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Construction of Defined Polytopic Integral Transmembrane Proteins

by

Richard E. Rothman

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Endocrinology

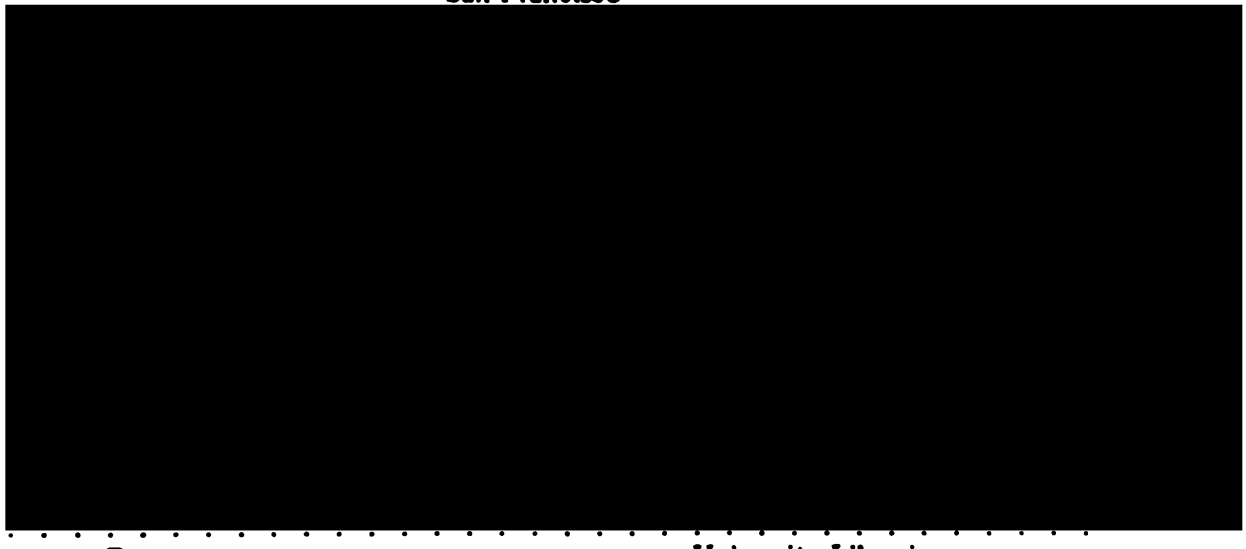
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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Construction of Defined Polytopic Integral Transmembrane Proteins
by Richard E. Rothman

Abstract

Signal and stop transfer sequences are discrete regions within a polypeptide chain able to initiate or terminate translocation of the protein across the membrane of the endoplasmic reticulum (ER). We have investigated the role of these topogenic sequences in the biogenesis of polytopic transmembrane proteins.

Since signal and stop transfer elements have recently been shown to have some similar activities, we first sought to systematically define the function of these elements, both alone and in the context of one another. Plasmids encoding various patterns of well-characterized signal and stop transfer sequences fused to a set of topogenically inert passenger domains were constructed. These molecules were expressed by transcription-translation in a cell-free system, or by microinjection of transcripts into *Xenopus oocytes*. The transmembrane disposition of the encoded products was assessed by protection of translocated domains from exogenously added protease, and the occurrence of post-translational modifications of proteins which take place exclusively on the lumenal side of the ER (i.e. signal cleavage and N-linked core glycosylation). Although the signal and stop transfer sequences shared some activities, they were found to be functionally distinct and noninterchangeable. Moreover, the observed orientation of the fusion proteins with respect to the ER membrane was dependent on the order of signal and stop transfer sequences in the coding region. These results were used to test the hypothesis that a protein can achieve polytopic transmembrane orientation using combinations of simple topogenic sequences. We conclude that some (but not all) patterns of signal and stop transfer sequences confer polytopic orientation to proteins across the membrane of the endoplasmic reticulum.

We also employed a gene fusion approach to explore the mechanism by which signal and stop transfer sequences direct translocation. Normally, segregation of proteins into the cisternae of the ER is tightly coupled to their synthesis. The experimental dissociation of translocation from translation described here demonstrates that transport of polypeptides into the ER can occur in the absence of chain elongation. Furthermore, translocation across the ER membrane was found to require energy substrates, independent of those necessary for protein synthesis.

J R Bingappa

This thesis is dedicated to those who have been a constant source of encouragement and inspiration: my parents, my sister, my life-long friend, Avi Zamir, and my advisor, Vishu Lingappa.

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It is difficult to express the extent of my appreciation for having had Vishu Lingappa as a graduate advisor. He has, and continues to challenge and influence my thinking and development - intellectually, socially and politically.

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Chapter 1:

Historical Background and Introduction

I. Overview

The compartmental organization of eukaryotic cells provides order for the complex array of activities that takes place within them. Intracellular membranes thereby serve as structural barriers, creating microenvironments which subserve specialized metabolic functions. For example, lysosomes house lysosomal hydrolases, proteins which are selectively sorted to this compartment and which carry out digestive activities in this membrane-delimited space.

Since nearly all proteins are synthesized in the cytoplasm, those proteins with noncytoplasmic destinations must either cross or enter a membrane in order to reach their site of action within an intracellular compartment or outside of the cell. Thus, the biophysical property of membranes that maintains the structural and enzymatic specificity of organelles also presents an access barrier for newly synthesized proteins. The membrane barrier that proteins with extracytoplasmic destinations face is initially overcome during protein synthesis, through interactions of the nascent chain with the endoplasmic reticulum (ER), and subsequently, by the sorting of the protein contents of the ER to their final destinations. How large hydrophilic proteins are specifically targeted to the surface of the endoplasmic reticulum and translocated across or integrated into the hydrophobic core of that bilayer is the subject of this dissertation.

II. Historical Background

1. Early Evidence For a Selective Sorting Pathway

Cell fractionation and morphological studies designed to trace the biogenesis of secretory proteins from their site of synthesis in the cytoplasm to their ultimate destination, implicated the endoplasmic reticulum as the intracellular organelle to which secretory proteins are first targeted. Proteins destined for export were found to be preferentially synthesized on polysomes attached to the surface of the ER (Siekevitz and Palade, 1960). The observation that labeled nascent secretory proteins are

initially associated with membrane-attached polysomes, and subsequently appear in the cavity of microsomal membranes (the in vitro equivalent of the ER), suggested that secretory polypeptides are extruded through the membrane into the lumen of the ER (Redman et al., 1966). Since either natural or puromycin-induced termination of protein synthesis resulted in the release of N-terminal fragments of secretory proteins into microsomal cavities and not into the cytoplasm (Redman and Sabatini, 1966), it was concluded that segregation occurs by vectorial discharge from membrane-bound polysomes into the cisternal space of the ER.

