Cassette Mutagenic Analysis of the Signal Peptide

of Yeast Invertase

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Abstract

The *SUC2* locus of *Saccharomyces cerevisiae* encodes two forms of invertase; a constitutively expressed cytoplasmic enzyme and a glucose-repressible secreted and glycosylated enzyme which is initially produced with an amino-terminal signal peptide. The coding sequence of the *SUC2* locus has been placed under the control of the constitutive *ADH1* promoter and transcription terminator in a centromere based yeast plasmid vector from which invertase is expressed in a Suc⁻ strain of yeast. Oligonucleotide-directed mutagenesis has been used to create a PstI site in the gene at the point encoding the signal peptide cleavage site. An internal methionine codon, the translation start for the cytoplasmic invertase, has been replaced by a serine codon. Mutants in the signal peptide sequence have been produced by replacing the region of the gene upstream of the PstI site with synthetic oligonucleotide cassettes with mixtures of nucleotides at several positions. The mutants could be divided into three classes based on their ability to secrete invertase. The first class of mutants produced secreted invertase, but in reduced amount. There is no obvious correlation between mutation and phenotype. The second class, represented by mutant 4-55B, also exhibited a reduced level of invertase, but a significant fraction (30%) of the enzyme is intracellular. This mutant had a delay in signal peptide cleavage which retards passage of invertase through the secretory pathway. The third class was defective in secretion. Most were defective in translocation from the cytoplasm to the lumen of the endoplasmic reticulum (ER), and produced enzymatically active, non-glycosylated pre-invertase in the cytoplasm. This class of mutant invertases, when transcribed and translated *in vitro*, was not processed by canine pancreas signal recognition particle (SRP) and microsomes. Comparison of the sequences of the mutant signal peptides of this non-translocating class identifies amino acids at the extreme amino-terminus as the causative defect.

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List of Abbreviations

A_{600}	absorbance at 600 nm
AP	alkaline phosphatase
ATP	adenosine triphosphate
Bb	Berkeley bodies
bp	base pair
BSA	bovine serum albumin
CCCP	carbonyl cyanide chlorophenylhydrazone
cm	centimeter
cpm	counts per minute
CTP	cytosine triphosphate
dATP	deoxyadenosine triphosphate
DNase	deoxyribonuclease
ddNTPs	dideoxynucleoside triphosphates
dNTPs	deoxynucleoside triphosphates
dol	dolichol
ds	double-stranded
Endo H	endoglycosidase H
ER	endoplasmic reticulum
Glc	glucose
GlcNAc	n-acetylglucosamine
GTP	guanosine triphosphate
h	hour
HRP	horseradish peroxidase
IgG	immunoglobulin G
kb	kilobase
kd	kilodalton

l	liter
M	molar
mA	milliampere
Man	mannose
MBq	megabecquerel
mg	milligram
μg	microgram
min	minute
ml	milliliter
μl	microliter
mm	millimeter
μm	micrometer
mM	millimolar
μM	micromolar
μmole	micromole
m.o.i.	multiplicity of infection
nm	nanometer
pfu	plaque forming unit
pmol	picomole
PMSF	phenyl methyl sulfonyl fluoride
psi	pound per square inch
RNase	ribonuclease
rpm	revolution per minute
S	Svedberg unit
SDS	sodium dodecyl sulfate
sec	second
SRP	signal recognition particle

TTC	2, 3, 5-triphenyl tetrazolium chloride
TTP	thymidine triphosphate
u	enzyme activity unit
UDP	uridine diphosphate
UTP	uridine triphosphate
V	volt

Introduction

Eukaryotic cells contain a variety of structurally distinct membrane systems and organelles. Within these are specific sets of proteins which are important for assembly and function. Many of the proteins are found only in a particular membrane system or organelle. This has led to the notion that there must exist mechanisms in the cell to correctly sort and target proteins from the site of synthesis in the cytoplasm to the various subcellular compartments or organelles. Furthermore, the cell must also possess mechanisms to ensure that the targeted proteins are selectively translocated across or inserted into hydrophobic membranes.

There are three major destinations for proteins in the cell: the nucleus, specialized organelles such as mitochondria, lysosomes and chloroplasts, and the secretory system which includes the endoplasmic reticulum (ER), Golgi complex, secretory vesicles and plasma membrane. Thus, there must exist at least three different types of targeting and localization signal. It has been widely accepted that the eventual fate of a protein is dictated by the type of signal it possesses, and that this signal is encoded within the protein structure. In support of this idea are studies showing that a cytoplasmic protein, such as β-galactosidase, can be directed into the secretory pathway (Silhavy et al. 1976; Emr et al. 1978; Lingappa et al. 1984), into the mitochondria (Schatz and Butow, 1983; Hay et al. 1984; Douglas et al. 1984) or into the nucleus (Hall et al. 1984; Silver et al. 1984; Barnes and Rine, 1985) by the addition of an appropriate targeting and localization signal. The work to be described in this thesis deals with the role of the structure of the signal peptide in targeting invertase to the secretory pathway of the budding yeast, *Saccharomyces cerevisiae*.

The Signal Hypothesis

According to the Signal Hypothesis, secreted proteins are synthesized with an amino-terminal extension known as the signal or leader peptide (Milstein et al. 1972; Schechter et al. 1974; Blobel and Dobberstein, 1975a, 1975b). In some cases, as in ovalbumin (Palmiter et al. 1978), the signal peptide is located within the mature sequence. The signal peptide, initially, was thought to directly cause the attachment of the nascent polysome to the membrane of the rough ER. This interaction then would result in the recruitment of two or more integral membrane proteins to form a transient pore or tunnel through which the polypeptide would be vectorially translocated. In recent years, isolation of two biochemical factors has advanced our understanding of the processes involved in the recognition of signal peptides and translocation of secreted proteins across the ER. One of these factors is an 11S ribonucleoprotein complex, purified from the canine pancreas, known as the signal recognition particle or SRP. (Walter and Blobel, 1980). This complex consists of a 7S RNA, also referred to as 7SL RNA by Ullu et al. (1982), and six polypeptides with apparent molecular weights of 9, 14, 19, 54, 68 and 72 kd (Walter and Blobel, 1980, 1982). The other factor, also purified from the canine pancreas, is the SRP receptor or docking protein (Gilmore et al. 1982a; 1982b; Meyer et al. 1982). This is a complex consisting of two distinct integral membrane proteins, a 69 kd α-subunit with a large (52 kd) cytoplasmic domain and a recently identified 30 kd β-subunit (Tajima et al. 1986). These discoveries have resulted in an updated version of the Signal Hypothesis (Walter et al. 1984). In this version, the signal peptide does not interact directly with the ER membrane. Instead, the signal peptide is recognized by the SRP as it emerges from the ribosome (Walter et al. 1981; Gilmore and Blobel, 1983). The interaction between the SRP, signal peptide and ribosome results in a transient arrest in elongation at a specific point in the nascent polypeptide chain. This elongation arrest is released upon interaction between the SRP and its receptor in the ER. Then, the SRP detaches from the ribosome complex and is free to interact with another signal peptide. Meanwhile, the targeted ribosome

remains associated with the ER membrane by binding to a putative ribosome receptor. The translocation of the protein through the membrane, immediately following this, is poorly understood, but is thought to be coupled to protein synthesis (Walter et al. 1984). Once translocated, the signal peptide is cleaved by the signal peptidase located on the lumenal side of the ER. Completion of protein synthesis then results in the dissociation of the membrane complex and the release of the polypeptide into the lumen of the ER.

One of the major attractions of the Signal Hypothesis is that it can be tested experimentally. Although it may not explain secretion by all cells, it has provided an invaluable starting point for a great number of ideas and experiments on secretion. The following is a review of some of those ideas as they apply to protein secretion in prokaryotes and eukaryotes.

Secretion by *Escherichia coli*

A Gram-negative bacterium such as *E. coli* consists of four morphologically distinct compartments. These are the cytoplasm, the inner (plasma) membrane, the periplasmic space and the outer membrane. The latter three are commonly referred to as the cell surface. Proteins exported to the outer membrane and periplasm are analogous to secreted proteins of eukaryotes in that they are synthesized with a transient amino-terminal signal peptide (for reviews, see Emr et al. 1980; Osborn et al. 1980). This raises the possibility that they are exported to the cell surface by mechanisms analogous to that described in the Signal Hypothesis which applies to eukaryote cell secretion.

Models for Secretion by *E. coli*

There are currently three models for secretion by *E. coli*. The first model is a modification of the eukaryotic Signal Hypothesis. A number of changes were made to accommodate the differences between prokaryotes and eukaryotes. One of these is the lack of a structurally distinct ER membrane in prokaryotes. However, it is likely that some of

the functions normally associated with the eukaryotic ER can be assumed by the bacterial plasma membrane. When viewed in the context of protein secretion, there is a strong analogy between the export of proteins by bacteria and the transfer of proteins across the ER membrane. Both systems involve processing of the precursor protein after translocation across a hydrophobic membrane. Therefore, it is reasonable to expect that proteins involved in the translocation and processing of exported proteins are integral parts of these membranes. One of these, the signal peptidase, has indeed been shown by Wolfe et al. (1983) to be localized in the bacterial plasma membrane.

The second model is known as the direct transfer model. This was proposed by von Heijne and Blomberg (1979) based on considerations of the energetics of protein structure and of protein interaction with the hydrophobic membrane. This model is similar to the Signal Hypothesis in that ribosome attachment to the membrane is essential for translocation. However, the attachment is mediated directly by the hydrophobic signal peptide as in the earlier version of the Signal Hypothesis. The main feature of this model is that it obviates the need for channel-forming proteins in the translocation of the nascent polypeptide chain. Hence, all that is required are two proteins: a ribosome receptor and the signal peptidase. One major criticism of this model is that it does not adequately address events preceding protein translocation (Randall and Hardy, 1984).

The third model is known as the membrane-triggered conformational change model. This was initially proposed by Wickner (1979) to explain the data collected for the M13 filamentous phage coat protein, and was subsequently expanded to include the translocation of larger proteins. The M13 coat protein is a small 5.3 kd peptide with its amino-terminus inserted into the plasma membrane and its carboxy-terminus exposed to the cytoplasm. It is synthesized as a precursor in the cytoplasm and inserted into the inner membrane post-translationally (Ito et al. 1979). In the model, the role of the signal peptide is not to mediate the attachment of the ribosome to the membrane, but rather to confer

upon the precursor a conformation that is competent for translocation across or insertion into the membrane. This membrane-triggered insertion or translocation process has been shown to be dependent on an energized membrane (Date and Wickner, 1981; Zimmermann and Wickner, 1983). Dissipation of the protonmotive force by uncouplers such as CCCP was shown by Randall (1983) to disrupt translocation, resulting in intracellular accumulation of the precursor. One yet unanswered question about this model is the nature and means of maintenance of the translocation-competent state (Randall and Hardy, 1984).

The Role of Signal Peptide in Secretion by *E. coli*

Studies illustrating the importance of the signal peptide in protein secretion can be divided into two groups, based on the following experimental approaches: mutation of the signal peptide of a secreted protein or signal peptide-directed export of a cytoplasmic protein. A number of studies have indicated that a functional signal peptide is essential for protein export. Deletion and missense mutations were shown to disrupt the secretion of the outer membrane proteins, LamB (Emr et al. 1978) and lipoprotein (Inouye et al. 1983a; 1983b); the periplasmic proteins, MalE (Bedouelle et al. 1980; Kumamoto et al. 1984), β-lactamase (Kadonaga et al. 1984), PhoA (Michaelis et al. 1983a), and RbsB (Iida et al. 1985). In the majority of cases, the missense mutations directed at charged residues at the amino-terminus, at residues within the hydrophobic core, and at residues at or near the cleavage site, resulted in a reduction in the rate of export and/or the cytoplasmic accumulation of the precursor protein. In addition, certain mutations also affected the synthesis of the exported protein.

The second approach involved the fusion of varying lengths of the amino-terminus of an exported protein to a cytoplasmic protein, β-galactosidase. Sequences from three different proteins were studied with this approach: LamB (Emr et al. 1980; Benson and Silhavy, 1983), MalE (Bassford et al. 1979; Bedouelle et al. 1980; Herrero et al. 1982),

and PhoA (Michaelis et al. 1983b). In the case of LamB, the signal peptide, alone, was found to be insufficient for export. A hybrid LamB-LacZ protein, containing either the signal peptide or the signal peptide and the first 27 residues of the mature LamB protein, was not exported and remained in the cytoplasm. Two other segments of the mature protein were shown to be required for export. The segment between residues 27 and 39 was shown by Benson et al. (1984) to be required for efficient insertion into the inner membrane, and that between residues 39 and 49 for transport to the outer membrane. In addition, removal of a segment from amino acid 70 to 183 prevented proteolytic cleavage of the signal peptide without affecting transport to the outer membrane (Benson and Silhavy, 1983). These segments do not exhibit any unusual structural features such as extended hydrophobic or charged region. Similar results were obtained in studies using the MalE-LacZ fusion (Bassford et al. 1979; Bankaitis et al. 1984). The important sequence in MalE was localized between residues 89 and 189 of the mature protein. Moreover, a defect in export caused by a methionine to arginine mutation at position -8 of MalE protein can be suppressed by a glycine to cysteine change at position 19 of the mature protein (Ryan et al. 1986). The simplest explanation for these fusion experiments is that sequences in the mature protein provide additional permissive information to the translocation apparatus and/or assembly at the target site. Alternatively, it is possible that they are required only in the translocation of large hydrophilic domains such as those present in β-galactosidase (von Heijne, 1980; Rapoport, 1987).

In contrast to the outer membrane and periplasmic proteins, inner membrane proteins are synthesized with or without an amino-terminal signal peptide. D-Alanine carboxypeptidase, DacA (Jackson et al. 1985) provides an example of an inner membrane protein synthesized with a signal peptide. Those synthesized without an amino-terminal signal peptide include lactose permease (Ehring et al. 1980), proton translocating ATPase (Brusilow et al. 1981), lactate dehydrogenase (Santos et al. 1982a) and signal peptidase (Wolfe et al. 1983). The transport of inner membrane proteins raises two interesting

questions: do these proteins follow the same export pathway as outer membrane and periplasmic proteins, and what is the nature of the information encoded by those without a signal peptide? In the case of DacA, Jackson et al. (1985) showed that its signal peptide can direct the export of an outer membrane protein, OmpF. They also showed that the export of the hybrid protein was disrupted by *secA*, a pleiotropic mutation shown to affect the export of several outer membrane and periplasmic proteins (Oliver and Beckwith, 1982). Thus, it can be concluded that the signal peptide of an inner membrane protein contains all the essential information necessary for initial recognition and targeting of the protein to the secretory apparatus. Furthermore, it would appear that inner membrane proteins containing a functional signal peptide are processed, at least initially, in the same pathway as outer membrane and periplasmic proteins.

Inner membrane proteins lacking a signal peptide are likely to use a different mode of transport. These proteins contain two common features: they are transmembrane proteins containing one or more hydrophobic domains inserted into the membrane, and they are inserted after complete synthesis by an energy dependent process (Wolfe et al. 1983). It is possible that one or more of the hydrophobic domains may serve as a signal peptide. The secretion of a protein containing an internal signal peptide has been shown to occur in at least two other albeit eukaryote cases: ovalbumin (Palmiter et al. 1978) and a globin-pre-prolactin fusion protein (Perara et al. 1985). If this is the case for bacterial inner membrane proteins, it would be interesting to examine what determines the targeting and lack of processing of an internal signal peptide, and whether proteins involved in the export of outer membrane and periplasmic proteins are also used for the insertion of inner membrane proteins.

Structure and Functional Analysis of Signal Peptides in *E. coli*

Although signal peptides show a lack of primary sequence homology (von Heijne, 1983), they do possess certain common features. First, the amino-terminus is hydrophilic

and usually contains one or more basic residues. Secondly, there is a central hydrophobic core consisting of 6-15 consecutive non-polar amino acids. Thirdly, the amino-terminus and the hydrophobic segment are predicted to assume an α-helical conformation (von Heijne, 1984b). α-Helix disrupting amino acids, such as proline, glycine, serine and threonine, are usually found in the region between the hydrophobic core and the cleavage site. Lastly, small neutral amino acids are often found adjacent to the cleavage site which lies about 20 amino acid residues away from the amino-terminus (Perlman and Halvorson, 1983; von Heijne, 1985).

Studies involving random and directed mutagenesis of the signal peptide of exported proteins have provided important insights into the structural features required for proper function. In summary, the following conclusions can be drawn. First, the hydrophobic core is absolutely essential for translocation across the plasma membrane. Partial deletion of the hydrophobic core of the lambda phage receptor protein, LamB, was shown by Emr et al. (1978) to abolish export and result in cytoplasmic accumulation. The introduction of an acidic or basic residue into this region was also shown to strongly disrupt signal peptide function in a number of proteins, including LamB (Emr et al. 1980), MalE (Bedouelle et al. 1980), PhoA (Michaelis et al. 1983a), and lipoprotein (Inouye et al. 1982). However, not all mutations produced the same degree of defectiveness. These studies showed that the severity of the defect is highly dependent on the position of the substituted residue. Charged residues introduced into the central region of the hydrophobic core were found to exert a much more deleterious effect than those inserted toward the hydrophilic amino- or carboxy-terminus. In all cases, the introduction of a single polar amino acid such as serine into this region produced only a minor defect and was generally tolerated by the secretory apparatus. The length of the hydrophobic core is another important parameter recognized by the secretory apparatus. Bankaitis et al. (1984) have isolated secondary intragenic mutations which suppressed the export deficiency caused by a seven amino acid deletion from methionine (-9) to leucine (-15) in the central hydrophobic

Escherichia coli

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                          -25        -20        -15        -10        -5        -1  +1
                           •          •          •          ○                 •    ○   •

dacA              M N T I F S A R I M K R L A L T T A L C T A F I S A A H A   D D
β-lactamase             M S I Q H F R V A L I P F F A A F C L P V F A   H P
lamB                  M M I T L R K L P L A V A V A A G V M S A Q A M A   V D
lipoprotein               M K A T K L V L G A V I L G S T L L A G   C S
M13 coat protein        M K K S L V L K A S V A V A T L V P M L S F A   A E
malE                M K I K T G A R I L A L S A L T T M M F S A S A L A   K I
ompA                    M K K T A I A I A V A L A G F A T V A Q A   A P
ompC                    M K V K V L S L L V P A L L V A G A A N A   A E
ompF                  M M K R N I L A V I V P A L L V A G T A N A   A E
phoA                    M K Q S T I A L A L L P L L F T P V T K A   R T
phoE                    M K K S T L A L V V M G I V A S A S V Q A   A E
rbsB              M N M K K L A T L V S A V A L S A T V S A N A M A   K

Saccharomyces cerevisiae
Acid phosphatase          M F K S V V Y S I L A A S L A N A   G T
Alpha-factor 1          M R F P S I F T A V L F A A S S A L A   A P
Alpha-factor 2        M K F I S T F L T F I L A A V S V T A S   S D
Invertase             M L L Q A F L F L L A G F A A K I S A   S M
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Figure 1. Amino-terminal sequence of secreted proteins from *Escherichia coli* and *Saccharomyces cerevisiae*.

region of MalE, the maltose-binding protein. These revertants fortuitously regenerated a hydrophobic segment of six to eight amino acids. Since a hydrophobic segment consisting of five consecutive non-polar amino acids is defective in secretion whereas one containing six consecutive non-polar amino acids is secretion-competent, one can conclude that a segment containing six consecutive non-polar amino acids is the minimum length required for efficient export. Using oligonucleotide-directed mutagenesis, Kendall et al. (1986) have demonstrated that the signal peptide of alkaline phosphatase can be made more efficient by replacing four amino acids in the hydrophobic core (a phenylalanine, proline and two alanines) with leucine residues. This resulted in a segment consisting of nine consecutive leucines and led to an accelerated rate of maturation of the precursor. When the length of the hydrophobic core was extended by six additional amino acids, one serine and five consecutive leucines, a significant reduction in the rate of precursor maturation was seen. This suggests that there is an optimal length of nine consecutive non-polar amino acids for the hydrophobic core.

Secondly, the maintenance of an α-helical conformation appears to be important but not absolutely required for export. Disruption of the predicted α-helical secondary structure was implicated in the export defect of the LamB signal peptide mutant containing a four amino acid deletion within the hydrophobic core (Emr and Silhavy, 1983). Two different secondary mutations, isolated by virtue of their ability to suppress the export defect of the deletion mutation, were shown to restore the predicted α-helical conformation in this region. A similar observation was made by Iida et al. (1985) in the ribose-binding protein, RbsB. They found that export of RbsB protein was completely abolished in a signal peptide mutant containing a single amino acid substitution in the hydrophobic core: a leucine to proline change at position -17. This defect was partially suppressed in a revertant carrying a second mutation in the signal peptide: a serine to phenylalanine change at position -15.

The third conclusion about structure-function is that the positive charge at the amino-terminus serves to facilitate secretory protein synthesis and translocation. Both the rate of synthesis and translocation of the outer membrane lipoprotein was shown to be affected by the number of positive charges in this region (Inouye et al. 1982; Vlasuk et al. 1983). These workers systematically replaced the two lysine residues at the amino-terminal segment resulting in a change in charge from +2 to +1, 0, -1 and -2. No change in the rate of export was seen in those mutations carrying a net charge of +1 or 0 on these two residues. However, those with a net negative charge on these two residues showed a five to ten fold decrease in the rate of secretion, resulting in transient cytoplasmic accumulation of the precursor. In all mutants, the rate of synthesis was significantly reduced, suggesting that the positive charges facilitate the synthesis of pre-lipoprotein.

Lastly, amino acids at the carboxy-terminus of the signal peptide serve to define the cleavage site. This is true for signal peptides processed by either one of the two E. coli signal peptidases, SPase I and II (Wolfe et al. 1983; Innis et al. 1984). Analysis of mutations affecting signal peptide cleavage in two proteins, β-lactamase (Kadonaga et al. 1984) and outer membrane lipoprotein (Inouye et al. 1983a; 1983b), suggests that the 5-9 residues at the carboxy-terminal of the signal peptide and 1-3 residues of the mature protein are involved in signal peptidase function. Mutants in either region caused a delay in signal peptide cleavage and accumulation of un-processed precursor in the periplasmic space. Thus, it can be concluded that the process of protein translocation precedes and is independent of signal peptide cleavage.

Despite the great variability in the primary sequence amongst signal peptides, it would appear that they are recognized by the secretory system through a common mechanism. Signal peptides from DacA, an inner membrane protein (Jackson et al. 1985), and β-lactamase, a periplasmic protein (Tommasson et al. 1983), efficiently direct the

localization of the outer membrane protein, OmpF. Thus, signal peptides from outer membrane, periplasmic and inner membrane proteins are functionally equivalent with respect to the initial stages of secretion. This ability to exchange signal peptides between two proteins can also be extended to include eukaryotic signal peptides. A number of studies have shown that *E. coli* can recognize and accurately process eukaryotic signal peptides (For example, see Talmadge et al. 1980; Gray et al. 1985). The reverse has also been shown to be true: signal peptide of β-lactamase is recognized by the mammalian SRP (Muller et al. 1982; Lingappa et al. 1984). Thus, the mechanism of signal peptide recognition appears to be functionally conserved throughout evolution.

Translocation Across the Bacterial Inner Membrane

According to the Signal Hypothesis and the direct transfer model, the nascent polypeptide chain is translocated across the hydrophobic membrane linearly as it is synthesized. In contrast, in the membrane-triggered conformational change model an entire domain is transferred after its synthesis. Thus, the crucial difference between the linear and the domain model of translocation is whether polypeptide chain elongation is coincident with translocation or precedes it. The linear mode of translocation is by necessity co-translational, whereas translocation in the domain mode may proceed after complete synthesis, as long as the domain is maintained in a translocation-competent state. The second point to consider is the source of energy for the translocation process. The Signal Hypothesis proposes the formation of a transient channel by integral ER membrane proteins which could reduce the energy required to propel the charged and polar residues of the nascent chain through the hydrophobic membrane (Blobel and Dobberstein, 1975a; 1975b). It also implies that the energy for translocation is derived in part from the energy of chain elongation on the ribosome. Hence, it is imperative that the ribosome remains tightly associated with the membrane throughout the translocation process. In the direct transfer model, a channel is not required for translocation. This model states that

the energy for translocation is inherent in the protein structure, based on estimation of the free energy difference for the transfer of residues from a random coil conformation in water to an α-helical conformation in a lipophilic environment (von Heijne and Blomberg, 1979). In the membrane-triggered conformational change model, an electrochemical potential across the inner membrane is essential for membrane insertion. Based on *in vitro* data, it was concluded that ATP is involved in the process (Chen and Tai, 1985). However, it is not clear whether ATP is utilized to propel the protein across the membrane or to maintain the electrochemical gradient.

Based on the experimental data available to date, it is clear that none of the proposed models is correct in its entirety. The following observations are often cited in support of the co-translational translocation models. First, there is a close association between ribosomes synthesizing exported proteins and the plasma membrane. Smith et al. (1977) found that nascent alkaline phosphatase can be radioactively labeled from outside the cell with a membrane-impermeable reagent, implying that translocation was initiated prior to complete synthesis. Using a different approach, Randall and Hardy (1977) showed that outer membrane and periplasmic proteins are predominantly synthesized by membrane-bound polysomes. Secondly, there is evidence of a feedback interaction between translocation of an exported protein and its synthesis. Oliver and Beckwith (1982) have shown that the absence of SecA protein greatly reduced the synthesis as well as the export of maltose-binding protein. This suggests that the function of the SecA protein is to couple the translation of secretory proteins to membrane translocation. In addition, Hall et al. (1983) showed that an arginine (-20) to serine mutation in the hydrophilic amino-terminus of the signal peptide of the LamB protein resulted in a block in translation as well as a significant reduction in export to the outer membrane.

Post-translational translocation is clearly evident in at least two proteins. M13 coat protein was shown by Date and Wickner (1981) to be synthesized by cytoplasmic

polysomes. Based on trypsin-accessibility of pulse-labeled β-lactamase, Koshland and Botstein (1980; 1982a; 1982b) concluded that this protein traverses the membrane after the completion of translation. Translocation of most other proteins, however, cannot be easily classified as co- or post-translational. For example, the precursor of maltose-binding protein was shown by Randall (1983) to be translocated after synthesis of approximately 80% of its entire length. Thus, translocation starts late in synthesis and is largely independent of chain elongation.

It is important to keep in mind that the size and structural features of the exported protein may play a significant role in determining the mode of translocation. Small peptides such as M13 coat protein may be post-translationally translocated by necessity. Due to their small size, translation is essentially complete at the time the signal peptide emerges from the ribosome. Their small size may also preclude the possibility of the signal peptide being masked by other polypeptide domains. Thus, the signal peptide can remain accessible to components of the translocation apparatus after complete synthesis of the preprotein. This interpretation is supported by the observation of a correlation between competence for translocation of the maltose-binding protein with the lack of stable tertiary structure (Randall and Hardy, 1986). It would appear that a kinetic competition exists between productive interaction of the signal peptide with the translocation apparatus and folding of the precursor into a conformation that is no longer compatible with translocation. Studies indicating that exported proteins are preferentially synthesized on membrane-bound ribosomes are consistent with either mode of transfer. The close association of ribosome and the membrane does not rule out the possibility of complete synthesis of polypeptide domains prior to translocation. The crucial issue appears to be the length of the nascent polypeptide chain and its three-dimensional structure at the time of translocation through the membrane. Most studies have not been directed at this issue.

It is conceivable that a secreted protein utilizes both modes of translocation. It may be that one is used under normal circumstances. Inhibition of the normal route of translocation could then divert the protein to the alternative but less favored route. This appears to be the case for the outer membrane lipoprotein. The translocation of this protein was shown by Inouye et al. (1983) to be coupled to its synthesis. Certain mutations in the signal peptide disrupted translocation, resulting in the cytoplasmic accumulation of the precursor. This precursor was subsequently post-translationally translocated, albeit at a much slower rate.

Components of the Export Apparatus of *E. coli*

Genes Involved in Secretion

One of the characteristic features of the Signal Hypothesis is the involvement of an array of proteins - both in the cytoplasm and the membrane - in the secretory process. In contrast, only the ribosome receptor and the signal peptidase are involved in the direct transfer model, and only the signal peptidase is required in the conformational change model. If the Signal Hypothesis is correct, it should be possible to isolate genetic mutants that are defective in protein secretion. However, if protein export were to occur as proposed by the other two models, the number of possible genetic mutants might be limited. Based on the number of genes isolated and characterized to date, the export of proteins appears to proceed as set forth by the Signal Hypothesis.

The genes involved in the secretory pathway include *secA* (Oliver and Beckwith, 1981), *secB* (Oliver et al. 1982; Kumamoto and Beckwith, 1983), *secC* (Ferro-Novick et al. 1984c), *secY* (Ito et al. 1984), *prlA*, *prlB* (Emr and Bassford, 1982), and *prlC* (Emr et al. 1981). It now appears that *prlA* is the same as *secY*, and that *prlB* and *prlC* are not involved directly in the normal secretory pathway. The following discussion is limited to the three genes characterized to date: *secA*, *secC* and *prlA* (*secY*).

The *secA* mutation was isolated by Oliver and Beckwith (1981) by virtue of its ability to block the export of the MalE-LacZ hybrid protein. Strains carrying this mutation were shown to accumulate precursors of normally exported proteins in the cytoplasm. The gene product was identified as a 92 kd protein peripherally associated with the cytoplasmic membrane (Oliver and Beckwith, 1982).

The *secC* mutant was isolated by Ferro-Novick et al. (1984c) as a temperature-sensitive suppressor of the secretion defect of *secA*. The mutation was shown to selectively block the synthesis of exported proteins but not of cytoplasmic proteins. At the non-permissive temperature (37°C), the synthesis of two outer membrane proteins, LamB and OmpF, and a periplasmic protein, ribose-binding protein, was abolished, while that of a cytoplasmic protein, elongation factor G, was unaffected. Screening of an *E. coli* gene library for *secC* complementation yielded a clone containing two previously known genes, *pnp* and *rpsO*. The *pnp* gene codes for polynucleotide phosphorylase and *rpsO* codes for ribosomal protein S15. Disruption of the *rpsO* gene destroyed the ability of the plasmid to complement the *secC* mutation, indicating that *secC* is the same as *rpsO*. The relationship between SecC protein and the synthesis of exported proteins remains unclear.

The *prlA* (*secY*) mutant was isolated by Emr et al. (1981) as a trans-acting suppressor of the LamB and MalE signal sequence mutations. The gene was initially mapped to the ribosomal gene cluster; between *rpsL*, the gene conferring streptomycin resistance, and *rpsE*, the gene conferring spectinomycin resistance. Schultz et al. (1982) subsequently showed that *prlA* is located immediately downstream of *rplO*, the structural gene for ribosomal protein L15. The *prlA* (*secY*) gene product was shown by Akiyama and Ito (1985) to be a 49 kd hydrophobic plasma membrane protein.

The involvement of the products of several genes in the synthesis and translocation of exported proteins suggests the existence of an export apparatus in *E. coli* that is analogous to the mammalian system. It is difficult, however, to identify the bacterial

equivalent of the mammalian proteins. Based on the ability of *secA* and *secC* mutations to block the synthesis of exported proteins but not of cytoplasmic proteins, it would appear that the *secA* and *secC* gene products relieve the bacterial analogue of a SRP-induced elongation arrest. The association of the *secA* gene product with the inner membrane makes it a good candidate for a component interacting with a SRP receptor or docking protein. The ability of *secC* to suppress the *secA* mutation suggests the involvement of the *secC* product, the ribosomal protein S15, in the release of the SRP-induced elongation arrest. As for *prlA* (*secY*), the ability of the mutant protein to suppress certain signal peptide mutations makes this gene product a likely candidate for either a component of the bacterial SRP or SRP receptor. The other mutations that were indirectly involved in the normal secretory pathway reflect the fact that translocation of proteins in *E. coli* is dependent on an electrochemical membrane potential. Hence, any mutations affecting the membrane potential or energy level of the cell can indirectly disrupt protein export.

Signal Peptidase

There are two distinct signal peptidases in *E. coli*, each with vastly different properties and substrate specificity. The signal peptidase (SPase I) characterized by Wickner's group is encoded by the *lep* gene and is responsible for the catalytic removal of signal peptide from almost all exported proteins. It is an inner membrane protein with the active site of the enzyme located on the periplasmic surface of the membrane (Zimmermann et al. 1982). This endopeptidase exhibits a low substrate specificity but cleaves at a very precise site in the precursor despite the lack of a unique sequence around this region. Its recognition sequence is thought to include the 5-9 carboxy-terminal residues of the signal peptide and 3-5 residues after the cleavage site (Dierstein and Wickner, 1986). The second signal peptidase (SPase II), encoded by the gene *lsp* (Innis et al. 1984), is responsible for the removal of signal peptide from the outer membrane pro-lipoprotein. SPase II is an inner membrane protein with four putative transmembrane domains. It

exhibits a much higher substrate specificity than SPase I, and cleaves between the glycine and cysteine residues at positions 20 and 21, respectively, of pro-lipoprotein (Inouye et al. 1983a). Deletion of the glycine residue completely abolished cleavage, while a conservative replacement with an alanine residue exerted no effect on cleavage. Cleavage is also dependent on the attachment of a diacylglycerol group onto the cysteine residue (Braun and Bosch, 1972). The cyclic peptidic antibiotic, globomycin, specifically inhibits this peptidase, resulting in the accumulation of diacylglyceride-modified pro-lipoprotein in the periplasmic space (Hussain et al. 1980). Processing of signal peptides by SPase I is not affected by globomycin (Inouye et al. 1983b).

Comparison of Secretion in Prokaryotes and Eukaryotes

Secretion in prokaryotes is relatively simple compared to eukaryotes. Once a protein has completely translocated across the prokaryotic inner membrane, it is by definition secreted. However, due to the presence of specialized membrane compartments in eukaryotes, secretory and plasma membrane proteins must be transported from one membrane compartment to another on route to the cell surface. Thus, intercompartmental transport is an integral part of eukaryotic secretion. The ability of a protein to translocate across the ER membrane does not ensure its secretion.

Eukaryotic secretory proteins, like all other proteins, are translated in the cytoplasm. The secretory pathway starts with the translocation of the protein across the ER membrane. After some modifications, the secretory, plasma membrane and lysosomal proteins are transported to the Golgi complex. Then they are sorted and packaged either into secretory or lysosomal vesicles. The eventual fusion of the secretory vesicles to the plasma membrane releases the lumenal contents into the external environment. A failure or delay at any point in the transport process can significantly affect secretion.

With the exception of the outer membrane lipoprotein, which contains a diacylglycerol group covalently linked to the amino-terminal cysteine, all secreted proteins in prokaryotes are unmodified. In contrast, almost all eukaryotic secreted proteins are processed and/or modified prior to secretion. In addition to signal peptide cleavage, some secretory proteins are processed by a second sequence-specific endopeptidase, as in the conversion of a zymogen to an enzyme or the conversion of a prohormone to a hormone. Another common modification in eukaryotic secreted proteins is the glycosylation of the polypeptide chain. This usually involves the transfer of a carbohydrate chain to an asparagine residue (N-linked) and serine or threonine residue (O-linked). Certain antibiotics (e.g. tunicamycin) and mutations (e.g. *alg*, a class of mutation in yeast affecting core oligosaccharide assembly) are known to block these modification reactions, resulting in a significant reduction in the rate of secretion. In the case of tunicamycin, a low level secretion of the unglycosylated protein is still evident. Thus, glycosylation is considered to have a facilitative role but is not absolutely essential for secretion (Schekman, 1985).

Another difference between prokaryotes and eukaryotes is the dependence of bacterial protein translocation on the presence of a membrane electrochemical potential (Date and Wickner, 1981; Zimmermann and Wickner, 1983; Bakker and Randall, 1984). In contrast, it is doubtful that such electrochemical potential exists across the eukaryotic ER membrane (Walter et al. 1984; Rapoport, 1987). However, the import of proteins into the mitochondrion is an energy dependent process requiring a membrane electrochemical potential (Schatz and Butow, 1983). It is not clear whether the energy required is expended in order to propel the protein across the membrane or is needed to maintain the membrane in a receptive state.

Topogenic Signals

Based on the observation that numerous proteins are specifically translocated across, integrated into or transported to distinct cellular membranes, Blobel (1980)

proposed that the information for these processes is encoded by "topogenic" sequences within the polypeptide chain. Theoretically, a secreted protein needs to contain two topogenic signals to reach its destination in the cell surface. First, it requires a signal to target the polypeptide chain to the translocation-competent membrane of the rough ER and to initiate the translocation process. This requirement is thought to be fulfilled by the signal peptide. Secondly, it must possess a sorting signal to direct its transport to the secretory organelles rather than to other destinations to which proteins entering the lumen of the ER can be directed. The nature of the sorting signal is not clear. It is thought to reside in one or more segments of the polypeptide chain. Its recognition may be based either on a contiguous amino acid sequence or a conformational domain of the protein. A sorting signal may not be an absolute requirement. It is possible for a protein to reach its final destination without a sorting signal by associating with a transit protein that is endowed with one (Blobel, 1980).

