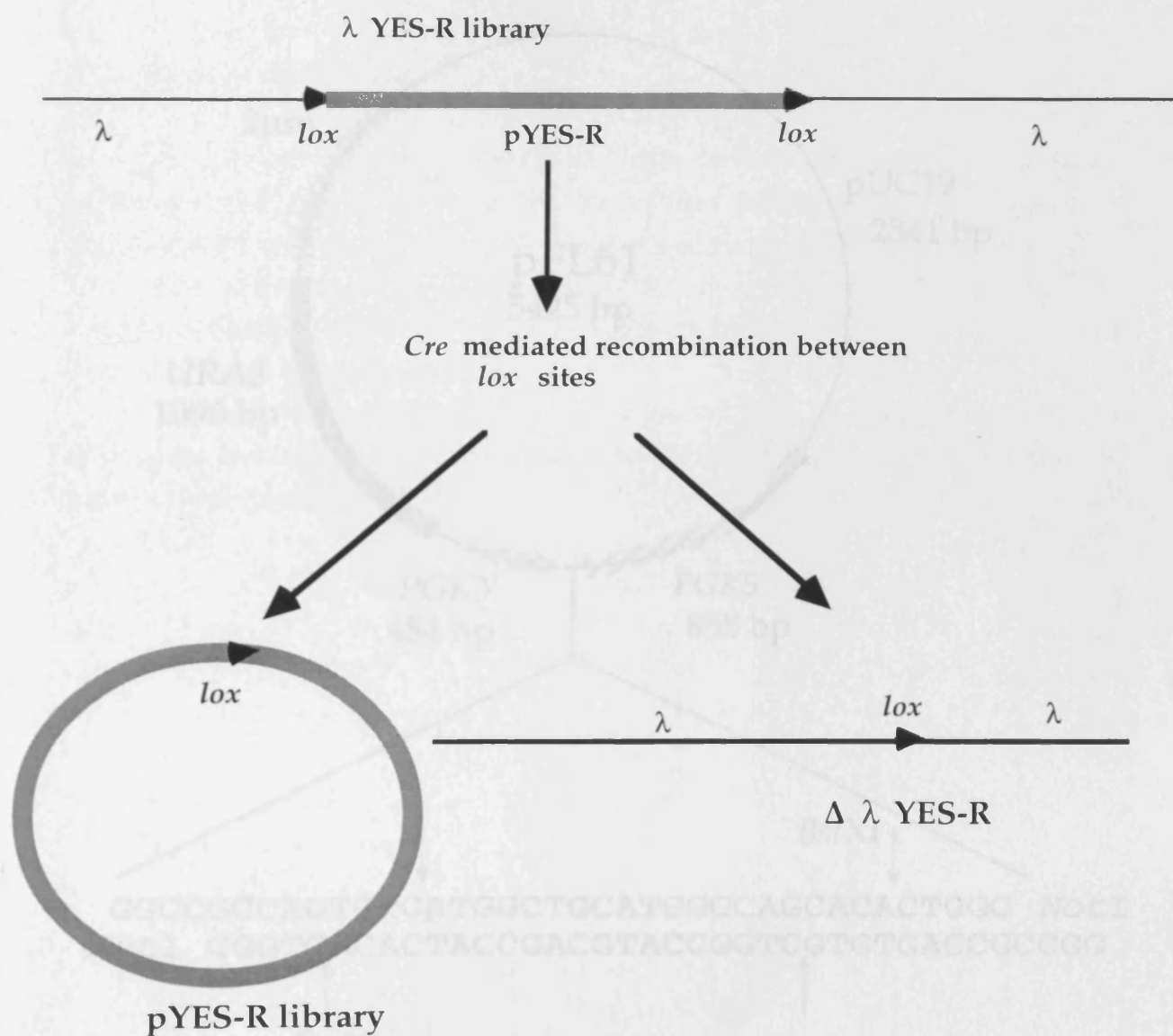
Thus the plasmid form of  $\lambda$ YES-R contains the ampicillin resistance *bla* gene and the bacterial origin of replication for selection and propagation in *E.coli*. For selection and maintenance in yeast it contains the *URA3* gene and the *ARS1* and *CEN4* sequences. The low copy number of this plasmid was seen as an advantage, in that it reduced the likelihood of encountering multicopy suppression or toxicity (Rose *et al.*, 1987). As the DNA cloning strategy employed in making the library is non-directional, half of the inserts are in the proper orientation for expression in yeast, driven by the *GAL1* inducible promoter and half for expression in *E.coli* driven by the *lac* promoter (Elledge *et al.*, 1991). Adjacent to the *lac* promoter is a termination sequence derived from the *HIS3* gene, in the same orientation as *GAL1* initiated transcripts.

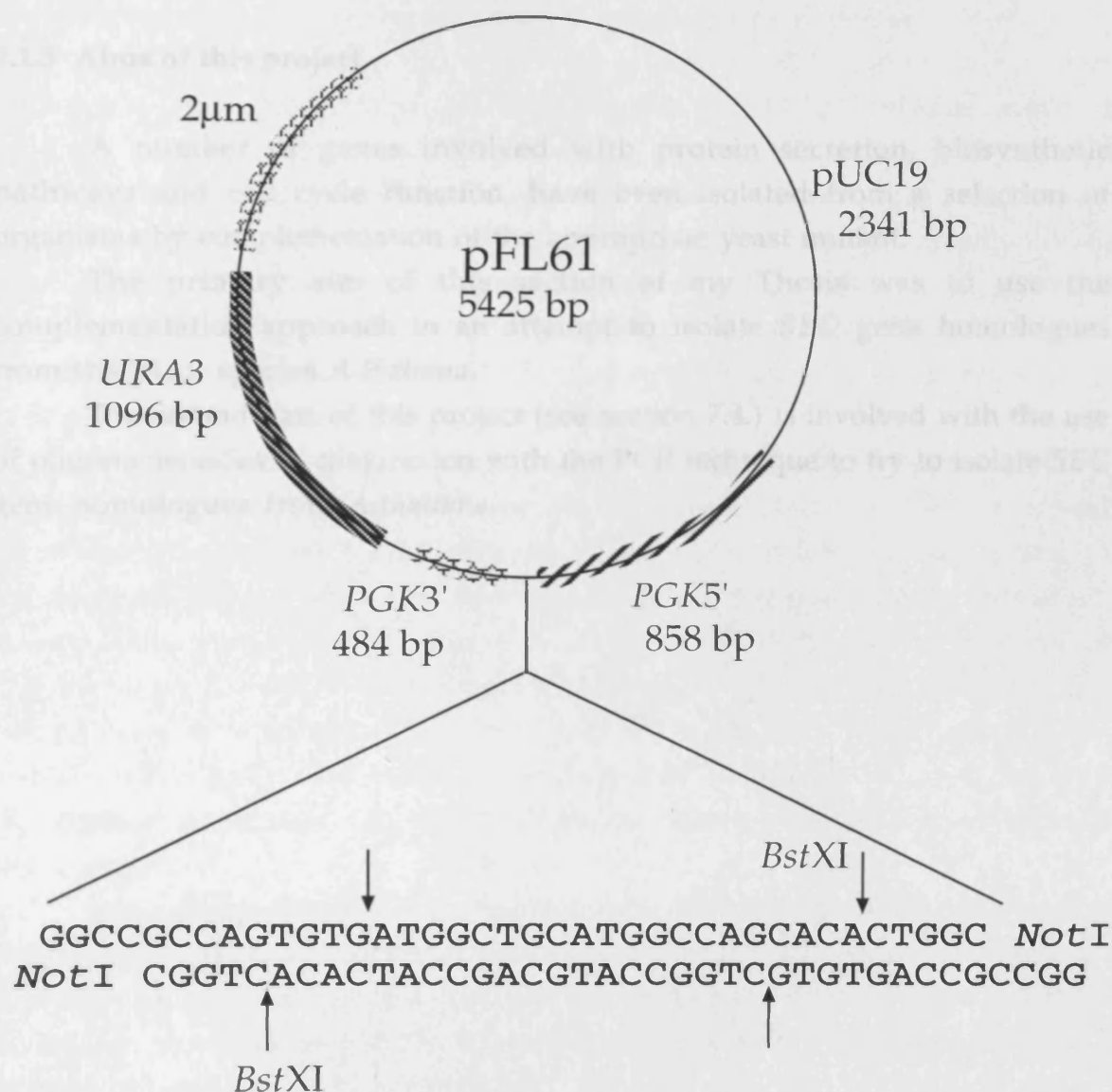
The  $\lambda$ YES-R cDNA library used in this study, was prepared with mRNA from total *Arabidopsis* plants and has been shown to complement a deletion of the *trpD* gene in *E.coli*, complementing clones being isolated at a frequency of approximately 1 in 100 000 (Elledge *et al.*, 1991). Elledge *et al* (1991) were also able to complement the *cdc28* mutation of *S.cerevisiae* with clones being isolated at a frequency of approximately 1 in 200 000.

#### 7.1.2.1.2 The *pFL61*cDNA expression library.

In this case a *A.thaliana* cDNA bank was constructed in a *E.coli/S.cerevisiae* plasmid shuttle vector *pFL61* (figure 7.2.) (Lacroute 1992). The cDNA's are expressed in *S.cerevisiae* from the phosphoglycerate kinase (PGK) promoter in conjunction with the PGK terminator. The *pFL61* vector contains the PGK promoter separated from its terminator by a multisite polylinker, the *URA3* gene and a small part of the 2 $\mu$ m plasmid for selection and multicopy maintenance in *S.cerevisiae*, and the vector *pUC19*, containing the *bla* gene and bacterial origin for selection and maintenance in *E.coli*. The library was constructed after the isolation of poly(A)<sup>+</sup> mRNA's from *A.thaliana* seedlings ( at the two leaf stage), including roots, and cDNA's

**Figure 7.1.** *Cre-lox* mediated excision of library plasmid from  $\lambda$  backbone.  $\lambda$ YES-R phage genomes are designed to enable the automatic subcloning of inserts in a *S.cerevisiae*/*E.coli* shuttle vector.





inserted into the *Bst*XI restriction sites after digestion of pFL61 with restriction endonuclease *Bst*XI (figure 7.2). This directional cloning of cDNA inserts provided the correct orientation for expression from the *PGK* promoter. cDNA inserts ranged in size from 0.2kb to 20kb and larger. Lacroute *et al* (1992) were able to complement eight auxotrophic marker genes from *S.cerevisiae* mutants, with complementing clones been isolated at a frequency ranging from 1 in 20 000 to 1 in 2000.

### **7.1.3 Aims of this project.**

A number of genes involved with protein secretion, biosynthetic pathways and cell cycle function, have been isolated from a selection of organisms by complementation of the appropriate yeast mutant.

The primary aim of this section of my Thesis was to use the complementation approach in an attempt to isolate *SEC* gene homologues from the plant species *A.thaliana*.

The second part of this project (see section 7.4.) is involved with the use of oligonucleotides in conjunction with the PCR technique to try to isolate *SEC* gene homologues from *A.thaliana*.



## 7.2 Results.

### 7.2.1 Complementation efficiencies for the two cDNA libraries.

After both the  $\lambda$  YES-R and pFL61 cDNA libraries had undergone transformation/infection into the appropriate bacterial strains, large scale plasmid preparations, using either caesium chloride gradients or Qiagen midi columns were carried out. To check the ability of each of these libraries to complement *S.cerevisiae* mutants, test transformations were performed.

The  $\lambda$  YES-R library was transformed into the *GAL1,ura3-52, trp1* and *leu2* yeast strain BJ2168 and identical aliquots of cells, plated onto minimal medium plates lacking uracil (*URA3* being the marker gene on the library plasmid) but containing galactose as the carbon source. The plates were incubated at 30°C for 2-4 days and the number of *URA3*<sup>+</sup> transformants noted. The transformation efficiency for strain BJ2168 was then calculated and found to be approximately  $5 \times 10^5$  transformants/ $\mu$ g of library DNA. This indicated that the preparation of the  $\lambda$ YES-R library could be used for complementation studies.

The next stage of the procedure was to check the ability of the  $\lambda$ YES-R library to complement the *leu2* mutation in strain BJ2168. This was achieved by replica plating the *URA3* transformants isolated above, onto minimal media plates lacking uracil and leucine and containing galactose as the carbon source. The plates were incubated at 30°C for 2-4 days and the number of *URA3*<sup>+</sup> and *LEU2*<sup>+</sup> transformants noted. Before calculating the complementation efficiency of the  $\lambda$ YES-R library, the *URA3*<sup>+</sup> and *LEU2*<sup>+</sup> transformants had to be checked to ensure that complementation of the *leu2* marker was dependent on the cDNA in the library plasmid and not due to reversion.

After plasmids had been rescued from the transformants, the strains were cultured on 5-FOA plates to select for *Ura*<sup>-</sup> cells that had lost the plasmid. A single colony was selected for each of the transformants from the 5-FOA plates and streaked onto minimal medium plates with and without uracil to ensure that the plasmid had been lost. Complementation of the *leu2* marker by the cDNA present in the original  $\lambda$ YES-R plasmids isolated was confirmed by transforming the isolated plasmids back into strain BJ2168, and selecting for transformants on minimal media plates lacking uracil and leucine and containing galactose. The frequency of complementing plasmids in the library containing the *LEU2* gene was found to be approximately 1 in  $10^5$  to 1 in  $10^6$ . This is slightly lower than that observed by Elledge *et al.*, (1991) for the

isolation of the human *CDC28* homolog (1 in 10<sup>5</sup>) by complementation of the *S.cerevisiae cdc28* mutant, and may be due to continual propagation of the library.

This procedure was repeated for the pFL61 cDNA library with a number of slight alterations. As the cDNAs in this library were under the constitutive expression of the *PGK* promoter it was not necessary to transform a galactose inducible *S.cerevisiae* strain. The strain BF307-10 was used as it had a number of auxotrophic markers; *trp1*, *ura3*, *his3*, *leu2* and *met4*. Selection of transformants was made on minimal medium plates lacking uracil and containing glucose, and the complementation ability of the library checked by utilising the *met4* marker of strain BF307-10. The frequency of plasmids in the library containing the *MET4* gene homologue was found to be approximately 1 in 30 000, which compares favorably with that observed by Lacroute *et al* (1992) for the biosynthetic pathway *URA3*, *URA5* and *URA7* homologues.

#### **7.2.2 Selection of *S.cerevisiae* sec strains for library transformations.**

As the only *sec* mutants available to use, that were also GAL<sup>+</sup> were the *sec1* and *sec18* strains, these were transformed with the  $\lambda$  YES-R galactose inducible cDNA library. It should have been possible to transform all of the 23 available *sec* mutant strains with the pFL61 cDNA library, so a selection procedure was needed as it was not possible for one person to transform all 23 strains (due to the time constraints). The criteria for selection were as follows; that the *SEC* gene homologue chosen had not already been reported from *A.thaliana* ; that the *SEC* gene homologues chosen had been isolated from other organisms, thereby indicating a degree of functional conservation between organisms; that each part of the secretion pathway had been covered. The mutant *sec* strains chosen were *sec13* (ER to Golgi), *sec7* and *sec14* (Golgi to secretory vesicles) and , *sec8* (secretory vesicles to plasma membrane). Unfortunately the *sec* mutant strains supplied by Dr.A.Boyd possessed no auxotrophic nutritional markers which would allow library plasmid selection after transformation. As both libraries utilised the *URA3* selectable marker for yeast transformation a *ura3::Cm<sup>R</sup>* disruption was introduced into the genome of each of the chosen strains.