The importance of understanding the mechanism of targeting to and translocation across the ER was highlighted by the recognition that the transfer of nascent chains across this membrane was the only point in the secretory pathway at which polypetides actually crossed a bilayer (Jamieson and Palade, 1967). All subsequent sorting occurs by vesicular transport (the mechanism for shuttling of lumenal or transmembrane proteins from one compartment to the next). Thus, the ER was recognized as the sole structural barrier for most proteins with noncytoplasmic destinations (i.e., secretory, lysosomal, ER, Golgi, and plasma membrane proteins).

The existence of a specialized signal in nascent secretory or membrane proteins containing the information for the selective recognition and localization of protein-polysome complexes to the rough ER was first suggested by Blobel and Sabatini in 1971. In vitro reconstitution of translation and translocation in a cell-free system provided experimental evidence for such a topogenic element. (The term 'topogenic element' will be used throughout this work to refer to a permanent or transient segment of a polypeptide chain which is a determinant of protein localization). When immunoglobulin light chain was synthesized in a cell-free translation system in the absence of microsomal membranes, a higher molecular weight precursor was detected.

The difference in size was found to be due to an amino terminal extension of the precursor of approximately 20 amino acids, relative to the secreted molecule (Milstein et al., 1972; Blobel and Dobberstein, 1975a). Co-translational addition of microsomal membranes resulted in cleavage of the amino terminal peptide extension, generating the authentic form of the secretory product which was protected from exogeneously added protease, indicative of sequestration into the lumen of the vessicle (Blobel and Dobberstein, 1975b). Determination of the amino terminal amino acid sequence of a number of secretory precursor polypeptides (Blobel et al., 1979) revealed that nearly all possess a cleaved amino terminal domain that is not present in the mature product. These sequences were termed 'signal sequences' (Blobel and Dobberstein, 1975a,b).

What is the molecular mechanism by which signal-bearing polysomes are targeted to the ER and how do these proteins overcome the structual barrier imposed by the lipid bilayer? What is the mechanism of the biogenesis of transmembrane proteins, which, instead of being transferred entirely into the lumen of the ER, achieve a precise transmembrane topology? Considerable progress has been made towards characterization of the components, and elucidation of the mechanism employed in targeting to the ER. On the other hand, our understanding of the machinery and molecular events involved in translocation across the ER, and the biogenesis of transmembrane proteins is incomplete, and remains a challenge for experimental analysis.

II. Models for Translocation

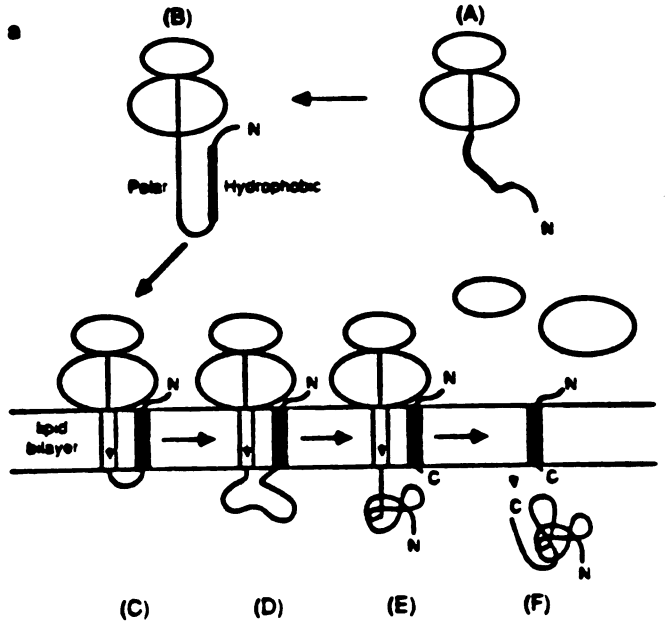
Two conceptually disparate views have evolved for discussing translocation across the ER, providing working models for experimental approaches. One school of thought asserts that the interactions between a protein destined for the ER and the bilayer can be explained by the thermodynamics of their interactions, without invoking protein

receptors or transport proteins (other than targeting proteins and signal peptidase, see iiib 1&3). The alternative model emphasizes the importance of protein-protein interactions in targeting to and translocation across the ER.

Several non-receptor mediated models for describing translocation exist. According to the direct transfer model (von Heijne and Blomberg, 1979) and the helical hairpin hypothesis (Engelman and Steitz, 1981), the energetics of transfer of polypeptide domains between the aqueous environment of the cytoplasm and the hydrophobic core of the bilayer can account for translocation. These theoretical hypotheses envision that nascent secretory chains in the cytoplasm form an antiparallel pair of helices, one of which is the hydrophobic region of the signal sequence (Fig. 1-1a). Insertion is initiated when the hydrophobic limb of the helix is buried into the membrane, resulting in a free energy gain. Secretion follows when the polar region of the protein enters the bilayer and is extruded into the lumen as the second limb of the helical hairpin. Continued translocation, driven by Brownian motion or the continued force of protein synthesis, can proceed if the free energy cost of transiently burying charged residues in the bilayer is exceeded by the free energy which arose from the initial insertion of the hydrophobic helical surface. For transmembrane proteins, emergence of a sufficiently hydrophobic segment would anchor the protein into the bilayer because of the energetic favorability of that state (see iv b). Cleavage of the signal sequence by a specific lumenal protease is proposed to result in secretory protein release into the cisternae of the ER.

The loop model of translocation (Halegoua and Inouye, 1979) does not not address the details of energetics, but is similar to the scheme described above in that translocation is said to occur directly across the lipid bilayer. According to this view, the positively charged residues at the amino terminal domain of the signal sequence bind to the

Figure 1-1. **a. Steps in the process of translocation according to spontaneous insertion models.** Translation begins on free cytoplasmic ribosomes (A); on emergence of a sufficient length of amino acid residues, marginally stable folding begins; (B). The hydrophobic limb of the hairpin inserts spontaneously into the membrane (C), pulling in the polar limb, as described in the text. As synthesis continues, the growing nascent polypeptide constitutes the relatively polar, thermodynamically unstable, limb of the hairpin and therefore passes through the membrane, folding in the extracytoplasmic space (D). Cleavage of the signal sequence occurs, releasing the amino terminus of the polypeptide into the lumen (E). **b. Steps in the process of translocation (subsequent to targeting) according to the signal hypothesis.** Synthesis begins on cytoplasmic ribosomes (A). Receptor-mediated targeting of the signal sequence-bearing ribosome to the ER membrane is described in the text and depicted in Fig. 2. Once targeted correctly, the signal sequence and the ribosome interact with their specific receptors in the ER membrane, resulting in the assembly of an aqueous, proteinaceous tunnel through the membrane (B). As protein synthesis continues, the chain passes through the tunnel to the lumen of the ER, and the signal sequence is removed by signal peptidase (C). Translocation (possibly of folded polypeptide domains) continues concomitant with protein synthesis (D). On termination of protein synthesis, the ribosomal subunits dissociate, the carboxy terminus passes through the tunnel, and the tunnel components disassemble (E), restoring the integrity of the bilayer. *Figure taken from Perara and Lingappa, 1988.*