A membrane protein requires a topogenic sequence for insertion into the lipophilic membrane. This is referred to as a stop-transfer or insertion sequence. One model for membrane insertion is that a stop-transfer sequence causes the immediate cessation of the translocation process that was initiated by the signal peptide. This results in the insertion of the protein into the membrane. A series of stop-transfer sequences can explain the insertion of integral membrane proteins containing multiple membrane spanning domains. Each hydrophobic domain is thought to act as an alternating on or off switch on the translocation apparatus, resulting in sequential insertion of the polypeptide by repeated initiation and termination of the translocation process (Blobel, 1980). The model assumes that each stop-transfer sequence determines the location of the polypeptide domain immediately following it. An alternative model is that translocation and insertion into the membrane may not necessarily be co-translational (Rapoport, 1987). This model assumes that two or more hydrophobic segments may interact to form a structure with a hydrophobic surface that is compatible with the lipophilic membrane either co- or post-

translationally. Hydrophilic portions of the insertion sequence could be retained in the membrane by interaction with hydrophilic regions of other insertion sequence(s) or with polar regions of the membrane channel. Failure to compensate for the charged or hydrophilic portions would result in expulsion of the polypeptide segment(s) to the aqueous phase on either side of the ER membrane (Rapoport, 1987).

Secretion by *Saccharomyces cerevisiae*

In recent years, much effort has been focused on protein secretion by yeast. Its main attractions lie in the availability of temperature-sensitive secretory mutants (Novick et al. 1980; Ferro-Novick et al. 1984a; 1984b) and the relative ease with which this organism can be manipulated genetically. More recently, the availability of an *in vitro* translation and translocation system (Hansen et al. 1986; Rothblatt and Meyer, 1986a; Waters and Blobel, 1986) has added an invaluable tool in studying the biochemical processes involved in the early stages of secretion.

A number of proteins are known to be transported to the cell surface of *S. cerevisiae*. These include acid phosphatase (Toh-e et al. 1973, Linnemans et al. 1977), chitin synthetase (Duran et al. 1975), α-galactosidase (Kew and Douglas, 1976), invertase (Carlson and Botstein, 1981a; 1981b), type I killer toxin (Bussey et al. 1982), α-factor pheromone (Julius et al. 1984), the *BAR1*-encoded α-factor peptidase (Manney, 1983), and a-factor pheromone (Brake et al. 1985). These proteins are either associated with the plasma membrane (e.g. chitin synthetase), transported to the periplasmic space (e.g. invertase and acid phosphatase) or secreted into the medium (e.g. α-factor, a-factor and *BAR1* peptidase). Most of the secreted proteins are glycosylated with oligosaccharides consisting mainly of mannose residues (see below) while a few undergo acylation (Wen and Schlesinger, 1984).

Order of Events in Yeast Secretion

Secretion in *S. cerevisiae* is restricted primarily to the region of the cell where there is rapid plasma membrane growth (Tkacz and Lampen, 1973; Field and Schekman, 1980). This region is typically located in the vicinity of the bud. The processes of secretion and of cell surface growth occur by exocytosis, with the fusion of secretory vesicles to the plasma membrane releasing the lumenal proteins into the periplasmic space and simultaneously replenishing the plasma membrane. Thus, mutations affecting the secretory process are likely to have a lethal effect on the cell due to the disruption of cell surface growth. However, when a mutant is placed under non-permissive conditions, it is possible that the synthesis of macromolecules may continue for some time before cell death thus producing dense cells. Based on this concept, Novick et al. (1980) subjected temperature-sensitive mutants to a density gradient enrichment step. The large number of temperature-sensitive secretory (*sec*) mutants isolated with this procedure belonged to at least 23 different complementation groups (Novick et al. 1980; Esmon et al. 1981). The mutants could be classed as: defective in transport from the ER to the Golgi complex; or they accumulated Golgi-like structures called Berkeley bodies (Bb); or they accumulated 80-100 nm vesicles. From these mutants, Novick et al. (1981) established that the order of events in the secretory pathway occurs in a sequence starting from the ER, passing to the Golgi stack to the secretory vesicles and finally to the cell surface (Figure 2). This order was determined by examining the organelles affected in double *sec* mutants. These genetic analyses showed that the ER phenotype was epistatic to both the Bb and vesicle classes, and the Bb phenotype was epistatic to the vesicle class.

Events Associated With the Endoplasmic Reticulum (ER)

Two processing events are associated with the ER: the catalytic removal of the signal peptide by signal peptidase and the transfer of core oligosaccharide chain to the polypeptide backbone (Watts et al. 1983; see reviews by Ballou, 1982; Schekman and

ORDER OF SECRETORY EVENTS IN YEAST

CYTOPLASM

↓

ENDOPLASMIC   — CORE GLYCOSYLATION

RETICULUM   — SIGNAL PEPTIDE CLEAVAGE

↓

GOLGI   — OUTER CHAIN GLYCOSYLATION

COMPLEX   — PROTEOLYTIC PROCESSING

↓

VESICLES   — PROTEOLYTIC PROCESSING

↓

PLASMA

MEMBRANE
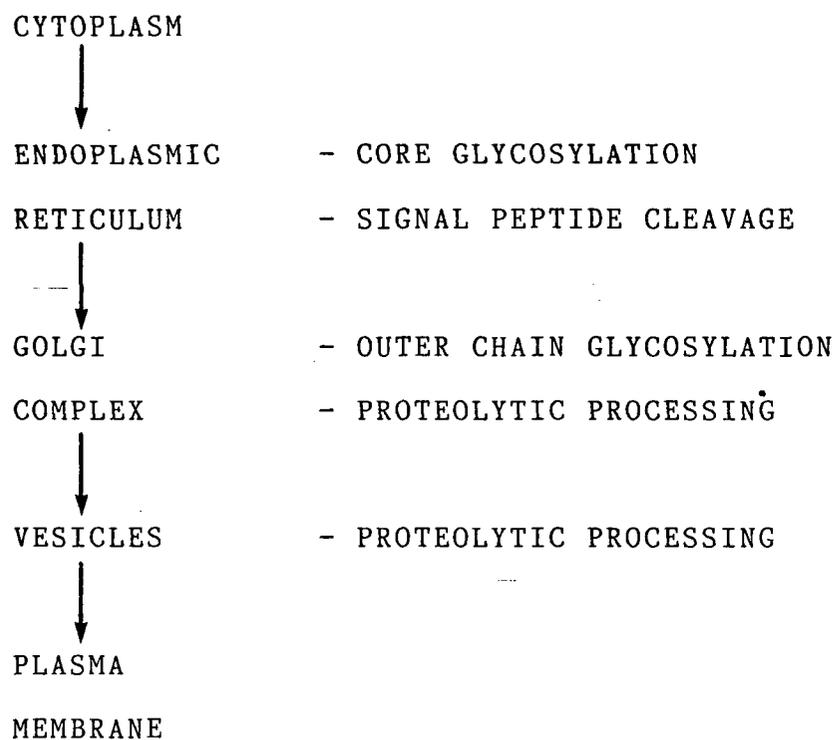
Figure 2. Order of secretory and processing events in yeast.

Novick, 1982; Schekman, 1985). Mutations affecting signal peptide cleavage of invertase (Schauer et al. 1985) and of acid phosphatase (Haguenauer-Tsapis et al. 1986) result in the accumulation of core glycosylated precursor in the ER. It appears that yeast signal peptidase has a preference for small neutral amino acids at the cleavage junction. A missense mutation of the alanine residue at position -1 of pre-invertase to a valine reduced the rate of signal peptide cleavage and subsequent transport (Schauer et al. 1985). One can infer from this that efficient transport from the ER to the Golgi complex is dependent on signal peptide cleavage.

The N-linked glycosylation of a polypeptide chain involves the transfer of a core oligosaccharide chain to the asparagine residue at the consensus Asn-X-Thr/Ser sequence (Neuberger et al. 1972). The synthesis of core oligosaccharide starts with the transfer of GlcNAc from UDP-GlcNAc to the lipid carrier, dolichol phosphate, to form dol-P-P-GlcNAc (for reviews, see Ballou, 1982; Kornfeld and Kornfeld, 1985). This step occurs in the cytoplasmic surface of the ER and is specifically inhibited by tunicamycin (Lehle and Tanner, 1976). After the addition of another GlcNAc from UDP-GlcNAc and five mannose residues from GDP-mannose, the $Man_5GlcNAc_2$ lipid-linked unit is translocated to the lumenal surface of the ER. Four additional mannose residues from dol-P-Man and three glucose residues from dol-P-Glc are added to form the core oligosaccharide $Glc_3Man_9GlcNAc_2$ which is then transferred to the polypeptide chain (Lehle, 1980; Trimble et al. 1980). At some point prior to transport of the core glycosylated protein to the Golgi complex, the three glucose and one mannose residues are removed by specific oligosaccharide glucosidases and mannosidase (Kilker et al. 1981; Byrd et al. 1982; Tsai et al. 1984).

Yeast proteins may also undergo O-linked glycosylation. This process initiates at the ER by the transfer of mannose from dol-P-Man to a serine or threonine residue (Lehle et al. 1979). After transport to the Golgi stack, more mannose residues are added,

resulting in a final product consisting of a 1-4 linked mannose chain. It is not clear what determines the O-linked or N-linked glycosylation of proteins. Invertase, for example, has numerous serine and threonine residues but contains exclusively N-linked oligosaccharide chains. In addition, only 9-10 of the 13 Asn-X-Thr/Ser sequences are N-glycosylated (Trimble et al. 1983).

Events Associated With the Golgi Complex

There are currently two prevailing models for protein sorting. One holds that the ER can efficiently retain the bulk of the integral ER proteins while allowing the passage of newly synthesized secretory, plasma membrane and lysosomal proteins. An example is the retention of β-glucuronidase by its interaction with an ER membrane protein (Medda and Swank, 1985). The second model is referred to as the distillation hypothesis (Rothman, 1981). This proposes that the sorting process in the ER is an imperfect one and results in the transport of some ER proteins to the Golgi stack. These misplaced ER proteins are then removed by the Golgi apparatus and returned to the ER. Hence, a continuous forward and backward flow of transport vesicles is predicted. This is supported by the observation that addition of the consensus Lys-Asp-Glu-Leu (KDEL) sequence, found in the carboxy-terminus of several ER proteins, resulted in the retention of lysozyme, a secreted protein, in the ER (Munro and Pellham, 1987). Both models invoke the existence of a topogenic signal within the transported protein that is recognized by receptors present either in the ER or Golgi stack.

In mammalian cells, sorting of lysosomal proteins from plasma membrane and secretory proteins has been shown conclusively to occur within the Golgi complex. Using organelle fractionation and chemical analysis of the oligosaccharide chains, Waheed et al. (1981) have shown that lysosomal glycoproteins are modified by the addition of GlcNAc-phosphate residues to the mannose residues, followed by the removal of the GlcNAc moiety. The resulting mannose-6-phosphate determinant is believed to be responsible in

part for the transport of the glycoprotein from the Golgi complex to lysosomes (Hasilik and Neufeld, 1980). However, there must exist a signal within the protein that is recognized prior to phosphorylation. This putative signal would then dictate the addition of GlcNAc-phosphate instead of outer chain oligosaccharide to the mannoprotein.

The vacuole in yeast is considered to be analogous to the mammalian lysosome. The yeast vacuole contains numerous hydrolytic enzymes. Of these, the serine-protease, carboxypeptidase Y (CPY), has been used extensively in the study of vacuolar protein targeting. CPY is produced as a 69 kd enzymatically inactive precursor and contains four N-linked oligosaccharide chains (Hasilik and Tanner, 1978). Conversion to the active enzyme occurs in the vacuole and involves the cleavage of an 8 kd amino-terminal pro-peptide by an endopeptidase encoded by the *PEP4* gene (Hemmings et al. 1981). Stevens et al. (1982) used the temperature-sensitive *sec* mutants to determine the involvement of the various organelles in the processing of CPY. They found that a block in transport from the ER (*sec18*) resulted in the accumulation of core glycosylated proenzyme (P1). A block in transport from the Golgi complex (*sec7*) resulted in a slightly more mature, glycosylated proenzyme (P2). The *sec* mutants that accumulated 80-100 nm vesicles (*sec1*) at the non-permissive temperature produced mature CPY. This indicates that CPY is transported from the ER to Golgi complex, and is then sorted and transported to the vacuole. Thus, the Golgi complex represents a branch point in the secretory pathway. It is not clear what protein determinant is recognized by the sorting apparatus. It is unlikely that the mannophosphate determinant is the sorting signal for lysosomal proteins in yeast. Although normally containing phosphate groups on the oligosaccharide chains (Stevens et al. 1982; Trimble et al. 1983), unglycosylated CPY synthesized in the presence of tunicamycin was shown by Schwaiger et al. (1982) to be faithfully and efficiently transported to the vacuole. Similarly, another vacuolar enzyme, alkaline phosphatase (AP), was transported to the vacuole in tunicamycin-treated cells (Onishi et al. 1979). Hence, it is more likely that vacuolar enzymes such as CPY and AP are sorted from

secretory and plasma membrane proteins prior to modification of the core oligosaccharide chains. Phosphorylation of vacuolar glycoproteins may be a consequence of segregating them to a different Golgi compartment.

Secretory and plasma membrane proteins transported through the Golgi complex usually undergo two other modifications: elongation of N-linked and O-linked carbohydrate chains and proteolytic processing. In mammalian cells, processing of protein-linked oligosaccharide chains involves trimming by glucosidases and mannosidases, and addition of modified sugars such as sialic acid, galactose and GlcNAc. In yeast, only multiple mannose oligosaccharides are added to the core oligosaccharide. These are commonly referred to as outer chains. The size of these oligosaccharide chains is highly variable and may range from 9 to over 50 mannose residues (Tarentino and Maley, 1974).

Proteolytic processing is the other prominent activity associated with the Golgi complex. This occurs after the addition of outer chain oligosaccharides to the glycoprotein. Two processing enzymes have been identified to date. Endoproteolytic cleavage at a pair of basic residues (e.g. Lys-Arg) is mediated by the *KEX2* gene product (Julius et al. 1984). Mutation at the *KEX2* locus causes α cells to be mating-deficient due to the inability to process the α-factor precursor (Liebowitz and Wickner, 1976). Maturation of α-factor also requires a membrane-bound dipeptidyl aminopeptidase (DPAPase) encoded by the gene, *STE13* (Julius et al. 1983). This peptidase specifically cleaves on the carboxyl side of repeating -X-Ala- sequences, where X is an acidic residue. A lesion in the *STE13* locus resulted in the secretion of biologically inactive α-factor containing the tetrapeptides H_2N-Glu-Ala-Glu-Ala- or H_2N-Asp-Ala-Glu-Ala- at the amino-terminus.

Transport from Golgi Complex to Cell Surface

In mammalian cells, secretion falls into two classes: constitutive and regulated (Tartaroff and Vassalli, 1978). In constitutive secretion, the newly synthesized proteins

reach the cell surface shortly after leaving the Golgi complex. Thus, they generally have a low intracellular pool. In contrast, regulated secretion refers to the ability of a cell to rapidly release large amounts of protein over a very short period of time. This release is usually in response to a stimulus. In order to achieve bolus release of proteins, the cell must be able to store the newly synthesized proteins in secretory vesicles over a period of time. Hence, one of the characteristic morphological features of these cells is the presence of numerous secretory vesicles in the cytoplasm. Yeast cells normally do not contain a significant amount of electron-dense secretory vesicles. Furthermore, none of the secreted yeast proteins identified to date is released in a regulated fashion. Thus, it is likely that secretion in yeast is constitutive and involves a continuous flow of secretory vesicles toward the cell surface.

Secretory Pathways

Secreted yeast proteins can be divided into three classes according to the types of post-translational processing events. The first is exemplified by external invertase and acid phosphatase. These proteins are synthesized as precursors containing an amino-terminal signal peptide which is cleaved shortly after translocating the ER membrane. They are then core glycosylated in the ER and transported to the Golgi complex. After the addition of outer chain oligosaccharides, the glycoproteins are packaged in secretory vesicles and transported to the cell surface. Thus, signal peptide cleavage is the only endoproteolytic event associated with this class.

The second class is similar to the first but involves additional processing steps in the Golgi complex or secretory vesicles. A member of this class is the α-factor. This pheromone is synthesized as a precursor containing a hydrophobic signal peptide at the amino-terminus, a pro-peptide with three potential N-linked glycosylation sites and four tandem copies of the 11 amino acid long α-factor (Kurjan and Herskowitz, 1982). Each α-factor cassette is separated by a dibasic residue and a spacer unit consisting of

repeating Glu-Ala or Asp-Ala residues. Based on its electrophoretic mobility on SDS polyacrylamide gel, Julius et al. (1984) concluded that the precursor was transported from the ER to the Golgi complex with an intact amino-terminal signal peptide. This implies that signal peptide cleavage is not a pre-requisite for transport out of the ER. However, more recent *in vitro* evidence indicates that the signal peptide is cleaved and that the confusion lies in the aberrant behavior of prepro-α-factor during SDS polyacrylamide gel electrophoresis (W. Hansen, personal communication). The pro-α-factor transported to the Golgi complex is subsequently processed by the two peptidases described previously: the endopeptidase encoded by the *KEX2* gene and the membrane-bound dipeptidyl aminopeptidase encoded by the *STE13* gene. It is not clear why only certain glycoproteins are cleaved by the *KEX2* endopeptidase. Invertase, for example, contains two potential *KEX2* cleavage sites but is not cleaved by the *KEX2* gene product. Differential processing at this type of cleavage site has been observed with mammalian secreted proteins (Nakamishi et al. 1979; see review by Lynch and Snyder, 1986).

Another yeast protein that undergoes multiple processing steps is the type I killer toxin. This secreted toxin kills sensitive cells by making the cytoplasmic membrane permeable to H^+ and K^+ ions (de la Pena et al. 1981). The determinant of the killer phenotype is the 1.9 kb M_1 dsRNA (Wickner, 1979) which is maintained in virus-like particles in the cytoplasm by capsid components provided by the L dsRNA (Bostian et al. 1980). The secreted toxin consists of two disulfide bonded subunits, α and β, with apparent molecular weights of 9.5 and 9.0 kd, respectively. The preprotoxin is synthesized as a 35 kd precursor consisting of a 44 amino acid amino-terminal domain, followed by α, γ and β domains. The γ subunit was previously thought to confer immunity to killer toxin (Bussey et al. 1983). However, recent evidence shows that immunity is conferred by the carboxy-terminal half of the α subunit and the amino-terminal part of the γ subunit (Boone et al. 1986). The current view is that immunity is a result of an incompletely processed precursor acting as a competitive inhibitor of the mature toxin by binding to the

killer toxin receptor. Processing of the secreted killer toxin starts in the ER by the removal of the amino-terminal signal peptide and addition of three core oligosaccharide units to the γ domain (Bostian et al. 1984; Lolle and Bussey, 1986). Three additional cleavage events are needed to produce mature toxin: generation of the amino-terminus of the α subunit; cleavage between the α and γ subunits which is dependent on the *KEX1* gene product, a chymotrypsin-like endopeptidase (Bussey et al. 1983; Boone et al. 1986); and cleavage between the γ and β subunits which is dependent on the *KEX2* gene product (Bussey et al. 1983). This multiple processing is reminiscent of the processing of insulin-like proteins in mammalian cells (Chance et al. 1968).

The third class of secretory pathway differs dramatically from the previous two. Proteins that use this secretory route are the two **a**-factor (Brake et al. 1985) and the two *RAS* gene products (De Feo-Jones et al. 1983; Kataoka et al. 1984). The a-factor is an 11 amino acid long mating pheromone produced by **a** cells and is encoded by two non-allelic genes, *MFa1* and *MFa2* (Brake et al. 1985). The pheromone is produced as a 36 or 38 amino acid long precursor with an amino-terminal extension that does not resemble the classical signal peptide structure. In contrast to α-factor, secretion of a-factor is not dependent on *KEX2* or *STE13* activities. Instead, a-factor secretion requires three different genes, *STE6*, *STE14* and *STE16*. One of these, *STE16*, is allelic to *SUPH*, a gene required for *RAS* functions (Powers et al. 1986). In mammalian cells, the maturation and localization of the Ras proteins to the inner surface of the plasma membrane requires the addition of a palmitic acid residue to the carboxy-terminus (Willumsen et al. 1984; Buss and Sefton, 1986). The putative acyltransferase recognition sequence is Cys-A-A-X, where A is an aliphatic amino acid, and X is the carboxy-terminal amino acid (Taparowsky et al. 1983; Powers et al. 1984, 1986). This sequence is present in the Ras proteins and the a-factor. Mutations in the *SUPH-STE16* locus, which has been redesignated as *RAM*, prevented acylation and membrane localization of the Ras proteins, and maturation of a-factor (Powers et al. 1986).

Secretory Mutants in Yeast

Class A and Class B Secretory (*sec*) Mutants

The class A *sec* mutants are the temperature-sensitive mutants isolated by Novick et al. (1980) described previously. These mutants accumulate intracellular membrane at the non-permissive temperature and affect transport events from the ER to the cell surface. The class B *sec* mutants, *sec53* and *sec59*, are also temperature-sensitive but only affect events associated with the ER (Ferro-Novick et al. 1984a, 1984b). At the non-permissive temperature (37°C), *sec53* produces little or no fully glycosylated invertase, and *sec59* causes accumulation of invertase with 0-3 instead of the 9-10 N-linked oligosaccharide chains normally added to the protein. These mutations seem to mimic the effect of the antibiotic, tunicamycin. However, it is unlikely that the major defect is due to a deficiency in core oligosaccharide synthesis or its transfer to the protein. In support of this is the observation that transport and assembly of unglycosylated plasma membrane proteins (e.g. the vanadate-sensitive ATPase) are blocked in *sec53* mutants but not in tunicamycin-treated cells (W. Hansen, personal communication). It is possible that *SEC53* and *SEC59* gene products are required either directly or indirectly for the completion of the membrane translocation process and glycosylation of the precursor in the ER lumen. If this were so, these proteins should be an integral component of, or closely associated with, the ER membrane. The *SEC53* gene has recently been cloned by complementation (Bernstein et al. 1986). Surprisingly, cell fractionation study showed that the 28 kd *SEC53* gene product was localized in the cytoplasm. This suggests that the *SEC53* protein is a cytoplasmic factor responsible in part for the translocation of proteins and/or of the lipid-bound $Man_5GlcNAc_2$ unit across the ER membrane. This is consistent with the finding that a mutation affecting N-linked glycosylation, *alg4*, is allelic to *sec53* (Huffaker and Robins, 1983). The *alg4* mutant at the non-permissive temperature accumulates lipid-linked oligosaccharides ranging from $Man_1GlcNAc_2$ to $Man_8GlcNAc_2$.

Mutations Affecting Oligosaccharide Synthesis

Using a $[^3H]$-mannose suicide selection, Huffaker and Robbins (1983) isolated mutants that are defective in the synthesis of precursor oligosaccharide. These mutations were designated *alg*, for asparagine-linked glycosylation. The *alg1* mutants block the addition of the first mannose residue to $GlcNAc_2$-lipid, *alg2* mutants accumulate $Man_{1-2}GlcNAc_2$, *alg3* mutants accumulate $Man_5GlcNAc_2$, *alg4* mutants accumulate $Man_{1-8}GlcNAc_2$, *alg5* mutants block the synthesis of dol-P-Glc and *alg6* mutants block the transfer of dol-P-Glc to $Man_9GlcNAc_2$. With the exception of *alg4*, none of the other *alg* mutations exert any adverse inhibitory effect on secretion.

Another set of mutations affecting mannan synthesis is *mnn1-10* (for reviews, see Ballou, 1976; 1982). These mutations affect different stages of mannan synthesis, resulting in the transfer of altered carbohydrate chains to the protein. They affect the extent of protein glycosylation, but do not prevent the secretion of proteins to the periplasmic space.

Glucosylation of the lipid-linked oligosaccharide precursor also affects the extent of protein glycosylation. The *dpg1* mutation, which has a similar phenotype but is nonallelic to *alg5*, blocks the synthesis of the glucose donor dol-P-Glc (Ballou et al. 1986). This inability to glucosylate the lipid-linked precursor results in a reduction in the efficiency of subsequent glycosylation. Thus, secreted glycoproteins such as invertase contain a reduced number of carbohydrate chains.

Mutations Affecting Vacuolar Protein Localization

Since secretory and vacuolar proteins share the same early processing events (Stevens et al. 1982), there must exist a mechanism by which these protein are sorted and transported to the appropriate organelles. A number of vacuolar protein-targeting mutants have been identified to date. Mutants identified by Bankaitis et al. (1986) were designated

vpt, for vacuolar protein targeting. These mutants were shown to belong to at least 8 different complementation groups. The mutants were isolated by screening for a Suc[+] phenotype in *suc* mutants carrying a *PRC1-SUC2* gene fusion. The hybrid protein, which contains the amino-terminal 433 amino acids of CPY and the carboxy-terminal 511 amino acids of invertase, is targeted to the vacuole. Thus, the ability to ferment sucrose is dependent on mistargeting of the hybrid protein to the cell surface. The *vpt* mutation also caused the mistargeting of CPY to the cell surface. Interestingly, the secreted CPY in these mutants retained its core glycosylation pattern and did not contain any outer chain oligosaccharide. This suggests that the secreted vacuolar protein has not been processed through the Golgi complex as would be a normal secretory protein. One possibility is that the mutations have caused the protein to be mistakenly packaged into secretory vesicles during its passage through the Golgi stack. Using a different approach, Rothman and Stevens (1986) identified a set of vacuolar protein-secreting mutants (designated *vpl*, for vacuolar protein localization) which behaved similarly to *vpt* mutants. In a *vpl* mutant, CPY traverses the late stages of the secretory pathway, requiring a functional *SEC1* gene. In addition, the finding that secretion of invertase has not been substantially affected suggests that *vpl* mutations perturb the vacuolar localization branch of the secretory pathway. Comparisons of the two sets of mutants suggest that as many as 14 different complementation groups are involved in vacuolar protein biogenesis. It remains to be determined how these gene products affect the protein sorting process in the Golgi complex or transport to the vacuole.

Relationship Between Exocytosis and Endocytosis

The mechanism by which a yeast cell controls the direction of flow of endocytotic and exocytotic vesicles is not clear. The finding that *sec* mutants which accumulate secretory vesicles but not those which accumulate ER or Golgi membranes are also defective in endocytosis suggests that secretion and endocytosis are coupled (Riezman,

1985). One possibility is that the endocytosis pathway and the late stages of the secretory pathway utilize the same set of proteins. For example, proteins necessary for endocytosis may rely on the secretory apparatus for initial transport to the cell surface. Several proteins and/or genes involved in endocytosis have been identified to date. One of these is the gene for clathrin heavy chain (Payne and Schekman, 1985). In mammalian cells, clathrin is involved in the receptor-mediated uptake of ligand (Goldstein et al. 1985), and it is likely to have a similar function in yeast. More recently, Chvatchko et al. (1986) identified two temperature-sensitive mutants, *end1* and *end2*, that were defective in lucifer yellow carbohydrazide accumulation at the non-permissive temperature (37^0C). The *end1* mutant was not only defective in endocytosis but also in internalization of α-factor and in vacuole biogenesis. These mutants exhibited no adverse defect in exocytosis or secretion.

Secretion of Non-homologous (Foreign) Proteins

There has been a considerable interest in recent years in the use of yeast for the production of proteins for biological and medical applications. In most cases, the emphasis has been on the secretion of the foreign protein into the culture medium, which would undoubtedly simplify subsequent purification procedures. The most widely used procedure for targeting a foreign protein for secretion by yeast is the in-frame fusion of the coding sequence of the protein of interest to the prepro-α-factor sequence. The rationale is that the amino-terminal sequence of α-factor contains all the information required to process the protein through the secretory pathway. The list of proteins successfully secreted into the culture medium using this approach includes human β-endorphin (Bitter et al. 1984), α-interferon (Bitter et al. 1984; Singh et al. 1984), epidermal growth factor (Brake et al. 1984), insulin (Thim et al. 1986) and tissue plasminogen activator (MacKay, 1986). In the case of -interferon, Singh et al. (1984) reported that the secreted interferon retained the repeating Glu-Ala residues plus four amino acids resulting from the linker used in the gene fusion. However, using a slightly different plasmid construction, the interferon isolated by

Bitter et al. (1984) was correctly processed. The difference may be a consequence of a delay in DPAPase processing caused either by the additional linker sequence present in the gene fusion or to the rate of interferon production. Processing by DPAPase appears to be the rate limiting step in α-factor secretion, as evident by the retention of Glu-Ala residues when the pheromone is overproduced, such as in strains carrying multiple copies of the *MFα1* gene (Julius et al. 1984).

An alternate approach is to utilize only the signal sequence of a secreted yeast protein to target the foreign protein to the secretory pathway. Both calf prochymosin (Smith et al. 1985) and human α-interferon (Chang et al. 1986) were secreted into the culture medium when fused to the *SUC2* signal sequence. Similarly, a bacterial cellulase was secreted when fused to the signal sequence of type I killer toxin (Skipper et al. 1985).

A third approach is to use the natural signal sequence of the foreign protein to direct its own secretion. This relies on the ability of the yeast secretory apparatus to recognize the foreign signal peptide. The *Aspergillus* glucoamylase, for example, is efficiently processed, glycosylated and secreted by yeast (Innis et al. 1985). Similarly, the human interferon signal peptide is recognized by the yeast secretory apparatus (Hitzeman et al. 1983). However, a fraction of the interferon isolated from the culture medium contained a different amino-terminal residue, which suggest that cleavage of the signal peptide had occurred at a site different from that cleaved by *Aspergillus*.

Two other foreign proteins expressed in yeast are of interest. The wheat pre-α-amylase gene, when expressed using the yeast phosphoglycerol kinase (PGK) promoter, was shown by Rothstein et al. (1984) to be processed, glycosylated and secreted into the medium in a biologically active form. The novelty of this expression system is that during the construction of the expression plasmid, the pre-α-amylase gene was placed downstream of a short coding sequence. This resulted in an internally localized signal peptide. Since the protein was secreted, one can conclude that the yeast secretory

apparatus must be capable of recognizing and processing an internal signal peptide. The second protein is human α_1-antitrypsin. When expressed under the control of the yeast *ARG3* promoter, the precursor protein was processed and glycosylated, but secreted inefficiently (Cabezon et al. 1984). The protein was expressed optimally in a *pep4* background. When intracellular α_1-antitrypsin was isolated, it contained predominantly core oligosaccharides. This suggests that transport of the protein from the ER to the Golgi complex is hindered.

There are numerous problems associated with secretion of heterologous proteins in yeast. It is clear that the secretory apparatus in yeast is capable of recognizing and correctly removing certain eukaryotic signal peptides. However, to ensure efficient processing and secretion, it may be prudent to utilize an endogenous yeast signal peptide. Proteins containing internal dibasic residues are susceptible in principle to *KEX2* endoproteolytic cleavage. This is perhaps desirable in certain cases, but it could be a nuisance. The problem may be circumvented by destroying the *KEX2* cleavage site or by expressing the protein in a *kex2* mutant strain. The nature of the desired product should also be taken into consideration before choosing yeast as a host organism. Proteins containing putative glycosylation signals are likely to have carbohydrate chains added to the polypeptide backbone during secretion. This may or may not have any effect on the desired biological activity of the protein. However, due to the antigenic nature of the outer chain oligosaccharide units, the secreted proteins are less likely to have any useful therapeutic applications.

Objectives of Thesis Project

The purpose of this study is to define the features encoded within the signal peptide of a yeast protein that are crucial for proper translocation and secretion. Yeast invertase was chosen in this study for the following reasons. First, yeast cells capable of secreting the enzyme have an easily selectable phenotype because invertase catalyses the hydrolysis

of sucrose into glucose and fructose. This represents the first step in sucrose utilization. Cells can be scored for the ability to ferment sucrose by simply examining their relative growth rate on a selective medium with sucrose as the sole source of carbon and energy (Carlson et al. 1981a; 1981b). Secondly, the protein is well characterized. The secreted form of invertase is heavily glycosylated and has an apparent molecular weight of 135 kd (Neumann and Lampen, 1967). Removal of the high mannose oligosaccharides with endoglycosidase H (Endo H) releases a protein moiety of approximately 60 kd (Tarentino et al. 1974). The enzyme normally exists as a dimer (Trimble and Maley, 1977) but aggregates into an octamer at its optimal pH of 5.0 (Chu et al. 1983). It is a very stable enzyme and renatures rapidly after complete denaturation with 4M guanidine-HCl (Chu et al. 1985). Thirdly, the invertase gene has been cloned and characterized genetically. It belongs to a dispersed multigene family encoded by the genetic loci *SUC1-5* and *SUC7* (Carlson et al. 1980). Two genes, *SUC2* and *SUC7*, have been cloned and sequenced (Taussig and Carlson, 1983; Sarokin and Carlson, 1985). Lastly, yeast carrying a SUC^+ gene possesses two forms of invertase. The cytoplasmic form is produced constitutively and is unglycosylated. The glycosylated secreted form is glucose-repressed and derepression is dependent upon a functional *SNF1* gene product (Celenza and Carlson, 1984). The two forms of invertase arise from differential 5' transcription start sites on the same *SUC2* locus (Carlson and Botstein, 1982; Carlson et al. 1983). The longer transcript encodes the signal peptide and gives rise to secreted invertase is repressed by glucose, whereas the shorter transcript is constitutive and when translated yields cytoplasmic invertase. Hence, one can conclude that the signal for secretion must reside in the amino-terminal sequence. An additional attractive feature of the yeast invertase gene is that it is expressed and the product glycosylated and secreted by a mammalian cell line (Bergh et al. 1987). Thus, signal sequence mutants may prove useful in the study of secretion by higher eukaryotes.

Cassette mutagenesis was used in this study to generate mutations in the signal sequence. There are two major advantages with this method. First, a large family of amino acid substitutions can be introduced at any desired position. Secondly, nonsense mutations can be avoided by carefully selecting the position of the target nucleotides in the codon or by choice of the mutant base compositions of the cassette oligonucleotides. Mutations were directed mainly at the amino- and carboxy-termini of the signal peptide, although some mutants were constructed in its hydrophobic core. To facilitate analysis of the mutants, the *SUC2* coding sequence was placed under the control of the constitutive *ADH1* promoter. The resulting mutants were then characterized by enzymatic assays, immunological detection, cell fractionation, *in vivo* pulse labeling, and *in vitro* translation and translocation.

Materials and methods

Materials

Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs, Promega Biotech., or Bethesda Research Laboratories. RNasin and SP6 RNA polymerase were from Promega Biotech. [^{32}P]-labeled nucleoside triphosphates and [^{35}S]-methionine were from Amersham. Alkaline phosphatase and horseradish peroxidase coupled goat anti-rabbit IgG antisera were from Bio-Rad. Reagents for growth media were purchased from Gibco. All other reagents were from Sigma and Boehringer-Mannheim.

Bacterial Strains and Growth Conditions

The following media were used to maintain the *Escherichia coli* strains:

2xYT	1.6% tryptone, 1% yeast extract, 0.5% NaCl
YT	0.8% tryptone, 0.5% yeast extract, 0.25% NaCl
M9*	50 mM Na_2HPO_4, 25 mM KH_2PO_4, 8.5 mM NaCl, 20 mM NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM glucose and 10 µg/ml thiamine

Bacterial vectors were propagated in *E. coli* strains RRI (Bolivar and Backman, 1979), JM101 (Messing, 1979) or RZ1032 (Kunkel, 1985). Unless stated otherwise, the strains were grown in 2xYT at 37^{0}C. This was supplemented with 100 µg/ml of ampicillin when selecting for ampicillin resistance. For plates, 1.5% agar was added to the liquid medium. All centrifugation of cells were carried out at 4^{0}C unless otherwise indicated.

When using JM101, the overnight culture was grown in M9* medium to maintain the f′ episome. To screen for M13 recombinants, the infected cells were plated in 3 ml of soft agar (YT with 0.6% agar) supplemented with 0.05 ml of 2 mg/ml 5-bromo-4-chloro-3-

indoyl-β-D-galactoside (XGAL) in dimethylformamide (DMF) and 0.01 ml of 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Transformation of *E. coli*

Competent cells were prepared as described by Dagert and Ehrlich (1979). A 50 ml solution of 2xYT with 0.4% glucose was inoculated with 0.5 ml of an overnight culture of *E. coli* and incubated at $37^{o}C$ to the exponential phase (A_{600} of 0.2 to 0.3). The culture was chilled on ice for 5 min and centrifuged at 3000 x g for 5 min. The cells were resuspended in 25 ml of ice-cold 50 mM $CaCl_2$ and incubated on ice for 30 min. The harvested cells were resuspended in 2 ml of ice-cold 50 mM $CaCl_2$. Plasmid DNA (1-100 ng) was added to 0.1 ml of competent cells and incubated on ice for 30-40 min. They were then transferred to a $42^{o}C$ water bath for 2 min. The cells were incubated with 1 ml of 2xYT in a $37^{o}C$ shaker bath for 30-40 min. Aliquots of 0.05 to 0.1 ml were plated on selective plates and incubated at $37^{o}C$ overnight.

Yeast Strains and Growth Conditions

The following media were used to maintain the *Saccharomyces cerevisiae* strains:

YPD	1% yeast extract, 2% peptone, 2% glucose
YPS	1% yeast extract, 2% peptone, 2% sucrose
YPR	1% yeast extract, 2% peptone, 2% raffinose
SD	0.67% yeast nitrogen base pH 5.8, 2% glucose, supplemented with the following: 20 mg/l each of adenine, uracil, tryptophan, histidine, arginine, methionine; 30 mg/l each of tyrosine, leucine, isoleucine, lysine; 50 mg/l of phenylalanine; 100 mg/l each of glutamic acid, aspartic acid; 150 mg/l of valine; 200 mg/l of threonine; 375 mg/l of serine. One or more of the above were excluded where indicated.
Spo	1% potassium acetate, 0.1% yeast extract, 0.05% glucose

SOS   3.7% YPD, 13 mM CaCl$_2$, 1 M sorbitol, supplemented with the
            appropriate amino acids at the final concentration listed above.

R-agar   SD with twice the concentration of the supplements in 1 M sorbitol
            and 3% agar.

For plates, 2% agar was added to the above media.