The plasmid YIp31::Cm<sup>R</sup> was digested by restriction endonuclease *HindIII* to yield a *ura3::Cm<sup>R</sup>* fragment approximately 3kb in size. This was checked by gel electrophoresis and the appropriate gel slice removed and

purified. This was then transformed into the appropriate mutant *sec* strain, to allow the fragment to integrate itself into the chromosome. Chloramphenicol-resistant transformants were isolated on YPE chloramphenicol medium after 5-6 days incubation at 30°C (the permissive temperature for *sec* strains). The uracil auxotrophy of the transformants was confirmed by streaking onto minimal medium lacking and containing uracil.

### 7.2.3 Transformation of *sec ura3::Cm<sup>R</sup>* strains.

Preliminary transformations were performed with each of the *sec ura3::Cm<sup>R</sup>* strains to determine their transformability. Each strain yielded between  $1 \times 10^4$  to  $1 \times 10^5$  transformants per microgram of cDNA library.

Various strategies were employed to optimise the possibility of isolation of complementing clones for each of the *sec* mutations;

(1) For each transformation performed with the cDNA libraries into the *sec ura3::Cm<sup>R</sup>* strains, a minimum of approximately one hundred thousand transformants were plated.

(2) Potential transformants were plated immediately after being heat shocked, the plates left at room temperature for 2-4 hours and then incubated at 37°C (the non-permissive temperature for *sec* strains) for up to ten days.

(3) Using the trial transformations as a guideline, it was attempted not to put more than ten thousand possible transformants on a plate .

Additionally conditions for the selection of transformants were varied as follows:

(a) With the galactose inducible  $\lambda$  YES-R plasmid library, cells were plated on to solid minimal medium lacking uracil and containing galactose and incubated at 30°C for 3-4 days. Potential transformants were replica plated on to the same media and incubated at 37°C for up to 6 days.

(b) For both libraries cells were grown for up to four hours at 30°C after transformation, to allow them to recover and express the genes contained within the library plasmid. These cells were pelleted and re-suspended in the appropriate amount of TE before being plated. Once the cells had been plated the plates were incubated at 37°C for up to 6 days.

(c) It had been shown by Denfert *et al.*, (1992) that complementation of a mutant *sec12* strain by a *A.thaliana* cDNA library had not been possible at 37°C (the non-permissive temperature) but had been possible at a lower

temperature of 33°C, which is also a non-permissive temperature for cell growth of the mutant *sec* strain. Therefore this approach was adopted for the *sec ura3::Cm<sup>R</sup>* strains.

#### **7.2.4 Complementation of the *sec1* and *sec18* mutations using the $\lambda$ YES-R library.**

At least four transformations for each of the *sec ura3::Cm<sup>R</sup>* strains, were performed in an attempt to isolate complementing clones from the cDNA library. The conditions were varied as described in section 7.3.3. and the number of potential positives colonies isolated after the initial transformation noted. These were rescreened by streaking on to galactose plates lacking uracil, and incubating these plates at the non-permissive temperature used for the original transformation procedure. Approximately 98% of the colonies for each transformation proved to be false positives. The remaining colonies that were able to grow at the non-permissive temperature underwent further analysis. Firstly this involved rescuing the library plasmid from each of the potential complementing clones. The rescued plasmids were transformed in to *E.coli* XL1-blue cells to allow their large scale preparation. Digestion of the possible complementing plasmids with restriction endonuclease *XhoI*, would have released the cDNA insert, if one was present. These inserts were visualised by gel electrophoresis.

The second step in the analysis involved eliminating the potential complementing plasmids from the original *sec ura3::Cm<sup>R</sup>* colonies that had grown at the non-permissive temperature. This was achieved using the 5FOA technique. Colonies that had grown on the 5FOA plates were taken and restreaked on uracil-deficient and uracil-containing glucose media, to confirm the loss of the plasmid. These Ura<sup>-</sup> colonies that had lost their plasmids were replica-plated onto uracil containing glucose medium and incubated at the non-permissive temperature. This was to confirm that growth at the non-permissive temperature was plasmid dependent.

The original *sec ura3::Cm<sup>R</sup>* strains used for the library transformations were retransformed with the previously rescued plasmids. Transformant selection was on uracil deficient, galactose solid medium after incubation at 30°C. Transformants were replica plated on to the same medium and incubated at the non-permissive temperature.

None of the possible complementing plasmids isolated from the *sec1ura3::Cm<sup>R</sup>* or *sec18ura3::Cm<sup>R</sup>* strains were able to complement after retransformation, indicating that complementation of the *sec1* and *sec18* mutations had not taken place.

#### 7.2.5 Complementation of the *sec* mutations using the *pFL61* library.

This process was almost the same as the one used for the  $\lambda$ YES-R library. The only differences were;

(1) potential positives colonies isolated after the initial transformation were rescreened by streaking on to glucose (not galactose) plates lacking uracil.

(2) approximately 95% of the colonies for each transformation proved to be false positives. The remaining colonies that were able to grow at the non-permissive temperature underwent further analysis.

(3) possible complementing plasmids were digested with restriction endonuclease *NotI*, to release the cDNA insert, if one was present. These inserts were visualised by gel electrophoresis.

The second step in the analysis was as described in section 7.3.4.

None of the possible complementing plasmids isolated from the *sec7ura3::Cm<sup>R</sup>* strain (see figure 7.3) were able to complement the original strain after retransformation, indicating that complementation of the *sec7* mutation had not taken place. This was also the case for the plasmids isolated from the *sec8ura3::Cm<sup>R</sup>* strain and the *sec13ura3::Cm<sup>R</sup>* and *sec14ura3::Cm<sup>R</sup>* strains, indicating that complementation of the *sec8*, *sec13* and *sec14* mutations had not taken place.

Small sections of the cDNA inserts in some of the plasmids were sequenced and the resulting DNA sequence analysed via the GCG molecular biology package. Although computer translations showed the presence of continuous open reading frames, when the deduced amino acid sequences were compared to the computer data bases using the BLAST and BLITZ searches, they failed to identify any previously reported homologues.

It was decided to analyse the potential complementing plasmids isolated from the *sec8, ura3::Cm<sup>R</sup>* strain transformations further, and see if the cDNA inserts they contained were similar to each other. This was achieved by isolating the cDNA insert from the plasmid in lane 3 (figure 7.4) and using it as a probe for Southern blot analysis of the other plasmid inserts, with lane 3

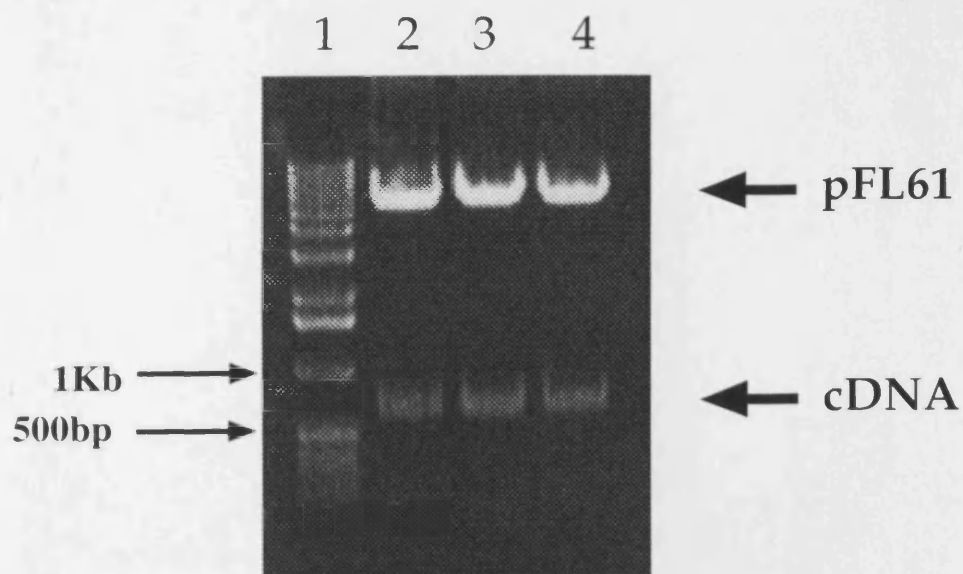
**Figure 7.3.** Possible complementing clones isolated from the *sec7ura3::Cm<sup>R</sup>* strain after transformation with the pFL61 cDNA library. Subjected to digestion with restriction endonuclease *NotI* and analysed via agarose gel electrophoresis.

- (a) Lane 1: 1Kb ladder (marker)  
Lanes 2-16: 15 different possible complementing clones.  
The vector (pFL61) and cDNA inserts are arrowed where present.
- (b) Lane 1: 1Kb ladder.  
Lanes 2-4: Another three possible complementing clones.  
The 1Kb and 500bp markers on the ladder are arrowed as well as the vector and cDNA insert.

**(a)**



**(b)**



**Figure 7.4.** Possible complementing clones isolated from the *sec8ura3::Cm<sup>R</sup>* strain after transformation with the pFL61 cDNA library.

- (a) Lanes 1-5: Possible complementing clones. Subjected to digestion with restriction endonuclease *NotI* and analysed via agarose gel electrophoresis.

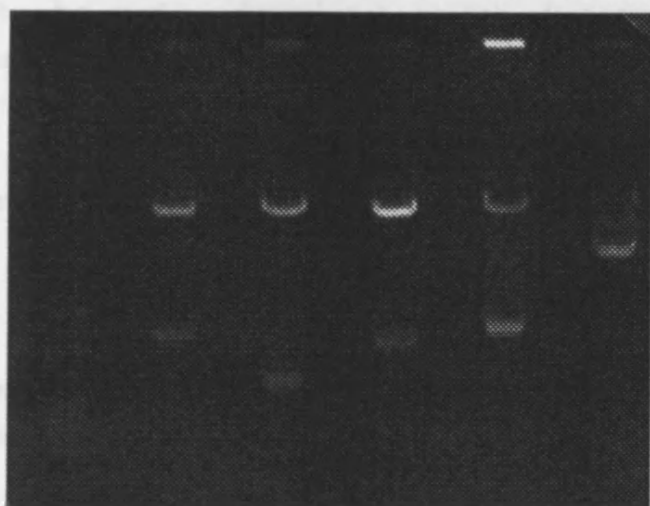
The vector is arrowed.

- (b) Southern blot analysis performed on the above agarose gel, after the DNA had been transferred onto nylon membrane. The cDNA insert from lane three was used as a probe, and cross hybridised with the cDNA insert in lane 1 (arrowed).



(a)

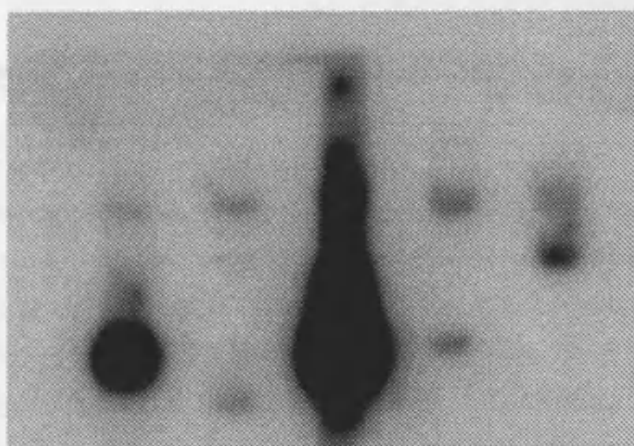
1 2 3 4 5



← pFL61

(b)

1 2 3 4 5



included as a positive control (figure 7.4.). As can be seen from the results that even though the plasmids were unable to complement the *sec8ura3::Cm<sup>R</sup>* strain on retransformation, two of the cDNA inserts did cross hybridise. This indicated that two of the cDNAs isolated originally as complementing the mutation shared a certain amount of homology at the DNA level. This seems unlikely to be just a fortuitous match due to the large number of transformants isolated after each transformation (approximately 10<sup>5</sup>). However it is not possible to draw any conclusions from this data as the plasmids were unable to complement the original mutation on retransformation.

#### **7.2.6 *Can the Polymerase Chain Reaction be used to isolate SEC gene homologues from A.thaliana?***

When homologous genes have been isolated from a number of different organisms often specific regions of similarity (known as consensus regions), within these proteins can be identified. These consensus regions can be utilised for the design of oligonucleotides to be used in the PCR, in an attempt to try to isolate the gene of interest from another organism. In this section, I describe the design of degenerate and homologous oligonucleotides, to try to isolate *NSF/SEC18* and *SEC4* homologues from *A.thaliana*.

#### **7.2.7 *Design of degenerate oligonucleotides.***

A protein sensitive to N-ethylmaleimide (NEM), known as NEM-sensitive fusion protein (NSF), catalyses the fusion of transport vesicles within Golgi cisternae. It has been shown that this protein is equivalent to the *SEC18* gene product of the yeast *S.cerevisiae*, which is essential for vesicle-mediated transport from the ER to the Golgi apparatus. A *SEC18* homologue has also been isolated from the dimorphic fungus *Candida albicans*, indicating that the mechanism of vesicular fusion is thus highly conserved between species.

All three proteins are between 750 amino acids to 790 amino acids in length and have two consensus regions which constitute a putative ATP binding site. The amino acid sequence and corresponding DNA sequence of the two consensus regions from *S.cerevisiae*, *C.albicans* and mammals, are represented in figure 7.5. These were used to design oligonucleotides to

**Figure 7.5.** The two consensus regions from *S.cerevisiae*, *C.albicans* and mamma that form the ATP binding site of the SEC18/NSF protein.

<b>Mammals</b>	<b>AA:</b>	Gly	Pro	Pro	Gly	Cys	Gly	Lys	Thr
	<b>DNA:</b>	GGC	CCC	CCT	GGT	TGT	GGT	AAG	ACT
<i>S.cerevisiae</i>	<b>AA:</b>	Gly	Pro	Pro	Gly	Thr	Gly	Lys	Thr
	<b>DNA:</b>	GGT	CCT	CCA	GGT	ACT	GGT	AAG	ACC
<i>C.albicans</i>	<b>AA:</b>	Gly	Pro	Pro	Gly	Thr	Gly	Lys	Thr
	<b>DNA:</b>	GGA	CCT	CCA	GGT	ACT	GGT	AAA	ACG
<b>Oligonucleotide 1:</b>		<b>GGI</b>	<b>CCC</b> <b>T</b>	<b>CCC</b> <b>T</b>	<b>GGT</b>	<b>ACT</b>	<b>GGT</b>	<b>AAG</b> <b>A</b>	<b>AC</b>

<b>Mammals AA:</b>	Gly	Leu	His	Ile	Ile	Ile	Phe	Asp
<b>DNA:</b>	GGC	TTA	CAC	ATC	ATC	ATC	TTT	GAT
<i>S.cerevisiae</i> AA:	Ser	Leu	His	Ile	Ile	Ile	Phe	Asp
<b>DNA:</b>	TCC	TTA	CAC	ATT	ATT	ATT	TTC	GAT
<i>C.albicans</i> AA:	Ser	Leu	His	Ile	Ile	Ile	Phe	Asp
<b>DNA:</b>	GAT	TTA	CAT	GTT	ATT	ATT	TTC	GAT
<b>Oligonucleotide 2:</b>	<b>CCG</b>	<b>AAT</b>	<b>GTG</b>	<b>TAG</b>	<b>TAG</b>	<b>TAG</b>	<b>AAA</b>	<b>CTA</b>
			<b>A</b>	<b>A</b>	<b>A</b>	<b>A</b>	<b>G</b>	

3' ← 5'

attempt isolation of the homologue from *A.thaliana* via the PCR. To do this it was necessary to take into account the degeneracy of the genetic code, with the utilisation of different codons in each of the organisms for the same amino acids. The oligonucleotides so designed are shown in figure 7.5.