a
(B)
(A)
N
Polar
Hydrophobic
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lipid bilayer
C
C
C
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(C)
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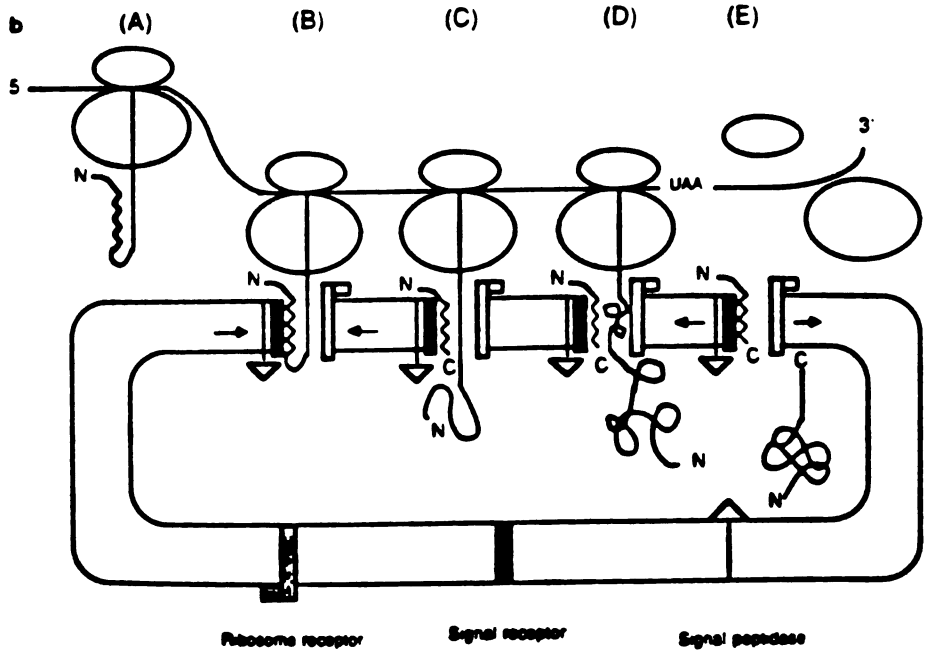

b
(A)
(B)
(C)
(D)
(E)
5'
3'
N
UAA
C
Ribosome receptor
Signal receptor
Signal peptidase

negatively charged phophatidylglycerol head groups at the membrane surface, causing the signal sequence to enter the membrane as a loop. Continued elongation results in the loop protruding into the cisternae of the ER with cleavage occuring on the lumenal side and the charged amino terminus remaining on the cytoplasmic face of the membrane.

The membrane trigger hypothesis is another example of a non-receptor mediated model which has been used to account for the translocation of secretory polypeptides. According to this model, the signal sequence facilitates proper folding as the protein encounters the bilayer and translocation is dictated by the thermodynamics of interaction between the folded substrate and the fatty acyl residues of the bilayer (Wickner, 1979).

The appealing aspects of these schemes, as described by their proponents, is the plausibility of a simple physio chemical model, which obviates the need for postulating the existence of additional protein catalysts. As described below, both cytosolic and ER membrane proteins which are required for translocation have since been isolated and characterized, so that certain tenets of a 'spontaneous insertion' model have been, and need to be revised. However, these models provide a valuable conceptual framework which presents an alternative perspective for assessing experimental findings that has often leading to more incisive experimental analysis.

The second model which attempts to account for how nascent proteins are targeted to and transported across the ER is described by the signal hypothesis. The central tenet of this proposal is that a set of specialized protein receptors direct intracellular sorting through their interactions with substrates, such as the signal sequence of a nascent secretory protein. In general terms, the receptor mediated view of translocation, initially elucidated to describe secretory protein translocation (Blobel and Dobberstein, 1975 a and b), and subsequently updated to include a description of transmembrane

protein biogenesis (Blobel, 1977; see iv b), is as follows (see Figure 1-1b): Newly synthesized secretory proteins contain specific trafficking sequences (e.g. signal sequences) that encode information which is recognized by specific receptors in the cytoplasm and the membrane of the ER; signal-receptor interactions promote both targeting to the surface of the ER and translocation across that bilayer; translocation across the membrane is facilitated by integral membrane proteins of the ER that form a channel or pore through which the nascent peptide passes; in most cases the signal peptide is cleaved from the nascent secretory protein and upon chain completion and detachment of the ribosomal subunits (which then join the recirculating free pool), the channel in the membrane is dissolved by disaggregation of component proteins in the plane of the bilayer.

In the past decade principles of the signal hypothesis have been directly tested, and a large body of experimental data has accumulated in its support. Biochemical fractionation of cell-free reconstitution systems has allowed the purification, characterization and reconstitution of many of the proposed components involved in translocation events. By employing purified components in reconstitution assays it has been demonstrated that at least some of the steps in translocation involve specific interactions between discrete ligands and their receptors. The experimental data supporting a receptor-mediated view of translocation is presented below. In addition some of the tenets of the spontaneous view of insertion are considered, particularly as related to the degeneracy of topogenic sequence information.

iii. Developing a Basic Model for Translocation

The application of in vitro translation systems to the study of secretory protein biogenesis resulted in the discovery of signal sequences, and suggested that a more detailed molecular analysis of targeting and translocation mechanisms was possible.

Studies employing in vitro reconstitution, recombinant DNA technology, and genetic manipulation in bacteria, have allowed not only a characterization of some of the receptors involved in targeting and translocation, but also the isolation and detailed analysis of the properties of the substrates which interact with these specialized receptors.

a. Characterization of Amino Terminal Signal Sequences

Since nearly all secreted and transmembrane eukaryotic proteins were found to be synthesized with an extra amino terminal segment whereas cytoplasmic proteins were not, it was appealing to search for consensus information among signal sequences. In an attempt to elucidate a structural-functional relationship, a large number of precursors of both eukaryotic (Blobel and Dobberstein, 1975 a & b; Schecter et al., 1975) and bacterial secretory proteins (Inouye and Beckwith, 1977; Randall et al., 1978; Sarvas et al., 1978) were subjected to sequence analysis. Comparison of these sequences revealed extreme variability, in both primary amino acid sequence and length (Watson, 1984; von Heijne, 1985).

In general, signal sequences range from 15-30 amino acids in length, and contain three structurally distinct regions: (1) a positively charged amino terminal region, consisting of 1-2 basic amino acids; (2) a central hydrophobic core of variable length made up of at least six amino acids, and; (3) a polar carboxy terminal region which defines the cleavage site. Amino acids with small neutral side chains such as Gly, Val or Ala are preferentially found at position -1 and -3 of the signal sequence. (The number refers to the position of the amino acid relative to the cleavage site.) (Watson, 1984; von Heijne, 1984 &1985; Briggs and Gierasch, 1986). Studies in a variety of systems both in vitro and in vivo demonstrate conservation of signal sequence function over a wide evolutionary distance (Muller et al., 1982). Bacterial and eukaryotic signal sequences are virtually indistinguishable and a secretory protein from either system

was found to be capable of directing translocation across the membrane of the other (Talmadge et al., 1980 a,b; Muller et al., 1982).