S. cerevisiae strain JNY4 (*MATa trp1 ade2-101 suc2-215*) was constructed by
crossing MCY36 (*MAT𝛼 ade2-101 suc2-215 gal2*), obtained from Dr. Marian Carlson,
with C1 (*MATa lys2 trp1*), obtained from the Cold Spring Harbor Laboratory stock. The
haploid strains were streaked out in 6-8 parallel lines on YPD plates and incubated at
30°C overnight. They were then replica plated on a fresh YPD plate such that the streaks
are perpendicular to each other. The cells in the intersections were allowed to mate by
incubating the plate at 30°C overnight. Diploid cells were selected by plating the cells in
the intersections on a SD Ade⁻, Trp⁻ plate. Cells from a single diploid colony were then
streaked on a Spo plate and incubated at 30°C for 3-5 days. A loopful of the culture was
resuspended in 0.2 ml of sterile TE10:1 (10 mM Tris-HCl pH 8, 1 mM EDTA) with 10 mM
dithiothreitol (DTT). Glusulase (5 μl) was added and the suspension incubated at 30°C for
60-80 min. The volume was adjusted to 1 ml with sterile distilled water and glass beads
(0.45 mm diameter) were added up to the meniscus. The cell suspension was vortexed at
top speed for three min. Sterile distilled water was added to 5 ml and 0.2 ml of a 1/100,
1/1,000 and 1/10,000 dilution was plated on YPD plates. After 3 days at 30°C, the
colonies were replica plated on SD Ade⁻ and SD Trp⁻ plates, and scored for Ade⁺ and
Trp⁺ phenotypes, respectively. To score for a Suc⁺ phenotype, the cells were replica
plated on a YPS or YPR plate and incubated at 30°C anaerobically (Carlson et al. 1981) or
on a YPS plate supplemented with 1 μg/ml of antimycin A.

The JNY10 strain (*MATa trp1 leu2 pep4::URA3 suc2-215 ade2-101*) was
constructed by mating JNY4 with DG5-1 (*MAT𝛼 pep4::URA3 leu2*) obtained from

Dr. David Goodin. After mating, the diploid cells were selected on a SD Ura⁻, Trp⁻, Leu⁻ plate and sporulated on a Spo plate. The haploid spores were selected for Trp⁻, Leu⁻, Suc⁻, Ade⁻ and Ura⁺ phenotypes. The Pep⁻ phenotype was confirmed by the screening procedures described by Jones (1977). A 3 ml solution of 0.6% agar kept at 55°C was added to 2 ml of 3 mg/ml N-acetyl DL-phenylalanine .β-naphthyl ester (APE) in DMF, mixed briefly, and gently poured over colonies grown on a YPD plate. The plates were incubated at room temperature for 10 min. Fast Garnet GBC (5 mg/ml) in 0.1 M Tris-HCl (pH 7.4) was then dispensed on top of the soft agar at 3-4 ml/plate. As soon as the wild-type *PEP4* colonies had begun to turn red, the dye solution was poured off and the plate rinsed with distilled water.

Transformation of *Saccharomyces cerevisiae*

Lithium Chloride or Lithium Acetate Method

Yeast cells were grown in 100 ml of YPD to exponential phase (A_{600} of 1.0) at 30°C. The cells were harvested by centrifugation at 3000 x g for 3 min and washed with 50 ml of TE(10:1). The cells were then incubated in 10 ml of 0.1 M LiCl or LiOAc at 30°C. After 60 min, the cells were harvested and resuspended in 1 ml of TE(10:1). Plasmid DNA (10-50 μg) and yeast tRNA (50 μg) were added in a volume of less than 10 μl to a 0.1 ml aliquot of the cell suspension. This was placed on ice for 10 min followed by 5 min in a 42°C water bath. The cells were returned to the ice bath for 10 min and incubated with 1 ml of ice-cold 40% polyethylene glycol (PEG) 3350 in 10 mM Tris-HCl (pH 7.4) for 10 min. The PEG solution was then removed by centrifugation at 1500 x g for 2-3 min. Cells were resuspended in 0.2 ml of SOS medium and 0.1 ml was plated on each of two selective plates. These were incubated at 30°C and transformed colonies were visible after 2-3 days.

Transformation of Yeast Spheroplasts

Exponentially growing yeast cells (A_{600} of 1.0) from 100 ml of YPD medium were harvested by centrifugation at 3000 x g for 5 min. The cells were resuspended in 10 ml of 1 M sorbitol, 50 mM DTT and kept at room temperature for 5 min. Glusulase (100 μl) or zymolase 60,000 (60 μg/ml) was added to the cells and incubated at 30°C for 45-90 min. The extent of spheroplast conversion was monitored periodically. After 30 min, a small sample of the cell suspension was removed every 5-10 min and diluted with 1% SDS. Under a phase-contrast microscope, cells with an intact chitin wall are refractile whereas spheroplasts appear translucent. When 70% or more of the cells had been converted to spheroplasts, the suspension was centrifuged at 3000 x g for 10 sec at speed or at 1000 x g for 3 min. The cells were then washed gently with 10 ml of 1 M sorbitol followed by 10 ml of STC (1 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$). The final pellet was resuspended in 1 ml of STC. Plasmid DNA (1-10 μg) in a volume less than 10 μl was added to 0.1 ml of the spheroplast suspension. After 20 min at room temperature, 0.5 ml of PEG-TC (40% PEG-3350, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$) was added and incubated for 20 min. The suspension was then transferred to 15 ml of R-agar lacking the selective amino acid (kept at 50°C), mixed briefly, and poured over a selective plate. Transformed colonies were visible after 2-3 days at 30°C.

Isolation of Plasmid DNA from *E. coli*

Large Scale Preparation

Plasmids were isolated by the alkaline lysis procedure (Birnboim and Doly, 1979) as described by Maniatis et al. (1982). The bacterial cells were grown to stationary phase in 100 ml of 2xYT supplemented with 100 μg/ml ampicillin. The cells were harvested by centrifugation at 5000 x g for 5 min and resuspended in 4 ml of ice-cold TEG (25 mM Tris-HCl pH 8, 10 mM EDTA, 50 mM glucose). Freshly made 0.2 M NaOH with 1% SDS (8

ml) was added to each tube. The contents were mixed gently and kept on ice. After 10 min, 6 ml of ice-cold 3 M KOac, 2 M HOAc (pH 4.8) was added. The lysate was mixed gently and kept on ice for another 10 min. This was then centrifuged at 10,000 x g for 30 min. The supernatant was carefully decanted into a clean centrifuge tube. One-half volume of isopropanol was added and the solution incubated at room temperature for 20-30 min. This was then centrifuged at 10,000 x g for 30 min at 20-25°C and the pellet redissolved in 2.5 ml TE(10:1). High molecular weight RNAs were removed by LiCl precipitation. Ice-cold 5 M LiCl was added to a final concentration of 2.5 M and the solution kept on ice for 10 min. The supernatant was recovered by centrifugation at 10,000 x g for 30 min. Ethanol (two volumes) was then added and chilled at -20°C for 20-30 min. The DNA precipitate was recovered by centrifugation at 10,000 x g for 30 min.

If necessary, plasmid DNAs were purified by centrifugation in CsCl density gradient or by gel filtration chromatography. For CsCl gradient centrifugation, the DNA was dissolved in 9.5 ml of TE(10:1) together with 9.4 g of CsCl. The solution was then transferred to a Beckman quick-seal polyallomer tube. Ethidium bromide, 0.9 ml of a 10 mg/ml solution, was layered on top with a 1 ml syringe using a 23 gauge hypodermic needle. The tubes were heat sealed and the contents mixed thoroughly. They were then centrifuged to equilibrium in a 50Ti rotor at 37,000 rpm for 40 h at 10°C, or in a 70.1Ti rotor at 60,000 rpm for 16 h at 10°C. After centrifugation, the tubes were illuminated with ultra-violet light and the lower DNA band was withdrawn through a 23 gauge hypodermic needle. Ethidium bromide was removed from the solution by repeated extraction with CsCl saturated 1-butanol. The DNA solution was then diluted with two volumes of TE(10:1) and recovered by ethanol precipitation (two volumes).

For gel filtration chromatography, the DNA was dissolved in 1 ml of TE(10:1). Pancreatic RNase A (40 μg/ml) and RNase T$_1$ (40 u/ml) were added and incubated at 37°C for 2 h. The solution was then adjusted with 5M NaCl to 100 mM and loaded onto a Bio-

gel A-5M column (1x40 cm) equilibrated in sterile TES (10 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl). Fractions (1 ml) were collected at a flow rate of 10-16 ml/h and monitored spectrophotometrically at 260 nm. The plasmid DNA, eluting at the void volume, was recovered by ethanol precipitation (two volumes).

Isolation of M13 Recombinant Replicative Form (RF) DNA

A 0.5 ml inoculum of *E. coli* JM101 was transferred to 100 ml of 2xYT medium and incubated at 37°C to exponential phase (10^8 cells/ml). At the same time, 10 μl of the overnight culture was added to 1 ml of 2xYT and infected with phage particles from a single plaque of freshly transfected JM101 cells. This was then incubated at 37°C for 3-4 h to approximately 10^{12} pfu/ml. Cells in the large culture were then infected with M13 phage at a multiplicity of infection (m.o.i.) of 10:1. This was incubated at 37°C for 3-4 h. Cells were collected by centrifugation at 5000 x g for 5 min and RF DNA was isolated by the alkaline lysis procedure.

Plasmid "Mini-preps"

Isolation of plasmid DNA from small cultures (1 to 1.5 ml) by the alkaline lysis procedure was as described by Maniatis et al. (1982). For DNA sequence determination, plasmids were prepared by a procedure developed by Ms. Caroline Beard in this laboratory. Plasmid DNA prepared by the alkaline lysis procedure was dissolved in 200 μl of TE(10:1) and 100 μl of 7.5 M NH_4OAc. The solution was kept on ice for 30 min and centrifuged in a microfuge for 15 min. The supernatant was removed and precipitated with two volumes of ethanol. The precipitate was collected by centrifugation for 5 min at 4°C and redissolved in 50 μl of TE(10:1). DNase-free pancreatic RNase A and RNase T_1 were added at a concentration of 40 μg/ml and 40 u/ml, respectively. After an incubation period of 2 h at 37°C, the solution was extracted with an equal volume of phenol/ chloroform (1:1). The DNA was precipitated with ethanol and redissolved in 50 μl of TE(10:1). A 5 μl

aliquot was used for DNA sequence determination by the double-stranded method (see below).

Isolation of Single Stranded DNA

From M13 Phage Recombinants

Single stranded M13 phage DNAs were isolated as described by Sanger et al. (1980). An inoculum (1 ml) of JM101, grown overnight in M9* at $37^{\circ}C$, was transferred to 50 ml of 2xYT. After 1 h at $37^{\circ}C$, 1.5 ml aliquots were dispensed into 13x100 mm tubes. Phage particles from a single plaque were transferred with a sterile capillary to each tube. The infected cultures were incubated at $37^{\circ}C$ for 4-6 h. Each culture was then transferred to an Eppendorf tube and the cells pelleted by centrifugation in a microfuge for 5 min. The supernatant was then poured into an Eppendorf tube containing 0.23 ml of 20% PEG-6000, 2.5 M NaCl. The contents were mixed and left at room temperature for 15-20 min. After centrifugation for 5 min, the supernatant was discarded. The tubes were centrifuged for another 2 min and the residual liquid removed with a drawn out Pasteur pipette. The phage pellet was resuspended in 150 μl of TES (10 mM Tris-HCl pH 8, 1 mM EDTA, 20 mM NaCl) and extracted with 150 μl of phenol/chloroform (1:1). After centrifugation for 2 min, the aqueous phase was transferred to a clean Eppendorf tube. The DNA was precipitated with sodium acetate (15 μl of a 3 M solution) and ethanol (0.75 ml) at $-20^{\circ}C$ for 1-12 h. The DNA precipitate was recovered by centrifugation at $4^{\circ}C$ for 5-10 min, washed with 70% ethanol, and dissolved in 35 μl of TE(10:1). A 5-8 ul aliquot was used for DNA sequence determination.

From pEMBL Recombinants

Single stranded pEMBL DNA was isolated as described by Dente et al. (1983). Cells from a single colony of *E. coli* JM101, freshly transformed by the pEMBL recombinant DNA, were transferred to 1.5 ml of 2xYT with ampicillin and incubated at

37°C to 1-5x10^8 cells/ml (approximately 1 h). The cells were then superinfected with IR1

phage at a m.o.i. of 20:1. The cultures were incubated at 37°C for 4-6 h. Phage particles

and single stranded DNAs were isolated as described for M13 phage.

Restriction Digestion and DNA Modifications

Restriction digest reactions were usually carried out in 20 μl volume at 37°C for

1-2 h. Restriction enzyme buffers were made in the following 10x stock solutions:

10x low salt	100 mM Tris-HCl pH 7.5, 100 mM MgCl$_2$, 10 mM DTT
10x medium salt	100 mM Tris-HCl pH 7.5, 100 mM MgCl$_2$, 10 mM DTT, 500 mM NaCl
10x high salt	100 mM Tris-HCl pH 7.5, 100 mM MgCl$_2$, 10 mM DTT, 1 M NaCl.

For SmaI, NaCl was replaced by 200 mM KCl in a 10x stock solution.

DNA fragments with 5' or 3' single-stranded ends were converted to flush ends by

incubation with T$_4$ DNA polymerase (1 u/μg DNA) at 37°C for 5-10 min in the following

buffer:

10x T$_4$ pol salt	0.33 M Tris-HOAc pH 7.9, 0.66 M KOAc, 0.1 M Mg(OAc)$_2$, 10 mM DTT, 10 mg/ml BSA.

The four dNTPs were added to the reaction mix at a final concentration of 250 μM each.

Alternatively, fragments with 3' single-stranded ends were converted to flush ends by

incubation with 0.5 u of DNA polymerase I (Klenow fragment) in 10 mM Tris-HCl pH 7.5,

10 mM MgCl$_2$, and 250 μM each of the four dNTPs at 37°C for 5 min.

DNA fragments were ligated with T$_4$ DNA ligase at room temperature for 3-6 h or

at 15°C overnight using the following buffer:

10xLK	0.6 M Tris-HCl pH 7.5, 150 mM DTT, 0.1 M MgCl$_2$, 2 mg/ml BSA.

ATP from a 10 mM stock solution was added to the reaction mix at a final concentration of 1 mM.

Stock solutions for DNA and RNA hybridization include:

20xSSC   3 M NaCl, 0.3 M sodium citrate pH 7

50xDenhardt's   1 g/100 ml of each of Ficoll, BSA and polyvinylpyrrolidone.

Recovery of DNA Fragments from Low-Melting Point Agarose Gel

The DNA solution was subjected to electrophoresis on low-melting point (LMP) agarose gel at 8 v/cm for 1-3 h. The gel was then stained for 10-15 min in a solution of 1 ug/ml ethidium bromide. Under long wavelength ultra-violet light, the gel containing the desired DNA band was quickly excised with a scalpel. The gel slice was placed in an Eppendorf tube with two volumes of TE(10:1). This was then incubated at $65^{O}C$ for 10-15 min. An equal volume of phenol, kept at $55^{O}C$, was added. After two min of centrifugation, the aqueous phase was transferred to a clean tube and extracted successively with equal volumes of phenol, phenol/chloroform (1:1), and chloroform. Sodium acetate (0.1 volume of a 3M solution, pH 5.5) and 2.5 volumes of ethanol were added. For small DNA fragments (<500 bp) or samples containing a low concentration of DNA (<100 ng/μl), 1-5 μg of yeast tRNA was added as carrier. After 30-60 min at $-20^{O}C$, the nucleic acids were recovered by centrifugation.

Oligonucleotide-Directed Mutagenesis

Purification of Oligonucleotides

The deoxyribo-oligonucleotides were synthesized on an Applied Biosystem 380A DNA synthesizer by Mr. T. Atkinson in this laboratory. The crude oligonucleotide was deprotected with an equal volume of concentrated NH_4OH at $55^{O}C$ overnight. The solution was then evaporated to dryness in a Speed-Vac. The pellet was dissolved in 50 μl of distilled water. A 10 μl aliquot was added to 20 μl of de-ionized formamide and denatured

by boiling for 2-3 min. The sample was rapidly chilled on ice and loaded onto a 20%

polyacrylamide gel (20 cm x 40 cm x 0.5 mm) containing 7M urea. A 12%

polyacrylamide/urea gel was used for longer (30-mer or longer) oligonucleotides.

Bromophenol blue (0.03%) and xylene cyanol (0.03%) in 60% formamide was loaded on an

adjacent lane as markers. Electrophoresis at 1500 volts was carried out until the

bromophenol blue dye front was at least two thirds down the gel. For longer

oligonucleotides, the xylene cyanol dye front was used as an indicator. The gel was then

carefully transferred to a sheet of Saran wrap. A TLC silica plate was placed beneath it

and the gel was illuminated with a hand held ultra-violet (254 nm) lamp. Regions in the

gel containing the oligonucleotides appeared as dark bands against a fluorescent

background. The gel containing the desired oligonucleotide was excised with a scalpel and

cut into small pieces. The oligonucleotide was eluted from the gel with 1 ml of 0.5 M

$NH_4(OAc)$ pH 5, 10 mM $Mg(OAc)_2$ at 37^oC overnight. The supernatant was recovered

after a brief centrifugation and filtered through a Millex HV4 filter. Urea and other

contaminants were removed by reverse phase chromatography. A C_{18} SEP-PAK cartridge

(Waters Scientific) was washed with 10 ml of HPLC grade acetonitrile followed by 10 ml of

distilled water. The oligonucleotide solution was passed through the C_{18} cartridge.

Contaminants were washed off the cartridge with 10 ml of distilled water. The

oligonucleotide was then eluted with three 1 ml aliquots of 20% acetonitrile. The

concentration was determined spectrophotometrically, with one A_{260} unit corresponding to

20 µg/ml. The solution was then evaporated to dryness in a Speed-Vac and kept at -20^oC.

The sequences of the mutagenic oligonucleotides used in this study are shown in Table I.

Mutagenesis With Two Primers

The procedure was as described by Zoller and Smith (1982). The mutagenic

oligonucleotide (50-100 pmol) was phosphorylated at the 5' end using T_4 polynucleotide

kinase (1u) in LK buffer with 1 mM ATP at 37^oC for 40-50 min. The enzyme was then

Table I. List of the mutagenic oligonucleotides used in this study.

Oligonucleotides Used in Site-directed Mutagenesis:

NAME	SEQUENCE	MUTATION	PLASMID
JN1	5'-GACGGGGGTCATGGTGGTGATG-3'	Delete signal sequence of bovine prolactin	BP-1
JN5	5'-CTTGTGCTCTGCAGGTGTG-3'	Create PstI site in signal sequence of prolactin	pS7
JN6	5'-CTTGTGCTCTGCAGGGTGT-3'	Create PstI site in signal sequence junction of SUC2	pS3
JN8	5'-TTAGTGAATTCAAAAGCT-3'	Create EcoRI site in 5'-untranslated region of SUC2	
JN9	5'-TCGTTTGTACTTGATGCA-3'	Change met(+2) to ser in SUC2	pS4
JN10	5'-TATCTGCAGCAAGTACA-3'	Create PstI site in pS4	pS5
JN11	5'-CCAAAATATCTGTTGCAAGTA-3'	Change ala(-1) to val in SUC2	pS9
JN12	5'-GATGATTTGATTAATTGG-3'	Change thr(+64) to ile in SUC2	pS10
JN13	5'-GCAGAAAGCTTCCCGCCT-3'	Create HindIII site in signal sequence of bovine prolactin	pS7-H pS8-H
JN14	5'-CAATCTGGACCCTTTTTGAG-3'	Destroy HindIII site of bovine prolactin plasmid pS7-1H	pS7-1

inactivated at 65°C for 10-15 min. In some cases, the reaction was carried out in the presence of 3.7 MBq of γ-[32P]-ATP at 37°C for 10 min prior to the addition of 1 mM of unlabeled ATP.

The target DNA was cloned into a M13 or pEMBL vector. Single stranded DNA was isolated as described previously. To 1 pmol of DNA template was added 3-5 pmol of the universal forward sequencing primer (5'-GTAAAACGACGGCCAGT-3'), 10-20 pmol of phosphorylated mutagenic primer, 1 μl of 10xLK buffer and distilled water to 10 μl. This was incubated at 65°C for 5 min and cooled at room temperature for 5 min. The primers were extended and ligated in 0.25 mM of the four dNTPs, 0.5 mM ATP, 1-2 u of DNA polymerase I (Klenow fragment), 1 u of T_4 DNA ligase, and 1xLK salts in 20 μl volume at 20-25°C for 4-6 h. Competent *E. coli* cells were then transformed with an aliquot of the reaction mix (1-5 μl) and plated on selective plates.

Single-Primer Method on Uracil Containing Template

This was modified from the procedure described by Kunkel (1985). Single-stranded DNA from either M13 phage or pEMBL recombinants was isolated as described previously, with the following modification: the vector was propagated in *E.coli* RZ1032 grown in 2xYT medium supplemented with 0.25 μg/ml uridine.

The single-primer mutagenesis procedure was essentially as described for the two-primer method. The phosphorylated mutagenic primer (5-10 pmol) was annealed to the single-stranded template (0.5-1 pmol) in 10 μl of LK buffer at 55°C for 5 min. After cooling to room temperature for 5 min, the primer was extended and ligated in a 20 μl volume with DNA polymerase I (Klenow fragment) (1-2 u), T_4 DNA ligase (1 u), 0.25 mM of the four dNTPs, 0.5 mM ATP, and 1xLK buffer. After 4-6 h of incubation at room temperature, an aliquot (1-5 μl) was used to transform competent JM101 as well as RZ1032 cells. As a control, the template was incubated with the enzymes and nucleoside

triphosphates in the absence of the mutagenic primer. The transformants were screened by phage blot, colony hybridization or direct DNA sequence determination.

Screening of Mutants Using Oligonucleotide Probes

Dot Blots with M13 Phage Recombinants

Phage particles were isolated from the 1.5 ml cultures by PEG precipitation. The pellets were resuspended in 20 μl of TE(10:1) and 1 μl of each was spotted on a nitrocellulose filter. This was baked in a vacuum oven at 80°C for 1-2 h. The filter was incubated in 6xSSC, 10x Denhardt's and 0.2% SDS in a volume of 2-3 ml/cm^2 filter at 65°C. After 1-2 h, the solution was replaced with fresh solution without SDS and the filter was then hybridized with the ^{32}P-labeled mutagenic oligonucleotide. The oligonucleotide (20 pmol) was phosphorylated with T_4 polynucleotide kinase (1 u) in the presence of 6.4-11.1 MBq of γ-[^{32}P]-ATP at 37°C for 40 min. The solution was then boiled for 3 min to inactivate the enzyme and to denature the oligonucleotide. One-half of the reaction mix was added to the filter and incubated at 20-25°C for 1 h. The filter was then subjected to three 5 min washes in 6xSSC at 30°C and autoradiographed for 1-4 h. The washes were repeated a number of times, with a 5-7°C increase in temperature. The filter was autoradiographed after each wash. Phage DNAs which continually gave a strong signal after the signal from the wild-type phage DNA has been removed were tentatively considered to carry the desired mutation. The sequence of the DNA was then determined to confirm the mutation.

pEMBL Recombinant-containing *E. coli* Colonies

Transformed *E. coli* colonies were transferred with a toothpick onto a nitrocellulose filter overlaid on a YT plate containing ampicillin. The plate was incubated at 37°C for 6-12 h or until the colonies were at least 1 mm in diameter. The cells were lysed by placing the filter face up, for 10 min, on a Whatman 3MM filter paper moistened with 1 M NaOH.

The NaOH was then neutralized by laying the filter on a Whatman 3MM filter paper moistened with 1 M Tris-HCl pH 7.5 for 1 min, and then onto one with 1 M Tris-HCl pH 7.5, 1.5 M NaCl for 5 min. The filter was air dried and baked in a vacuum oven at 80°C for 1-2 h to immobilize the DNA onto the filter. Pre-hybridization and hybridization with the oligonucleotide probe of the filter were as described for M13 phage screening.

DNA Sequence Determination

DNA sequences were determined by the chain terminator method (Sanger et al. 1977, 1980). Approximately 0.5 μg of single-stranded DNA was annealed to 3-5 pmol of primer in 10 μl volume containing 10 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$ at 55°C for 5 min, and subsequently cooled to room temperature. For double stranded DNA, the plasmid was digested in 10 μl volume with a restriction enzyme that cuts the DNA at a site or sites away from the area of interest. The sequencing primer (3-5 pmol) was then added directly into the digest. The mixture was heated to 100°C for 5 min and quickly chilled on ice. α-[^{32}P]-dATP (1 μl of 3700 MBq/ml at >1.11 x 10^6 MBq/mol) and unlabeled dATP (1 μl of 12.5 μM) were added to the primer/template solution, and 2 μl aliquots were then transferred to 4 tubes containing 2 μl of C,T,A or G terminator mix (see below). The tubes were transferred to a 37°C water bath and the extension reaction was started by addition of 2 μl of DNA polymerase I (Klenow fragment), diluted to 0.25 u/μl in 10 mM Tris-HCl pH 7.5, 1 mM DTT, 10% glycerol and 50 μg/ml BSA. The reaction was allowed to proceed for 15 min, after which 2 μl of a solution containing 0.5 mM each of the 4 dNTPs was added. The reaction was terminated after 15 min with 4 μl of formamide dye mix (90% formamide, 20 mM EDTA, 0.03% bromophenol blue and 0.03% xylene cyanol). The mixture was heated to 100°C for 3 min, chilled on ice, and 2-4 μl was loaded onto a 20 cm x 40 cm x 0.3 mm polyacrylamide gel (6% or 8%) containing 7 M urea. In most cases, a wedge gel was used to reduce the rate of migration of the bands near the bottom of the gel. The wedge gel was constructed by inserting a 3 cm long piece of the spacer material

between the two glass plates at the bottom along with the regular spacer. The electrophoresis was carried out at 1200-1500 V (maximum of 20 mA/gel) for 1.5 to 2 h, after which the gel was separated from the glass plates, dried and autoradiographed.

The terminator mixes were prepared as follow (all volumes are in μl):

	C	T	A	G
0.5 mM dCTP	1	20	20	20
0.5 mM dTTP	20	1	20	20
0.5 mM dGTP	20	20	20	1
TE(10:1)	5	5	5	5
0.5 mM ddCTP	46	-	-	-
1 mM ddTTP	-	46	-	-
0.1 mM ddATP	-	-	65	-
0.6 mM ddGTP	-	-	-	46

Construction of Plasmids and M13 Recombinants

M13mp18(H/S) Cloning Vector

This derivative of the phage vector M13mp18 was used to clone the cassettes oligonucleotides. The SphI and HindIII sites were destroyed while maintaining the β-galactosidase reading frame for selection on IPTG/XGAL plates. The M13mp18 replicative form DNA (0.5 μg) was digested to completion with HindIII and SphI. The digest was extracted with an equal volume of phenol/chloroform (1:1) and the DNA was recovered by ethanol precipitation. The single-stranded ends were made blunt by incubating with 0.5 u of T_4 DNA polymerase in 10 μl volume containing T_4 pol buffer and 0.25 mM each of the 4 dNTPs at 37°C for 5-10 min. After phenol/chloroform (1:1) extraction, the DNA was precipitated with ethanol. Ligation of the ends was carried out in a 10 μl reaction volume containing 0.25 mM ATP and 1 u T_4 DNA ligase in LK buffer at

room temperature for 4 h. An aliquot was then used to transfect competent JM101 cells. The cells were plated on IPTG/XGAL plates and incubated at 37°C overnight. Single stranded phage DNA from several blue plaques were isolated and their DNA sequence determined to confirm the mutation.

Plasmids YCp75 and pJ1

The construction of YCp75 is outlined in Figure 3. The two EcoRI sites in YRp7 (Stinchcomb et al. 1979) were destroyed by digesting the plasmid with EcoRI. The two EcoRI fragments were isolated from low melting point agarose gel and the larger fragment was dephosphorylated with bacterial alkaline phosphatase. The single-stranded ends were repaired with T_4 DNA polymerase (1 u/µg of DNA), and the blunt-ended fragments were re-ligated with 0.5 u of T_4 DNA ligase, as described previously. An aliquot of the ligation mixture was then used to transform competent *E. coli* RRI to ampicillin resistance.

The PstI site within the *ARS1* region was destroyed by partial digestion of the plasmid with PstI, conversion of the 3' protruding ends to blunt ends with T_4 DNA polymerase, and re-ligation of the fragments (Figure 3). The recombinant plasmids were then used to transform *E. coli* RRI to ampicillin resistance. The remaining PstI site within the β-lactamase gene was removed by replacing the ScaI to PvuII fragment with that from pUC13 (Vieira and Messing, 1982). Two other DNA fragments were then cloned into this plasmid. The yeast *CEN3* fragment from the plasmid pYe(CEN3)30 (Clarke and Carbon, 1980) was cloned into the BamHI to ClaI site. This recombinant plasmid was designated YCp75. The yeast *ADH1* promoter and terminator with an EcoRI insertion site, contained in a 1.8 kb BamHI fragment in plasmid pAAR6 (Ammerer, 1983), was isolated from LMP-agarose gel and cloned into the unique BamHI site of YCp75 to give plasmid pJ1.
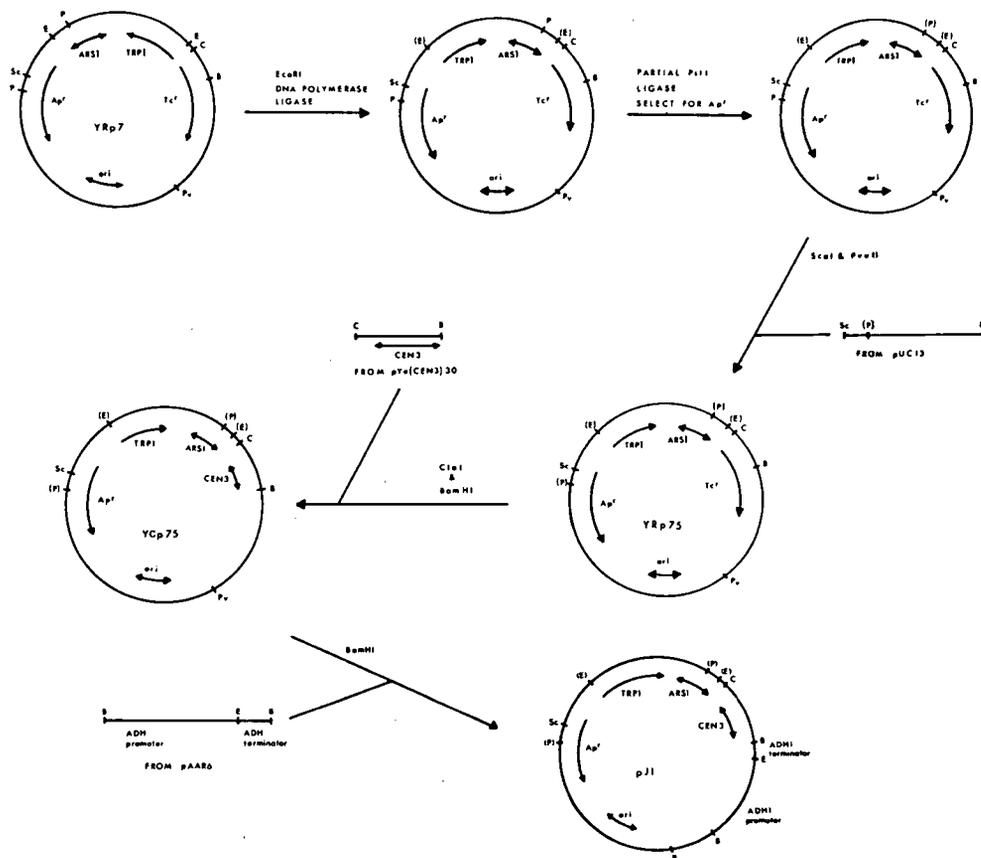
Figure 3. Construction of plasmids YRp75, YCp75 and pJ1.

The EcoRI and PstI sites in YRp7 were destroyed, as described in the text, to give plasmid YRp75. Plasmid YCp75 was derived from plasmid YRp75 by inserting the CEN3 fragment from pYe(CEN3)30 into the BamHI and ClaI site. The ADH1 promoter and terminator fragment from pAAR6 was inserted into the BamHI site of YCp75 to give plasmid pJ1. Restriction sites are: B, BamHI; C, ClaI; E, EcoRI; P, PstI; Pv, PvuII; Sc, ScaI. A letter in parentheses indicates that the endonuclease recognition site has been destroyed.

pS1 to pS6 Series of Plasmids

The 4.3 kb EcoRI fragment from plasmid pRB58 (Carlson and Botstein, 1982) containing the *SUC2* coding sequence was subcloned into pEMBL8+ (Dente et al. 1983). Oligonucleotide-directed mutagenesis was used to introduce an EcoRI site in the 5' untranslated region of *SUC2* (Figure 4). The HindIII site, 3' to the coding sequence, was converted into an EcoRI site by partial digestion of the *SUC2*-containing pEMBL8+ plasmid with HindIII, converting the 3' single-stranded ends to blunt ends with DNA polymerase I (Klenow fragment), and re-ligation of the fragments in the presence of 20 molar excess of phosphorylated EcoRI linkers. The resulting 2.2 kb EcoRI fragment was then isolated from LMP-agarose gel and cloned into the EcoRI site of pJ1 to give the plasmid pS1. To facilitate subsequent manipulations, the EcoRI site at the junction of the *SUC2* and *ADH1* terminator was destroyed by partial digestion of the plasmid with EcoRI, conversion of the single-stranded ends with DNA polymerase I (Klenow fragment) and re-ligation of the ends with T_4 DNA ligase (plasmid pS2).

A series of oligonucleotide-directed mutageneses were carried out on the *SUC2* gene, as shown in Figure 5. A PstI site was created adjacent to the signal peptide cleavage junction in plasmid pS3. The methionine residue at position +2 of the mature invertase was mutated to a serine in plasmid pS4. Plasmid pS5 combines the above two changes. The oligonucleotides used to generate these mutations are shown in Table I. The DNA sequence coding for the invertase signal peptide was deleted in plasmid pS6. This deletion was constructed from plasmid pS3 by digestion of the plasmid with EcoRI and PstI, repair of the ends with T_4 DNA polymerase, and re-ligation of the DNA in the presence of 20 molar excess of phosphorylated EcoRI linker. Plasmids containing EcoRI linkers were selected and excess linkers were removed with the restriction endonuclease.
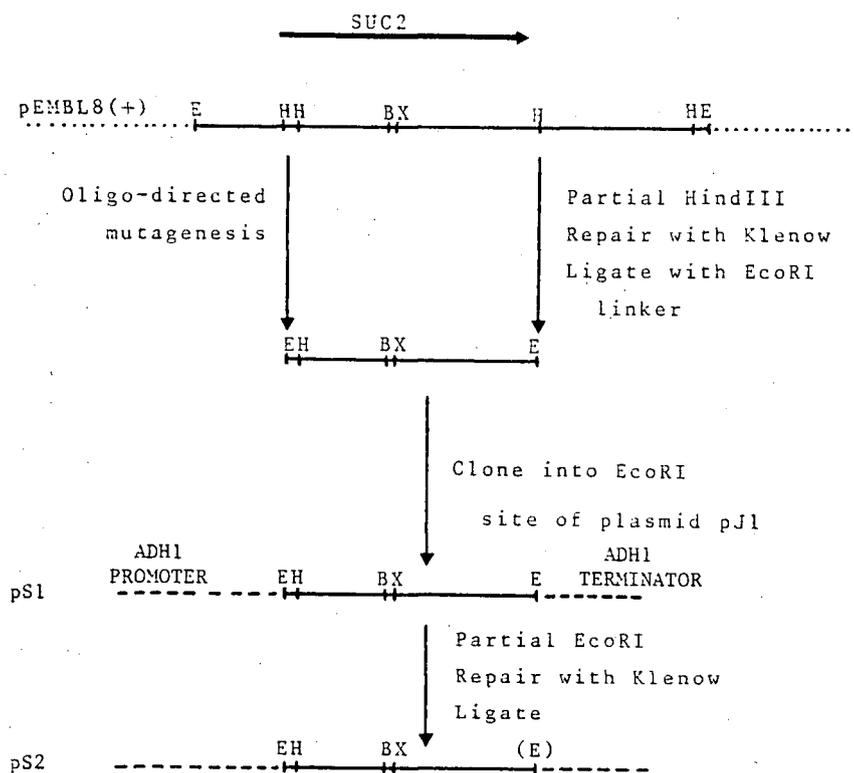
Figure 4. Construction of plasmids pS1 and pS2.