#### **7.2.8 Optimisation of the PCR conditions for product isolation.**

The PCR conditions were set up in accordance with the guidelines provided by the suppliers of the Deep Vent Polymerase used in the reaction, and the calculated melting temperatures of the oligonucleotide sequences. The Deep Vent Polymerase has a 3'-5' proof reading activity which removes misincorporated bases from the 3' end of the growing chain. These conditions were varied on numerous occasions in an attempt to isolate the PCR product from *A.thaliana*.

The products of the PCR were examined by agarose gel electrophoresis. Two products of approximately 350bp and 220bp were isolated after the PCR using genomic *S.cerevisiae* DNA. No products were isolated after the PCR using genomic *A.thaliana* DNA, only a smear of DNA was seen. This indicated that using degenerate oligonucleotides and the PCR to isolate homologues from *A.thaliana* was not very effective, possibly due to non-specific binding of the oligonucleotides to the genomic DNA. This is probably borne out by the fact that two products are amplified when using genomic *S.cerevisiae* DNA.

#### **7.2.9 Design of homologous oligonucleotides.**

Small GTP binding proteins are encoded by *ras*-related genes and play a central role in vesicle transport. The *SEC4* gene of *S.cerevisiae* encodes a 24kDA GTP-binding protein involved in the final stage of secretion. *Ras* and *ras*-related proteins constitute a superfamily of eukaryotic proteins which, by a cycle of GDP and GTP binding, are thought to act as regulators of diverse cellular processes. All of these proteins are around 200 amino acids residues long, and contain highly conserved segments, which are known to interact with the bound guanine nucleotide.

In mammals at least three sub families of small GTP-binding proteins can be distinguished: *Ras*, *Rho* and *Rab*. The yeast counterparts of the *Rab* proteins have been called *Ypt* proteins. *Ras* related proteins have been cloned

from a wide range of evolutionary distinct organisms, including mammals, birds, *Drosophila* and plants.

Homologous oligonucleotides were designed to the DNA sequence of the *S.pombe* *YPT2* gene (a *SEC4* homolog) that is able to complement a *S.cerevisiae* *sec4* mutant (Haubruck *et al.* 1990). There were two reasons for taking this approach: (i) degenerate oligonucleotides had already been used with poor effect, due to non-specific binding (see previous section); (ii) *S.pombe* is more closely related to *A.thaliana* on the phylogenetic tree than is *S.cerevisiae*.

The oligonucleotides were specifically designed to three of the four regions implicated in the binding of GTP in the *SEC4* gene (see figure 7.6.). These oligonucleotides were used in the following combinations; priming with oligonucleotides 4.1 and 4.2 should yield a product approximately 160bp in size after the PCR reaction; priming with oligonucleotides 4.1 and 4.3 should yield a PCR product of approximately 320bp.

#### **7.2.10 Optimisation of the PCR conditions for product isolation and cloning.**

The original PCR conditions were set up in accordance with the guidelines provided by the manufacturers of the Deep Vent Polymerase used in the reaction, and the calculated melting temperatures of the oligonucleotide sequences. Test PCRs were performed to check that the oligonucleotides were able to generate the correct sized DNA product from genomic *S.pombe* DNA. The products of the PCR were examined by agarose gel electrophoresis (figure 7.7.). As can be seen from the gel electrophoresis products of approximately the correct sizes are obtained using the oligonucleotides 4.1 with 4.2 and 4.1 with 4.3. The PCR was performed using genomic *S.pombe*, *A.thaliana* and *K.lactis* DNA templates as well as the pFL61 *A.thaliana* cDNA library. Both conditions and quantities of genomic DNA used in the PCR were altered until the correct sized DNA products were obtained (see figure 7.7.).

However, it was only possible to produce the correct sized product in large enough quantities using oligonucleotides 4.1 and 4.2. Different sets of reaction conditions were tried in an attempt to isolate the 320bp DNA fragment from *A.thaliana* using the 4.1 and 4.3 oligonucleotides. However it was not possible to do so. The products of the PCR were examined by agarose gel electrophoresis (figure 7.7.). As can be seen from the gel electrophoresis a product of approximately the correct size was obtained using the oligonucleotides 4.1 with 4.2. The 165bp DNA fragments were removed from

**Figure 7.6.**

The amino acid sequences of the *S.cerevisiae* (*S.cerev*) Sec4 protein and its functional homologue from *S.pombe*, ypt2 (Salminen and Novick 1987). The bold print indicates the exact regions to which the oligonucleotides were designed. The arrows indicate the 5' to 3' direction of the oligonucleotides and the two CC indicate the carboxy terminus.

		4.1	→
<i>S.pombe</i> ypt2: 1	MSTKSYDYLIKLLLI	<b>GDSGVGKS</b>	SCLLLRFSEDS
<i>S.cerev</i> Sec4: 1	MSGLRTVSASSGNGKSYDSIMKILLI	<b>GDSGVGKS</b>	SCLLVRFVEDK
		← 4.2	
<i>S.pombe</i> ypt2:	FTPSFITTIGIDFKIRTIELDGKRIKLQIW	<b>DTAGOER</b>	FRTITTA
<i>S.cerev</i> Sec4:	FNPSFITTIGIDFKIKTVDINGKKVKLQLW	<b>DTAGQER</b>	FRTITTA
<i>S.pombe</i> ypt2:	YYRGAMGILLLYDVTDKKSFDNVRTWFSNVEQHASENVYKIL	<b>IIG</b>	
<i>S.cerev</i> Sec4:	YYTGAMGIILVYDVTDERFTFTNIKQWFKTVNEHANDEAQLL	<b>LVG</b>	
		← 4.3	
<i>S.pombe</i> ypt2:	<b>NKDC</b> EDQRQVSFEQQALADELGVKFLEASAKTNVNVDEAFFT		
<i>S.cerev</i> Sec4:	<b>NKSDME</b> -TRVVTADQGEALAKELGIPFIESSAKNDDNVNEIFFT		
<i>S.pombe</i> ypt2:	LAREIKKQKIDAENEFSNQANNVDLGNDRTVKR-----		CC
<i>S.cerev</i> Sec4:	LAKLI-QEKIDSNKLVGVGNGKEGNISINSGSGNSSKSN-		CC

**Figure 7.7.** Products of PCR examined by gel electrophoresis.

(a) Agarose gel of test PCR's to check the oligonucleotides 4.1, 4.2 and 4.3 (designated as simply 4.1, 4.2 or 4.3 below) were able to generate the correct sized DNA product from genomic *S.pombe* DNA.

Lane 1: 1Kb ladder

Lane 2: 4.1 and 4.2 negative control.

Lane 3: 4.2 and 4.3 negative control.

Lane 4: Reaction mix containing 50ng of genomic *S.pombe* DNA (4.1 and 4.2).

Lane 5: Reaction mix containing 200ng of genomic *S.pombe* DNA (4.1 and 4.2).

Lane 6: Reaction mix containing 400ng of genomic *S.pombe* DNA (4.1 and 4.2).

Lane 7: Blank lane.

Lane 8: Reaction mix containing 50ng of genomic *S.pombe* DNA (4.1 and 4.3).

Lane 9: Reaction mix containing 200ng of genomic *S.pombe* DNA (4.1 and 4.3).

Lane 10: Reaction mix containing 400ng of genomic *S.pombe* DNA (4.1 and 4.3).

The products are arrowed for each of the set of oligonucleotides.

(b) Agarose gel of PCR's performed using genomic *S.pombe*, *K.lactis* and *A.thaliana* DNA and the pFL61 *A.thaliana* cDNA library.

Lane 1: 1Kb ladder

Lane 2: 4.1 and 4.2 negative control.

Lane 3: Reaction mix containing 200ng of genomic *S.pombe* DNA (4.1 and 4.2).

Lane 4: Reaction mix containing 200ng of genomic *K.lactis* DNA (4.1 and 4.2).

Lane 5: Reaction mix containing 200ng of genomic *A.thaliana* DNA (4.1 and 4.2).

Lane 6: Reaction mix containing 200ng of pFL61 *A.thaliana* cDNA (4.1 and 4.2).

Lane 7: Reaction mix containing 400ng of pFL61 *A.thaliana* cDNA (4.1 and 4.2).

Lane 8: 4.1 and 4.3 negative control.

Lane 9: 4.1 and 4.3 negative control.

Lane 10: Reaction mix containing 200ng of genomic *S.pombe* DNA (4.1 and 4.3).

Lane 11: Reaction mix containing 200ng of genomic *K.lactis* DNA (4.1 and 4.3).

Lane 12: Reaction mix containing 200ng of genomic *A.thaliana* DNA (4.1 and 4.3).

Lane 13: Reaction mix containing 200ng of pFL61 *A.thaliana* cDNA (4.1 and 4.3).

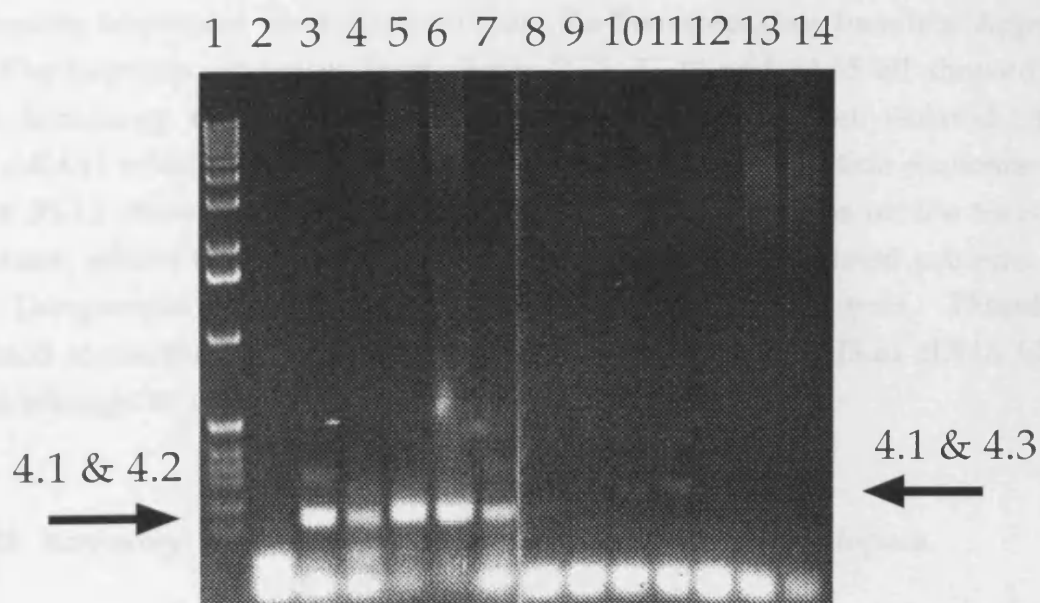
Lane 14: Reaction mix containing 400ng of pFL61 *A.thaliana* cDNA (4.1 and 4.3).

The products are arrowed for each of the set of oligonucleotides.

(a)



(b)





the gel lanes where the PCR products had been derived from *A.thaliana* genomic DNA template and the *A.thaliana* cDNA library and isolated by electroelution. The DNA fragments isolated were ligated into the cloning vector pUC18 which had been digested with restriction endonuclease *Sma*I and phosphatased.

After being cloned into the *Sma*I restriction site of pUC18, positive clones containing PCR products derived from *A.thaliana* genomic DNA template or the *A.thaliana* cDNA library were identified by colony PCR. Three clones were selected containing genomic DNA inserts and three containing cDNA inserts, and subjected to automated DNA sequence analysis. The sequences obtained were analysed via the GCG molecular biology package. All six DNA sequences were different. Consequently when the DNA sequence was translated into the resulting amino acid sequence, it was different for each clone (see figure 7.8.). One of the isolates (36.4) did not produce a translated product of a reasonable size in any of the possible six reading frames, so it was not used for further analysis.

#### **7.2.11 *The identification of homologous protein sequences.***

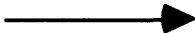
Potential homologues to the protein sequences shown in Appendix 2, were obtained using the BLAST server on the NCBI network and the BLITZ programme at EBI . The BLAST and BLITZ searches show that a whole range of peptide sequences were obtained from the Swissprot data base (see Appendix 2). The peptide sequences from clones 30.2, 31.14 and 34.25 all showed very high homology to *Arabidopsis* genes that have already been isolated (*ARA1* and *ARA4*) which are small GTP binding proteins. The peptide sequence from clone 33.13 showed little homology with any of the proteins on the Swissprot database, whilst the 32.16 clone showed homology to *ras* related proteins from rice, *Dictyostelium discoideum*, humans, yeast and *Arabidopsis*. Therefore I decided to use the 32.16 DNA insert as a probe against the pFL61 cDNA library in an attempt to isolate a complete *SEC4* homologue.

#### **7.2.12 *Screening the pFL61 cDNA library for putative homologues.***

The pFL61 cDNA plasmid library was transformed into *E.coli* strain XL1-blue and cells were plated onto large LB-agar plates containing ampicillin. On average 20 000 colonies were put on each plate and grown overnight at 37°C.

**Figure 7.8.** The peptide sequences of six clones selected after transformation in *E.coli*. The first three contained *A.thaliana* genomic DNA and the last three contained *A.thaliana* cDNA from the pFL61 library. The bold print denotes the peptide sequence of the original oligonucleotide 4.1 used in the PCR. The arrow indicates 5' to 3' translation.

30.2  **GDSGVGKSNL** LSRFTRNDFS HDSRSTIGVE FAHVASRSTT RLSKLRYGTL LARN

31.14  **GDSGVGKSNL** LSRFSRDEFD TNSKATIGVE FQTQLVEIEG KEVKAQIWXT TGQEREFQ

32.16  **GDSGVGKSNL** LSRFTRNDFS HDSRSTIGVE FGTRRSRSTT RLSKLRYGTL LAGTGYR

33.13  **GDSGVGKSNW** RSISRVVVRV RPSVTGTSGQ KVPDLELTLL PFDLARLRSF TRCRIGQS

34.25  **GDSGVGKSNL** LSRFSRDEFD TNSKATIGVE FQTQLVEIEG KEVKAQIWTL LARN

36.4  V\*VI\*AAATE VTAAQDAAR MPEKLFRLSP VTKTIY\*PIT TMHVLHYVCM ILSKPWC

Duplicate lifts were taken from each of the plates using Hybond-N filters. Southern blot analysis were performed on these filters using the DNA insert from clone 32.16 as a probe.