Bacterial genetic studies (which allow precise deletions or mutations in specific amino acids to be made) tested the importance of the conserved features of signal sequences. The requirement for the amino terminal charged domain was tested genetically by replacing the basic amino acids of the bacterial outer membrane lipoprotein signal peptide with acidic residues (Inouye et al.,1982; Vlasuk et al., 1983). The finding that secretion occured, but in a delayed fashion, suggested that the positively charged domain is not essential for translocation but rather may exert a regulatory role in secretion.

The absolute requirement for a central hydrophobic domain for signal sequence function was highlighted by the finding that disruption of this domain, by the introduction of charged residues or small deletions, abolished export in bacteria (Emr and Silvahy, 1982). Furthermore, specific alterations which changed the conformation of the hydrophobic core were shown to significantly disrupt signal seqeunce function (for reviews, see Silvahy et al., 1983 and Briggs and Giersasch, 1986). Fine structural analysis of the signal peptidase cleavage site indicated that the only requirement for cleavage is the appearance of a small neutral amino acid side chain at the first and third positions before the cleavage site. Substitution of a Val for an Ala at position -1 of the yeast invertase signal peptide resulted in lack of cleavage at the correct site (Schauer et al., 1985). One unifying characteristic of all signal sequences therefore appeared to be extreme hydrophobicity in the central core region.

Interestingly, while most signal sequences occur at the extreme amino terminus of a protein and are cleaved during translocation with efficiency and specificity, proteolytic removal of the signal sequence is not an absolute requirement for translocation. Both

secretory and transmembrane proteins have been described which contain functional, internal uncleaved signal sequences (see iv e 1,2). The precise requirements for signal sequence function therefore, are not well understoood. Neither a positively charged domain nor signal cleavage are absolute requirements for signal sequence function. Taken together with the lack of any clear primary amino acid sequence homology, these findings have led to the suggestion that recognition and function of signal sequences is determined by secondary structural features.

Based on the general pattern of degeneracy observed among signal sequences and evidence from biophysical studies with synthetic signal peptides (reviewed in Briggs and Gierasch, 1986), some investigators have suggested that signal sequences act as amphiphiles that are integrated into and possibly perturb the lipid bilayer. According to von Heijne, signal sequences are 'too variable' in length and amino acid sequence to allow for specific protein-protein interactions, so it is more likely that targeting and translocation occur by unspecific binding of the N-terminal region to the surface of the ER membrane and interaction of the hydrophobic region directly with the bilayer (von Heijne, 1979, 1985). No experimental evidence exists however, that would suggest that the general mechanism for translocation across the ER is by direct interaction of the signal sequence with the hydrophobic core of the ER membrane. On the other hand, several lines of evidence do suggest that signal sequences interact directly with proteins.

c. Proteins Involved in Translocation

The signal hypothesis states that translocation events are mediated by direct interactions between nascent secretory proteins and receptors, postulating the existence of both cytoplasmic and membrane-localized receptors. Several proteins have been isolated and purified from eukaryotic cell extracts, and their activity has been characterized in reconstitution experiments. Two components, signal recognition

particle (SRP) and signal recognition particle receptor (SRP R) have been definitively shown to be required for translocation across the ER. In vitro studies with fusion proteins have also been exploited to assess the physical nature of the environment across which the nascent protein is transferred. Finally, additional components of the cellular secretion machinery have been identified, although their precise role in translocation is still being investigated.

1. Identification of SRP and SRP R: Developing a Working Model for Translocation

The model in figure 2 summarizes the key events mediated by SRP and its receptor, as determined by detailed in vitro reconstitution studies. A brief overview of the experiments which led to the identification and characterization of these two components follows.

Signal Recognition Particle (SRP)

The first demonstration that a protein component of the ER membrane is required for translocation was made when a salt-wash extract from microsomal membranes was shown to restore translocation to a wheat germ (WG) cell-free translation system in which translocation had previously been abolished by high salt extraction of the microsomal membranes (Warren and Dobberstein, 1978). Purification of the active component of the high salt extract resulted in the isolation of SRP, an 11 S ribonucleoprotein complex, consisting of a 7S, 300 nucleotide RNA molecule (Walter and Blobel, 1982), and 6 nonidentical polypeptide chains, including a 19 kDa and 54 kDa monomer, as well as two heterodimers, one composed of a 9 kDa and 14 kDa polypeptide, and the other comprised of a 68 kDa and 72 kDa polypeptide, which are associated with the 7 S RNA (Siegel and Walter, 1988 a & b). Neither the protein nor RNA fractions were active alone, but reconstitution of translocation activity was

possible with both protein and RNA constituents (Walter and Blobel, 1983a).

Functional studies suggest that SRP acts as an 'adapter' between the cytoplasmic protein synthesizing apparatus engaged in the translation of secretory proteins, and the protein translocation machinery in the ER (Meyer and Dobberstein, 1980 a & b; Walter and Blobel, 1981 a & b). The role of SRP in the selective recognition of secretory proteins was demonstrated in a reconstitution system, in which purified SRP was found to bind to polysomes synthesizing secretory, but not cytoplasmic proteins (Walter et al., 1981a). Emergence of the signal sequence from polysomes synthesizing secretory proteins resulted in a 4-5 fold increase in the affinity of SRP for the ribosome nascent chain complex (Walter and Blobel, 1981b). The SRP-ribosome nascent chain complex is then targeted to the surface of the ER (Walter and Blobel, 1981b). A roughly equal distribution of SRP between the cytoplasm and membrane fraction was demonstrated by subcellular fractionation (Walter and Blobel, 1983b).

Direct interaction between a signal sequence and SRP was suggested by the finding that incorporation of the leucine analog, hydroxyleucine into leucine-rich signal sequences blocked signal recognition by SRP (Walter and Blobel, 1981b) . More recently, a direct interaction between the 54 kDa component of SRP and the signal sequence has been demonstrated by experiments in which incorporation of a photoactivatable amino acid into a nascent secretory protein resulted in cross-linking of the signal sequence to the 54 kDA subunit of SRP (Krieg et al., 1986 ; Kurzchalia et al., 1986).

In addition to targeting secretory proteins to the surface of the ER, another function of SRP has been described. When translation was carried out in vitro, in the absence of microsomal membranes, but in the presence of SRP, a selective block of synthesis was observed for polysomes engaged in the synthesis of secretory but not cytoplasmic

proteins (Walter and Blobel, 1981b). This block, termed elongation arrest, correlated with SRP binding as demonstrated by both cross-linking and hydroxyleucine incorporation (as described above). Elongation arrest was released upon SRP-mediated binding of the arrested nascent chain to the ER, thereby allowing translocation of the protein into the lumen of the vesicle (Walter and Blobel, 1981b).