The EcoRI fragment containing the *SUC2* gene was cloned into pEMBL8(+). Oligonucleotide-directed mutagenesis was used to generate an EcoRI site between the two closely spaced HindIII sites at the 5' end of *SUC2*. The HindIII site at the 3' end was converted to an EcoRI site. The *SUC2* coding sequence was then placed under the control of the *ADH1* promoter and terminator by subcloning the resulting EcoRI fragment into plasmid pJ1 to produce pS1. Finally the EcoRI site at the 3' end of *SUC2* was destroyed to produce pS2. The restriction sites are: B, BamHI; E, EcoRI; H, HindIII; X, XbaI.
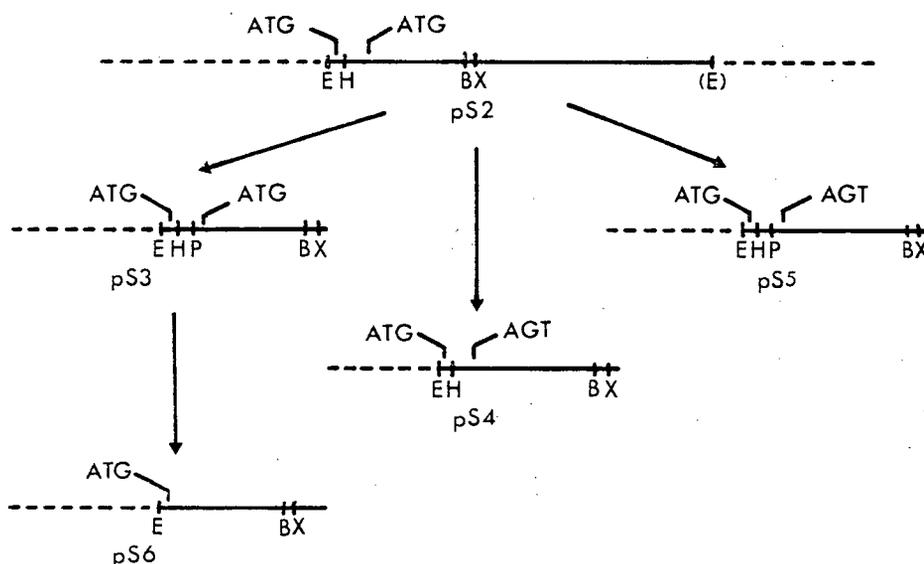
Figure 5. Construction of plasmids pS3 to pS6.

Using oligonucleotide-directed mutagenesis, a PstI site was generated near the signal peptide cleavage junction of *SUC2* (plasmid pS3); the internal ATG codon was mutated to an AGT codon (plasmid pS4); plasmid pS5 combined the above two changes. The signal peptide was deleted in pS6 by restricting plasmid pS3 with EcoRI and PstI, repairing the recessed ends with T_4 DNA polymerase, and re-ligating the plasmid in the presence of excess EcoRI linkers. The restriction sites are: B, BamHI; E, EcoRI; H, HindIII; P, Pst; X, XbaI.

Plasmids pS7 and pS7-H

Plasmid pBPRL72, containing the bovine prolactin cDNA cloned by poly-(GC) tailing into the PstI site of pBR322 (Sasavage et al. 1982), was generously provided by Dr. Fritz Rottman. The PstI fragment (0.5 µg) shown in Figure 6(A) was isolated from low melting point agarose gel and partially restricted with AluI (1 u) for 10 min. A 0.8-0.9 kb fragment, excluding the poly-(GC) tail as well as part of the 5' and 3' untranslated sequences, was isolated from low melting point agarose gel (Figure 6A). The fragment was then ligated into the filled in EcoRI site of pEMBL8+. This resulted in the regeneration of EcoRI sites on both ends of the cDNA insert (Figure 6B). The sequence of the EcoRI fragment containing the prolactin cDNA was confirmed by the Sanger et al. (1977) method. Oligonucleotide-directed mutagenesis was then used to generate a unique PstI site next to the signal peptide cleavage junction. The resulting EcoRI to PstI fragment was subcloned into plasmid pS5 to give plasmid pS7.

A HindIII site was generated within the signal sequence of bovine prolactin by oligonucleotide-directed mutagenesis. The mutagenesis was carried out on the EcoRI to KpnI fragment of plasmid pS7 cloned into M13mp19. After DNA sequence confirmation, the mutated fragment was returned to the EcoRI to KpnI site of plasmid pS7 and the plasmid was re-named pS7-H (Figure 7). The newly generated HindIII site also gave rise to a glycine to alanine missense mutation.

Plasmids pS7-1 and pS7-1H

Plasmid pS7-1H, which contains the sequence of the oligonucleotides shown in Figure 10, was isolated by screening the bovine prolactin signal sequence mutations created by cassette mutagenesis (see below). The EcoRI to KpnI fragment was subcloned into M13mp19. The HindIII site was then destroyed by oligonucleotide-directed mutagenesis to give plasmid pS7-1 (Figure 7).

A.

BOVINE PROLACTIN cDNA

```
      *  ATG                                          TAA  *
  P  A  |                          A    A          A  |    AA   P
  ▨━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━▨
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━━ 100 BP ━━

B.

```
5'── GAATTC ──3'              5'────AGCT────────AGCT────3'
3'── CTTAAG ──5'              3'────TCGA────────TCGA────5'

        │ EcoRI                         │ AluI
        ▼                               ▼

5'──G        AATTC──3'         5'  CT──────────AG  3'
3'──CTTAA        G──5'         3'  GA──────────TC  5'

        │ END-FILLING
        │ REACTION
        ▼

5'──GAATT        AATTC──3'
3'──CTTAA        TTAAG──5'

        └──────────── LIGATION ────────────┘
                         │
                         ▼

5'──── GAATTCT──────────────AGAATTC ──3'
3'──── CTTAAGA────────────── TCTTAAG──5'
         EcoRI                  EcoRI
```
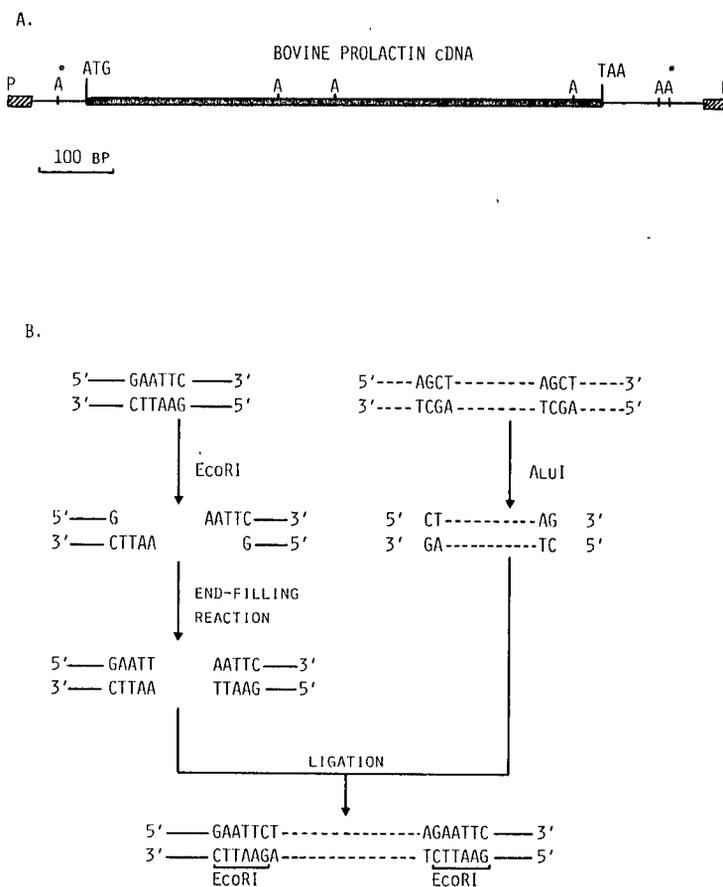
Figure 6. (A) Schematic diagram of the bovine prolactin cDNA and (B) regeneration of the EcoRI restriction sites.

(A) The hatched area represents the poly-GC tail, the thin line represents 5' and 3' untranslated sequences, and the heavy line represents the coding sequence. Sequence between the AluI sites indicated with an asterisk was isolated from low melting point agarose and subcloned into the filled in EcoRI site of pEMBL8+. The restriction sites are A, AluI and P, PstI.

(B) Ligation of the AluI fragment from prolactin cDNA onto the blunt-ended EcoRI site of the cloning vector regenerates the EcoRI recognition sites.
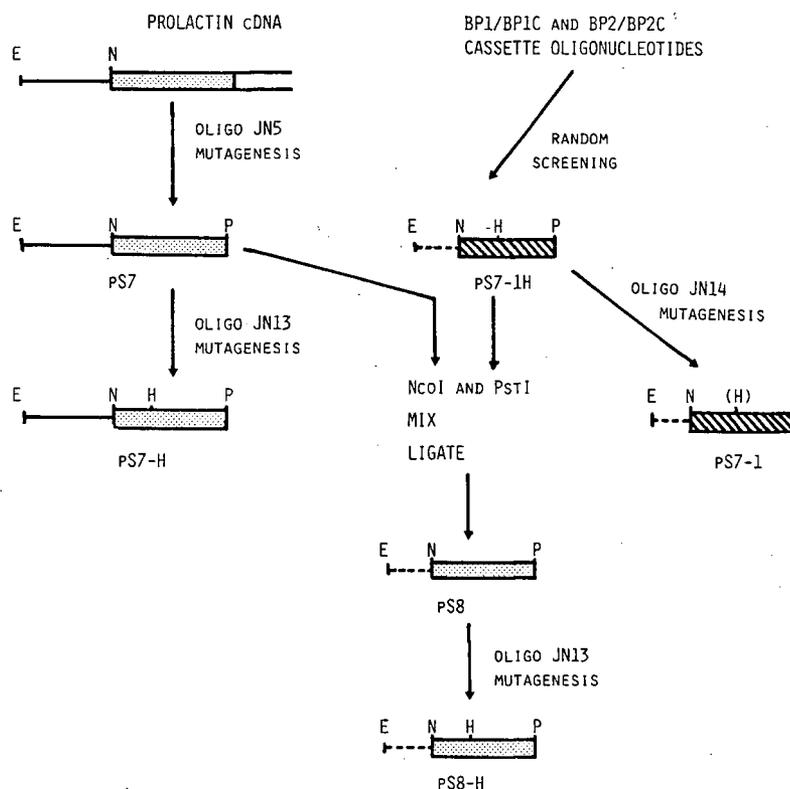
Figure 7. Construction of pS7 and pS8 series of plasmids.

A PstI site was introduced by oligonucleotide-directed mutagenesis into the signal peptide of bovine prolactin to give plasmid pS7. Plasmid pS7-H was derived from pS7 by the generation of a HindIII site in the signal peptide. Plasmid pS7-1H was isolated from DNA sequence determination of a random sample of the BP1/BP1C and BP2/BP2C cassette oligonucleotides mixture. Plasmid pS7-1 was derived from pS7-1H after destroying the HindIII site. Plasmid pS8 contained the nucleotide sequence of the wild-type prolactin signal peptide (pS7) and the truncated 5' untranslated sequence of pS7-1H. Introduction of a HindIII site in plasmid pS8 yielded plasmid pS8-H. The sequences of the mutagenic oligonucleotides are shown in Table I. The restriction sites are: E, EcoRI; H, HindIII; N, NcoI and P, PstI.

Plasmids pS8 and pS8-H

The EcoRI to PstI fragments of pS7 and pS7-1H were subcloned into pEMBL8+. The resulting plasmids were then digested with NcoI and PstI, pooled and re-ligated (Figure 7). The recombinant plasmids were screened for the NcoI to PstI fragment from pS7 ligated onto the 3' end of pS7-1H. This plasmid was named pS8. Plasmid pS8-H was derived from pS8 after destroying the HindIII site of plasmid pS8-H by oligonucleotide-directed mutagenesis (Figure 7).

Cassette Mutagenesis

Complementary sets of oligonucleotides homologous to the signal sequence of invertase were synthesized with the bases at several pre-determined positions substituted with a mixture of the wild-type and two or three of the other bases. The position of each target base and the composition of the substituted bases were chosen such that mutant clones would not contain in-frame nonsense codons. The ratio of the wild-type to mutant bases used was calculated to give an optimum of 2 mutant bases per complementary sets of oligonucleotides (McNeil and Smith, 1985; Porter and Smith, 1986; Hutchison et al. 1986). Figure 8 shows the sequence of the cassette oligonucleotides. The purified SU1 and SU1-C oligonucleotides (10 pmol of each) were phosphorylated using T_4 polynucleotide kinase in two separate 5 ul reactions. The two oligonucleotides were pooled and boiled for 5 min. After annealing at $55^{O}C$ for 10 min, they were inserted between the EcoRI and KpnI sites of M13mp18(H/S), as shown in Figure 9. Competent JM101 cells were transfected and plated on IPTG/XGAL plates. Approximately 200 white plaques were pooled and the infected cells were incubated in 25 ml of 2xYT at $37^{O}C$ for 4-6 h. Replicative form (RF) DNA from this pool was isolated by the alkaline lysis method. Oligonucleotides SU2 and SU2-C (10 pmol each) were phosphorylated and annealed as above. They were then inserted between the HindIII and PstI sites of the pooled RF DNA. The re-ligated DNA was restricted with KpnI prior to transfection to linearize those clones not possessing the

```
              EcoRI              ** *   *HindIII KpnI
SU1      5'-AATTCATCACCATGCTTTTGCAAGCTTGGTAC-3'
SU1C        3'-GTAGAGGTACGAAAACGTTCGAAC-5'
                               ** *   *
```

* Mixture of 75% wild type and 25% mutant bases

```
              HindIII            **       *       **  ****     *PstI
SU2      5'-AGCTTTCCTTTTCCTTCTTGCTGGTTTTGCAGCCAAAATATCTGCA-3'
SU2C        3'-AAGGAAAAGGAAGAACGACCAAAACGTCGGTTTTATAG-5'
                               **       *       **  ****    *
```

* Mixture of 80% wild type and 20% mutant bases

Figure 8. Sequence of the *SUC2* cassette oligonucleotides.

During their synthesis, mixtures of two or three of the other nucleotides were added to the wild-type nucleotide at the ratio indicated. The position of the target nucleotide in the sequence is indicated with an asterisk.

M13mp18(ΔSphI/HindIII)
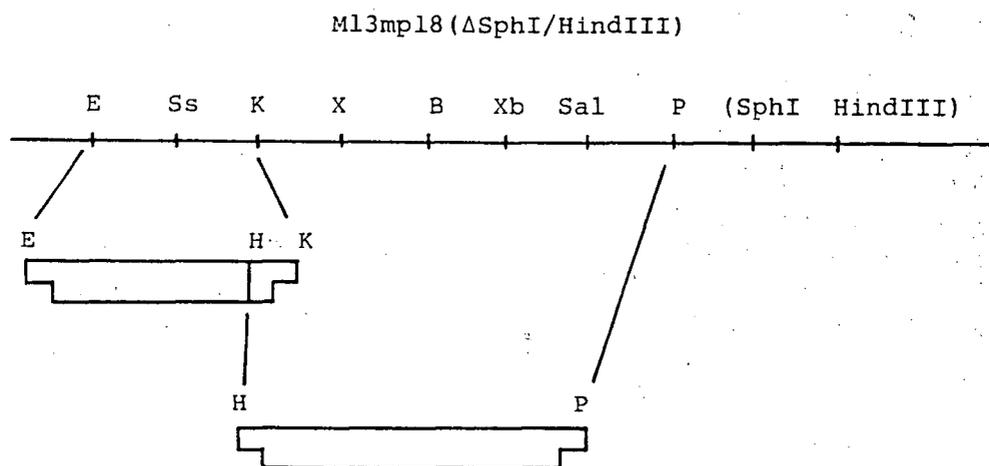
1. CLONE SU1 AND SU1-C INTO EcoRI/KpnI SITES
2. ISOLATE RF'S POOL (APPROX. 200 CLONES)
3. CLONE SU2 AND SU2-C INTO HindIII/PstI SITES
4. RESTRICT WITH KpnI PRIOR TO TRANSFECTION
5. TRANSFER EcoRI TO PstI FRAGMENT TO EXPRESSION VECTOR

Figure 9. Cloning of the *SUC2* cassette oligonucleotides into M13mp18(H/S).

```
              EcoRI           ***   ***           ****HindIII KpnI
BP1    5'-AATTCATCACCATGGACTCCAAAGGTTCTTCTCAAAAAGCTTGGTAC-3'
BP1C      3'-GTAGTGGTACCTGAGGTTTCCAAGAAGAGTTTTTCGAAC-5'
                            ***   ***           ****
```

* Mixture of 80% wild type and 20% mutant bases