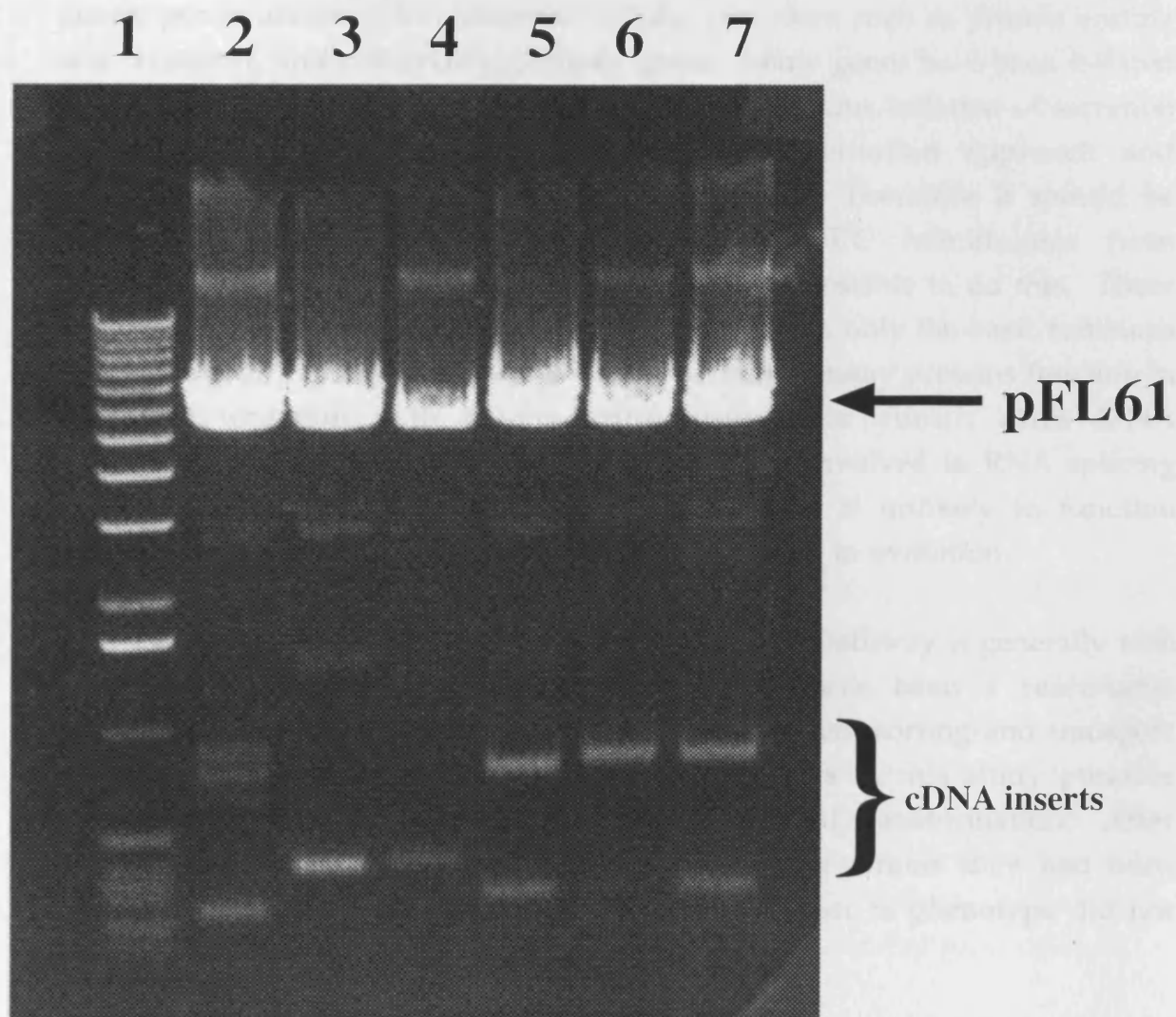
Positive clones were isolated using the duplicate autoradiographs produced from the filters screened, and a secondary screen performed on these putative positives. Those that were still positive on the secondary screen were streaked onto LB-ampicillin agar plates, before a tertiary screen was performed on them. Using the autoradiograph produced by the tertiary screen, six positive clones were selected (named GTP1-6) and the pFL61 library plasmids isolated from them. Large scale preparations were made of these clones using Qiagen columns. These plasmids were digested with restriction endonuclease *NotI* to release the cDNA insert, and these were examined by gel electrophoresis (see figure 7.9). As a final check to see if the clones contained the partial GTP-binding site, a PCR was performed using the oligonucleotides 4.1 and 4.2 and the plasmid isolates. All the clones GTP1-6 contained the partial GTP-binding site as they produced the correct sized 165bp fragment after PCR.

#### **7.2.13 Transformation of a *sec4 ura3* mutant.**

A *sec4 ura3* yeast strain was obtained from Prof.D.Gallwitz and each of the clones GTP1-6 were individually transformed into this strain to see if complementation of the temperature sensitive phenotype would take place. The conditions were varied as described in section 7.4.2. None of the clones GTP1-6 were able to complement the *sec4* ts phenotype. There was however insufficient time to sequence all the clones in order to complete their characterisation.

**Figure 7.9.** Positive clones GTP1-6 digested with restriction endonuclease *NotI* to release the cDNA inserts.

Lane 1: 1 Kb ladder  
Lanes 2-7: Clones GTP1-6



### 7.3 Discussion.

Although interspecies complementation cannot isolate every gene of interest from higher organisms, a very large number of genes are potentially accessible using this approach, including many of the general biosynthetic genes, genes involved in conserved cellular processes such as protein sorting and transport, and cell cycle regulatory genes. Many genes have been isolated by the complementation of yeast mutants. The previous isolation of secretion pathway gene homologues using the complementation approach and *A.thaliana* cDNA libraries has been documented. Therefore it should be possible to use this method to isolate further *SEC* homologues from *A.thaliana*. In this study however it has not been possible to do this. There could have been a number of reasons for this; firstly, only the basic functions of a eukaryotic cell are expressed in yeast; secondly, many proteins function in narrow interaction with neighbouring proteins or nucleic acids (RNA Polymerase subunits, ribosomal proteins, proteins involved in RNA splicing etc). In this case heterospecific complementation is unlikely to function effectively in organisms which have widely diverged in evolution.

It has been shown however, that the secretion pathway is generally well conserved between species, therefore it may have been a reasonable assumption to make that the genes involved in protein sorting and transport could be isolated by complementation. In all cases in this study putative complementing clones were isolated after the initial transformation. After plasmid rescue and then retransformation into the strains they had been isolated from however, the complementation of the *sec* ts phenotype did not occur.

As this work was being brought to a conclusion an interesting publication was released by Shevell *et al.* (1994) concerning the isolation of *EMB30*, a gene involved in apical-basal pattern formation in the *Arabidopsis* embryo. *EMB30* is essential for normal cell division, cell expansion and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to the yeast Sec7 protein and to two other open reading frames identified in clones from humans and *C.elegans*. This region of similarity among these four sequences has been referred to as the Sec7 domain.

The *emb30-1* allele has a mutation in the Sec7 domain that alters a residue conserved in all four of these sequences, suggesting that this domain

may be important for *EMB30* function. Since yeast temperature-sensitive *sec7* mutants accumulate exaggerated Golgi cisternae at the non-permissive temperature (Novick *et al.*, 1981), Shevell *et al.*, (1994) examined *emb30* mutants and WT seedlings using transmission electron microscopy. No obvious changes in the structure of the Golgi apparatus of *emb30* cells compared with that of WT were observed by Shevell, indicating that although the two genes had areas of homology, their cellular functions probably differed.

The procedure of using homologous and degenerate oligonucleotides for the isolation of other gene homologues has become an accepted laboratory procedure. It should therefore have been feasible to isolate gene homologues to at least the *SEC4* gene if not the *SEC18* gene. The results indicated however that using degenerate oligonucleotides to try to isolate the *SEC18* gene homologue from *A.thaliana* was not possible. This was probably due to non-specific binding of the oligonucleotides to the *A.thaliana* genomic and cDNA from the library.

It should have been possible to isolate gene homologues using the techniques mentioned above. That this has not been possible may be due to species divergence between *S.cerevisiae*, *S.pombe* and *A.thaliana*, but is more likely to be as a result of the interactions of the proteins of the genes selected (as gross secretion pathway function has been shown to be very well conserved between species). i.e. A homologue may have been isolated using the PCR technique, but may function in a slightly different manner in the *A.thaliana* secretion pathway, interacting with the same gene products but with a different specificity, therefore being unable to complement the yeast mutant.

It was however possible to isolate DNA products using the PCR technique. Although these were similar to many of the GTP-binding proteins that had already been isolated from many species, when plasmid clones were isolated from the pFL61 *A.thaliana* cDNA library, they were unable to complement the *sec4* ts mutant. Due to time restrictions it was not possible to sequence these clones and confirm that they did indeed encode GTP binding proteins.

## CHAPTER 8.

### EXPERIMENTAL PROCEDURES

#### 8.1 Growth Media and Conditions.

##### 8.1.1 Luria broth (LB)/ agar.

*E.coli* strains were grown in complex LB medium containing 1% (w/v) bacto-peptone, 0.5% (w/v) bacto-yeast extract and 0.5% (w/v) NaCl, pH adjusted to 7.2 with NaOH. BBL agar was added to a final concentration of 2% for luria agar plates.

##### 8.1.2 *E.coli* transformant selection.

The selection of plasmid-carrying *E.coli* cells was achieved via incorporation of either ampicillin or kanamycin (100mg/ml stocks) in the growth medium at a final concentration of 100µg/ml. Both inducer IPTG and the chromogenic substrate X-gal (100mg/ml stocks) were used at a final concentration of 100µg/ml, for the specific selection of recombinant plasmids in *E.coli* by insertional inactivation of the *lacZ* gene.

##### 8.1.3 Yeast Peptone D-glucose (YPD), SD media and X-gal media.

Yeast strains were grown in complex YPD medium consisting of 1% (w/v) yeast extract, 2% (w/v) peptone and 2% glucose (added after autoclaving from a 40% stock), or in minimal SD media. SD medium was composed of 6.7% (w/v) Difco Yeast Nitrogen Base (containing (NH)<sub>2</sub>SO<sub>4</sub> but not amino acids) and 2% (w/v) glucose. In order to supplement the auxotrophic marker genes of the strains used in this study the following amino acids and bases were added to SD medium after autoclaving, to final concentrations of 40µg/ml : L-tryptophan, L-leucine, L-histidine, adenine sulphate, L-arginine, L-methionine and uracil. S.D. galactose medium was made with 2% galactose instead of glucose.

X-gal was added to SD media with the appropriate amino acids to a final concentration of 40µg/ml from a stock of 20mg/ml.

Starch media consisted of 2% BBL agar, 6.7% (w/v) Difco Yeast Nitrogen Base, 2% (w/v) glucose plus the appropriate amino acids and the pH adjusted to 6.4 with sodium phosphate buffer. The media was autoclaved at 10psi, 121°C for 20 mins.

#### 8.1.4 Yeast peptone glycerol chloramphenicol medium (YPGE).

Yeast cells that had been transformed to uracil auxotrophy by the homologous recombination of a *URA3* gene disruption (*ura3::Cm<sup>R</sup>*) that contained the Chlorophenicol resistance marker, were grown on complex YPGE media consisting of 1% (w/v) yeast extract , 2% (w/v) peptone, 1.5% glycerol (v/v), 2mg/ml chloramphenicol (made as a 100mg/ml stock in ethanol) and 1% ethanol.

#### 8.1.5 5-Fluoro orotic acid (5-FOA) medium.

Yeast cells were streaked on 5-FOA plates (SD media containing 1 mg/ml 5-FOA and the appropriate amino acids) prepared as describe by Boeke *et al* (1984) to recover uracil auxotrophs.

#### 8.1.6 Sporulation media.

Diploid strains to be sporulated were first grown overnight in pre-sporulation medium (YP glucose 4%). Strains were then streaked onto sporulation plates (1% KOAc, 0.1% yeast extract, 0.05% dextrose, 2% agar and the appropriate amino acids and bases).

#### 8.2 Bacterial and yeast strains.

All *E. coli* and yeast strains provided, used and constructed throughout this study are listed and characterized in Table 8.2.

**Table 8.2** Bacterial and yeast strains used in this study.

Strain	Genotype	Reference/source
<i>E. coli</i> :		
NM522	F' ( <i>proAB</i> <sup>+</sup> <i>lacI</i> <sup>q</sup> <i>lacZ</i> ΔM15) Δ( <i>lac proAB</i> ) Δ( <i>hsdMS-mcrB</i> )5 <i>r</i> <sub>k</sub> <sup>-</sup> <i>m</i> <sub>k</sub> <sup>-</sup> <i>mcrB</i> <i>thi</i> <i>supE</i>	Gough and Murray (1983)
XL1-Blue	F':Tn10 <i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> <i>lacI</i> <sup>q</sup> Δ( <i>lacZ</i> ) M15/ <i>recA1</i> <i>endA1</i> <i>gyrA</i> 96 (Nal <sup>r</sup> ) <i>thi</i> <i>hsdR</i> 17 ( <i>r</i> <sub>k</sub> <sup>-</sup> <i>m</i> <sub>k</sub> <sup>+</sup> ) <i>supE</i> 44 <i>relA1</i> <i>lac</i>	Stratagene



BNN132	<i>endA1 gyr96 thi hsdR17 supE44 relA1 Δ</i> ( <i>lac-proAB</i> ) F' <i>traD36 proAB + lacI</i> $\phi$ ZΔM15	Elledge <i>et al</i> (1991)
<u><i>S. cerevisiae</i>:</u>		
BF307-10	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4</i>	Futcher and Carbon (1986)
JHRY188	<i>MATα sir3-8 leu2-3 ura12 his3-52 rme4</i>	Brake <i>et al</i> (1984)
BJ2168	<i>MATa leu2 trp1 ura3 prb1 pep4 prc1 gal2</i>	Shore <i>et al</i> (1987)
DBY 746	<i>MATα leu2 trp1 ura3 his3 can1</i>	D.Botstein
JHRY20-2C	<i>MATa leu2 ura3 his3 can1</i>	NYCC
74/2	<i>MATa leu2 his4</i>	NYCC
S150-2B	<i>MATa leu2 trp1 ura3 his3</i>	NYCC
<i>esi1α</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 esi1</i>	This Study
<i>esi4α</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 esi4</i>	This Study
<i>esi9α</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 esi9</i>	This Study
<i>esi1a</i>	<i>MATa leu2 ura3 his3 trp1 arg4 ade6 met4 esi1</i>	This Study
<i>esi4a</i>	<i>MATa leu2 ura3 his3 trp1 arg4 ade6 met4 esi4</i>	This Study
BF307-10-U	<i>MATα leu2 URA3 his3 trp1 arg4 ade6 met4</i>	This Study
<i>esi1-U</i>	<i>MATα leu2 URA3 his3 trp1 arg4 ade6 met4 esi1</i>	This Study
<i>esi4-U</i>	<i>MATα leu2 URA3 his3 trp1 arg4 ade6 met4 esi4</i>	This Study
<i>esi9-U</i>	<i>MATα leu2 URA3 his3 trp1 arg4 ade6 met4 esi9</i>	This Study
<i>esg1</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 esg1</i>	This Study
<i>esg2</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 esg2</i>	This Study
<i>esg3</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 esg3</i>	This Study
<i>esg4</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 esg4</i>	This Study
<i>dsg1</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 dsg1</i>	This Study
<i>dsg2</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 dsg2</i>	This Study
<i>dsg3</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 dsg3</i>	This Study
<i>dsg4</i>	<i>MATα leu2 ura3 his3 trp1 arg4 ade6 met4 dsg4</i>	This Study
HMSF1	<i>MATa gal2 sec1-1</i>	A.Boyd
SF294-2B	<i>MATa sec7-1</i>	A.Boyd
HMSF95	<i>MATα sec8-6</i>	A.Boyd
HMSF163	<i>MATa sec13-1</i>	A.Boyd
HMSF169	<i>MATa sec14-3</i>	A.Boyd
HMSF176	<i>MATa gal2 sec18-1</i>	A.Boyd
<i>sec1-ura</i>	<i>MATa ura3 gal2 sec1-1</i>	This Study
<i>sec7-urs</i>	<i>MATa ura3 sec7-1</i>	This Study
<i>sec8-ura</i>	<i>MATa ura3 sec8-6</i>	This Study

<i>sec13-ura</i>	<i>MATa ura3 sec13-1</i>	This Study
<i>sec14-ura</i>	<i>MATa ura3 sec14-3</i>	This Study
<i>sec18-ura</i>	<i>MATa ura3 gal2 sec18-1</i>	This Study
NY405	<i>MATa ura3-52 sec4-8</i>	Gallwitz <i>et al</i> (1994)
MPC1	<i>MAT<math>\alpha</math> his4</i>	M.Pocklington
MPC6	<i>MATa his4</i>	M.Pocklington

### *S.pombe*

NCYC 972	<i>ura3 gal</i>	NCYC
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### 8.3 Bacterial and yeast plasmids.