The exact mechanism and the physiological significance of the elongation arrest function of SRP remains controversial although its activity has been extensively characterized in vitro. In the WG cell-free protein synthesizing system, translation arrest produced either a discretely-sized protein fragment or a broad smear of fragments (as visualized by separation on an SDS gel), indicating that SRP recognizes signal sequences, and arrest synthesis within a range of chain lengths (Walter and Blobel, 1981b; Meyer et al., 1982a). Variability in the extent of arrest has been observed with different polypeptides (some show only a transient pause in synthesis), as well as in different in vitro translation systems (Meyer, 1985). In the rabbit reticulocyte lysate (RRL) cell-free system, a less tight arrest of synthesis, or kinetic delay occurs with addition of pancreatic canine SRP, as opposed to the more permanent arrest seen in the WG system (Walter and Lingappa, 1986). It has been suggested that these variations may reflect the proposed in vivo role of SRP, as a rate controlling factor of nascent secretory protein synthesis which serves to maintain nascent chains in a translocation competent state.

Disassembly and reconstitution of SRP has provided further insight into its function (Walter and Blobel, 1983). The 9/14 kDa proteins and the 7S RNA component were shown to be responsible for the elongation arrest activity. Perturbed SRP's, prepared either by nucleolytic dissection (Siegel and Walter, 1986), or by reconstitution from fractionated and purified protein and RNA components (Siegel and Walter, 1985) generated molecules lacking elongation arrest activity but retaining signal recognition,

targeting and translocation function. Interestingly, the elongation arrest-defective SRP molecules were functional only transiently during nascent chain growth. This finding supports the proposed role for SRP in maintaining nascent chains in a translocation competent state by delaying further elongation in the cytoplasmic space.

Alkylation of the 54 kDa subunit of SRP destroyed the high affinity binding activity of SRP to signal sequences (Siegel and Walter, 1988a). Taken together with the cross-linking data, the signal recognition function of SRP has been assigned to this subunit. The membrane targeting region of SRP was localized to the 68/72 kDA domain by similar alkylation inactivation experiments (Siegel and Walter, 1988a).

The protein domains responsible for signal recognition and elongation arrest were localized to opposite ends of SRP by footprint analysis of the SRP proteins on its RNA (Siegel and Walter, 1988b). Because the secondary structure of the 7 S RNA resembles that of a tRNA that is missing the anticodon stem it has been suggested that elongation arrest could involve binding of the 7 S RNA to the A site of the ribosome. Thus the block in translation might be caused by the RNA component of SRP preventing the next amino acyl tRNA from binding. The dimensions of the SRP molecule (an elongated rod about 24 nm in length and 5-6 nm in diameter [Andrews et al., 1985, 1987]), are consistent with a model in which the 54 kDa subunit directly binds the signal sequence at the site where the signal sequence emerges from the large ribosomal subunit, while the 9/14 kDa proteins and the 7 S RNA bind at the elongation site betweeen the two ribosomal subunits (Andrews et al, 1985; 1987).

Signal Recognition Particle Receptor (SRP R)

The existence of a protein which serves as a specific membrane receptor for SRP was suggested by the finding that a salt-washed extract from microsomal membranes functioned to release SRP arrest, thereby allowing completion of translation and

sequestration of the nascent secretory protein into the lumen of the ER (Walter and Blobel, 1981b). Identification and purification of SRP R (or docking protein) resulted from two distinct experimental approaches: (1) reconstitution of translocation across proteolyzed microsomal membranes was accomplished by addition of a 52 kDa proteolytic fragment, which was subsequently determined to be derived from a 69 kDa integral membrane protein of the ER (Meyer et al., 1982 a & b); and, (2) the SRP-induced translation arrest was released by addition of a purified component of a detergent extract of microsomal membranes, which was isolated by affinity chromatography on an SRP-Sepharose column (Gilmore et al, 1982b). Peptide-mapping and immunological analysis demonstrated that the affinity purified fraction was identical to the 69 kDa integral membrane protein identified by proteolytic dissection (Gilmore et al., 1982a,b). More recent data has identified a second subunit of the SRP receptor, a 30 kDa ER membrane protein which is tightly associated with the 69 kDa polypeptide (Tajima et al., 1986).

In addition to the role of SRP R in releasing translation arrest it functions independently as an ER specific targeting site for polysomes complexes engaged in the synthesis of secretory proteins. Nascent secretory-polysomes which employed perturbed SRP molecules deficient in elongation arrest were demonstrated to still require SRP R for translocation (Siegel and Walter, 1985). Analysis of the primary amino acid sequence of the SRP R from its cognate cloned DNA shows three unsually hydrophilic domains in the 69 kDA subunit which are rich in positively charged amino acids and which resemble nucleic acid binding proteins (Lauffer et al., 1985). This has led to speculation that interaction with SRP might occur directly via its 7 S RNA.

The SRP/SRP R Cycle

Based on the data presented and the estimated in vivo concentrations of SRP and its

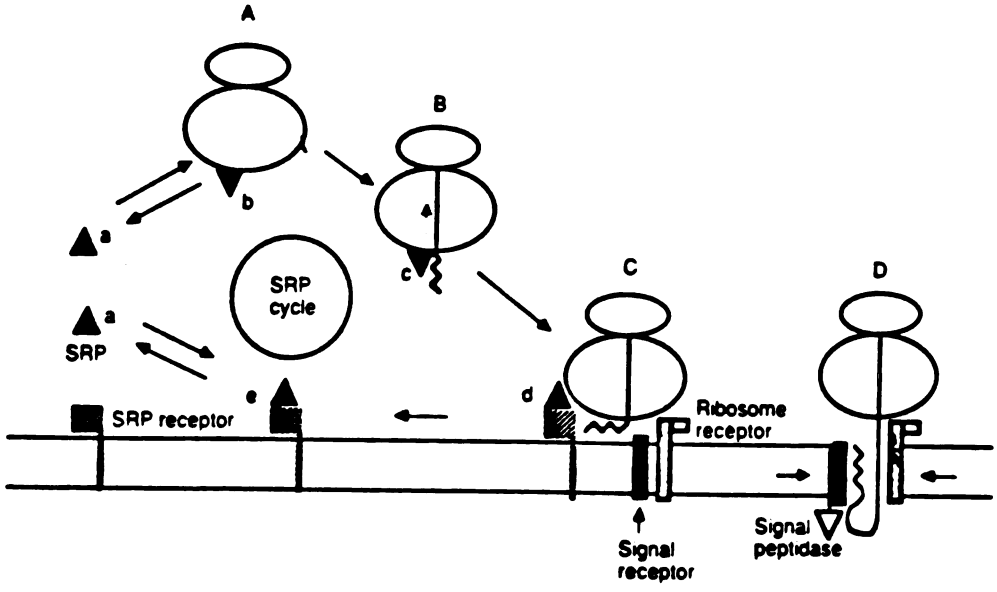
receptor a working model describing the role of these two components in translocation has been proposed (see Fig. 1-2). SRP exists in equilibrium between a soluble and membrane bound form. The emergence of a signal sequence from polysomes results in SRP binding to the signal sequence and elongation arrest. Substoichiometric levels of SRP and SRP R in pancreatic cell extracts relative to membrane bound ribosomes (Gilmore et al., 1982b; Walter and Blobel, 1980), suggests a catalytic role for these components. Thus, following specific targeting of nascent secretory polysome complexes to the ER membrane via SRP's interaction with the integral membrane protein SRP R, elongation arrest is released and SRP loses its high affinity to the signal sequence-bearing polysome complex. The ribosome does not bind SRP R (Gilmore and Blobel, 1983) so that SRP and SRP R are free to recycle once targeting has occurred. Additional protein components in the ER membrane, distinct from SRP and its receptor are proposed to interact with the targeted polysome complex and facilitate subsequent translocation (see below).

