Characterization of the *SEC*18 Gene of *S. cerevisiae*: Identification of a Protein Involved in Yeast Secretion.

Thesis By

Kurt Andrew Eakle

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1989

(Submitted October 1988)

Abstract

*SEC*18 gene function is required for secretory protein transport between the endoplasmic reticulum and the Golgi complex. We have cloned the *SEC*18 gene by complementation of the *sec*18-1 mutation. Deletion/disruption of this gene has shown that *SEC*18 is essential for yeast cell growth. Sequence analysis of the gene revealed a 2271 bp open reading frame which would code for a protein of 83.9 kd. The predicted protein sequence showed no significant homology to other known protein sequences. *In vitro* transcription and translation of *SEC*18 led to the synthesis of two proteins of approximately 84 and 82 kd. Antisera raised against a Sec18-β-galactosidase fusion protein, detects two proteins from *in vivo* ^{35}S labeled yeast cells identical in size to those seen by *in vitro* translation. Although potential sites for N-linked glycosylation are present in the Sec18p sequence, the sizes of the *in vivo SEC*18 gene products are unaffected by the drug tunicamycin. Hydrophobicity analysis indicated that the protein is hydrophilic in nature and lacks any region that would be predicted to serve as a signal sequence or transmembrane anchor. These results suggest that the Sec18p resides in the cell cytoplasm. Pulse-chase experiments indicate that the two forms of Sec18 protein are not the result of post-translational processing. Mapping of the 5' end of the *SEC*18 mRNA revealed only one major start site for transcription, which indicates that the multiple forms of Sec18 protein do not arise from mRNAs with different 5' ends. We suggest that translation initiating at different in-frame AUG start codons is likely to account for the presence of two forms of the Sec18 protein. While cell fractionation studies show that the Sec18p are not associated with ER or Golgi compartments, association with a 100,000 $\times g$ pellet fraction has been observed suggesting that Sec18p may bind transiently to small vesicles such as those presumed to participate in ER to Golgi transport.

TABLE OF CONTENTS

Introduction

As little as 30 or 40 years ago, a "yawning gulf between morphology and biochemistry" (1) existed in our understanding of biology. Living organisms could be described in terms of the morphologies and behavior of cells and organs, or the chemical makeup of cell constituents and the biochemical reactions that took place. The biochemistry of the synthesis of many cellular components such as amino acids, proteins and lipids was understood in detail. Yet one could not begin to tell how these pieces were assembled into functional units. While subcellular organelles could be observed with the light microscope, it was virtually impossible to assign particular functions to an organelle or to determine the ways in which organelles interacted. Many of the important activities of the cell, such as locomotion, cellular division, and the assembly and function of intracellular organelles, lie in this gap of our understanding of events on a molecular level and a morphological level.

Eukaryotic cells contain a number of morphologically and biochemically distinct subcellular organelles. These compartments provide the cell with different environments which are specialized for performing particular cellular functions. Many of the important processes in eukaryotic cells depend as much on the structural organization of the cell as they do on the chemical reactions involved. For example, the generation of ATP by mitochondria is dependant as much upon the structural features of the mitochondria which form separate compartments with different pHs, as the enzymatic reactions of electron transport and the chemiosmotic generation of ATP. The production of enzymes that are potentially deleterious to the cell, such as lysosomal proteases, again requires that they be segregated in a separate compartment before they are activated. The ordered nature of biochemical events, such as the generation of complex patterns of protein gylcosylation, means that substrates must be exposed to a progression of different compartments each containing dif-

ferent enzymes. In all of these cases, the cell's ability to generate different environments with specific sets of proteins is crucial to the functional process. This raises a daunting question for cellular biologists, namely, how are the components of each compartment directed to their correct locations within the cell?

Much of what we know about the process of protein localization has developed from the study of how proteins are secreted from eukaryotic cells. Certain cells in higher eukaryotes, such as the exocrine cells of dog pancreas and liver, are highly specialized for the production and extracellular secretion of large amounts of particular proteins. Yet this posed a fundamental problem - these proteins were unable to cross cellular membranes spontaneously (at least in their secreted form) while protein synthesis was known to be a cytoplasmic process. The initial characterization of the secretory pathway revealed that proteins were extruded across the membrane as they were being synthesized, and passed through multiple distinct subcellular compartments before their release from the cell by exocytosis. This description was a product of the correlation of information from multiple levels: correlating morphological and molecular information by following the progress of newly synthesized proteins through intracellular structures using electron microscopy and autoradiography; correlating biochemical and morphological information by the characterization of the chemical activities associated with subcellular structures isolated by cell fractionation.

In my view, science is driven by the techniques and methodologies available for looking at a problem. While a large part of science involves the application of established techniques to a spectrum of subjects, rapid progress in understanding comes with the development of new techniques for addressing a problem. At the same time, it is a truism that what we see is a function of how we look; that the limitations of techniques themselves play a part in what is observed and must be carefully considered in the experimental interpretation. We live in a time where rapid advances in technology have led to equally great advances in

our understanding of biology. Yet, this advance also places greater burdens on the scientist, who must be able to utilize an ever greater spectrum of techniques, and evaluate and correlate an ever wider range of information. Even so, it is still true that "gaps" remain between our ability to characterize components on different levels - molecular, biochemical and morphological - and our ability to correlate the information so as to understand the functional significance of these different pieces.

This study of the *SEC*18 gene illustrates that this problem is still the basic challenge of our science. The *SEC*18 gene was first isolated as a genetic lesion that caused a temperature sensitive block in the cellular process of protein localization through the traditional secretory pathway of eukaryotic cells. With the tools available to us, we have been able to isolate this gene and characterize both the gene and its protein product in great detail, i.e., the DNA sequence of the gene, the start and stop positions of the mRNA transcribed from the gene, and the amino acid sequence and general characteristics of the proteins made by this gene. These tools have also allowed us to examine certain aspects of the Sec18 proteins *in vivo*, their production by the yeast cell, and something of their intracellular location. Likewise, other studies have been able to characterize many facets of the overall process of cellular secretion. Only recently, however, have techniques become available to specifically address the nature of the vesicular transport of proteins from the endoplasmic reticulum to the Golgi apparatues. Thus, a real understanding of the functional role of the *SEC*18 gene product and the mechanisms that drive secretory transport, still lies in this "gap" where we cannot yet see. Rapid progress on many fronts, however, suggests that this will not long remain true.

Reference.

1. de Duve, C. Exploring cells with a centrifuge. Science 189:186-189 1975.

Chapter 1: Protein Secretion

The pathway of organellar traffic, which eukaryotic cells use to secrete proteins from the cell, was worked out in the 1950s and 1960s, largely by George Palade and co-workers (1-6). By detailed microscopic examination of pancreatic exocrine cells, which are highly specialized for the production and secretion of proteins, they discovered that secretory proteins follow a defined ordered pathway through a number of distinct subcellular structures. Using a combination of electron microscopy and autoradiography, they found that the radiolabeled secretory proteins, identified by exposed silver grains, first appeared in the lumen of the endoplasmic reticulum (ER). Pulse-chase experiments showed that these proteins were later passed to the Golgi complex, and finally to secretory or zymogen granules before being secreted from the cell. These results were later confirmed by subcellular fractionation of cells, and characterization of the covalent modifications of secretory proteins that take place as they pass through the secretory organelles.

In contrast to cytoplasmic proteins, which are synthesized on free ribosomes, secretory proteins are synthesized on ribosomes tightly bound to the endoplasmic reticulum (ER) of the cell. The ER is a membrane-bound organelle that forms networks of flattened vesicles or tubules, some studded with ribosomes on their cytoplasmic surface (rough ER) and some without (smooth ER). In addition to being a major site of protein synthesis within the cell, the ER is also the principal site of membrane phospholipid synthesis and assembly (7). At the ER, secretory proteins are co-translationally translocated across the membrane and released into the lumen. From the ER, proteins transit to the Golgi apparatus by the formation and transfer of vesicles. Covalent modifications to the structure of secretory proteins, particularly the addition of carbohydrates, occur in both the ER and Golgi. The Golgi apparatus is a series of stacked disc-shaped organelles that are ordered from the *cis* side where proteins from the ER enter, to the *trans* side where they are sorted and packaged

for export. In exocrine cells, secretory proteins are stored intracellularly in the form of condensed zymogen granules, and finally discharged from the cell by exocytosis.

The processes involved in this basic pathway of secretion have come to be recognized as important not just for the secretion of a few proteins from higher eukaryotic cells, but as basic processes in the localization of proteins to many destinations inside and outside of all cells. A closer examination of what is known about the individual steps in this pathway shows that while much is known about the basic pathways followed by secretory proteins, little is known about the molecules that form the machinery guiding protein localization and how they function.

Targeting of Proteins to the Secretion Pathway.

The initial step in protein localization involves the specific targeting of secretory proteins to the proper membrane and initiating the process of protein transfer across the lipid barrier. Secretory proteins are synthesized selectively on ribosomes tightly bound to the ER membrane (8). Release of these ribosomes requires both high salt and puromycin, a drug that terminates protein synthesis and breaks down the structure of the translating ribosome. This suggests that ribosomes are held in place both by ionic interactions with the membrane and by the nascent chain of the translated protein being threaded across the membrane. What is the signal that directs secretory proteins to the ER membrane?

In vitro translation of mRNA for the light chain of immunoglobulins showed that they encoded a protein that was larger than the mature form secreted from the cell (9). This precursor had an amino acid extension at its amino terminus. Blobel and Dobberstein showed that both the precursor and mature forms of Ig light chain could be observed in extracts from the completion of nascent chains formed on polysomes isolated from detergent-treated rough microsomes (10). In contrast, only mature proteins were observed during

translation in the presence of intact microsomes. This showed that the extra amino acids at the amino-terminus of the precursor form of the protein were proteolytically removed even before the completion of protein synthesis. This amino terminal extension was dubbed the signal sequence (10), and was hypothesized to be a unique amino acid sequence, which was responsible for attachment of the ribosome to the ER. The formation of a membrane pore was postulated through which the nascent chain passed during transfer into the lumen of the ER.

The presence of an amino terminal extension on the mature domain of a secreted protein, which functions in targeting the protein to the proper membrane and is then proteolytically removed after membrane insertion/translocation has begun, has become a general rule for secretory proteins. However, far from being a unique sequence of amino acids, which would be recognized by a specific receptor, signal sequences have been shown to vary widely in their primary amino acid sequence (11,12). Two general structural motifs have come to be recognized (13) - signal sequences that target proteins to the ER in eukaryotes and the plasma membrane in prokaryotes, and leader peptides that target proteins to mitochondria and chloroplasts.

Signal Sequences.

The first motif is that of the signal sequence, which is found on secretory and integral membrane proteins in both prokaryotes and eukaryotes. In prokaryotes, this signal serves to mark a protein for insertion into the inner plasma membrane of the cell, and initiates the process of translocation across the membrane for periplasmic and outer membrane proteins. (see review 11) In eukaryotes, the signal sequence is present not only on secreted proteins, but also on proteins that follow the secretory pathway to a number of intracellular organelles including the ER, Golgi, lysosomes (14) and integral membrane proteins of the cell's plasma membrane (15). The common nature of the eukaryotic and prokaryotic signal sequence has been demonstrated in a number of ways. When eukaryotic secreted proteins

are expressed in prokaryotes, they are targeted to the inner membrane and often secreted (16,17). Likewise, when prokaryotic secreted proteins are expressed in eukaryotes, they are targeted to the lumen of the ER. In addition, signal sequences of prokaryotic proteins have been shown to interact with purified components of the mammalian signal sequence recognition machinery (18).

Fusion of DNA segments coding for various domains of cytoplasmic and secretory proteins to create hybrid genes, has been an important tool for investigating the targeting properties of particular protein sequences (19). The fusion proteins produced from such hybrid genes can be localized either by the enzymatic activity or immunological properties of one of the protein domains. Fusion proteins comprised of a eukaryotic signal sequence fused to a prokaryotic enzyme (or vice versa) are targeted to the inner membrane when expressed in prokaryotes or to the ER when expressed in eukaryotes (17), again demonstrating the functional homology between these segments. In a few cases, mature domains of a hybrid protein appeared to be necessary for signal sequence function (21,22). These can now be explained by improper folding of the hybrid protein so as to mask the signal sequence. In most cases, however, the signal sequence regions have been shown to be both necessary and sufficient for membrane targeting.

The basic structure of the signal sequence contains three regions (12). At the amino-terminus, there are usually 2-4 positively charged amino acids that may interact with the negatively charged head groups of membrane phospholipids. This region is followed by a central core region of at least 9 hydrophobic amino acids that appears to be most critical to signal sequence function. Finally, the C-terminal region of the signal sequence contains more polar amino acids and defines a site for the cleavage of the signal sequence to form the N-terminus of the mature protein. A great deal of effort has gone into characterizing the important features of the signal sequence, by the selection of mutants which block export and further selection of pseudorevertants that restore export (22,23). The overwhelming majority of mutants and pseudorevertants obtained were found to be altered in the signal

sequence region. Insertion of random sequences in place of the signal sequence of the yeast periplasmic enzyme, invertase, indicates that a wide variety of primary sequences will suffice if the structural motif of a signal sequence is maintained (24). In particular, the hydrophobic core region of the signal sequence appears to be critical for function. A minimum signal sequence seems to require a hydrophobic domain of at least 9 amino acids in length, with no charged residues, that is able to fold into a stable alpha helical conformation. The importance of this structural motif may reflect a functional role that the signal sequence plays in the insertion/translocation across the membrane barrier. Both chemically synthesized signal peptides, and precursor secretory proteins which still have their signal peptide region, have been shown to spontaneously insert into and in some cases partially cross lipid bilayers (25, 26). Also, the lack of specific sequence requirements may reflect the diversity of proteins which need to be inserted into or translocated across the membrane, and thus the need for a generalized recognition system rather than a specific receptor.

Leader Sequences.

The second structural motif of an amino terminal protein extension, which directs membrane targeting, is that of the leader peptide regions of mitochondrial and chloroplast proteins that are encoded by nuclear genes and subsequently imported into the organelle. Like signal sequences, sequence comparisons of the leader regions for a number of these proteins do not reveal primary sequence similarity, however they are able to form similar secondary structures (27). The structural motif in this case, is that the leader region forms an amphiphilic alpha helix with hydrophobic amino acids on one face and positively charged residues on the other. Here too, mutational studies and the insertion of random sequences has demonstrated the importance of maintaining the structural motif rather than demanding a particular sequence of amino acids (28,29). The functional homology between mitochondrial and chloroplast leader sequences has been demonstrated by the fusion

of a chloroplast leader sequence to both a cytoplasmic protein (DHFR) or a mitochondrial protein (Cox IV) (30). In both cases, the chloroplast leader was able to direct targeting to the mitochondrial matrix (DHFR) or inner membrane (COX IV), although with reduced efficiency. This targeting raises interesting questions of how proteins produced in the cytoplasm are correctly sorted to mitochondria and choloroplasts, which are both present in plant cells (see 31). The evolutionary model that chloroplasts and mitochondria both evolved as intracellular symbionts (32) may shed light on why they share a similar type of targeting signal.

Chemically synthesized leader peptides have also been shown to insert spontaneously into lipid bilayers (33). Like signal sequences, this suggests a functional role for the leader peptide regions in targeting to the mitochondrial or chloroplast membrane and initiating import. However, the fact that this structure is very different from that of ER signal sequences suggests that there may be basic differences in the mechanisms involved in import in these two systems. Both mitochondria and chloroplasts have very complex membrane topologies, and an imported protein may need to cross a membrane barrier two or three times to arrive at its proper destination. Protein import at contact sites between inner and outer membranes is thought to accomplish this feat (34). In a similar vein, systems of protein export directly from the cytoplasm of bacteria to the extracellular medium through both the bacterial inner and outer membranes, are beginning to be characterized (35). It is possible that the mitochondrial import system evolved from the reversal of a direct protein export system in their bacterial ancestors. Further characterization of these systems may shed light on the evolutionary origins of mitochondria and chloroplasts.

Variability in Membrane-Targeting Signals.

While a cleaved amino-terminal signal or leader sequence is the most common targeting signal for membrane translocation, there are variations of this signal that function in both prokaryotic and eukaryotic export. In some cases, the N-terminal signal sequence is not

proteolytically removed and the protein becomes anchored in the membrane rather than translocated across, as in the case of influenza neuraminidase (36). A wide variety of integral membrane proteins are known to initially insert in the ER membrane and are then transported to the cell surface via the secretory pathway (37). Some secretory proteins, such as ovalbumin, lack an amino-terminal signal sequence, but are secreted none the less (38). Here, a signal sequence-like region internal to the mature domain of the protein is responsible for membrane translocation (39,40). It is interesting to note that the placement of a signal sequence type region internal to a protein can, in some cases, allow the transfer of domains on both sides of the signal sequence (e.g., ovalbumin), while in other cases only domains C-terminal to the region are translocated across the membrane (see 41). There are no cases where an internal signal is known to direct the translocation of protein domains only N-terminal to that signal. The presence of charged residues next to the signal sequence may be important in determining which domains are translocated. Another possibility is that this "sidedness" results from the folding of N terminal domains into translocation-incompetent forms before the translocation signal emerges from the ribosome, while initiation of translocation may occur before a C terminal domain has a chance to fold up completely. Translocation of protein domains on only one side of the signal results in the insertion of a protein into the membrane with a specific membrane orientation (42).

Structural Signals that Determine the Membrane Topology of a Protein.

The precise topology with which an integral membrane protein is inserted into the lipid bilayer is often critically important for function (see reviews 11,37). A protein may traverse the membrane multiple times, with functional domains on both sides of the lipid bilayer. For example, the band III anion transport protein of erythrocytes has a large cytoplasmic domain at its amino terminus and is anchored in the membrane by carboxy-terminal domains that cross the membrane multiple times (43). Complex topologies such as this are thought to be generated by structural motifs within the protein, which govern its insertion

into the membrane (44). Stop-transfer sequences were originally postulated by Blobel and Dobberstein (10) to interact with the proposed translocation pore and cause its dissociation, leaving the protein anchored in the membrane with an untranslocated C-terminal tail. However, the N-terminus of the protein can also remain on the cytoplasmic side of the membrane if translocation is initiated by an internal signal sequence. In this case, a later stop-transfer sequence would give a protein that crosses the membrane twice with both termini on the cytoplasmic side. For proteins that cross the membrane multiple times, a succession of signal sequences and stop-transfers can generate any desired topology (45). Signal sequences in this context have often been considered as insertion sequences, that appear not to require cellular machinery to insert and initiate translocation, as opposed to the original signal sequences that do require cellular machinery (44).

The nature of topology signals such as insertion sequences and stop-transfer sequences is unclear. In both cases, these sequences are highly hydrophobic and lack charged amino acids in their hydrophobic domains, similar to signal sequences (although they may be slightly longer in length than signal sequences). Like signal sequences, insertion signals and stop-transfer domains are often flanked by positively charged amino acids. Here too, it appears that maintaining a structural motif like the signal sequence is more important than the primary sequence of amino acids. The hydrophobic nature of these signals suggests that they function by directly interacting with the hydrophobic domain of the lipid bilayer, either by halting further transfer through the membrane or re-inserting naturally into the membrane. Recent work suggests that the exact function of such sequences may be due in part to the context in which they are seen, and may depend on whether translocation has been previously initiated or not (41,46,47). In one case, the C-terminal transmembrane segments that normally serve as stop transfer signals, were found to function as internal signal sequences when they replaced this region in the human transferrin receptor (46). In another study, hybrid proteins were generated using the stop transfer region from the membrane form of IgM, and hydrophilic domains of B-globin, lactamase (with and without

its signal sequence region), and prolactin in various ordered combinations (41). They found that the stop transfer region would halt translocation of a hybrid protein previously initiated by an amino-terminal signal sequence. However, if no signal sequence was present on the hybrid protein, the stop transfer region would serve as an internal signal sequence causing protein domains on both sides of the region to be translocated across the membrane. In a similar fashion, hydrophobic domains in the targeting regions of imported mitochondrial proteins are thought to interrupt transfer and thus sort proteins between the inner and outer mitochondrial membranes (34). In general, hydrophobic domains could be thought of as on/off switches for translocation, either causing the nascent chain to interact with the membrane and engage the machinery to initiate translocation, or causing the nascent chain to disengage from the machinery and halting translocation.

The Membrane Translocation Machinery - Protein and RNA Components That Function in Secretion.

Some proteins, such as bacterial toxins and the components of the complement pathway, are able to spontaneously insert into or cross biological membranes (11). The insertion of these proteins requires neither energy nor a cellular machinery for insertion/translocation. However, with the exception of these few proteins, the lipid bilayer is an absolute barrier that proteins are unable to cross spontaneously. In general, the process of the insertion of proteins into membranes and/or the translocation of proteins across membranes, is an active process that requires a source of biological energy either in the form of ATP or an electrochemical gradient across the membrane. This process has been shown to require a translocation machinery composed of both cytosolic and integral membrane components. A number of proteins have been identified, which form part of the cellular translocation machinery in various systems. Still others have been inferred from the

sensitivity of unfractionated components in *in vitro* translocation assays to agents that modify or degrade proteins. In some instances, an RNA species has also been shown to be an integral part of a component of the translocation machinery.

The original observations of the process of secretion in pancreas or liver cells showed that secreted proteins are synthesized selectively on ribosomes tightly bound to the ER membrane. Blobel and Dobberstein were able to reconstitute the process of membrane translocation *in vitro* (48). By adding stripped ER microsomes at various times after translation was initiated, they showed that the process of translocation was obligately co-translational in this system. That is, if membranes were added too late after protein synthesis had begun, yet still before synthesis of the nascent chain was completed, proteins would remain outside the lumen of ER microsomes. This led them to propose that when the signal sequence emerged from the ribosome, it was recognized by a receptor protein in the membrane of the ER that caused the formation of a transient proteinaceous membrane pore through which the nascent chain passed. They theorized that the process of passing the nascent chain across the membrane barrier was driven by the energy of protein elongation from the ribosome that is tightly coupled to the membrane.

The Signal Recognition Particle and Its Receptor.

A detailed picture has emerged in the past few years of the initial steps of targeting and transport across the ER membrane in higher eukaryotes, including the isolation and biochemical characterization of some of the cellular components for this process. As the signal peptide of the targeted protein emerges from the ribosome, it is recognized by a macromolecular complex known as the signal recognition particle (SRP). The SRP is a macromolecular complex of 6 peptides and a 7S RNA, which was first isolated from a salt wash of microsomal membranes from dog pancreas (49). The SRP binds selectively to ribosomes synthesizing a secreted protein (50). In early work using a wheat germ translation extract, the SRP was shown to cause the process of protein translation to arrest (51). Later

studies have shown that translation arrest is peculiar to the combination of mammalian SRP and wheat germ ribosomes (52); SRP present in reticulocyte or HeLa cell-free systems does not arrest translation of secretory proteins. However, translation arrest by SRP in the wheat germ system has been an important assay for the purification of the SRP and its receptor.

After binding to ribosomes synthesizing secretory proteins, the SRP mediates their binding to the membrane of the ER (53), by interacting with a 72 kd integral membrane protein present in the ER, known as the docking protein (54) or the SRP receptor (55). In this system, the ribosome is unable to bind to the ER membrane or translocate the nascent chain without the presence of SRP and its receptor. Interaction of the SRP with its receptor removes the translational arrest by releasing both components from interacting with the ribosome (56). At the same time, the ribosome becomes bound to the ER membrane, and insertion and cotranslational translocation of the nascent chain proceeds. An integral membrane protein has been tentatively identified by photo-crosslinking between the signal sequence of a nascent chain and the protein, which may function as a receptor for the signal sequence during membrane insertion (57). The endopeptidase activity responsible for removing signal peptides from the mature domain of the protein has also been purified (58). It consists of a membrane protein complex that contains six polypeptides between 12 and 25 kd, two of which are glycosylated. The subunit responsible for signal peptide cleavage has not yet been identified.

The structure of the SRP and its receptor have been extensively studied, along with the interactions between the ribosome and the SRP, and the SRP and its receptor. The SRP itself has been dissected into different functional components by disassembly of the complex into its individual molecules, chemical modification of these components, and subsequent assay of the reassembled particle for its different activities (59). The SRP complex has 6 different polypeptides: monomer proteins of 19 kd and 54 kd, heterodimers composed of a 9 kd and a 14 kd polypeptide, and a 68 kd and a 72 kd polypeptide (49). In

addition, there is a 300 nucleotide RNA that is identical to the small cytoplasmic 7SL RNA, a relatively abundant small RNA species found throughout the cell (60). The RNA is essential for SRP activity and serves as the structural backbone of the SRP (61). The RNA has a domain that is homologous with the Alu-family of repetitive sequences (62). Digestion of the SRP with micrococcal nuclease removes the Alu-like domain along with the 9/14 heterodimer. This subparticle no longer arrests elongation, but is able to promote translocation across the membrane if it is present early in translation (63). The 54 kd protein has been implicated in the recognition of the signal sequence, since it has been shown to cross link to the signal sequence of the nascent chain of preprolactin by photoaffinity labeling (64). In addition, chemical modification of the 54 kd protein by alkylation leads to an SRP that fails to recognize the signal sequence (65). Similar alkylation of the 68/72 kd heterodimer gives an SRP that can arrest translation of the secreted protein, but no longer interacts with the SRP receptor and thus fails to mediate translocation (65).

The SRP receptor was first identified as a 60-kd protein fragment that could be removed from microsomal membranes by limited proteolysis and high salt (66). Addition of this purified fragment to stripped membranes showed that it could reconstitute translocation across the ER membrane. This fragment represents the cytoplasmic domain of a 72 kd integral membrane protein of the ER (67). This protein has been shown to bind to the SRP, release its elongation arrest of secretory protein translation (54), and promote the co-translational translocation of the nascent chain. The SRP receptor has been cloned and sequenced (55), and contains regions with a high density of basic amino acids that could possibly interact with nucleic acid. Other components of an SRP receptor complex have been tentatively identified by their co-purification with the SRP receptor (68), including a 30 kd protein subunit that is tightly associated with the receptor.

The SRP was first purified as a peripheral membrane complex associated with the ER membrane of dog pancreas cells (49). However, the SRP complex is also present in the post-microsomal supernatant of dog pancreas, and is found cytoplasmically in rabbit

reticulocyte *in vitro* translation extracts (54,69). Thus, the SRP is thought to cycle on and off the ER membrane, first binding ribosomes synthesizing secretory proteins, then interacting with the SRP receptor at the ER membrane and being released (70). SRP receptor, on the other hand, does not interact directly with the ribosome. Instead it binds to the SRP complex on the ribosome (56). In the initial studies, interaction of the SRP with its receptor was required to release elongation arrest and resulted in displacement of the SRP from the ribosome (56).

Despite the intense characterization of the SRP and its receptor, it is unclear whether they play functional roles in the insertion of the signal peptide into the membrane, and in the initiation of the process of translocation. It is possible that SRP mediated targeting serves primarily to selectively target secretory proteins to the ER membrane with high fidelity. For a long time, the translation arrest caused by SRP was thought to be an integral part of the mechanism of translocation, coupling the process to that of translation and allowing the energy of protein elongation to drive the nascent chain across the membrane. However, removal of the Alu-domain of the SRP showed that elongation arrest was not required for protein translocation (71). Later studies showed that elongation arrest is an artifact of the interaction of mammalian SRP with the wheat germ translation system (52,63). The demonstration that translocation can occur post-translationally in another eukaryotic system (yeast - 72,73) suggests that translocation into the ER is not obligately co-translational, and that the energy driving translocation comes from the hydrolysis of ATP (73,74). Termination of translation and release of the nascent chain from the ribosome removes the ability of proteins to be post-translationally imported into mammalian microsomes (75,76). This suggests that SRP mediated targeting to the ER is still required in higher eukaryotes even if elongation of the nascent chain is complete. Translocation into dog pancreas microsomes in a post-translational fashion is possible if sufficient dithiothre-

itol is present in the translation reaction (77)[1]. Thus, protein folding and the formation of disulfide bonds may be the major barriers to post-translational translocation in the *in vitro* mammalian system.

Is SRP Mediated Targeting Present in Lower Organisms?

The similar nature of prokaryotic and eukaryotic signal sequences, and their ability to functionally substitute for one another both *in vitro* and *in vivo*, suggests that components similar to the SRP and SRP receptor should be present in these systems. However, homologous proteins to the SRP and SRP receptor have yet to be identified in either prokaryotic secretion, or ER targeting in lower eukaryotic organisms such as yeast[2]. SRP mediated targeting may have evolved from simpler systems seen in bacteria and yeast, as the need for cells specialized in the production and secretion of proteins evolved in higher organisms. The high level of secretory protein production in these cells may have evolved beyond the cell's tolerance for mis-targeted proteins, necessitating a more stringent targeting system. One piece of evidence that SRP targeting may differ from targeting in prokaryotic secretion, is that mutant signal sequences that greatly delay protein export in *E. coli* are recognized and translocated in an SRP-dependent system with the same efficiency as the wild-type signal sequence (18). This suggests that SRP mediated targeting may be specialized to more closely couple the processes of translation and translocation, perhaps to give the cell a greater tolerance for signal sequence mutations. Once in place, this system

---

[1] The authors suggest that such a reducing environment more closely resembles in vivo conditions created by the high concentration of glutathione in the cellular cytoplasm (77).

[2] One recent report suggests that an RNA homologous to the 7SL RNA of the SRP has been identified in *Schizosaccharomyces pombe* (78).

also could relieve the cell's requirement for cytosolic factors which keep the nascent chain from folding into translocation incompetent forms. A variety of such factors, many of that are related to the heat shock family of proteins, have recently been demonstrated to be present throughout the cell and appear to have roles in translocation and protein assembly (see below).

Other Membrane Components.

The mechanism of how the ribosome is bound to the ER membrane, and how translocation is initiated remains unclear. Two ER transmembrane glycoproteins called ribophorin I and II have been characterized by cross-linking to 80S ribosomes (79). This may be artifactual, however, since protease treatment of microsomal membranes suggests that protein translocation activity is abolished long before ribophorins are subject to significant degradation (80). In these studies, *in vitro* translocation could be reconstituted by adding back the cytoplasmic fragment of the SRP receptor to ER membranes that had been stripped of this component. Further controlled proteolysis of these stripped membranes abolished the ability of the cytoplasmic fragment of SRP receptor to bind to the membranes and restore translocation. In addition, treatment of stripped membranes with N-ethylmaleimide resulted in membranes that were still capable of binding the cytoplasmic SRP receptor fragment and ribosomes, but were unable to initiate translocation (80). These results suggest that other components of the translocation machinery, particularly integral membrane components, remain to be identified. Whether these components form a pore with an aqueous channel for the translocation of the nascent chain, or function in unfolding the nascent chain and assisting its passage directly through the membrane remains to be determined. Characterization of these components will greatly clarify the biophysical mechanism(s) used in membrane translocation.

Translocation in Yeast.