All *E. coli* and yeast plasmids used and constructed throughout this study are listed and characterized in Table 8.3.

**Table 8.3** Bacterial and yeast plasmids used in this study.

Plasmid	Description	Reference
pUC18/19	General cloning vectors	Pharmacia
pUC18/ <i>Sma</i> I BAP	pUC18 digested with restriction endonuclease <i>Sma</i> I and treated with bovine Alkaline Phosphatase.	Pharmacia
pBS SK-/+	General cloning vector	Stratagene
pLG669-Z	Yeast/ <i>E.coli</i> shuttle vector for the expression of <i>E.coli</i> $\beta$ -galactosidase ( <i>CYC1-LacZ</i> fusion).	Guarente and Ptashne (1980)
pDP314	Yeast/ <i>E.coli</i> shuttle vector for the expression of mini-proinsulin.	D.Pioli. (1991).
pDP315	Yeast/ <i>E.coli</i> shuttle vector for the expression of mini-proinsulin.	D.Pioli. (1991).
pVK1.1	Yeast/ <i>E.coli</i> shuttle vector for the expression of <i>A.niger</i> $\beta$ -galactosidase.	Kumar <i>et al</i> (1992)
pBEJ15	Yeast expression vector utilising the <i>PGK</i> promoter.	Hadfield <i>et al</i> (1988)

pYCDE-2	Yeast expression vector utilising the ADH1 promoter.	Purvis <i>et al</i> (1991)
YEppGKareBRNSX	Yeast/ <i>E.coli</i> shuttle vector carrying androgen response elements(are)	Purvis <i>et al</i> (1991)
pPGK-hARI	Yeast integrating vector carrying the human androgen receptor gene (hAR)	Purvis <i>et al</i> (1991)
pCH100	Yeast/ <i>E.coli</i> shuttle vector for the expression of CAT.	Hadfield <i>et al</i> (1986)
pBG2-βgal	Yeast/ <i>E.coli</i> shuttle vector for the expression of the <i>A.niger</i> β-galactosidase.	This study
pYIp31	Yeast integrating vector containing the <i>URA3</i> marker gene.	Hadfield (1987)
pFL61cDNA Lib	Yeast/ <i>E.coli</i> shuttle vector containing a cDNA library from <i>A.thaliana</i> , and utilising the <i>PGK</i> promoter for expression of the cDNA's.	Lacroute <i>et al</i> (1992)
λ-YES-R cDNA Lib	A multifunctional cDNA expression vector, containing a library from <i>A.thaliana</i> .	Elledge <i>et al</i> (1991)
pBG1	Yeast/ <i>E.coli</i> shuttle vector for the expression of mini-proinsulin.	This study
pJK-αw	Yeast/ <i>E.coli</i> shuttle vector for the expression of wheat α-amylase.	J.Kirham (1989)

## 8.4 Yeast Nucleic Acid Isolation.

### 8.4.1 Isolation of genomic DNA.

Based on Cryer *et al* (1975)

Overnight 10 ml YPD cultures were set up of the strains of interest. The cultures were back diluted to an  $A_{600}$  of approximately 0.1-0.2. The cells were grown up to mid-log phase and then harvested in a Herus centrifuge at 6000 rpm for 5 mins. The pellet was resuspended in 3 ml of 1.2M Sorbitol, 25mM EDTA pH 8.0 for each gramme wet weight of cells , 175 µl of 1M DTT was added and the tube incubated at 30°C for 30 mins. The tube was centrifuged at 6000 rpm for 5 mins and resuspended in 5 ml of 1.2M Sorbitol, 0.1M Na citrate, 10mM EDTA pH 8.0 and 1mg of yeast lytic enzyme added. The cells were

incubated at 30°C with gentle shaking until they had spheroplasted. After spheroplasting the cells were washed three times in 5 ml of 1.2 M Sorbitol and then resuspended in 2 ml of 5% Sarkosyl, 0.5M Tris and 0.2M EDTA pH 7.6. 0.2mg of proteinase K was added and the cells incubated at 55°C for an hour. After incubation the volume was made up to 5 ml with 1.2M Sorbitol and the lysate extracted twice with chloroform/iso-amyl alcohol, then twice with phenol/chloroform and then again with chloroform/iso-amyl alcohol. The nucleic acids were recovered by ethanol precipitation, by the addition of 2 volumes of cold absolute ethanol, and centrifugation at 5000 rpm for 5 mins. The pellet was resuspended in 500 µl of TE, 20 µl of 10mg/ml RNase I added and the tube incubated at 37°C for 2 hours. The DNA was ethanol precipitated by the addition of 2 volumes of cold absolute ethanol and centrifuged at 7000 rpm for 5 mins. The pellet was air dried, resuspended in 500 µl of TE and the DNA analysed by UV spectrophotometry and gel electrophoresis.

#### **8.4.2 Yeast RNA preparation.**

Modified from Schmitt *et al.* (1990).

Ten ml cultures of the strains of interest were grown in YPD to an  $A_{600}$  of 2.5-5. The cultures were harvested at 5000 rpm for 5 mins and resuspended in 1 ml of 50mM Na acetate pH5.3 and 10mM EDTA (AE buffer), 40 µl of 10% SDS was added and the tube vortexed for 30s. An equal volume of AE buffer equilibrated phenol was added, the tube vortexed for 30 secs and incubated at 65°C for 4 mins. The tube was then rapidly chilled in a dry ice/ethanol bath until phenol crystals appeared. The tube was then placed back at 65°C and the chilling/heating process repeated twice more. The tube was then centrifuged at 13000 rpm for 2 mins, the upper aqueous phase transferred to a fresh tube and phenol chloroform extracted twice. The RNA was precipitated by the addition of 40 µl of 3M Na acetate pH 5.3 and 2.5 volumes of absolute ethanol. The tube was incubated in the -20°C freezer for an hour and the RNA collected by centrifugation at 13000 rpm for 5 mins, washed in 80% ethanol and resuspended in 50 µl of double distilled water. The RNA was analysed by UV spectrophotometry and gel electrophoresis.

#### **8.4.3 Rapid preparation of plasmid DNA.**

Crude preparations of *S.cerevisiae* plasmid DNA for subsequent transformation into *E.coli* were obtained by lysis of yeast spheroplasts.

##### Solutions:

BME: 0.9M sorbitol, 0.05M NaPO<sub>4</sub> (pH7.5), 0.1% (v/v) β-mercaptoethanol (added just before use)

4-5 ml yeast cells grown overnight in selective media were pelleted for 1min in a microfuge at top speed and resuspended in 800µl BME. 25µl 10mg/ml yeast lytic enzyme (YLE) was added, the tubes mixed by inversion and incubated at 37°C for 30-45 mins until cells were spheroplasting. Tubes were placed at 70°C for 20 mins to denature the YLE, 200µl 5M K acetate added, mixed and left on ice for 45 mins, followed by pelleting in a microfuge at top speed for 30sec. The supernatant was transferred to a fresh tube and the plasmid DNA precipitated with 550µl isopropanol, at room temperature for 5 mins. DNA was then pelleted (13K rpm, 5 mins, room temperature) washed in 70% rt EtOH and resuspended in 20µl TE at 65°C for 5 mins. 10µl samples could then be used directly for the transformation of *E.coli*

## **8.5 Yeast transformation , mutagenesis, matings and tetrad dissections.**

### **8.5.1 Lithium acetate high efficiency transformations.**

Based on Schiestl and Gietz (1989).

Yeast cultures were grown overnight in YPD from a single colony and back diluted to a cell density of  $5 \times 10^6$  cells/ml in 10 mls of fresh YPD. The cultures were grown up to a cell density of  $2 \times 10^7$  cells/ml and then harvested at 3000 rpm for 5 mins. The spent broth was removed and the cells resuspended in 1 ml of 10mM Tris.HCL, 0.1mM EDTA pH7.0 (TE) and then spun at 7000 rpm for 2s. The supernatant was removed and the pellet was then resuspended in 1 ml TE, 0.1M lithium acetate and respun at 7000 rpm. The supernatant was removed and the cells resuspended in TE, 0.1M lithium acetate to give a cell density of  $2 \times 10^8$  cells/ml.

50 µl of cells were added to 7.5mg of single stranded Salmon sperm carrier DNA and 0.4-1mg of transforming DNA and the tube mixed by flicking. 300 µl of fresh filter sterilized 40% PEG3350 in TE, 0.1M lithium acetate was added and the tube mixed. The tube was placed on a "rock and roller" mixer at room temperature for 20 mins, heat shocked at 42°C for 20 mins and then plated directly onto selective medium . The plates were incubated at the optimal temperature for 2-4 days.

### **8.5.2 One step lithium acetate low efficiency transformation.**

Yeast cultures were grown up overnight in 10 mls of YPD from a single cell. 500 µl of culture pelleted at 5000 rpm, the spent broth discarded and the pellet resuspended in 100 µl of 40% (w/v) PEG4000, 0.2M lithium acetate pH5, 0.1M DTT (one step buffer). The transforming DNA (up to 3 µg) was added, the tube

mixed well and incubated at 45°C for 30 mins. The cells were then plated straight onto selective medium and incubated at the appropriate temperature for 3-4 days.

#### **8.5.3 Mutagenesis of yeast cells. (Based on Sleep *et al* 1991)**

Yeast cells to be mutated were grown overnight in 10mls of YPD and the cell density calculated using a haemocytometer. The cells were harvested by centrifugation at 4000 rpm for 5 mins and resuspended in 10 mls of distilled water. This process was repeated. To 1 ml aliquots of washed cells 0, 10 µl, 20 µl, 40 µl, 80 µl or 160 µl of the mutagen stock solution was added. Following incubation at 30°C for 30 mins, the mutated cells were washed in Na thiosulphate, then distilled water and finally resuspended in 1 ml of distilled water. To determine the percentage of cells that had survived the mutagenic treatment, an aliquot of the appropriate dilution was plated onto YPD plates and incubated at 30°C for 2 days. The % killing was then determined for each mutagen concentration and the amount of mutagen required to give 40% killing calculated. Mutagen stock solutions: EMS supplied as a liquid from Sigma and used without dilution. NTG supplied as a powder and used at between 20-100mM.

#### **8.5.4 Tetrad dissections.**

Tetrad dissections were performed using a Singer MSM Series 200 system micromanipulator by following the manufacturer's instructions.

Asci were digested with 5 µl of β-glucuronidase (90 000 unit/ml) in 250 µl of distilled water for 5 mins before being plated.

#### **8.5.5 Yeast matings.**

The strains of interest were grown o/n in YPD medium. Inoculated into fresh YPD medium at a cell density of  $1 \times 10^5$ /ml and grown until they had reached a cell density of  $1 \times 10^6$ /ml. Cultures were mated by placing 5 µl of the first strain onto selective minimal media plates (that would allow only diploid cells to grow), allowing this spot to dry and then placing 5 µl of the second strain on top of the first. As controls 5 µl aliquots of each strain were also placed separately on the selective plate. Plates were incubated at 28°C for 2-3 days.

## **8.6 Cloning and manipulation of plasmids in *E.coli*.**

### **8.6.1 Small scale plasmid preparations.**

Modified from Birnboim and Doly, 1979.

1.5ml of overnight culture grown in LB plus antibiotic in the 37°C shaker was spun down in the microfuge for 5 mins. The spent broth was removed and the bacterial pellet resuspended in 100 µl of 50mM glucose, 25mM TE pH8.0, 10mM EDTA pH8.0 (solution I) . 200 µl of 0.2N NaOH, 0.1% SDS (solution II) added and the contents mixed by inversion. Following the addition of 150 µl 60% 5M KOAc, 11.5% glacial acetic acid (solution III), the tubes were shaken briefly to mix the solutions. The precipitate was removed by centrifugation and the supernatant pipetted to a new tube carefully. Following extraction with phenol chloroform the plasmid DNA was precipitated by the addition of 2.5 volumes of cold absolute ethanol, and recovered by centrifugation for 10 mins. The ethanol was removed and the pellet washed in 70% ethanol, air dried and resuspended in 20 ml of double distilled water. 3-4 µl were then used in restriction analysis. RNase I was added to the restriction digest to remove any RNA present.

### **8.6.2 Large scale plasmid preparations.**

Larger amounts of purified plasmid DNA were obtained by combining the alkaline lysis method (Birnham & Doly 1979) to ion exchange chromatography using P100 Quiagen kits according to the manufacturers instructions.

### **8.6.3 Highly purified *E. coli* plasmid DNA maxi-preparation**

Plasmid DNA of highly purified quality for specific DNA manipulations, yeast transformation and DNA sequencing reactions was prepared by buoyant density ultra-centrifugation in CsCl gradients adopted from Sambrook *et al.* (1989) as follows.

#### **Solutions:**

SUT: 25% (w/v) sucrose, 50 mM Tris-HCl, pH 8.0

TES: 50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0

LS: 0.2% (v/v) Triton, 100 mM EDTA, 20 mM Tris-HCl, pH 8.0

TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Bacterial cells grown o/n in 400 ml selective medium were pelleted (6K rpm, 10 mins, 4°C), resuspended in 20 ml SUT, re-pelleted as before and suspended in 10 ml TES containing freshly added lysozyme (1 mg/ml). After 10 mins incubation on ice and addition of 0.3 ml 0.5 M EDTA (pH 8.0) cells were kept on ice for a further 10 mins and finally lysed by addition of 10 ml LS and gentle

swirling for 30 mins. Clear cell lysates obtained after centrifugation (35K rpm, 35 mins, 10°C) were phenol extracted by addition of 8 ml phenol and 3 ml 1 M Tris-HCl (pH 8.0) for 5 mins followed by phenol-chloroform extraction in 16 ml phenol/chloroform (1:1 v/v). To the supernatant (10K rpm, 10 mins, 4°C) 2g ammonium acetate was added and total DNA was precipitated in 0.8 vol. ice cold isopropanol for 30 mins on ice. After centrifugation (10K rpm, 10 mins, 4°C) the pellet was allowed to dry at rt, well dissolved in 5 ml TE containing RNase (1 mg/ml) and incubated at 37°C for 30 mins. Further 16 ml TE and 0.2 ml of 0.5M EDTA (pH 8.0) were added and after dissolving 20g ultrapure CsCl (Sigma) the solution was decanted into Sorvall TV850 tubes using a 20 ml-syringe as a funnel. Following addition of 0.7 ml EtBr (10 mg/ml) the tubes were filled up with paraffin oil, precisely balanced, crimp-sealed and ultracentrifuged (45K rpm, 20h, 20°C). Plasmid DNA was gently harvested under UV light using wide bore syringe needles and EtBr was removed by several extractions with an equal volume of water-saturated isobutanol. After addition of 1 vol. water and 2 vol. rt absolute EtOH and incubation at room temperature for 1h plasmid DNA was precipitated (13K rpm, 30 mins, room temperature), washed twice in 70% rt EtOH, air dried and usually resuspended in 0.3 ml TE.