2. Evidence for a Protein Channel

In addition to describing receptor mediated targeting of proteins destined for translocation to the surface of the ER, the signal hypothesis proposes that the nascent chain crosses the lipid bilayer via an aqueous pore or channel formed by integral membrane proteins (Walter et al., 1984). The following experiments lend support for this idea.

In order to address the nature of signal sequence interaction with the bilayer the fate of an internally engineered signal sequence was assessed in a hybrid fusion protein encoding the cytoplasmic passenger alpha-globin attached directly to the amino terminus of preprolactin, a secretory protein with an amino terminal signal sequence (Perara and Lingappa, 1985). In vitro expression in a cell-free reconstitution system resulted in

Figure 1-2. **Model of signal recognition particle (SRP) cycle for targeting nascent secretory and transmembrane proteins to the ER membrane.** Soluble SRP (a) exists in equilibrium with a membrane-bound form, presumably bound to SRP receptor (e), and a ribosome-bound form (b). On translation of mRNA encoding a signal sequence for targeting to the ER membrane (zigzag lines), the affinity of SRP for the translating ribosome is enhanced (represented by dashed arrow B), and SRP binds to the signal sequence directly (c), effecting elongation arrest (B-C). On interaction with ER membranes, elongation arrest is released and SRP and SRP receptor are free to be recyicled (SRP cycle, a-e), the synthesizing ribosome interacts with other transmembrane proteins, leading to formation of a functional ribosome-membrane junction, translation resumes, and translocation across the membrane occurs (D). (Taken from *Perara and Lingappa, 1988*).


A
B
C
D
a
b
c
d
e
SRP
SRP cycle
SRP receptor
Ribosome receptor
Signal receptor
Signal peptidase

translocation of both globin and prolactin domains and signal cleavage, leaving the amino terminal signal sequence attached to the carboxy terminus of alpha-globin. The finding that both domains were carbonate extractable (see Chapter 2, Fig. 2-5 for an explanation of this procedure) indicated that they were localized in the lumen of the vesicle either free, or bound to lumenal proteins. These results suggest that signal sequences do not bind directly to the bilayer, but rather interact with protein machinery in the membrane (Perara and Lingappa, 1985).

A more direct demonstration that nascent proteins are transported through a proteinaceous channel in the bilayer was provided by experiments in which the accessibility of partially translocated nascent secretory peptides to aqueous solutes was tested. Using a WG cell-free translation system programmed with prolactin mRNA and supplemented with microsomal membranes, a secretory protein which spanned the membrane (with the amino terminus in the lumen and the carboxy terminal domain in the cytoplasmic space) was generated, by an oligonucleotide-mediated hybrid arrest of translation (Gilmore and Blobel, 1985). The extractibility of this substrate from membranes with protein denaturants such as urea, lends credence to a translocation mechanism involving interaction of the nascent chain with integral membrane proteins of the ER.

Definitive proof that translocation across the ER occurs via a protein channel however, requires identification, purification and reconstitution of those components. It is possible that the translocation site may be composed of both protein and lipid constituents, such that the nascent polypeptide is in contact with both, either throughout, or at different stages of the translocation process (Weidmann et al., 1987b; Walter et al., 1984).

3. Potential Components of a Membrane Associated Protein Complex

Ribosome Receptor

Several proteins have been implicated as components of a complex that might form the proposed proteinaceous site for transport of nascent polypepetides across the membrane. Because substochiometric amounts of SRP and its receptor, relative to membrane bound ribosomes are found, a specific ribosome binding protein has been suggested to be responsible for the functional ribosome-membrane junction. Support for the existence of such a ribosome receptor is provided by several findings: ribosome binding to the ER is required for translocation (Gilmore and Blobel, 1985); GTP is required for the formation of a functional ribosome membrane junction (Connolly and Gilmore, 1986); and, ribosome binding to the ER is saturable and sensitive to protease (Hortsch et al., 1986).

The ribophorins are two integral membrane proteins of the ER which have been suggested to play the role of ribosome receptors for polysomes engaged in the synthesis of secretory proteins (Kreibich et al., 1978a). A direct association of ribosomes and ribophorins was demonstrated by chemical cross-linking of rough microsomal membranes (Kreibich et al., 1978b). Further support for the involvement of the ribophorins in binding of secretory polysome complexes to the ER was suggested by the finding that the molar ratios of each of the ribophorins to bound ribosomes is approximately one (Marcantonio et al., 1984). However, the demonstration that the ribophorins also bind polysomes synthesizing cytoplasmic proteins (Amar-Costesec et al., 1984), and that some secretory proteins can be translocated across smooth microsomes which lack ribophorins (Bielinska et al., 1979), suggests that the ribosome binding proteins involved in mediating secretory protein translocation remain to be identified. That protein components of the ER other than the ribophorins are required for translocation is further supported by the finding that rough microsomes

lose ribosome binding activity following protease treatment, to which the ribophorins are resistant (Hortsch et al., 1986). It remains to be determined what role, if any, the ribophorins have in translocation.

Signal Peptidase

Cleavage of nascent secretory proteins occurs on the lumenal side of microsomal membranes by the enzyme signal peptidase (Jackson and Blobel, 1977). This enzyme has recently been purified from canine pancreatic rough microsomes as a complex of 4-6 polypeptides which exists in stoichiometric amounts relative to membrane-bound ribosomes (Evans et al., 1986). It is not known whether all or only one of these polypeptides serves the signal cleavage function, although the bacterial counter-part leader peptidase I, purifies as a single polypeptide (Wolfe et al., 1982 and 1983). This raises the possibility that one or more components of the *eukaryotic* complex may serve some function other than that of signal cleavage. Studies in progress are characterizing these subunits for possible translocation-related functions. One possibility is that these proteins may be components of the channel, or 'translocon' proposed by the signal hypothesis.

Signal Sequence Receptor (SSR)

Following displacement of the nascent secretory protein from SRP and its receptor, the signal sequence interacts directly with another protein in the ER membrane, the signal sequence receptor (SSR). This was recently demonstrated by analog cross-linking experiments, which previously were used to show that signal sequences interact directly with SRP (Krieg et al., 1986; Kurzchalia et al., 1986). By monitoring the fate of the signal sequence of a nascent chain after its release from SRP, binding to a 35 kDa integral membrane protein of the ER was detected (Weidmann et al., 1987 a,b). Precedent for the existence of such a receptor was suggested by two earlier observations: (1) a signal sequence, either as part of a nascent protein (Prehn et al., 1980) or as a

synthetic peptide (Robinson et al., 1987; Majzoub et al., 1980) acted as a competitive inhibitor of translocation when preincubated with microsomal membranes; (2) a protein-protein interaction between short nascent chains that were targeted but not yet translocated or cleaved by signal peptidase, was sustained following release of ribosomes from the ER membrane by puromycin (Gilmore and Blobel, 1983).