Recently, *in vitro* systems of protein translocation into yeast ER microsomes have been established using protein translation extracts from wheat germ (73) and yeast (73,74,81). In contrast to the higher mammalian system, post-translational translocation of secretory proteins was easily demonstrated. Translocation into yeast microsomes was independent of the presence or absence of canine SRP. Furthermore, yeast ER microsomes were unable to release the translational arrest imposed by mammalian SRP on the wheat germ translation system. These results suggest that components homologous to SRP and SRP receptor are not present in yeast (73). Translocation required the hydrolysis of ATP (73,74), while ionophores and uncouplers of membrane potential had no effect (72,73). Translocation activity of yeast microsomes was sensitive to proteolysis and alkylation by N-ethylmaleimide (73), indicating that integral membrane proteins of the yeast ER were required.

A requirement for cytosolic components for translocation into yeast ER has also been demonstrated (82). These components are sensitive to protease, alkylation and heat treatment, but not to RNase (82). At least some of these soluble factors appear to be common to both prokaryotes and eukaryotes, since post-ribosomal supernatants from either *E. coli* or *S. cerevisiae* can stimulate post-translational translocation across both yeast microsomes and inverted vesicles of the *E. coli* plasma membrane (83). Purification of one of these cytosolic factors revealed that it consists of two constitutively expressed yeast proteins, which are both 70 kd in size (84). These proteins are identical to members of the hsp70 heat shock proteins (hsps) in yeast (84).

Heat Shock Proteins Modulate Folding During Translocation.

Heat shock proteins form a large family of genes, which are highly conserved throughout evolution in both their protein-coding and regulatory sequences (for review see 85). While some hsps are constitutively expressed, others are induced in response to environ-

mental stress. Hsp70 is the most highly conserved of the hsps, and has been found in all organisms studied so far including *E. coli* (dnaK gene product), yeast, Drosophila, humans, and plants. In yeast, there are at least 8 genes coding for members of the hsp70 family, of which six are constitutively expressed. In addition to their biochemical purification from yeast as cytosolic translocation factors, genetic tests also indicate that these gene products are involved in translocation of both secretory proteins into the yeast ER, and of precursor proteins into yeast mitochondria (86). Hsp70 proteins are found not only in the cytoplasm, but also in the nucleus, particularly in the nucleolus, and the ER in the case of immunoglobulin binding protein (BiP) (87). Hsp70 proteins have been shown to bind to abnormal or incompletely assembled proteins in an ATP-reversible manner (87). Current models suggest that hsp70 proteins are involved in unfolding and renaturation of proteins damaged by temperature elevation (88). Under normal conditions, hsp70 proteins might function either to prevent folding of translocated proteins in the cytoplasm and/or to unfold such proteins as they are passed across the membrane. Within the ER, hsp70 proteins might serve to correctly fold translocated proteins and assemble them into multi-subunit complexes (89). Homologues from another highly conserved family of heat shock proteins, termed chaperonins, are found in *E. coli* (*groEL*), mitochondria and chloroplasts (90). Like hsp70s, these proteins bind to newly synthesized unassembled subunits of oligomeric complexes. In a similar fashion, chaperonins are thought to assist in the correct folding of proteins and their assembly into multisubunit complexes (90). Other cytosolic factors for protein translocation remain to be characterized. However, protein folding and assembly before, during, and after membrane translocation is coming to be recognized as an important part of localization. This data suggests that many proteins may require an active mechanism for correct folding and assembly, instead of such processes occurring spontaneously as had long been thought .

Genetic Approaches to the Study of Translocation.

There have been a number of attempts to genetically identify proteins that play functional roles in translocation. In yeast, a series of conditional lethal mutants in the secretion process have been isolated by their increase in density upon shift to the non-permissive temperature (91). Using an easily assayed secretory enzyme, invertase, these mutants have been classified into those that accumulate active enzymes internally (class A *SEC* mutants) and those that accumulate inactive enzymes internally (class B *SEC* mutants) (91). Invertase requires the addition of carbohydrates for its activity (92), a function carried out in the lumen of the ER (93). Thus, class B *sec* mutants are candidates for components of the ER membrane targeting and translocation machinery. To date, four complementation groups of Class B *sec* mutants have been identified- *sec*53, *sec*59, *sec*61 and *sec*62 (94,95,96). The most thoroughly characterized of these is the *sec*53 mutation. The *SEC53* gene was cloned by complementation in yeast (97). It encodes a 29 kd protein, which is thought to be located in the cell cytoplasm (97) or loosely attached to the cytoplasmic face of the ER (98). However, secretory proteins are fully translocated into the lumen of the ER in *sec*53 and *sec*59 mutants (as shown by the removal of their signal sequences), suggesting that these genes may encode products that function in the maturation of proteins rather than their translocation into the ER (99). The *SEC*53 gene is allelic to the yeast *alg*4 mutation, and encodes the phosphomannomutase enzyme that is involved in the glycosylation of proteins within the ER (100,101,102). The *sec*61 and *sec*62 mutations were identified by selection for cytoplasmic localization of an enzyme that had been engineered to localize to the ER by the addition of a signal sequence (96). These mutants accumulate precursor forms of a number of secretory enzymes which, although associated with the ER membrane, remain sensitive to exogenous proteases. (96) The fact that secretory proteins retain their signal peptides in *sec*61 and *sec*62 mutants suggests that they have a direct role in translocation. The addition of the power of genetic selection to the efforts at

biochemical characterization and reconstitution of *in vitro* translocation, should greatly enhance progress in this area.

In *E. coli*, signal sequence mutations and gene fusions that block export of a secretory protein required for growth on a substrate have been used to select for compensating mutations in components of the translocation machinery. For example, a fusion of the signal sequence of maltose binding protein to β-galactosidase created strains that were unable to grow on lactose (Lac^-) because of low uninduced levels of β-gal activity (103). Selection of these strains for the Lac^+ phenotype led to the identification of 2 loci, *secA* and *secB*, which accumulate precursor forms of a number of secreted proteins at 30° (104). *SecA* encodes a 92,000 dalton protein, which appears to be loosely associated with the cytoplasmic face of the plasma membrane (105,106). There is data that the *E. coli secB* gene product (a 12 kd protein) performs functions similar to hsp70s in delaying protein folding until secretory proteins are translocated across the bacterial membrane (107). Other genetic loci have been identified, which cause pleiotropic defects in protein secretion including *secC*, *secD*, *prlA* (*secY*) and *prlD* (see review 108). One of the surprising results in this system is that these mutations appear to affect different subsets of secreted proteins, suggesting that there may be multiple pathways for export. Similarly, the overproduction of certain secretion defective proteins will interfere with the normal export of only a subset of other secretory proteins (108). In light of the recent data on the role of cytosolic factors in preventing folding of precursor proteins prior to translocation, these effects on subsets of secretory proteins may reflect substrate specificities of such "unfoldases."

Other potential components of the *E coli* secretory machinery include the *prlA* (*secY*) gene, located in the spc operon, which codes for ribosomal subunits (109,110). Both biochemical and sequence data indicate that the *secY* gene product is a 49,000 dalton integral membrane protein in the *E coli* plasma membrane (111,112). *SecY* is one of the more promising candidates for a component of a translocation pore. The *E. coli* leader peptidase that removes signal peptides from most secreted proteins has been purified biochemically

and characterized in detail (114,115). A second endopeptidase, which is specific for the signal peptides of lipoproteins, has also been characterized (see 108 for references).

In Vitro Translocation in E. coli.

In vitro systems of protein translocation across the plasma membrane of *E. coli* have also been established (115,116). These systems require the production of inverted vesicles of the *E. coli* plasma membrane, and translocation is demonstrated by removal of the signal peptide and protection from externally added proteases. Precursor proteins can be translocated into such vesicles either co-translationally or post-translationally (115,116). Translocation is sensitive to protease treatment, N-ethylmaleimide, and requires ATP hydrolysis (117). An electrochemical gradient is necessary for optimal activity (118). Using these *in vitro* systems, a 12S complex of 4 proteins has been identified from both *E.coli* (119) and *B. subtilis* (120) that interacts with the ribosome and is required for translocation. While this complex is a good candidate for a bacterial homologue of the mammalian SRP, it does not contain the bacterial 6S RNA that was thought to be a homologue of the mammalian 7SL RNA (119). A soluble protein (trigger factor) has also been identified, which is required for *in vitro* translocation of pro-OmpA (121). This protein must be complexed with the denatured precursor in order to have activity, suggesting that it is involved in preventing the precursor from folding into a translocation incompetent form (121). The relationship of this factor to the heat shock proteins, which perform similar functions, remains to be seen.

In Vitro Import into Mitochondria and Chloroplasts.

In vitro import of nuclear-encoded proteins into mitochondria and chloroplasts has also been used to characterize the components involved in these processes. In addition to the hsp70 proteins, which have been shown to stimulate import into yeast mitochondria (discussed above, see 86), a cytoplasmic RNA species (122) and a protein factor of ap-

proximately 40,000 daltons (123) are also required for import into mitochondria. While protease treatment of isolated organelles demonstrated early on that integral membrane proteins were necessary for import (124,125), characterization of these components has proven extremely difficult. An interesting approach has recently led to the identification of an integral membrane protein, which serves as the leader sequence receptor for protein import into chloroplasts (126). Antibodies were raised against a 30-residue peptide corresponding to the leader segment for the small subunit of ribulose-1,5-biphosphate carboxylase (Rubisco-S). By molecular complementarity, the binding site of these antibodies should resemble the binding site for the chloroplast leader sequence receptor. Anti-idiotypic antibodies, raised against the first antiserum, should subsequently be able to bind the receptor itself. A 30-kd integral membrane protein was identified, which specifically binds the precursor form of Rubisco-S. Using immuno-electron microscopy, this receptor has been localized to contact zones between the inner and outer chloroplast membranes (126). Import at these sites has long been hypothesized to account for protein import into the inner matrix, rather than a mechanism that requires multiple membrane translocation steps (127). These *in vitro* systems have also shown that ATP hydrolysis is required for import into both mitochondria (125,128) and chloroplasts (129,130). Part of this ATP requirement may be for use by cytosolic factors, which maintain the precursors in a translocation competent form and/or unfold the precursor prior to translocation (see review 131, also 90,132,133,134). However, the requirement for an internal chloroplast ATPase (130) suggests that hydrolysis may also be required for translocation itself. While an electrochemical gradient is not necessary for import into chloroplasts (130), the presence of a membrane potential across the inner membrane is required in mitochondria (126,129).

The Mechanism of Translocation Across the Membrane Barrier.

The biophysical mechanism whereby hydrophilic domains of a protein are transported across the hydrophobic barrier of a membrane has been the subject of intense speculation and investigation. The original signal hypothesis proposed that a proteinaceous membrane pore structure assembled in the ER membrane at the site of ribosome binding (10). This pore would shield the nascent chain from the hydrophobic domain of the lipid bilayer, allowing it to be translocated across. The energy from elongation of the nascent chain during translation was thought to be the driving force for translocation and thus the reason that translocation was only seen co-translationally. The importance of the co-translational nature of ER import was reinforced by the finding that components that target secretory proteins to the ER membrane (the SRP) arrest translation of cytoplasmic ribosomes in the original *in vitro* system, and that this arrest is relieved upon interaction with the membrane and initiation of translocation (135).

Most of the proteins in mitochondria and chloroplasts are encoded by nuclear genes (136), translated by cytoplasmic ribosomes (137,138), and must be subsequently imported post-translationally into these organelles. Such post-translational import would rule out elongation of the nascent chain as the driving force for translocation, suggesting that a very different mechanism is at work here. Post-translational import into mitochondria and chloroplasts has been demonstrated both *in vitro* (see reviews 139,140) and *in vivo* (141). Like secretory proteins, most mitochondrial proteins are synthesized in a precursor form with an amino-terminal extension that is removed during or shortly after import. However, while the import signal varies widely between mitochondrial proteins, it is structurally distinct from the signal peptides of secretory proteins (142). These results led to a general belief that protein import into mitochondria and chloroplasts is fundamentally different from the process that translocates secretory proteins across the membrane of the ER.

Early work on protein translocation across the *E. coli* plasma membrane showed that post-translational translocation was also possible in this system (119,143). While similar in this aspect to mitochondrial import, the signal peptide regions of exported bacterial proteins were structurally and functionally like those of eukaryotic secretory proteins. This led people to question if the mechanism of bacterial secretion was like mitochondrial import or like ER translocation. The relevance of post-translational translocation *in vivo* in bacteria has also been questioned, since many bacterial secretory proteins are preferentially synthesized on membrane-bound polysomes (144) and co-translationally exported (115,145). In some cases, particularly with signal sequence mutants, it has been difficult to distinguish whether export is proceeding post-translationally or is initiated co-translationally and proceeds with very slow kinetics.

The significance of co-translational versus post-translational translocation, and the translocation mechanism(s) used in different systems have been widely debated. The view that translocation proceeds by fundamentally different mechanisms in these different systems has been challenged recently (146). The demonstration that post-translational import of secretory proteins into yeast ER is possible (73,74,81), has shown that ER import is not obligately co-translational. Earlier work had proven that protein elongation is not necessary for translocation into mammalian ER (71), and thus that the energy for translocation is not derived from protein translation. While attachment of the ribosome to the nascent chain still appears to be required for import into mammalian ER (75), this seems to be for the purpose of membrane targeting rather than translocation itself. In addition, cytosolic factors have been characterized that are able to stimulate both mitochondrial import and ER translocation (84,86). Cytosolic factors such as these are common to both prokaryotes and eukaryotes (83) suggesting a high degree of conservation in both structure and function. It is starting to become clear that while there may be unique facets to the translocation process in all these systems, at least some of the components and the underlying mechanisms may be common to all systems.

A number of different models have been proposed for how proteins are translocated across membranes. One of the first alternatives to the model of nascent chain translocation through a membrane pore proposed in the signal hypothesis (10) was the membrane trigger theory proposed by Wickner (147). Comparison of signal sequences from a number of different proteins led to the recognition that the signal sequence was not a specific array of amino acids, suggesting that recognition was not mediated by a specific receptor. The structural characteristics of signal sequences suggested that they might spontaneously associate with the hydrophobic domain of the lipid bilayer and thus initiate insertion. This led Wickner to propose that as the nascent chain emerged from the ribosome, it folded into a configuration that spontaneously inserted the signal peptide into the membrane. Presumably as it did so, hydrophilic domains of the protein were pulled across the membrane. Further folding of the nascent chain on the opposite side of the membrane provided the energy for continued translocation. Cleavage of the signal peptide and the folding of the protein into its mature form rendered the process of translocation unidirectional and irreversible. The key features of this hypothesis are 1) the lack of a membrane transport system or pore complex for the process of translocation and 2) that the structural features of the signal peptide and the folding of the nascent chain are functionally important for driving the process of translocation rather than the process of elongation.

The signal hypothesis and the membrane trigger hypothesis represent the extremes in the spectrum of thought on the process of translocation ranging from a spontaneous process driven by the process of protein folding and requiring no cellular machinery, to a highly mechanistic, enzymatically driven process. The difficulty in demonstrating the existence of a membrane pore complex has led to a number of proposed mechanisms where the nascent chain interacts directly with the membrane (13,148-151). Like the membrane trigger hypothesis, the helical hairpin model postulates that insertion and translocation are spontaneous processes that do not require additional cellular machinery (151). In this model, the hydrophobic signal sequence and adjacent sequences of the mature portion of

the protein form a pair of alpha-helices that spontaneously insert into the membrane. Hydrophilic domains of the protein are threaded across the membrane as an alpha helix, remaining in contact with the alpha helix of the signal peptide. The direct transfer model (150) invokes the tight attachment of the ribosome to the membrane, along with the energy of protein elongation, as providing sufficient energy to force strongly hydrophilic residues through the membrane. Both of these models include a "pulling" force generated by the folding of the nascent chain on the opposite side of the membrane as contributing to further translocation. Folding and cleavage of the signal sequence are presumed to make the process irreversible. There is general agreement that peptide sequences within the translocated protein itself can halt and/or re-initiate membrane transfer. The order in which these occur on the protein is presumably responsible for the final topology of the protein in the membrane (11,37,149).

Experiments have shown that there is wide variation in the time frame in which individual proteins cross the membrane (152), and in their requirements for energy and accessory molecules. A number of proteins, such as bee venom mellitin, will spontaneously insert into membranes or even into artificial liposomes totally lacking in proteins (11). In general, however, most translocated proteins have been shown to require both cytosolic and integral membrane factors to transfer hydrophilic domains across the membrane barrier (above). The specificity of such factors for the proteins they assist and differences in their kinetics may result in the appearance of different requirements or kinetics in the translocation of particular proteins even though the basic mechanism of translocation is largely the same. Likewise, ATP hydrolysis is, in general, required for translocation (131). Whether ATP is used for the unfolding of a translocated protein or is directly used by a pore complex to pass the protein across the membrane will probably be difficult to distinguish. In mitochondria, an electrochemical potential across the inner membrane is also required for import into the matrix. Similarly, in bacteria, proton-motive force is required for optimal rates of protein export.

The existence of a membrane pore complex to shield the nascent chain from the hydrophobic environment of the lipid bilayer remains an open question. The importance of the hydrophobic nature of signal/leader regions and membrane topology signals strongly suggests that they interact directly with the membrane. However, there is still disagreement about whether it is energetically possible to pass hydrophilic protein domains across the bilayer without a pore of some type. Likewise, any mechanism for translocation must also be able to account for those proteins with uncleaved internal signal sequences such as ovalbumin (39,40), or other large hydrophobic domains such as *E. coli* hemolysin (154), which are translocated completely across the membrane. How are the hydrophobic domains of these proteins either prevented from interacting from the membrane, or actively removed from the membrane during translocation? The importance of cytosolic and/or integral membrane factors in translocation, and an understanding of their role in unfolding the translocated protein prior to passage across the membrane is only starting to emerge. New studies looking at the folding state of a protein indicate the importance of maintaining the protein in an unfolded or loosely folded configuration before translocation (77,108,154-156). Likewise, recent evidence suggests that proteins within the lumen of the ER, (for example BiP) or the matrix of mitochondria and chloroplasts (chaperonins) may be actively involved in re-folding proteins after membrane translocation and assembling them into larger complexes. The role of such "unfoldases," particularly if any are found which are integral membrane proteins, may be difficult to distinguish from that of a membrane pore for translocation without a crystal structure of the membrane translocating complex.

While similar in their use of cytoplasmic factors for unfolding translocated proteins, pathways of translocation that pass proteins across more than one membrane at a time may be very different in some aspects. Import into the mitochondria or chloroplast matrix involves a dual membrane translocation, which occurs at contact sites between the inner and outer membranes (126,157). Likewise, recent studies have begun to suggest that bacteria are able to export proteins directly from the cytoplasm to the extracellular medium

(35,153). Here too, export is thought to occur at contact points between the inner and outer membranes. The structure of such contact regions, and mechanisms of how they are generated and maintained, is not understood. One well-studied example of import at such sites involves the Fe/S protein of the ubiquinol-cytochrome c reductase complex, which is localized to the outer surface of the mitochondrial inner membrane (158). A prokaryotic homologue of this protein is made in the bacterial cytoplasm, contains a typical signal sequence, and is translocated to the outer surface of the bacterial inner membrane. The eukaryotic protein has an amino-terminal leader sequence attached to the signal peptide region. This conformation was thought to function by first using the leader sequence to insert through the mitochondrial outer membrane, and then halting translocation at inner membrane by using the signal sequence region as a stop transfer sequence (34). However, studies indicate that this protein is translocated completely across the mitochondrial outer and inner membranes in a process that is dependent on a membrane potential across the inner membrane (158). The signal sequence in this case does not halt translocation, suggesting that it is not allowed to interact directly with the lipid bilayers. Once in the mitochondrial matrix, the leader region of the Fe/S protein is then proteolytically removed, exposing the signal peptide region. This protein is further translocated back across the mitochondrial inner membrane, like the translocation of the prokaryotic homologue, with the concomitant removal of the signal peptide region. The authors suggest that such a two step process may reflect the evolutionary origins of mitochondria (158). It is possible that the import step in this case may have evolved from a prokaryotic pathway of export directly from the cytoplasm to the extracellular medium. In both cases, an electrochemical potential seems to be utilized in the translocation process. Shielding of hydrophobic domains from interaction with the lipid bilayers is also common to both systems (153,158), suggesting the involvement of a membrane pore at sites of contact between the two membranes. In a similar vein, when stop transfer regions are engineered into a chloroplast matrix protein

they do not cause a halt in translocation. This suggests that they, too, are shielded from interaction with the membrane (159).

Passage Through the Organelles of the Eukaryotic Secretory Pathway.

Compared to the large volume of detailed information on the initial membrane targeting and translocation steps of secretion, relatively little is known about how proteins are transported from compartment to compartment within the cell to finally arrive at their correct destinations. There are primarily two recognized functions performed by the remaining secretory compartments; the modification and processing of proteins to their final structural conformations, and the task of sorting proteins for delivery to their correct final destination inside or outside of the cell. Both of these activities are accomplished with a high degree of fidelity. Individual proteins are correctly modified to their final forms through a precise ordered sequence of covalent modifications such as proteolytic cleavages and oligosaccharide additions. Likewise, subcellular organelles have distinctive subsets of proteins in their membranes and enclosed within their lumenal space, a process crucial to forming functionally differentiated compartments within the cell. This implies the ability to precisely sort both integral membrane and membrane bound proteins, as well as those proteins that are free in the lumen of secretory organelles. Indeed, sorting by the secretory pathway is probably an integral part of the process of organelle biogenesis.

The processing pathways of a number of individual proteins have been characterized in detail. Both the kinds of modifications that occur, and the extent to which they occur, are often unique to individual proteins and the type of cell in which synthesis occurs. Little is currently known about the mechanism(s) that determines which modifications will occur on a particular protein, or what determines the extent of modification of a particular protein. However, studies that detail the kinds of modifications, the order in which they occur, and

the locations where they take place, have provided useful model systems for looking at 1) the number and kinds of compartments that make up the secretory pathway and 2) the transfer of proteins from one compartment to the next. In particular, the addition of oligosaccharides to the side chains of asparagine residues and subsequent maturation of the glycosidic side chains has been well studied and provides useful markers for progress along the secretory pathway. Through use of recombinant DNA and gene expression techniques, progress has also been made in studying the sorting of proteins to particular compartments and the signals that mark proteins for their final destinations.

Since the initial characterization of the secretory pathway, which followed the progress of *in vivo* labeled proteins through organelles by electron microscopy, autoradiography and cell fractionation (1-6), characterization of the individual transport steps that make up the pathway has proven difficult. Some progress has been made by *in vivo* studies, which utilize cell fusions to characterize transport between successive compartments. *In vitro* systems, which reconstitute transfer between the ER and Golgi complex, or between successive compartments in the Golgi, have only recently been established. These systems hold the promise of being able to biochemically dissect the process of secretion. Finally, a system of conditional-lethal mutations that disrupt the secretion process in yeast have been characterized. This adds the power of genetic selection to identify components of the secretion machinery, and the ability to easily clone and characterize the products of these genes, to the efforts aimed at achieving a functional understanding of the secretion process.

Protein Maturation During the Process of Secretion.

Glycosylation.

Beyond the proteolytic removal of the signal sequence, which usually occurs during the process of translocation across the ER membrane, proteins can undergo a variety of cova-

lent modifications as they progress through the eukaryotic secretory pathway. One of the most thoroughly studied of these modifications is the addition of complex carbohydrates in an N-linked fashion to asparagine side-chains of the protein (reviewed 93,160-162). For the most part, the significance of glycosylation - the diversity of carbohydrate structures and the precise way that individual proteins are modified - remains somewhat enigmatic since many of these modifications can be eliminated in mutant cell lines with no detectable defect in cell growth (163). However, some of the roles that carbohydrate side chains serve in glycoprotein structure and function are known. For some proteins, such as carboxypeptidase Y and invertase in yeast, glycosylation is required for enzymatic function (93). The function of insulin receptors in higher eukaryotes is also dependent upon glycosylation (164). Glycosylation also plays roles in the targeting and secretion of proteins. Mannose-6-phosphate residues on lysosomal enzymes are used as a sorting signal for correct localization (165). In some cases, the effects of glycosylation on secretion may be more subtle. N-linked glycosylation has been shown to be important for the formation of octamers of invertase (166). Lacking the correct quaternary structure, unglycosylated invertase is secreted very slowly and is not properly retained in the periplasmic space between the yeast plasma membrane and its outer cell wall (166). In higher eukaryotes, glycoproteins are thought to play important roles in mediating cell-cell interactions during development (167). A general role for glycosylation in cell-cell recognition has been proposed, with differences in outer chain structures forming the signals that are communicated. However, the inability to modulate particular glycosylation events in a controlled fashion has made this a difficult subject to approach.

The addition of N-linked oligosaccharides begins in the ER. A core oligosaccharide complex of 14 monosaccharides attached to a dolichol-phosphate carrier [$Glc_3Man_9GlcNAc_2$-P-P-Dol] is synthesized on the membrane of the ER (162). This structure is transferred as a unit to asparagine side-chains of secretory proteins once they reach the lumen of the ER. The sequence -Asn-X-Ser/Thr-, where X can be any amino

acid except Asp or Pro (168), serves as the structural determinant on the secretory protein which is recognized by the oligosaccharide transferase. While this sequence is required for N-linked glycosylation, only about 1/3 of such sites are actually glycosylated (169). Experiments *in vitro* with denatured proteins and oligopeptide fragments suggest that protein folding around the site can inhibit its recognition as a substrate (see 160 for discussion). The transfer reaction is restricted to the lumen of the ER (170), and in most cases occurs concomitant with the processes of protein translation and membrane translocation (160,161). The role, if any, that glycosylation plays in the translocation process is unclear. In some cases, defects in glycosylation have been shown to affect the folding of secretory proteins and their transit from the ER (94,166,170); however, the processes of translation and translocation are not directly affected (94). In fact, when glycosylation is either inhibited by mutation (99,170) or saturated by overexpression of a glycoprotein (95), unglycosylated precursors can be detected that are then glycosylated in a post-translational, post-translocational fashion. Thus, the processes of translation/translocation and glycosylation do not appear to be mechanistically linked.

Following transfer to the peptide chain, a trimming process begins that eventually reduces the core oligosaccharide structure to $Man_3GlcNAc_2$ in higher eukaryotes (160) or $Man_8GlcNAc_2$ in yeast (171). First, the three outer glucose residues are removed from the core oligosaccharide unit. This appears to be counter-productive; however the glucosylated $Glc_3Man_9GlcNAc_2$ oligosaccharide is highly preferred over $Glc_2Man_9GlcNAc_2$ or $GlcMan_9GlcNAc_2$ as the donor oligosaccharide for transfer to proteins (172). The glucosidases that are responsible for glucose removal have been shown to be integral membrane proteins in the ER of a number of different eukaryotic cells from yeast to human fibroblasts (161). Following glucose removal, the four outer mannose units that are linked in an α-1,2 fashion are removed to leave a $Man_5GlcNAc_2$ structure. The removal of the first α-1,2 Man residue occurs in the ER (173), while the remaining α-1,2 Man residues are removed in the Golgi compartment. A kinetic delay between the removal of the first

mannose residue and the remaining α-1,2 mannose residues has been observed (174), which presumably corresponds to the time for transfer between the ER and Golgi.

At this stage, the processing of Asn-linked carbohydrate chains begins to diverge, leading to heterogeneous populations of glycosylated molecules. In higher eukaryotes, this process generally leads to two major types of outer chain oligosaccharide structures: a "high mannose" type of structure that has only mannose residues added to form the outer chains, and a "complex" type to which the sugars N-acetylglucosamine, fucose, galactose and sialic acid are added (in roughly that order - see 161,175) to form outer chain branches. The sequential nature of these processing steps, and localization of the corresponding enzymes to particular stacks within the Golgi, has been important in dividing the Golgi into three subcompartments - *cis*, *medial*, and *trans* (see reviews 163,175). In yeast, the trimming process removes only the outer three glucose residues and one of the mannose residues (171), and only the "high mannose" type of outer chain structures are formed (93). Other types of modifications to N-linked oligosaccharides also occur along this pathway. For example, lysosomal enzymes are phosphorylated at the $Man_8GlcNAc_2$ stage shortly after arrival at the *cis* Golgi compartment (176). Outer chain mannose residues are first modified with GlcNAc-phosphate, followed by removal of the GlcNAc residue, leaving Man-6-phosphate (176,177). A receptor for Man-6-P has been characterized, which is largely responsible for sorting lysosomal enzymes for transport to that compartment (see review 165). The addition of sulfate residues to certain secretory and membrane glycoproteins is another important process, which also takes place during Golgi transit (6,161,175).

Both the extent and kinds of outer chain additions that occur are dependent on a variety of factors. While a wide variety of final structures are possible, a given glycoprotein usually contains only one or a limited subset of oligosaccharide outer chains (161). The protein structure itself appears to be the major factor determining the structure of the outer chain that is formed. Whether this involves the recognition of protein determinants by particular oligosaccharide processing enzymes, or is simply due to steric interference of the

protein structure with the site of addition probably varies from case to case (161). Sorting processes may also prevent certain proteins, such as those retained in the ER, from being exposed to enzymes responsible for certain outer chain additions that occur later in the pathway (161). Finally, in different cell types and under different growth conditions, the reactions that occur on a particular protein substrate can vary in a stochastic fashion; i.e., only 80% of the core units may have the final α-1,4 Man residue removed, mannotransferases may add only 5-10 outer chain mannoses or up to 50-100 mannoses. This variability may reflect the availability of enzymes and substrates for particular reactions, as well as the substrate preferences of particular enzymes. The heterogenity that arises from these variations is cumulative, and can lead to a very diverse population of final products, such as those seen in the case of yeast invertase (178).

Another covalent modification that occurs during passage through the secretory pathway is the O-linked addition of oligosaccharides, primarily mannose residues, to serine or threonine side-chains of a protein. While O-linked oligosaccharides are approximately as prevalent as N-linked oligosaccharides (179), the process of their formation has been less thoroughly studied. The features that mark a Ser or Thr residue for O-linked mannose addition are currently unknown (180). The first mannose residue of the chain is transferred from Dol-P-Man to the acceptor side chain in the ER. In the Golgi, up to four additional mannose residues are transferred from GDP-Man. While only mannose residues are found in O-linked oligosaccharides in yeast, higher eukaryotes often have sialylated O-linked chains (93).

Proteolytic Maturation.

Proteolytic cleavages, which are necessary to mature a protein to its active form, also occur during passage through the secretory pathway. A number of proteases, which are targeted to the lysosome, are synthesized with a pro-segment that keeps the enzyme inactive during its passage through the ER and Golgi. During or shortly after transit to the

lysosome, this pro-segment is cleaved off to convert the enzyme to its active conformation. In a similar fashion, an internal pro-segment of insulin is proteolyticly removed once the protein is packaged into secretory granules (181). A number of peptide hormones such as α-factor and killer toxin in yeast (182,183) and enkephalins in mammals, are synthesized as larger precursor proteins containing either multiple copies of the active peptide, or as a precursor from which several different active peptides are evolved (184). Maturation of most of these peptides involves specific endopeptidase cleavage at pairs of basic residues (commonly Lys-Arg), as well as cleavage by amino- or carboxy- peptidases to give the correct termini (182). These enzymes are thought to act late in the Golgi (185) or in secretory granules (181).

Using Protein Maturation to Study Secretion.