```
              HindIII ***          *  *  *  *        **    *      ** * PstI
BP2    5'-AGCTTCCAGATTGTTGTTGTTGTTGGTCGTCTCCAACTTGTTGTTGTGCTCTGCA-3'
BP2C      3'-AGGTCTAACAACAACAACAACCAGCAGAGGTTGAACAACAACACGAG-5'
                       ***          *  *  *  *       **    *      ** *
```

* Mixture of 85% wild type and 15% mutant bases

Figure 10. Sequence of the prolactin cassette oligonucleotides.

The position of the target nucleotide in the sequence is indicated with an asterisk.

second set of oligonucleotides. Again, RF DNA from a pool of approximately 200 plaques was isolated by the alkaline lysis method. The EcoRI to PstI fragment from the pooled RF DNA was then transferred to the *SUC2* containing yeast vector pS5. This was used to transform competent *E. coli* RRI cells to ampicillin resistance. Cells from transformant colonies (400-600) were washed off the plates with TE(10:1) and collected by centrifugation at 5000 x g for 5 min. Plasmid DNA from the pool of clones was isolated by the alkaline lysis method and used to transform the yeast strain JNY4 to tryptophan prototrophy. Individual transformed clones were then isolated for further analysis.

The procedures described above also were used to generate mutations in the signal peptide of bovine prolactin. Oligonucleotides BP1 and its complement BP1-C (Figure 10) were treated as described above and inserted between the EcoRI to KpnI sites of M13mp18(H/S). Replicative form DNA from approximately 200 clear plaques was isolated and oligonucleotides BP2 and BP2-C were then inserted between the HindIII to PstI sites. As in the *SUC2* signal sequence oligonucleotides, the EcoRI to PstI fragment from an RF pool was transferred to plasmid pS5 and the recombinant plasmid was used to transform JNY4 yeast strain to tryptophan prototrophy, after which individual clones of transformants were isolated.

Colony Screening of Invertase Secretory Mutants in Yeast

The JNY4 transformants were transferred with sterile toothpicks onto duplicate sets of nitrocellulose filters overlaid on SD Trp⁻ plates and incubated at 30°C for 16-24 h. To assay for total invertase, one of the filters was placed in a chloroform saturated chamber for 10 min at 20°C. The other filter, for the assay of secreted invertase, was kept in air at 20°C during this period. The filters were then gently placed side by side on top of a Whatman 3MM filter paper moistened with 100 mM NaOAc (pH 5), 250 mM sucrose and 10 mM NaN$_3$ at room temperature. After 10 min, the nitrocellulose filters were

removed, dried briefly and stained with 0.2% 2,3,5-triphenyl tetrazolium chloride (TTC) in 0.5 M NaOH for 2-5 min.

Quantitative Assay of Invertase in Yeast Transformants

Invertase activity was determined on intact yeast cells as described by Goldstein and Lampen (1975). The assay consisted of two stages: the hydrolysis of sucrose to fructose and glucose, and the determination of the amount of glucose generated by the glucose oxidase/horseradish peroxidase method. Two or more independent isolates of each mutant were assayed; the assay was carried out in duplicate. Yeast cells were grown to exponential phase (A_{600} of 0.5 to 1.0) in 5 ml of SD Trp⁻ medium at 30°C. After measuring the cell density by absorbance at 600 nm, 0.2 ml aliquots of each were dispensed into 4 Eppendorf tubes. The cells were harvested by centrifugation for 1 min, washed with 0.2 ml of ice-cold 10 mM NaN_3, and resuspended in 50 µl of 0.2 M sodium acetate (pH 4.9), 10 mM NaN_3. Two of the four tubes were assayed for external invertase and the remaining two for total (external plus internal) invertase. For the latter assay, 10 µl of chloroform was added to the cell suspension to render the cell membrane permeable to sucrose. The tubes were pre-incubated at 37°C for 1 min. The first stage of the assay was initiated by adding 50 µl of freshly prepared 0.5 M sucrose. After 5 min, the reaction was stopped with 100 µl of 0.5 M sodium phosphate buffer (pH 7) and the tube was immediately transferred to a boiling water bath. After 3 min, the tube was removed and cooled to room temperature. In most cases, the solution was deproteinized with 150 µl of 0.3 M $Ba(OH)_2$ followed by 150 µl of 0.3 M $ZnSO_4$. The resultant precipitate was then removed by centrifugation for 2 min. An aliquot of the supernatant (5-200 µl) was transferred to a 12x75 mm test tube and distilled water was added, if necessary, to adjust the volume to 200 µl. After pre-incubating at 37°C for 1 min, 1 ml of a solution containing 50 mM potassium phosphate (pH 7.5), 12.5 u/ml glucose oxidase, 1.25 u/ml horseradish peroxidase and 0.25 mg/ml o-dianisidine was added. The reaction

was allowed to proceed in the dark at 37°C for 20-25 min, after which it was stopped with 1 ml of 6 M HCl. The absorbance at 540 nm was measured. Invertase activity was expressed in units per A_{600} unit of cells, with one invertase unit defined as that required to generate one umole of glucose per min at 37°C.

Detection of Invertase Activity After Gel Electrophoresis

Crude extract was prepared from 10 ml of exponentially growing cells (see Preparation of Yeast Extract). The invertase activity was assayed as described above. An aliquot containing 1 u of invertase activity was added to an equal volume of 2x SDS loading buffer and immediately loaded onto a 1.5 mm thick SDS polyacrylamide gel (3% stacking and 6% separating). Current was applied to the gel at 15 mA and 20 mA while the protein was passing through the stacking and separating gel, respectively. After completion of the electrophoretic separation, the gel was washed at room temperature with six changes of 0.1 M NaOAc (pH 5), for 5-10 min each. It was then incubated in 100 ml of 0.1 M NaOAc (pH 5), 0.25 M sucrose at 30°C for 20 min. The gel was rinsed 2-3 times with distilled water and a solution containing 0.25% TTC in 0.5 M NaOH was added. Upon the appearance of a strong red color (usually after 10-15 min), the gel was removed from the stain solution, rinsed with distilled water and fixed for 20-30 min in 10% HOAc. The gel was then rinsed with distilled water and dried under vacuum.

Recovery of Plasmids From Yeast

Yeast cells harboring the *TRP1*-based plasmid were grown in 5 ml of SD Trp⁻ at 30°C to late-log stage ($1-5 \times 10^{8}$ cells/ml). Cells from each culture were harvested in a clinical centrifuge, resuspended in 0.5 ml of 1M sorbitol, 0.1 M EDTA pH 7.5, 10 mM DTT, and transferred to an Eppendorf tube. Zymolase 60,000 was added to a final concentration of 40 µg/ml and the cell suspension was incubated at 37°C for 1 h. The cells were then pelleted in a microfuge (30 sec) and resuspended in 0.45 ml of TE(50:20). SDS

was added to a final concentration of 1% and the cell suspension was incubated at 65°C for 30 min. Ice-cold KOAc (0.2 ml of a 5 M solution) was added to each tube and chilled on ice for 30-60 min. After centrifugation in a microfuge for 5 min, the supernatant from each tube was carefully decanted into a fresh tube. An equal volume of isopropanol was added and the tube was kept at room temperature for 3-5 min. The DNA pellet was recovered by a very brief centrifugation (10 sec) and washed with 70% ethanol. The pellet was then dried under vacuum and redissolved in 15 μl of TE(10:1). An aliquot (5-10 μl) was used to transform competent *E. coli* RRI cells to ampicillin resistance. Plasmid DNA was isolated from the transformants by the alkaline lysis "mini-prep" method. The EcoRI to KpnI fragment was then subcloned into M13mp19 and the recombinant DNAs transfected into *E. coli* JM101. Single stranded DNA was isolated from the transfected cells and the sequence determined by the Sanger method.

Northern (mRNA) Analysis

Isolation of Yeast RNA

This procedure was developed by Dr. Andrew Spence (Ph.D. thesis; the University of British Columbia). Cells transformed with TRP1-containing plasmids were grown to mid-log phase in 10 ml of SD Trp⁻ at 30°C. Cycloheximide was added from a 10 mg/ml stock in ethanol to a final concentration of 0.1 mg/ml. After 5 min, the cultures were quickly chilled on ice. Cells were harvested by centrifugation at 5000 x g for 1 min, rinsed with 1 ml of ice-cold 0.1 mg/ml cycloheximide in distilled water into an Eppendorf tube, and pelleted in a microfuge for 30 sec. The cells were resuspended in 0.2 ml of ice-cold extraction buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl) and 10 μl of vanadyl ribonucleoside complex (VRC). Glass beads (0.45 mm diameter) were added to the level of the meniscus. The tube was vortexed at top speed in six 15 sec periods, each followed by 45 sec on ice. Another 0.2 ml aliquot of extraction buffer was added to each tube. After a brief centrifugation (10 sec), the supernatant was transferred to a clean tube. Extraction

buffer (0.2 ml) and VRC (10 μl) were added to the pellet, and the vortexing and centrifugation were repeated. The second supernatant was pooled with the first and SDS was added to a final concentration of 0.5%. The extract was then incubated with 0.5 mg/ml of proteinase K at 37°C for 1 h, after which an equal volume of 4 M LiCl was added. The tube was chilled on ice for 6-16 h. The precipitate was collected by centrifugation in a microfuge for 15 min. The pellet was redissolved in 0.5 ml of 0.1% diethyl pyrocarbonate (DEP) treated distilled water and the LiCl precipitation was repeated. The pellet was washed twice with 2 M LiCl in 10 mM EDTA pH 7.5, and redissolved in 0.4 ml of DEP-treated distilled water. The RNA concentration was estimated by absorbance at 260 nm, with one A_{260} corresponding to 40 μg/ml. Sodium acetate was added to 0.3 M and the RNA precipitated with 2.5 volumes of ethanol at -20°C for 4-12 h. The pellet was recovered by centrifugation, washed with 70% ethanol and dissolve in DEP-treated distilled water at 5 mg/ml based on ultra-violet absorbance.

Formaldehyde Gel Electrophoresis of RNA and Northern (mRNA) Analysis

RNA was electrophoresed on a formaldehyde agarose gel as described by Lehrach et al. (1977) and Maniatis et al. (1982). RNA (up to 20 μg) was denatured in a volume of 20 μl containing 2.2 M formaldehyde, 50% formamide and 1/2 x MOPS buffer (1x MOPS buffer contained 40 mM NaMOPS, 10 mM NaOAc, 1 mM EDTA pH 7) at 55°C for 15 min. A 2 μl aliquot of sample loading buffer (50% glycerol, 1 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol) was added, and the sample was loaded on a 1-1.2% agarose gel containing 1x MOPS buffer and 2.2 M formaldehyde. Electrophoresis in 1x MOPS buffer at 0.5-1 V/cm was carried out for 6-12 h at room temperature. After the electrophoresis, the gel was rinsed a few times with distilled water and soaked in 20xSSC for 1 h. The RNA was transferred to a nylon based filter, Gene Screen Plus (New England Nuclear), in 20xSSC as described by Thomas (1980). After 12-16 h, the filter was baked at 80°C for 1-2 h to immobilize the RNA. The filter was pre-hybridized for 4-6 h at 42°C

in a solution containing 50% formamide, 5xSSC, 1x Denhardt's solution, 50 mM sodium phosphate pH 7.0, 0.1% SDS and 250 µg/ml of sheared, denatured calf thymus DNA. ^{32}P-Labeled RNA probe (0.5-1 x 10^6 cpm) was then added and the mixture incubated at 55°C for 12-16 h. The filter was washed six times with 0.1xSSC, 0.1% SDS at 65°C at 15-20 min/wash and autoradiographed.

Preparation of RNA Probe

Plasmid pS5 was digested to completion with HindIII and XbaI. A 0.43 kb fragment containing the yeast *TRP1* coding sequence and a 1.3 kb fragment containing part of the *SUC2* coding sequence were each isolated after electrophoresis in a low melting point agarose gel, and cloned into plasmid pSP64. The RNA probe was synthesized using SP6 RNA polymerase as described by Melton et al. (1984). The two plasmids (0.5 µg each) were digested with EcoRI and transcribed in 40 mM Tris-HCl pH 7.5, 6 mM MgCl$_2$, 2 mM spermidine, 100 µg/ml BSA, 0.5 mM each of ATP, CTP, and GTP, 0.1 mM UTP, 200 u/ml RNasin, 3.7 MBq α-[^{32}P]-UTP, 10 mM DTT, and 500 u/ml SP6 RNA polymerase at 40°C for 60 min. Following RNA synthesis, the DNA template was removed by the addition of 20 u of RNasin and 1 u of RNase-free DNase. After a 10 min incubation at 37°C, the reaction mixture was extracted with phenol/chloroform (1:1) and the RNA was recovered by ethanol precipitation.

The *CYC1* transcript was analyzed using the 600 bp EcoRI to HindIII DNA fragment of pYeCYC1. The probe was prepared by nick translation of the fragment in the presence of 11.1 MBq of α-[^{32}P]-dATP.

Preparation of Yeast Extract for Invertase Assay and SDS Polyacrylamide Gel Electrophoresis

Yeast cells grown to mid-log at 30°C in 10 ml of SD Trp$^-$ medium were harvested in a clinical centrifuge. The cells were resuspended in 1 ml of ice-cold 10 mM NaN$_3$ and

transferred to an Eppendorf tube. After a brief centrifugation (30 sec), the cells were resuspended in 0.15 ml of lysis buffer (50 mM Tris-HCl pH 8, 50 mM DTT, 2 mM PMSF, 2 mM benzamidine, 5 mM 1,10-phenanthroline, 20 μM pepstatin A, 2 μg/ml leupeptin), and glass beads (0.45 mm diameter) were added to the level of the meniscus. The tube was vortexed at top speed in six 15 sec periods, each followed by 45 sec on ice. After a 30 sec centrifugation, the supernatant was transferred to a clean Eppendorf tube. The pellet was extracted a second time with 0.15 ml of lysis buffer. The supernatants were pooled and centrifuged in a microfuge (12,000 x g) for 10 min at 4°C to remove the cell debris. The supernatant was then dispensed in 0.1 ml aliquots and stored at -70°C.

Large Scale Purification of Yeast Invertase

This was modified from the procedures described by Williams et al. (1985). Cells grown to late-log (1x10^8 cells/ml) in 2 l of SD Trp$^-$ medium were harvested by centrifugation at 5000 x g for 5 min. After resuspending the cells in an equal wet weight to volume ratio of ice-cold lysis buffer (described in the previous section), the cell suspension was passed through a French press at 1200 psi. This procedure was repeated three times. Figure 11 shows a flow chart of the purification procedure. The homogenate was centrifuged at 10,000 x g for 10 min and the pellet was washed with one volume of lysis buffer. The combined supernatants were pooled and centrifuged at 30,000 x g for 30 min. Streptomycin sulfate was then added to the supernatant to a final concentration of 10 mg/ml. After 1 h at 4°C, the precipitate formed was removed by centrifugation at 10,000 x g for 30 min. The specific conductance of the supernatant was then adjusted to less than 2 mMHO/cm with ice-cold 1 mM Tris-HCl pH 7.5 containing the protease inhibitors, and loaded onto a DEAE-Sepharose column (2.5 x 20 cm) equilibrated in TEE (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA). The column was washed with 3 volumes of TEE and developed with an increasing linear salt gradient generated with 500 ml of TEE and 500 ml of 0.6 M NaCl in TEE. The glycosylated invertase was eluted from the column at a salt

Purification of Invertase:

Harvest yeast at late
exponential phase

↓

French press at 1200 psi

↓

Differential centrifugation
10,000 × g for 10 min
30,000 × g for 20 min

↓

Add streptomycin to supernatant
to final concentration of 1%

↓

Centrifuge 10,000 × g for 20 min

↓

DEAE-Sepharose CL-6B chromatography
elute with 0-0.6 M NaCl gradient

External invertase ←   → Internal invertase

↓   ↓

70% ammonium sulfate   20% ammonium sulfate

↓   ↓

dialyse   Pheny-Sepharose CL-4B
                                                elute with 20%-0% ammonium sulfate

↓   ↓

Concentrate with   Concentrate with
Amicon YM-30   Amicon YM-30

↓   ↓

Sephacryl S-300 chromatography   Sephacryl S-300 chromatography

Figure 11. Flow chart for the large scale purification of yeast invertase.

concentration of 50 mM NaCl and the unglycosylated invertase at 150 mM NaCl. Both were detected by invertase assay of the column fractions.

Ammonium sulfate was added to the pooled glycosylated invertase fractions to 70% saturation. After 4-8 h at 4^{0}C, the precipitate was removed by centrifugation at 10,000 x g for 30 min. The supernatant was then dialysed overnight at 4^{0}C in TEE buffer containing 0.5 mM PMSF. The protein solution was concentrated with an Amicon YM-30 filter to 2-3 ml and loaded onto a Sephacryl S-300 column (2.5 x 90 cm) equilibrated in TS buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl). The invertase-containing fractions were pooled, concentrated with an Amicon YM-30 filter, and washed three times with TEE to remove the salt. The protein solution was lyophilized and stored at -20^{0}C.

Ammonium sulfate was added to the pooled unglycosylated fractions to 20% saturation and the precipitate was removed by centrifugation at 10,000 x g for 30 min. The supernatant was then loaded onto a phenyl-Sepharose column (0.9 x 10 cm) equilibrated in 20% saturated $(NH_4)_2SO_4$ in TEE. The column was developed with a gradient formed with 50 ml of 20% saturated $(NH_4)_2SO_4$ in TEE and 50 ml of 10% ethylene glycol in TEE. The pooled invertase fractions were concentrated with an Amicon YM-30 filter and purified through a Sephacryl S-300 column (2.5 x 90 cm) equilibrated in TS. The invertase-containing fractions were concentrated with an Amicon YM-30 filter, washed three times with TEE to remove the salt, and lyophilized.

SDS Polyacrylamide Gel Electrophoresis (PAGE) of Proteins

This was modified from the procedures described by Laemmli (1970) and O'Farrell (1975). The stock solutions were as follow:

(a) 50% acrylamide/1.3% bis-acrylamide solution was stirred with Bio-Rad AG1x8 mixed bed resin for 10 min and filtered through a 0.45 um Millipore filter. The solution was stored at 4^{0}C in an amber bottle.

(b) 4x separating gel buffer contained 1.5 M Tris-HCl pH 8.8 and 0.4% SDS.

(c) 4x stacking gel buffer contained 0.5 M Tris-HCl pH 6.8 and 0.4% SDS.

(d) 1.6% ammonium persulfate (APS).

(e) The running buffer contained 0.05 M Tris base, 0.384 M glycine and 0.1% SDS at pH 8.1.

(f) 2x DTT sample buffer was made by adding 120 μl of 1 M DTT to 480 μl of dye solution (83 mM Tris base, 30% glycerol and 7.5% SDS). For TCA precipitated protein samples, a solution made up of 400 μl of 0.2 M Tris base, 0.02 M EDTA added to 600 μl of 2xDTT sample buffer was used.

(g) 5x stain stock contained 2.5 g of Coomassie blue R-250 dissolved in 400 ml methanol. The solution was filtered through a Whatman 1 filter. The working stain solution was made by adding 0.4 l of the 5x stain stock to 0.6 l methanol, 0.2 l HOAc and 0.8 l distilled water.

(h) Destaining solution contained 35% methanol and 10% HOAc.

The gels were made by mixing the gel buffer with the desired amount of acrylamide stock solution; ammonium persulfate (32 mg/ml) and TEMED (1 μl/ml gel solution) were added to catalyze polymerization. For gradient gels, 5% glycerol was added to the more concentrated gel solution. Sucrose (0.15 M) was added to the stacking gel. To allow proper stacking of the proteins, the gel was run at a lower current (15 mA) through the stacking gel. The current was then increased to 20 mA after the bromophenol blue dye had reached the separating gel. For protein "mini-gels", the current was reduced to 12 mA and 16 mA through the stacking and separating gel, respectively.

Immunological Techniques

Production of Invertase Antiserum

Female New Zealand white rabbits were immunized with biweekly doses of glycosylated invertase in complete Freund's adjuvant. The glycoprotein (200 μg) was

dissolved in 0.5 ml of sterile 150 mM NaCl and emulsified with an equal volume of complete Freund's adjuvant. The emulsion was conveniently prepared by repeatedly forcing the solution through a nylon three-way stopcock connected to two 3 ml syringes. The 1 ml oil emulsion was injected into the rabbit at 4 subcutaneous sites. Two weeks after the first booster dose, a 5 ml blood sample was collected from the ear vein. The blood sample was allowed to clot in a glass test tube at $4^{\circ}C$ for 2-4 h. Serum was collected after a 5 min centrifugation at 3000 x g and stored in convenient aliquots at $-20^{\circ}C$.

Affinity Purification of Invertase Antibodies

Antibodies to invertase were purified by affinity chromatography using cytoplasmic invertase immobilized on Sepharose 4B support, followed by immunoadsorption with a crude extract of the SUC^- yeast strain JNY4 to remove antibodies which interact with other yeast proteins.

Cyanogen bromide-activated Sepharose 4B (1 g dry weight) was suspended in 200 ml 1 mM HCl for 5 min and collected by filtration through a Buchner funnel. The gel beads were then suspended in 5 ml of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3). Cytoplasmic invertase (1 mg), purified from the JNY4 yeast strain carrying the pS6 plasmid, was dissolved in 1 ml coupling buffer and added to the gel suspension. This was incubated with gentle agitation at $4^{\circ}C$ overnight. The gel beads were collected after centrifugation at 1500 x g for 2-3 min, and resuspended in 5 ml of 0.2 M glycine (pH 8.0). After a 3 h incubation at room temperature, the suspension was centrifuged and the supernatant was discarded. The gel beads were then washed with four alternating cycles of 5 ml coupling buffer and 5 ml acetate buffer (0.1 M NaOAc pH 4.0, 0.5 M NaCl), starting and ending with coupling buffer. For column chromatography, the gel beads were resuspended in PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 0.14 M NaCl adjusted to pH 7.4) containing 10 mM NaN_3, and packed into a 0.7 x 20 cm column. The rabbit serum (5 ml) was diluted 5 fold with PBS, 10 mM NaN_3 and passed through the

column at 1-5 ml/h. After the column was washed with 80-90 ml of PBS supplemented with 0.2 M NaCl, the bound invertase-specific antibodies were eluted with 0.1 M HOAc (pH 2.9) at 4°C. The collected fractions were immediately neutralized to pH 7-7.5 with 1 M Tris base. Fractions containing the antibodies, as monitored by the absorbance at 280 nm, were pooled and diluted 3 fold with PBS containing 1 mg/ml BSA. Residual cross-reacting species were then removed by immunoadsorption.

A crude protein extract was prepared from a 100 ml culture of JNY4. The cells were homogenized with glass beads as described previously and the supernatant after centrifugation at 30,000 x g for 10 min was precipitated with 1% streptomycin sulfate. Following the removal of the precipitate by centrifugation, the supernatant was passed through a Sephadex G-100 column (1.4 x 20 cm) equilibrated in bicarbonate coupling buffer. The fractions at the void volume with an A_{280} of more than 2 were pooled and added to 1 g of acid-washed CNBr-activated Sepharose 4B. After the blocking and washing steps, the gel beads were packed into a 0.7 x 20 cm column and equilibrated with PBS. The invertase antibody solution described above was then passed through the column at 1-5 ml/h and washed with 40 ml of PBS. The protein-containing fractions, as determined by absorbance at 280 nm, were pooled and concentrated with an Amicon YM-30 filter. The antibodies were stored at -20°C in a solution containing PBS, 5 mg/ml BSA and 50% glycerol.

Electrophoresis and Western (Protein) Analysis

Crude extracts from 10 ml of yeast cultures were assayed for protein content (Bio-Rad protein assay kit) and for invertase activity. An equal volume of 2x SDS sample loading buffer was added to an aliquot of the extract containing 0.08-0.1 unit of invertase activity. The sample was boiled for 5 min and immediately loaded onto a 7.5% to 12.5% gradient SDS polyacrylamide gel for electrophoretic separation. The proteins were electroblotted onto a nitrocellulose filter in 20 mM Tris base, 150 mM glycine pH 8.3, 20%

methanol (Towbin et al. 1979) at 4^{0}C for 12-16 h at 150 mA or for 2-4 h at 0.5 A. The filter was then incubated at room temperature with Tris-saline (10 mM Tris-HCl pH 7.4, 150 mM NaCl) with 5% skim milk powder (Carnation brand) for 0.5-2 h. Affinity-purified invertase antiserum at 1:1000 dilution in Tris-saline, 10 mM NaN$_3$, 2.5% skim milk was added and incubated at room temperature for 60-90 min. The antibody solution was re-used 15-20 times without appreciable loss in sensitivity. The filter was washed with three changes of Tris-saline, 0.25% n-lauryl sarcosine, at 3-5 min per wash. The bound antibodies were detected by one of the following three secondary antibodies using the assays described below. Affinity purified goat anti-rabbit IgG antisera coupled to horseradish peroxidase or to alkaline phosphatase (Bio-Rad) were used at a 1:3000 dilution in Tris-saline, 2.5% skimmed milk. The alkaline phosphatase-coupled antiserum had a 10 fold greater sensitivity relative to the horseradish peroxidase-coupled antiserum. The third secondary antibody is [^{125}I]-labeled goat anti-rabbit IgG antiserum. After a 60-90 min incubation period at room temperature with the secondary antibody, the filter was washed with Tris-saline, 0.25% n-lauryl sarcosine as before.

Horseradish Peroxidase-Coupled Antiserum

After thorough washing, the filter was transferred to a clean petri dish. A freshly prepared solution of 24 mg of 4-chloro-1-naphthol in 2 ml of methanol was added to 40 ml of Tris-saline and quickly poured into the dish containing the filter. Hydrogen peroxide (12 μl of a 30% solution) was added to the mixture. Upon the appearance of purple-colored bands (usually 10-30 min), the dye solution was poured off and the filter was washed thoroughly with distilled water.

Alkaline Phosphatase-Coupled Antiserum

The washed filter was placed in a clean dish containing 50 ml of carbonate buffer (0.1 M NaHCO$_3$, 1 mM MgCl$_2$, pH 9.8). The filter was developed with 0.3 ml of 50 mg/ml

nitro blue tetrazolium (NBT) in 70% DMF and 0.15 ml of 25 mg/ml 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in DMF. Upon the appearance of suitably intense red-colored bands, the dye solution was discarded and the filter was washed thoroughly with distilled water.

^{125}I-labeled Antibody

The iodination procedure was modified from that described by Markham (1982). Three iodobeads (Pierce) were added to 370 MBq of ^{125}I in 0.25 ml of 0.1 M sodium phosphate buffer (pH 7.0). To the radioactive solution was added 1 mg of goat anti-rabbit IgG antibody (Cappel) in 0.25 ml of 0.1 M phosphate buffer. After 5-10 min at room temperature, the reaction was terminated by removing the solution from the iodobeads. The unreacted ^{125}I was separated from the ^{125}I-labeled protein by ion-exchange chromatography. Bio-Rad AG1X8 anion exchanger (0.5 g) was packed into a Centrex disposable column (Schleicher and Schuell). The column was washed with three 1 ml aliquots of 0.1 M phosphate buffer. The iodination solution was added to the column and centrifuged at 1000 x g for 1 min. The iodinated antibody was stored at 4°C and was active for at least three months.

The ^{125}I-labeled antibody was diluted in Tris-saline, 2.5% skim milk and added to the nitrocellulose filter at 5 x 10^4 cpm per lane. After 1-2 h at room temperature, the filter was washed as before and autoradiographed at -70°C with an intensifying screen.

Isolation of Yeast Microsomal Invertase and Protection from Proteolysis

The procedure for isolation of yeast microsomal membranes described by Hansen et al. (1986) was modified to accommodate 100 ml cultures. Cells harvested at exponential phase were treated with zymolase 60,000 (60 μg/ml) in 2 ml of 50 mM potassium phosphate buffer pH 7.5, 1.2 M sorbitol and 10 mM DTT to produce spheroplasts. The cell suspension was then gently loaded on 6 ml of sorbitol cushion (50 mM potassium

phosphate buffer pH 7.5, 1.6 M sorbitol) and centrifuged at 3000 x g for 5 min. The cells were regenerated in 2 ml of YPD supplemented with 0.4 M $MgSO_4$. After 90 min at room temperature, the cells were collected by centrifugation through a sorbitol cushion. The spheroplasts were then resuspended in 0.2 ml of lysis buffer (20 mM HEPES pH 7.5, 0.5 M sucrose, 3 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and glass beads (0.45 mm diameter) were added to the level of the meniscus. The tube was vortexed at low speed in two 30 sec periods, each followed by 60 sec on ice. The glass beads and cell debris were removed by centrifugation at 3000 x g for 5 min. The supernatant was removed and centrifuged at 10,000 x g for 10 min. The S-10 supernatant was carefully layered on top of 4 ml 35% Percoll in lysis buffer without the protease inhibitors. The gradients were centrifuged in a SW50.1 rotor at 27,000 rpm for 60 min. Fractions were collected from the bottom of the tube and were assayed for invertase and NADPH-cytochrome c reductase (Kubota et al. 1977). The ER enriched fractions were pooled and an aliquot (2-10 μl) was diluted two fold with ice-cold 20 mM HEPES (pH 7.5), 250 mM sucrose, 10 mM $CaCl_2$, 2 mM DTT. TPCK-trypsin and proteinase K, both pre-incubated at 37°C for 15 min, were added to a final concentration of 0.75 mg/ml and 0.2 mg/ml, respectively. The digestion was carried out at 0°C for 60 min, after which it was stopped with 2 mM PMSF. An equal volume of boiling 2x SDS sample loading buffer was immediately added to the digest and the tube was kept at 100°C for 5 min. The sample was centrifuged for 1 min to pellet the silica particles and the supernatant was then fractionated on a SDS polyacrylamide gel. The proteins were transferred to a nitrocellulose filter and subjected to Western analysis.

Enzymatic Deglycosylation of Yeast Invertase

The polysaccharides were removed using the enzyme N-glycanase[TM] (Genzyme Corp.). SDS and 2-mercaptoethanol were added to the Percoll gradient fractionated sample to a final concentration of 0.5% and 1%, respectively. The sample was heated at 100°C for

5 min to denature the protein. After centrifugation to remove the silica particles, the sample was added to a solution containing 0.2 M sodium phosphate buffer pH 8.6, 10 mM 1,10-phenanthroline, and 1.25% nonidet NP-40. N-glycanase was added to a final concentration of 1 u/ml and the mixture was incubated at 37°C for 12-18 h. The protein was then precipitated with 10% ice-cold TCA for 5-10 min. The precipitate was collected by centrifugation, redissolved in SDS sample loading buffer, fractionated by electrophoresis on a SDS polyacrylamide gel, and subjected to Western analysis.

In Vivo ^{35}S-labeling of Invertase

Cells were grown in 5 ml SD Trp⁻ medium to mid-log phase. An aliquot was removed, centrifuged and resuspended at 10 A_{600} units/ml in 50 mM potassium phosphate buffer pH 7.5, 1.2 M sorbitol, 10 mM DTT. Zymolase 60,000 was added to a final concentration of 60 μg/ml. After 30-45 min, the spheroplasts were centrifuged through 3 volumes of sorbitol cushion (50 mM potassium phosphate buffer pH 7.5, 1.6 M sorbitol) at 1500 x g for 5 min. The cells were resuspended in SD Met⁻, Trp⁻ medium maintained isotonically with 1.2 M sorbitol at 5 A_{600}/ml. After 45-60 min at 30°C, the cells were labeled for 4 min with [^{35}S]-methionine (>3.7 x 10^5 MBq/mol) at 7.4 MBq/A_{600} unit of cells. The chase was initiated by the addition of 0.2 mM unlabeled methionine and 0.1 mg/ml cycloheximide. Aliquots of cells were removed at various times and chilled on ice with 10 mM NaN$_3$, 1.2 M sorbitol until all samples had been collected. The cells were centrifuged through 3 volumes of sorbitol cushion as described above. The supernatant was removed and adjusted to 1 ml volume in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mg/ml BSA, 1% Aprotinin and 10 mM NaN$_3$. The cell pellet was extracted in 150 μl of lysis buffer and glass beads as described earlier. The volume of the crude extract was then adjusted to 1 ml with 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mg/ml BSA, 1% Aprotinin (Sigma). Affinity-purified invertase antibody (10 μl), enough to precipitate all the invertase in the sample, was added and incubated at 0°C overnight. Protein A-

Sepharose (30 μl of a 1:8 dilution) was added. After 60 min at room temperature, the beads were washed twice with 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.5 M urea, 0.5% n-lauryl sarcosine; once with 10 mM Tris-HCl (pH 7.5), 200 mM NaCl; and once with 0.5% 2-mercaptoethanol. SDS sample loading buffer was added to the beads and boiled for 5 min. After a brief centrifugation to pellet the beads, the supernatant was fractionated by electrophoresis on a 7.5% to 12.5% gradient SDS polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie blue solution heated to 60-80°C for 10-15 min and destained for 20-30 min with pre-heated destaining solution. The gel was then incubated with Enlightning (New England Nuclear) for 30-60 min, dried under vacuum, and autoradiographed with Kodak X-Omat XAR-5 film at -70°C. The autoradiograms were quantified with a Bio-Rad 620 densitometer.

In Vitro Translation Systems

In Vitro Transcription Using SP6 RNA Polymerase

The BamHI to HindIII invertase-coding DNA fragment of plasmid pRB58 was cloned into the pSP65 vector. The amino-terminal region of all invertase mutants was subcloned between the EcoRI and BamHI sites of the plasmid. The resulting vectors (Figure 12) were linearized either at the AccI or BamHI site prior to transcription with SP6 RNA polymerase (Melton et al. 1984). To obtain transcripts coding for the truncated version of invertase, the vectors were linearized with BamHI, the 5' extensions were filled in with DNA polymerase I (Klenow fragment) and the plasmid was recircularized with T_4 DNA ligase. The plasmids were linearized with AccI prior to SP6 RNA polymerase transcription.

Preparation of Wheat Germ Translation Extract

This was prepared as described by Erickson and Blobel (1983). The wheat germ was floated for 2 min on a mixture of 600 ml CCl_4 and 240 ml cyclohexane. The floated
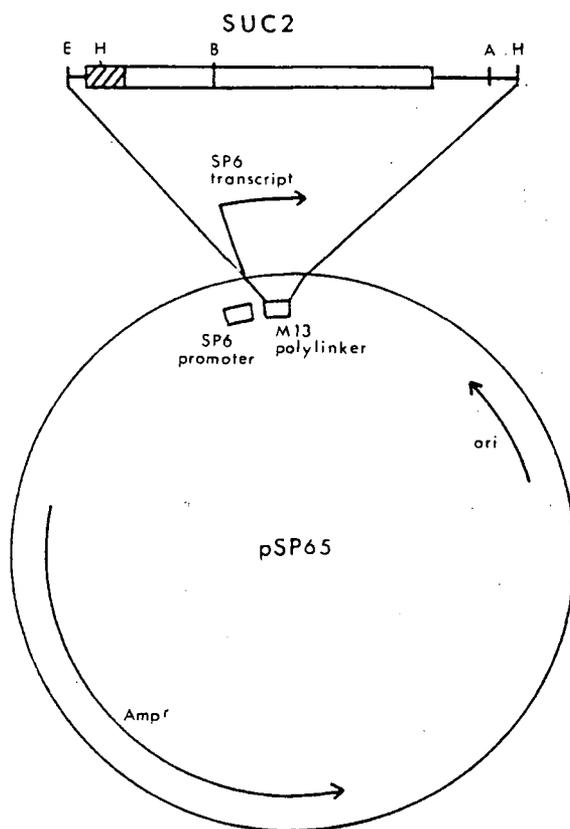
Figure 12. Restriction map of the pSP65 plasmid containing the *SUC2* sequence.

The boxed area represents the coding sequence of the mature invertase and the dashed area represents the coding sequence of the signal peptide. For transcript production with SP6 RNA polymerase, the plasmid was linearized with either BamHI or AccI. The restriction sites are: A, AccI; B, BamHI; E, EcoRI; H, HindIII.