In addition to its proposed role in providing the specificity and fidelity required for targeting of nascent secretory-polysome complexes to the correct intracellular membrane, SSR is an obvious candidate as a component of the translocation machinery. Its may mediate interaction of the signal sequence with the lipid, induce or open a channel through which the nascent protein crosses the membrane, or serve as a component of the channel itself (Weidmann et al., 1987b; Walter, 1987).

c. Maintenance of Specificity in a Degenerate Signaling System

Studies with fusion proteins provided definitive evidence that a signal sequence may be sufficient for directing translocation, since a signal sequence for beta-lactamase was found to direct translocation of normally cytoplasmic globin when fused to the amino terminus of this protein, both in vitro and in vivo (Lingappa et al.,1984; Simon et al., 1987). More recent experiments however, demonstrate that the passenger domains downstream of the signal sequence can exert profound influences on the function of a topogenic element. Several examples have been reported in which amino terminal signal sequences did not direct the translocation of a passenger domain in a simple fusion protein (Moreno et al., 1980; Kadanoga et al., 1984). These findings suggested that although signal sequences may be required for translocation, some sequences or conformations may exist which inhibit translocation in a non-specific way. One possible explanation for this is that the observed inhibitory effects on translocation may be a result of alterations in signal-receptor interactions.

The interaction of a signal sequence with one or more of its receptors (see section iiib) has been shown to be influenced by the context in which it is found (Andrews et al., 1988). Progressive deletions in the mature domain of mature prolactin resulted in a decrease in translocation efficiency, and were accompanied by significant changes in the interactions of the signal sequence with both cytoplasmic and membrane receptors. Thus, the interactions between substrates and receptors, which provides selectivity in translocation across the ER, are likely to be mediated by positive features, which are responsible for signal recognition, and negative influences, which might mask or interfere with the interaction between the signal sequence and its receptor(s) (Andrews et al., 1988). Further support for this idea is provided by the results of Randall and Hardy (1986), which demonstrate that a nascent protein must be in a particular conformation to allow translocation.

It is probable that the signal sequences of secretory proteins have evolved together with their passengers to achieve optimum interaction with receptors for translocation. The observed selectivity and fidelity of translocation events is therefore most likely a consequence of the sum of both the positive and negative influences discussed above, and is effective enough to ensure that only those proteins intended to be exported are translocated across the ER (i.e. the cytoplasm is not secreted). Thus, the recent finding that approximately 20% of protein fusions generated by replacing wild-type signal sequences with random peptide fragments, were translocated (Kaiser et al., 1987), is not surprising, based on the known pattern of degeneracy of signal sequences, and the determinants of translocation function described above. For example, if bona fide signal sequences (either amino terminal or internal) must be on the surface of a protein in order to be functional, latent signal sequences (such as those unmasked in this study) might normally be forced into the interior of a protein domain and therefore never be

expressed (Andrews et al., 1988). The information encoded by 'degenerate' signal sequences along with a requirement for proper presentation, could together provide enough specificity for translocation receptors to discriminate between cytoplasmic proteins and those destined for translocation across the ER. Relevant to this discussion is the fact that the fusion proteins examined in the study by Kaiser et al., were translocated with very low efficiency compared to wild-type secretory proteins.

IV. Biogenesis of Transmembrane Proteins (TMPs)

a. Evidence for a Common Translocation Mechanism

Early theories of membrane biogenesis, correctly predicting that the plasma membrane is a derivative of the ER, which has been processed through the Golgi apparatus (Whaley et al., 1972), suggested that transmembrane proteins might be considered a special class of secretory proteins. According to this model, since TMPs must overcome the same structural barrier as that encountered by secretory proteins (i.e. the bilayer of the ER), they might share common steps in their biogenesis.

The classical biochemical and morphological studies dedicated to defining the steps involved in protein export recognized that two subclasses of ER membrane-attached polysomes exist: one synthesizes proteins for export and the other is involved in translation of transmembrane proteins (TMPs) (Dallner et al., 1966). Employing the techniques of cell fractionation, in vitro expression and gene fusion, these studies resulted in definitive demonstration that membrane and secretory proteins employ a shared set of substrates and receptors for initial steps in intracellular sorting (i.e. targeting and initiation of translocation across the ER).

In cells infected with vesicular stomatitis virus (VSV), the membrane protein VSV G was found to be synthesized exclusively on polysomes bound to the ER membrane (Morrison and Lodish, 1975). Further analysis in vitro showed that glycosylation

(indicative of translocation) occurred only if microsomal membranes were present co-translationally. This observation suggested that as with secretory protein biogenesis, translocation of membrane proteins occurs by vectorial discharge into the ER lumen (Katz et al., 1977; Toneguzzo and Ghosh, 1977). The orientation of VSV G that was achieved in microsomal membranes was shown to be identical to that seen on the surface of the plasma membrane (Lodish et al., 1981).

Amino amino acid sequence analysis of the amino terminus of the VSV G protein expressed in a cell-free system showed that this TMP is synthesized with a transient amino terminal signal sequence which is cleaved during translocation across the ER (Lingappa et al., 1978). This finding supported the idea that the initial step in sorting TMPs occurs via the same mechanism described by the signal hypothesis for secretory protein sorting. A direct demonstration that common ligands and receptors operate at the level of the ER was provided by the finding that polysomes engaged in the synthesis of VSV G, but not those synthesizing the cytoplasmic protein globin, competed with polysomes synthesizing a secretory polypeptide for membrane-associated components of the ER required for translocation (Lingappa et al., 1978). More recently it has been shown that the shared cellular machinery includes SRP and SRP R (Walter and Blobel, 1981 a & b; Meyer et al., 1982 a & b; Gilmore et al., 1982 a & b), as well as a requirement for nucleoside triphosphate hydrolysis (Mueckler and Lodish, 1986b).

b. Models for Membrane Protein Biogenesis

If initial targeting and translocation events are identical for secretory and membrane proteins, what causes transmembrane proteins to be retained in the membrane? The demonstration that all copies of a given TMP achieve the same transmembrane disposition indicates that the mechanism by which TMPs are oriented asymmetrically into the ER operates with high fidelity (Katz and Lodish, 1979). Two general theories have been presented to account for how cells faithfully direct TMPs into their

characteristic transmembrane topologies. As described for secretory protein biogenesis, both receptor mediated (protein-protein) and spontaneous (protein-lipid) processes have been proposed.