The processing steps, which occur during transit through the secretory pathway, provide a number of structural intermediates that can be used to follow the progress of proteins from compartment to compartment. In particular, changes in molecular weight that accompany proteolytic cleavage events, or the addition and modification of oligosaccharides, are usually easy to observe as differences in the mobility of the protein on SDS-PAGE. The enzymatic reactions of processing are usually fast compared to transport between compartments. Thus, intermediate structures representing the final modified form that a protein achieves in a particular compartment often appear as a single discrete band. Kinetic studies of the transport and maturation of pulse-labeled proteins often can be easily followed by looking at the appearance and disappearance of different molecular weight forms during a "chase" with unlabeled substrates.

Another method of detecting transport into a particular compartment, or the stage at which a particular carbohydrate addition occurs, is by the incorporation of specific radiolabeled sugars such as [^{3}H]GlcNAc or [^{3}H] sialic acid (186). Lectins, such as Con A or wheat germ agglutanin, are able to bind glycoproteins that have specific kinds of carbo-

hydrates and oligosaccharide linkages. These, too, have been used to characterize the compartments where particular carbohydrate additions occur, as well as to confirm the structures present on a given glycoprotein intermediate. The enzyme endo-β-N-acetylglucosaminidase H (EndoH), which cleaves between the GlcNAc residues by the Asp side chain, is specific for high mannose-type oligosaccharides (160). Conversion of the initial core oligosaccharide unit to complex-type outer chains in higher eukaryotes confers EndoH resistance to the oligosaccharide structure. This is used as a marker for transport into the *medial* Golgi compartments where these modifications take place (163). Finally, mutants that are defective in particular glycosylation events have been used to generate "donor" compartments both *in vivo* and *in vitro* (187,188). Transfer to a wild-type "acceptor" compartment, either *in vivo* by cell fusion or *in vitro*, then is detectable by the restoration of normal processing of intermediates.

Compartmental Organization of the Golgi Complex.

Until the advent of electron microscopy in the 1950s, the existence of the Golgi apparatus as a morphologically distinct cellular compartment was widely debated (6). Now, it is clear that the Golgi is comprised of at least three functionally distinct subcompartments (163). The Golgi complex plays a central role in the protein processing, sorting, and membrane transport activities within the cell (6,164,189). In addition to the question of how secretory proteins are efficiently passed from compartment to compartment during transfer through the Golgi, the complex structure of the Golgi and the many different vesicular transport events that occur in the Golgi raise questions of how such subcompartments are generated and maintained as separate entities.

Golgi Structure.

In higher eukaryotes, the Golgi complex usually appears as a stack of 4-10 smooth-surfaced platelike cisternae ("rigid lamellae") with dilated rims (6). This structure is generally located between the ER and the plasma membrane, and has a distinct polarity with larger cisternae on the ER side (*cis* side) usually wrapping around the smaller cisternae on the plasma membrane side (*trans* side) to form a concave-shaped structure. This orientation would suggest that newly synthesized secretory proteins enter the Golgi at the *cis* face and are packaged into secretory vesicles at the *trans* face. A profusion of small vesicles (40-50 nm) is usually present on the *cis* side of the Golgi, while the *trans* face is often distorted into an array of tubules and sacs (the *trans*-Golgi network - 190) with secretory granules commonly found condensing here. Somewhat larger vesicles (60-70 nm) are commonly seen at the dilated rims of cisternae (6). While the number of cisternae, their exact size, and shape vary in different types of eukaryotic cells, the general features of the Golgi complex are discernable in most cases.

Cisternal Progression Vs.Vectorial Transport.

The morphological characteristics of the Golgi complex suggested that individual cisternae were initially formed by condensation of vesicles at the *cis* face, progressed as distinct entities through the stack, and were finally used up in packaging proteins into secretory vesicles (191). This "cisternal progression" model for Golgi function would imply that the secretory contents of a given cisternae remain constant, and that most components would be common to all cisternae. Early histochemical studies, however, showed that cisternae vary histochemically from *cis* to *trans*. For example, osmium tetroxide was found to stain only 1-2 cisternae at the *cis* Golgi face in many different cell types. Likewise, stains for thymidine pyrophosphatase (TPPase) usually detect only the trans-most 1-2 cisternae. This heterogenity suggested that different cisternae are maintained as distinct subcompartments within the Golgi rather than progressing through the stack as a unit. Characterization

of the processes of protein maturation that takes place within the Golgi, and localization of the corresponding processing enzymes has, indeed, shown that different cisternae within the Golgi are functionally specialized. In addition, Golgi membrane proteins have been shown to turn over at a much slower rate than is required for secretory protein passage through the Golgi (192). This provided strong evidence against the cisternal progression model for secretory protein passage through the Golgi. Instead, a vectorial transfer model for Golgi function (6,193) has come to be widely accepted, that postulates that secretory proteins enter at the *cis* face of the Golgi, are passed directionally from *cis* to *trans* cisternae, and are finally packaged into secretory vesicles at the *trans* Golgi face. Small vesicles are thought to mediate transfer between compartments.

Subcompartments Within the Golgi.

Current evidence indicates that there are at least three functionally distinct subcompartments within the Golgi complex (163), usually called *cis*, *medial*, and *trans* in agreement with the histological designations of cisternal orientation. In addition to the early histochemical evidence for different compartments, a number of different lines of evidence have come to show that these compartments comprise functionally distinct domains with different biochemical activities. Characterization of the glycosylation process has given workers a number of enzymatic markers for Golgi compartments (6). Localization of such enzymes within the Golgi by subcellular fractionation on sucrose density gradients showed that different activities were present in compartments of different densities (194). These were found to correspond to different cisternae within the Golgi, with vesicles of higher density corresponding to *cis* Golgi and lighter vesicles corresponding to *trans* Golgi (see review 163). Enzymes responsible for modifications that occur late in the glycosylation pathway, such as the addition of galactose and sialic acid, were present in fractions of light density corresponding to the trans-most cisternae of the Golgi (194). These could be separated from enzymes responsible for earlier modifications in the glycosylation pathway, such as

Golgi mannosidase I and GlcNAc transferase, which were present in fractions of somewhat higher density (*medial* Golgi - 163,194). Enzymes responsible for the modification of lysosomal enzymes with Man-6-P were present in even denser fractions corresponding to the *cis* most cisternae of the Golgi (195). These results have been confirmed by localization of these enzymes using immuno-electron microscopy and correlations of immunolocalization with earlier methods of histochemical characterization (163). Further evidence for the compartment structure of the Golgi comes from the use of lectins to identify the compartments where particular sugar linkages are present in oligosaccharide side chains. This evidence also confirms the existence of three Golgi subcompartments (reviewed 163). Overall, there is an excellent correlation between the processes of protein maturation that occur during passage through the Golgi, with the order of enzymatic activities present in Golgi subcompartments arranged *cis* to *trans*.

The Role of Vesicles in the Secretory Transport.

Membrane enclosed vesicles are generally present in profusion throughout the cytoplasm of eukaryotic cells, and form a diverse spectrum in terms of their size, shape, membrane characteristics, and contents. While the role of some of these vesicles in endocytosis and exocytosis is well established, many others are present whose functions are difficult to discern. As characterization of the secretion process has progressed, it is increasingly clear that small vesicles are a general means of transport between cellular compartments (i.e., ER to Golgi transport - 196), and even between subcompartments such as the *cis*, *medial* and *trans* Golgi cisternae. While the role of small vesicles in such transport has long been widely accepted, direct evidence for vesicle-mediated transport is sparse. Because of the diversity of small vesicles within the cell and the large number of different possible trans-

port routes between compartments, little progress has been made in characterizing the features that target a vesicle to a particular destination.

The process of vesicle mediated transport presumably generates a net flow of membranes to the cell surface or other terminal compartments (1), which must be balanced either by degradation or recycling of membranes to the ER and Golgi. While recycling of membrane receptors (and presumably membranes also) between the cell surface and lysosome during endocytosis is well documented (197,198), there is currently little evidence for membrane recycling to the Golgi and ER. Experiments have shown some evidence for recycling between the cell surface and Golgi. Briefly, transferrin and transferrin receptors that have been desialyted *in vitro*, can be shown to bind to the plasma membrane of cells and can be recycled between the lysosome and cell surface. At a very slow rate, transferrin and transferrin receptors become resialyted; a function that occurs in the *trans* Golgi (199,200). Because of the rate at which this occurs, it is unclear if this represents a significant membrane recycling or if it is indicative of a low degree of mistargeting.

The role of small vesicles in transport between the ER and Golgi has long been inferred from morphological studies (1-5) and is generally well accepted. Vesicles budding off from the smooth ER have been shown to contain secretory products by autoradiographic, cytochemical and immunocytochemical means (175). Likewise, the budding of secretory vesicles from the *trans* face of the Golgi is well established (6), and the role of vesicles in exocytosis is also well accepted. Lysosomal enzymes are transported through the secretory pathway (201), and in at least some cases, are known to pass through the *trans* Golgi subcompartment because they are modified with complex type outer chain oligosaccharides (165). Like secreted proteins, protein transport between the Golgi and lysosomes is presumed to be mediated by small vesicles (190,201). However, some disagreement exists over whether all lysosomal proteins are exported from the *trans* Golgi (190), or if some proteins are transported directly from *cis* or *medial* Golgi to lysosomes (202).

The role of vesicles in intra-Golgi transport has been more difficult to demonstrate. The stacked structure of successive Golgi compartments makes it difficult to exclude the possibility that there are direct means of secretory protein transfer between cisternae through membrane pores. However, efficient transport between entirely separate Golgi stacks has been demonstrated *in vivo*, using cell fusions between a mutant cell line with Golgi that is incapable of terminal glycosylation (donor) and a cell line with wild type Golgi (acceptor) (187). Control experiments show that the two Golgi structures are not intermingled during cell fusion (188). Thus, the transfer process definitely involves both exit from the donor compartment and entry into an acceptor compartment that is not contiguous with the donor (187). Kinetic studies of such transfer both *in vitro* and *in vivo* demonstrate that if labeled proteins are allowed to progress too far within the donor Golgi, they are unable to transfer to an exogenous Golgi acceptor compartment (187,188,203). This suggests that secretory protein traffic is uni-directional, i.e., that incompletely modified proteins cannot return to the *medial* Golgi compartment of the wild-type acceptor once they have entered the *trans* Golgi compartment of the donor (187). A similar assay for intra-Golgi transport has been established *in vitro* (203), and has shown that the conditions that support transfer *in vitro* lead to budding of small vesicles at the rim of Golgi cisternae (204). The dissociative, uni-directional nature of vesicular transport between cellular compartments is generally well accepted. However, whether the uni-directional nature of such transport is absolute, or instead reflects a higher probability of transport in one direction is still debatable. Resolution of this question will undoubtedly require further characterization of such transport vesicles and some elucidation of the signals that mediate their target recognition.

Protein Sorting During Secretory Transport.

Besides lysosomal and secreted proteins, plasma membrane proteins and proteins that reside in the ER and Golgi all enter the secretory pathway at the same point. To date, there is no evidence that subdomains exist with the ER where proteins destined for different targets might be pre-sorted for delivery by, for example, translocation into a particular subdomain. In fact, all current evidence suggests that these proteins enter the ER compartment randomly and are transported together through most of the secretory pathway (190). Thus, mechanisms must exist that cause a protein to be selectively transported to its correct organelle, or retained in its correct compartment as in the case of ER and Golgi proteins.

Several features of the sorting process are apparent from the studies of secretion (189). Protein secretion in some cell types is highly regulated both in terms of when a particular protein is secreted (i.e., hormones such as insulin) and also the precise location where a particular protein is secreted (i.e., neurotransmitters in nerve cells). Such regulation can be quite dramatic, as in the case of polarized epithelial cells that are present in many different types of tissues (see reviews 205,206). Tight gap junctions formed between these cells differentiate their plasma membranes into apical and basolateral domains. These cells are able to sort specific proteins for delivery to each surface, while other proteins are delivered non-specifically to both. In some cases, these delivery processes are thought to work in concert with intracellular transport systems that take up materials at one surface and deliver them to another (206). In a similar fashion, secretion in *S. cerevisiae* appears to be regulated in a spatial fashion since it is restricted to the bud site during most of the division cycle (207).

Besides these regulated pathways, constitutive pathways must be present within the same cell (189) to deliver proteins to the plasma membrane and lysosomes in order to maintain cellular function. Thus, sorting must not only separate proteins on the basis of their target destination, but also must be able to separate products whose release is regu-

lated from constitutively expressed proteins (189). Furthermore, the amount of time it takes for different proteins to move from compartment to compartment can vary widely (208). In particular, transport from the ER to Golgi is the rate-limiting step for most secretory proteins (209,210). There is currently a debate as to whether such differences in the rate of protein secretion reflect a positive selection for the fastest moving proteins, utilizing some type of "export receptor" to facilitate transport, or some negative selection against more slowly moving proteins such as an inhibitory protein conformation or aggregation (see review 211).

The sorting process is undoubtedly intimately involved with the processes of vesicle formation and targeting, which operate in secretory transport. The basic process of vesicle formation is beginning to be understood in some detail, at least in the case of clathrin coated vesicles formed during receptor-mediated endocytosis (212,213). There is evidence to suggest that the endocytosis and exocytosis pathways may share some of the same functional components. Conditional yeast mutants, which appear to affect specific steps in the secretory pathway, are also temperature sensitive for endocytosis of a fluid phase marker that accumulates in the yeast vacuole. (214) However, the degree of similarity between secretory vesicle formation and clathrin coated vesicle formation, which has been described for receptor-mediated endocytosis, is unknown. Both clathrin coated and non-clathrin coated vesicles have been observed budding from the *trans*-Golgi network (190). Secretory products that are released in a regulated fashion, such as insulin, are found concentrated in clathrin coated secretory granules (215). However, yeast mutants, which delete the clathrin gene (216), demonstrate that a basal level of secretion that allows cell viability and growth is possible even in its absence. There is evidence that proteins that follow the constitutive secretion pathway from the Golgi to the cell surface are not clathrin coated (217). Intra-Golgi transport appears to be mediated by a coated vesicle that does not contain clathrin, based on both morphology and inability to react with anti-clathrin antibodies (218). Recently, the isolation of vesicles that are involved in transport from ER to Golgi

has been reported (196). However, there is no characterization yet of whether these vesicles are clathrin coated or not. Practically nothing is known about the mechanisms that selectively sort (or exclude) both membrane bound and soluble proteins for packaging into secretory transport vesicles. Finally, little is known about the mechanisms that mediate the processes of vesicle transport from donor to acceptor compartments, making the transfer specific and uni-directional.

An excellent theoretical framework has developed for looking at protein sorting in the secretion process (reviewed by Pfeffer and Rothman - 211). Overall, the default route that most proteins follow after entering the ER is considered to be a "bulk flow" pathway from the ER through the *cis*, *medial*, and *trans* Golgi compartments finally reaching the cell surface. This bulk flow pathway is thought to be responsible for cell surface delivery of constitutively expressed secretory and plasma membrane proteins. Proteins that are destined for any other compartment must presumably have some kind of sorting signal responsible for their targeting; generally thought to be some kind of conformational domain of the protein or glycoprotein structure (211). The main site of secretory protein sorting has long been thought to be the *trans* Golgi compartment (176,190), primarily because this is the final compartment where proteins destined for the different sorting pathways can be co-localized. However, direct evidence for this has been obtained only recently (219). Sorting also occurs in the compartments prior to the *trans* Golgi, in that there is specific retention of those proteins that are resident in the ER and Golgi subcompartments (220).

Bulk Flow to the Cell Surface.

Initially, the processes of protein transport and sorting within the secretory pathway were thought to be mediated by transport receptors at each stage (44), similar in nature to the receptors that mediate endocytosis. This model presumes that there are positive sorting signals present on all secreted proteins, or on assembled multi-protein complexes, which are required for transport from the ER. The finding that different proteins move from the

ER to the Golgi at different characteristic rates (209,210), was taken as support for the concept that different integral membrane transport receptors are present in the ER that specifically mediate the transport of one or a few proteins (44).

The concept that proteins, once having entered the ER, are swept along by the bulk flow of materials through the secretory pathway, was developed from the characterization of deletion mutants (220). Deletion of an internal hydrophobic domain that serves as the membrane anchor of a viral glycoprotein that normally resides in the ER, resulted in its passage through the Golgi apparatus and secretion from the cell (221). This suggested that rather than requiring a positive signal for export, the protein was swept along the constitutive secretion pathway. Since the wild type protein had no positive signal that led to its export, the membrane domain presumably served as a signal for ER retention. A similar result was shown in the case of adenovirus E19 (222). However, in this case only a cytoplasmically oriented tail of the protein was deleted, with the resulting membrane-bound protein found in the plasma membrane. Thus, membrane-bound proteins as well as proteins that are soluble in the lumen of the ER are subject to bulk flow transport.

Convincing evidence of bulk flow was shown with the use of a small tripeptide (N-acyl-Asn-Tyr-Thr-NH_2) that can readily diffuse across membranes (223). When incubated with isolated ER microsomes, such a peptide becomes core glycosylated and is trapped inside. Since this small glycopeptide structure is found in both secreted and retained proteins, it presumably carries no transport signals itself. When this peptide is incubated with CHO cells *in vivo*, glycosylated forms of the peptide rapidly begin appearing in the medium. Analysis of the glycosylation indicates that the peptide entered the ER, was core glycosylated, and was transported through the Golgi apparatus (receiving Endo H resistant modifications to the oligosaccharide) en route to the cell surface (223). The half-time for secretory transport was about 10 minutes, much faster (2-3x) than that of other known proteins in this system (223). It has been suggested that the slower secretion kinetics seen for most proteins reflect the recent findings that proteins require significant re-folding and

subunit assembly after translocation into the ER, before they are ready for export to the Golgi (220). These bulk flow experiments suggest that transport along the constitutive secretion pathway is an unselective process resulting in the default delivery of most products to the cell surface. Deviations from this pathway, such as retention in the ER or packaging into regulated secretory vesicles, thus requires a positive signal on the protein structure.

Protein Determinants Responsible for Targeting - Sorting Signals.

Most attempts at identifying sorting signals have looked at modifications of protein structure that alter intracellular targeting, such as deletions, point mutations, and the generation of protein fusions between a targeted protein and one that presumably lacks targeting signals (224). For example, deletion mutants that remove a targeting signal and lead to constitutive secretion, such as those discussed above in regard to the recognition of bulk flow in the secretion pathway, have suggested that these amino acids are part of the targeting domain of the protein (221,222). However, these studies are complicated by their unknown effects on overall protein structure, which may lead to grossly abnormal protein conformations that could interfere with protein targeting in a number of ways. For example, the fusion of invertase with *E. coli* β-galactosidase results in a protein that fails to be transported from the ER (225). It has been suggested that this is due to mis-folding and protein aggregation that prevents ER export (225). Changes in protein structure can also lead to kinetic defects in transport, which may prove misleading in determining localization (226). In other cases, altering the position of a targeting signal may cause it to be masked by the folding of adjacent sequences (227). Sorting signals may also be contained in "patches" of structure on the surface of the folded molecule, comprised of amino acid residues which are not contiguous in the primary structure (211). Thus, deletions or alterations in widely spaced parts of the sequence could conceivably lead to similar defects. In

these studies, it has been important to verify the effects of changes in protein structure on intracellular targeting in multiple ways, before conclusively identifying a sorting domain.

Comparisons of the primary amino acid sequence of proteins that are retained in the ER, identified a common C-terminal tetrapeptide [Lys-Asp-Glu-Leu] (228). The function of this sequence in targeting proteins for retention in the ER has been convincingly demonstrated (227). Deletion or mutation of these amino acids in the grp78 protein, which normally resides in the lumen of the ER, led to its secretion into the medium. Likewise, the addition of this sequence to a constitutively secreted protein (lysozyme) led to its retention in the ER (227). The carboxy-terminal location of this sequence appears to be essential, since the addition of as little as two amino acids to the carboxy-terminus led to efficient secretion.

Certain disease states, which arise from incorrect targeting, such as the deficiency of lysosomal enzymes seen in I-cell disease, have led to characterization of the sorting signal. For most lysosomal enzymes, the presence of mannose-6-phosphate residues on outer chain oligosaccharides is used to mediate the targeting process for both protein transport from the Golgi to the lysosome and receptor-mediated endocytosis of exogenous lysosomal enzymes (reviewed 165). Two receptors have been identified; one is a 215-kd glycoprotein that functions in receptor-mediated endocytosis (165), and the other is a 46-kd protein thought to function primarily in Golgi to lysosome targeting (229). However, other lysosomal enzymes have been characterized that lack phosphorylated residues altogether (165). Presumably, other sorting signals are used in these cases.

The delivery of hydrolases to the yeast vacuole, the compartment that is presumed to be the yeast equivalent of a lysosome, occurs efficiently even in the absence of oligosaccharide addition (230). The sorting signal for the yeast vacuolar protease carboxypeptidase Y (CPY), has been identified by the analysis of point mutations and deletions (226) and generation of protein fusions between CPY and yeast invertase (231). Both of these studies indicate that amino acids in the pro-segment of the enzyme, which keeps the pro-

tease inactive until its removal in the vacuole, are important for targeting. Similar results from a study of yeast proteinase A (232), suggests that the use of targeting signals within the pro-region of a zymogen may be a common theme in yeast vacuolar targeting.

Characterization of Secretory Transport *In vitro.*

Early work, which defined the secretory pathway (1-6), provided little insight into the biochemistry of vesicular transport between secretory compartments. Secretory transport was shown to be an active process, which requires a continuing supply of ATP, by treatment of cells with inhibitors of oxidative phosphorylation such as CCCP. This resulted in the accumulation of secretory proteins in transitional regions of the smooth ER where secretory vesicles bud (5). However, inhibition of protein synthesis with cycloheximide did not interrupt secretory transport (4), indicating that this process is not driven by continuing protein synthesis.

A biochemical characterization of secretory transport is only starting to emerge, with the generation of *in vitro* systems that can serve as assays for characterizing the requirements for transport. There have been several barriers to *in vitro* characterization. Because of the precise ordered fashion in which secretory proteins are passed through successive compartments, there were expectations that cytoskeletal elements might be involved in secretory transport. Thus, reconstitution of transport *in vitro* might not occur after disruption of the subcellular structure during cell fractionation. Another major problem in establishing *in vitro* transport systems, is the difficulty involved in isolating a well characterized, homogeneous population of donor compartments that contain a suitable marker substrate to monitor transport. Methods that separate ER and Golgi microsomes on sucrose density gradients unfortunately still give significant cross-contamination between the fractions (see 203). Thus, an inefficient transport system could not necessarily be distinguished from contami-

nation. As discussed above, there are a number of covalent modifications that can serve as assayable markers for transport between compartments. However, strategies must insure that the assayed modifications of the substrate are not simply the result of fusion between donor and acceptor compartments that might occur during cell lysis and fractionation.

In vitro Transport Within the Golgi Complex.

In higher eukaryotic cell lines, the vesicular stomatitis virus (VSV) G protein provides a good marker substrate because it is the only glycoprotein synthesized in VSV-infected cells. A mutant cell line (clone 15B) of Chinese hamster ovary (CHO) cells exists, which lacks UDP-GlcNAc glycosyltransferase (233). This enzyme, present in the medial compartment of the Golgi, confers EndoH resistance to core oligosaccharides that were added to proteins in the ER. Fries and Rothman were able to show that a crude cellular lysate containing newly synthesized G protein, which had been labeled by a brief pulse of [^{35}S]-methionine *in vivo* in clone 15B CHO cells, could subsequently be modified to EndoH resistance *in vitro* by the addition of a wild-type membrane fraction (234). There were several lines of evidence that this reaction resembles the *in vivo* process of vesicle mediated secretory transport. First of all, both the EndoH sensitive form of G protein (which served as the starting substrate) and the EndoH resistant form (which marked transport to a new compartment) were insensitive to exogenous trypsin digestion, indicating that they remained within sealed vesicles (234). Secondly, the conversion to EndoH resistance resembled transfer *in vivo* in that it required ATP, UDP-GlcNAc, and cell cytosol. The reaction was also sensitive to ionophores, which halt secretory transport *in vivo*, and proceeded with similar kinetics to G protein maturation *in vivo* (234,235).

Initially, this conversion to EndoH resistance was thought to represent transfer between the ER and Golgi (234). However, later experiments showed that transfer was taking place between successive compartments within the Golgi (203). Experiments where cells were chased for brief periods (0-20 min) after labeling, showed that there was a window of time

during which the substrate became available for transfer, and after which it was unable to be transferred. Extracts from cells, which had no chase *in vivo* after the 5 minute labeling period, could not serve as substrate donors and suggested that transport did not involve ER vesicles. Likewise, extracts from cells after 20 minutes of chase, by which time most of the G protein was delivered to the plasma membrane, were also incapable of serving as donors. These results indicate that modification to EndoH resistance does not result simply from non-specific fusion of donor and acceptor vesicles, and that ER and plasma membrane vesicles do not function as donors (203). The characterization of the donor as an early Golgi compartment was indicated by the fact that the time of chase where there is maximal donor activity (10 min) correlates to the time *in vivo* that it takes for passage to the Golgi (203,236). Closer examination of G protein within a functional donor compartment suggested that it had undergone trimming modifications of the core oligosaccharide, which accompany transfer to the *cis* Golgi (203). Finally, fractionation of donor vesicles on a sucrose density gradient showed that donor activity coincided with Golgi markers (203).

Subsequently, this assay system has been refined by procedures, which achieve approximately a 20 fold purification of both the donor and acceptor Golgi compartments on sucrose density step gradients (237). In addition, instead of looking at the conversion of EndoH sensitivity to EndoH resistance on a labeled G protein substrate, donor Golgi with unlabeled G protein is isolated and transfer to the acceptor compartment is measured by the incorporation of [^{3}H]-GlcNAc. These results provide further evidence that this system efficiently reconstitutes transfer from the *cis* to the *medial* Golgi subcompartments. Examination of the Golgi fractions, using electron microscopy and autoradiography (238), indicate that only a subpopulation of intact Golgi stacks are radiolabeled. Using rat liver Golgi as the acceptor compartment, which can be distinguished by its smaller median size, Braell and colleagues have shown that it is specifically the acceptor Golgi fraction that becomes radiolabeled (238).

Biochemical characterization of this assay has revealed that at least three successive steps are involved in the vesicular transport process by looking at the kinetics of transfer and the sensitivity to NEM (204). Preincubation of the donor compartment with ATP and cytosol, "primes" the reaction and significantly reduces the lag observed in incorporation following the addition of acceptor membranes. This priming step has been correlated by electron microscopy with the appearance of vesicle buds at the rim of donor Golgi cisternae. A second stage in the transfer process presumably involves completion of vesicles and their transfer by diffusion to acceptor membranes. This step requires cytosolic components and is sensitive to NEM treatment. Finally, there appears to be a significant delay in the fusion of transfer vesicles with the acceptor compartment. A pre-fusion state can be demonstrated, which no longer requires cytosol and is NEM insensitive, requiring only ATP to complete delivery (204,239). Balch et al suggest that recognition events, which specifically insure fusion with the correct acceptor subcompartment, may be responsible for the delay between vesicle binding to the acceptor Golgi stack and membrane fusion (204).

Fractionation of the cytosol required for transfer, has shown that there are multiple components that are transport-active (240). A 25-kd component, isolated from calf brain cytosol, has been identified that functions in a late stage of transport, just prior to fusion with the target cisternae (240). A role for fatty acyl-coenzyme A has also been proposed, with ATP dependent acylation and deacylation of an NEM sensitive protein required for each cycle of transport (241). Use of cytosol from a variety of different eukaryotic organisms has demonstrated that the cytosolic components required for intra-Golgi transport *in vitro* are highly conserved between mammals, yeast (242), and even plants (243). Transport from the *medial* to the *trans* Golgi has also been demonstrated recently , using a similar *in vitro* system that incorporates terminal sialic acid resides (a *trans* Golgi function) on the VSV G protein (186). In addition, each of the compartmental transfer reactions, from the

ER to the Golgi, through the Golgi subcompartments, and from the Golgi to the cell surface, have been shown to require ATP *in vitro* (244).

Reconstitution of ER to Golgi Transport In vitro.

The development of a similar *in vitro* system, which reconstitutes ER to Golgi transport, has been particularly slow in coming. There are a number of possible approaches to generating an ER donor fraction containing a suitable marker substrate, either *in vitro* by co- or post-translational translocation of newly synthesized proteins into ER microsomes, or *in vivo* utilizing temperature sensitive mutants that accumulate proteins at the ER level. However, it appears that disruption of the ER cisternal structure, which occurs during microsome isolation, destroys their ability to accomplish vesicle mediated transport to exogenous Golgi. One report of ER microsomes, isolated from a *sec*18^{ts} yeast mutant, showed some evidence of *in vitro* transport to Golgi (245). However, the efficiency of this transfer was extremely poor (<5%) suggesting that it may be the result of non-specific vesicle fusions.

Balch and co-workers have found that ER microsomes, isolated from a synchronized culture of CHO cells during mitosis, will support *in vitro* transport from the ER to the Golgi (246,247). Using a temperature sensitive mutant of VSV whose G protein accumulates in the ER at the non-permissive temperature (248), transport from the ER to the Golgi was monitored by the trimming of the [Man$_9$] core-glycosylated G protein to the [Man$_5$] form. Transfer in this system is remarkably efficient (80-90%) and depends on cytosol and ATP. The difference between this system and earlier attempts at reconstitution using ER microsomes is the use of cells in mitosis where the nuclear envelope, ER and Golgi are disassembled before cell homogenization. The authors suggest that such disassembled ER may be able to reassemble *in vitro*, and thus may be able to reconstitute a functional compartment (247). However, differences between transport in this *in vitro* system and *in vivo* transport, particularly that the transport of the ts form of G protein is

just as efficient at the non-permissive temperature, suggests that this system may be subtly different from normal secretory transport (247). In addition, it is not clear whether biochemical requirements in this system represent those involved in secretory transport or those involved in organelle re-assembly after mitosis.

The key to the establishment of other *in vitro* ER to Golgi transport assays has been the development of new techniques, which permeabilize the plasma membrane of whole cells to create "semi-intact" cells (see 249). While these methods release the cytosolic contents of the cell, intracellular organelles including the ER and Golgi are left largely intact (as judged by electron microscopy) and the plasma membrane remains permeable to macromolecules (250,251). Balch and Beckers have shown that CHO cells, which are first swollen in hypotonic buffer and then scraped from the culture dish, have their plasma membranes sheared open (250). Simons and Virta have been able to selectively perforate the apical membrane of polarized epithelial cells by laying a sheet of nitrocellulose on a confluent lawn of cells, drying and removing it carefully (251). Both of these methods result in approximately 95% of the cells being lysed. Likewise, yeast spheroplasts that are gently lysed by freeze thawing (252) or osmotic shock (253) become permeablized and are able to support ER to Golgi transport.