#### **8.6.4 Estimation of DNA concentration**

DNA concentrations were calculated using a Shimadzu spectrophotometer and quartz cuvettes of 1 cm path length. OD<sub>260</sub> and OD<sub>280</sub> of appropriately diluted DNA solutions were measured and concentrations deduced given that OD<sub>260</sub> values of 1 equal 50 µg/ml for dsDNA and 20 µg/ml for oligonucleotides of about 20 mer, respectively (Sambrook *et al.*, 1989). OD<sub>260</sub> /280 ratios were calculated as well in order to deduce DNA quality considering that ratios of 1.8 are optimal in terms of reduced protein contamination.

#### **8.6.5 Transformation of Competent *E.coli*.**

Competent *E.coli* XL1-blue and NM522 cells were prepared using the CaCl<sub>2</sub> method described by Sambrook *et al* (1989). The competent cells were stored at -80°C until required.

Ligated DNA was transformed by adding half of the ligation mix to a 100 µl aliquot of competent cells. The transformation mix was incubated on ice for 30 mins, placed in a water bath at 37°C for 2 mins to heat shock the cells. 1ml of LB was then added and the cells incubated at 37°C for an hour in order to express the antibiotic resistance genes carried on the newly received cloning vector. Following expression the cells were collected by centrifugation, the



spent medium removed and the cells resuspended in 100 µl of LB and 10 and 90 µl of these cells plated out onto the appropriate selection plates.

## **8.7 Nucleic acid electrophoresis and blotting.**

### **8.7.1 DNA agarose gel electrophoresis.**

Electrophoresis was carried out at 1-10V/cm in 1 x TAE buffer (0.04 M Tris-acetate, 0.5 mg/ml ethidium bromide and 0.001 M EDTA). A 0.6% to 1.5% agarose gel, depending on the size range of separation required, was prepared in 1 x TAE buffer. A fifth volume of 10 x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water) was added to the DNA sample prior to loading and running. Marker DNAs of known molecular mass were run alongside the DNA samples to enable rapid size estimation of the DNA bands when visualised on a UV trans-illuminator. The gel was then photographed using a Mitsubishi video camera and video processor K65HM.

### **8.7.2 RNA formaldehyde gel electrophoresis.**

A 3% agarose was prepared in water, cooled to 70°C, and 10 x MOPS buffer (0.2 M MOPS pH 7.0, 80 mM Sodium acetate and 10 mM EDTA pH 8.0) and formaldehyde added to give final concentrations of 1 x MOPS and 2.2 M formaldehyde. The gel was poured immediately in a fume hood and allowed to set for 30 mins.

The RNA samples were prepared in a sterile microfuge tube by mixing the following;

RNA (up to 20 mg)	4.5 µl
5 x MOPS	2.0 µl
Deionised formamide	10.0 µl
Formaldehyde	3.5 µl

The sample were heated to 65°C for 10 mins and then placed on ice immediately. Three microlitres of sterile RNA loading buffer (50% glycerol, 1mM EDTApH8.0, 0.25% bromophenol blue and 0.25% xylene cyanol FF) was added to each sample, and the samples run in 1 x MOPS, 13% formaldehyde at 3-6V/cm. Following staining with ethidium bromide (0.5 mg/ml) for 30-60 mins the gel was photographed alongside a transparent ruler.

### **8.7.3 Northern Blotting (Thomas 1980).**

Following electrophoresis, staining and photography the RNA gels require no further treatment and are blotted directly.

A tray was filled with 10 x SSC and a heavy duty sponge placed in the tray and allowed to soak up the 10 x SSC. A piece of 3MM paper cut to size was placed on the sponge and the gel placed on top, a piece of Hybond-N cut to size and placed on the gel. The Hybond-N was then masked fully around the edges to prevent the SSC from passing around the edges rather than through the Hybond-N. Four more pieces of 3MM paper were placed on top of the filter and then a stack of paper towels on top. A weight (approx. 500 g) was placed on top of the towels; the nucleic acids were transferred to the membrane by capillary action overnight.

The nucleic acids were fixed on the Hybond-N membrane by placing the filter face down onto a UV transilluminator for 2 mins.

### **8.7.4 Southern Blotting.**

DNA was transferred from agarose gels to nylon membranes by the method of Southern (1975). After blotting, the DNA on the membrane was immobilised by exposure to UV on a transilluminator for 5 mins.

### **8.7.5 Preparation of high specific activity probes by random hexamer priming.**

(Based on Feinberg and Vogelstein 1983.)

DNA probes were radiolabelled using random hexanucleotide priming. Labelling reactions of 15 µl contained 7.5 µl DNA solution with a total of 100-200 ng DNA, 3 µl oligonucleotide labelling buffer (OLB-C), 1 µl BSA, 1 µl (6 U) Klenow fragment and 2.5 µl <sup>32</sup>P-dCTP. OLB-C is a mixture of solutions A:B:C in the ratio 10:25:15 with solution A: 18 µl β-mercapto-EtOH, 5 µl of each 100 mM dATP, dGTP and dTTP; solution B: 2 M HEPES - NaOH (pH 6.6) and solution C: hexanucleotides (Pharmacia, in TE buffer). The reaction was incubated for 6-20 h at room temperature. The unincorporated label was removed by running the reaction mix down a 1 ml G50 Sephadex column in a pasteur pipette, with a polyallomer wool plug. The column was equilibrated with TE and the sample added after it had been made up to 100 µl with TE pH8.0. The probe was eluted with 100ul aliquots of TE and 100 µl fractions collected from the bottom of the column into separate eppendorf tubes. Fractions were tested with a Gieger counter and the fractions giving peak readings were pooled. Prior to use in hybridisations radiolabelled probes were denatured at 95°C for 5 mins and placed on ice immediately afterwards.

#### **8.7.6 Pre-hybridization and hybridization of nylon membranes.**

The filters were placed into a hybridization chamber with 5ml of Church buffer (Church and Gilbert 1986) (7% SDS, 1mM EDTA, 0.5M Sodium hydrogen phosphate buffer pH7.4) for one or two hours at 65°C. The probe was added to the chamber and the hybridization was carried out at 65°C overnight.

Washing stringency conditions;

The filters were washed three times in 2 x SSC, 0.1% SDS at 65°C. The filters were partially dried and wrapped in Saran wrap. Autoradiography was carried out at -80°C using X-ray film in a cassette, for a period ranging from a few hours to one week depending on the radio-activity detectable on the filter after washing.

#### **8.7.7 Autoradiography and Phosphor-Imager**

When using <sup>35</sup>S-dATP in DNA sequencing reactions, gels were dried prior to autoradiography, and exposed naked to X-ray film (Kodak XAR5 13x18 cm, Fuji NIF RX 18x24 cm or Amersham Hyperfilm) at room temperature. In the case of <sup>32</sup>P-dCTP, filters were wrapped in clingfilm and exposed to X-ray film between two intensifying screens at -70°C. For single copy genes exposure was 24-72 h. Autoradiograms were developed using an Agfa-Gevaert Gevamatc automatic film processor. For mRNA analysis, hybridisation signals were quantified with a Phosphor-Imager (Molecular Dynamics) using the Image-Quant™ software package (Johnston *et al.*, 1990).

### **8.8 DNA manipulation and modification.**

#### **8.8.1 DNA ligations.**

For general ligations of molecules with compatible cohesive termini the following conditions were used; 100-200ng of digested dephosphorylated vector and 300-400ng of digested gel purified fragment were mixed with a fifth volume of 5 x ligation buffer (Gibco-BRL), 1-10 Weiss units of T4 DNA ligase and made up to a final volume of 10-50 µl with double distilled water. Incubations were carried out at 15°C overnight. Where blunt end ligations needed to be performed the Pharmacia T4 DNA ligase (8U/ µl) was used.

#### **8.8.2 DNA Restriction endonuclease digestion and dephosphorylation**

These were carried out according to the manufacturers recommendations for each individual enzyme. Generally a tenfold excess of restriction enzyme was used. Restriction enzyme buffers were supplied by the manufacturer.

Vector DNA that was digested with a single restriction enzyme was dephosphorylated prior to ligation with the fragment of interest to prevent self ligation of the vector. 1-5µg of vector were digested with the relevant restriction enzyme. The digest was then mixed with a tenth final volume of 10 x CIP buffer (100mM Tris.Cl pH8.5, 10mM MgCl<sub>2</sub> and 10mM ZnCl<sub>2</sub>), one unit of Calf Intestinal Alkaline Phosphatase was added and the incubation allowed to proceed at 37°C for 30 mins. The dephosphorylated vector was then extracted with phenol/chloroform, ethanol precipitated, dried and redissolved in double distilled water.

### **8.8.3 Purification of DNA fragments.**

DNA fragments were purified from agarose gels directly using the "Gene Clean" kit (BI0 101 Inc) according to the manufacturers instructions. Gel slices were melted in 6M NaI at 65°C, the DNA adsorbed onto silica glass beads, washed and subsequently dissociated from the glass in TE buffer at 65°C. One tenth of the purified DNA was used in agarose gel electrophoresis to estimate quality and quantity of DNA and the remainder used or stored at -20°C.

### **8.8.4 DNA phosphorylation**

DNA fragments were phosphorylated mainly to facilitate direct cloning of PCR products using dephosphatased pUC vectors. In all cases the enzyme of choice was Polynucleotide Kinase supplied with reaction buffer by NEB. Kinase reactions of 100 µl contained 20 units enzyme, ATP at a final concentration of 1 mM, and up to 5 µg DNA. They were incubated at 37°C for 30 mins and inactivated by direct phenol extraction. Phosphorylated DNA was recovered by standard EtOH precipitation.

### **8.8.5 DNA sequencing.**

#### **8.8.5.1 Manual sequencing.**

DNA sequence was derived directly from double stranded plasmid DNA by using the T7 sequencing kit (Pharmacia LKB Biotechnology) according to the manufacturer's instructions. DNA fragments were labelled with <sup>35</sup>S-dATP using both standard pUC universal and synthesised oligonucleotide primers. Sequencing reaction products were analysed by PAGE on a 0.5 mm x 45 cm x 20 cm (50 ml) denaturing acrylamide gel. Commercially available sequencing grade 40% (w/v) acrylamide (Accugel 40, 19:1 acrylamide: bisacrylamide) was used for making the gel. The urea stock (46% (w/v)) was deionised prior to

use, and was stored, for up to two weeks at 4°C. Sequencing gels (1 x TBE, 23% (w/v) urea, 7% (w/v) acrylamide, 0.1% (w/v) ammonium persulphate, 0.1% v/v TEMED) were electrophoresed in TBE buffer at a constant 1000-1400 V for 2-6 h. The gel was then transferred to a sheet of 3 MM Whatman paper, covered with Saranwrap and dried under vacuum at 80°C for 2 hrs. The was then exposed to Kodak XAR-5 film at room temperature for between 16 and 72 hrs.

#### **8.8.5.2 Automated sequencing.**

DNA was sequenced using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, and analysed on a ABI model 373A DNA Sequencer.

Terminator premix: 1.58μM A-DyeDeoxy, 94.74μM T-DyeDeoxy, 0.42μM G-DyeDeoxy, 47.37μM C-DyeDeoxy, 78.95μM dITP, 15.79μM dATP, dCTP, dTTP, 168.42mM Tris-HCl (pH9.0), 4.21mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 42.1mM MgCl<sub>2</sub>, 0.42 units/μl AmpliTaq DNA polymerase.

Sequencing reactions were carried out in a total volume of 20μl (overlaid with liquid paraffin) containing 9.5μl terminator premix, 1μg template DNA and 3.2pmol primer DNA. The cycling reaction was carried out in a Hybaid Omn-E thermal cycler, and consisted of 25 cycles of a denaturation step (96°C, 30"), an annealing incubation (50°C, 15") and an extension incubation (60°C, 4 mins). After PCR the reaction mix was isolated from under the paraffin layer by pipetting, made up to 100μl with water and extracted twice in phenol : H<sub>2</sub>O : Chloroform (68:18:14). Extension products were precipitated with 15μl 2M Na acetate (pH4.5), 300μl 100% EtOH, washed in 70% EtOH, then analysed on a ABI model 373A DNA Sequencer.

#### **8.8.6 Polymerase chain reaction**

PCR was used both for engineering novel restriction enzyme sites into DNA fragments and for amplification of DNA fragments which were not amenable to direct cloning. PCR was performed using a Techne PHC-3 Programmable Heating Block. Initially AmpliTaq *Taq* DNA polymerase was used (with a reaction buffer of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% (w/v) gelatine) but later Promega *Taq* DNA polymerase and NEB Vent Polymerase were used. dNTPs (Pharmacia, Ultrapure, 5 mM stock stored at -20°C) were used at a final concentration (for each dNTP) of 200 μM. The final concentration of target DNA present in each reaction was less than 10 ng. All oligonucleotide primers, listed in Table 8.3., used for PCR or DNA sequencing were synthesised by J. Keyte and D. Langton (Department of Biochemistry,

University of Leicester) using an Applied Biosystems 380B Synthesiser according to the  $\beta$ -cyanoethyl-phosphoramidite method (Winnacker and Dörper, 1982). Supplied as approximately 1 ml aqueous ammonia solutions oligonucleotide purification was carried out using standard EtOH precipitation followed by spectrophotometric calculation of concentration. Primers were used in equimolar concentrations at final concentrations of 0.2-1  $\mu$ M. PCR reactions were carried according to the manufacturer's instructions and methods outlined by Ehrlich (1989). A typical reaction profile consisted of 25-30 cycles of a denaturation step (1 min, 95°C), an annealing incubation (2 mins, 40-55°C) and an extension incubation (1 min, 72°C). All reactions were carried out in a total vol. of 50 or 100  $\mu$ l and were overlaid with liquid paraffin to prevent evaporation. Tubes were heated to 95°C for 5 mins to destroy any DNAases, and cooled on ice prior to the addition of polymerase. PCR results were analysed by horizontal TAE-agarose slab gel electrophoresis.

## ***8.9 Preparation of soluble protein extracts and culture supernatants.***

### **8.9.1 Preparation of soluble protein extracts.**

Soluble protein extracts were required to perform a number of enzymic assays. For each enzymic assay a different extraction buffer was required to optimise the conditions for that particular enzyme. The buffers used were as follows:

Chloramphenicol Transferase Extraction Buffer (CEB);

50mM Tris-HCl pH7.5, 100mM sodium chloride,  
0.1mM chloramphenicol,  
1mM Phenylmethanesulfonyl fluoride (PMSF).

$\alpha$ -amylase extraction buffer;

10mM Tris-HCl pH7.0,  
50mM sodium acetate pH5.5, 1mM PMSF.

Alcohol dehydrogenase extraction buffer;

10mM magnesium chloride, 1mM dithiothreitol,  
0.4M sodium chloride, 1mM PMSF.

All strains were cultured in YPD to a cell density of  $1 \times 10^7$ /ml for producing the soluble protein extracts, except for the CAT assays, where strains were cultured in YPGE-chloramphenicol medium.

10 mls of culture ( $10^8$  cells) was harvested by centrifugation at 4000 rpm in a Heraeus centrifuge, and the supernatant discarded. Cells were resuspended in 5 ml of the appropriate ice cold extraction buffer. Cells were harvested as before and resuspended in 250  $\mu$ l of the appropriate ice cold extraction buffer and transferred to Eppendorf tubes. 0.5g of acid-washed glass beads were added and the mixture vortexed for three periods of 30s, interspaced with 30s on ice. Cell breakage was confirmed by microscopic examination and if intact cells remained the vortex treatment was repeated. 0.75 mls of the appropriate extraction buffer was added and the mixture vortexed for 10s. Cell debris was harvested by centrifugation at 5000 rpm for 5 mins and the supernatant transferred to a fresh Eppendorf tube. Finally the tube was centrifuged at 13 000 rpm for 5 mins and the supernatant collected in a fresh Eppendorf. Protein concentrations were estimated for extracts as described in 8.10.2. Soluble protein extracts were either stored on ice until assays were performed on them, or frozen and stored at  $-20^{\circ}\text{C}$  for assay at a later date.