wheat germ was decanted into a Buchner funnel and dried under vacuum at $4^{\circ}C$. The wheat germ (15 g) was ground in a mortar under liquid nitrogen and 30 ml of ice-cold, sterile homogenization buffer (40 mM HEPES pH 7.5, 100 mM KOAc, 1 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, 4 mM DTT) was added. The homogenate was centrifuged at 23,000 x g for 10 min. The lipid layer was aspirated with a drawn out Pasteur pipette and the supernatant was transferred to a sterile Corex tube. This was centrifuged a second time at 23,000 x g for 10 min. The pooled supernatant was loaded on a Sephadex G-25 column (2.5 x 80 cm) equilibrated in elution buffer (40 mM HEPES pH 7.5, 100 mM KOAc, 5 mM $Mg(OAc)_2$, 4 mM DTT). The first turbid peak to elute from the column was collected. Calcium chloride (1 mM) and staphylococcal nuclease (2 u/ml) were added and incubated at $25^{\circ}C$. After 10 min, the sample was chilled on ice and EGTA was added to a final concentration of 2 mM. The extract was then centrifuged at 23,000 x g for 10 min. The supernatant was divided into aliquots and stored at $-70^{\circ}C$.

The composition of the reaction mixture and the conditions for the *in vitro* translation were as described by Erickson and Blobel (1983). For protein translocation assay, canine pancreatic microsomal membranes and SRP, generously provided by Dr. Peter Walter, were added to the translation reaction as described by Walter and Blobel (1983b).

Preparation of Yeast Translation Extract

This was prepared according to procedures described by Hansen et al. (1986). The yeast strain JNY6 (*MATa trp1 leu2 suc2 pep4::URA3*) was grown in 4 l of YPD to an A_{600} of 2. Cells were harvested at 3000 x g for 5 min, washed with distilled water, and resuspended in 100 ml of 50 mM potassium phosphate buffer pH 7.5, 1.4 M sorbitol, 10 mM DTT. Zymolase 60,000 was added to a final concentration of 60 µg/ml, and the suspension was incubated at room temperature for 60 min. Spheroplasts were collected by centrifugation through 3 volumes of sorbitol cushion (50 mM potassium phosphate buffer

pH 7.5, 1.6 M sorbitol) at 3000 x g for 5 min, and resuspended in 400 ml of YM-5 (0.1%
yeast extract, 0.2% peptone, 0.1% glucose, 1x yeast nitrogen base, 40 mM sodium
succinate pH 5.8 and amino acid supplements) containing 0.4 M $MgSO_4$ at room
temperature for 90 min. The regenerated spheroplasts were harvested as before and
resuspended in 30 ml lysis buffer (20 mM HEPES pH 7.5, 100 nM NH_4OAc, 2 mM
$Mg(OAc)_2$, 2 mM DTT, 0.5 mM PMSF). The suspension was homogenized with 10 strokes
in a motor-driven Potter homogenizer and centrifuged in a Ti60 rotor at 19,500 rpm for 15
min. The lipid layer was aspirated with a drawn out Pasteur pipette and the supernatant
was centrifuged in the same rotor at 37,500 rpm for 30 min. The supernatant was loaded
onto a Sephadex G-25 column (12 x 90 cm) equilibrated in lysis buffer with 20% glycerol.
Fractions with an A_{260} of more than 20 were pooled and adjusted to 1 mM $CaCl_2$ and 2
u/ml staphylococcal nuclease. After 15 min at $25^{\circ}C$, the extract was chilled on ice,
adjusted to 1.8 mM EGTA, divided into 0.05-0.1 ml aliquots, and stored at $-70^{\circ}C$.

The translation reactions (10 µl) contained 40% by volume of the yeast extract
with the following additions: 1 mM ATP, 80 uM GTP, 17.5 mM creatine phosphate, 30 µM
of each of the 19 amino acids excluding methionine, 0.2 mg/ml creatine phosphate kinase,
2.4 mM DTT, 3.1 mM $Mg(OAc)_2$, 150 mM NH_4OAc, 20 mM HEPES pH 7.5, 185 MBq/ml
$[^{35}S]$-methionine, in vitro transcribed mRNA, and 100 ug/ml yeast tRNA. The reactions
were carried out at $20^{\circ}C$ for 60 min.

Preparation of Yeast Microsomes

The same yeast strain was grown in 1 l YPD to an A_{600} of 1.5-2. Cells were
converted to spheroplasts with zymolase 60,000 and regenerated in YM-5 plus $MgSO_4$
medium as before. Cells collected by centrifugation were resuspended in 4 ml
homogenization buffer (20 mM HEPES pH7.5, 0.5 M sucrose, 3 mM $Mg(OAc)_2$, 1 mM
EDTA, 1 mM EGTA, 1 mM DTT, 100 u/ml Aprotinin, 0.5 mM PMSF, 2 µg/ml pepstatin
A, 2 µg/ml leupeptin). The suspension was homogenized with a Potter homogenizer. The

extract was centrifuged at 10,000 x g for 10 min and the supernatant was transferred to a clean tube. The pellet was resuspended in 4 ml lysis buffer, homogenized, and centrifuged as before. The supernatants were pooled; 1.3 ml was loaded on top of 3.5 ml of 35% Percoll in homogenization buffer excluding the protease inhibitors and centrifuged in a SW50.1 rotor at 27,000 rpm for 60 min. The lipid layer was aspirated with a drawn out Pasteur pipette. The upper turbid band was withdrawn and adjusted to 1.2 mM $CaCl_2$. This was incubated at 20^oC for 20 min with 1 u of staphylococcal nuclease per A_{280} unit of extract. The sample was then chilled on ice and adjusted to 0.5 mM EGTA. Aliquots of 3 ml were loaded on top of a two-step gradient containing 1.2 ml of 20 mM HEPES pH 7.5, 0.5 M sucrose, 1 mM DTT, and 0.8 ml of 50% Percoll, 20 mM HEPES pH 7.5, 250 mM sucrose, 1 mM DTT. The gradients were centrifuged in a SW50.1 rotor at 39,000 rpm for 50 min. The turbid band on top of the Percoll cushion was collected and diluted with 20 mM HEPES pH 7.5, 250 mM sucrose, 1 mM DTT to a final concentration of 25 A_{280} units/ml. The membranes were divided into aliquots and stored at -70^oC.

Results and Discussion

Construction of Plasmid for Constitutive Expression of Invertase in Yeast

The plasmid (pS5) shown in Figure 13 was derived from YRp7 (Stinchcomb et al.
1979), a yeast-bacteria shuttle vector containing the β-lactamase gene for antibiotic
selection in *E. coli*, and the yeast *TRP1-ARS1* fragment for Trp^+ prototrophic selection
and plasmid replication in *S. cerevisiae*. The EcoRI and PstI sites in the plasmid were
removed by standard DNA recombinant techniques before inserting the *SUC2* coding
sequence into YRp7. This modified plasmid (YRp75) was then tested for stability in yeast.
Although maintained at a moderately high copy number (8-20 copies per cell) under
selective conditions, the plasmid was extremely unstable under non-selective conditions,
being lost at a frequency of 80-90% per generation. The yeast centromeric *CEN3*
fragment, shown by Clarke and Carbon (1980) to stabilize plasmids at a copy number of 1-
2 copies per cell, was added to the plasmid. The newly constructed *CEN3-TRP1* plasmid
(YCp75) was stably maintained and lost at a frequency of less than 2% per generation
under non-selective conditions.

Modification of the *SUC2* Gene

In this study, the invertase coding sequence was inserted between the constitutive
yeast *ADH1* promoter and terminator, because the wild-type *SUC2* promoter responsible
for the expression of the secreted form of invertase is repressed in the presence of glucose
(Carlson and Botstein, 1982). It is more convenient for screening of invertase activity to
have a constitutively expressed enzyme. Replacement of the wild-type promoter by the
ADH1 promoter also eliminates the constitutive *SUC2* promoter which produces
cytoplasmic invertase.
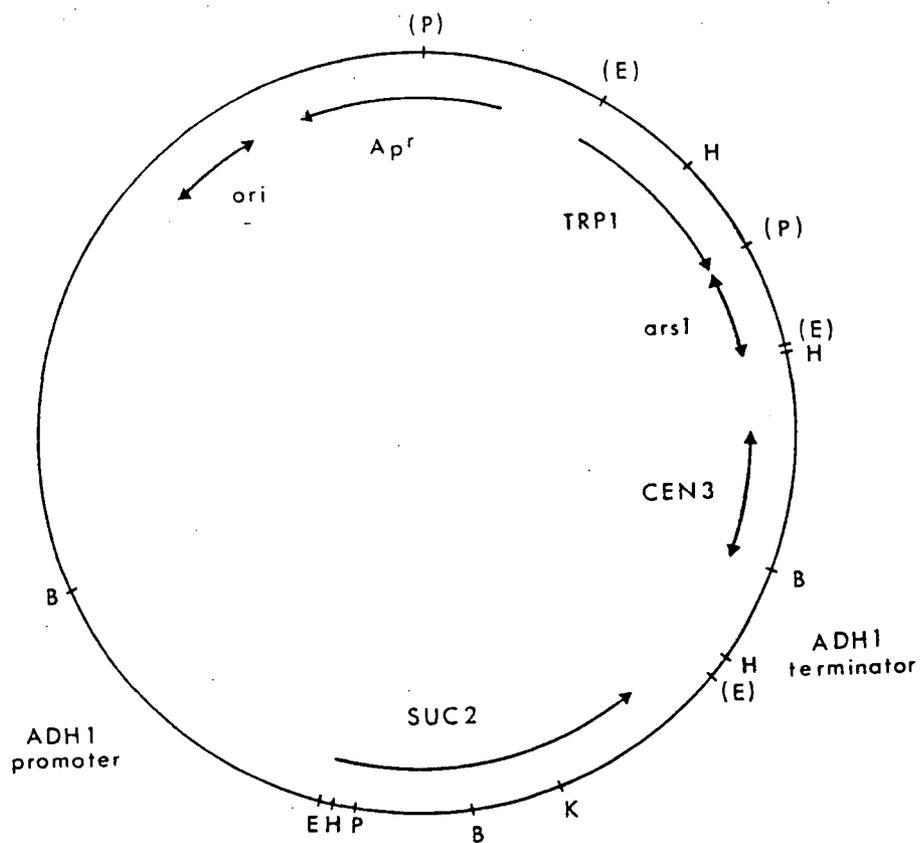
Figure 13. Restriction map of the expression plasmid pS5.

The restriction sites are: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; P, PstI. A letter in parentheses indicates a deleted restriction site.

Several other modifications in the *SUC2* gene were introduced to facilitate cassette mutagenesis of the signal sequence and to eliminate the internal methionine codon responsible for the *SUC2* encoded cytoplasmic form of the enzyme. The structure of the fully modified plasmid, pS5, is shown in Figure 13 and the details of the modifications in Figure 14. First, a PstI site was constructed adjacent to the normal signal peptide cleavage site. This resulted in a missense mutation of the codon for serine at position +1 of the mature sequence to that of an alanine (plasmid pS3). Second, the internal methionine (ATG) codon used in the translation initiation of the cytoplasmic form of invertase was mutated to a serine (AGT) codon (plasmid pS4). This change ensured that all invertase produced by the cell initially contained the signal peptide, even if mutagenesis of the signal sequence generated new transcript start sites downstream of the pre-invertase translation initiation codon. Third, the above two mutations were inserted together in plasmid pS5 which now contains the amino acid sequence -Ser-Ala-Ala-Ser- at the signal peptide cleavage junction (Figure 14). These three plasmids should produce large amounts of a protein with a fully functional signal peptide which would be rapidly processed and transported through the secretory pathway with little or no significant steady state internal accumulation. As shown in Figure 14, almost all invertase activity was secreted indicating that the above three changes had no significant effect on invertase secretion. As expected, when the signal peptide was deleted (plasmid pS6), invertase secretion was abolished and all invertase activity was localized intracellularly. Plasmid pS5 was subsequently used as the target for cassette mutagenesis of the signal peptide. Since the synthesis of the cytoplasmic form of invertase was abolished by replacement of the internal methionine and its cognate promoter, and all invertase produced initially contained the signal peptide, any internal accumulation of the protein resulting from cassette mutagenesis must be due to a failure at some stage of the secretory pathway to recognize the signal peptide and process the protein for secretion.
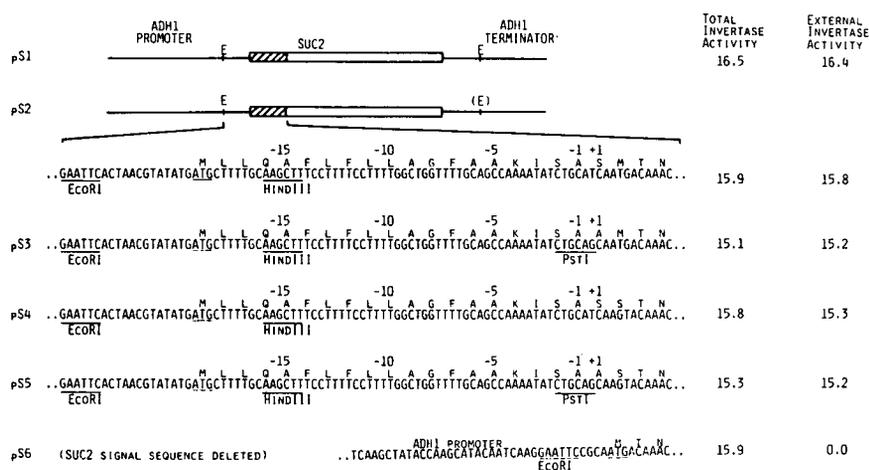
Figure 14. Invertase activities of the modified *SUC2*.

The modifications were introduced by oligonucleotide-directed mutagenesis. The modified

fragments were cloned into the expression plasmid and transformed into the *SUC⁻* yeast

strain, JNY4. A PstI site generated in plasmid pS3 resulted in a Ser to Ala missense

mutation at position +1. The internal methionine (ATG) codon was changed to a serine

(AGT) codon in plasmid pS4. Plasmid pS5 combined the above two changes, and the signal

peptide was deleted in plasmid pS6. Invertase activities are expressed in μmole of glucose

generated per min for one A_{600} unit of cells at 37°C.

Cassette Mutagenesis of *SUC2*

Construction of *SUC2* Signal Peptide Mutants

The cassette oligonucleotides shown in Figure 8 were used to generate the signal peptide mutations. Mutagenesis was directed mainly at the amino- and carboxy-termini of the signal peptide, with limited amount of mutagenesis of the hydrophobic core. The design of the oligonucleotides was based on the following criteria. First, the position of the target nucleotide in a codon was selected in order to avoid the generation of a termination codon. For example, a cytosine in any one of the three positions of a codon was usually retained, as this would eliminate the possibility of creating a nonsense mutation. Similarly, the introduction of a thymine residue in the first, and an adenine or guanine residue in the second and third positions of a codon was avoided in order to insure against nonsense mutations. Second, the above criterion was overridden if the nucleotide changes resulted in a very limited number of amino acid substitutions or conservative changes. In this case, nonsense mutations were avoided by altering the mutant base compositions of the oligonucleotides during synthesis (e.g. the incorporation of only two of the three mutant bases). Third, to facilitate subsequent cloning, the complementary cassette oligonucleotides were inserted into sites with protruding ends. At the time of design of this experiment, the efficiency of synthesis of long oligonucleotides was low. Hence, the cassettes were synthesized as two pairs which were assembled in two steps (Figure 9). The two restriction sites introduced into plasmid pS5 by oligonucleotide-directed mutagenesis, EcoRI and PstI, were used for the cassette replacement. The pool of mutated *SUC2* plasmids were then transformed into the *suc2* yeast strain, JNY4.

Fractional Composition of Mutagenic Nucleotide Mixtures

The oligonucleotides were synthesized to give an average frequency of two mutations per mutated clone. The ratio of mutant bases to wild-type base used in the

synthesis of the oligonucleotides was estimated by assuming a linear relationship between this ratio and the number of mutations generated for any given length of oligonucleotide. The probability of mutation (P_x) was calculated with the general equation for a binomial distribution:

$$P_x = k \, (q)^{r-x} \, (1-q)^x$$

where $k = r! \, / \, x! \, (r-x)!$; q is the fractional concentration of the wild-type base; $(1-q)$ is the fractional concentration of the mutant bases; r is the total number of targeted nucleotides; x is the number of mutations per oligonucleotide.

To test the accuracy of the prediction, the complementary oligonucleotides were cloned into M13mp18(H/S) as described in Materials and Methods. A random sample of single-stranded DNAs were isolated and their sequences determined. A good correlation was found between the observed and expected frequencies (Table II).

Screening for Secretion-Defective Mutants

Transformants containing mutated plasmids were screened for external and total (external plus internal) invertase activities by colony transfer to nitrocellulose filters. This screening procedure was chosen because of its ability to discriminate between secretion-defective mutants and those with a reduced level of the secreted enzyme. Four different phenotypes emerged from an initial screen of 220 yeast transformants. The overwhelming majority (197 transformants) exhibited qualitatively wild-type invertase levels. Since the mutagenesis was very efficient (Table III) and was predominantly directed at the amino- and carboxy-termini of the signal peptide, this suggests that amino acid sequences of these regions are not critical for secretion. Another possibility is that functionality of the signal peptide may depend on secondary or tertiary structural features, rather than a specific amino acid sequence. Thus, diversity at the primary amino acid sequence level may be tolerated. A representative sample from this group was analyzed further by DNA sequence determination and quantitative invertase assays. The other three phenotypes

Table II. Expected and observed mutagenic frequencies with the cassette oligonucleotides. BP1/BP1-C and BP2/BP2-C are complementary oligonucleotides containing the bovine prolactin signal sequence; SU1/SU1-C and SU2/SU2-C are complementary oligonucleotides containing the yeast invertase signal sequence.

Oligo	BP1/BP1-C		BP2/BP2-C		SU1/SU1-C		SU2/SU2-C	
# of mutation	Expected frequency	Observed frequency	Expected frequency	Observed frequency	Expected frequency	Observed frequency	Expected frequency	Observed frequency
0	0.14	0.20	0.10	0.10	0.32	0.24	0.14	0.24
1	0.30	0.30	0.26	0.30	0.42	0.24	0.30	0.18
2	0.30	0.30	0.29	0.40	0.21	0.35	0.30	0.41
3	0.17	0.20	0.20	0.15	0.09	0.18	0.17	0.06
4	0.07	0.0	0.10	0.05	0.09	0.0	0.07	0.06
Total number analyzed		20		20		17		17

included secretion of invertase at a reduced level, defective secretion, and total lack of

invertase activity (4 mutants). Restriction endonuclease and DNA sequence analyses of the

rescued plasmids from the last group of mutants indicated that three of the four contained

multiple copies of the cassette oligonucleotide and one carried a single nucleotide insertion.

Since they resulted in frameshift mutations, these mutants were discarded. The following

experiments focus mainly on the second and third mutant phenotypes.

Charaterization of *SUC2* Signal Peptide Mutants

Quantitative Assay of External and Total Invertase Activities

Quantitative assays of invertase activity were carried out on a number of the

transformants which had been identified as producing functional invertase by the

qualitative screen. This identified three classes of mutant. Most mutants belonged to a

group (Class I) in which virtually all invertase was secreted, although the enzyme was

produced in differing amounts (Table III; mutants 4-6 to 7-27). All members of this group

were able to grow on sucrose under anaerobic conditions, a test used to select for a SUC^+

phenotype (Carlson and Botstein, 1981a; 1981b). This group is representative of those

mutants which were identified as being phenotypically wild-type in the qualitative screen

and those which secreted invertase in reduced amount.

The Class II mutants have one representative (Table III; mutant 4-55B). This

mutant has a reduced level of invertase, similar to that of the Class I mutants, but a

significant fraction (30%) of the enzyme is intracellular. This mutant also grows on sucrose

under anaerobic conditions.

The Class III mutants (Table III; mutants 28* to 4-56) also produce a reduced

amount of invertase, but the major portion of the enzyme is intracellular. While the Class

II mutant (4-55B) would grow on sucrose under anaerobic conditions, the Class III

mutants would not grow under these conditions and would be classified as SUC^- by this

Table III. Invertase activities of the *SUC2* signal peptide mutants.

One unit of invertase activity is defined as one µmole of glucose generated per minute for one A_{600} unit of cells at 37°C.

	-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	Total Invertase Activity	External Invertase Activity
pS5	M	L	L	Q	A	F	L	F	L	L	A	G	F	A	A	K	I	S	A	15.3	15.2
4-6	H																			15.9	14.8
31	H		L													N				13.2	12.9
4-56B										D										12.4	12.9
27																H				12.1	12.1
30	H	V																		11.1	10.7
34	N																			10.7	10.1
32	G		E													R				10.5	10.5
25	V		K												E					10.2	8.9
M-3	V															N	P			9.8	9.8
4-3	V														V	T				9.4	9.4
3-15	F	M	K													N	P			9.3	8.3
M-2	H								F											9.2	9.5
4-56A	N	V																		9.0	8.7
35	F		E							H										9.0	8.3
26	R		E							F						G				8.7	8.4
3-75	H															T	P			8.3	8.3
3-6																	P			7.4	7.4
3-12			K													D				7.2	6.8
3-13	P									T										6.6	6.1
7-27	N									F										5.0	5.0
4-55B										R					E	Q				8.6	5.8
28*	N		E							P					A	K	I	S	A	5.4	0.3
28	N		E							P										4.9	0.3
M-1	G									D										4.5	0.7
4-55	G									R					E	Q				4.0	0.2
M-5	V									R					E	Q				2.7	0.1
4-56	N	V								D										2.6	0.2

test. One of the Class III mutants (28*) has a five amino acid duplication near the signal peptide cleavage site, but behaved in the same way as a mutant (28) lacking this duplication.

Some general features regarding the functional role of the signal peptide are evident. First, a variety of single and multiple amino acid substitutions do not block secretion although they can reduce the total production of invertase by up to three fold. Possible reasons for the reduction in enzyme production could be a reduction in the steady state level of mRNA, a reduction in translation efficiency or a decrease in enzyme stability. Second, the secretion of invertase as exemplified by the Class III mutants can be blocked by some additional single amino acid replacements. Thus, the Class III, non-secreted mutant M-1 has Gly(-18) whereas the Class II, secreted mutant 4-55B has Leu(-18). Similarly, the Class III mutant 4-56 has Asp(-11) whereas the secreted mutant 4-56A has Leu(-11). But, these same substitutions in other situations (Table III) do not result in non-secretion.

Northern Analysis of Steady State Invertase mRNA

Northern analysis was carried out on electrophoretically fractionated RNA isolated from exponentially growing cells, to determine whether the difference in the level of enzyme activity of signal mutants was due to changes in the amount or stability of the *SUC2* transcript. Total RNA was fractionated on a formaldehyde-agarose gel and transferred to nitrocellulose filter. The *CYC1*, *SUC2* and *TRP1* transcripts were identified using gene-specific ^{32}P-labeled probes. The estimated lengths of the transcripts were 0.7 kb for *CYC1*, 1.9 kb for *SUC2* and 2.4 kb for *TRP1*. The intensities of the bands were determined by densitometric scanning. The amount of *SUC2* mRNA was estimated by comparing the intensity of its hybridization signal to that from the chromosomal *CYC1* and plasmid-derived *TRP1* transcripts. Table IV shows the ratio of the *SUC2* to *CYC1* or *TRP1* transcript. Fluctuations in the steady state level of *SUC2* mRNA were detected in

Table IV. Steady state level of *SUC2* transcript.

Total cellular RNA was subjected to Northern analysis. The intensities of the bands were determined by densitometric scanning and the amount of transcripts was estimated from the intensity of the hybridization signals. The values represent the ratio of the *SUC2* transcript to that of the chromosomal *CYC1* transcript or to the plasmid-derived *TRP1* transcript.

PLASMID	SUC2:CYC1	SUC2:TRP1
PS5	1.83	1.39
PS6	1.30	1.05
4-6	1.32	ND*
4-56B	1.08	ND
4-56A	2.20	ND
3-13	1.00	0.75
7-27	ND	2.00
4-55B	1.40	1.90
4-55	1.91	0.89
4-56	2.65	0.90

* ND = NOT DETERMINED

some of the mutants (e.g. mutants 4-56A, 3-13 and 4-56). However, these changes showed no correlation to the level of enzyme activity (compare the mRNA levels for 4-56, 4-56A and 4-56B in Table IV with their invertase activities in Table III). Thus, it can be concluded that the steady state level of the transcript was not the limiting factor for the signal peptide mutations.

Invertase Activities in a *pep4* Mutant Strain of *S. cerevisiae*

Since secretory and vacuolar proteins share the same early processing events (Stevens et al. 1982), mutation in the signal peptide may conceivably alter the protein sorting process, resulting in the misdirection of secretory proteins to the vacuole. Such a misdirected protein could have a very short life span because of degradation by the vacuolar proteolytic enzymes. Hence, the possibility of vacuolar proteolysis as a cause of a reduction in the level of detectable invertase was examined. A representative set of the signal peptide mutants was introduced into JNY10, a *pep4* mutant yeast strain. The *PEP4* gene has been shown to be essential for the activation of a number of vacuolar proteolytic enzymes (Jones, 1977; Zubenko et al. 1983) and mutation in the *PEP4* locus significantly reduces the level of enzymatically active vacuolar proteases (Hemmings et al. 1981). If invertase were transported to the vacuole, then an appreciable increase in invertase activity would be anticipated in a mutant *pep4* background. Table V compares the invertase activities present in a wild-type *PEP4* strain (JNY4) to those present in a *pep4* mutant strain (JNY10). Only a slight increase in invertase levels was observed when the plasmids were propagated in the *pep4* background. This implies that intracellular proteolysis by vacuolar enzymes is not responsible for the reduced levels of invertase for the signal peptide mutants. This is consistent with the current view on secretion and vacuolar protein localization in yeast. Secretory and vacuolar proteins are sorted in the Golgi stack prior to transport to their target membrane compartments (Stevens et al. 1982). The absence of increased amounts of invertase when the signal peptide mutants are

Table V. Comparison of invertase activities in the wild-type *PEP4* (JNY4) and mutant *pep4* (JNY10) background.

The genotypes of the two yeast strains are:

JNY4   *MATα trp1 ade2-101 suc2-215^{am} PEP4*

JNY10   *MATα trp1 ade2-101 leu2 suc2-215^{am} pep4::URA3*

Plasmid	JNY4		JNY10	
	External	Total	External	Total
pS5	15.4	15.4	12.9	12.9
pS6	0	15.6	0	12.9
4-3	9.4	9.4	11.7	12.2
4-56A	8.7	9.0	7.3	7.8
3-6	7.4	7.4	10.0	10.0
3-12	6.8	7.2	9.0	9.0
3-13	6.1	6.6	6.7	7.4
4-55B	5.8	8.6	5.2	7.5
28*	0.3	5.4	0	3.8
28	0.3	4.9	0	4.0
4-55	0.2	4.0	0.4	4.5
4-56	0.2	2.6	0	2.4

expressed in a *pep4* host supports the proposition that the function of the signal peptide is restricted to its role in the ER.

Detection of Invertase Activity After Gel Electrophoresis

A representative set of mutants was analyzed for the presence of glycosylated and unglycosylated invertase in a modified SDS polyacrylamide gel assay. Cellular extracts were subjected to SDS polyacrylamide gel electrophoresis without prior heat denaturation, and enzymatic activity was detected in the gel by the procedure described by Gabriel and Wang (1969). As shown in Figure 15, no invertase activity was detected in JNY4 cells transformed with plasmid pJ1, a *CEN3*-containing plasmid lacking the *SUC2* coding sequence. Cells carrying the parental *SUC2* plasmid (pS5) contained fully glycosylated invertase. This appeared as a very broad band of activity of low electrophoretic mobility. The Class I mutant, 4-56B, gave a similar glycosylated product, whereas the Class II mutant, 4-55B, exhibited a significantly broader band of glycosylated enzyme together with a sharp band of unglycosylated invertase of slightly higher molecular weight than the intracellular unglycosylated enzyme produced by the signal-deleted *SUC2* plasmid, pS6. The two Class III mutants, 4-55 and 4-56, produced three bands of enzyme activity with mobilities corresponding to apparent molecular weights of 58 kd, 180 kd and 240 kd. The two larger forms of the enzyme are not seen on denaturing SDS gel electrophoresis (see below) and are presumed to be aggregates of the monomeric enzyme. The fact that the low molecular weight enzyme is slightly larger than the enzyme produced by plasmid pS6 suggests that the former has retained its signal sequence. That the enzyme produced by mutant 4-55 and 4-56 is different from that produced by pS6 is also evident from the very small amount of high molecular weight bands in the latter (Figure 15).
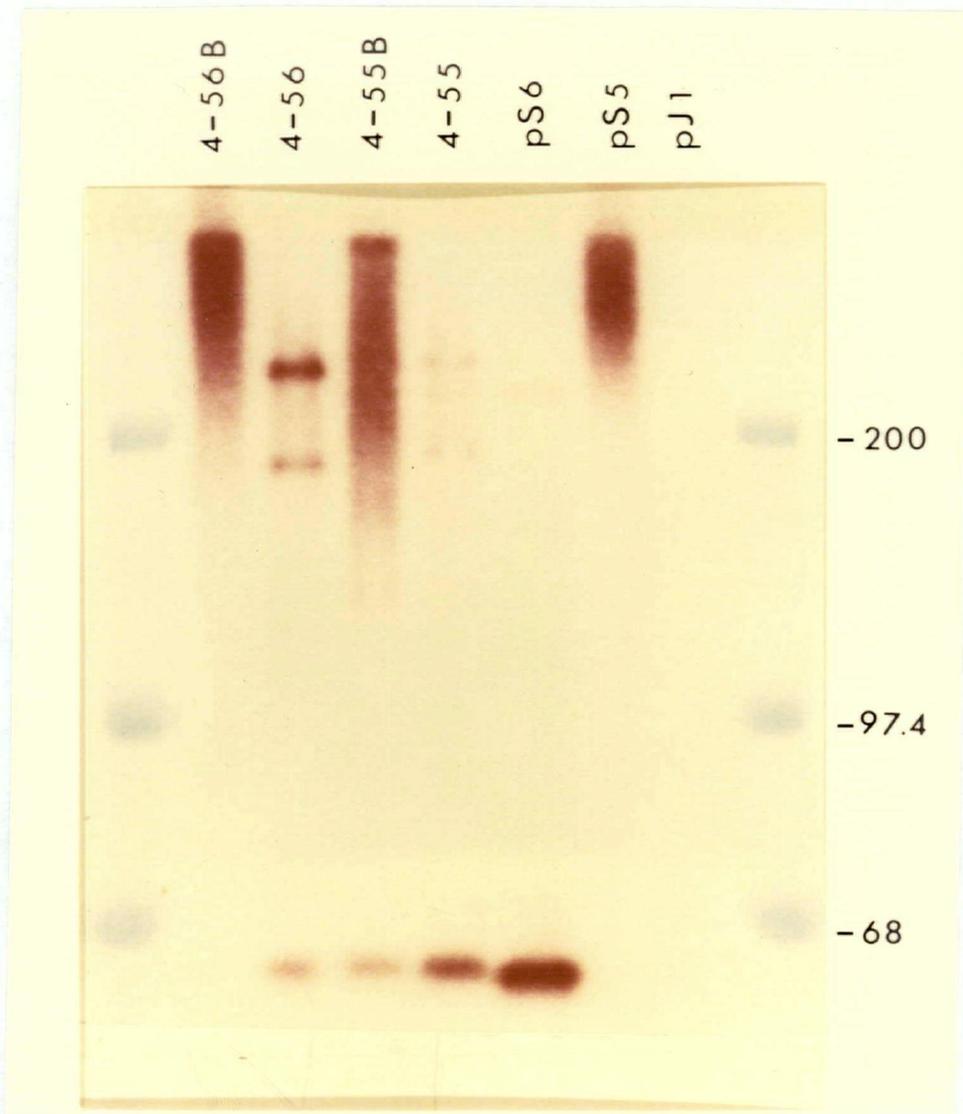
Figure 15. Detection of invertase activity after SDS polyacrylamide gel electrophoresis.

The enzyme was renatured in 0.1 M NaOAc buffer (pH 5) upon the completion of the electrophoretic separation. The gel was then incubated with 0.25 M sucrose in 0.1 M NaOAc (pH 5) and developed with 0.2% 2,3,5-triphenyl tetrazolium chloride (TTC) in 0.5 M NaOH. Plasmid pJ1 is a plasmid lacking the *SUC2* coding sequence. Plasmid pS5 contains the wild-type invertase signal sequence and pS6 carries the signal peptide deleted form. Glycosylated invertase appears as a smear at the top of the gel and the unglycosylated invertase has an apparent molecular weight of either 56 or 58 kd. The higher molecular weight bands in mutants 4-55 and 4-56 probably represent oligomeric forms of invertase. The pre-stained molecular weight markers are: myosin, 200 kd; phosphorylase b, 97.4 kd; bovine serum albumin, 68 kd.

Western (Protein) Analysis of Invertase

The nature of the total invertase protein produced by the mutants was determined by Western analysis. When a fixed amount of extract (15 μg protein) was analyzed, mutants from all three classes exhibiting a low invertase activity also showed a low or undetectable level of immunoreactive invertase (data not shown). Thus, it is highly unlikely that substantial amounts of enzymatically inactive invertase are produced. Figure 16 shows the Western analysis of cell extracts in which a fixed amount of invertase was analyzed. No immunoreactive invertase was detected in an extract from the host strain carrying a plasmid lacking the *SUC2* coding sequence (pJ1). Fully glycosylated invertase, appearing as a broad band due to its heterogeneous nature, was produced from the parental plasmid, pS5. As expected, only the unglycosylated form of invertase, with an apparent molecular weight of 56 kd, was detected in the absence of signal peptide (plasmid pS6). As in the invertase activity assay, the Class I mutants such as 3-12, 3-75, 7-27, 4-56A and 4-56B showed the same pattern as the parental *SUC2* plasmid: all immunoreactive invertase appeared glycosylated with no detectable unglycosylated form. However, mutant 3-13 produced fully glycosylated invertase as well as a trace amount of the unglycosylated form (this is seen in Figure 18). This unglycosylated protein had a lower electrophoretic mobility than the normal cytoplasmic form, suggesting the presence of an uncleaved signal peptide. This may reflect a slight deficiency in the ability of the protein to translocate across to the lumenal side of the ER, the site of signal peptide cleavage. Alternatively, the defect may be due to a delay in signal peptide cleavage and core glycosylation of the translocated enzyme. This issue will be addressed in the next section.

In the secretory defective mutants, 4-55B (Class II) and 4-55, M-5, 4-56, M-1 and 28 (Class III), both unglycosylated and partially glycosylated forms of invertase were evident (Figures 16 and 18). Mutant 4-55B produced relatively more partially glycosylated
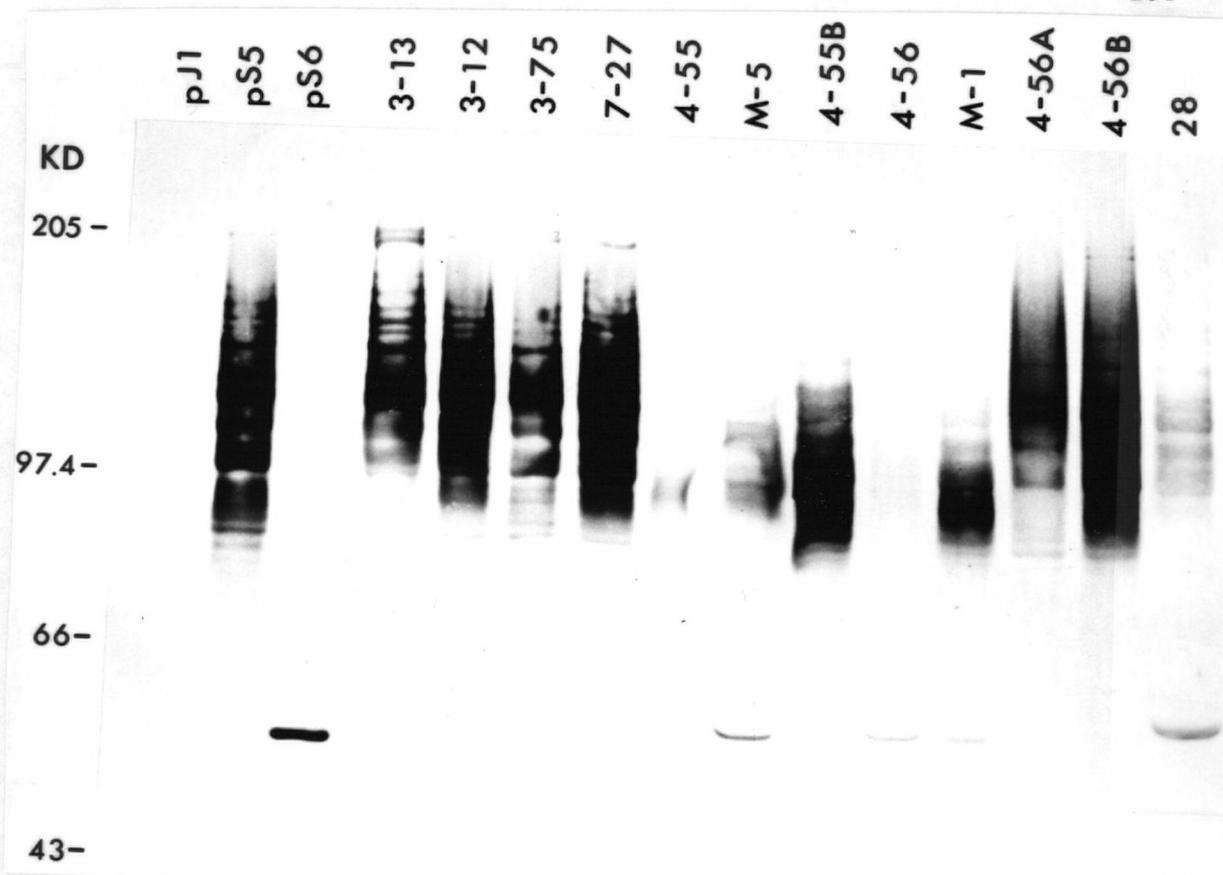
Figure 16. Western analysis of the signal peptide mutants.

Cell extracts fractionated by SDS polyacrylamide gel electrophoresis were transferred to a nitrocellulose filter. Immunoreactive invertase was detected with an affinity-purified antiserum. No immunoreactive invertase was detected in the strain harboring a plasmid lacking the *SUC2* coding sequence (pJ1). Fully glycosylated invertase appears as a smear due to the heterogeneous nature of the oligosaccharide chains (e.g. in pS5, 3-13, 3-12, 3-75, 7-27, 4-56A and 4-56B). Cytoplasmic invertase in the signal peptide deleted plasmid (pS6) has an apparent molecular weight of 56 kd. A larger unglycosylated form (58 kd) is present in some of the mutants (e.g. in 3-13, 4-55, M-5, 4-56, M-1 and 28). Core glycosylated invertase has an apparent molecular weight of 70-90 kd (e.g. in 4-55B and M-1). The protein molecular weight markers are: myosin H-chain, 200 kd, phosphorylase b, 97.4 kd; bovine serum albumin, 66 kd; ovalbumin, 43 kd.

invertase than the Class III mutants. The unglycosylated form was clearly of lower electrophoretic mobility than the plasmid pS6 protein. The glycosylated protein of Class II and III mutants had the characteristic electrophoretic pattern seen when *SUC2* is expressed in a *sec18* mutant of yeast, a temperature-sensitive mutant blocked at the transport between the ER and Golgi complex, and where the enzyme accumulates as a core glycosylated form (Esmon et al. 1981; see Figure 18). In the Class II mutant, 4-55B, this is the major form of the enzyme whereas it is a minor component in the Class III mutants. Within this latter group, mutants 28 and 28* are distinct in that the 58 kd protein is partially processed to the 56 kd form.

Isolation of Yeast Microsomal Membranes and Protection of Invertase from Proteolysis

Cell fractionation studies were undertaken to determine the cellular localization of the different forms of invertase. Extracts were prepared from exponentially growing cells and centrifuged through a Percoll gradient as described by Hansen et al. (1986). The gradient was fractionated, and assayed for invertase and NADPH-cytochrome c reductase, an ER membrane marker enzyme (Kubota et al. 1977). Invertase and NADPH-cytochrome c reductase assays revealed two enzymatically active peaks (Figure 17). One remained at the top of the gradient and corresponded to the loading sample. This is most likely to represent enzymes dislodged from the intracellular membrane compartments during homogenization or enzymes associated with membrane fragments. The second peak corresponded to a turbid, membrane-enriched band midway down the Percoll gradient. Western analysis of the membrane-enriched fractions from mutants 4-55B (Class II), 4-55, 4-56, 28 and 28* (Class III) showed the presence of core glycosylated and unglycosylated invertase precursor, while only outer-chain glycosylated invertase was detected in Class I mutants 4-56A, 4-56B and 7-27 (Figure 18). With a temperature-sensitive *sec18 SUC2*^{+} strain of yeast, only core glycosylated invertase was produced at the non-permissive
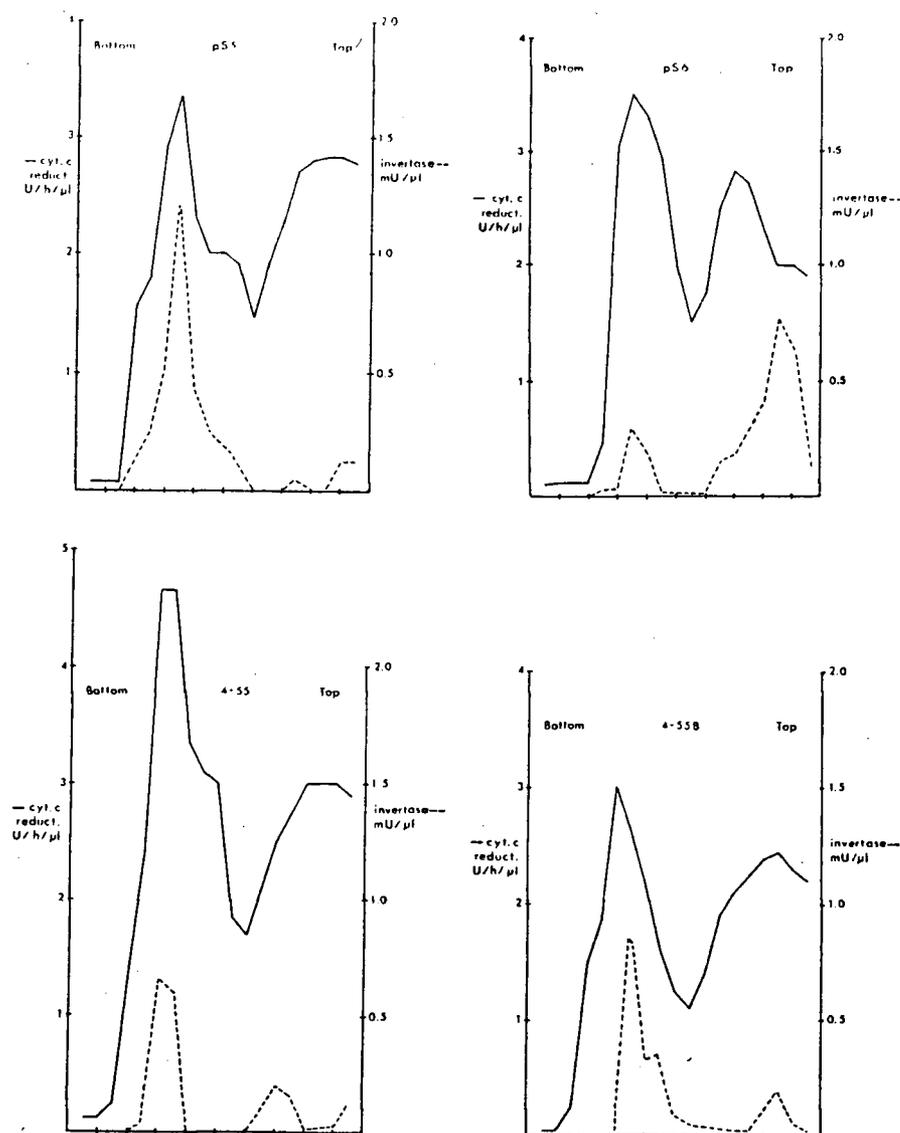
Figure 17. Fractionation of cell extracts on Percoll gradient.

Cell extracts fractionated on Percoll gradient were assayed for invertase (dashed line) and

NADPH-cytochrome c reductase (solid line) activities. The enzyme peak located at the top

of the gradient corresponded to the load volume. The second peak corresponded to a visibly

turbid, membrane-enriched band midway down the gradient. Each mark on the horizontal

axis represents a 0.3 ml fraction.
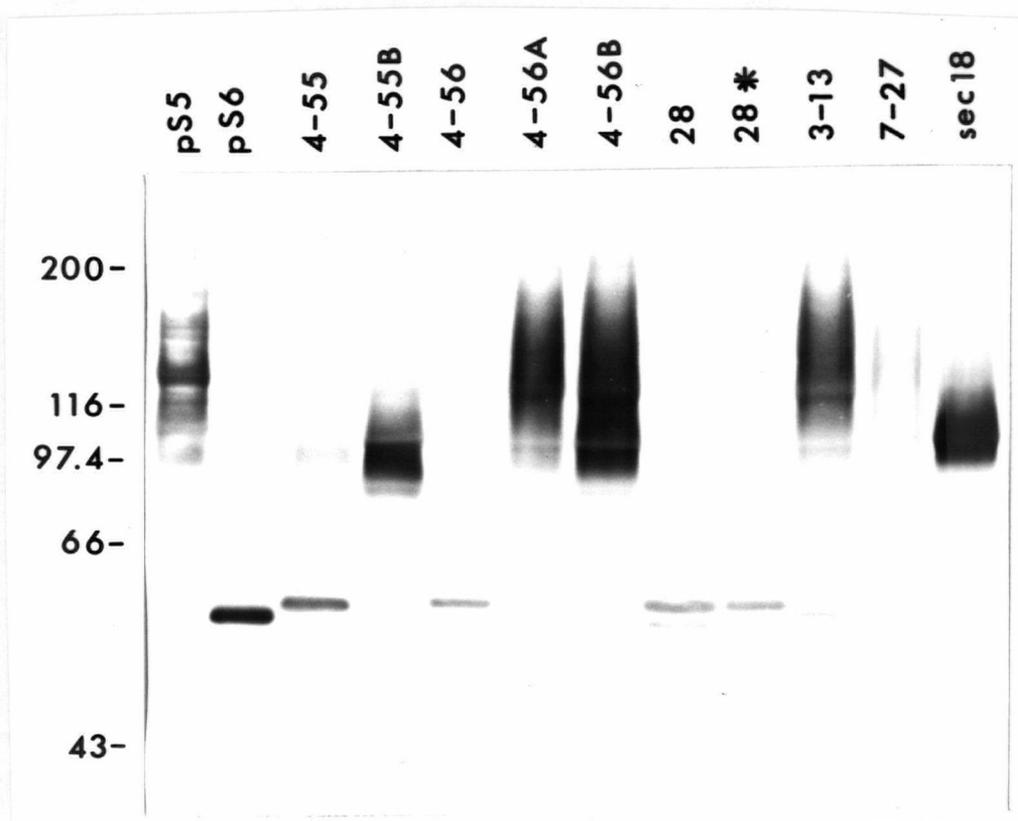
Figure 18. Western analysis of membrane-enriched Percoll gradient fractions.

Extracts were prepared from cells grown to mid-log phase in selective medium. The *sec18* strain was incubated at the non-permissive temperature (37°C) for two hours prior to preparation of the cell extract. The membrane-enriched Percoll gradient fractions were subjected to SDS polyacrylamide gel electrophoresis and Western analysis using affinity-purified anti-invertase antibodies.

temperature (37°C). Since the addition of core oligosaccharide chains occurs only at the lumenal side of the ER (Neuberger et al. 1979), it can be concluded that invertase which is core glycosylated must have translocated across the ER membrane. Furthermore, it is implicit that Class I proteins containing outer-chain oligosaccharides (mutants 3-13, 4-56A, 4-56B and 7-27) must have translocated across the ER membrane and on to the Golgi stack, the site of outer-chain oligosaccharide addition.

The association of 58 kd unglycosylated invertase with the membrane fraction is of interest. One interpretation for its co-purification with the membrane fraction is that the protein is non-specifically associated with cytoplasmic components of the ER membrane. This would also explain the presence of cytoplasmic invertase from plasmid pS6 in the membrane fraction. Another interpretation is that the precursor protein is partially or fully translocated across the ER membrane, but has neither undergone processing by the signal peptidase nor core glycosylation. To resolve this question, the membrane fractions were treated with two proteases, trypsin and proteinase K, in the presence or absence of the detergent, saponin. Proteins that have completely translocated the membrane bilayer will be resistant to proteolysis unless the integrity of the membrane is disrupted by detergent. Preliminary studies using trypsin revealed a protease-resistant unglycosylated species with an apparent molecular weight of 43 kd. This suggests that the mutant pre-invertase was partially inserted into the membrane bilayer. However, the following observations contradicted this conclusion. First, a protected invertase species, approximately 2 kd smaller, was observed on trypsin treatment of the membrane preparation derived from plasmid pS6 (data not shown). Second, this protected species was also observed when purified cytoplasmic invertase was added to the membrane-enriched fractions and treated with trypsin (data not shown). Third, these trypsin-derived species were degraded upon the addition of proteinase K to the digest. Hence, a more plausible interpretation of the data is that invertase contains a trypsin-resistant core which can be degraded by proteinase K. The unglycosylated form of invertase was degraded by the two