In CHO cells, a temperature sensitive form of VSV G protein that accumulates in the ER at the non-permissive temperature (248) has been used to monitor transport to the Golgi. Infected cells are first labeled with [^{35}S]-Met at the non-permissive temperature, permeablized, and washed. Transfer to the Golgi is monitored by the modification of core glycosylated [Man_{8-9}] EndoD-resistant G protein to a [Man_5] EndoD-sensitive structure by the trimming of oligosaccharides, which occurs upon transfer to the *cis* Golgi (250). This reaction is sensitive to 1-deoxymannorjirimycin, an inhibitor of the α-mannosidase I enzyme responsible for trimming in the *cis* Golgi (247). As in intra-Golgi transport, G protein is inaccessible to exogenous trypsin digestion both before and after the reaction indicating that it remains with sealed, membrane bound compartments (250). ER to Golgi

transport in this system requires the addition of cytosol and ATP (250). In addition, here the ts form of G protein remains temperature sensitive for *in vitro* ER to Golgi transport (250). Using a CHO cell line, which is deficient in the *cis* Golgi enzyme α-1,2-mannosidase I, responsible for conversion to the EndoD-sensitive form, transport from the ER of semi-intact cells to exogenously added Golgi isolated from wild-type cells has been demonstrated (250). Thus, transport of vesicles between the two compartments by simple diffusion is possible; structural elements such as microtubules do not appear to be required for vesicle transport. Finally, these results suggest that structural elements of ER cisternae, which are disrupted during homogenization, are important components in the process of vesicle formation.

In vitro ER to Golgi transport has also been demonstrated using semi-intact cells of the yeast *Saccharomyces cerevisiae* (252,253). In both cases, the oligosaccharide processing of [^{35}S]-Met labeled, *in vitro* translated, yeast prepro-α-factor was used to follow both translocation into the ER (by the addition of core glycosylation) and transport to the Golgi (by modification to high mannose outer chains - see 182). The discovery that prepro-α-factor can be efficiently translocated into ER post-translationally (72-74) allows the labeled substrate to be loaded into the ER under conditions, such as low temperature (10^{o}C-252), which are not able to support transport to the Golgi. Thus, the requirements for transport can be examined independently of translation and translocation. Transfer to the Golgi, in these yeast systems, has been shown to require cytosolic components, ATP, and can be inhibited by GTPγS (252,253). Both cytosolic and membrane components of this transport have been shown to be sensitive to agents that modify or degrade proteins such as NEM or trypsin (252,253). Like the mammalian systems, transport here involved sealed compartments, which were inaccessible to exogenous proteases both before and after the reaction (252,253).

Eleven complementation groups of temperature sensitive yeast *sec* mutants have been characterized, which appear to specifically block secretory transport from the ER to the

Golgi (91,254). Semi-intact cells from one of these strains (*sec*23) are also temperature sensitive for secretory transport *in vitro* and can be complemented by the addition of cytosol (100,000 xg supernatant) from a *sec*23 yeast strain containing the wild-type *SEC*23 gene on a single copy plasmid (252). Somewhat surprisingly, if *sec23* cells are incubated at the non-permissive temperature (37°C) before lysis, they are unable to support transport from the ER to the Golgi (252). Many of the *sec* mutants undergo extensive morphological alterations *in vivo* at the non-permissive temperature (252), suggesting that these mutations may have pleotropic effects on cellular structure and metabolism. Semi-intact cells from other yeast *sec* strains are often unable to support *in vitro* ER to Golgi transfer even when manipulated at the permissive temperature at all times (252).

In another report (253), *in vitro* transfer from ER was shown to be directed to the exogenous Golgi present in a 3,000 ×*g* supernatant of a yeast lysate. In contrast to other systems, the donor ER compartment could be provided by isolated yeast microsomes (253). Here, too, components from a *sec*23 mutant were temperature sensitive for transport *in vitro*. However, in contrast to the other report, the *sec*23 defect was associated with the acceptor Golgi compartment (253) rather than the cytosolic fraction (252).

Finally, it is hoped that separate characterizations of the processes of vesicle formation at a donor compartment, and vesicle fusion at the acceptor compartment, will eventually be possible. A recent report of the isolation of vesicles containing secretory proteins in transit from the ER to the Golgi could perhaps serve as a starting point for the study of vesicle target binding and fusion reactions alone (255).

Secretion in Yeast: A Genetic System for Identifying and Characterizing Components of the Secretion Machinery.

The lower eukaryote yeast (*Saccharomyces cerevisiae*) has recently become a popular system for studies of the secretory process (see reviews 207,256). However, until the characterization of yeast secretory mutants a few years ago, the existence of a eukaryotic secretory pathway in yeast was debated. There were several reasons for this. First of all, secretion is not a primary function of yeast cells as it is in pancreatic exocrine cells. There is no evidence for a regulated secretory pathway in yeast that releases products stored in secretory granules in response to environmental stimuli. Changes in the levels of secreted proteins by yeast cells in response to environmental stimuli must be accompanied by increases in protein synthesis. Thus, only low concentrations of secretory precursors are normally found within yeast cells. Secretory organelles are, likewise, not found in abundance. Moreover, the capacity of the yeast secretory system to adjust for increased levels of secretory protein production seems somewhat limited, since for example overexpression of the vacuolar protein CPY leads to missorting and secretion (257).

Second, yeast are generally poor subjects for morphological examination by techniques such as electron microscopy. The outer cell wall is thick, inhibiting fixative agents, and its disruption requires forces that can obliterate internal organelles. The high density of cytoplasmic ribosomes in yeast also tends to obscure structures (207). Later work with yeast secretory mutants has shown that the characteristic morphologies of secretory organelles, especially the Golgi complex (91), do not seem to be the same in yeast as those seen in higher eukaryotes. Thus, yeast often show little direct morphological evidence of secretory organelles.

Third, the process of protein glycosylation in yeast is different from higher eukaryotes, with yeast forming only high-mannose outer chain oligosaccharides (see above). Many of the activities involved in formation of complex-type outer chains - associated with mam-

malian Golgi - are not found in yeast. While the existence of an ER-like vesicle fraction could be demonstrated in yeast lysates, there was little evidence for a yeast Golgi compartment (207).

The isolation of conditional yeast mutants, which appear to block the process of secretion at specific stages in the transport between compartments, has been the key to demonstrating the existence of a eukaryotic secretory pathway in yeast and characterizing the maturation of secretory proteins that occurs in transit. The mutant genes themselves, which presumably provide components of the secretion machinery, are only starting to be characterized. This, along with the development of *in vitro* transport systems, holds great promise for advancing the understanding of the secretory process.

Isolation Class A sec Mutants and Characterization of Phenotypes.

In 1979, Novick and Schekman first reported the isolation of two conditional yeast mutants (*sec*1-1 and *sec*2-1) which appeared to specifically block the secretory process (258). These two *sec* mutants were found from a screen of only 87 clones that were first identified as being temperature-sensitive for growth. In addition to the growth defect, these mutants were also temperature-sensitive for secretion of acid phosphatase and invertase, as well as the incorporation of sulfate permease into the plasma membrane (98). At the non-permissive temperature, these mutants continued protein synthesis and accumulated active forms of the secreted enzyme markers intracellularly (98). These intracellular pools could be secreted later, when cells were shifted back to the permissive temperature and cycloheximide was added to block new protein synthesis. Finally, these mutants showed a marked accumulation of small vesicles (50-70nm) at the non-permissive temperature, which stained for the acid phosphatase marker (258) and resembled secretory vesicles - rarely seen in yeast.

In a later paper, Novick et al (91) found that these secretory mutants showed an increase in density at the non-permissive temperature and could be selectively enriched on a

Luddox density gradient. Using this method, a total of 485 secretion-defective temperature sensitive mutants were isolated and put into three classes. In one class, protein synthesis was shown to be temperature-sensitive (class C). In another, cells accumulated inactive forms of the secreted enzymes intracellularly. These class B mutants formed two complementation groups (*sec*53 and *sec*59), initially thought to be involved in the translocation of proteins across the ER membrane (see above and 95,96). Finally, 188 mutant alleles were designated as class A *sec* mutants which, like the earlier *sec*1 and *sec*2 mutants, accumulated active secretory proteins intracellularly at the non-permissive temperature (91). These mutations fell into 23 complementation groups (*sec*1-23). Again, like *sec*1 and *sec*2 mutants, representative alleles of most of these *sec* mutants were able to resume secretion of intracellular pools, accumulated at the non-permissive temperature, upon return to the permissive temperature and in the presence of cycloheximide to inhibit new protein synthesis (91).

Electron microscopy of *sec* mutants suggested that they could be divided into three subgroups based on the morphologies of the intracellular structures that they accumulated at the non-permissive temperature (91). Nine complementation groups (*sec*12, *sec*13, *sec*16, *sec*17, *sec*18, *sec*20, *sec*21, *sec*22, *sec*23) accumulated ER, which was seen as single thin tubules that were often contiguous with the nuclear membrane. Two additional complementation groups with this phenotype have been generated using a [^{3}H] mannose suicide selection (*bet*1 and *bet*2 - 254). Ten complementation groups (*sec*1, *sec*2, *sec*3, *sec*4, *sec*5, *sec*6, *sec*8, *sec*9, *sec*10, *sec*15) accumulated numerous small vesicles. Two complementation groups (*sec*7, *sec*14) showed a previously unseen bulbous structure, which the authors named a Berkeley body (91). The two remaining complementation groups (*sec*11, *sec*19) showed an indeterminant morphology. The authors suggested that the Berkeley body structure represented the yeast equivalent of the Golgi apparatus. Furthermore, they postulated that these mutants defined a pathway of ER -> Berkeley body -> vesicles -> cell surface, completely analogous to the secretion pathway in higher eukaryotes

(91). The order of these subgroups in the secretion pathway was confirmed by further morphological studies of the phenotypes of double *sec* mutants (259). In addition, these studies confirmed that the order in the pathway of these mutants also correlates to the process of protein maturation that takes place during secretion. For example, the subgroup of *sec* mutants, which appear to accumulate ER morphologically, likewise accumulate only core-glycosylated forms of invertase (259,260). The other subgroups, which are later in the pathway, accumulate invertase whose outer chains have been hyper-glycosylated (259,260) analogous to the maturation of outer chain oligosaccharides, which takes place in the Golgi of higher eukaryotes. Finally, under certain conditions (0.1% glucose) one of the mutants that accumulated Berkeley bodies at the non-permissive temperature(*sec*7), shows the presence of Golgi bodies with normal eukaryotic morphology (259). This supports the hypothesis that the secretory pathway of higher eukaryotes is conserved in yeast.

The yeast *sec* mutants have been used extensively to examine the processing events, which modify proteins as they follow the secretory pathway in yeast (see reviews 93,256). These studies have shown that, in addition to plasma membrane and secreted proteins, vacuolar proteases such as CPY (261) are also targeted through the yeast secretory pathway. This is analogous to the delivery of hydrolases to the lysosome, through the secretory pathway in higher eukaryotes. The *sec* mutants have proven invaluable in numerous studies for interrupting the secretory process at particular stages and allowing characterization of processing intermediates (i.e., 182). In particular, the *sec*18 and *sec*7 mutants are widely used as representative *sec* mutants to characterize the ER and Golgi stages of the secretory pathway (respectively), because they provide a rapid and complete block in the secretory process.

Cloning and Characterization of SEC Genes.

The temperature-sensitive growth phenotype of *sec* mutants has been used to clone a number of the *sec* genes. This is done by transformation of a *sec* mutant strain with a li-

brary of wild-type yeast genomic fragments, and subsequent selection for growth at the non-permissive temperature indicating complementation of the *sec* mutation. To date, genes for at least 7 of the 25 complementation groups of Class A *sec* (and *bet*) mutants have been cloned and characterized to various degrees. These include the genes for the ER accumulating mutants *sec* 12 (262), *sec*18 (263), and *sec*23 (264); the Golgi accumulating mutants *sec*7 (265) and *sec*14 (266); and the secretory vesicle accumulating mutant *sec*4 (267,268). The *SEC*11 gene, corresponding to a mutant whose order in the secretion pathway was not clear from earlier studies, has also been cloned and sequenced (269). Gene disruption has shown that all of the *sec* genes cloned so far are essential for yeast cell growth (262-269).

The most thoroughly characterized *SEC* gene is *SEC*4. This gene codes for a 23,479 kd protein that shows significant homology to the G-protein (or *ras*) gene family. This gene family is known to function in signal transduction and the regulation of cellular metabolism (270). Upon stimulation, G-proteins have been shown to bind GTP and activate an effector enzyme such as adenylate cyclase. Inactivation, then, involves the slow hydrolysis of GTP. Different G-proteins can be permanently activated or inactivated by non-hydrolyzable GTP analogs, such as GTPγS, which subsequently has been shown to inhibit secretory transport *in vitro* (252,253,271). Sec4p shares homology with G proteins in regions of the proteins thought to constitute a GTP binding site (267), and has been directly shown to bind [32P]GTP (268). These similarities have led to suggestions that Sec4p may regulate the secretory process in yeast (267,268). There are indications that members of the G protein family may commonly function in the regulation of secretion (272,273), including evidence that inhibition of G protein function by GTPγS halts transfer between Golgi subcompartments *in vitro* and leads to accumulation of non-clathrin coated buds on Golgi cisternae (271).

From the DNA sequence, the predicted *SEC*4 protein structure is uncharged at neutral pH and contains no long hydrophobic stretches of amino acids suggesting that the protein

should remain soluble in the cellular cytoplasm (267). However, characterization of the intracellular location of Sec4p by cell fractionation and immunofluorescence, indicates that it associates primarily with the cytoplasmic face of secretory vesicles and the plasma membrane (268). Kinetic studies suggest that newly synthesized Sec4p becomes rapidly associated with the membrane ($t_{1/2} \leq 1$ min.) (268). The authors suggest that this association may be the result of some covalent modification such as the fatty acid acylation, like that seen at the carboxy-terminus of the *ras* protein (274). In spite of this, a significant portion of the total pool of Sec4p (15%) remains in the cytoplasm at steady state, suggesting the protein may function by cycling on and off the membrane of transport vesicles (268).

Another yeast gene (*ypt*1) has been identified that codes for a member of the G-protein family, and causes defects in secretion somewhat similar to the *sec* mutants (275). Like Sec4p, the YPT1 protein binds GTP and appears to associate with intracellular membrane bound structures. Mammalian homologs of the YPT1 protein have been identified that are over 70% homologous to the yeast protein (276). Immunofluorescence using YPT1 antisera to stain mammalian cells shows that it is associated with the Golgi apparatus, which suggests that it may perform a function similar to Sec4p at an earlier point in the secretion pathway (275).

Preliminary characterizations of genes corresponding to the Golgi accumulating mutants *sec*7 and *sec*14 are in progress (265,266). The DNA sequence for the *SEC*7 gene has an exceptionally long open reading frame (2008 aa), which could encode a 230 kd protein. One stretch of 125 amino acids within this predicted sequence contains approximately 70% negatively charged amino acids (265). The sequence of the *SEC*14 gene predicts that it would encode a 35 kd hydrophilic protein. Antibodies raised against a Sec14-T_4 lysozyme fusion protein have identified an unglycosylated 37 kd protein in yeast (266).

While the initial studies of the *sec* mutants were unable to clearly identify the position of the *sec*11 defect in the secretory pathway (259), further characterization of *sec*11 mutants

indicate that they are defective in the removal of the signal peptide from the amino-terminus of soluble secretory proteins (269). The DNA sequence of the *SEC*11 gene shows that it codes for an 18.8 kd protein that is very basic (pI ≈ 9.5) and has an amino-terminal hydrophobic domain, which may serve as a membrane anchor. The size and charge characteristics of the predicted Sec11p are similar to subunits of the canine and hen oviduct signal peptidases (269).

Finally, three of the *SEC* genes from ER accumulating yeast mutants have been characterized. The *SEC*23 gene encodes an abundant 84 kd protein in yeast (264). In contrast, the *SEC*12 and *SEC*18 genes are only expressed at very low levels (262,263). A 70 kd glycoprotein has been identified using antibodies to a Sec12p-β-galactosidase fusion protein. This protein appears to be an integral membrane protein, consistent with the presence of a possible membrane-spanning hydrophobic domain predicted by the DNA sequence (262). The data suggest that Sec12p is an integral membrane protein of the yeast ER (262). Our lab has been involved with the characterization of the *SEC*18 gene. While there is only a single open reading frame in the DNA sequence of *SEC*18, two Sec18p protein products (82 and 84 kd) have been identified both *in vitro* and *in vivo*. Both Sec18p proteins remain cytoplasmic, but there appears to be some association of Sec18p with a low density fraction of cellular vesicles (263), suggesting that Sec18p may function by interacting with transport vesicles between the ER and Golgi.

References

1. Palade, G. E. Intracellular aspects of the process of proteins synthesis. Science 189:347-358 1975.

2. Jamieson, J.D. and Palade, G. E. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. J. Cell Biol. 34:577-596 1967.

3. Jamieson, J.D. and Palade, G. E. Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transporting to condensing vacuoles and zymogen granules. J. Cell Biol. 34:597-615 1967.

4. Jamieson, J.D. and Palade, G. E. Intracellular transport of secretory proteins in the pancreatic exocrine cell. III. Dissociation of intracellular transport from protein synthesis. J. Cell Biol. 39:580-588 1968.

5. Jamieson, J.D. and Palade, G. E. Intracellular transport of secretory proteins in the pancreatic exocrine cell. IV. Metabolic requirements. J. Cell Biol. 39:589-603 1968.

6. Farquhar, M.G. and Palade, G. E. The Golgi apparatus (complex) - (1954-1981) - from artifact to center stage. J. Cell Biol. 91:77s-103s 1981.

7. Dawidowicz, E. A. Dynamics of Membrane Lipid Metabolism and Turnover. Ann. Rev. Biochem. 56:43-61 1987.

8. Rolleston, F. S. Membrane bound and free ribosomes. Sub-Cell. Biochem. 3:91-117 1974.

9. Milstein, C., Brownlee, G. G., Harrison, T. M. and Mathews, M. B. A possible precursor of immunoglobulin light chains. Nature New Biology 239:117-120 1972.

10. Blobel, G. and Dobberstein, B. Transfer of proteins across membranes: I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. 67:835-851 1975.

11. Wickner, W. Assembly of Proteins into membranes. Science 210:861-868 1980.

12. von Heijne, G. Signal sequences: The limits of variation. J. Mol. Biol. 184:99-105 1985.

13. Kreil, G. Transfer of proteins across membranes. Ann. Rev. Biochem. 50:317-348 1981.

14. Erickson, A. H., Conner, G. E. and Blobel, G. Biosynthesis of a lysosomal enzyme. J. Biol. Chem. 256:11224-11231 1981.

15. Lingappa, V. R., Katz, F. N., Lodish, H. F. and Blobel, G. A signal sequence for the insertion of a transmembrane glycoprotein. J. Biol. Chem. 253:8667-8670 1978.

16. Talmadge, K., Brosius, J. and Gilbert, W. An 'internal' signal sequence directs secretion and processing of proinsulin in bacteria. Nature 294:176-178 1981.

17. Talmadge, K., Stahl, S. and Gilbert, W. Eukaryotic signal sequence transports insulin antigen in E. coli. PNAS 77:3369-3373 1980.

18. Garcia, P. D., Ghrayeb, J., Inouye, M. and Walter, P. Wild type and mutant signal peptides of E. coli outer membrane lipoprotein interact with equal efficiency with mammalian signal recognition particle. J. Biol. Chem. 262:9463-9468 1987.

19. Garoff, H. Using recombinant DNA techniques to study protein targeting in the eukaryotic cell. Ann. Rev. Cell Biol. 1:403-45 1985.

20. Moreno, F., Fowler, A. V., Hall, M., Silhavy, T. J., Zabin, I. and Schwartz, M. A signal sequence is not sufficient to lead β-galactosidase out of the cytoplasm. Nature 286:356-359 1980.

21. Emr, S. D., Hall, M. and Silhavy, T. J. A mechanism of protein localization: the signal hypothesis and bacteria. J. Cell Biol. 86:701-711 1980.

22. Emr, S. D. and Silhavy, T. J. Molecular components of the signal sequence that function in the initiation of protein export. J. Cell Biol. 95:689-696 1982.

23. Emr, S. D. and Silhavy, T. J. The importance of secondary structure in the signal sequence for protein secretion. PNAS 80:4599-4603 1983.

24. Kaiser, C. A., Preuss, D., Grisafi, P. and Botstein, D. Many random sequences functionally replace the secretion signal sequence of yeast invertase. Science 235:312-317 1987.

25. Briggs, M. S., Gierasch, L. M., Zlotnick, A., Lear, J. D. and DeGrado, W. F. In vivo function and membrane binding properties are correlated for E. coli Lam B signal peptides. Science 228:1096-1099 1985.

26. Ohno-Iwasita, Y., Wolfe, P. and Wickner, W. Processing of preproteins by liposomes bearing leader peptidase. Biochemistry 23:6178-6184 1984.

27. von Heijne, G. Mitochondrial targeting sequences may form amphiphilic helices. EMBO J. 5:1335-1342 1986.

28. Allison, D. S. and Schatz, G. Artificial Mitochondrial Presequences. PNAS 83:9011-9015 1986.

29. Baker, A. and Schatz, G. Sequences from a prokaryotic genome or the mouse dihydrofolate reductase gene can restore the import of a truncated precursor protein into yeast mitochondria. PNAS 84:3117-3121 1987.

30. Hurt, E. C., Soltanifar, N., Goldschmidt-Clermont, M., Rochaix, J. and Schatz. G. The cleavable pre-sequence of an imported chloroplast protein directs attached polypeptides into yeast mitochondria. EMBO J. 5:1343-1350 1986.

31. Pfanner, N., Pfaller, R. and Neupert, W. How finicky is mitochondrial protein import? TIBS 13:165-167 1988.

32. Gray, M. W. and Doolittle, W. F. Has the endosymbiont hypothesis been proven? Microbiol. Rev. 46:1-42 1982.

33. Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H. and Schatz, G. A chemically synthesized pre-sequence of an imported mitochondrial protein can form an amphiphilic helix and perturb natural and artificial phospholipid bilayers. EMBO J. 5:1327-1334 1986.

34. Hurt, E. C. and van Loon, A. P. G. M. How proteins find mitochondria and intramitochondrial compartments. TIBS 11:204-207 1986.

35. Hirst, T. R. and Welch, R. A. Mechanisms for secretion of extracellular proteins by gram-negative bacteria. TIBS 13:265-269 1988.

36. Fields, S., Winter, G. and Brownlee, G. G. Structure of the neuraminidase gene in human influenza virus A/PR/8/34. Nature 290:213-217 1981.

37. Wickner, W. T. and Lodish, H. F. Multiple mechanisms of protein insertion into and across membranes. Science 230:400-407 1985.

38. Gagnon, J., Palmiter, R. D. and Walsh, K. A. Comparison of the NH_2-terminal sequence of ovalbumin as synthesized *in vitro* and *in vivo*. J. Biol. Chem. 253:7464-7468 1978.

39. Braell, W. A. and Lodish, H. F. Ovalbumin utilizes an NH_2-terminal signal sequence. J. Biol. Chem. 257:4578-4582 1982.

40. Lingappa, V. R., Lingappa, J. R. and Blobel, G. Chicken ovalbumin contains an internal signal sequence. Nature 281:117-121 1979.

41. Mize, N. C., Andrews, D. W. and Lingappa, V. R. A stop transfer sequence recognizes receptors for nascent chain translocation across the endoplasmic reticulum. Cell 47:711-719 1986.

42. Spiess, M. and Lodish, H. F. An internal signal sequence: the asialoglycoprotein receptor membrane anchor. Cell 44:177-185 1986.

43. Braell, W. A. and Lodish, H. F. The erythrocyte anion transport protein is cotranslationally inserted in microsomes. Cell 28:23-31 1982.

44. Blobel, G. Intracellular protein topogenesis. PNAS 77:1496-1500 1980.

45. Friedlander, M. and Blobel, G. Bovine opsin has more than one signal sequence. Nature 318:338-343 1985.

46. Zerial, M., Huylebroeck, D. and Garoff, H. Foreign transmembrane peptides replacing the internal signal sequence of transferrin receptor allow its translocation and membrane binding. Cell 48:147-155 1987.

47. Coleman, J., Inukai, M. and Inouye, M. Dual functions of the signal peptide in protein transfer across the membrane. Cell 43:351-360 1985.

48. Blobel, G. and Dobberstein, B. Transfer of proteins across membranes: II. Reconstitution of Functional Rough Microsomes from Heterologous Components. J. Cell Biol. 67:852-862 1975.

49. Walter, P. and Blobel, G. Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. PNAS 77:7112-7116 1980.

50. Walter, P., Ibrahimi, I. and Blobel, G. Translocation of proteins across the endoplasmic reticulum I. Signal recognition protein (SRP) binds to *in vitro* assembled polysomes synthesizing secretory protein. J. Cell Biol. 91:545-550 1981.

51. Walter, P. and Blobel, G. Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence dependent and site-specific arrest of chain elongation that is released by microsomal membranes. J. Cell Biol. 91:557-561 1981.

52. Meyer, D. I. Signal recognition particle (SRP) does not mediate a translational arrest of nascent secretory proteins in mammalian cell-free systems. EMBO J. 4:2031-2033 1985.

53. Walter, P. and Blobel, G. Translocation of proteins across the endoplasmic reticulum II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of *in vitro* assembled polysomes synthesizing secretory protein. J. Cell Biol. 91:551-556 1981.

54. Meyer, D. I., Krause, E. and Dobberstein, B. Secretory protein translocation across membranes - the role of the 'docking protein.' Nature 297:647-650 1982.

55. Lauffer, L., Garcia, P. D., Harkins, R. N., Coussens, L., Ullrich, A. and Walter, P. Topology of the signal recognition particle receptor in endoplasmic reticulum membrane. Nature 318:334-338 1985.

56. Gilmore, R. and Blobel, G. Transient involvement of signal recognition particle and its receptor in the microsomal membrane prior to protein translocation. Cell 35:677-685 1983.

57. Wiedmann, M., Kurzchalia, T. V., Hartmann, E. and Rapoport, T. A. A signal sequence receptor in the endoplasmic reticulum. Nature 328:830-833 1987.

58. Evans, E. A., Gilmore, R. and Blobel, G. Purification of microsomal signal peptidase as a complex. PNAS 83:581-585 1986.

59. Walter, P. and Blobel, G. Disassembly and reconstitution of signal recognition particle. Cell 34:525-533 1983.

60. Busch, H., Reddy, R., Rothblum, L. and Choi, Y. C. SnRNAs, SnRNPs, and RNA processing. Ann. Rev. Biochem. 51:617-653 1982.

61. Walter, P. and Blobel, G. Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature 299:691-698 1982.

62. Ullu, E., Murphy, S. and Melli, M. Human 7SL RNA consists of a 140 nucleotide middle-repetitive sequence inserted in an Alu sequence. Cell 29:195-202 1982.

63. Siegel, V. and Walter, P. Removal of the Alu structural domain from signal recognition particle leaves its protein translocation activity intact. Nature 320:81-84 1986.

64. Kurzchalia, T. V., Wiedman, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H. and Rapoport, T. A. The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. Nature 320:634-636 1986.

65. Siegel, V. and Walter, P. Each of the activities of signal recognition particle (SRP) is contained within a distinct domain: Analysis of biochemical mutants of SRP. Cell 52:39-49 1988.

66. Meyer, D. I. and Dobberstein, B. Identification and characterization of a membrane component essential for the translocation of nascent proteins across the membrane of the endoplasmic reticulum. J. Cell Biol. 87:503-508 1980.

67. Meyer, D. I., Louvard, D. and Dobberstein, B. Characterization of molecules involved in protein translocation using specific antibody. J. Cell Biol. 92:579-583 1982.

68. Tajima, S., Lauffer, L., Rath, V. L. and Walter, P. The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. J. Cell Biol. 103:1167-1178 1986.

69. Walter, P. and Blobel, G. Subcellular distribution of Signal Recognition Particle and 7SL-RNA determined with polypeptide-specific antibodies and complementary DNA probe. J. Cell Biol. 97:1693-1699 1983.

70. Walter, P., Gilmore, R. and Blobel, G. Protein translocation across the endoplasmic reticulum. Cell 38:5-8 1984.

71. Siegal, V. and Walter, P. Elongation arrest is not a prerequisite for secretory protein translocation across the microsomal membrane. J. Cell Biol. 100:1913-1915 1985.

72. Rothblatt, J. A. and Meyer, D. I. Secretion in yeast: translocation and glycosylation of prepro-α-factor *in vitro* can occur via an ATP-dependent post-translational mechanism. EMBO J. 5:1031-1036 1986.

73. Hansen, W., Garcia, P. D. and Walter, P. *In vitro* protein translocation across the yeast endoplasmic reticulum: ATP-dependent post-translational translocation of the prepro-α-factor. Cell 45:397-406 1986.

74. Waters, M. G. and Blobel, G. Secretory protein translocation in a yeast cell-free system can occur posttranslationally and requires ATP hydrolysis. J. Cell Biol. 102:1543-1550 1986.

75. Garcia, P. D. and Walter, P. Full-length prepro-α-factor can be translocated across the mammalian microsomal membrane only if translation has not terminated. J. Cell Biol. 106:1043-1048 1988.

76. Perara, E., Rothman, R. E. and Lingappa, V. R. Uncoupling translocation from translation: Implications for transport of proteins across membranes. Science 232:348-352 1986.

77. Maher, P. A. and Singer, S. J. Disulfide bonds and the translocation of proteins across membranes. PNAS 83:9001-9005 1986.

78. Breenwald, P., Liao, X., Holm, K., Porter, G. and Wise, J. A. Identification of an essential Schizosaccharomyces pombe RNA homologous to the 7SL component of the signal recognition particle. Mol. Cel. Biol. 8:1580-1590 1988.

79. Kreibich, G., Frienstein, C. M., Pereyra, N., Ulrich, B. L. and Sabatini, D. D. Proteins of rough microsomal membranes related to ribosome binding. II Cross-linking of bound ribosomes to specific membrane proteins exposed at the binding sites. J. Cell Biol. 77:488-506 1978.

80. Hortsch, M., Avossa, D. and Meyer, D. I. Characterization of secretory protein translocation: ribosome-membrane interaction in endoplasmic reticulum. J. Cell Biol. 103:241-253 1986.

81. Rothblatt, J. A. and Meyer, D. I. Secretion in yeast: reconstitution of the translocation and glycosylation of a-factor and invertase in a homologous cell-free system. Cell 44:619-628 1986.

82. Waters, M. G., Chirico, W. J. and Blobel G. Protein translocation across the yeast microsomal membrane is stimulated by a soluble factor. J. Cell Biol. 103:2629-2636 1986.

83. Fecycz, I. T. and Blobel, G. Soluble factors stimulating secretory protein translocation in bacteria and yeast can substitute for each other. PNAS 84:3723-3727 1987.

84. Chirico, W. J., Waters, M. G. and Blobel, G. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature 322:805-810 1988.

85. Lindquist, S. The heat-shock response. Ann. Rev. Biochem. 55:1151-1191 1986.

86. Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. and Schekman, R. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature 332:800-805 1988.

87. Kassenbrock, C. K., Garcia, P. D., Walter, P. and Kelly, R. B. Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*. Nature 333:90-93 1988.

88. Pelham, H. Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell 46:959-961 1986.

89. Pelham, H. Heat shock proteins: coming in from the cold. Nature 332:776-777 1988.

90. Hemmingsen , S. M., Woolford, C., van der Vies, S., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W. and Ellis, R. J. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature 333:330-334 1988.

91. Novick, P., Field, C. and Schekman, R. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21:205-215 1981.

92. Gallili, G. and Lampen, O. Large and small invertases and the yeast cell cycle: pattern of synthesis and sensitivity to tunicamycin. Biochem. Biophys. Acta 475:113-122 1977.