### **8.9.2 Preparation of culture supernatants.**

For the  $\alpha$ -amylase and  $\beta$ -galactosidase reporter proteins, assays were performed on culture supernatants. When cells were originally harvested for soluble protein preparation, 1 ml of the supernatant was saved in a Eppendorf tube and either placed on ice for immediate assay or frozen at  $-20^{\circ}\text{C}$ , for assay at a later date.

### **8.9.3 Calculation of protein concentrations.**

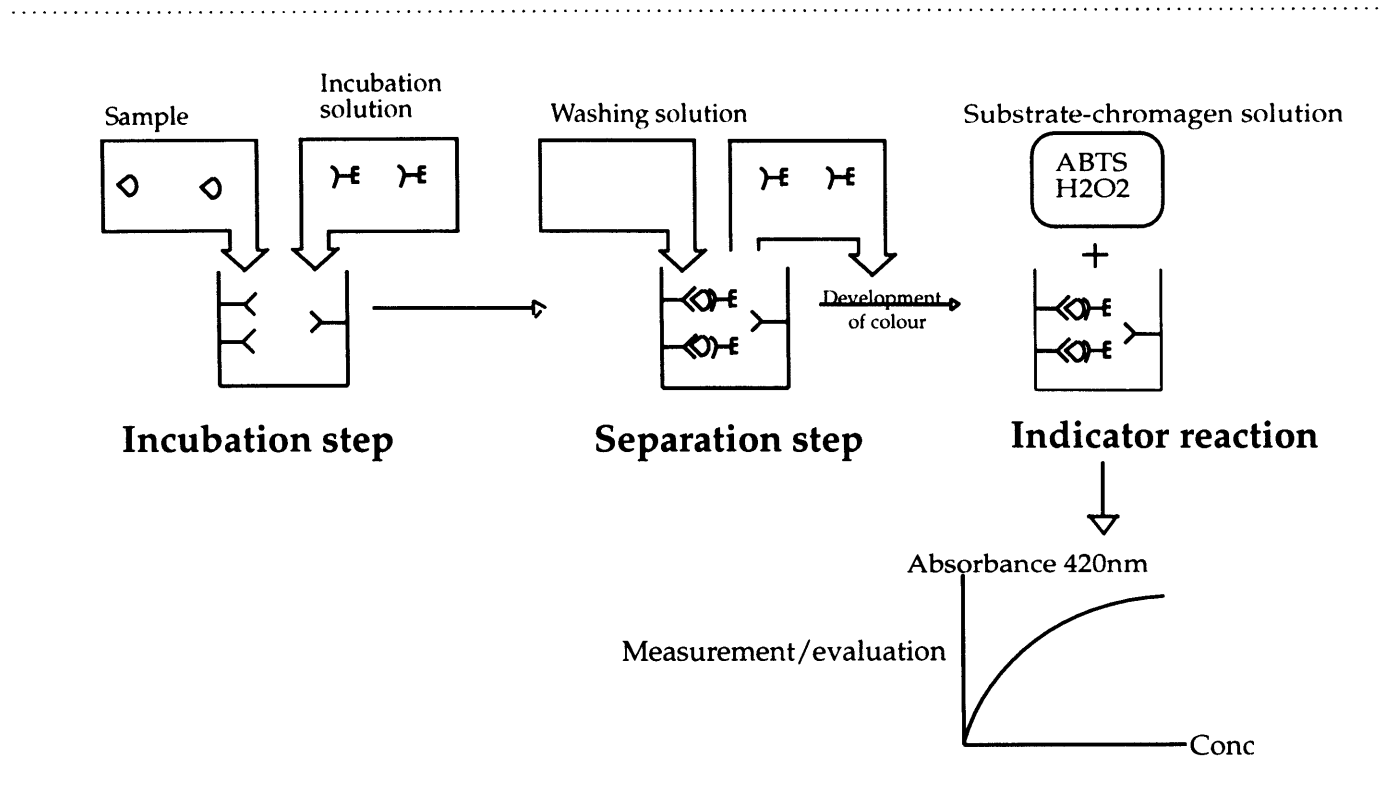
Protein concentration was estimated by the method of Bradford (1976) using a BSA or ovalbumin standard with the Biorad Protein Assay, according to the manufacturer's instructions.

## **8.10 Enzymic and ELISA assays.**

### **8.10.1 Insulin ELISA assays**

Insulin assays were performed using the Boehringer Mannheim Immunodiagnosics Kit, catalogue number 1 289 101. Assays were carried out according to manufacturers instructions. A schematic representation of the assay is shown in figure 8.10.1. As we can see from the diagram, during the incubation step (2 hours at room temperature) the plastic tubes coated with monoclonal anti-insulin antibodies bind to the mini-proinsulin within the supernatant (sample). A blank was prepared without supernatant. Standards

**Figure 8.10.1** ELISA/One step sandwich assay.



**KEY**

- ◊ Insulin in sample
- Y Anti-insulin antibodies
- Y-E Anti-insulin antibody-POD conjugate



(containing known units of insulin activity ) supplied with the kit were used to prepare a standard curve. The incubation solution also contained anti-insulin antibodies conjugated to the enzyme peroxidase. These bind to the mini-proinsulin bound to the tubes. The peroxidase is required for development of the chromagen ABTS (di-ammonium 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonate). The tubes are washed to remove any excess anti-insulin-peroxidase conjugate. The chromagen solution is then added and the tubes incubated at room temperature for 1 hour. The contents of the are mixed and their absorbance measured against the blank at OD<sub>420nm</sub>, using a Philips Pye Unicam SP6-550 UV/VIS spectrophotometer. Mini-proinsulin activity was calculated using the standard curve (see Appendix 3).

### 8.10.2 $\beta$ -galactosidase assays.

Solutions; Z buffer:

60mM disodium phosphate, 40mM sodium dihydrogen phosphate,  
10mM potassium chloride, 1mM magnesium sulphate pH7.0.

ONPG: 4mg/ml o-nitrophenol- $\beta$ -D-galactosidase in  
100mM potassium phosphate buffer pH7.0.

Strains to be assayed were cultured overnight in YPD medium and cells permeabilised for cell enzymic assays as follows:

1 ml of cells were harvested in Eppendorf tubes by centrifugation at 13 000 rpm for 5 mins. Supernatants were collected and saved for assay immediately or frozen at -20°C for assay at a later date.

Harvested cells were washed twice in 1 ml of distilled water and resuspended in a final volume of 1 ml of Z buffer. The OD of the cells was measured at 600nm. 50  $\mu$ l of cells were added to 900  $\mu$ l of Z buffer, 50  $\mu$ l of sodium dodecyl sulphate (SDS)(0.7%) and 50  $\mu$ l of chloroform. A blank containing 950  $\mu$ l of Z buffer, 50  $\mu$ l SDS (0.7%) and 50  $\mu$ l of chloroform, was prepared to zero the spectrophotometer. After vortexing for 10s samples and blank were placed in a 30°C waterbath for 15 mins. 180  $\mu$ l of ONPG was added, the tubes mixed and returned to the waterbath. The time taken for the samples to turn a medium yellow colour was noted and the reaction stopped by the addition of 450  $\mu$ l of sodium carbonate. The tubes were centrifuged at 13 000 rpm for 5 mins and 1 ml of sample transferred to a plastic cuvette. The absorbance of the samples were measured against the blank at OD<sub>420nm</sub>, using a Philips Pye Unicam SP6-550 UV/VIS spectrophotometer.  $\beta$ -galactosidase activity was calculated as follows:

$n \text{ mol ONPG converted/min } (\beta\text{-gal Units}) = 1000 \times \text{OD}_{420\text{nm}} / T \times V \times \text{OD}_{600\text{nm}}$

Where T= reaction time at 30°C and V= volume of cells used.

Culture supernatants were assayed as follows: 100 µl of culture supernatant was added to an Eppendorf tube containing 1 ml of Z buffer and vortexed for 10s. A blank containing 1 ml of Z buffer and 100 µl of YPD was prepared and the procedure followed, the same as described for the cell extracts.

β-galactosidase activity was calculated as follows:

$$\beta\text{-gal units} = 1000 \times \text{OD}_{420\text{nm}} / T \times V \times \text{OD}_{600\text{nm}}$$

Where V= volume of supernatant used and T= reaction time at 30°C.

### 8.10.3 α-amylase assays.

α-amylase activity was measured via the 3,5-dinitrosalicylic acid (DNS) method. This method measures the appearance of reducing sugars as starch is broken down into maltose (causing an increase in absorbance). Either 100 µl of soluble protein extracts from 2 mls of culture or 100 µl of supernatant were incubated with an equal volume of 1% w/w starch in 50mM sodium acetate pH5.5 for 3 hrs at 37°C. Blanks were also prepared without protein extracts or supernatants. After incubation 400 µl of DNS (1%w/v 3,5-dinitrosalicylic acid, 1.5%w/v sodium hydroxide, 30% sodium potassium tartrate) reagent was added and samples boiled in a heating block for 5 mins. Samples were made up to 1 ml with dd water and their absorbance measured against the blanks at 540nm using a Philips Pye Unicam SP6-550 UV/VIS spectrophotometer.

α-amylase activity was calculated by taking the difference between the blank at  $A^{540\text{nm}}$  and the sample at  $A^{540\text{nm}}$  and converting this value into mg of maltose liberated from soluble starch, using a standard maltose curve (Appendix 4).

The value obtained from the standard curve was divided by two for the cell extracts (as 2 mls of culture were used for the assay) and multiplied by ten for the culture supernatants (as 0.1 ml of culture was used for the assay) to give mg of maltose equivalents liberated from soluble starch per ml of culture.

### 8.10.4 Chloramphenicol acetyl transferase assays.

Solution; CAT assay buffer (CAB):

50mM Tris-HCl pH7.5, 100mM EDTA, 1mM 5,5'-dithiobis-2-benzoic acid, 0.1mM Chloramphenicol.

Before assays could be performed two blanks were prepared to zero the Shimadzu UV-visible recording UV-240 spectrophotometer. In two 0.5 ml

quartz cuvettes (1 cm pathlength) 360 µl of CAB and 40 µl of acetyl CoA were aliquoted. The spectrophotometer was set at a wavelength of 412nm, chart speed of 20mm/minute and a absorbance range of 0-0.5. After zeroing one cuvette was removed to be used for sample assays. 350 µl of CAB was aliquoted together with 10 µl of cell extract (0.2-5.0µg protein) and 40 µl of 4mM acetyl CoA into the cuvette. A piece of Parafilm was placed over the top of the cuvette and the cuvette quickly inverted three times. The cuvette was placed into the spectrophotometer and the initial rate of increase in absorbance at 412nm ( $\Delta A^{412}/\text{min}$ ) was measured for a period of 3 mins.

The CAT activity was calculated as follows:

$$\begin{aligned}\Delta A^{412}/\text{min} \times 2 &= \Delta A^{412}/\text{min/ml} \\ (\Delta A^{412}/\text{min/ml})/\mu\text{g protein} \times 10^3 &= \Delta A^{412}/\text{min/ml/mg protein} \\ (\Delta A^{412}/\text{min/ml/mg})/0.136 &= \text{CAT U/mg protein}\end{aligned}$$

Where 0.136= *M* extinction coefficient of DTNB at 412  $\times 10^{-3}$  (for 1 ml volume).

One enzyme unit is defined as the amount of enzyme that catalyses the production of 1µM DNTB/min. The units are expressed relative to the cellular protein.

#### 8.10.5 Alcohol dehydrogenase enzyme assays.

Blanks were prepared as follows: In two 1.7 ml quartz cuvettes 50-100µg of protein extract in a 100 µl sample was added together with 0.7 mls of 10mM Tris-HCl pH9.5 and 0.1 mls of 1.5mM NAD.

A piece of Parafilm was placed over the top of the cuvettes and the cuvettes inverted. The samples were zeroed for 2 mins at 315nm in a Shimadzu UV-visible recording UV-240 spectrophotometer, set at a chart speed of 10mm/min and range 0-0.5. 0.1 ml of 3M ethanol was added to the reaction cuvette and the cuvette quickly mixed before returning to the spectrophotometer. The change in absorbance was measured at 315nm (*E*/minute) for up to 3 mins and ADH units representing ADH specific activity were calculated as follows:

$$\frac{\text{E/minute} \times \text{Reaction volume}}{\text{E} \times \text{path length} \times \text{Sample volume}}$$

Where *E*= extinction coefficient (6.622) of ethanol

path length= 1cm

Sample volume= 0.1 ml

Reaction volume= 1 ml

## 8.11 Miscellaneous.

### 8.11.1 Strain conservation

#### Storage of *E. coli* strains:

Single colonies of bacteria were grown overnight in 2 ml of selective medium and an equal volume of sterile preservation mix (LB, 15% glycerol) was added and the cells were snap frozen in ldry ice-IMS. Cells were stored at -70°C.

#### Storage of yeast strains:

*S. cerevisiae* strains were grown overnight in 10 ml of minimal medium. To 1.5 ml aliquots an equal volume of sterile preservation mix (12.6 g/l K<sub>2</sub>HPO<sub>4</sub>, 3.6 g/l KH<sub>2</sub>PO<sub>4</sub>, 0.9 g/l trisodium citrate, 0.18 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.8 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20% v/v glycerol) was added and the cells were snap frozen in dry ice/IMS. Samples were then stored at -70°C.

### 8.11.2 Colony blots.

A nylon filter cut to the shape of the petri-dish was placed on top of *E.coli* cells, on LB-amp plates after they had been incubated at 37°C o/n, for 30s. Four plastic boxes were lined with 2 sheets of 3MM whatman paper and soaked in the solutions listed below. The filter was then placed in each of the boxes, colony side up for the times listed below:

Box 1	0.5M NaOH	7 mins
Box 2	1.0M Tris-HCl (pH7.4)	2 mins
Box 3	1.0M Tris-HCl (pH7.4)	2 mins
Box 4	0.5M Tris-HCl (pH7.4), 1.5M NaCl	4 mins

The filters were carefully blotted dry and DNA fixed as described in section 8.7.4. As colony blotts were performed in duplicate a second filter was placed on the LB-amp plate for 2 mins and subjected to the above procedure.

### 8.11.3 Library screening.

The *A.thaliana* pFL61 cDNA library was screened as follows: plasmid DNA containing the library was transformed into *E.coli* as described in section 8.6.5. Transformed cells were plated onto large (18 cm diameter) LB petri-dishes

containing 100µg/ml ampicillin (LB amp), at approximately 10 000 cells/petri-dish. Duplicate colony filter lifts were taken from each plate (section 8.10.5). The nylon filters pre-hybridised and hybridised with radioactive probe (8.7.5), washed and autoradiographed as described in section 8.7.6. Using the duplicate autoradiographs, areas containing putative positives were from the plates, washed in 1 ml of LB and cultured o/n in LB amp. Cells from these cultures were plated onto a small LB amp plates and re-screened using the duplicate colony filter procedure (secondary screen). Positives on the secondary screen were confirmed via a tertiary screen. Positives isolated on the tertiary screen were used to transform the *S.cerevisiae* strain NYCC405.

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## Appendix 1. A summary of the BLITZ and BLAST searches for five of the six GTP clones.