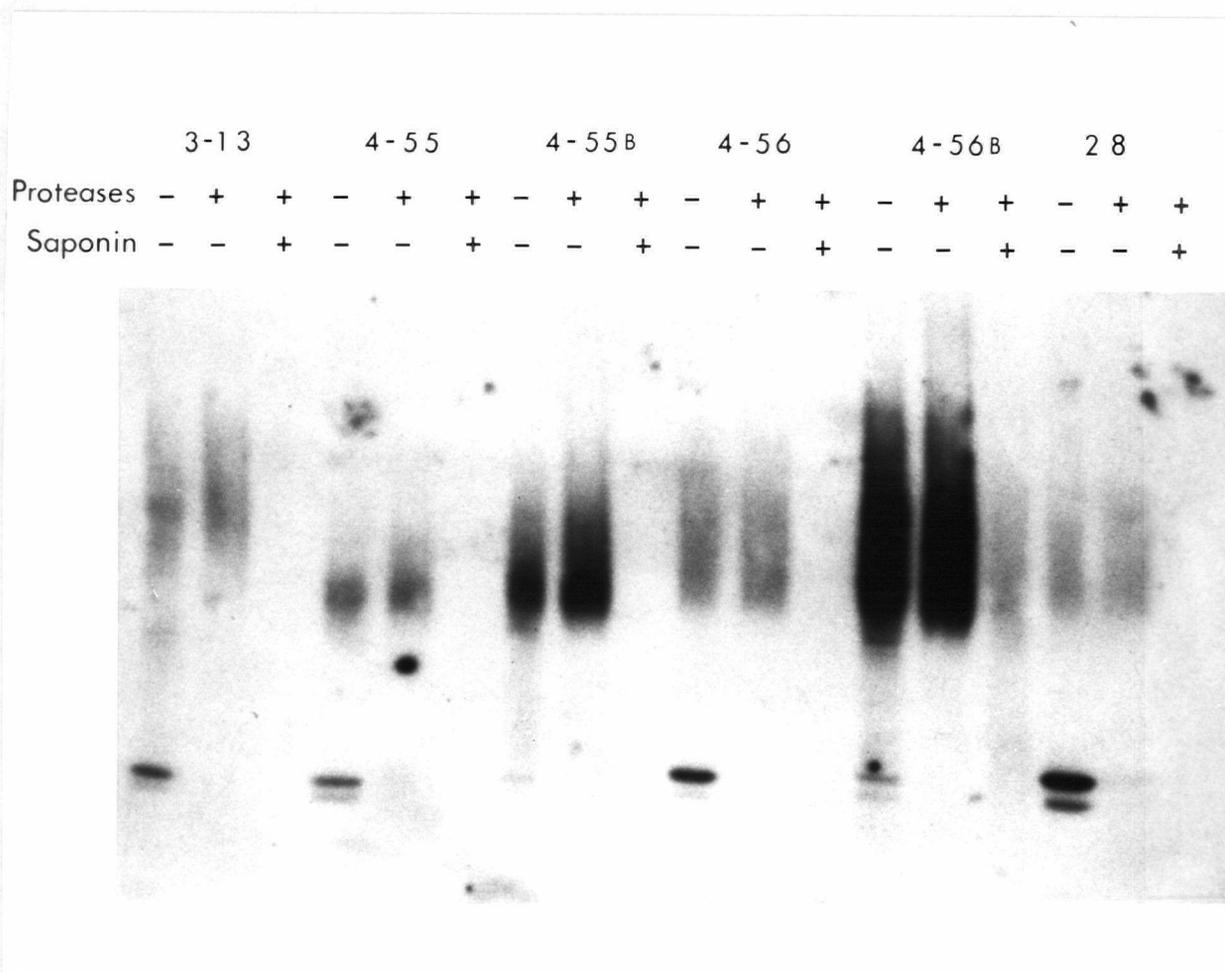
Figure 19. Trypsin and proteinase K treatment of membrane associated invertase.

The membrane-enriched Percoll gradient fractions were digested with 0.75 mg/ml TPCK-trypsin and 0.2 mg/ml proteinase K at 0^0C for 60 min. To disrupt the integrity of the microsomal membrane, 0.3% saponin was added. The proteases were inactivated with 2 mM PMSF, an equal volume of pre-heated 2xSDS sample loading buffer and boiled for 5 min. The proteins were subjected to Western analysis after SDS polyacrylamide gel fractionation. Mutants 3-13 and 4-56B represent the Class I mutants; 4-55B is a Class II mutant; mutants 4-55, 4-56 and 28 belong to Class III.

proteases in all three classes of mutant (Figure 19). In contrast, proteolysis of the fully glycosylated form of invertase in Class I mutants (3-13 and 4-56B) and of core glycosylated invertase in Class II (4-55B) and Class III (4-55, 4-56 and 28) mutants only occurred in the presence of detergent (Figure 19). Thus, it can be concluded that the unglycosylated precursor is not sequestered in membrane vesicles whereas the glycosylated forms are. Furthermore, the accumulation of the unglycosylated form cannot be caused by a failure to glycosylate the enzyme after its translocation across the ER membrane. This experiment reveals that mutants of all three classes produce detectable amounts of unglycosylated protein. This suggests that the differences between mutants represent quantitative rather than qualitative differences in their abilities to transit the secretory pathway. A smaller 56 kd unglycosylated invertase was present in some of the mutants after the mock protease incubation period (Figure 19; mutants 3-13, 4-55, 4-56, 4-56B and 28). This is likely an artifact generated by endogenous protease, since the membranes were prepared in the absence of protease inhibitors and the 56 kd species was not detectable in the membrane preparations with these mutants prior to the incubation period (Figure 18). In the case of mutants 28 and 28*, the 56 kd was detectable without incubation (Figure 18), suggesting that the mutations had rendered the 58 kd form of these mutants more susceptible to limited proteolysis than for the other mutants.

Cleavage of Signal Peptide in Pre-invertase Mutants

The signal peptide can be detected on unglycosylated invertase by SDS gel electrophoresis because of the differing mobilities of pre-invertase (58 kd) and unglycosylated invertase (56 kd), but it cannot be detected directly on the heterogeneous glycosylated invertase. Therefore, carbohydrate chains were removed by treatment of the membrane-enriched Percoll gradient fractions with N-glycosidase F (N-glycanase^{TM}), an enzyme that hydrolyses protein N-linked oligosaccharides (Plummer et al. 1984). This enzyme was chosen because of its broad specificity (Plummer et al. 1984). It completely

hydrolyses the carbohydrate moiety, leaving a protein containing an aspartic acid at the asparagine glycosylation site (Plummer et al. 1984). After complete deglycosylation, the protein samples were fractionated on an SDS polyacrylamide gel and subjected to Western analysis (Figure 20). The wild-type invertase produced by the parental plasmid (pS5) when deglycosylated has an electrophoretic mobility between that of the 56 kd cytoplasmic form produced by plasmid pS6 and the 58 kd pre-invertase (Figure 20). This reduced mobility compared to the cytoplasmic form may be due to the effect of the additional negative charges (aspartate residues) generated after deglycosylation. Of the mutants treated with N-glycanase, the Class I mutant, 4-56B, exhibited this mobility after deglycosylation indicating normal signal peptide cleavage. The Class II mutant, 4-55B, gave a product larger than the deglycosylated wild-type invertase (pS5). The Class III mutants, 4-55, 4-56 and 28, did not have a noticeable change in mobility as a consequence of N-glycanase treatment.

These experiments show that the Class I mutants, 4-56B, whose phenotype is a reduced level of secreted invertase, produces enzyme from which the signal peptide is cleaved. In the Class II mutant, 4-55B, the protein released by N-glycanase treatment has a slightly lower mobility than that released from wild-type and Class I enzyme. Thus, it may be that the core glycosylated enzyme, which is the major product of this mutant, retains the whole or part of its signal peptide. The fact that there is no change in the mobility of the proteins of the Class III mutants (4-55, 4-56 and 28) indicates that only a minor fraction is glycosylated and supports the interpretation that the 58 kd protein is unprocessed pre-invertase.

Pulse-chase Studies on Invertase Synthesis

The progression of invertase through the secretory pathway can be monitored radioactively for temperature-sensitive mutants which block transport of secretory proteins from intracellular organelles to the plasma membrane (Novick et al. 1981; Esmon et al.
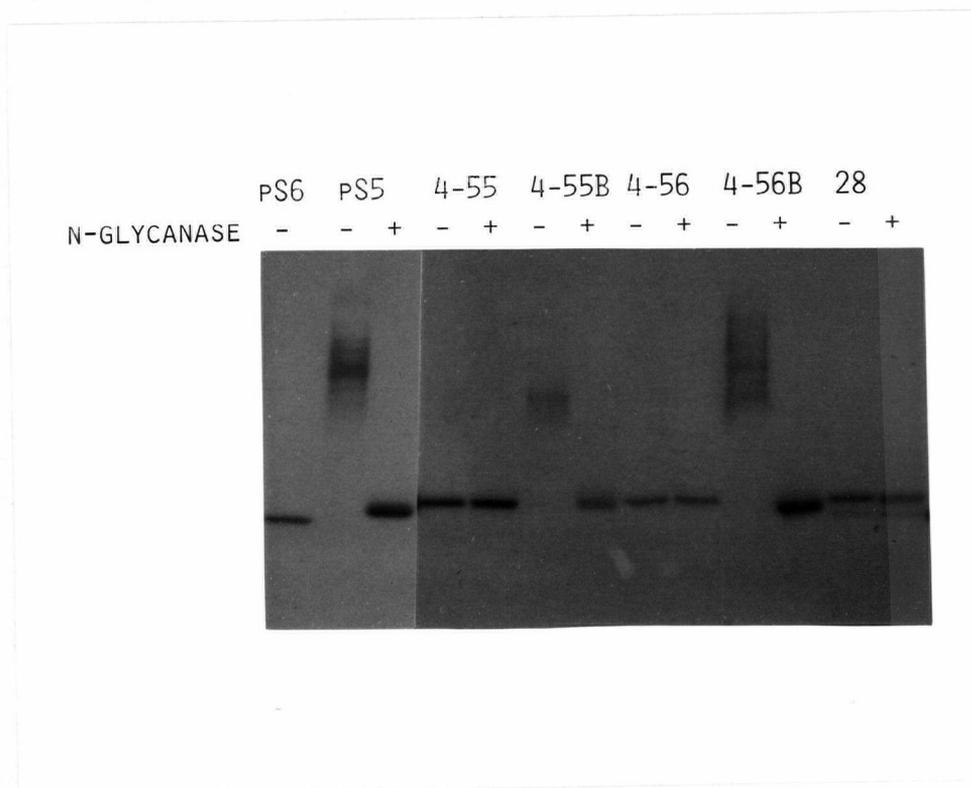
Figure 20. Deglycosylation of glycosylated invertase with N-glycanase to determine signal peptide cleavage.

The membrane-enriched Percoll gradient fractions were treated with N-glycanase and subjected to Western analysis. The wild-type enzyme (pS5) upon deglycosylation gave a product with a mobility between that of cytoplasmic, 56 kd invertase (pS6) and of 58 kd pre-invertase (as in mutant 4-55). Mutants examined were: Class I, 4-56B; Class II, 4-55B and Class III, 4-55, 4-56 and 28.

1981), and for *SUC2* mutations affecting the rate of signal peptide cleavage (Schauer et al. 1985; Haguenauer-Tsapis et al. 1986). Pulse-chase studies were undertaken in an attempt to identify the step(s) in biosynthesis affected by the signal peptide mutations. Cells transformed with plasmids carrying the wild-type or mutant *SUC2* genes were converted to spheroplasts, labeled with [^{35}S]-methionine for 4 min, and chased for varying lengths of time with unlabeled methionine and cycloheximide. Periplasmic (external) and intracellular protein fractions were immunoprecipitated with affinity-purified invertase antiserum and subjected to SDS polyacrylamide gel electrophoresis. The sites of invertase accumulation can be deduced from the degree of glycosylation of the protein and the kinetics of passage of the intermediates through the secretory pathway can be quantitated. Pulse-chase study on a strain harboring the wild-type plasmid, pS5, detected only fully glycosylated invertase in the intracellular fraction; no intermediate unglycosylated or core glycosylated invertase was evident even at 0 min of the chase (data not shown). Fully glycosylated invertase was detectable in the external medium after a chase period of 1 min. Because fully glycosylated invertase is disperse and hard to quantify immunologically, immunoprecipitated intracellular and extracellular samples obtained at different times after the start of the chase were deglycosylated with endoglycosylase H (Endo H) prior to electrophoresis (Figure 21). The intensities of the bands in the autoradiogram were determined with a densitometer. A rapid disappearance of intracellular invertase coincident with the appearance of extracellular invertase indicates that the enzyme was rapidly transported to the cell surface. The half-life of radioactive intracellular enzyme was approximately 1.5 min (Table VI). This is in agreement with the half-life reported by Schauer et al. (1985).

Pulse-chase studies were carried out on two Class I mutants, 4-56B and 3-13 (Figure 22). Mutant 4-56B, which exhibited a wild-type phenotype on quantitative enzymatic assay and in Western and cell fractionation analyses, has transiently detectable core glycosylated invertase and barely detectable unglycosylated 58 kd pre-invertase. The
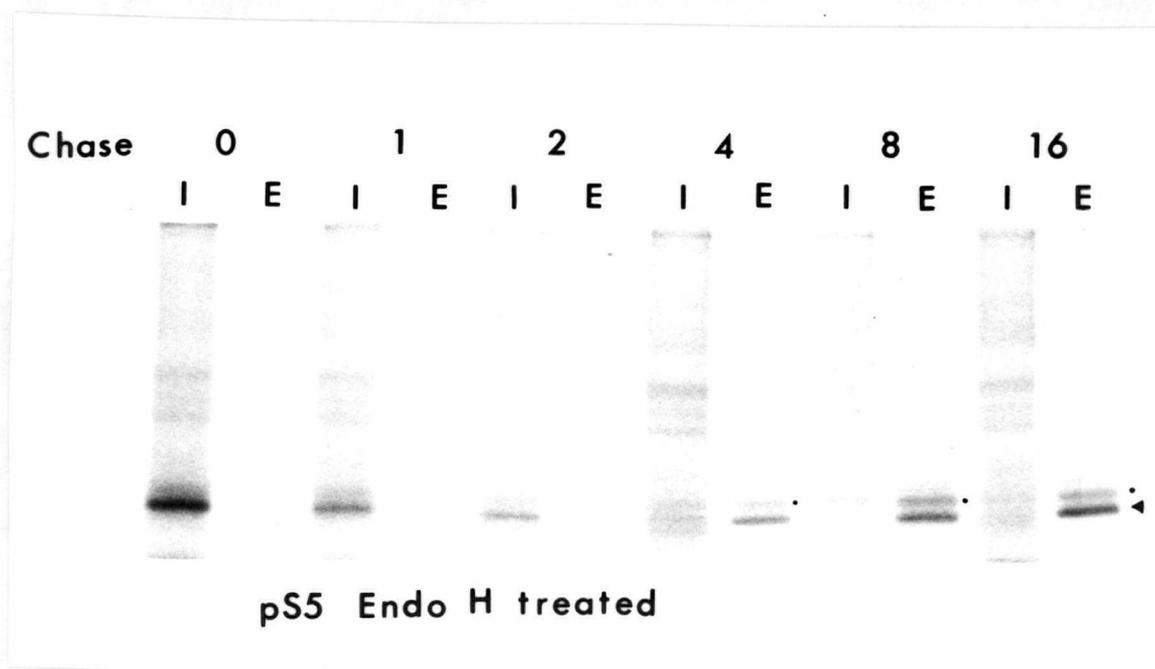
Figure 21. Pulse labeling of JNY4 strain harboring the parental plasmid, pS5.

Yeast cells were grown to exponential phase and the cell wall was removed with zymolase 60,000. The cells were labeled with [^{35}S]-methionine for 4 min, after which unlabeled methionine and cycloheximide were added to initiate the chase period (0, 1, 2, 4, 8 and 16 min). Invertase immunoprecipitated from the cells (intracellular fraction, I) and from the medium (extracellular fraction, E) was deglycosylated with Endo H, and subjected to SDS polyacrylamide gel electrophoresis. The deglycosylated invertase appears as a sharp band with apparent molecular weight slightly larger than 56 kd (arrow head). The higher molecular weight band in the extracellular fractions (dotted; chase 4, 8 and 16 min) represents incompletely deglycosylated invertase.
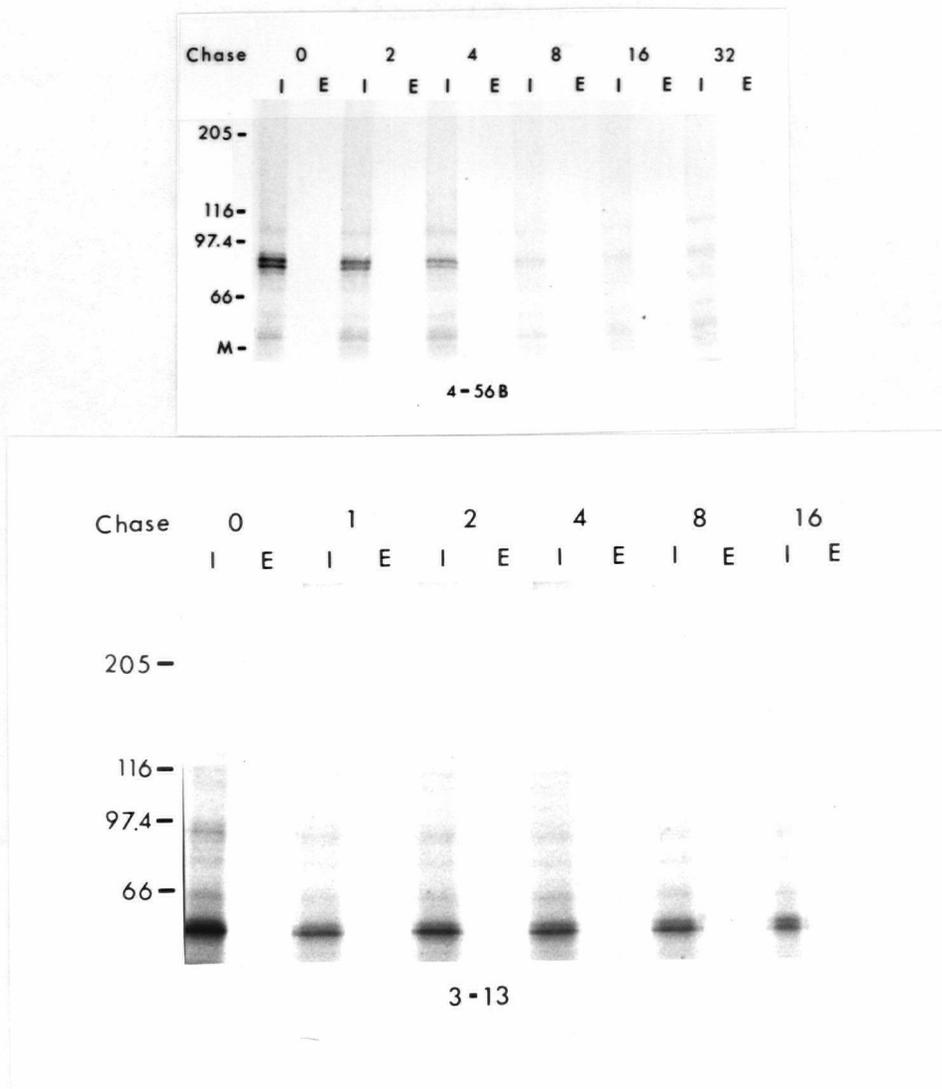
Figure 22. Pulse labeling of the Class I mutants 4-56B and 3-13.

The spheroplasts were labeled with ^{35}S-methionine for 4 min and chased with unlabeled methionine and cycloheximide for varying lengths of time (in min). Intracellular invertase (I) and invertase present in the medium (E) were immunoprecipitated with affinity-purified invertase antiserum. The positions of the Coomassie blue stained protein molecular weight standards (in kd) and cytoplasmic invertase purified from plasmid pS6 (marked M) are indicated. The unglycosylated, cytoplasmic invertase in mutant 3-13 appears as a doublet in which the lower mobility band has an apparent molecular weight of 56 kd. Secreted invertase appears as a very faint broad band in the (E) fraction of chase periods 4, 8 and 16 min. Core glycosylated invertase appears as a series of bands with apparent molecular weights of 70-90 kd in both mutants. The samples were not treated with Endo H.

$t_{1/2}$ for the core glycosylated form was slightly greater than for pS5 derived enzyme, suggesting a slight delay in transit from the ER to the Golgi complex (Figure 22; Table VI).

Two interesting features were observed in the pulse-chase study of the other Class I mutant, 3-13 (Figure 22). First, although it produces greatly reduced levels of invertase (Table III), the defect in this mutant was not as severe as the Class II and Class III mutants (see below) in that the majority of the enzyme was fully glycosylated and transported to the cell surface. However, external invertase, which only becomes apparent after a chase period of 4 min, was transported to the cell surface at a reduced rate compared to the wild-type plasmid (pS5) where secreted invertase was detectable after a 1 min chase. The delay in transport to the cell surface was not associated with substantial accumulation of core glycosylated enzyme. Second, the presence of unglycosylated pre-invertase, as was evident in earlier experiments with this mutant (Figures 18 and 19), indicates that the precursor enzyme was not efficiently translocated across the ER membrane. Two unglycosylated intermediates with apparent molecular weights of 56 kd and 58 kd are noticeable. The larger 58 kd form is most likely the unglycosylated cytoplasmic pre-invertase.

In the Class II mutant, 4-55B, both core glycosylated and the unglycosylated 58 kd intermediates were evident (Figure 23), suggesting delays in translocation across the ER membrane and transit from the ER on to the Golgi complex. The $t_{1/2}$ of the core glycosylated form was 2.5 times that of the wild-type pS5 enzyme (Table VI). Although the fate of the core glycosylated invertase is not apparent in Figure 23, the presence of secreted invertase in this mutant, as determined by quantitative enzymatic assay (Table III), indicates that a fraction of the glycosylated enzyme must eventually reach the cell surface. Taking into account the results in the N-glycanase studies (Figure 20), the data suggest that the pre-invertase is inefficiently translocated across the ER membrane and
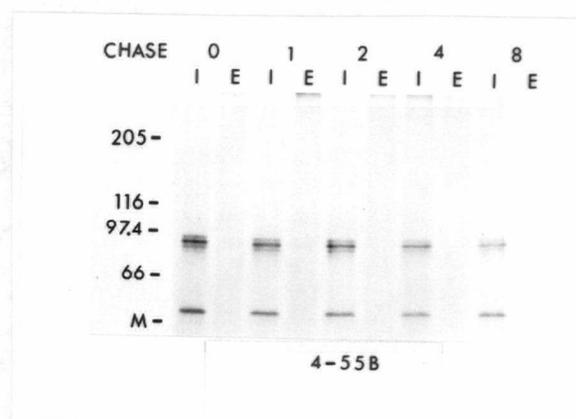
Figure 23. Pulse labeling of the Class II mutant 4-55B.

Legend as in Figure 22. The unglycosylated pre-invertase has an apparent molecular weight of 58 kd and core glycosylated invertase appears as a series of bands with apparent molecular weights of 70-90 kd. The samples were not treated with Endo H.

that the signal peptide is cleaved slowly. There does not appear to be any defect in addition of core oligosaccharide chains to the protein.

In the Class III mutants, 4-55 and 28, the unglycosylated 58 kd pre-invertase was the major intermediate detectable at all times (Figure 24). This pre-invertase was found to be less stable in mutant 28 ($T_{1/2}$ of 9 min) than that produced by mutant 4-55 ($T_{1/2} > 16$ min). This was also the major intermediate detected in another Class III mutant, 4-56, although a small amount of core glycosylated enzyme was also transiently apparent (Figure 24). Since the results from the protease protection experiments indicate that the 58 kd pre-invertase of the three mutants is a cytoplasmic protein (Figure 19), this suggests that the major defect is the inability of the pre-invertase to translocate across the ER membrane.

Comparison of the deduced amino acid sequences of these mutants indicates that residues at the amino-terminus play an important role in the efficiency of translocation across the ER membrane. The Class II mutant, 4-55B [K(-4)Q, A(-6)E, L(-11)R], differs from the Class III mutant, 4-55 [K(-4)Q, A(-6)E, L(-11)R, L(-18)G], by a single amino acid at position -18. As shown in Figure 24, only the unglycosylated 58 kd form was detectable at all times in mutant 4-55, while both core glycosylated and unglycosylated 58 kd forms were present in mutant 4-55B (Figure 23). Hence, the replacement of the leucine residue at position -18 by glycine in the signal peptide of mutant 4-55 has reduced the efficiency with which the rest of the protein can translocate across the ER membrane. The effect of mutations at the amino-terminus on pre-invertase translocation is also evident by comparing the Class I mutant, 4-56B [L(-11)D], and the Class III mutant, 4-56 [L(-11)D, L(-17)V, L(-18)N]. In mutant 4-56, mutation of two amino acids at the amino-terminus [L(-17)V; L(-18)N] resulted in a greatly increased $t_{1/2}$ for pre-invertase and a barely detectable amount of core glycosylated enzyme. Thus, the major effect of these N-terminal
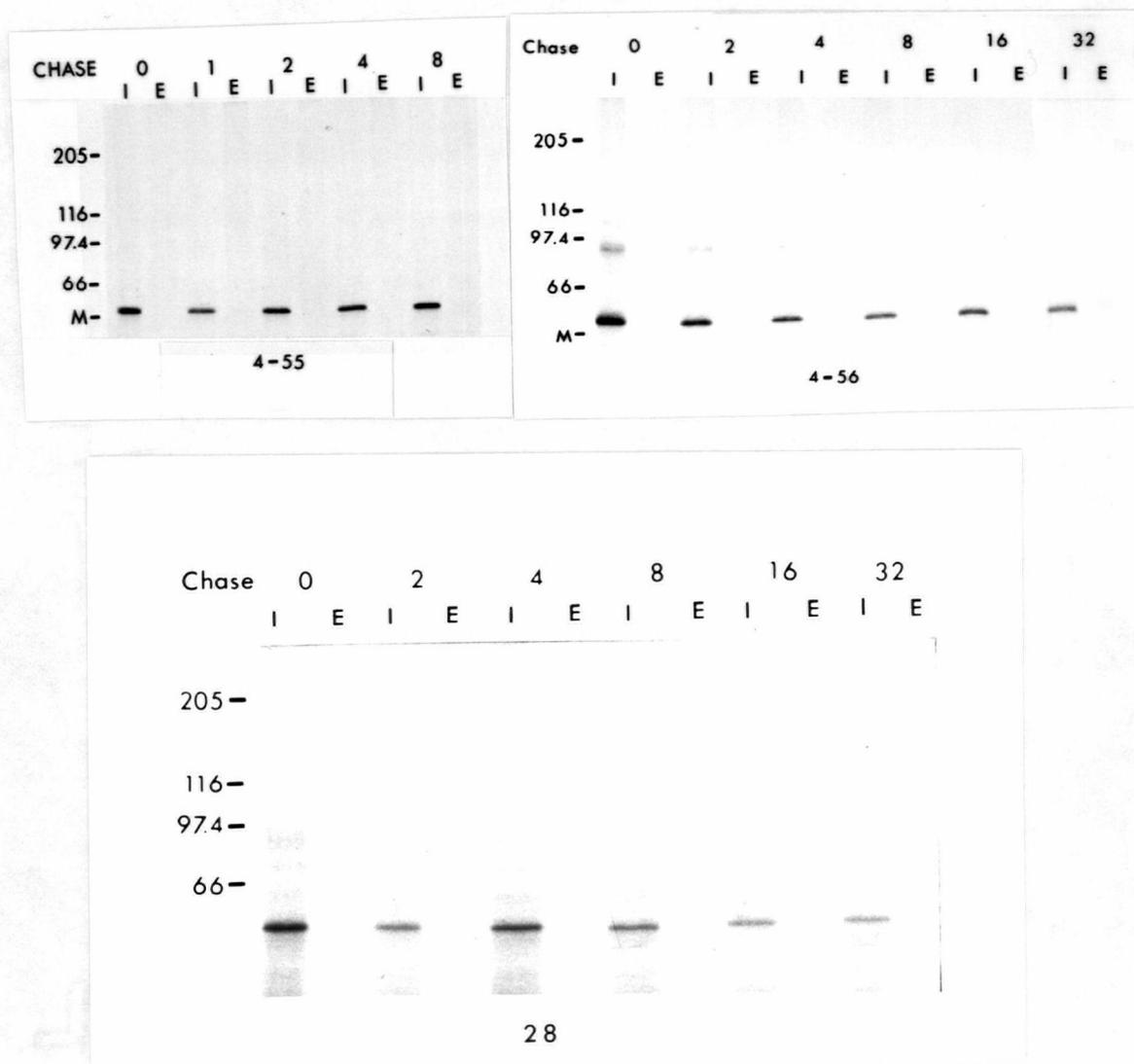
Figure 24. Pulse labeling of the Class III mutants 4-55, 4-56 and 28.

Legend as in Figure 22. The protein molecular standards (in kd) and cytoplasmic pS6 invertase (M) are indicated. Unglycosylated pre-invertase and core glycosylated invertase have apparent molecular weights of 58 kd and 70-90 kd, respectively.

Table VI. Half-lives of invertase mutants.

The intensities of the [^{35}S]-labeled invertase bands were determined with a densitometer and plotted against the chase period. The half-life is defined as the time for the signal to decrease by 50% in intensity from 0 min chase period.

PLASMID		CYTOPLASMIC 58 kd FORM	CORE GLYCOSYLATED 70-90 kd FORM
wild-type	pS5	ND*	1.5 min
Class I	4-56B	ND	4.5 min
Class II	4-55B	>16 min	4.5 min
Class III	4-55	>16 min	ND
	4-56	>16 min	3.8 min
	28	9 min	ND

* ND = NOT DETERMINED

amino acid replacements again is a reduced efficiency of the pre-invertase translocation across the ER membrane.

Ability of the Mutant Pre-invertase to Translocate Across the ER Membrane *in vitro*

The yeast (Hansen et al. 1986) and the combined wheat germ and canine pancreas (Walter et al. 1981a; 1981b; Erickson and Blobel, 1983) *in vitro* translation and translocation systems next were used to examine the possibility that the defect in the Class II and Class III mutants resides in the ability of the protein to be recognized by components of the secretory apparatus and to translocate across the ER. The mutant *SUC2* sequences were subcloned into the pSP65 vector (Figure 12) and capped transcript was produced with SP6 RNA polymerase (Melton et al. 1984). Two different series of *SUC2* transcripts were used. Transcripts coding for the full length pre-invertase mutants were synthesized from AccI linearized pSP65-*SUC2* plasmids. A series of pSP65 plasmids containing truncated versions of pre-invertase mutants were constructed by linearizing the pSP65-*SUC2* plasmids at the BamHI site, repairing the single-stranded ends with DNA polymerase I (Klenow fragment) and recircularization. The manipulation of the BamHI site generated a translation termination signal eight codons downstream of the site, and produces a truncated pre-invertase and invertase of 271 and 252 amino acids, respectively. Transcripts from this series also were synthesized from AccI linearized plasmids.

Canine SRP Mediated Elongation Arrest

According to the Signal Hypothesis, the signal peptide is recognized by the SRP as it emerges from the ribosome (Walter et al. 1981a). The binding of SRP to the signal peptide results in a transient arrest in elongation of the nascent polypeptide chain, and in the absence of releasing components from the ER membrane, results in a reduced

synthesis of the full length product (Walter et al. 1981; 1984). The canine SRP has been purified to homogeneity (Walter and Blobel, 1980; 1982) and shown to interact directly with the signal peptide (Gilmore and Blobel, 1983; Kurzchalia et al. 1986; Krieg et al. 1986). This provides the opportunity to examine the ability of canine SRP to recognize the mutant *SUC2* pre-invertases produced *in vitro* by the wheat germ translation system. One of the properties of this system is the ability of exogenously added canine SRP to impose, in many cases, a transient block in translation elongation at specific sites during synthesis of the nascent chain of secreted proteins (Lipp et al. 1987). The extent of elongation arrest in the absence of microsomal membranes was used in the following experiments to assess the interaction between canine SRP and the *SUC2* mutant signal peptides. A decrease in SRP binding affinity of the mutant signal peptide should require a higher concentration of the canine SRP for arrest in polypeptide chain elongation. In Figure 25, full length *SUC2* transcripts were translated with wheat germ extract containing increasing amounts of canine SRP (generously provided by Dr. Peter Walter). Synthesis of the invertase possessing the wild-type signal peptide (pS5) was noticeably inhibited by canine SRP at a concentration of 15 nM (Figure 25). This inhibition was completely abolished when the signal peptide was deleted (pS6). Thus, the wild-type invertase signal peptide was effectively recognized by the canine SRP. The translation of the Class I mutant, 4-56B, required a high concentration of canine SRP for significant inhibition (Figure 25). This is surprising because, *in vivo* in yeast, this mutant invertase was secreted almost as efficiently as the wild-type enzyme (Table III). The Class III mutants, 4-55, 4-56, M-1 and 28 revealed no major reduction in pre-invertase with any tested level of SRP. These experiments demonstrate that a variety of Class III invertase signal peptide mutants can prevent the inhibition by canine SRP of *in vitro* translation.
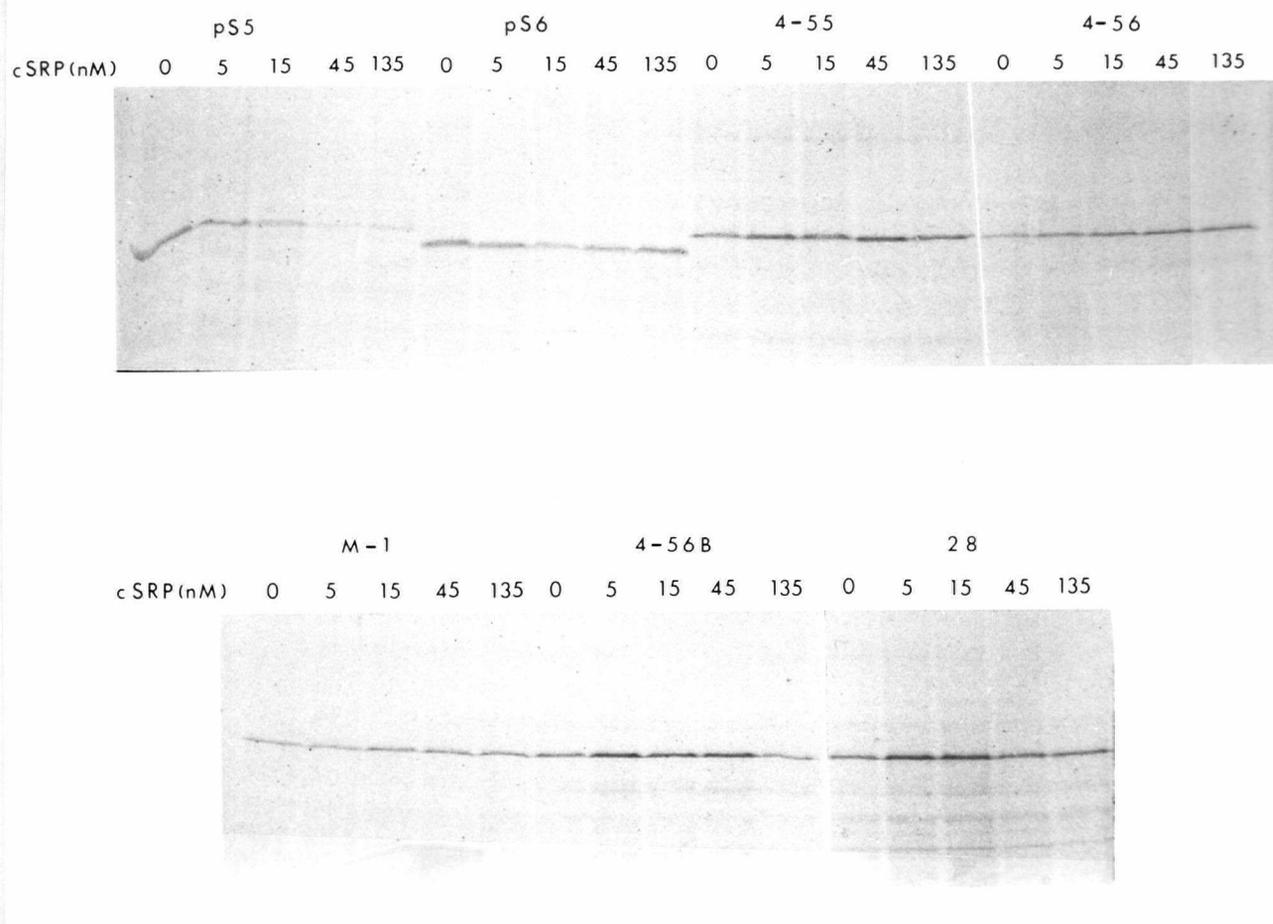
Figure 25. Canine SRP-mediated elongation arrest.

The SP6 RNA polymerase synthesized transcripts coding for the full length pre-invertases were added to the *in vitro* wheat germ translation system in the presence of [^{35}S]-methionine. Varying amounts (0 to 135 nM) of purified canine SRP were added to the translation mix and the reaction was carried out at 20°C for 60 min. The translated products were fractionated on a 10-15% SDS polyacrylamide gradient gel and autoradiographed. The binding of SRP to the signal peptide causes a transient inhibition in translation, resulting in a reduction in the amount of full length invertase (e.g. the wild-type plasmid, pS5, and mutant 4-56B at 135 nM canine SRP). The signal peptide was deleted in plasmid pS6 resulting in a lower molecular weight protein compared to the wild-type or mutant pre-invertase.

Ability of Pre-invertase Mutants to Translocate Across the Canine Microsomal Membrane

Some signal peptides of secreted proteins are competent in translocation despite the absence of a noticeable translation arrest (Siegel and Walter, 1985). Moreover, translocation of proteins in the absence of translational inhibition has been predicted by a mathematical treatment of the data on the translocation process (Rapoport et al. 1987). This provides a possible explanation for the properties of the Class I mutant, 4-56B, which secretes invertase almost as efficiently as the wild-type enzyme in yeast but exhibits a drastically reduced translation arrest in the presence of SRP *in vitro*. Hence, *SUC2* transcripts encoding full length mutant pre-invertases were added to the wheat germ translation system containing canine SRP and canine microsomal membranes. Core glycosylation of the protein, seen as a shift in the apparent molecular weight to 70-90 kd on SDS polyacrylamide gel, was taken as a criterion for translocation. Invertase containing the wild-type signal peptide (plasmid pS5) was efficiently translocated across the membrane and was dependent on exogenously added SRP (Figure 26). As would be expected, no SRP mediated translocation was observed in the signal peptide deleted invertase (plasmid pS6). The Class I mutant, 4-56B, also required SRP for translocation, but was translocated less efficiently than the wild-type pre-invertase. Likewise, the Class II mutant, 4-55B, was translocated with reduced efficiency in the presence of canine SRP. The Class III mutants, 4-55, 4-56, 28 and 28*, exhibited no detectable translocation, even with the highest level of added SRP (45 nM). Thus, the present experiments suggest that a defect in all three classes of mutants is a reduction in the efficiency of translocation across the ER membrane, with the translocation of the Class III mutants being most severely inhibited. It is interesting that the Class I mutant, 4-56B, and the Class II mutant, 4-55B, appear to be translocated with similar efficiencies while subsequent processing of the Class II mutant is clearly less efficient.
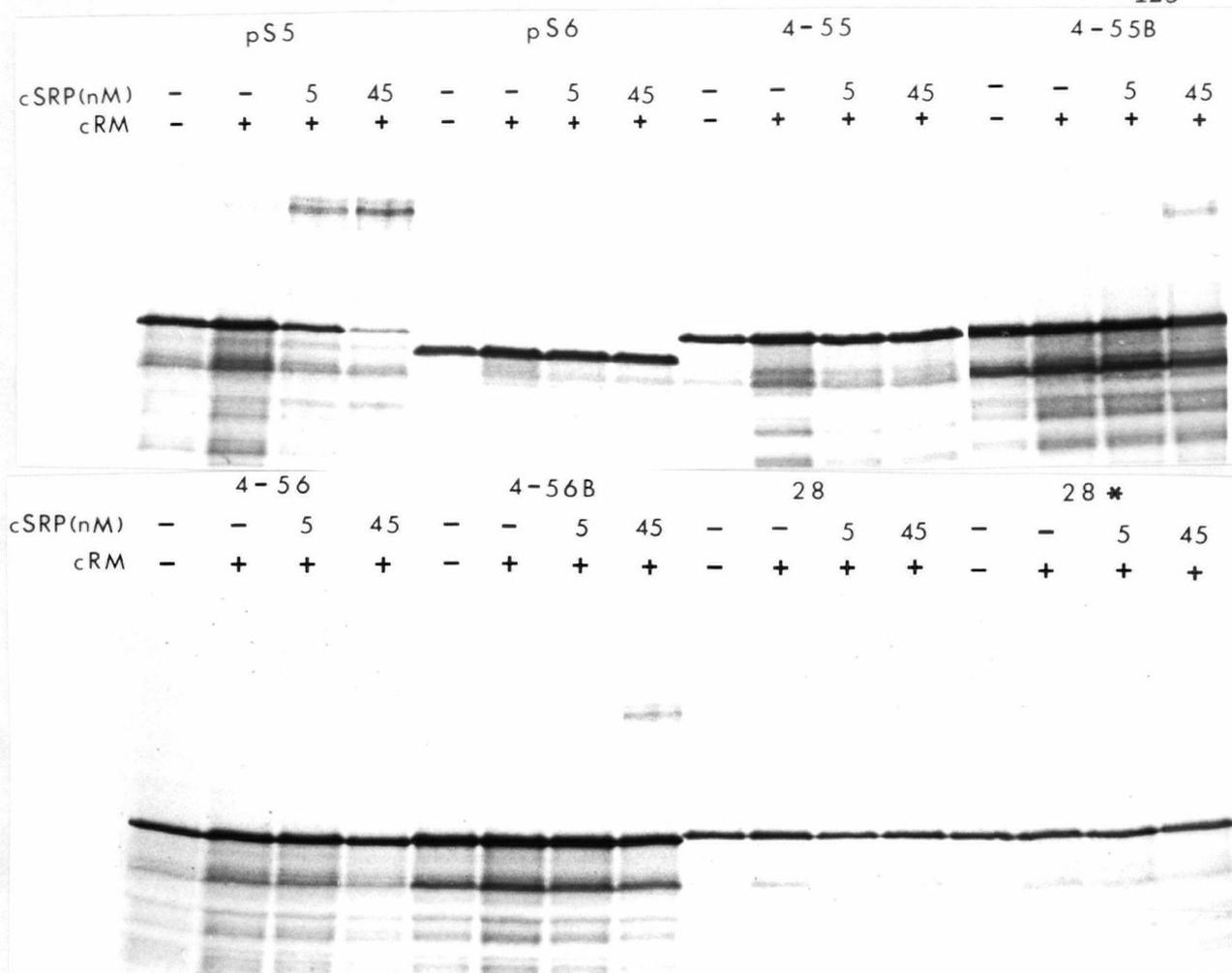
Figure 26. *In vitro* translocation of invertase across canine microsomal membranes.

Canine SRP at two concentrations (5 and 45 nM) and canine microsomal membranes (cRM) were added to the *in vitro* wheat germ translation system in the presence of [^{35}S]-methionine and SP6 RNA polymerase synthesized pre-invertase transcripts. The samples were fractionated on a 10-15% SDS gradient gel and autoradiographed. The major product seen in the wild-type (pS5) and mutant plasmids corresponds to the unglycosylated 58 kd pre-invertase, and deletion of the signal peptide produces a 56 kd product (pS6). Translocated products, which have a higher apparent molecular weight due to the addition of core oligosaccharide units, can be seen in the wild-type pS5; the Class I mutant, 4-56B; and the Class II mutant, 4-55B. The Class III mutants, 4-55, 4-56, 28 and 28*, did not produce translocatable products.

Yeast *in vitro* Translation and Translocation

It might be argued that there are differences between the yeast and mammalian systems, hence, the capped mutant *SUC2* transcripts were translated in the yeast *in vitro* translation system (Hansen et al. 1986) in the presence or absence of yeast microsomal membranes. Since full length invertase is heavily glycosylated, transcripts coding for truncated pre-invertases were used in order to simplify analysis of the translocated products. Core glycosylation was observed in the wild-type pre-invertase (plasmid pS5) in the presence of yeast rough microsomes (Figure 27). The efficiency of translocation is poor compared to the *in vivo* conditions, as only 5-10% of the translated product was translocated. It is likely that factors essential for protein translocation are missing or inactive in the *in vitro* system. No glycosylated products were evident in the signal peptide deleted plasmid, pS6. Class I (4-56B and 3-13), Class II (4-55B) and Class III (4-55, 4-56, 28*) mutants were tested, but none showed any core glycosylated protein. It is probable that the inefficiency of the system precluded the detection of any lower level of translocation such as might be anticipated with the Class I and Class II mutants.

Characterization of Bovine Prolactin Signal Peptide-Invertase Mutants

To determine whether yeast can effectively recognize and process signal peptides from higher eukaryotes, a hybrid precursor protein containing the bovine prolactin signal peptide and the mature invertase sequence was constructed, and placed under the control of the constitutive yeast *ADH1* promoter (plasmid pS7). All the resultant invertase is secreted indicating that the prolactin signal peptide was recognized and processed by the yeast secretory apparatus (Figure 28). However, the amount of enzyme is much reduced relative to that produced with the wild-type invertase signal peptide (plasmid pS5). This reduced level was raised over six-fold to that produced by the wild-type invertase signal by a single amino acid substitution: a glycine to alanine change at position -18 (plasmid pS7-H). Some possible causes for this effect were evaluated. First, there is the possibility
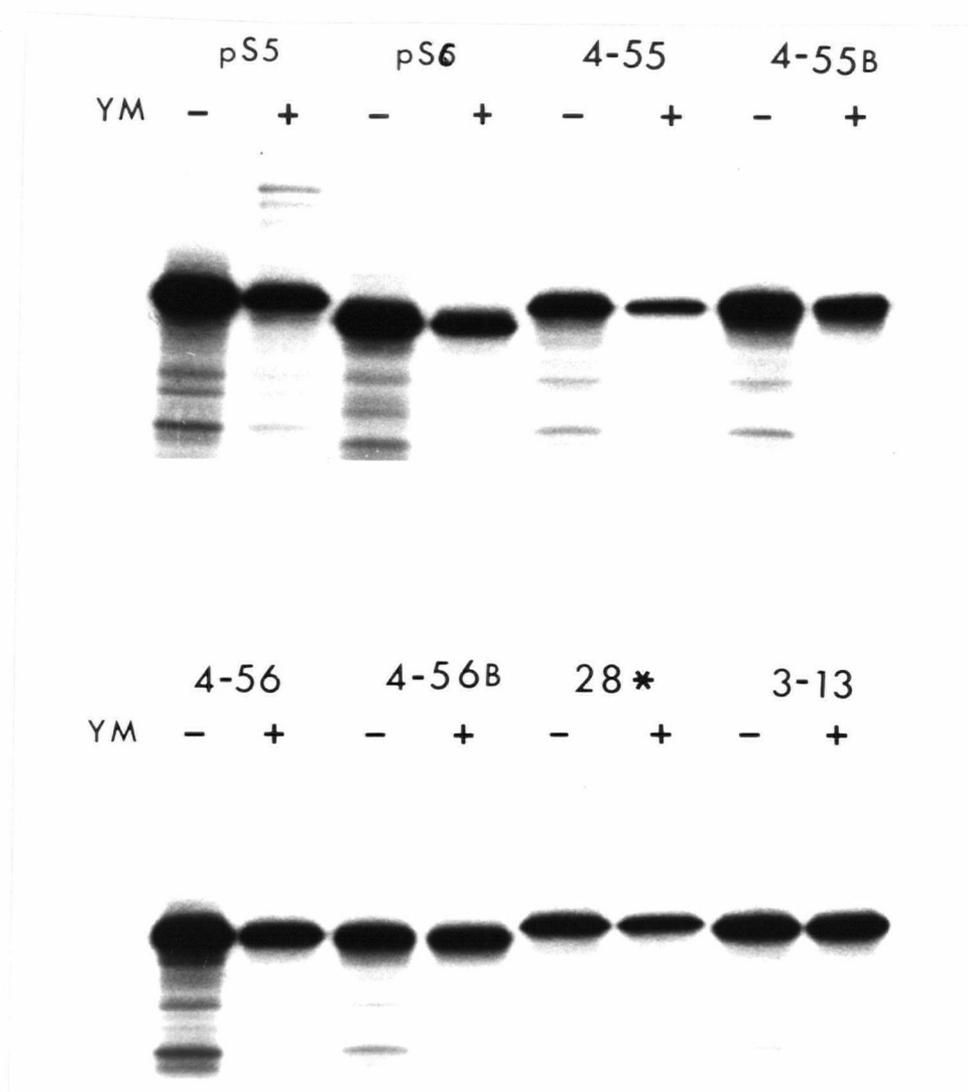
Figure 27. Translocation across yeast rough membranes.

The SP6 RNA polymerase synthesized capped transcripts coding for the truncated mutant pre-invertases were added to the yeast *in vitro* translation extract containing [^{35}S]-methionine in the presence (+) or absence (-) of yeast microsomal membranes (YM). The [^{35}S]-labeled invertase was immunoprecipitated with affinity-purified antiserum. The samples were fractionated on a 10-15% SDS gradient gel, fluorographed with Enlightning and autoradiographed at -70°C. The intense radioactive bands are unglycosylated polypeptides and the core glycosylated products appear as more slowly migrating bands.

		External Invertase Activity	Total Invertase Activity

pS5
```
            M  L  L  Q  A  F  L  F  L  L  A  G  F  A  A  K  I  S  A↓A  S  T  N
..GAATTCACTAACGTATATGATGCTTTTGCAAGCTTTCCTTTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCAGCAAGTACAAAC..
  EcoRI                    HindIII                                 PstI
```
15.4   15.4

pS6
```
    M  T  N
..GAATTCCGCAATGACAAAC...    (signal peptide deleted)
  EcoRI
```
0   15.6

pS7
```
                    M  D  S  K  G  S  S  Q  K  G  S  R  L  L  L  L
..GAATTCCTTCCTGGTGAAGTGTGTTTCTTGAAATCATCACCATGGACAGCAAAGGTTCGTCGCAGAAAGGGTCCCGCCTGCTCCTGCTG
  EcoRI                                NcoI
        L  V  V  S  N  L  L  L  C  S  A↓A  S  T  N
        CTGGTGGTGTGTCAAATCTACTCTTGTGCTCTGCAGCAAGTACAAAC..
                                    PstI
```
2.6   2.7

pS7-H
```
                    M  D  S  K  G  S  S  Q  K  [A] S  R  L  L  L  L
..GAATTCCTTCCTGGTGAAGTGTGTTTCTTGAAATCATCACCATGGACAGCAAAGGTTCGTCGCAGAAAGCTTCCCGCCTGCTCCTGCTG
  EcoRI                                NcoI                      HindIII
        L  V  V  S  N  L  L  L  C  S  A↓A  S  T  N
        CTGGTGGTGTGTCAAATCTACTCTTGTGCTCTGCAGCAAGTACAAAC..
                                    PstI
```
16.6   16.6

pS8
```
        M  D  S  K  G  S  S  Q  K  G  S  R  L  L  L  L  L  L  V  V  S  N  L  L  L  C
..GAATTCATCACCATGGACAGCAAAGGTTCGTCGCAGAAAGGGTCCCGCCTGCTCCTGCTGCTGGTGGTGTCAAATCTACTCTTGTGC
  EcoRI    NcoI
        S  A↓A  S  T  N
        TCTGCAGCAAGTACAAAC..
          PstI
```
6.5   8.3

pS8-H
```
        M  D  S  K  G  S  S  Q  K  [A] S  R  L  L  L  L  L  L  V  V  S  N  L  L  L  C
..GAATTCATCACCATGGACAGCAAAGCTTCGTCGCAGAAAGCTTCCCGCCTGCTCCTGCTGCTGGTGGTGTCAAATCTACTCTTGTGC
  EcoRI    NcoI              HindIII
        S  A↓A  S  T  N
        TCTGCAGCAAGTACAAAC..
          PstI
```
14.2   14.5

pS7-1
```
        M  D  S  K  G  S  S  Q  K  G  S  R  L  L  L  L  L  L  V  V  S  N  L  L  L  C
..GAATTCATCACCATGGACTCCAAAGGTTCTTCTCAAAAAGGGTCCAGATTGTTGTTGTTGTTGGTCGTCTCCAACTTGTTGTTGTGC
  EcoRI    NcoI
        S  A↓A  S  T  N
        TCTGCAGCAAGTACAAAC..
          PstI
```
6.4   6.9

pS7-1H
```
        M  D  S  K  G  S  S  Q  K  [A] S  R  L  L  L  L  L  L  V  V  S  N  L  L  L  C
..GAATTCATCACCATGGACTCCAAAGGTTCTTCTCAAAAAGCTTCCAGATTGTTGTTGTTGTTGGTCGTCTCCAACTTGTTGTTGTGC
  EcoRI    NcoI              HindIII
        S  A↓A  S  T  N
        TCTGCAGCAAGTACAAAC..
          PstI
```
15.0   15.6

Figure 28. Sequence and invertase activities of the bovine prolactin signal peptide-*SUC2* gene fusion.

Plasmid pS5 contained the signal peptide sequence of yeast invertase. The signal peptide was deleted in plasmid pS6. Plasmid pS7 contained the bovine prolactin signal peptide. The introduction of a HindIII recognition site within the signal sequence of prolactin generated a glycine to alanine (boxed) missense mutation (plasmid pS7-H). The 5' untranslated sequence was truncated in plasmids pS8 and pS8-H. Preferred yeast codons (Bennetzen and Hall, 1982) were introduced into the signal peptide coding sequence in plasmids pS7-1 and pS7-1H. The putative signal peptide cleavage site is indicated with an arrow. Invertase activities are expressed in umole of glucose generated per minute for one A_{600} unit of cells at $37^{\circ}C$.

of mRNA secondary structure affecting translation efficiency in yeast. Thus, in the *CYC1* gene, mutations introduced in the early coding region significantly reduced the amount of detectable cytochrome c protein (Baim et al. 1985). This effect was attributed to a diminished translation efficiency, presumably caused by the formation of a stable secondary structure in the coding region of the mRNA. The possibility of stable secondary structure within the mRNA for the signal sequence of bovine prolactin was examined. There is no obvious secondary structure in the mRNA that would be changed by the glycine to alanine codon replacement (Figure 28). Next, the effect of the length of the mRNA leader sequence on translation efficiency was examined. The 5' untranslated sequences of plasmids pS7 and pS7-H were truncated by 29 nucleotides in plasmids pS8 and pS8-H. Plasmid pS8 contained the amino acid sequence of the wild-type prolactin signal peptide, whereas pS8-H contained the glycine to alanine missense mutation at position -18. As shown in Figure 28, a truncated 5' untranslated sequence significantly increased the level of invertase (over two-fold) with the wild-type prolactin signal peptide (compare the invertase activity of pS7 with pS8); only a slight difference in enzyme activity was detected in the plasmid carrying the alanyl variant of the prolactin signal peptide (compare pS7-H with pS8-H). Thus, it appears that the low level of invertase seen with the wild-type prolactin signal peptide was partially caused by the nucleotide sequence in the 5' untranslated region of prolactin mRNA. This effect could be mediated either through mRNA stability or translation efficiency. A bias in codon usage, which appears to correlate with the abundance of the corresponding isoacceptor tRNA species, has been observed in a number of highly expressed yeast genes (Bennetzen and Hall, 1982). To determine whether the presence of preferred yeast codons has any effect on expression, the signal peptide coding sequences of plasmids pS8 and pS8-H were replaced with cassette oligonucleotides containing preferred yeast codons (plasmids pS7-1 and pS7-1H). Plasmid pS7-1 retained the wild-type amino acid sequence of the prolactin signal peptide and plasmid pS7-1H carried the glycine to alanine mutation at position -18. As shown in

Figure 28, no improvement in the expression of invertase relative to that produced with the bovine codons was observed (compare pS7-1 to pS8 and pS7-1H to pS8-H). Finally, the possibility that the glycine to alanine missense mutation at position -18 has a direct effect on the structure of the signal peptide and hence on the level of expression and/or secretion was examined. The probable secondary conformation of the two signal peptides was calculated using the procedure described by Garnier et al. (1978). A higher probability for an α-helical conformation was obtained when the glycine residue at position -18 was substituted with an alanine. Since signal peptides are thought to assume an α-helical conformation (von Heijne, 1984b), it might be argued that the disruption of the α-helix imposed by the glycine residue has altered the ability of the signal peptide to interact with components of the secretory apparatus. The reduced level of invertase produced with the wild-type prolactin signal peptide may be a direct consequence of a delay in the translocation process which exerts a negative influence on the rate of polypeptide chain elongation.

Cassette Mutagenesis of Bovine Prolactin Signal Peptide

As in the case of invertase signal peptide, structural features of the bovine prolactin signal peptide was examined by cassette mutagenesis. The bovine prolactin signal peptide was replaced with the cassette oligonucleotides shown in Figure 10. The design and cloning of the oligonucleotides were as described for the *SUC2* cassettes. All mutants carried the alanine missense mutation at position -18. Based on their ability to secrete invertase, the prolactin signal peptide mutants were divided into two classes (Table VII). Class I (mutants 5-39B to 5-39) has a reduction in the total level of invertase, with almost all of the enzyme activity being secreted. Class II (mutants 12 to 2-5) has a slight

Table VII. Activities of the prolactin signal peptide-invertase mutants.

One unit of invertase activity is defined as one μmole of glucose generated per minute for

one A_{600} unit of cells at 37°C.