93. Kukuruzinska, M. A., Bergh, M. L. E. and Jackson, B. J. Protein glycosylation in yeast. Ann. Rev. Biochem. 56:915-944 1987.

94. Ferro-Novick, S., Novick, P., Field, C. and Schekman, R. Yeast secretory mutants that block the formation of active cell surface enzymes. J. Cell Biol. 98:35-43 1984.

95. Ferro-Novick, S., Hansen, W., Schauer, I. and Schekman, R. Genes required for completion of import of proteins into the endoplasmic reticulum in yeast. J. Cell Biol. 98:44-53 1984.

96. Deshaies, R. J. and Schekman, R. A yeast mutant defective at an early stage in import of secretory protein presursors into the endoplasmic reticulum. J. Cell Biol. 105:633-645 1987.

97. Bernstein, M., Hoffmann, W., Ammerer, G. and Schekman, R. Characterization of a gene product (Sec53p) required for protein assembly in the yeast endoplasmic reticulum. J. Cell Biol. 101:2374-2382 1985.

98. Ruohola, H. and Ferro-Novick, S. Sec53, a protein required for an early step in secretory protein processing and transport in yeast, interacts with the cytoplasmic surface of the endoplasmic reticulum. PNAS 84:8468-8472 1987.

99. Feldman, R.I., Bernstein, M. and Schekman, R. Product of the SEC53 gene is required for folding and glycosylation of secretory proteins in the lumen of the yeast endoplasmic reticulum. J. Biol. Chem. 262:9332-9339 1987.

100. Huffaker, T. C. and Robbins, P. W. Yeast mutants deficient in protein glycosylation. PNAS 80:7466-7470 1983.

101. Runge, K. W. and Robbins P. W. *Saccharomyces cerevisiae* mutants in the early stages of protein glycosylation. Microbiology-1986 312-316 1986.

102. Kepes, F. and Schekman, R. The yeast SEC53 gene encodes phosphomannomutase. J. Biol. Chem. 263:9155-9161 1988.

103. Bassford, P. J. Jr., Silhavy, T. J. and Beckwith, J. R. Use of gene fusion to study secretion of maltose-binding protein into E. coli periplasm. J. Bact. 139:19-31 1979.

104. Oliver, D. B. and Beckwith, J. E. coli mutant pleiotropically defective in the export of secreted proteins. Cell 25:765-772 1981.

105. Oliver, D. B. and Beckwith, J. Identification of a new gene (SecA) and gene product involved in the secretion of envelope proteins in E. coli. J. Bact. 150:686-691 1982.

106. Oliver, D. B. and Beckwith, J. Regulation of a membrane component required for protein secretion in E. coli. Cell 30:311-319 1982.

107. Collier, D. N., Bankaitis, V. A., Weiss, J. B. and Bassford, P. J. Jr. The antifolding activity of SecB promotes export of the E. coli maltose binding protein. Cell 53:273-283 1988.

108. Randall, L. L., Hardy, S. J. S. and Thom, J. R. Export of protein: a biochemical view. Ann. Rev. Microbiol. 41:507-541 1987.

109. Emr, S. D., Hanley-Way, W. and Silhavy, T. J. Suppressor mutations that restore export of a protein with defective signal sequence. Cell 32:79-88 1981.

110. Shultz, J., Silhavy, T. J., Berman, M. L., Fill, N. and Emr, S.D. A previously unidentified gene in the *spc* operon of Escherichia coli K12 specifies a component of the protein exprot machinery. Cell 31:227-235 1982.

111. Cerretti, D. P., Dean, D., Davis, G. R., Bedwell, D. M. and Nomura, M. The *spc* ribosomal protein operon of *E. coli*: sequence and cotranscription of the riblsomal protein genes and a protein export gene. Nuc. Acids Res. 11:2599-2616 1983.

112. Akiyama, Y. and Ito, K. The SecY membrane component of the bacterial protein export machinery: analysis by new electrophoretic methods for integral membrane proteins. EMBO J. 4:3351-3356 1985.

113. Zwizinski, C. and Wickner, W. Purification and characterization of leader (signal) peptidase from E. coli. J. Biol. Chem. 255:7973-7977 1980.

114. Wolfe, P. B., Silver, P. and Wickner, W. The isolation of homogeneous leader peptidase from a strain of E. coli which overproduces the enzyme. J. Biol. Chem. 257:7898-7902 1982.

115. Smith, W. P. Cotranslational secretion of diphtheria toxin and alkaline phosphatase *in vitro*: involvement of membrane protein(s). J. Bact. 141:1142-1147 1980.

116. Muller, M. and Blobel, G. *In vitro* translocation of bacterial proteins across the plasma membrane of E. coli. PNAS 81:7421-7125 1984.

117. Chen, L. and Tai, P. C. ATP is essential for protein translocation into E. coli membrane vesicles. PNAS 82:4384-4388 1985.

118. Geller, B. L., Movva, N. R. and Wickner, W. Both ATP and the electrochemical potential are required for optimal assembly of pro-OmpA into E. coli inner membrane vesicles. PNAS 83:4219-4222 1986.

119. Muller, M. and Blobel, G. Protein export in E. coli requires a soluble activity. PNAS 81:7737-7741 1984.

120. Caulfield, M. P., Furlong, D., Tai, P. C. and Davis, B. D. Secretory S complex of B. subtilis forms a large, organized structure when released from ribosomes. PNAS 82:4031-4035 1985.

121. Crooke, E. and Wickner, W. Trigger factor: a soluble protein that folds pro-OmpA into a membrane-assembly-competent form. PNAS 84:5216-5220 1987.

122. Firgaira, F. A., Hendrick, J. P., Kalousek, F., Kraus, J. P. and Rosenberg, L. E. RNA required for import of precursor proteins into mitochondria. Science 226:1319-1322 1984.

123. Ohta, S. and Schatz, G. A purified precursor polypeptide requires a cytosolic protein fraction for import into mitochondria. EMBO J. 3:651-657 1984.

124. Chua, N. H. and Schmidt, G. W. Transport of proteins into mitochondria and chloroplasts. J. Cell Biol. 81:461-483 1979.

125. Gasser, S. M., Daum, G. and Schatz, G. Import of proteins into mitochondria: energy dependent uptake of precursors by isolated mitochondria. J. Biol. Chem. 257:13034-13041 1982.

126. Pain, D., Kanwar, Y. S. and Blobel, G. Identification of a receptor for protein import into chloroplasts and its localization to envelope contact zones. Nature 331:232-237 1988.

127. Dobberstein, B., Blobel, G. and Chua, N. H. *In vitro* synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-bisphosphate carboxylase of *Chlamydomonas reinhardtii*. PNAS 74:1082-1085 1977.

128. Eilers, M., Oppliger, W. and Schatz, G. Both ATP and an energized inner membrane are required to import a purified precursor into mitochondria. EMBO J. 6:1073-1077 1987.

129. Grossman, A., Bartlett, S. and Chua, N. H. Energy-dependent uptake of cytoplasmically synthesized polypeptides by chloroplasts. Nature 285:625-628 1980.

130. Pain, D. and Blobel, G. Protein import into chloroplasts requires a chloroplast ATPase. PNAS 84:3288-3292 1987.

131. Eilers, M. and Schatz, G. Protein unfolding and the energetics of protein translocation across biological membranes. Cell 52:481-483 1988.

132. Chen, W. J. and Douglas, M. G. Phosphodiester bond cleavage outside mitochondria is required for the completion of protein import into the mitochondrial matrix. Cell 49:651-658 1987.

133. Chen, W. J. and Douglas, M. G. The role of protein structure in the mitochondrial import pathway: unfolding of mitochondrially bound precursors is required for membrane translocation. J. Biol. Chem. 262:15605-15609 1987.

134. Pfanner, N., Tropschug, M. and Neupert, W. Mitochondrial protein import: nucleoside triphosphates are iinvolved in conferring import-competence to precursors. Cell 49:815-823 1987.

135. Warren, G. Stop-go proteins. Nature 297:624-625 1982.

136. Douglas, M. and Takeda, M. Nuclear genes encoding mitochondrial proteins in yeast. TIBS 10:192-194 1985.

137. Maccecehini, M. L., Rudin, Y., Blobel, G. and Schatz, G. Import of proteins into mitochondria: precursor forms of the extramitochondrially made F_1-ATPase subunits in yeast. PNAS 76:343-347 1979.

138. Suissa, M. and Schatz, G. Import of proteins into mitochondria: translatable mRNAs for imported mitochondrial proteins are present in free as well as mitochondria-bound cytoplasmic polysomes. J. Biol. Chem. 257:13048-13055 1982.

139. Schatz, G. How mitochondria import proteins from the cytoplasm. FEBS Letters 103:201-211 1979.

140. Neupert, W. and Schatz, G. How proteins are transported into mitochondria. TIBS 6:1-4 1981.

141. Reid, G. A. and Schatz, G. Import of proteins into mitochondria: extramitochondrial pools and post-translational import of mitochondrial precursors *in vivo*. J. Biol. Chem. 257:13062-13067 1982.

142. Schatz, G. and Butow, R. A. How are proteins imported into mitochondria? Cell 32:316-318 1983.

143. van Tiel-Menkveld, G. J., Rezee, A. and de Graff, F. K. Production and excretion of cloacin DF13 by E. coli harboring plasmid CloDF13. J. Bact. 140:415-423 1979.

144. Cancedda, R. and Schlessinger, M. J. Localization of polyribosomes containing alkaline phosphatase nascent polypeptides on membranes of E. coli. J. Bact. 117:290-301 1974.

145. Smith, W. P., Tai, P. C., Thompson, R. C. and Davis, B. Extracellular labeling of nascent polypeptides traversing the membrane of E. coli. PNAS 74:2830-2834 1977.

146. Schatz, G. A common mechanism for different membrane systems? Nature 321:108-109 1986

147. Wickner, W. The assembly of proteins into biological membranes: The membrane trigger hypothesis. Ann. Rev. Biochem. 48:23-45 1979.

148. Davis, B. D. and Tai, P. C. The mechanism of protein secretion across membranes. Nature 283:433-438 1980.

149. Sabatini, D. D., Kreibich , G., Morimoto, T. and Adesnik, M. Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. 92:1-22 1982.

150. Von Hejne, G. and Blomberg, C. Trans-membrane translocation of proteins: The direct transfer model. Eur. J. Biochem. 97:175-181 1979.

151. Engelman, D. M. and Steitz, T. A. The spontaneous insertion of proteins into and across membranes: the helical hairpin hypothesis. Cell 23:411-422 1981.

152. Josefsson, L. G. and Randall, L. L. Different exported proteins in *E. coli* show differences in the temporal mode of processing *in vivo*. Cell 25:151-157 1981.

153. Felmlee, T., Pellett, S., Lee, E. Y. and Welch, R. A. *E. coli* hemolysin is released extracellularly without cleavage of a signal peptide. J. Bact. 163:88-93 1985.

154. Eilers, M. and Schatz, G. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. Nature 322:228-232 1986.

155. Park, S., Liu, G., Topping, T. B., Cover, W. H. and Randall, L. L. Modulation of folding pathways of exported proteins by the leader sequence. Science 239:1033-1035 1988.

156. Collier, D. N., Bankaitis, V. A., Weiss, J. B. and Bassford, P. J. Jr. The antifolding activity of SecB promotes the export of the E. coli maltose-binding protein. Cell 53:273-283 1988.

157. Schleyer, S. and Neupert, W. Transport of proteins into mitochondria: translocation intermediates spanning contact sites between outer and inner membranes. Cell 43:339-350 1985.

158. Hartl, F. U., Schmidt, B., Wachter, E., Weiss, H. and Neupert, W. Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. Cell 47:939-951 1986.

159. Lubben, T. H., Bansberg, J. and Keegstra, K. Stop-transfer regions do not halt translocation of proteins into chloroplasts. Science 238:1112-1113 1987.

160. Hubbard, S. C. and Ivatt, R. J. Synthesis and processing of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 50:555-583 1981.

161. Kornfeld, R. and Kornfeld, S. Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 54:631-664 1985.

162. Hirschberg, C. B. and Snider, M. D. Topography of glycosylation in the rough endoplasmic reticulum and golgi apparatus. Ann. Rev. Biochem. 56:63-87 1987.

163. Dunphy, W. G. and Rothman, J. E. Compartmental organization of the Golgi stack. Cell 42:13-21 1985.

165. Ronnett, G. V. and Lane, M. D. Post-translational glycosylation-induced activation of aglycoinsulin receptor accumulated during tunicamycin treatment. J. Biol Chem. 256:4704-4707 1981.

165. von Figura, K. and Hasilik, A. Lysosomal enzymes and their receptors. Ann. Rev. Biochem. 55:167-193 1986.

166. Esmon, P. C., Esmon, B. E., Schauer, I. E., Taylor, A. and Schekman, R. Structure, assembly, and secretion of octameric invertase. J. Biol. Chem. 262:4387-4394 1987.

167. Carson, D. D. and Lenarz, W. J. Relationship of dolichol synthesis to glycoprotein synthesis during embryonic development. J. Biol. Chem. 256:4679-4686 1981.

168. Bause, E. and Hettkamp, H. Primary structural requirements for N-glycosylation of peptides in rat liver. FEBS Lett. 108:341-344 1979.

169. Kronquist, K. E. and Lennarz, W. J. Enzymatic conversion of proteins to glycoproteins by lipid-linked saccharides: a study of potential exogenous acceptor proteins. J. Supramol. Struct. 8:51-65 1978.

170. Hanover, J. A. and Lennarz, W. J. N-linked glycoprotein assembly: evidence that oligosaccharide attachment occurs within the lumen of the endoplasmic reticulum. J. Biol. Chem. 255:3600-3604 1980.

171. Byrd, J. C., Tarentino, A. L., Maley, F., Atkinson, P. H. and Trimble, R. B. Glycoprotein synthesis in yeast: identification of $Man_8GlcNAc_2$ as an essential intermediate in oligosaccharide processing. J. Biol. Chem. 257:14657-14666 1982.

172. Trimble, R. B., Maley, F. and Tarentino, A. L. Characterization of large oligosaccharide-lipids synthesized in vitro by microsomes from Saccharomyces cerevisiae. J. Biol. Chem. 255:10232-10238 1980.

173. Bishoff, J. and Kornfeld, R. Evidence for an α-mannosidase in endoplasmic reticulum of rat liver. J. Biol. Chem. 258:7907-7910 1983.

174. Hubbard, S. C. and Robbins, P. W. Synthesis and processing of protein-linked oligosaccharides *in vivo*. J. Biol. Chem. 254:4568-4576 1979.

175. Farquhar, M. G. Progress in unraveling pathways of Golgi traffic. Ann. Rev. Cell Biol. 1:447-488 1985.

176. Goldberg, D. E. and Kornfeld, S. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. J. Biol. Chem. 258:3159-3165 1983.

177. Reitman, M. and Kornfeld, S. UDP-N-acetylglucosamine: glyprotein N-acetylglucosamine-1-phosphotransferase. Proposed enzyme for the phosphorylation of the high mannose oligosaccharide units of lysosomal enzymes. J. Biol. Chem. 256:4275-4281 1980.

178. Trimble, R. B. and Maley, F. Subunit structure of external invertase from *Saccharomyces cerevisiae*. J. Biol. Chem. 252:4409-4412 1977.

179. Orlean, P., Schwaiger, H., Appertauer, U., Haselbeck, A. and Tanner, W. Saccharomyces cerevisiae mating pheromones specifically inhibit synthesis of proteins destined to be N-glycosylated. Eur. J. Biochem. 140:183-189 1984.

180. Bause, E. and Lehle, L. Enzymatic N-glycosylation and O-glycosylation of synthetic peptide acceptors by dolichol-linked sugar derivatives in yeast. Eur. J. Biochem. 101:531-540 1979.

181. Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Vassalli, J. D. and Perrelet, A. Direct identification of prohormone conversion site in insulin-secreting cells. Cell 42:671-681 1985.

182. Julius, D. Schekman, R. and Thorner, J. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. Cell 36:309-318 1984.

183. Bostian, K. A., Jayachandran, S. and Tipper, D. J. A glycosylated protoxin in killer yeast: models for its structure and maturation. Cell 32:169-180 1983.

184. Herbert, E. and Uhler, M. Biosynthesis of polyprotein precursors to regulatory peptides. Cell 30:1-2 1982.

185. Julius, D., Brake, A., Blair, L., Kunisawa, R. and Thorner, J. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. Cell 37:1075-1089 1984.

186. Rothman, J. E. Transport of the vesicular stomatitis glycoprotein to *trans* Golgi membranes in a cell-free system. J. Biol. Chem. 262:12502-12510 1987.

187. Rothman, J. E., Miller, R. L. and Urbani, L.J. Intercompartmental transport in the Golgi complex is a dissociative process: facile transfer of membrane protein between two Golgi populations. J. Cell Biol. 99:260-71 1984.

188. Rothman, J. E., Urbani, L. J. and Brands, R. Transport of protein between cytoplasmic membranes of fused cells: correspondence to processes reconstituted in a cell-free system. J. Cell Biol. 99:248-259 1984.

189. Kelly, R. B. Pathways of protein secretion in eukaryotes. Science 230:25-32 1985.

190. Griffiths, G. and Simons, K. The *trans* Golgi network: sorting at the exit site of the Golgi complex. Science 234:438-443 1986.

191. Grasse, P. P. Ultrastructure polarite et reproduction de l'appareil de Golgi. C.R. Acad. Sci. 245:1228-1281 1957.

192. Meldolesi, J., Borgese, N., DeCamilli, P. and Ceccarelli, B. Cytoplasmic membranes and the secretory process. In *Membrane Fusion* , ed. G. Poste and G. L. Nicolson, pp 509-627 Elsevier/North Holland Biomedical Press, Amsterdam. 1978.

193. Farquhar, M. G. Traffic of products and membranes through the Golgi complex. In *Transport of Macromolecules in Cellular Systems*, ed. S. Silverstein, pp 341-362 Dahlem Konferenzen, Berlin. 1978.

194. Dunphy, W. G., Fries, E., Urbani, L.J.and Rothman, J. E. Early and late functions associated with the Golgi apparatus reside in distinct compartments. PNAS 78:7453-7457 1981.

195. Goldberg, D. E. and Kornfeld, S. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. J. Biol. Chem. 258:3159-3165 1983.

196. Lodish, H. F., Kong, N., Hirani, S. and Rasmussen, J. A vesicular intermediate in the transport of hepatoma secretory proteins form the rough endoplasmic reticulum to the Golgi complex. J. Cell Biol. 104:221-230 1987.

197. Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Lodish, H. F. and Schwartz, A. L. Intracellular site of asialoglycoprotein receptor ligand uncoupling: double-label immuno-electron microscopy during receptor-mediated endocytosis. Cell 32:277-287 1983.

198. Yamashiro, D. J., Tycko, B., Fluss, S. R. and Maxfield, F. R. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. Cell 37:789-800 1984.

199. Regoeczi, E., Chindemi, P. A., Debanne, M. T. and Charlwood, P. A. Partial re-sialylation of human asialotransferrin type 3 in the rat. PNAS 79:2226-2230 1982.

200. Snider, M. D. and Rogers, O. C. Intracellular movement of cell surface receptors after endocytosis: resialylation of asialo-transferrin receptor in hyman erythroleukemia cells. J. Cell Biol. 100:826-834 1985.

201. Kornfeld, S. Trafficking of lysosomal enzymes. FASEB J. 1:462-468 1987.

202. Brown, W. J. and Farquhar, M. G. The mannose-6-phosphate receptor for lysosomal enzymes is concentrated in *cis* Golgi cisternae. Cell 36:295-307 1984.

203. Fries, E. and Rothman, J. E. Transient activity of Golgi-like membranes as donors of vesicular stomatitis viral glycoprotein *in vitro*. J. Cell Biol. 90:697-704 1981.

204. Balch, W. E., Glick, B. S. and Rothman, J. E. Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. Cell 39:525-536 1984.

205. Simons, K. and Fuller, S. D. Cell surface polarity in epithelia. Ann. Rev. Cell Biol. 1:243-288 1985.

206. Bartles, J. R. and Hubbard, A. L. Plasma membrane protein sorting in epithelial cells: do secretory pathways hold the key? TIBS 13:181-184 1988.

207. Schekman, R. and Novick, P. The secretory process and yeast cell-surface assembly. In *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression*, eds. J. N. Strathern, E. W. Jones and J. R. Broach, pp 361-393 Cold Spring Harbor Laboratory, New York. 1982.

208. Fitting, T. and Kabat ,D. Evidence for a glycoprotein "signal" involved in transport between subcellular organelles: two membrane glycoproteins encoded by murine leukemia virus reach the cell surface at different rates. J. Biol. Chem. 257:14011-14017 1982.

209. Lodish, H. F., Kong, N., Snider, M. and Strous, G. J. A. M. Hepatoma secretory proteins migrate from rough endoplasmic retivulum to Golgi at characteristic rates. Nature 304:80-83 1983.

210. Fries, E., Gustaffson, L. and Peterson, P. A. Four secretory proteins synthesized by hepatocytes are transported from endoplasmic reticulum to Golgi complex at different rates. EMBO J. 3:147-152 1984.

211. Pfeffer, S. R. and Rothman, J. E. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Ann. Rev. Biochem. 56:829-52 1987.

212. Goldstein, J. L., Anderson, R. G. W. and Brown, M. S. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature 279:679-685 1979.

213. Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W. and Schneider, W. J. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Ann. Rev. Cell Biol. 1:1-39 1985.

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

215. Orci, L., Halban, P., Amherdt, M., Ravazzola, M., Vassalli, J. D. and Perrelet, A. A clathrin-coated, Golgi-related compartment of the insulin secreting cell accumulates proinsulin in the presence of monesin. Cell 39:39-47 1984.

216. Payne, G. S. and Schekman, R. A test of clathrin function in protein secretion and cell growth. Science 230:1009-1014 1985.

217. Griffiths, G., Pfeiffer, S., Simons, K. and Matlin, K. Exit of newly synthesized membrane proteins from the *trans* cisterna of the Golgi to the plasma membrane. J. Cell Biol. 101:949-964 1985.

218. Orci, L., Glick, B. S. and Rothman, J. E. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. Cell 46:171-184 1986.

219. Orci, L., Ravazzola, M., Amherdt, M., Perrelet, A., Powell, S. K., Quinn, D. L. and Moore, H. P. H. The *trans*-most cisternae of the Golgi complex: a compartment for sorting of secretory and plasma membrane proteins. Cell 51:1039-1051 1987.

220. Rothman, J. E. Protein sorting by selective retention in the endoplasmic reticulum and Golgi stack. Cell 50:521-522 1987.

221. Poruchynsky, M. S., Tyndall, C., Both, G. W., Sato, F., Bellamy, A. R. and Atkinson, P. H. Deletions into an NH2-terminal hydrophobic domain result in secretion of rotavirus VP7, a resident endoplasmic reticulum glycoprotein. J. Cell Biol. 101:2199-2209 1985.

222. Pääbo, S., Bhat, B. M., Wold, W. S. M. and Peterson, P.A. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. Cell 50:311-317 1987.

223. Wieland, F. T., Gleason, M. L., Serafini, T. A. and Rothman, J. E. The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell 50:289-300 1987.

224. Garoff, H. Using recombinant DNA techniques to study protein targeting in the eucaryotic cell. Ann. Rev. Cell Biol. 1:403-45 1985.

225. Emr, S. D., Schauer, I., Hansen, W., Esmon, P. and Schekman, R. Invertase β-galactosidase hybrid proteins fail to be transported from the endoplasmic reticulum in Saccharomyces cerevisiae. Mol. Cell. Biol. 4:2347-2355 1984.

226. Valls, L.A., Hunter, C. P., Rothman, J. H. and Stevens, T. H. Protein sorting in yeast: the localization determinant of yeast carboxypeptidase Y resides in the propeptide. Cell 48:887-897 1987.

227. Munro, S. and Pelham, H. R. B. A C-terminal signal prevents secretion of luminal ER proteins. Cell 48:899-907 1987.

228. Munro, S. and Pelham, H. R. B. An hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46:291-300 1986.

229. Stein, M., Zijderhand-Bleekemolen, J. E., Geuze, H., Hasilik, A. and von Figura, K. M_r 46,000 mannose 6-phosphate specific: its role in targeting of lysosomal enzymes. EMBO J. 6:2677-2681 1987.

230. Haslik, A. and Tanner, W. Carbohydrate moiety of carboxypeptidase Y and perturbation of its synthesis. Eur. J. Biochem. 91:567-575 1978.

231. Johnson, L. M., Bankaitis, V. A and Emr, S. D. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. Cell 48:875-885 1987.

232. Klionsky, D. J., Banta, L. M. and Emr, S. D. Intracellular sorting and processing of a yeast vaculolar hydrolase: proteinase A propeptide contains vacuolar targeting information. Mol. Cell. Biol. 8:2105-2116 1988.

233. Tabas, I. and Kornfeld, S. Purification and characterization of a rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. J. Biol. Chem. 254:11655-11663 1979.

234. Fries, E. and Rothman, J. E. Transport of vesicular stomatitis viral glycoproteinin a cell-free extract. PNAS 77:3870-3874 1980.

235. Rothman, J. E. and Fries, E. Transport of newly synthesized vesicular stomatitis viral glycoprotein to purified Golgi membranes. J. Cell Biol. 89:162-168 1981.

236. Bergman, J. E., Tokuyasu, K. T. and Singer, S. J. Passage of an integral membrane protein, the vesicular stomatitis viral glycoprotein, through the Golgi apparatus en route to the plasm membrane. PNAS 78:1746-1750 1981.

237. Balch, W. E., Dunphy, W. G., Braell, W. A. and Rothman, J. E. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. Cell 39:405-416 1984.

238. Braell, W. A., Balch, W. E., Dobbertin, D. C. and Rothman, J. E. The glycoprotein that is transported between successive compartments of the Golgi in a cell-free system resides in stacks of cisternae. Cell 39:511-524 1984.

239. Wattenberg, B. W., Balch, W. E. and Rothman, J. E. A novel prefusion complex formed during protein transport between Golgi cisternae in a cell-free system. J. Biol. Chem. 261:2202-2207 1986.

240. Wattenberg, B. W. and Rothman, J. E. Multiple cytosolic components promote intra-Golgi protein transport. J. Biol. Chem. 261:2208-2213 1986.

241. Glick, B. S. and Rothman, J. E. Possible role for fatty acyl-coenzyme A in intracellular protein transport. Nature 326:309-312 1987.

242. Dunphy, W. G., Pfeffer, S. R., Clary, D. O., Wattenberg, B.W., Glick, B. S. and Rothman, J. E. Yeast and mammals utilize similar cytosolic components to drive protein transport through the Golgi complex. PNAS 83:1622-26 1986.

243. Paquet, M. R., Pfeffer, S. R., Burczak, J. D., Glick, B. S. and Rothman, J. E. Components responsible for transport between successive Golgi cisternae are highly conserved in evolution. J. Biol. Chem. 261:4367-4370 1986.

244. Balch, W. E. and Keller, D. S. ATP-coupled transport of vesicular stomatitis virus G protein: functional boundaries of secretory compartments. J. Biol. Chem. 261:14690-14696 1986.

245. Haselbeck, A. and Schekman, R. Interorganelle transfer and glycosylation of yeast invertase *in vitro*. PNAS 83:2017-22 1986.

246. Balch, W. E., Elliott, M. M. and Keller, D. S. ATP-coupled transport of vesicular stomatitis virus G protein between the endoplasmic reticulum and the Golgi. J. Biol. Chem. 261:14681-14689 1986.

247. Balch, W. E., Wagner, K. R. and Keller, D. S. Reconstitution of transport of vesicular stomatitis virus G protein from the endoplasmic reticulum to the Golgi complex using a cell-free system. J. Cell Biol. 104:749-760 1987.

248. Zilberstein, A., Snider, S. D., Porter, M. and Lodish, H. F. Mutants of vesicular stomatitis virus blocked at different stages in maturation of the viral glycoprotein. Cell 21:417-427 1980.

249. Featherstone, C. Perforated cell systems to study membrane transport. TIBS 13:284-286 1988.

250. Beckers, C. J. M., Keller, D.S. and Balch, W.E. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. Cell 50:523-534 1987.

251. Simons, K. and Virta, H. Perforated MDCK cells support intracellular transport. EMBO J. 6:2241-2247 1987.

252. Baker, D., Hicke, L., Rexach, M., Schleyer, M. and Schekman, R. Reconstitution of *SEC* gene product-dependent intercompartmental protein transport. Cell 54:335-344 1988.

253. Ruohola, H., Kabcenell, A. K. and Ferro-Novick, S. Reconstitution of protein transport from the ER to the Golgi complex in yeast: the acceptor Golgi compartment is defective in the *sec*23 mutant. J. Cell Biol. 107:1465-1476 1988.

254. Newman, A. P. and Ferro-Novick, S. Characterization of new mutants in the early part of the yeast secretory pathway isolated by a [^{3}H] mannose suicide selection. J. Cell Biol. 105:1587-1594 1987.

255. Lodish, H. F., Kong, N., Hirani, S. and Rasmussen, J. A vesicular intermediate in the transport of hepatoma secretory proteins from the rough endoplasmic reticulum to the Golgi complex. J. Cell Biol. 104:221-230 1987.

256. Schekman, R. Protein localization and membrane traffic in yeast. Ann. Rev. Cell Biol. 1:115-143 1985.

257. Stevens, T. H., Rothman, J. H., Payne, G. S. and Schekman, R. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. J. Cell Biol. 102:1551-1557 1986.

258. Novick, P. and Schekman, R. Secretion and cell surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. PNAS 76:1858-1862 1979.

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

260. Esmon, B., Novick, P. and Schekman, R. Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell 25:451-460 1981.

261. Stevens, T., Esmon, B. and Schekman, R. Early stages of the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell 30:439-448 1982.

262. Nakano, A., Brada, D. and Schekman, R. A membrane glycoprotein, Sec12p, required for protein transport from the endoplasmic reticulum to the golgi apparatus in yeast. J. Cell Biol. 107:851-863 1988.

263. Eakle, K. A., Bernstein, M. and Emr, S. Characterization of a component of the yeast secretion machinery: identification of the *SEC*18 gene product. Mol. Cell. Biol. 8:4098-4109 1988.

264. Hicke, L.A. and Schekman, R.W. SEC23P: A yeast protein required for intracellular transport. Abstract of papers presented at: Yeast Genetics and Molecular Biology Meeting, San Francisco, Cal., p 30 1987

265. Achstetter, T., Franzusoff, A., Field, C. and Schekman, R. *SEC7* encodes an unusual, high-molecular-weight protein required for membrane traffic from the yeast Golgi apparatus. Submitted.

266. Bankaitis, V. A. and Malehorn, D. E. Identification of the *S. cerevisiae SEC*14 gene product: a polypeptide that is required for transport of secretory proteins from the yeast Golgi complex. Submitted.

267. Salminen, A. and Novick, P. J. A *ras*-like protein is required for a post-Golgi event in yeast secretion. Cell 49:527-538 1987.

268. Goud, B., Salminen, A., Walworth, N. C. and Novick, P. J. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. Cell 53:753-768 1988.