### Sequence 30.2.pep:

1 GDSGVGKSNLLSRFSRDEFD...LVEIEGKEVKAQIWTLARN 54

ID ARA1\_ARATH STANDARD; PRT; 218 AA.  
DE RAS-RELATED PROTEIN ARA-1.

Matches 40; Mismatches 4; Partial 4; Indels 0; Gaps 0;

```
*** *****..** *****
Db 19 GDSAVGKSNLLSRFYARNEFSANSKATIGVEFQTQSMEIEGKEVKAQIW 66
Qy 1 GDSGVGKSNLLSRFSRDEFDTNSKATIGVEFQTQLVEIEGKEVKAQIW 48
```

RESULT 2 Score 191; Match 0.0%; Predicted No. 2.94e-35;

ID ARA4\_ARATH STANDARD; PRT; 214 AA.  
DE RAS-RELATED PROTEIN ARA-4.

Matches 37; Mismatches 4; Partial 7; Indels 0; Gaps 0;

```
*** *****..** *****
Db 19 GDSAVGKSNLLTRYARNEFNPNKATIGVEFQTQSMLIDGKEVKAQIW 66
Qy 1 GDSGVGKSNLLSRFSRDEFDTNSKATIGVEFQTQLVEIEGKEVKAQIW 48
```

RESULT 3 Score 185; Match 0.0%; Predicted No. 1.49e-33;

ID YPT8\_YEAST STANDARD; PRT; 223 AA.  
DE GTP-BINDING PROTEIN YPT8.

Matches 34; Mismatches 4; Partial 10; Indels 0; Gaps 0;

```
*****..**..**..*****
Db 20 GDSGVGKSNLLSRFTKNEFNMDKSTIGVEFATRTLEIDGKRIKAQIW 67
Qy 1 GDSGVGKSNLLSRFSRDEFDTNSKATIGVEFQTQLVEIEGKEVKAQIW 48
```

Sequence 31.14.pep

1 GDSGVGKSNLLSRFSRDEFD.....EGKEVKAQIWXTTGQEREF 58

RESULT 1 Score 230; Match 0.0%; Predicted No. 0.00e+00;

ID ARA1\_ARATH STANDARD; PRT; 218 AA.  
DE RAS-RELATED PROTEIN ARA-1.

Matches 45; Mismatches 6; Partial 4; Indels 0; Gaps 0;

\*\*\* \*\*\*\*\*..\*.\* \*\*\*\*\* \*\*\*\*\* \* \*\*\*\*

Db 19 GDSAVGKSNLLSRFYARNEFSANSKATIGVEFQTQSMEIEGKEVKAQIWDTAGQER 73  
Qy 1 GDSGVGKSNLLSRFSRDEFDTNSKATIGVEFQTQLVEIEGKEVKAQIWXTTGQER 55

RESULT 2 Score 216; Match 0.0%; Predicted No. 0.00e+00;

ID ARA4\_ARATH STANDARD; PRT; 214 AA.  
DE RAS-RELATED PROTEIN ARA-4.

Matches 42; Mismatches 6; Partial 7; Indels 0; Gaps 0;

\*\*\* \*\*\*\*\*..\*.\*.\* \*\*\*\*\* .\*.\*\*\*\*\* \* \*\*\*\*

Db 19 GDSAVGKSNLLTRYARNEFNPNKATIGVEFQTQSMLIDGKEVKAQIWDTAGQER 73  
Qy 1 GDSGVGKSNLLSRFSRDEFDTNSKATIGVEFQTQLVEIEGKEVKAQIWXTTGQER 55

RESULT 3 Score 210; Match 0.0%; Predicted No. 6.09e-41;

ID YPT8\_YEAST STANDARD; PRT; 223 AA.  
DE GTP-BINDING PROTEIN YPT8.

Matches 39; Mismatches 6; Partial 10; Indels 0; Gaps 0;

\*\*\*\*\*.....\*.\*.\* \*\*\*\*\* \*.\*\*.\* \*\*\*\*\* \* \*\*\*\*

Db 20 GDSGVGKSNLLSRFTKNEFNMDSKSTIGVEFATRTLEIDGKRIKAQIWDTAGQER 74  
Qy 1 GDSGVGKSNLLSRFSRDEFDTNSKATIGVEFQTQLVEIEGKEVKAQIWXTTGQER 55

**Sequence 32.16.pep:**

**1 GDSGVGKSNLLSRFTRNDFS.....STTRL SKLRYGTLLAGTGYR 57**

RESULT 1 Score 147; Match 0.0%; Predicted No. 7.02e-25;

ID RIC2\_ORYSA STANDARD; PRT; 217 AA.  
DE RAS-RELATED PROTEIN RIC2.

Matches 29; Mismatches 2; Partial 3; Indels 0; Gaps 0;

\*\*\*\*\*.\*\*\*.\*\*\*\*\* \*\*

Db 21 GDSGVGKSNLLSRFTRNEFSLESKSTIGVEFATR 54  
Qy 1 GDSGVGKSNLLSRFTRNDFSHDRSTIGVEFGTR 34

RESULT 2 Score 146; Match 0.0%; Predicted No. 1.37e-24;

ID YPT8\_YEAST STANDARD; PRT; 223 AA.  
DE GTP-BINDING PROTEIN YPT8.

Matches 28; Mismatches 2; Partial 4; Indels 0; Gaps 0;

\*\*\*\*\*.\*\*\*.\*\*\*\*\* \*\*

Db 20 GDSGVGKSNLLSRFTKNEFNMDSKSTIGVEFATR 53  
Qy 1 GDSGVGKSNLLSRFTRNDFSHDRSTIGVEFGTR 34

RESULT 3 Score 145; Match 0.0%; Predicted No. 2.68e-24;

ID RB11\_DICDI STANDARD; PRT; 214 AA.  
DE RAS-RELATED PROTEIN RAB11.

Matches 31; Mismatches 18; Partial 8; Indels 0; Gaps 0;

\*\*\*\*\*.\*\*\*.\*\*\*\*\* \*\* .. \* . \*\*

Db 20 GDSGVGKSNLLSRFTRNEFSLETGSTIGVEFATRTIQTEGKTIKAQVWDTAGQERYR 76  
Qy 1 GDSGVGKSNLLSRFTRNDFSHDRSTIGVEFGTRRSRSTTRL SKLRYGTLLAGTGYR 57

**Sequence 33.13.pep:**

**1 GDSGVGKSNWRSISRVPVRV.....LLPFDLARLRSFTRCRIGQS 58**

RESULT 1 Score 63; Match 0.0%; Predicted No. 5.27e-03;

ID NIFA\_BRAJA STANDARD; PRT; 582 AA.  
DE NIF-SPECIFIC REGULATORY PROTEIN.

Matches 17; Mismatches 15; Partial 10; Indels 2; Gaps 2;

Db 145 VPIRV DSTVVGTLTIDRIPEGSSSLLEYD-ARLLAMVANVIGQT 187  
Qy 16 VPVRVRPSVTGT-SGQKVPDLELTLLPFDLARLRSFTRCRIGQS 58

RESULT 2 Score 60; Match 0.0%; Predicted No. 2.69e-02;

ID PSM\_HUMAN STANDARD; PRT; 750 AA.  
DE PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSM).

Matches 15; Mismatches 15; Partial 2; Indels 1; Gaps 1;

Db 310 GGSAPPDSSWRGSLKVPYNVGPFGFTGNFSTQKV 342  
Qy 1 GDSGVGKSNWRSISRVPVRVRPSVTGT-SGQKV 32

RESULT 3 Score 58; Match 0.0%; Predicted No. 7.76e-02;

ID RNH1\_CRIFA STANDARD; PRT; 494 AA.  
DE RIBONUCLEASE H (EC 3.1.26.4) (RNASE H).

Matches 17; Mismatches 19; Partial 3; Indels 0; Gaps 0;

Db 338 VDAGVPPAALGTSHCVEPDWELSELPLRRLVIYTDSR 376  
Qy 16 VPVRVRPSVTGTSGQKVPDLELTLLPFDLARLRSFTRCR 54

**Sequence 34.25.pap.**

>sp|P19892|ARA1\_ARATH RAS-RELATED PROTEIN ARA-1.  
Length = 218

Score = 205 (92.3 bits), Expect = 1.3e-22, P = 1.3e-22  
Identities = 40/48 (83%), Positives = 44/48 (91%)

Query: 1 GDSGVGKSNLLSRFSRDEFDTNSKATIGVEFQTQLVEIEGKEVKAQIW 48  
GDS VGKSNLLSR++R+EF NSKATIGVEFQTQ +EIEGKEVKAQIW  
Sbjct: 19 GDSAVGKSNLLSRYARNEFSANSKATIGVEFQTQSMEIEGKEVKAQIW 66

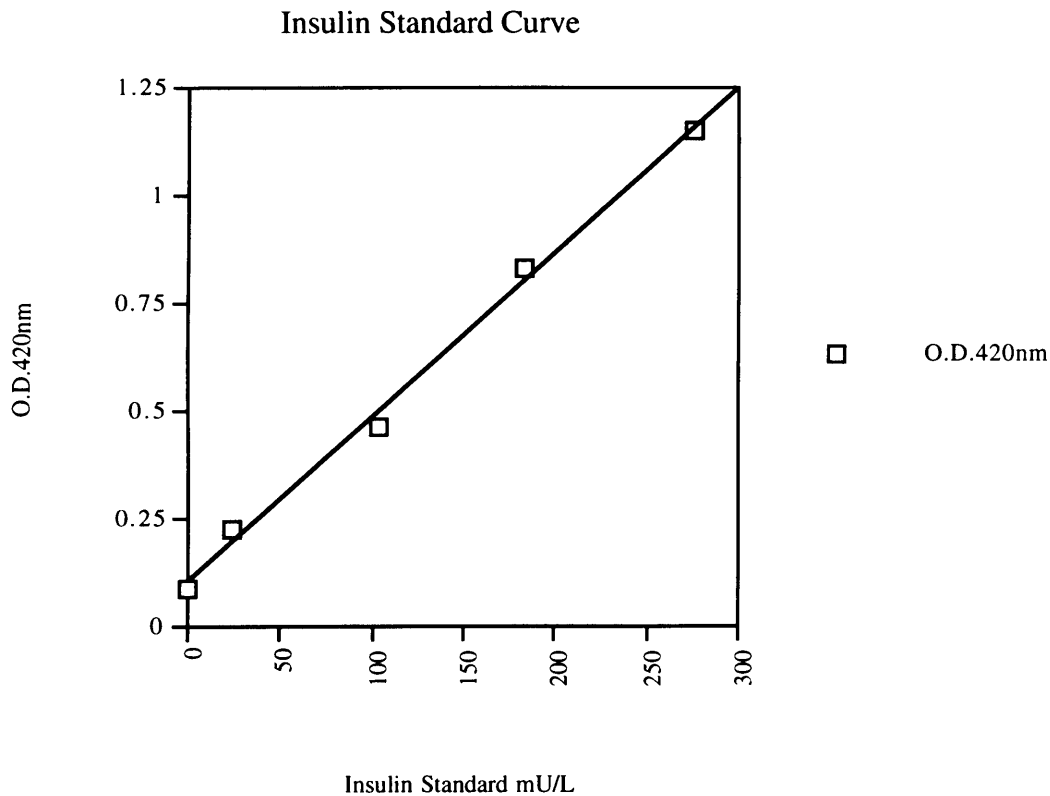
>sp|P28187|ARA4\_ARATH RAS-RELATED PROTEIN ARA-4.  
Length = 214

Score = 191 (86.0 bits), Expect = 1.3e-20, P = 1.3e-20  
Identities = 37/48 (77%), Positives = 44/48 (91%)

Query: 1 GDSGVGKSNLLSRFSRDEFDTNSKATIGVEFQTQLVEIEGKEVKAQIW 48  
GDS VGKSNLL+R++R+EF+ NSKATIGVEFQTQ + I+GKEVKAQIW  
Sbjct: 19 GDSAVGKSNLLTRYARNEFPNSKATIGVEFQTQSMLIDGKEVKAQIW 66

## Appendix 2. Human Insulin Standard Curve.

The insulin zero standard seems to interact with the chromogen giving rise to a small change in optical density at 420nm. However within the insulin concentrations indicated below the curve is a straight line. Therefore test solutions can be diluted to fall within this concentration range, to allow their accurate quantification.

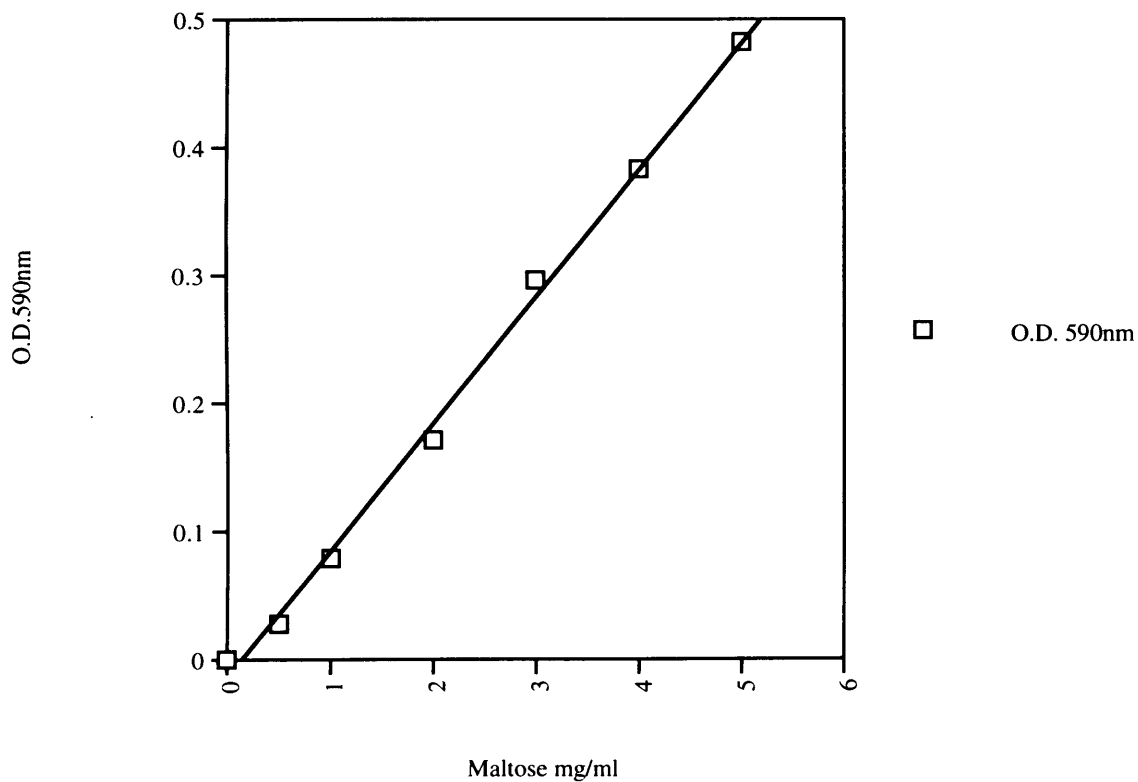


$$OD.420nm = 0.004(Insulin) + 0.103(C)$$

$$\frac{O.D.420nm - 0.1}{0.004} = \text{Insulin}$$

### Appendix 3. Maltose Standard Curve

With the maltose concentrations indicated below the standard curve is a straight line. Test solutions can be diluted to fall within this concentration range, to allow their accurate quantification.



$$OD\ 590nm = 0.099x - 0.014(c)$$

$$\frac{OD\ 590nm}{0.099} + 0.014 = Mg\ Maltose/ml$$