```
               -25        -20       -15       -10        -5       -1    Total      External
                                                                        Invertase  Invertase
                                                                        Activity   Activity
pS7-H  M D S K G S S Q K A S R L L L L L V V S N L L L C S A            16.6       16.6
pS7                        G                                            2.7        2.6

5-39B                              I                     T              16.0       16.8
8          A                                   F                        15.1       13.9
4            Q                                       F     Y            13.6       12.7
2-61B                                        A     D                    12.5       12.8
6          Y                       G                       R            12.4       11.9
2          E                                                 A          10.4       10.5
7                      K           I                       G            10.4       10.3
9          Y           T           G           F           G            11.8       14.9
2-61A                    Q                                              11.0       11.1
2-9                    K                                     S          9.7        9.6
2-10                                       L                            8.8        8.9
5-54A      E           E                                                3.6        3.6
2-54       E                           V   G         M                  2.6        2.2
2-61                     Q                   A     D                    2.4        2.3
5-54       E             E         T   V   G         M                  1.6        1.2
5-31                               K   V               Y                1.5        1.5
5-39       E           P E         I                     T              0.9        0.7

12         A                                                Y          9.8        8.9
5          E           P E         M                                   8.8        7.3
6-65       A                                                           6.6        5.0
10           E         R                             D                 6.0        5.3
11         E           P E                                             7.6        6.7
2-5        E                                                 S         2.2        1.6
```

defect in secretion with about 10-30% of the detectable enzyme in the cytoplasm. This class showed a striking similarity to the Class II invertase signal peptide mutant, 4-55B, and suggests a similar mechanism for the deficiency. No mutants like the Class III pre-invertase mutants were observed.

Defects in *SUC2* Signal Mutants and Relationship to Amino Acid Sequence

The series of experiments described in this thesis demonstrate a range of consequences of replacement of residues in the *SUC2* signal sequence. Thus, mutants 4-6 [L(-18)H] and 4-56B [L(-11)D] produce completely secreted invertase in close to wild-type amount whereas mutants 28 [L(-11)P; Q(-16)E; L(-18)N] and M-1 [L(-11)D; L(-18)G] produce completely internal invertase at about 25% of the secreted wild-type level. In general, other mutants lie between these two extremes both in the amount of enzyme produced and in the points in the secretory pathway at which the enzymes accumulate. Thus, while the results will be discussed in terms of the three classes defined earlier, it is clear that these groupings are probably quite arbitrary and that there is a gradation of mutant properties.

Class I Mutants

Quantitative assay (Table III), gel electrophoresis (Figure 15), and Western analysis (Figure 16) show that the characteristic of this group is that it produces fully glycosylated, totally secreted invertase but in amounts that can be as low as 33% of normal. The group includes mutants with amino acid replacements near the signal cleavage (residues -2, -4 and -6) in the hydrophobic region (residue -11) and near the amino-terminus (residue -16, -17 and -18). There is no obvious pattern to the mutations which correlates with phenotype. This confirms earlier observations that qualitatively functional signal peptides have a great amount of sequence variability (von Heijne, 1983; Kaiser et al. 1987). It is clear from the wild-type and the mutant sequences that a basic

residue (arginine or lysine) close to the amino-terminus of the signal is not essential for efficient function. This raises a question as to the reason for the widespread occurrence of such a residue in other signal sequences (von Heijne, 1983) and contrasts with the apparent requirement for such a residue in the bacterial signal sequences (Inouye et al. 1982; Vlasuk et al. 1983).

There are several possible reasons for reduced production of secreted invertase without concurrent production of intracellular enzyme. These include reduced mRNA levels (because of reduced rates of transcription, transport from the nucleus to the cytoplasm or of reduced stability), reduced rate of protein synthesis (due to unfavorable mRNA structure or to presence of unfavorable codons), and reduced protein stability or reduced efficiency of transit through the secretory pathway (coupled with feedback repression of protein synthesis or feedback induction of precursor proteolysis). It is unlikely that variation in invertase levels is due to changes in transcription efficiency since all mutations were introduced downstream of the *ADH1* promoter and its transcription start sites. Furthermore, Northern analysis revealed no correlation between the steady state level of the invertase transcript and enzymatic activity. A change in efficiency of transport of mature mRNA from nucleus to cytoplasm cannot be excluded. While mRNA structural changes affecting translation efficiency cannot be ruled out, there were no obvious increases in the potential for secondary structure resulting from the mutations. Also, no codon bias was shown by those mutants with a high or a low level of expression. Moreover, data from experiments with the bovine prolactin signal peptide indicate that invertase levels were not influenced by the selective usage of preferred yeast codons. Generalized intracellular proteolysis probably is not involved because enzymatically active intermediates have been shown to accumulate intracellularly when signal peptide cleavage and transport to the Golgi complex are hindered (Schauer et al. 1985; Haguenauer-Tsapis et al. 1986). Furthermore, expression of the mutants in a *pep4* strain does not modify the pattern of enzyme production (Table V). Although it is unlikely that amino acid

substitutions at the amino-terminal region, which in some cases involve one or two changes, can render the rest of the protein hypersensitive to proteolytic degradation, the possibility that a fraction of the intermediate may be degraded rather than transported to the cell surface is not precluded. In a recent study by Bachmair et al. (1986), the half-life of a cytoplasmic protein in yeast was found to be significantly affected by its amino-terminal residue (the "N-end" rule). Addition of a small, uncharged amino acid (such as alanine, glycine and serine) to the amino-terminus appeared to confer stability to the cytoplasmic protein, β-galactosidase, whereas addition of a charged (arginine and lysine) or large non-polar residue (tyrosine and phenylalanine) destabilized the protein. Although unlikely to affect secretory proteins that have translocated across the ER membrane, proteolytic degradation could affect the cytoplasmic accumulation of a translocation-defective invertase precursor. Signal peptide mutants from all three classes were analyzed according to this rule. Since all newly synthesized proteins contained a methionine residue at the amino-terminus (one of the stabilizing amino acids in the N-end rule), the analysis was performed on the penultimate amino acid on the assumption that there was removal of the amino-terminal methionine by the sequence-specific methionyl aminopeptidase (Tsunasawa et al. 1985). A stabilizing amino acid was present in most of the mutants. Of those containing destabilizing amino acids, no consistent correlation can be drawn between the presence of a destabilizing amino-terminal residue and the level of invertase or the cytoplasmic accumulation of pre-invertase.

Further characterization of Class I mutants shows that they translocate across the ER membrane more slowly than does the wild-type pre-invertase (mutants 4-56B and 3-13; Figure 22). In the case of mutant 4-56B, the transit of core glycosylated invertase from the lumen of the ER to the Golgi stack is slightly slower than that of the wild-type enzyme without any accumulation of pre-invertase. This could be explained by a reduced rate of cleavage by the signal peptidase. Mutant 3-13 has one of the most extreme reductions in invertase production of the Class I mutants, and it is clear from the pulse-

chase experiments as well as studies on membrane-bound invertase (Figures 18, 19 and 22) that there is a delay in pre-invertase processing at a stage prior to core glycosylation. This could be a consequence of a reduction in the rate of translocation. In fact, the quantitative assays of enzyme activity suggest that 3-13 may accumulate a small but significant amount of intracellular invertase (Table III). Thus, it may lie on the border between Class I and Class II mutants.

The *in vitro* translocation studies with mutant 4-56B (Figure 26) shows that it has a slightly reduced ability to be translocated across the ER membrane. This suggests that there may be an internal accumulation of pre-invertase for this mutant, but that the amount is too little to be detectable in total extracts of cells even though it is detectable in membrane preparations (Figure 19). However, these experiments have not resolved the question of why the Class I mutants (with the exception of 3-13) produce reduced amounts of completely secreted and glycosylated invertase. Perhaps there exists a mechanism in yeast whereby translation of a secretory protein is dependent on its ability to translocate across the ER membrane. This may serve as a means for preventing the synthesis and accumulation of pre-protein in the cytoplasmic compartment. It should be noted that there are several secreted proteins produced by *E. coli* where protein synthesis is coupled to translocation (Inouye et al. 1982; Silhavy et al. 1983; Hall et al. 1983; Vlasuk et al. 1983).

Class II Mutants

Mutant 4-55B, the only invertase signal mutant in this class, has a distinct phenotype in that it produces significant amounts of intracellular and extracellular invertases (Table III). It should be noted that a number of mutants of this class have emerged in the studies on prolactin signal peptide (Table VII). This class of mutants secretes fully glycosylated invertase as well as the more extreme mutants of Class I, but differs in that it accumulates intracellular invertase (Figures 15 and 16). This intracellular

invertase is clearly intact, unglycosylated pre-invertase (Figure 15). Thus, the feedback repression control of intracellular pre-invertase synthesis, postulated to explain the properties of the Class I mutants, may be non-functional for the Class II mutants. Another feature of mutant 4-55B, revealed by pulse-chase experiments, is that processing of core glycosylated invertase to fully glycosylated enzyme is hindered (Figure 23). This correlates with the retention of the signal peptide (Figure 20). However, this delay in transport from the lumen of the ER to the Golgi stack is not as severe as with the signal peptide cleavage mutant reported by Schauer et al. (1985). Thus, the Class II mutant appears to be defective in ER transport and signal peptide cleavage.

Class III Mutants

The third class of mutants showed a complete lack of invertase secretion coupled with an internal accumulation of enzymatically active invertase and a reduction in the amount of total invertase (Table III). The internal invertase exists as a 58 kd unglycosylated precursor (mutants 4-55 and 4-56, Figure 16) or as this form and 70-90 kd core glycosylated enzyme (mutants 28, M-1, M-5, Figure 16). This latter group has these features in common with the Class II mutant, 4-55B. From the *in vivo* pulse-chase experiments (Figure 24), the primary defect in mutants M-5 and 4-55 appears to be the inability of the precursor to translocate across the ER membrane. This results in the accumulation of unglycosylated pre-invertase in the cytoplasm. It is interesting to note that the Class III mutants, M-5 and 4-55, differ from the Class II mutant, 4-55B, by only a single amino acid [L(-18)V in M-5; L(-18)G in 4-55]. Another example of the subtlety of signal peptide sequence or structure on function can be seen by comparing the Class III mutants, 4-56 and M-1, to the Class I mutants, 4-56A and 4-56B. Amino acid substitutions either at the amino-terminal region [L(-17)V, L(-18)N in mutant 4-56A] or within the hydrophobic core [L(-11)D in mutant 4-56B] exerted only a minor defect on

secretion or the level of invertase. However, when combined, as in mutants M-1 [L(-11)D, L(-18)G] and 4-56 [L(-11)D, L(-17)V, L(-18)N], a severe defect in secretion was observed.

In summary, the study of the invertase signal peptide mutants has revealed three types of biochemical defect. These are: (i) reduced production of fully glycosylated and secreted invertase, (ii) accumulation of core glycosylated enzyme which appears to retain the signal peptide and (iii) accumulation of unprocessed pre-invertase. A given mutant may have one or more of these phenotypes. The Class I mutants have predominantly the first defect, the Class II mutant has all three and the Class III mutants have the second and third or just the third type of defect.

Structural and Functional Features of Signal Peptides

Despite a lack of homology at the primary sequence level, signal peptides show certain common features (von Heijne, 1983; Perlman and Halvorson, 1983). First, one or more basic residues are usually found near the amino-terminus. This has been proposed to target the secretory protein to the membrane (Inouye et al. 1982). Second, there is a central lipophilic core consisting of 6-15 consecutive hydrophobic amino acids, which may be essential for insertion into the membrane (von Heijne and Blomberg, 1979). Third, the amino-terminus and the hydrophobic core are predicted to assume an α-helical conformation (von Heijne, 1984b). Lastly, small neutral and/or α-helix disrupting amino acids are often found adjacent to the cleavage site (Perlman and Halvorson, 1983; von Heijne, 1985).

Several conclusions can be drawn that are pertinent to the functional features of yeast signal peptides. First, a large number of signal peptides contain one or more basic amino acids preceding the hydrophobic core. In *E. coli*, this basic residue has been shown to be essential for efficient secretion of exported proteins (Inouye et al. 1982; Hall et al. 1983) and for secretion-translation coupling (Rasmussen et al. 1987). However, the

significance of an equivalent basic residue to yeast secretion is unclear as the wild-type yeast invertase signal does not contain any basic amino acid near the amino-terminus. The replacement of other residues with basic residues, such as in the Class I mutants 25 [A(-6)E, Q(-16)K, L(-18)V], 3-15 [S(-2)P, K(-4)N, Q(-16)K, L(-17)M, L(-18)F] and 3-12 [K(-4)D, Q(-16)K], or with acidic residues, as in mutants 32 [K(-4)R, Q(-16)E, L(-18)G] and 35 [L(-11)H, Q(-16)E, L(-18)F], did not produce any dramatic effect on secretion. In the case of the signal peptide from bovine prolactin, it appears that basic residues play a facilitative role as their replacement with acidic residues has exerted a moderately disruptive influence on invertase levels. When one or two of the three basic residues of the bovine prolactin signal at the amino-terminus were mutated to acidic or neutral residues, a reduction in the steady state level of the enzyme was seen in the Class I mutants 5-54A [K(-19)E, D(-26)E], 5-54 [L(-5)M, V(-10)G, L(-12)V, R(-16)T, K(-19)E, D(-26)E] and 2-61A [K(-19)Q].

Data from the Class III mutants of invertase indicate that, while a basic residue may not be essential, the nature of the amino acids at the extreme amino-terminus can play a role in the secretory process. The Class III mutants, M-5 and 4-55, differs from the Class II mutant, 4-55B [K(-4)Q, A(-6)E, L(-11)R], by a single amino acid [L(-18)V in M-5 and L(-18)G in 4-55]. As shown in Figures 16 and 18, only the unglycosylated form was detectable at all times in mutant 4-55, while both core glycosylated and unglycosylated 58 kd forms were present in mutants 4-55B and M-5. Hence, the replacement of the leucine at position -18 in mutant 4-55B with valine (mutant 4-55) or with glycine (mutant M-5) has resulted in a reduced efficiency of translocation. A similar situation was seen in the Class I mutant, 4-56B [L(-11)D], and the Class III mutants, 4-56 [L(-11)D, L(-17)V, L(-18)N] and M-1 [L(-11)D, L(-18)G]. Mutant 4-56B exhibited an essentially wild-type phenotype on quantitative enzyme assay (Table III) and Western analysis (Figure 16), but has transiently detectable core glycosylated and barely detectable cytoplasmic pre-invertase in the pulse-chase experiments (Figure 22). Thus, a difference of two amino

acids at the amino-terminus of mutant 4-56 [L(-17)V, L(-18)N] and a single amino acid in mutant M-1 [L(-18)G] resulted in a decrease in invertase level (Table III), accumulation of cytoplasmic pre-invertase (Figure 18) and accumulation of core glycosylated enzyme (Figures 18 and 24). Mutant M-1 contained more core glycosylated invertase relative to cytoplasmic pre-invertase compared to that in mutant 4-56. This suggests that the pre-invertase of mutant M-1 [L(-11)D, L(-18)G] is more efficiently translocated across the ER membrane than that of mutant 4-56 [L(-11)D, L(-17)V, L(-18)N].

Second, the most essential feature of signal peptides appears to be the presence of a hydrophobic core of 6-15 amino acids. In *E. coli*, introduction of a charged residue into the hydrophobic core disrupted signal peptide function (Emr et al. 1980; Bedouelle et al. 1980; Inouye et al. 1982; Michaelis et al. 1983a; 1983b). In *S. cerevisiae*, the importance of a hydrophobic core in invertase secretion has been demonstrated by partially or completely deleting this segment (Kaiser and Botstein, 1986; Perlman et al. 1986) and by inserting random DNA fragments coding for short stretches of hydrophobic amino acids (Kaiser et al. 1987). However, mutant 4-56B, which contains an aspartate(-11) residue in the middle of the hydrophobic core, clearly shows that a continuous stretch of hydrophobic amino acids is not a prerequisite for signal peptide function. This is consistent with the observation reported by Kaiser et al. (1987) that, providing a minimum number of hydrophobic residues are present in the center of the signal, the sequence can be interrupted by non-hydrophobic residues. It would be interesting to extend this study through an examination of whether the position of the charged aspartate residue within the hydrophobic core has any effect on signal peptide recognition.

A third feature of signal peptides is the presence of small neutral and α-helix disrupting amino acids in the vicinity of the cleavage site. In *E. coli*, signal peptide cleavage defective mutants contain substitutions clustered between the cleavage site and the preceding 5-6 amino acids (Koshland et al. 1982b; Kuhn and Wickner, 1985). In

addition, cleavage by the *E. coli* signal peptidase, SPase I, can only be demonstrated in peptide fragments containing the carboxy-terminal 5-9 residues of the signal peptide and at least three residues of the mature sequence (Dierstein and Wickner, 1986). In yeast invertase, substitution of the alanine residue at position -1 has been shown to alter the signal peptide cleavage site as well as the rate of cleavage and the rate of transport from the ER to the Golgi stack (Schauer et al. 1985). The accumulation of core glycosylated invertase in the Class II mutant, 4-55B, and the Class III mutant, M-5, may result from a similar reduction in the rate of signal peptide cleavage. The amino acid substitutions in mutant 4-55B are located at position -4 to -11 of the signal peptide [K(-4)Q, A(-6)E, L(-11)R], and extend as far as position -18 in mutant M-5 [K(-4)Q, A(-6)E, L(-11)R, L(-18)V]. If the delay in transit to the Golgi complex is indeed due to a slower rate of signal peptide cleavage, then one can infer that a mutation as far away as 18 residues from the cleavage site can influence signal peptidase function.

Fourth, the diversity in primary sequence amongst signal peptides suggests that the information essential for recognition is likely contained within the secondary or tertiary structure. Analysis of signal sequences in *E. coli* suggests that the maintenance of an α-helical conformation at the central hydrophobic region of the signal peptide is essential for proper function (Emr and Silhavy, 1983). Substitution of α-helix disrupting amino acids, particularly the proline at position -11, may be primarily responsible for the defect seen in mutants 28 and 28*. This is supported by the isolation of an intragenic revertant in which the proline(-11) residue in mutant 28* was mutated to a leucine residue (data not shown). The wild-type residue is leucine(-11). This revertant exhibited a Class I phenotype with a secreted invertase level approximately 1/3 of that with the wild-type *SUC2* signal peptide (pS5). The inefficient translocation of the pre-invertase produced by mutant 3-13 [L(-11)T, L(-18)P] also might be attributed to the disruption of the predicted α-helical conformation by the amino-terminal proline(-18) residue. From the behavior of mutants 28, 28* and 3-13, it appears that the position of the proline residue in the signal peptide

plays a role in determining the severity of the defect and that maintenance of the α-helical conformation through the hydrophobic segment of the signal peptide is particularly important. Disruption of the α-helix may also be responsible for the lower effectiveness of the bovine prolactin signal peptide (plasmids pS7, pS7-1 and pS8). The increased level of invertase observed when the α-helix disrupting glycine(-18) residue was replaced with an alanine residue (plasmids pS7-H, pS7-1H and pS8-H) may be a direct consequence of a stabilized α-helical conformation.

Future Perspectives

The data in this study indicate that the early stages of invertase secretion in yeast are similar to that stated in the Signal Hypothesis. It is likely to involve the recognition of the signal peptide by components present either in the cytoplasm or in association with the ER membrane. Most of the components of the yeast secretory system have yet to be identified. Of particular interest is the yeast equivalent of the mammalian SRP. If the proposed defect for the Class III mutants was correct, it should be possible to isolate genetic mutants capable of suppressing the signal peptide mutation. Since the Class III mutants exhibited a *SUC*⁻ phenotype, one approach is to screen for *trans*-acting suppressors capable of producing a SUC^+ phenotype. Based on our current understanding of secretion in yeast, these suppressors are likely to involve stages in the secretory process preceding events in the ER and may affect the rate of protein translation, rate of proteolysis or degree of translation-translocation coupling. Since vacuolar and plasma membrane proteins also utilize this early secretory pathway, these suppressors are likely to diminish, if not completely abolish, the transport and assembly of these proteins. Thus, the screening procedure would have to be modified to select for conditional lethal mutants or carried out in a diploid strain.

One can also utilize these signal peptide mutants to assay for the biochemical factors involved in co- and post-translational translocation of proteins. The observation

that mutations in the signal peptide can alter the efficiency of co-translational translocation across the yeast microsomal membranes *in vitro* implies that this process is dependent on the interaction of a SRP-like component with features of the signal peptide. It might be possible to isolate biochemical factors which could increase the efficiency of translocation of the Class III mutant pre-invertases. It also would be interesting to examine the effects of the signal peptide mutations on the efficiency of post-translational translocation, and whether the same biochemical factors are required for the two modes of translocation.

The nature of the interaction between the signal peptide and components of the secretory apparatus remains unclear. The data suggest that recognition involves the hydrophobic core, sequences at the extreme amino-terminus and the maintenance of an α-helical secondary structure. Since the mammalian SRP has been purified to homogeneity and shown to interact with invertase signal peptide, perhaps analysis of the binding affinity of SRP for the mutant *SUC2* signal peptides may clarify the structural requirements for signal peptide function.

Appendix A

Oligonucleotide-Directed Mutagenesis

Table I summarizes the mutagenic oligonucleotides and the mutations generated in this study. The length of the oligonucleotides varied from a 17-mer (JN10) to a 22-mer (JN1). Two of the oligonucleotides were used to generate a single point mutation (JN6 and JN12), four to generate a 2 consecutive nucleotide change (JN9, JN11, JN13 and JN14), two to generate a 3 consecutive nucleotide change (JN8 and JN10), one to generate a 4 nucleotide insertion (JN5), and one to delete a 90 nucleotide segment (JN1). The target DNA was cloned in one of two single-stranded vectors: M13mp series and pEMBL series. The choice of vector was strictly a matter of convenience, as no significant difference in mutational frequency was observed. The desired mutation was screened either by differential hybridization with the ^{32}P-labeled mutagenic oligonucleotide or by DNA sequence analysis (Table VIII). Figure 29 shows a representative M13 phage blot and a pEMBL colony blot hybridized with the corresponding ^{32}P-labeled oligonucleotide. In each case, the desired mutation was identified by progressively increasing the stringency of the wash procedure (i.e. higher temperature) until the mismatched mutagenic oligonucleotide had been removed from the matrix-bound wild-type template DNA. Phage DNA retaining the hybridization signal at this point was tentatively considered to carry the desired mutation. Final confirmation was dependent on DNA sequence analysis of this region.

Two different mutagenic procedures were used in this study: the two-primer method (Zoller and Smith, 1982; Norris et al. 1983) and single-primer method on uracil-containing template (Kunkel, 1984). There are several differences between the two procedures, with the most important being the phenotypic selection offered by the latter. The template DNA used in this procedure was isolated from a *dut⁻ ung⁻* strain of *E. coli* (RZ1032). Uracil was incorporated into the DNA in place of thymine due to a high

Table VIII. Summary of oligonucleotide-directed mutagenesis.

| Oligo	Mutagenic Method	Screening Method	% Mutant
JN1	M13; 2-primer	Plaque lift	7.4
JN5	M13; 2-primer	Dot blot	5.6
JN6	pEMBL; 2-primer	Colony hybridization	3.9
JN8	pEMBL; 2-primer	Colony hybridization	6.6
JN9	pEMBL; 2-primer	Colony hybridization	10.5
JN10	pEMBL; 2-primer	Colony hybridization	15.8
JN11	pEMBL; 2-primer; U-template	Colony hybridization	10.5
JN12	pEMBL; 2-primer; U-template	Colony hybridization	21.1
JN13	M13; 1-primer; U-template	Dot blot	25.0
JN14	M13; 1-primer; U-template	DNA sequencing	75.0
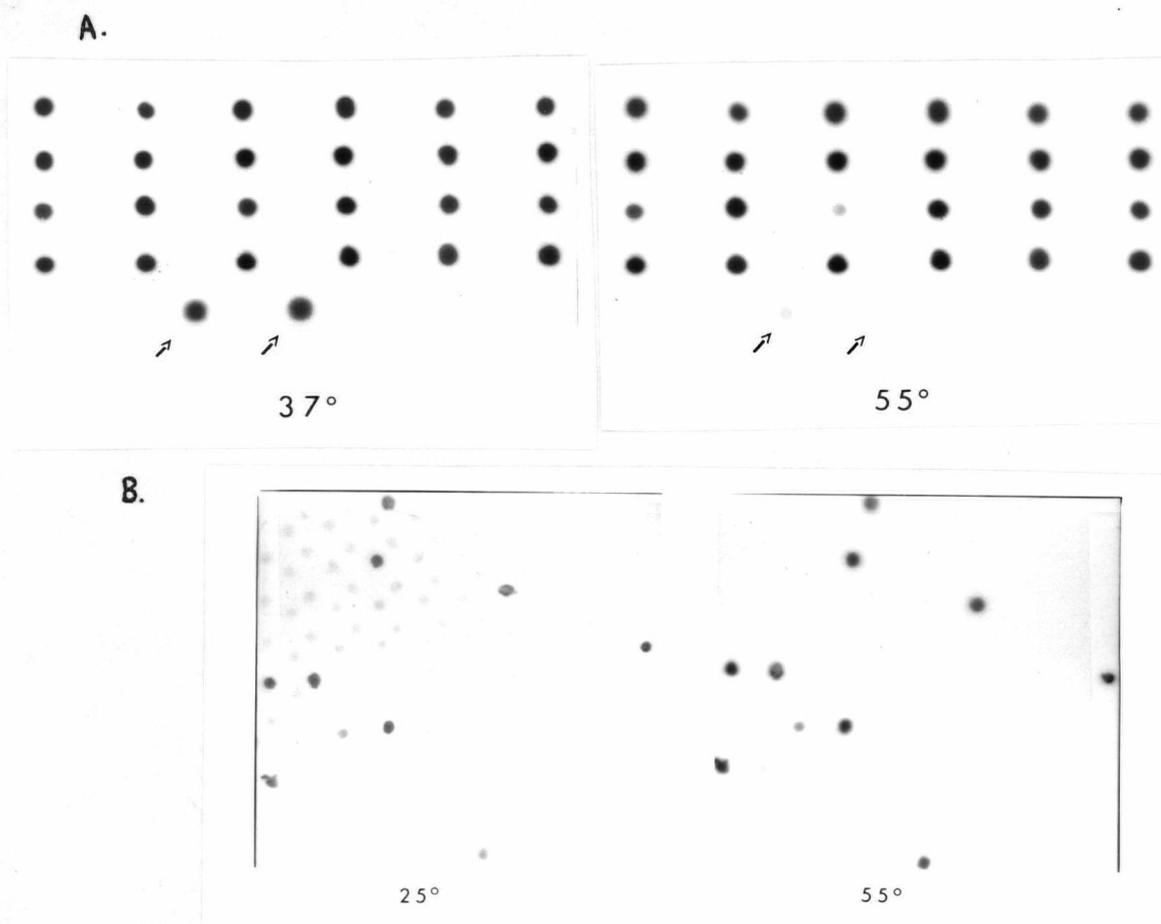
Figure 29. Screening of mutants with oligonucleotide probes.

A. An autoradiogram of a M13 dot blot. M13 phage DNA immobilized on nitrocellulose filter was hybridized with ^{32}P-labeled mutagenic primer, JN1 (panel A). All phage DNAs appeared positive at the lower wash temperature (37°C). Subsequent wash at a higher temperature (55°C) resulted in the removal of the hybridization signal from the wild-type DNA (arrows), but not from the mutant DNA.

B. Screening by colony hybridization. *E. coli* DNA immobilized on the nitrocellulose filter was hybridized with the ^{32}P-labeled mutagenic primer, JN8. The mutant clones retained the hybridization signal after a 55°C wash.

intracellular dUTP pool caused by the lack of dUTPase, and a failure to remove the incorporated uracil as a result of the absence of uracil N-glycosylase. After completion of the *in vitro* reactions, uracil residues were removed from the template strand by transfecting the unfractionated products into a wild-type ung^+ strain (JM101). Apyrimidinic sites, which had an inhibitory effect on DNA replication, were generated upon the removal of the uracil residues by uracil N-glycosylase. Consequently, the template strand was rendered biologically inactive and the majority of the packaged single-stranded DNA must have originated from the newly synthesized complementary strand. Mutational frequencies of 3.9-15.8% were obtained using the two-primer method. In contrast, a mutational frequency of as high as 75% was achieved with the second method (see Table VIII). One major advantage of this higher mutational frequency is that it has made possible the identification and confirmation of the desired mutation in a single step by DNA sequencing.

Variable efficiency in single-stranded DNA packaging was occasionally encountered with the pEMBL vector. In some cases, only infectious IR1 phage particles were recovered from the culture medium. This phenomenon was most prevalent after prolonged storage (1-3 days) of the *E. coli* transformants at 4°C. Although the low yield of packaged single-stranded DNA can be attributed to the acquisition of host cell resistance to IR1 phage infection, the mechanism underlying preferential phage DNA packaging remains unknown. The possibility of a spontaneous mutation within the pEMBL plasmid was ruled out because double stranded plasmid DNA recovered from a packaging-defective clone, when re-transfected into another cell, was able to extrude single-stranded DNA into the culture medium. In practice, this problem was alleviated by isolating the single-stranded DNA from freshly transformed cells or by sequencing the double-stranded pEMBL plasmid.

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