269. Bohni, P. C., Deshaies, R. J. and Schekman, R. W. *SEC*11 is required for signal peptide processing and yeast cell growth. J. Cell Biol. 106:1035-1042 1988.

270. Bourne, H. One molecular machine can transduce diverse signals. Nature 321:814-816 1986.

271. Melancon, P., Glick, B. S., Malhotra, V., Weldman, P. J., Serafini, T., Gleason, M. L., Orci, L. and Rothman, J. E. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. Cell 51:1053-1062 1987.

272. Bourne, H. R. Do GTPases direct membrane traffic in secretion? Cell 53:669-671 1988.

273. Burgoyne, R. D. Control of exocytosis. Nature 328:112-113 1987.

274. Buss and Sefton, B. Direct identification of palmitic acid as the lipid attached to p21 *ras*. Mol. Cell Biol. 6:116-122 1986.

275. Segev, N., Mulholland, J. and Botstein, D. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. Cell 52:915-924 1988.

276. Haubruck, H., Disela, C., Wagner, P. and Gallwitz, D. The *ras*-related *ypt* protein is an ubiquitous eukaryotic protein: isolation and sequence analysis of mouse cDNA clones highly homologous to the yeast *YPT1* gene. EMBO J. 6:4049-4053 1987.

Chapter 2

Characterization of a Component of the Yeast Secretion Machinery: Identification of the SEC18 Gene Product.

KURT A. EAKLE*, MITCHELL BERNSTEIN+§, and SCOTT D. EMR*

*Division of Biology, 147-75, California Institute of Technology

Pasadena, CA 91125

and

+Department of Biochemistry, University of California

Berkeley, CA 94720

§Present address: Department of Biology, Princeton University

Princeton, NJ 08544

Running title: Characterization of *SEC18*

As Published in: Molecular and Cellular Biology, Volume 8, pp. 4098-4909 1988.

ABSTRACT

SEC18 gene function is required for secretory protein transport between the endoplasmic reticulum (ER) and the Golgi complex. We have cloned the *SEC18* gene by complementation of the *sec18-1* mutation. Gene disruption has shown that *SEC18* is essential for yeast cell growth. Sequence analysis of the gene revealed a 2,271-base-pair open reading frame, which could code for a protein of 83.9 kilodaltons. The predicted protein sequence showed no significant similarity to other known protein sequences. In vitro transcription and translation of *SEC18* led to the synthesis of two proteins of approximately 84 and 82 kilodaltons. Antisera raised against a Sec18-β-galactosidase fusion protein also detected two proteins (collectively referred to as Sec18p) in extracts of ^{35}S-labeled yeast cells identical in size to those seen by *in vitro* translation. Mapping of the 5' end of the *SEC18* mRNA revealed only one major start site for transcription, which indicates that the multiple forms of Sec18p do not arise from mRNAs with different 5' ends. Results of pulse-chase experiments indicated that the two forms of Sec18p are not the result of post-translational processing. We suggest that translation initiating at different in-frame AUG start codons is likely to account for the presence of two forms of Sec18p. Hydrophobicity analysis indicates that the proteins were hydrophilic in nature and lacked any region that would be predicted to serve as a signal sequence or transmembrane anchor. Although potential sites for N-linked glycosylation were present in the Sec18p sequence, the sizes of the in vivo SEC18 gene products were unaffected by the drug tunicamycin, indicating that Sec18p does not enter the secretory pathway. These results suggest that Sec18p resides in the cell cytoplasm. While preliminary cell fractionation studies showed that Sec18p is not associated with the ER or Golgi complex, association with a 100,000 ×*g* pellet fraction was observed. This suggests that Sec18p may bind transiently to small vesicles such as those presumed to participate in secretory protein transport between ER and the Golgi complex.

INTRODUCTION

The temperature conditional yeast *sec* mutants define a set of functions required for secretory protein transport (20, 25). Nine *sec* genes, including *sec18* have been shown to cause a block in the transport of secretory proteins between the yeast endoplasmic reticulum (ER) and the Golgi complex (7, 19). In *sec18-1* mutant yeast cells, secretory protein traffic is blocked at the level of the ER on a shift to the nonpermissive growth temperature (37°C). This is true for both secreted enzymes, such as invertase, and vacuolar proteases, like carboxypeptidase Y (CPY), which use the secretory pathway for intracellular localization (28). In *sec18* mutant cells, ER function per se is not impaired. Protein translocation across the ER membrane continues at the nonpermissive temperature, as do secretory protein processing activities such as signal sequence cleavage and core oligosaccharide addition. However, protein modifications characteristic of the Golgi complex, such as the addition of outer chain mannose residues, are not observed on the accumulated secretory proteins (7, 19). Electron microscopy has shown that *sec18* mutant cells accumulate exaggerated forms of the ER (19, 25). When *sec18* mutant cells are returned to the permissive temperature (23°C), accumulated secretory proteins undergo transport and processing through the remainder of the secretory pathway (20). Accumulated invertase is released at the cell surface as the normal, active, highly glycosylated enzyme. Together, the specificity of the *sec18-1* defect as well as its reversibility indicate that the *sec18* mutant accumulates an authentic intermediate in the protein secretion pathway. Because interorganelle traffic between secretory compartments likely occurs via vesicle carriers, the *SEC18* gene is presumed to encode a function involved either in the selective packaging of secretory proteins into carrier vesicles, or the transport and fusion of such vesicles with the correct Golgi complex target membrane.

We report the cloning and sequencing of the *SEC18* gene. In addition, we have characterized both the mRNA and protein products (referred to collectively as sec18p) encoded

by this gene. Together, our observations suggest that Sec18p acts on the vesicle carriers that shuttle membrane and protein between ER and Golgi complex compartments.

MATERIALS AND METHODS

Strains. *Escherichia coli* strain MC1061 [F⁻ *araD139* Δ(*araABOIC-leu*)*7679* Δ*lacX74 galU galK rpsL hsdR*) (3) was used for cloning and fusion protein production, and JM101 was used for M13 phage growth for sequencing. *Saccharomyces cerevisiae* strains used were SEY2101 (*MATa ura3-52 leu2-3,112 suc2-Δ9 ade2-1*) (6), SEY5186 (*MATα sec18-1 ura3-52 leu2-3,112*) (6), and SEY6201 (*MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901suc2-Δ9 ade2-101*) (this study). Strains were grown in standard media preparations (16,27).

Materials. Restriction enzymes, T4 DNA ligase, *Bal*31 nuclease, mung bean nuclease, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal) were purchased from either New England Biolabs, Inc. (Beverly, Mass.) or Bethesda Research Laboratories (Gaithersburg, Md.) and were used as recommended by the suppliers. Deoxy and dideoxynucleotides were purchased from Pharmacia Fine Chemicals (Piscataway, N. J.). Universal sequencing primer was a gift from M. Simon. [^{32}P]dATP, [α-thio-^{35}S]dATP, Na $^{35}SO_4$, and Trans ^{35}S label (a hydrolysate of *E. coli* grown in $^{35}SO_4$ containing ~70% [^{35}S]~methionine and 20% [^{35}S] cysteine) were purchased from ICN Biochemicals (Irvine, Calif.). GeneScreen was purchased from Dupont, NEN Research Products (Boston, Mass.). Sodium dodecyl sulfate (SDS) Polyacrylamide gel electrophoresis (PAGE) supplies and Western blot reagents (horseradish peroxidase [HRP] system) were from Bio-Rad Laboratories (Richmond, Calif.). The RNA cap analog [m7G(5')ppp(5')G], ribonucleotides, and yeast translation extracts were a gift from E. Greyhack. SP6 RNA polymerase and RNasin were from Promega Biotech. Oxylyticase was purchased from Enzogenetics (Corvalis, OR). Antiserum against β-galactosidase was

a gift from T. Silhavy. Antiserum against the β subunit of mitochondrial F_1-ATPase was a gift from M. Douglas. Antiserum against CPY was from Klionsky et. al. (10).

Recombinant DNA constructions. Preparation of DNA, restriction digestions, agarose gel electrophoresis, and cloning of DNA fragments were done as described previously (14).

DNA sequencing. DNA sequencing was done by standard dideoxy chain termination methods (24) using *Bal*31 deletion subclones of *SEC18* in M13mp19. DNA and protein sequences were compiled and analyzed with computer programs written by K. Eakle (unpublished data). Homology searches of the National Biomedical Research Foundation Protein Sequence data base were done on BIONET using the XFASTP program (13).

Antisera production. *SEC18-lacZ* fusions were generated by cloning *Bal* 31 nuclease digestions of the 3.0 kilobase (kb) *Bam*HI-*Hin*d III fragment into *Sma*I cut pORF5. Clones were screened for overproduction of β-galactosidase activity on ampicillin-containing plates with Xgal. Plasmid DNA was prepared from candidate clones and checked for *SEC18* inserts by restriction mapping. Whole cell extracts of *E. coli* with candidate plasmids were run on SDS-polyacrylamide gels (11) and evaluated by both Western blotting (30) with primary antisera against β-galactosidase (visualized by the HRP color reaction system), and staining with Coomassie blue to identify fusion protein bands and estimate the extent of overproduction.

Fusion proteins were purified by growing *E. coli* with the fusion construction pORF18-40 to the stationary phase. Cells (250 OD_{600} units; 1 OD_{600} unit of cells equals the cells in 1 ml of a culture grown to an optical density at 600 nm of 1.0) were pelleted and suspended at 10 OD_{600} units per ml in 50 mM Tris (pH 8.0)-10 mM EDTA-10 mM dithiothreitol. Cells were lysed with a French press and spun at 6000 ×*g* for 20 min at 4°C in a rotor (JA-20), and the supernatant was spun at 170,000 ×*g* for 30 min at 15°C in a rotor (Ti70.1). Membrane pellets were suspended in a small volume of buffer, and sam-

ples of the fractions were assayed for β-galactosidase activity at appropriate dilutions (16). Greater than 50% of the total β-galactosidase activity of the fusion proteins was associated with the membrane pellet, which yielded the equivalent of 2 to 3 mg of β-galactosidase activity. The largest fusion band was fragment purified from 5% SDS-polyacrylamide preparative gels that were stained with Coomassie blue. Commercially available β-galactosidase was also run on the preparative gels and was used to estimate the amount of fusion protein recovered. Fusion bands were homogenized, dialysed against phosphate-buffered saline, and mixed with an equal volume of 1% agarose in phosphate-buffered saline. Approximately 50μg of fusion protein was injected subcutaneously into young New Zealand White male rabbits weekly for 4 weeks, and then once every two weeks for 4 months. Antisera were initially evaluated for their ability to react in a Western blot procedure to the Sec18-β-galactosidase fusion protein that was present in a whole cell SDS-polyacrylamide extract *E. coli* Mc1061 transformed with the pORF18-40 fusion construct. Attempts at identifying the yeast *SEC18* gene product in a similar fashion from extracts of whole yeast cells by Western blotting were unsuccessful. Following identification of the Sec18 protein by in vitro translation, the antisera were found to specifically immunoprecipitate the [^{35}S]-methionine-labeled in vitro translation product, as well as proteins with the same mobility on the SDS-PAGE from extracts of yeast cells labeled with $^{35}SO_4$. A titration curve was generated for the *SEC18* antisera, as well as other antisera used in this study, by immunoprecipitating $^{35}SO_4$ labeled whole yeast cell extracts (1 OD_{600} unit per sample) with increasing amounts of antiserum. With this information, levels of antiserum were chosen that were in excess over the amount of the labeled proteins to be precipitated along with an amount of protein A-Sepharose CL4B sufficient to bind all the antiserum present, in order to insure quantitative recovery. Precipitation using this same amount of antiserum against the Sec18p produced in yeast cells containing the *SEC18* gene on a multicopy plasmid with a 2μm origin of replication, showed that this level of antiserum was sufficient to precipitate an eightfold excess of labeled *SEC18* gene product. Greater amounts of antiserum

showed that the total level of Sec18p overproduction was about 20-fold with the *SEC18* gene on a multicopy plasmid.

Northern blot and 5' and 3' end mapping. Yeast RNA was prepared by a modification of a previously described procedure (4). Yeast cells were grown in YNB-glucose medium (27) to mid-log phase. Yeast (50 OD_{600} units) were pelleted, washed with distilled H_2O and suspended in 2 ml of 0.5M NaCl-0.2M Tris-hydrochloride (pH 7.5)-10 mM EDTA-1% SDS. Cells were added to 15-ml polypropylene tubes with 2 g of acid-washed glass beads and 2 ml of phenol-$CHCl_3$ (1:1 [vol/vol]). Cells were chilled on ice, vortexed extensively, and spun at 12000 ×*g*, 4°C for 10 min in a rotor (JA-20). The supernatant was re-extracted twice more with phenol-$CHCl_3$ and the RNA was precipitated with 2.5 volumes of cold ethanol. Pellets were washed with 70% ethanol, dried, and redissolved in 10 mM Tris-hydrochloride (pH 7.5)-1 mM EDTA (TE). RNA recovery was measured by determining the optical density at 260nm. Sodium acetate was added to 0.3 M, ethyl alcohol was added to 70%, and RNA was stored at -80C. Poly(A)$^+$ RNA was isolated by binding to oligo(dT)-cellulose (Sigma Chemical Co., St. Louis, Mo.) in the presence of 0.5 M LiCl, washing extensively with the high salt buffer, and eluting with TE. RNA was run on formaldehyde 1% agarose gels and transferred to GeneScreen (14). Size standards were generated by ^{32}P end-labeling lambda DNA, which was cut with *Eco*RI and *Hin*d III. [^{32}P]RNA probes were made by transcribing the noncoding strand of our *SEC18* clone in pSP64 with SP6 RNA polymerase using [^{32}P]UTP and were hybridized to the blot as described previously (15).

Mapping at the 5' end was done using a ^{32}P-end-labeled oligonucleotide (5'-GGGAAATCATTTGGT-3', complementary to bases 681 to 695 of the DNA sequence), which was hybridized to a single stranded M13-*SEC18* subclone and extended using the Klenow fragment of *E. coli* DNA polymerase I. The reaction was then digested with *Hin*d III, and the probe was fragment purified on a 6 M urea-5% acrylamide gel. The probe (10^5 cpm) was hybridized to 25 μg of total yeast RNA in 80% formamide-40 mM PIPES

[piperazine-*N*, *N'*-bis(2-ethanesulfonic acid); pH 6.8]-400 mM NaCl-1 mM EDTA in a total volume of 25 μl overlaid with 50 μl mineral oil. The hybridization reaction mixture was heated at 85°C for 5 min and then incubated at 42°C overnight (12). The reaction mixture was diluted with 400 μl of 50 mM sodium Acetate (pH 5.0)-10 mM NaCl-1 mM $ZnSO_4$ and digested with 50 U of mung bean nuclease for 1 hr at 37°C (8). The reaction was phenol extracted twice and ethanol precipitated with glycogen as a carrier. To identify the size of the digestion products of the hybridization reaction, dideoxy sequencing reactions were carried out with the ^{32}P-end-labeled oligonucleotide as the primer and label in the reactions. The sequencing reactions and the digestion products of the hybridization reaction were run on a 6% sequencing gel.

Mapping at the 3' end was performed as described above for the mapping of the 5' end. The probe was a [^{32}P]UTP-labeled SP6 RNA polymerase transcript labeled from the *Hin*d III site of our *SEC18* clone to the *Pst*I site at position 2235. Hybridization was done as described above for the 5' end mapping, except that overnight incubation was done at 50°C. Samples were digested with mung bean nuclease as described above and run on 5% sequencing gel, with ^{32}P-end-labeled lambda phage fragments and undigested probe as size standards.

In vitro transcription and translation. Yeast in vitro translation extracts were prepared as described previously (31) and treated with micrococcal nuclease. RNA was generated by SP6 RNA polymerase runoff transcription of the minimum complementing subclone of *SEC18* cloned in pSP65 using ribonucleotide mixes with and without 0.5 mM m^7G(5')ppp(5')G to give a capped mRNA (15). In vitro translation was performed as described previously (31) with 10 to 15 μCi of [^{35}S]methionine per reaction. Translation products were run on SDS-12%-polyacrylamide gels (5% stacking gel). Gels were fixed, stained, treated with AutoFlour (National Diagnostics, Somerville, N. J.), and dried before autoradiography on XAR film (Eastman Kodak Co., Rochester, N. Y.).

Immunoprecipitation. Yeast cells were grown to the mid-log phase in a defined medium (10) containing 100 μM SO_4. One OD_{600} unit of cells per immunoprecipitation sample were pelleted, washed with distilled H_2O, and suspended in 0.5 ml of sulfate-free medium-1 mg of bovine serum albumin per ml. Samples were incubated for 30 min at 30°C with shaking, and 100 to 200 μCi $^{35}SO_4$ per OD_{600} units of cells was added. Cells were labeled for 30 min, 30°C with shaking and unlabeled SO_4 was added to a final concentration of 50 mM. Labeling or chase was stopped by the addition of trichloroacetic acid to 5%, and samples were put on ice for 20 min. Samples were pelleted in the microfuge for 5 min, and the pellets were washed twice with cold acetone and dried. SDS Boiling Buffer (1% SDS, 50 mM Tris [pH 7.5], 1 mM EDTA, 100 μl per sample) and glass beads were added, and the samples were vortexed extensively and boiled for 3 min. One milliliter of Tween-IP buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 0.5% [wt/vol] Tween-20) was added. Samples were vortexed and microfuged for 15 min in the cold. Supernatant (0.9 ml) was carefully removed and 1 to 20 μl of appropriate antiserum was added. Samples were rocked gently at 4°C for 3 hr and 100 μl of 3.6% (wt/vol) protein A-Sepharose CL4B slurry was added. Samples were rocked an additional 0.5 h 4°C and pelleted. The pellets were washed (1 ml per wash) twice with Tween-IP buffer, twice with Tween-urea buffer (100 mM Tris [pH 7.5], 200 mM NaCl, 0.5% [wt/vol] Tween-20, 2 M urea), once with 1% ß-mercaptoethanol, and once with 0.1% SDS. SDS-PAGE sample buffer (50 μl) was added, and the samples boiled for 5 min before they were loaded onto an SDS-8% polyacrylamide gel (4% stacking gel).

Fractionation. Yeast cells to be labeled were pelleted, washed with distilled H_2O, and incubated with 100 mM Tris (pH 8.5)-25 mM dithiothreitol for 5 min at 30°C. Cells were pelleted, washed with distilled H_20 and spheroplasted with 1 μg of oxylyticase per OD_{600} units of cells in sulfate-free medium, which was adjusted to pH 7.5 and which contained 1.2 M sorbitol for osmotic support (22). Spheroplasts were pelleted gently, washed and suspended with sulfate-free medium (pH 5.5) containing 1.2 M sorbitol, and

labeled with 50 to 100 μCi of Trans ^{35}S label per OD_{600} units of cells for 30 min at 30°C. Cells were pelleted at 500 ×*g*, suspended gently in 100 μl of 1.2 M sorbitol-100 mM KPO_4 (pH 7.5)-10 mM EDTA, and lysed by rapid dilution with 1 ml 0.25 M sucrose-10 mM Tris (pH 7.5)-10 mM EDTA-1 mM phenylmethylsulfonyl fluoride-1 mg bovine serum albumin per ml. One OD_{600} unit of the lysed cells was precipitated with trichloroacetic acid for a whole cell sample. Unlysed cells were pelleted at 2000 ×*g* for 2 min, and the supernatant was centrifuged at 13000 ×*g* for 15 min at 4°C. Supernatant (0.8 ml) from the microfuge spin was carefully removed and spun at 100,000 ×*g* in a rotor (Ti70.1). Excess supernatant was removed carefully from the microfuge pellet, which was suspended in 100 μl SDS boiling buffer, and boiled for 3 min. The supernatant from the 100,000 ×*g* spin was precipitated with trichloroacetic acid on ice 20 min, and the 100,000 ×*g* pellet was suspended in 100 μl SDS boiling buffer and boiled for 3 min. Samples were immunoprecipitated as described above.

RESULTS

Characterization and cloning of the SEC18 locus. The *sec18-1* mutation *S. cerevisiae* has been shown to cause a block in the transfer of proteins from the ER to the Golgi complex apparatus on a shift to the nonpermissive temperature. This block results in the accumulation of ER, as identified by thin section electron microscopy (19, 20, 25). Proteins that transit the secretory pathway also accumulate with modifications that are consistent with their presence inside the ER, that is, with signal sequences removed and only core glycosylation units added to the protein backbone. This block is also reversible. The invertase accumulated under these conditions has been shown to resume transport through the secretory pathway when it is returned to the permissive temperature (20). The speed with which ER transport is blocked by the *sec18-1* mutation is demonstrated in Fig. 1. Invertase has been immunoprecipitated from *sec18-1* yeast cells that were grown under

various conditions. In Fig. 1, lane 1 shows the size of unglycosylated invertase produced at the permissive temperature in the presence of tunicamycin. To determine the rate of onset of the block in secretory protein traffic in *sec18-1* mutant yeast cells, we shifted cells to 37°C for only 10 minutes before they labeled with $^{35}SO_4$. Under these conditions, invertase accumulates as three distinct bands between 78,000 and 84,000 daltons (Fig. 1, lane 2). These bands corresponded to invertase, which varies in the total number of core oligosaccharide units added to the protein backbone (each core increases the apparent molecular weight of the protein by 2-2.5 kilodaltons). At the permissive temperature (Fig. 1, lane 3), *sec18-1* mutant cells process the labeled invertase to its highly glycosylated secreted form during passage through the Golgi complex. Thus, the *sec18-1* mutation results in a rapid block in secretory protein traffic at some stage after ER translocation and core oligosaccharide modification of secretory proteins.

Based on the results of morphology studies, the rapid and specific block in secretory protein movement, as well as the reversibility of the *sec18* mutant block, we reasoned that the *SEC18* gene is likely to code for a function that is directly involved in protein transfer between the ER and Golgi complex. To investigate this function, the *SEC18* gene was cloned by complementation of the *sec18-1* mutation with a genomic library of yeast DNA fragments in the multicopy vector YEp13 (18). Subcloning and further complementation analysis showed that the complementing activity resides on a 3.0 kb *Bam*HI-*Hin*d III fragment. Because of a lack of restriction sites on one side of the clone, a library of *Bal*31 deletion subclones was constructed, which extended from either end of the *Bam*HI-*Hin*d III fragment. Complementation analysis with these fragments cloned into the low copy number shuttle vector pSEYC58 (*CEN IV, ARS I*) revealed that 500 base pairs of DNA at the *Bam*HI side of the fragment could be deleted without destroying the complementing activity (Fig. 2B). Deletion of as little as 200 bp from the *Hin*d III side of the fragment was sufficient to destroy complementing activity.

To confirm that our clone carried the authentic *SEC18* gene and not a suppressor locus, we substituted the *LEU2* gene on a 2.1 kb *Hpa*I fragment for a 300 bp *Hpa*I fragment in the central region of the clone. A linear fragment with *SEC18* homology at both ends was transformed into the diploid strain SEY6201/SEY5186 (**a**/α *leu2-3, 112/leu2-3, 112 SEC18/sec18-1*) and *Leu*$^+$ transformants were selected. Approximately 50% of the *Leu*$^+$ transformants simultaneously acquired the recessive Ts phenotype, indicating that the *SEC18* gene of one homolog was disrupted (23). Tetrad dissections of spores from the Ts diploid strains showed 2:2 segregation of *sec18*(Ts)/*Leu*$^-$: dead(*Leu*$^+$) spores per tetrad (n=7). In addition, viable random spores from such diploids were all found to be *sec18*(Ts)/*Leu*$^-$ (200 haploid spores). These results indicated that our clone maps to the *SEC18* chromosomal locus and that *SEC18* is an essential gene. Examination of the dead spores showed that although many germinated and went through one cell division, none grew beyond the two cell stage suggesting that the intracellular pool of *SEC18* gene product is sufficient for spore germination but not large enough to sustain vegetative growth.

Further evidence that our clone contains the *SEC18* gene was obtained by transforming *sec18-1* mutant yeast with a yeast integrating plasmid (YIp5 vector) containing a noncomplementing fragment of the *SEC18* clone. This plasmid was restricted within the *SEC18* DNA to direct integration to the homologous chromosomal region (21). Following selection of *Ura*$^+$ transformants (all of which remained temperature-sensitive), genomic DNA was prepared from integrants and restriction digested with an enzyme (*Eco*RI), which was expected to release the YIp5 vector together with adjacent *SEC18* sequences. The resulting DNA fragments were then ligated in dilute solution to recircularize the DNA, transformed into *E. coli*, and selected on the basis of ampicillin resistance. Restriction mapping of the resultant clones showed that they had, in fact, captured the entire *sec18* locus. These clones were shown to contain a functional temperature-sensitive allele of the *sec18* gene by transferring the gene to a yeast replicating vector (pSEYC58) and transformation into the *SEC18*::*LEU2*/*sec18*(Ts) diploid strain. These transformants remained temperature sensi-

tive and upon sporulation were now able to give *Leu*$^+$, *Ura*$^+$, (Ts) haploids, indicating functional expression of a temperature-sensitive copy of *sec18* from the plasmid. To identify the approximate position of the temperature-sensitive lesion in the *sec18-1* mutant gene, restriction fragments were exchanged between the wild-type and temperature-sensitive *sec18*(Ts) clones. After transformation into *sec18-1* mutant yeast cells, the hybrid gene constructs were scored for whether they encoded a temperature-sensitive or wild-type form of Sec18p. Based on these studies, the *sec18-1* mutation was found to unambiguously map to a 351 bp *Cla*I fragment (Fig. 2A). This clearly placed the Ts mutation within our *SEC18* clone.

DNA sequence of SEC18. The entire 3,042 bp *Bam*HI-*Hin*d III *SEC18* complementing DNA clone was sequenced using standard dideoxy sequencing techniques and the library of *Bal*31 deletion subclones described above (Fig. 2C). The sequence (Fig. 3) revealed a 2,271-bp open reading frame, which could potentially code for a protein product of 83,903 daltons. A search of the NBRF protein sequence database showed no significant similarity between the predicted protein sequence and other known proteins (13). The position of the start of the smallest complementing subclone was only 35 bp upstream of the first ATG codon of the open reading frame, leaving little room for a SEC18 promoter. Yeast gene promoters normally consist of a TATA sequence element usually 40 to 120 bases upstream from the site of transcription initiation and an additional enhancerlike element (upstream activating sequence) 5' to the TATA box (29). Possible TATA sequences are found at positions 516 to 522 and 529 to 533, which are only 10 to 20 bp upstream of the open reading frame. No candidate sequences corresponding to an upstream activating sequence element in the region 5' to the TATA boxes included in the smallest complementing clone were identified. Two additional ATG codons were present in the open reading frame 54 and 63 bp downstream of the first ATG codon. Combined, this information led us to question whether the transcriptional start point for the gene included the first ATG of the open reading frame.

Analysis of SEC18 mRNA. Northern blots (Fig. 4A) with single-stranded probes from either strand of the *SEC18* clone confirmed that the only transcript from this region of DNA was a poly(A)$^+$, 2,500-nucleotide mRNA in the orientation of the open reading frame seen in the DNA sequence. End mapping experiments of the 5' end of the the *SEC18* transcript were done using a ^{32}P-end-labeled single stranded probe and mung bean nuclease (Fig. 4B). Results of these experiments revealed that there was only one major initiation point for genomic transcription, beginning almost exactly at the position of the start of the smallest complementing subclone. This places our predicted TATA sequences within the transcribed region. Only the DNA that corresponds to the transcribed region appears to be necessary for complementation of the *sec18*(Ts) defect. Because other single-copy and multi-copy vectors were used to test complementation of this subclone, it seems unlikely that complementation was due to run-on transcripts from other genes on our initial yeast shuttle vector. While aberrant expression of *SEC18* on a plasmid vector cannot be ruled out, the observation that a complementing subclone contained so little upstream sequence suggests that sequences that function in transcription initiation may be located within the transcript or may not be necessary for weak expression. The latter might be explained if only very low levels of SEC18 transcription are required for complementation of the *sec18*(Ts) defect.

End mapping of the 3' end of the gene (data not shown) showed two major sites of transcription termination at approximately positions 2942 and 2956. Analysis of the sequence downstream from the termination of the open reading frame showed that this region conforms well to the consensus sequence ([T-rich region] ... TAG ... TAGT/TATGT ... [AT-rich region]) for yeast termination and polyadenylation (32). The presence of three TAGT-like sequences suggests that these may function as two overlapping pairs of transcription terminators and, thus, lead to the two major sites of termination. Note also that both transcription termination sites are well beyond the TAA codon at position 2812, which would terminate translation of the open reading frames.

Identification of Sec18p in vitro and in vivo. The protein products of the *SEC18* gene were identified by in vitro translation using a yeast translation extract and an SP6 RNA polymerase-generated template RNA corresponding to the minimum complementing *SEC18* subclone. Two protein products (sec18p) were detected, one of 84,000 daltons and one of 82,000 daltons (Fig. 5). The larger product corresponds well to the size predicted for translation of our entire open reading frame. The smaller translation product may result either from premature termination of protein translation or initiation of translation at one of the two ATGs located at positions 54 bp and 63 bp downstream from the start of the open reading frame. From the DNA sequence, these smaller open reading frames were predicted to encode proteins of 82,024 or 81,678 daltons. The yeast extract translation system we used does not normally perform any posttranslational modifications of the protein products such as signal or prosequence cleavages or addition of core oligosaccharides, which could account for the multiple forms of proteins that were produced. Transcription by SP6 RNA polymerase also has high fidelity in terms of the site of transcription initiation, so variation in the 5' end of the RNA template does not seem likely (15).

In order to test if the protein(s) produced from the *SEC18* open reading frame are also produced in vivo, we raised antisera to the *SEC18* protein. A gene fusion of the *SEC18* open reading frame to the 5' end of the *E. coli lacZ* gene was generated by ligating random *Bal*31 digestions of the 3.0 kb *Bam*HI-*Hin*d III fragment into the vector pORF5 (27) (Fig. 6A). Clones that overproduced β-galactosidase activity were initially picked by screening on Xgal plates. Plasmids from these clones were screened for inserts corresponding to the *SEC18* gene by restriction site analysis. In addition, SDS-PAGE of whole cell *E. coli* extracts expressing high levels of β-galactosidase activity were stained with Coomassie Blue to visualize the extent of overproduction and to identify the size of the hybrid proteins (see Figure 6B). Western blotting of these gels using β-galactosidase- specific antibodies confirmed the identity of hybrid protein bands. Several gene fusions were created that over-

produced fusion proteins in *E. coli* up to 40,000 daltons larger than β-galactosidase itself. The fusion joints between five of these clones and the *lacZ* sequences were determined by DNA sequencing, which confirmed that the large open reading frame of *SEC18* was being utilized. The largest fusion protein was purified using preparative SDS-PAGE and injected into rabbits to raise antiserum that was reactive to Sec18p. Antisera were tested initially for reaction to the Sec18-β-galactosidase fusion protein on Western blots, and later confirmed to react with the in vitro translation products of *SEC18*.

Immunoprecipitation from extracts of ^{35}S-labeled whole yeast cells with the Sec18p antiserum was able to detect in vivo production of the putative *SEC18* gene products. The in vivo forms of Sec18p are identical in size to those seen by in vitro translation (Fig. 5). Tunicamycin, which inhibits N-linked glycosylation, had no effect on the size of the in vivo proteins that were observed, even though potential sites for glycosylation were present in the predicted amino acid sequence at amino acid positions 13, 36, 455, 474, and 689. This suggests that Sec18p is not translocated across the ER membrane into the secretory pathway, and thus, it never comes in contact with the oligosaccharide transferase enzyme. This is further supported by the hydrophobicity profile for the predicted Sec18p sequence (Fig. 7), which shows no evidence for a characteristic hydrophobic signal sequence at the amino terminus of either the full length protein or a protein whose translation began at one of the two downstream ATG codons at the 5' end of the open reading frame. In addition, the hydrophobicity profile did not show any internal regions that would be predicted to span a membrane or function as internal signal sequences. These data are most consistent with the fact that Sec18p remained in the cell cytoplasm. However, these results do not exclude the association of Sec18p with the cytoplasmic face of an intracellular organelle membrane.

The two forms of Sec18p seen in vivo could also result from a posttranslational processing event other than glycosylation. To address this question, yeast cells were pulse-labeled for 20 min with $^{35}SO_4$ and chased for various times following the addition of excess

cold SO_4. Whole-cell extracts were immunoprecipitated using antisera directed against both Sec18p and CPY, a vacuolar protease that undergoes processing in the ER, Golgi complex, and vacuole to give forms that can be distinguished on SDS-polyacrylamide gels (28). The results (Fig. 8) indicated that while CPY is processed normally with a half-time of approximately 5 min, the two forms of Sec18p are maintained in a constant ratio. This is true for chase periods up to 2 h (data not shown). Thus, the two forms of Sec18p do not appear to result from posttranslational processing of the larger form into the smaller form or vice versa. It appears that messages containing the entire open reading frame are leading directly to the production of two forms of the same protein. It is reasonable to propose that the two forms arise from translation initiating at different points in the open reading frame of the mRNA.

Results of this study indicate that Sec18p represents a relatively minor fraction of the total protein production in yeast cells. From quantitation of the relative levels of production of Sec18p and CPY by densitometry, we estimate that Sec18p is produced at only 1/5th to 1/10th of the level of CPY. Twentyfold overproduction of Sec18p was observed in cells harboring the *SEC18* gene on a multicopy plasmid (pSEY8). This suggests that *Sec18* is expressed constitutively and is subject to simple gene dosage control. Experiments looking at the production of Sec18p from the mutant *sec18-1*(Ts) allele show that the temperature-sensitive defect does not result from a decrease in Sec18p expression. In addition, the temperature-sensitive forms of the protein appear to show the same stability at nonpermissive temperature as wild-type proteins in pulse chase labeling experiments (data not shown).

Intracellular location of Sec18p. Our data predict that Sec18p is probably a cytoplasmic protein. To determine this directly, we used cell fractionation techniques with differential centrifugations to separate yeast cells into membrane enclosed and cytoplasmic compartments. Because of its role in transport between the ER and Golgi complex, we were especially interested to see whether Sec18p might be associated with these compart-

ments. A procedure giving gentle osmotic lysis was developed, which appeared to maintain the integrity of small organelles such as mitochondria, ER and the Golgi complex. Cells were spheroplasted extensively before they were labeled, and then they were washed and labeled with Trans ^{35}S label in the presence of full osmotic support (1.2 M sorbitol). To lyse the cells osmotically, cells were pelleted gently, suspended in a small volume of buffer with full osmotic support and then quickly diluted in a buffer with partial osmotic support (0.25 M sucrose). After an initial low-speed spin to pellet any unlysed cells, the cleared lysate was centrifuged sequentially in the microfuge (13,000 $\times g$ for 15 min) and the ultracentrifuge (100,000 $\times g$ for 30 min). Judging from trichloroacetic acid-precipitable counts, only 30 to 50% of cells were lysed by the procedure. However, no enhancement of any particular form of the markers used was observed, which suggests that the cleared lysate is representative of the contents of the cell.

Fractions were immunoprecipitated with Sec18p antisera under conditions of antibody excess to ensure that comparisons of the level of Sec18p present in the various fractions were valid. Likewise, intermediates in the maturation of CPY were monitored using CPY-specific antisera to give markers for the ER, Golgi complex and vacuolar compartments (28). Immunoprecipitation with antisera against the β-subunit of F_1-ATPase was also done to assess the fractionation and intactness of mitochondria in this procedure. Figure 9 shows an autoradiograph of immunoprecipitates of CPY and Sec18p from such an experiment, which is quantitated in Table 1. The p1 and p2 forms of CPY, which should be localized within the ER and Golgi complex, respectively, were mainly present (80%) in the 13,000 $\times g$ pellet. A small amount of p1 and p2 (20%) was found in the 100,000 $\times g$ pellet fraction, perhaps due to some fragmentation of these compartments or the material present in small vesicles in transit between secretory organelles. Greater than 95% of the β-subunit of mitochondrial F_1-ATPase was also pelleted in the microfuge. These results indicate that organelles such as ER, Golgi complex, and mitochondria appear to remain largely intact with this lysis procedure and are recovered predominantly in the 13,000 $\times g$ pellet. In con-

trast, lysis of whole cells by a more vigorous procedure (vortexing in the presence of glass beads) led to 60 to 80% release of the ER, Golgi complex, and mitochondrial markers into the supernatant fraction (data not shown). Very little (<5%) of the mature CPY, a protease present in the lumen of the yeast vacuole, was associated with the 13,000 ×*g* pellet fraction. The yeast vacuole is a very fragile compartment which we would expect to be lysed without full osmotic support. This was confirmed by the presence of mature CPY predominantly (>95%) in the 100,000 ×*g* supernatant fraction.

Sec18p was found in significant amounts in both the 100,000 ×*g* pellet (44%) and supernatant (56%) fractions. Little (<1%), if any, of Sec18p was associated with the microfuge pellet fraction. Since Sec18 is implicated in transport between the ER and the Golgi complex, it is somewhat surprising that it would be found in a high speed pellet fraction in which small vesicles would presumably be recovered and yet not be associated with a fraction (the 13,000 ×*g* pellet) in which the bulk of the ER and the Golgi complex are represented. The fact that mature CPY is not associated with the high speed pellet fraction indicates that the Sec18p found in the pellet is not the result of nonspecific trapping. The data would suggest that Sec18p does not interact directly with ER or the Golgi complex within the cell, or does so only very transiently. The presence of significant amounts of Sec18p in the 100,000 ×*g* pellet fraction, along with a small proportion of the ER and Golgi complex forms of CPY, suggests that Sec18p may associate with small vesicles in transit from the ER to the Golgi complex. It is also possible that Sec18p binds to some large macromolecular complex such as the cytoskeletal network, which could be pelleted at the higher *g* force. Of course, the possibility remains that the association of Sec18p with the 100,000 ×*g* pellet may be an artifact due to aggregation of the protein or binding to membrane fragments. Nevertheless, the association of Sec18p with the high speed pellet may provide a clue to the functional role of Sec18 within the cell. Note also that there is not a preferential association of one or the other form of Sec18p with the pellet fraction, which suggests that the two protein forms may be functionally equivalent.

DISCUSSION

As a first step in understanding the role of the yeast *SEC18* gene product in secretory protein transport between the ER and the Golgi complex, we cloned and sequenced the gene. A single 3.0-kb DNA fragment was isolated that complements the temperature- sensitive defect exhibited by *sec18-1* mutant yeast cells. This DNA segment mapped genetically to the *SEC18* locus. RNA probes derived from the cloned segment detected one major 2,500-nucleotide poly(A)$^+$ mRNA on Northern blots (Figure 4A). Consistent with this, the major open reading frame (2,271 bp) in the *SEC18* clone was predicted to encode a protein of 84 kilodaltons. Somewhat surprisingly, two protein products of this open reading frame were observed both from in vitro translation extracts programmed with *SEC18* mRNA and from in vivo ^{35}S-labeled yeast cells extracts (Figure 5). Disruption of this open reading frame indicates that the function of Sec18p is essential for cell viability. Spores containing a disrupted *SEC18* gene are able to germinate but only undergo, at most, one round of cell division in the absence of *SEC18* expression.

Several aspects of *SEC18* gene expression are novel and unexpected. First, sequences upstream from the site of transcription initiation did not appear to be necessary for *SEC18* expression. A subclone, which contained only five nucleotides preceding the start point of transcription, was sufficient for the expression of adequate levels of Sec18p for the complementation of the *sec18-1* mutation. Complementation with this subclone was observed when several different high- and low-copy number yeast shuttle vectors were used, suggesting that aberrant expression of this subclone in a particular plasmid context is not the cause of the observed complementation.

Second, there were two sequences just upstream of the open reading frame, TATATT at bases 516 to 522 and TATAAA at 529 to 535, which could possibly function as TATA elements for transcriptional control. Yet, these sequences are actually within the transcribed region itself rather than the normal position TATA elements usually occupy 40 to 120 bp 5' of the site of transcription initiation (29). This, along with the fact that the tran-

scribed region alone appears to be sufficient for gene expression, raises the possibility that *SEC18* promoter elements may function from within the gene itself. Alternatively, these TATA elements formed part of a cryptic promoter leading to expression of the minimum complementing fragment.

Third, the *SEC18* gene produced two proteins from transcripts that appeared to start at a single site. Other cases of the expression of multiple forms of protein from a single yeast gene have been observed, such as the production of cytoplasmic and secreted forms of invertase from the yeast *SUC2* gene (2). However, these were caused by multiple sites of transcription initiation leading to messages that have different AUG codons of the open reading frame at their 5'-ends. There is a precedent for translation initiating at multiple AUG codons in a yeast mRNA. The *GCN4* gene of *S. cerevisiae*, whose protein product controls the transcription of a large number of amino acid biosynthesis genes, uses translation initiation at multiple AUG codons in the mRNA as a translational control of its expression (17). Four short open reading frames of an AUG codon followed by two to three codons are present in a ~600 nucleotide leader region of the *GCN4* mRNA and act to inhibit the translation of *GCN4* under non-starvation conditions (17). In contrast, *SEC18*, appeared to have only one major class of mRNA with a short 5'-untranslated leader and a set of three AUG codons early in the mRNA that were all in frame with the major open reading frame of the gene, yet two forms of the same protein with different sizes were produced (see Figs. 4 and 5). The two forms of the protein did not appear to be the result of posttranslational processing events (Fig. 8). The size of the smaller protein product corresponded well to the predicted size of translation products initiated from AUGs that were 19 or 21 codons downstream of the first AUG codon of the open reading frame, leading us to suggest that the smaller protein product arises from translation initiating at one of these downstream codons. In vitro mutagenesis of the different AUG codons in *SEC18* should reveal if translation is indeed started at more than one site or whether another mechanism is responsible for the multiple forms of protein being produced. In addition, such in

vitro mutagenesis should permit one to test whether the two Sec18 proteins are functionally equivalent. It is tempting to speculate that the two isoforms of Sec18p could have different functional roles in directing secretory protein traffic.

The intracellular location of Sec18p was analyzed to gain insight into the functional role the Sec18 plays in the secretory pathway. The predicted amino acid sequences of Sec18 proteins did not indicate any targeting signal that would allow Sec18p to be translocated across the ER membrane. The conclusion that Sec18p remains cytoplasmic was reinforced by the observation that Sec18p was not modified with core oligosaccharides (Fig. 5), a function that is carried out within the ER, even though potential sites for N-linked glycosylation are present in the amino acid sequence. Results of cellular fractionation experiments by an osmotic lysis procedure, which was able to maintain the integrity of organelles such as mitochondria, ER, and the Golgi complex, indicated that Sec18p is not tightly associated with the bulk of ER and the Golgi complex (Fig. 9). However, centrifugation at higher *g* forces revealed a distribution of Sec18p between the pellet and soluble fractions. This observation suggests that Sec18p may be associated with small vesicles or a macromolecular complex that is important in intracellular secretory protein transport.

The phenotype of *sec18-1* mutant yeast cells suggests a direct role for Sec18p in the transport of secretory proteins between the ER and the Golgi complex. At the nonpermissive temperature, there was a rapid accumulation of newly synthesized secretory proteins that underwent all of the modifications characteristic of the ER (signal sequence cleavage and addition of core oligosaccharides). No modification or processing events known to occur after that of the ER are observed in the *sec18-1* mutant. Therefore, it appears that a block exists in the transfer of secretory proteins from the ER to the Golgi complex. Possible functions for Sec18p might include the packaging of proteins into transport vesicles, a role in the budding of such vesicles, the transport of vesicles to the target organelle, recognition or fusion of these vesicles with the Golgi complex, or the recycling of transport components back to the ER. The cytoplasmic location of Sec18p rules out any direct role

in the sorting and selection of proteins from the lumen of the ER for transport to the Golgi complex. Our data, which show an association of Sec18p with a high-speed pellet fraction, suggest that Sec18p may function in the formation of small transport vesicles at the ER or in their transport and targeting to Golgi complex membranes. The recent development of efficient in vitro transport assays for ER to Golgi complex protein traffic (1) may provide a means to directly examine the functional role of Sec18p in this critical interorganelle transport event, potentially in both yeast and mammalian reconstituted systems.

ACKNOWLEDGEMENTS

We would like to thank Elizabeth Greyhack for her help with in vitro translation, Lois Banta and Jean Edens for electron microscopy and Michael Clark for his expertise and help with microscopy in general. We also thank Shawn Westway for assistance with BIONET and Cathy Elkins for typing the manuscript. We thank Randy Schekman for his support, especially in the early stages of cloning SEC18, and for continued interest throughout.

This study was supported by Public Health Service Grant GM-32703 from the National Institutes of Health (to S.D.E.). K.A.E. was supported in part by a National Research Service Award 1 T32 GM07616 from the National Institute of General Medical Sciences. S.D.E. is a Presidential Young Investigator supported by grant DCB-8451633 from the National Science Foundation.

LITREATURE CITED

1. Beckers, C. J. M., Keller, D. S. and Balch, W. E. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. Cell 50:523-534 1987

2. Carlson, M. and Botstein, D. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell 28:145-154 1982.

3. Casadaban, M. J. and Cohen, S. N. Analysis of gene control signals by DNA fusion and cloning in E. coli. J. Mol. Biol. 138:179-207 1980.

4. Elder, R. T., Loh, E. Y. and Davis, R. W. RNA from the yeast transposable element Ty1 has both ends in the direct repeats, a structure similar to retrovirus RNA. Proc. Natl. Acad. Sci. USA 80:2432-2436 1983.

5. Emr, S. D., Schauer, I., Hansen, W., Esmon, P. and Schekman, R. Invertase b-galactosidase hybrid proteins fail to be transported from the endoplasmic reticulum in *Saccharomyces cerevisiae*. Molec. Cell. Biol. 4:2347-2355 1984.

6. Emr, S. D., Schekman, R., Flessel, M. C. and Thorner, J. An MFα-SUC2 (α factor-invertase) gene fusion for study of protein localization and gene expression in yeast. Proc. Natl. Acad. Sci. USA 80:7080-7084 1983.

7. Esmon, B., Novick, P. and Schekman, R. Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell 25:451-460 1981.

8. Green, M. R. and Roeder, R. G. Definition of a novel promoter for the major adenovirus-associated virus mRNA. Cell 22:231-242 1980.

9. Hopp, T. and Woods, K. R. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA 78:3824-3828 1981.

10. Klionsky, D., Banta, L. M. and Emr, S. D. Intracellular sorting and processing of a yeast vacuolar hydrolase: Proteinase A propeptide contains vacuolar targeting information. Mol. Cell. Biol. 8:2105-2116 1988.

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

12. Lee, J. J., Calzone, F. J., Britten, R. J., Angerer, R. C. and Davidson, E. H. Activation of sea urchin actin genes during embryogenesis: measurement of transcript accumulation from five different genes in Strongylocentrotus purpuratus. J. Mol. Biol. 188:173-183 1986.

13. Lipman, D. J. and Pearson, W. R. Rapid and sensitive protein similarity searches. Science 227:1435-1441 1985.

14. Maniatis, T., Fritsch, E. F. and Sambrook, J. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1982.

15. Melton, D., Kreig, P., Rebaglianti, T., Maniatis, T., Kinn, T. and Green, M. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing an SP6 promoter. Nucl. Acids Res. 18:7035-7056 1984.

16. Miller, J. Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1972.

17. Mueller, P. P., and Hinnebusch, A. G. Multiple upstream AUG codons mediate translational control of GCN4. Cell 45:201-207 1986.

18. Nasmyth, K. A., and Reed, S. T. Isolation of genes by complementation in yeast; Molecular cloning of a cell-cycle gene. Proc. Natl. Acad. Sci. USA 77:2119-2123 1980.

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

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

21. Orr-Weaver, T. L., Szostak, J. W. and Rothstein, R. J. Genetic application of yeast transformation with linear and gapped plasmids. In: Cloning of Genes into Yeast Cells. Methods in Enzymology 101:228-245 1983.

22. Reid, G. A. Pulse-labeling of yeast cells and spheroplasts. In: Methods in Enzymology 97:324-329 1983.

23. Rothstein, R. J. One-step gene disruption in yeast. In: Cloning of Genes into Yeast Cells. Methods in Enzymology 101:202-211 1983.

24. Sanger, F., Nicklen, S. and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467 1977.

25. Schekman, R. and Novick, P. The secretory process and yeast cell-surface assembly. In: The Molecular Biology of the Yeast Saccharomyces Metabolism and Gene Expression (Strathern, Jones, and Broach, eds.), pp. 361-398. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1982.

26. Schultz, J., Silhavy, T. J., Berman, M. L., Fiil, N. and Emr, S. D. A previously unidentified gene in the spc operon of Escherichia coli K12 specifies a component of the protein export machinery. Cell 31:227-235 1982.

27. Sherman, F., Fink, G. R. and Lawrence, L. W. Methods in Yeast Genetics: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1979.

28. Stevens, T., Esmon, B., and Schekman, R. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell 30:439-448 1982.

29. Struhl, K. Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell 49:295-297 1987.

30. Towbin, H., Staehelin, T. and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354 1979.

31. Tuite, M.F., Plesset, J., Moldave, K. and McLaughlin, C. S. Faithful and efficient translation of homologous and heterologous mRNAs in an mRNA-dependent cell-free system from Saccharomyces cerevisiae. J. Biol. Chem. 255:8761-8766 1980.

32. Zaret, K. S. and Sherman, F. DNA sequence required for efficient transcription termination in yeast. Cell 28:563-573 1982.

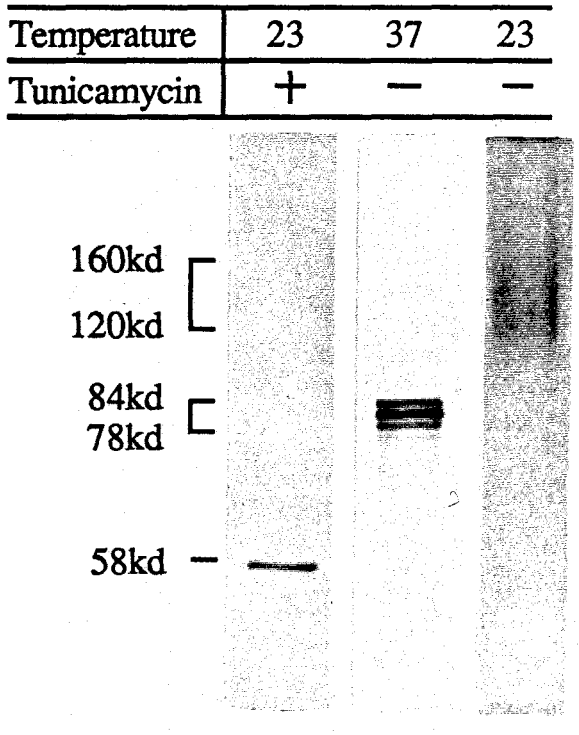
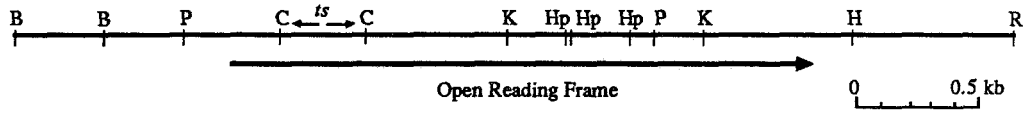
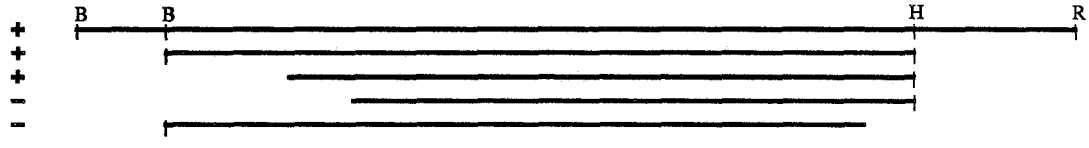

Temperature
23
37
23
Tunicamycin
+
—
—
160kd
120kd
84kd
78kd
58kd

Figure 1. *sec18-1* mutant cells were grown in 100 μM SO_4 medium with 2% glucose. Cells were pelleted, washed with distilled H_2O, and suspended in sulfate-free medium with 0.2% glucose for 30 min before they were labeled, to induce invertase production. Cells were labeled with 0.5 mCi/ml $^{35}SO_4$ per ml for 20 min and chased for 20 min following the addition of $(NH_4)_2SO_4$ to 50 mM.

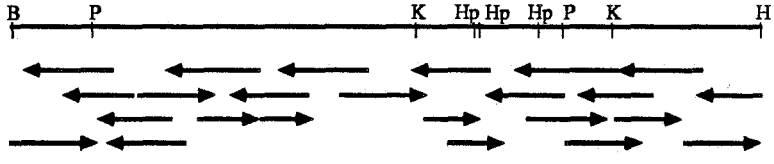
A) **SEC 18 Gene Clone**



B) Complementation Analysis



C) Sequencing Strategy



D) mRNA Transcript End-Mapping

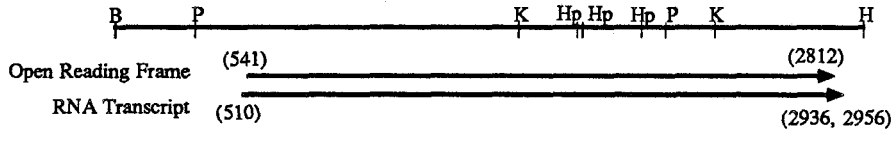


Figure 2. (A) Restriction map of *SEC18* clone showing the position of the open reading frame as determined by DNA sequencing. Restriction enzymes B, *Bam*HI; P, *Pst*I; K, *Kpn*I; Hp, *Hpa*I; H, *Hin*d III; C, *Cla*I; R, *Eco*RI. The position of the mutation leading to the temperature sensitive phenotype of *sec18-1* is indicated with double arrows. (B) Complementation analysis of *SEC18* subclones. Restriction fragments or *Bal*31 deletions were cloned into pSEYC58 (*CENIV-ARSI, URA3*) and transformed into strain SEY5186 by LiCl yeast transformation. Transformants were selected for Ura^+ and tested for growth at 25 and 37°C. A plus sign indicates temperature-resistant transformants, and a minus sign indicates temperature-sensitive transformants. (C) Sequencing strategy used for DNA sequencing. Each arrow indicates independent subclones obtained by *Bal*31 digestion of the 3.0 kb *Bam*HI-*Hin*d III from either end cloned into M13mp19. The entire *Bam*HI-*Hin*d III *SEC18* fragment was cloned into M13mp8 and M13mp9 to obtain sequence from the *Bam*HI site at the 5' end of the clone and from the *Hin*d III site at the 3' end of the clone, respectively. (D) Comparison of the results of end mapping of the *SEC18* mRNA with the open reading frame of the DNA sequence. Numbers in parenthesis represent the position in the nucleotide sequence. The position of the 5' end is mapped to ±2 nucleotides, the position of the 3' ends are ±15 nucleotides.

SEC 18 Genomic Sequence

```
                                                                    Bam HI
                                                                1   GGATCCAACA ACAACTGTAA ACGCCAAAGT GGGTGCAGAA    40

   41  AATGATGGAC GATTCTTCCT TATTTTTGCG AACGCCTCAC ACCTTTAGAT CCTTTGGATA TGGATGATGC TTTTGATGAT ACCAATATGG GCAGCAATAG   140

  141  TTCATTTAGC TTGAGCCTTC CTCGCCTTAA TCAATAATGA GCGCGCTAAA TAGTTGAATA TTTTATCTTT AATTAAATAC ATTTGCTTAT AAAGGTTGTA   240

                                                                                          Pst I
  241  CTATTTACTT GTAGTTGATG TAGGATTCCA ATTCAATGCA CTTTAATACT CAGAGTACGC TTTATACTTC TGCAGTACCC TCTCTAAAAT GTCACCTAGT   340

  341  ATTTTTTTCT CTTTACACAA CGTAGCGTCA CTAAAAAAAG GTATTTCCCA TGGGTTAATT CGGTATCACA CTATACGTTA GGCGTACCAA ATTTCGAGAT   440

                                                                          *-> Minimum Complementing Fragment
  441  TCATGAAATT GGCTTATTCG TAGCCTTTAA GTACTACCGT GTGTTTTCTC TCAAAGATAA GAGCAAATAG TACCGTATAT TCAGCCTTTA TAAATTGAGT   540
                                                                                *-> mRNA Start

  541  ATG TTC AAG ATA CCT GGT TTT GGA AAA GCT GCT GCA AAT CAT ACT CCA CCA GAT ATG ACA AAC ATG GAT ACC CGT ACA CGC CAT   624
    1  MET Phe Lys Ile Pro Gly Phe Gly Lys Ala Ala Ala Asn His Thr Pro Pro Asp MET Thr Asn MET Asp Thr Arg Thr Arg His    28

  625  TTA AAG GTG TCA AAC TGT CCA AAT AAC TCC TAT GCA CTC GCA AAC GTA GCT GCT GTC TCA CCA AAT GAT TTC CCT AAT AAC ATT   708
   29  Leu Lys Val Ser Asn Cys Pro Asn Asn Ser Tyr Ala Leu Ala Asn Val Ala Ala Val Ser Pro Asn Asp Phe Pro Asn Asn Ile    56

  709  TAT ATT ATT ATC GAT AAT TTA TTT GTT TTC ACA ACT AGA CAC TCC AAC GAC ATT CCA CCG GGA ACC ATT GGA TTT AAC GGT AAC   792
   57  Tyr Ile Ile Ile Asp Asn Leu Phe Val Phe Thr Thr Arg His Ser Asn Asp Ile Pro Pro Gly Thr Ile Gly Phe Asn Gly Asn    84

  793  CAG CGT ACC TGG GGT GGT TGG TCC CTA AAT CAA GAC GTG CAA GCA AAA GCA TTT GAT TTA TTC AAG TAT TCC GGT AAG CAA TCG   876
   85  Gln Arg Thr Trp Gly Gly Trp Ser Leu Asn Gln Asp Val Gln Ala Lys Ala Phe Asp Leu Phe Lys Tyr Ser Gly Lys Gln Ser   112

  877  TAT CTT GGT TCA ATA GAT ATA GAT ATC TCA TTC AGA GCT AGA GGT AAG GCG GTA AGC ACG GTA TTC GAT CAA GAT GAG TTA GCC   960
  113  Tyr Leu Gly Ser Ile Asp Ile Asp Ile Ser Phe Arg Ala Arg Gly Lys Ala Val Ser Thr Val Phe Asp Gln Asp Glu Leu Ala   140

  961  AAA CAA TTT GTT CGT TGC TAC GAA TCT CAA ATA TTT TCT CCC ACC CAG TAC CTT ATC ATG GAG TTC CAA GGC CAT TTC TTT GAC  1044
  141  Lys Gln Phe Val Arg Cys Tyr Glu Ser Gln Ile Phe Ser Pro Thr Gln Tyr Leu Ile Met Glu Phe Gln Gly His Phe Phe Asp   168

 1045  TTA AAA ATT AGA AAT GTC CAA GCA ATC GAT TTG GGT GAT ATT GAA CCA ACC TCC GCT GTT GCA ACT GGG ATA GAG ACA AAG GGA  1128
  169  Leu Lys Ile Arg Asn Val Gln Ala Ile Asp Leu Gly Asp Ile Glu Pro Thr Ser Ala Val Ala Thr Gly Ile Glu Thr Lys Gly   196

 1129  ATT TTG ACA AAA CAA ACA CAA ATT AAT TTT TTC AAA GGA AGA GAT GGT TTA GTT AAT TTG AAA TCA TCA AAT TCA TTA AGA CCA  1212
  197  Ile Leu Thr Lys Gln Thr Gln Ile Asn Phe Phe Lys Gly Arg Asp Gly Leu Val Asn Leu Lys Ser Ser Asn Ser Leu Arg Pro   224

 1213  AGA TCA AAT GCT GTG ATC AGA CCG GAT TTC AAG TTT GAA GAT TTG GGT GTC GGT GGT TTG GAT AAA GAG TTT ACT AAA ATT TTC  1296
  225  Arg Ser Asn Ala Val Ile Arg Pro Asp Phe Lys Phe Glu Asp Leu Gly Val Gly Gly Leu Asp Lys Glu Phe Thr Lys Ile Phe   252

 1297  AGA AGA GCG TTT GCA AGT CGA ATC TTT CCT CCT TCA GTT ATA GAA AAA CTG GGT ATT TCT CAT GTT AAA GGT TTG CTA TTG TAC  1380
  253  Arg Arg Ala Phe Ala Ser Arg Ile Phe Pro Pro Ser Val Ile Glu Lys Leu Gly Ile Ser His Val Lys Gly Leu Leu Leu Tyr   280

 1381  GGT CCT CCA GGT ACT GGT AAG ACC TTA ATT GCA AGA AAG ATT GGT ACA ATG CTG AAT GCC AAA GAG CCC AAA ATC GTC AAT GGT  1464
  281  Gly Pro Pro Gly Thr Gly Lys Thr Leu Ile Ala Arg Lys Ile Gly Thr Met Leu Asn Ala Lys Glu Pro Lys Ile Val Asn Gly   308

 1465  CCA GAA ATT TTG AGT AAG TAC GTT GGT TCT TCA GAA GAA AAC ATT CGT AAT TTA TTT AAG GAT GCA GAA GCA GAA TAT AGG GCC  1548
  309  Pro Glu Ile Leu Ser Lys Tyr Val Gly Ser Ser Glu Glu Asn Ile Arg Asn Leu Phe Lys Asp Ala Glu Ala Glu Tyr Arg Ala   336

                                                                                                        Kpn I
 1549  AAG GGT GAG GAA TCT TCC TTA CAT ATT ATT ATT TTC GAT GAG CTG GAT TCT GTT TTC AAG CAG AGA GGT TCA AGA GGT GAT GGT  1632
  337  Lys Gly Glu Glu Ser Ser Leu His Ile Ile Ile Phe Asp Glu Leu Asp Ser Val Phe Lys Gln Arg Gly Ser Arg Gly Asp Gly   364

 1633  ACC GGT GTA GGG GAC AAT GTA GTT AAT CAA TTG TTA GCT AAA ATG GAT GTT GAT CAA TTG AAT AAT ATT TTG GTT ATT GGT ATG  1716
  365  Thr Gly Val Gly Asp Asn Val Val Asn Gln Leu Leu Ala Lys Met Asp Val Asp Gln Leu Asn Asn Ile Leu Val Ile Gly Met   392

 1717  ACC AAT CGT AAA GAT TTA ATA GAC AGT GCT CTT TTG CGT CCA GGT AGA TTT GAA GTC CAA GTT GAA ATT CAT TTA CCC GAT GAA  1800
  393  Thr Asn Arg Lys Asp Leu Ile Asp Ser Ala Leu Leu Arg Pro Gly Arg Phe Glu Val Gln Val Glu Ile His Leu Pro Asp Glu   420

                                                                                                  Hpa I
 1801  AAA GGA AGA CTC CAA ATT TTC GAC ATT CAG ACG AAG AAA ATG AGG GAA AAT AAT ATG ATG AGC GAC GAT GTT AAC TTA GCT GAG  1884
  421  Lys Gly Arg Leu Gln Ile Phe Asp Ile Gln Thr Lys Lys Met Arg Glu Asn Asn Met Met Ser Asp Asp Val Asn Leu Ala Glu   448

                  Hpa I
 1885  TTA GCT GCG TTA ACA AAA AAC TTC TCT GGT GCT GAG ATT GAG GGT TTA GTG AAG AGT GCA AGT TCT TTT GCA ATC AAC AAA ACC  1968
  449  Leu Ala Ala Leu Thr Lys Asn Phe Ser Gly Ala Glu Ile Glu Gly Leu Val Lys Ser Ala Ser Ser Phe Ala Ile Asn Lys Thr   476

 1969  GTC AAC ATC GGG AAA GGT GCC ACA AAA CTT AAC ACT AAA GAT ATA GCA AAA CTT AAA GTA ACA AGA GAA GAC TTT TTA AAT GCA  2052
  477  Val Asn Ile Gly Lys Gly Ala Thr Lys Leu Asn Thr Lys Asp Ile Ala Lys Leu Lys Val Thr Arg Glu Asp Phe Leu Asn Ala   504

 2053  CTC AAC GAT GTT ACT CCC GCT TTT GGG ATT AGT GAA GAA GAT TTG AAA ACA TGT GTG GAA GGT GGA ATG ATG CTT TAT TCC GAA  2136
  505  Leu Asn Asp Val Thr Pro Ala Phe Gly Ile Ser Glu Glu Asp Leu Lys Thr Cys Val Glu Gly Gly Met Met Leu Tyr Ser Glu   532

       Hpa I
 2137  CGA GTT AAC TCA ATA TTG AAG AAC GGA GCC CGT TAC GTC CGC CAA GTT CGC GAG AGT GAT AAA TCC AGG TTA GTA TCT CTA TTA  2220
  533  Arg Val Asn Ser Ile Leu Lys Asn Gly Ala Arg Tyr Val Arg Gln Val Arg Glu Ser Asp Lys Ser Arg Leu Val Ser Leu Leu   560

                  Pst I
 2221  ATC CAC GGC CCT GCA GGG TCC GGT AAA ACA GCT TTA GCC GCT GAA ATT GCT TTA AAA TCT GGA TTC CCA TTC ATC AGG TTA ATT  2304
  561  Ile His Gly Pro Ala Gly Ser Gly Lys Thr Ala Leu Ala Ala Glu Ile Ala Leu Lys Ser Gly Phe Pro Phe Ile Arg Leu Ile   588

 2305  TCT CCC AAC GAG TTG TCA GGC ATG TCA GAA AGC GCA AAA ATT GCC TAT ATT GAT AAC ACT TTC AGA GAT GCG TAT AAA TCT CCA  2388
  589  Ser Pro Asn Glu Leu Ser Gly Met Ser Glu Ser Ala Lys Ile Ala Tyr Ile Asp Asn Thr Phe Arg Asp Ala Tyr Lys Ser Pro   616

                                                                                  Kpn I
 2389  CTA AAC ATT CTT GTT ATT GAT TCG TTA GAG ACT CTA GTT GAT TGG GTA CCA ATT GGT CCA AGA TTC TCT AAT AAC ATT TTA CAA  2472
  617  Leu Asn Ile Leu Val Ile Asp Ser Leu Glu Thr Leu Val Asp Trp Val Pro Ile Gly Pro Arg Phe Ser Asn Asn Ile Leu Gln   644

 2473  ATG CTA AAG GTT GCA TTG AAG CGT AAA CCC CCA CAA GAC CGT CGT TTA TTG ATC ATG ACT ACT ACA TCA GCT TAT TCG GTA CTT  2556
  645  Met Leu Lys Val Ala Leu Lys Arg Lys Pro Pro Gln Asp Arg Arg Leu Leu Ile Met Thr Thr Thr Ser Ala Tyr Ser Val Leu   672

 2557  CAA CAA ATG GAT ATC TTG AGT TGC TTC GAC AAT GAG ATA GCA GTT CCA AAT ATG ACC AAT TTA GAT GAA TTG AAC AAC GTC ATG  2640
  673  Gln Gln Met Asp Ile Leu Ser Cys Phe Asp Asn Glu Ile Ala Val Pro Asn Met Thr Asn Leu Asp Glu Leu Asn Asn Val Met   700

 2641  ATA GAA TCA AAC TTT CTT GAC GAT GCT GGT AGA GTT AAA GTT ATT AAT GAA TTA TCA AGG AGC TGT CCT AAC TTC AAT GTC GGT  2724
  701  Ile Glu Ser Asn Phe Leu Asp Asp Ala Gly Arg Val Lys Val Ile Asn Glu Leu Ser Arg Ser Cys Pro Asn Phe Asn Val Gly   728

 2725  ATT AAA AAG ACC TTG ACC AAC ATT GAA ACC GCA AGG CAC GAT GAA GAT CCC GTG AAC GAG CTT GTT GAG TTG ATG ACC CAA TCC  2808
  729  Ile Lys Lys Thr Leu Thr Asn Ile Glu Thr Ala Arg His Asp Glu Asp Pro Val Asn Glu Leu Val Glu Leu Met Thr Gln Ser   756

                                                                                    Termination Recognition
 2809  GCA TAA TTATTT CAAATTTTTC ATGTTCTTGT ATTTTTATTC TTTATCCCTT CCAATCAGAA AGGATCTAGT GAACAAGTTC TTTCCTCTAT  2900
  757  Ala TER                                                                    ****                ***

    Sequences                          Approximate Poly-Adenylation Addition Sites
 2901  GGTATATATT TTAGTGATAA ATTTTATAAA ATTATAAAAC CAAGGCATCC TTTCCTTTTA TTCTGTCATT GGATTCTGCC TGTCATAATT ATCACCACCG  3000
       ***       ****                ++                  ++

                                  Hind III
 3001  GGTAAAGATG ATAATTTTTC AGTCGCTTTG CCCGAGAAGC TT  3042
```

Figure 3. Nucleotide Sequence of *SEC18*. The nucleotide sequence with translation of the open reading frame. Positions of sites identified by restriction mapping prior to sequencing are indicated in bold face, as are the positions of initiator methionine codons at the 5' end of the open reading frame, position of the start of the smallest complementing subclone, position of the transcription start point for *SEC18* mRNA, the translation termination codon (TRM), and the transcription recognition sequences and approximate poly-(A) addition sites at the 3' end of the clone.

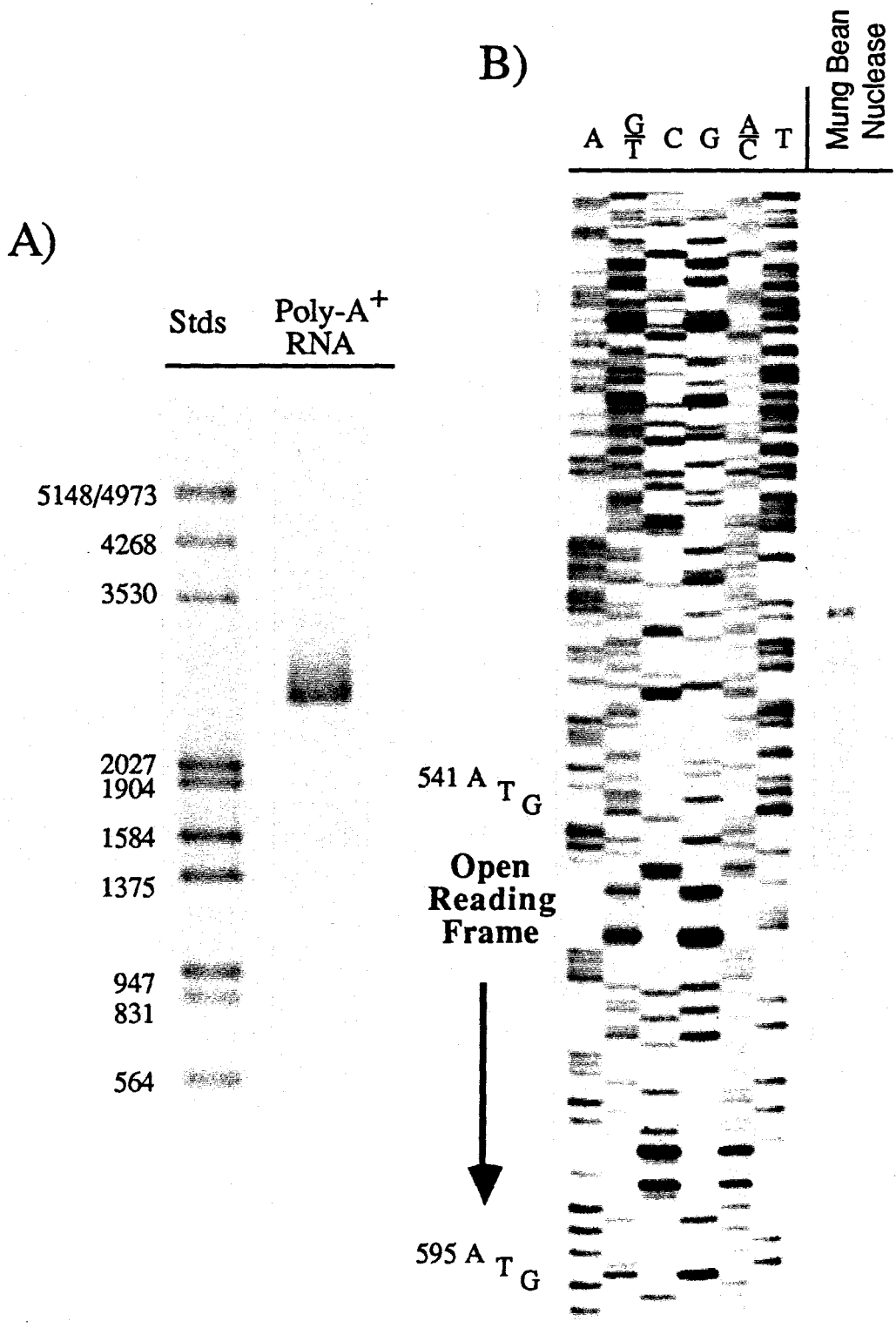

A)
Stds
Poly-A+ RNA
5148/4973
4268
3530
2027
1904
1584
1375
947
831
564
B)
A G/T C G A/C T
Mung Bean Nuclease
541 A T G
Open Reading Frame
595 A T G

Figure 4. (A) Northern blot of *SEC18* mRNA. Poly(A)$^+$ RNA (1 μg) from mid-log phase SEY2101 cells was run on a 1% agarose formaldehyde gel, transferred to Gene-Screen, and probed with an anti-sense *SEC18*[^{32}P]RNA that was made with SP6 RNA polymerase. Standards (Stds.) were *Eco*RI-and *Hin*d III-cut lambda DNA that was end-labeled by treating it with [γ ^{32}P]ATP and T4 polynucleotide kinase. Sizes (in nucleotides) are indicated to the left of the gel. (B) Mapping of the 5' end of *SEC18* mRNA. An oligonucleotide complementary to bp 681 to 695 of the DNA sequence was end-labeled with ^{32}P, hybridized to a single-stranded *SEC18*-M13mp19 template, and extended with the Klenow fragment to the *Bam*HI site. The single-stranded probe was fragment purified, hybridized to 25 μg of total RNA, and digested with mung bean nuclease. The DNA ladder was obtained by deoxy- and dideoxy sequencing reactions by using the end-labeled oligo as primer. The positions of the first two ATG codons of the open reading frame of the DNA sequence are indicated to the left of the gel.

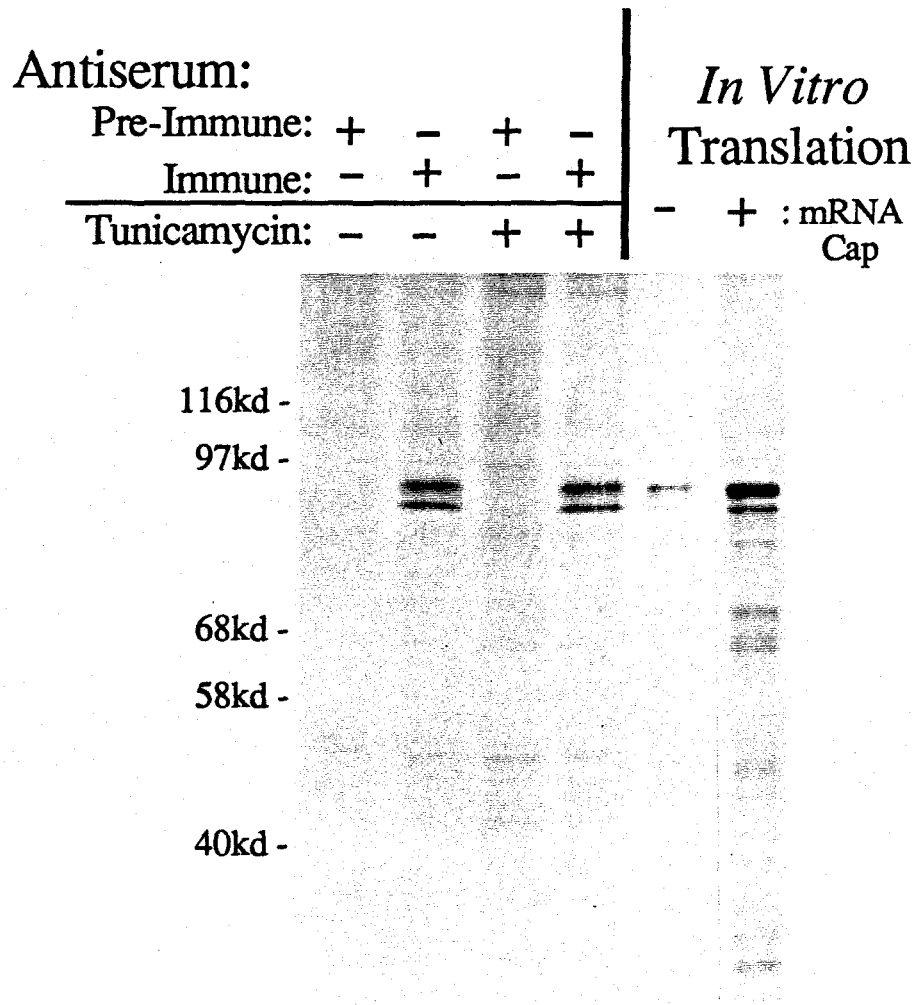
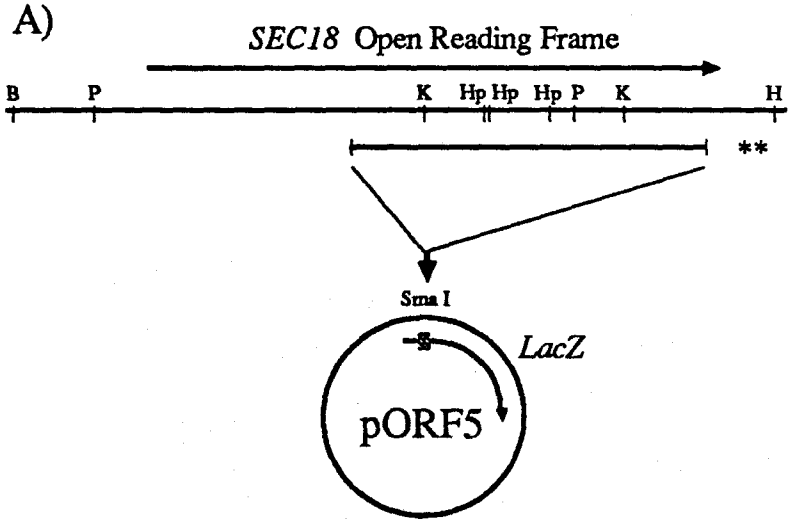

Antiserum:
Pre-Immune: + − + −
Immune: − + − +
Tunicamycin: − − + +
In Vitro Translation
− + : mRNA Cap
116kd -
97kd -
68kd -
58kd -
40kd -

Figure 5. Identification of Sec18p in vitro and in vivo. (A) Immunoprecipitations of Sec18p from ^{35}S-labeled whole-cell yeast extracts done with pre-immune or immune serum in the presence or absence of tunicamycin, as indicated. (B) Products of in vitro translation of SP6 RNA polymerase transcripts of the *SEC18* open reading frame. RNAs were made with and without a mRNA cap analog [$m^7G(5')ppp(5')G$] in the transcription reaction as indicated. Translation of the capped message proceeded with much higher efficiency. kd, Kilodaltons.


A)
SEC18 Open Reading Frame
B
P
K
Hp Hp
Hp
P
K
H
**
Sma I
LacZ
pORF5

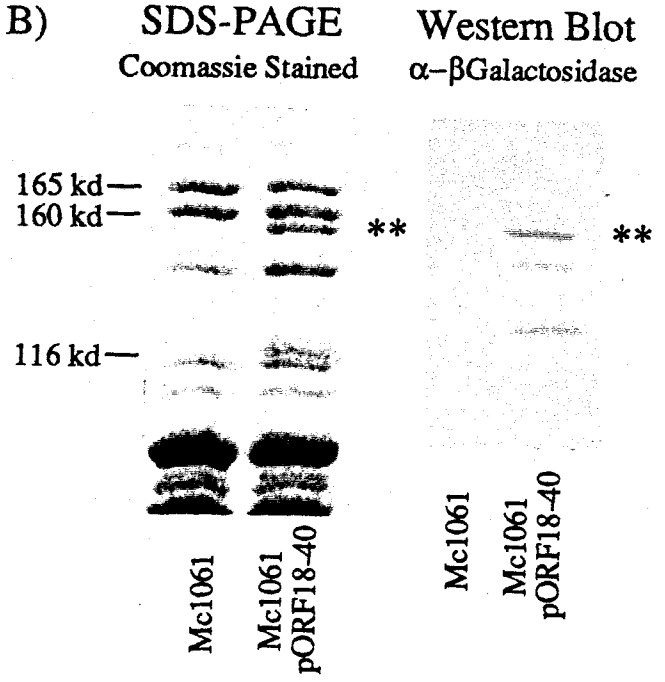

B)
SDS-PAGE
Coomassie Stained
Western Blot
α–βGalactosidase
165 kd
160 kd
116 kd
**
**
Mc1061
Mc1061 pORF18-40
Mc1061
Mc1061 pORF18-40

Figure 6. Production of *SEC18-lacZ* fusion proteins. (A) *Bal*31 digestions of the 3.0-kb *Bam*HI-*Hin*d III fragment were cloned into the *Sma*I site of pORF5, a *LacZ* fusion vector in which a *Eco*RI-*Sma*I-*Bam*HI polylinker has been inserted at the amino acid at position 7 to interrupt the reading frame of the *LacZ* gene. The fragment marked with double asterisks is clone pORF18-40, which expressed the largest fusion protein observed. Restriction enzyme abbreviations: B, *Bam*HI; P, *Pst*I; K, *Kpn*I; Hp, *Hpa*I; H, *Hin*d III; C, *Cla*I; R, *Eco*RI. (B) Whole-cell extracts of *E. coli* Mc1061 with and without the pORF18-40 fusion plasmid, were run on SDS-polyacrylamide gels. A gel stained with Coomassie blue G-250 is shown on the left, and the right is a Western blot of a gel identical to that shown on the left whose proteins were transferred to nitrocellulose by Western blot and probed with anti-β-galactosidase antisera. The largest fusion protein is marked with double asterisks. kd, Kilodaltons.

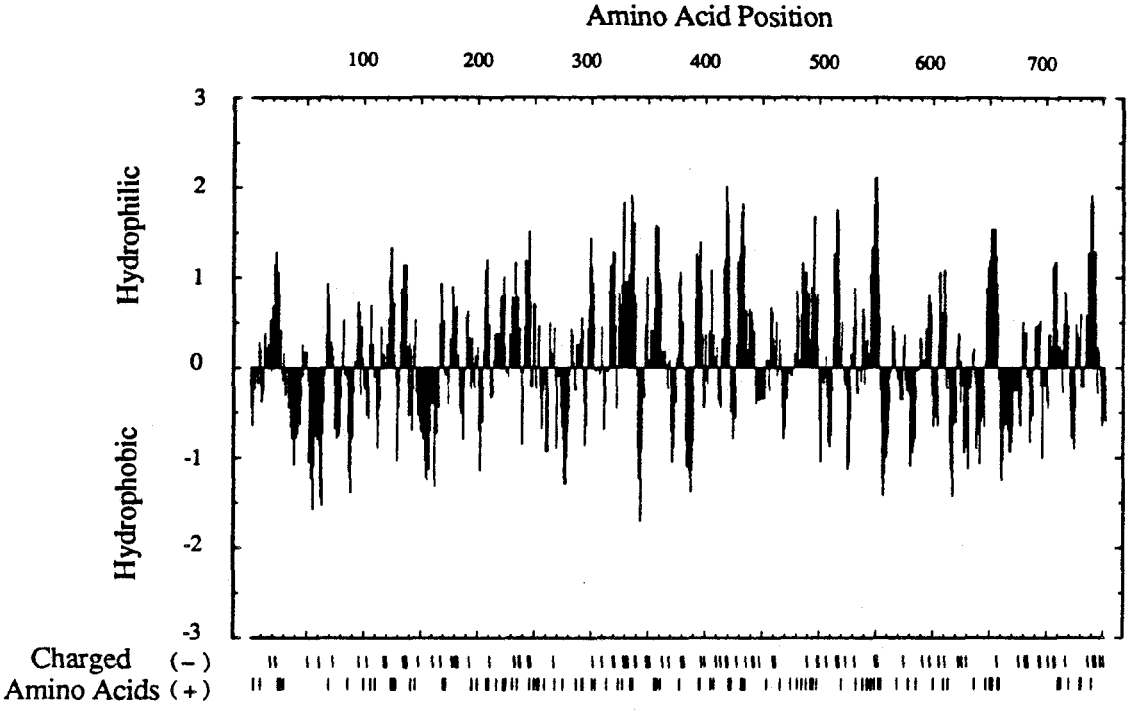

Amino Acid Position
100
200
300
400
500
600
700
3
2
1
0
-1
-2
-3
Hydrophilic
Hydrophobic
Charged (–)
Amino Acids (+)

Figure 7. Hydrophobicity analysis of Sec18p. A hydrophobicity profile of the predicted protein sequence of the *SEC18* open reading frame was calculated using the values of Hopp and Wood (9) and a window of six amino acids. The position of charged amino acid residues is indicated by tics at the bottom of the figure. Neither the entire open reading frame nor open reading frames starting at 19 or 21 amino acids into the protein sequence revealed a satisfactory signal sequence or membrane-spanning domain.

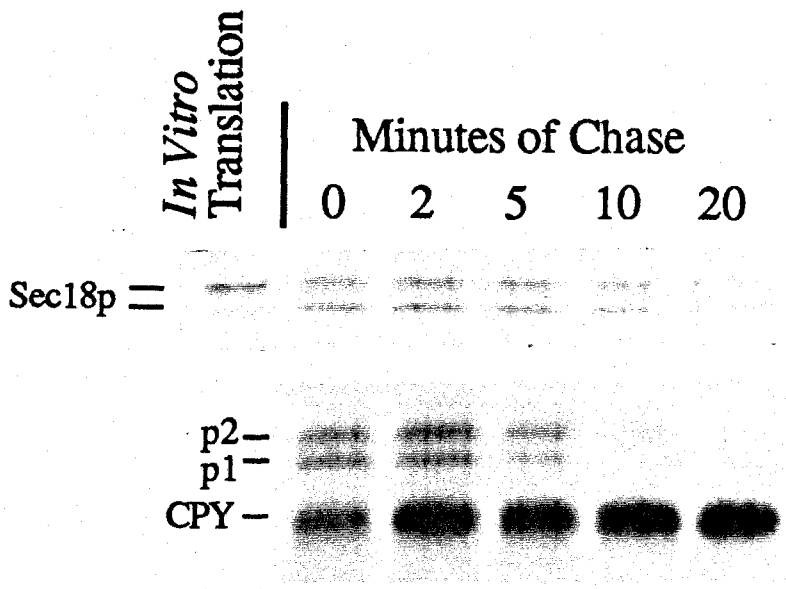

In Vitro Translation
Minutes of Chase
0
2
5
10
20
Sec18p
p2
p1
CPY

Figure 8. Pulse-chase labeling of Sec18p and CPY. Yeast cells (Strain SEY2101; 1 OD_{600} unit per lane) were labeled for 30 min with $^{35}SO_4$ at 30°C and chased for the indicated times following the addition of $(NH_4)_2SO_4$ to a final concentration of 50 mM. Labeling was stopped by the addition of trichloroacetic acid to 5%, and cell extracts were immunoprecipitated simultaneously with saturating amounts of both Sec18p and CPY antisera. (A) Two forms of Sec18p are not post-translationally modified into a single species; (B) p1 (ER) and p2 (Golgi complex) forms of CPY are chased posttranslationally into the mature vacuolar form. In panel A, exposure of the gel was 2 times longer than that in panel B. From densitometry of the bands, we estimate that Sec18 is produced at approximately 1/10th the level of CPY.

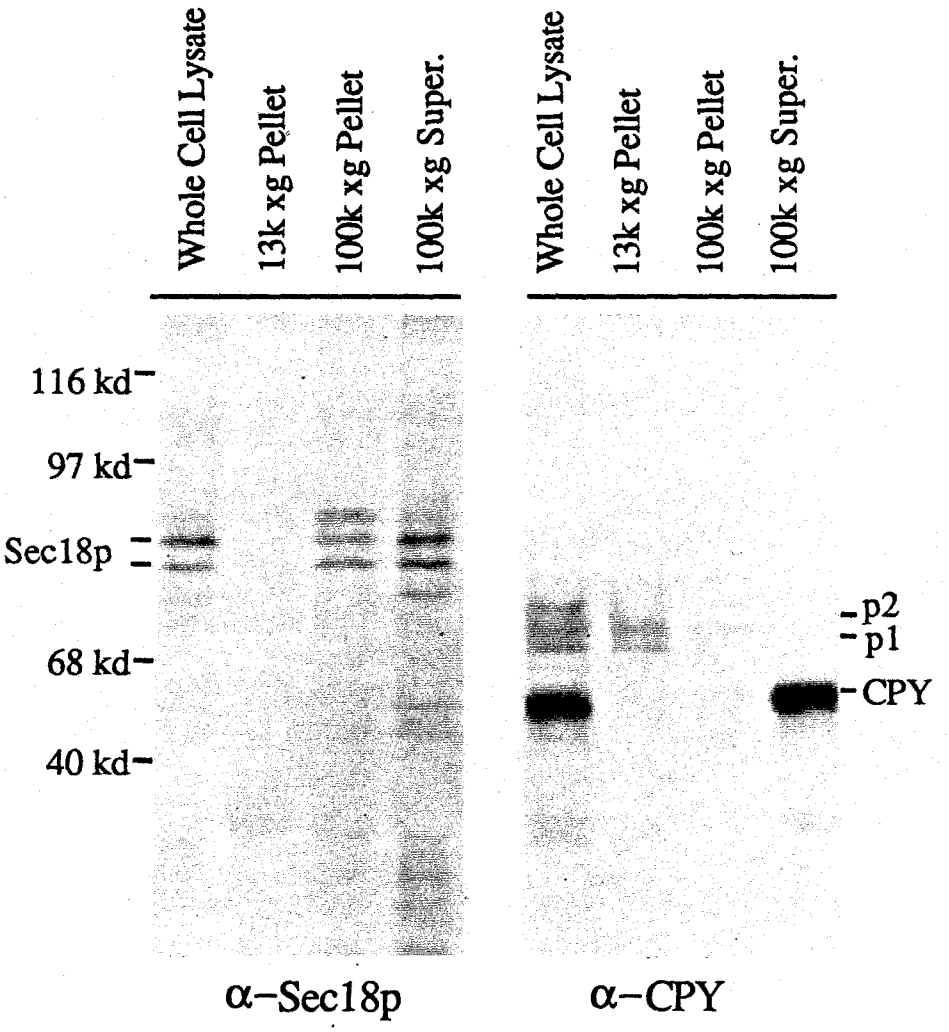

Whole Cell Lysate
13k xg Pellet
100k xg Pellet
100k xg Super.
Whole Cell Lysate
13k xg Pellet
100k xg Pellet
100k xg Super.
116 kd
97 kd
Sec18p
68 kd
40 kd
p2
p1
CPY
α–Sec18p
α–CPY

Figure 9. Sec18p fractionates between a high-speed membrane and supernatant fractions. Yeast cells (strain SEY2101) were spheroplasted with oxylyticase, labeled with Trans ^{35}S label, and lysed osmotically. The lysate was pelleted at 2000 $\times g$ to remove any unbroken cells, and the supernatant was pelleted sequentially at 13,000 $\times g$ and 100,000 $\times g$. From left to right, the first lane of each panel shows immunoprecipitation from a fraction of the whole-cell lysate, the second lane from a 13,000 $\times g$ pellet, the third lane from a 100,000 $\times g$ pellet and the fourth lane from trichloroacetic acid-precipitated proteins from the 100,000 $\times g$ supernatant fraction. (A) Immunoprecipitation with Sec18p antiserum; (B) subsequent immunoprecipitation with CPY antiserum. p1 and p2 represent the ER and Golgi complex intermediates, respectively, in the processing of CPY to its mature form. Extra bands that were present in the lane with the Sec18p 100,000 $\times g$ pellet and the lane with the CPY whole-cell lysate were not reproducible and probably represented some nonspecific cross-reaction of the antiserum that was used for immunoprecipitation. kd, Kilodaltons.

Table 1. Quantitation of Cell Fractionation

Fraction	% of fraction in[b]:		
	Pellet from:		Supernatant from
	13,000 xg Centrifugation	100,000 xg Centrifugation	100,000 xg Centrifugation
Sec18p	<1	44	56
p1 and p2 CPY	80	20	<1
Mature CPY	<5	<1	>95
Mitochondrial F_1-ATPase β	>95	<1	<5

[a] Quantitation was done by scanning autoradiographs on a laser densitometer (2202; LKB Instruments Inc., Rockville, Md.) and integration with a program (GelScan; LKB).

[b] Percentages represent the amount present in a given fraction divided by the total of all three fractions. In all cases, the quantitative sum of all three fractions was within 10% of the quantitation of a similar whole-cell lysate sample. Samples which are <5% or <1% were either too faint for accurate quantitation or difficult to resolve because of the presence of contaminating background bands.