Thermodynamic principles have been employed to attempt to account for the interruption of transfer of TMPs across the bilayer of the ER. According to one model, following insertion of a helical hairpin into the bilayer, the polar domain of the second helix is transferred across the membrane until a nonpolar structure is encountered. At that point export ceases and the remainder of the carboxy terminus is left in the cytoplasm (Engelman and Steitz, 1981). Thus, the thermodynamic stability of a hydrophobic domain in the bilayer is proposed to be responsible for interrupting translocation, as well as causing integration of the protein into the membrane. Applications of these principles are proposed to be sufficient to account for all categories of transmembrane proteins. Several variations of this model have been described which also emphasize a direct interaction between the hydrophobic core of the bilayer and the hydrophobic domains of membrane proteins, in the absence of any peptide transport system. The direct transfer model (von Heijne and Blomberg, 1979) focuses on physio-chemical principles and the energetics of transfer, while the membrane trigger hypothesis (Wickner, 1979), proposes that the proper assembly of TMPs is determined by folding of the nascent polypeptide into a conformation that spans the membrane, triggered by interactions of the lipid bilayer with the leader peptide and additional domains in the nascent protein.

The signal hypothesis extends the receptor mediated model of translocation to account for the topology of TMPs. As described for secretory protein sorting, information for addressing each TMP is proposed to be contained in discrete segments of the polypeptide chain that act independently, interacting with receptor proteins to

initiate (or terminate) translocation. Thus, subsequent to initiation of translocation (see II: ii), emergence of a unique topogenic element, termed a 'stop transfer sequence', would result in interaction with a protein receptor which would abrogate transfer, perhaps by the disengagement of the translocation machinery previously engaged by the signal sequence. According to this view, following the receptor-mediated (protein-protein) termination of translocation, integration of the chain into the bilayer would occur, leading to stabilization of the transmembrane disposed chain. This second event is proposed to be achieved through hydrophobic interactions of the non-polar side chains of the membrane spanning domain with the tails of membrane lipids. Continued synthesis would then proceed, with the ribosome now converted into the functional equivalent of a free ribosome, remaining tethered to the membrane by the nascent chain.

It has been suggested that the actual mechanism employed for the biogenesis of transmembrane proteins likely involves aspects of both these models (for reviews see Wickner and Lodish, 1985; Perara and Lingappa, 1988). Principles of each hypothesis therefore are valuable for guiding experimentation and interpretation of results. Furthermore, as will become evident, each model may be an accurate description of a subset of cases, such that a protein can stop in a membrane either through receptor interactions, or because of extreme hydrophobicity.

c. Classses of Transmembrane Proteins

Membrane proteins exist in a wide variety of orientations with respect to the membrane bilayer. Thus, the study of TMP biogenesis is much more complex than that of secretory protein biogenesis, where proteins assume only a single disposition with respect to the membrane (i.e. in the lumen). The simplest class of transmembrane proteins are called bitopic TMPs, and cross the bilayer only once. When categorized with respect to topology, two subclasses of bitopic membrane proteins are recognized.

Those with their amino terminus in the ER lumen or extracytoplasmic space are termed Group I TMPs (e.g.. VSV G [Katz et al., 1977] and immunoglobulin M {IgM} heavy chain [McCune et al., 1980]). Group II TMPs are in the opposite orientation, with their carboxy terminal domain in the extracytoplasmic space (e.g. asialoglycoprotein receptor {ASGPR} [Spiess and Lodish, 1985, 1986] and transferrin receptor {TR} [Schneider et al., 1984; McCelland et al., 1984; Zerial et al., 1986]).

Proteins which cross the bilayer more than once belong to the general class termed polytopic transmembrane proteins, and exist in diverse orientations with two or more membrane spanning domains. Polytopic TMPs are described according to the disposition of their amino and carboxy terminus relative to the bilayer; those with amino and carboxy terminus on the same side of the membrane are referred to as cis (e.g., erythrocyte band III [Kopito and Lodish, 1985; Wickner and Lodish, 1985] and hepatitis B surface antigen [Eble et al., 1986, 1987]), while those proteins which express amino and carboxy terminus on opposite sides of the membrane are trans (e.g., rhodopsin [Nathans and Hogness, 1983] and acetylcholine receptor {AcH R} [Young et al., 1985]). The side of the membrane may be specified by E (extracytoplasmic) or C (cytoplasmic).

The simplest bitopic transmembrane proteins like VSV G (Group I), encode an amino terminal signal sequence, and were the focus of early studies on membrane protein biogenesis.

d. Stop Transfer Sequences

1. Identification

Many Group I TMPs were recognized to possess a hydrophobic region at or near their carboxy terminus. Preliminary analysis of transmembrane protein biogenesis assessed the functional properties of this region because sequence and structural studies demonstrated that these regions traverse the bilayer.

The existence of two forms of immunoglobulin M heavy chains, one secreted and the other membrane bound, provided a useful model for experimental analysis. The only difference between the secreted and membrane bound form was the presence in the membrane form, of a carboxy terminal hydrophobic segment (which spanned the bilayer) and a small cytoplasmic tail of 3 amino acids (Vasalli et al, 1979; Kehry et al., 1980; Singer and Williamson, 1980; McCune et al., 1980).

In order to test the function of the transmembrane sequence directly, a fusion protein was engineered in which the carboxy terminal membrane spanning domain of IgM heavy chain was inserted into the coding region of a protein which normally was completely translocated. When expressed in a cell-free translation system complemented with microsomal membranes, the product encoded by the fusion gave rise to a TMP of predicted topology, determined by the position of the transmembrane insert (Yost et al., 1983). This result demonstrated that a discrete domain, termed a stop transfer sequence, is sufficient to terminate translocation and direct integration of the product into the bilayer. Furthermore, the suggestion that the transmembrane domain had a passive function, capable of anchoring a protein only if presented at the extreme carboxy terminus (Lodish et al., 1981) was ruled out, because the stop transfer sequence was expressed in an internal position in the encoded fusion, such that temination of translocation left a large carboxy passenger domain in the cytoplasmic space. That the transmembrane domain of Group I TMPs is required for terminating translocation was further suggested by experiments in which deletion of the transmembrane encoding sequence resulted in the expressed product being completely translocated (Gething and Sambrook, 1982; Boeke and Modell, 1982; Rose and Bergmann, 1982).

2. Stop Transfer Sequences: Structure/Function Correlations

Although deletion and fusion studies clearly established that a cassette which includes

the transmembrane domain of Group I TMPs, is required for their anchoring in the lipid bilayer, the mechanism by which this region halted translocation remains unclear. Several studies have been undertaken to characterize the structural requirements for stop transfer function. While these efforts have resulted in the characterization of the minimal requirements for a membrane anchoring domain, they have not allowed a definitive determination of whether translocation termination involves the participation of protein receptors or is dictated solely by thermodynamic interactions between the nascent chain and the bilayer.

In general, membrane spanning domains consist of about 20-30 uncharged amino acids which are largely hydrophobic in character, followed by one or more positively charged residues which remain on the cytoplasmic side of the membrane (Sabatini et al., 1982). Based on these characteristics and the thickness of the bilayer it was suggested that the hydrophobic region spans the membrane as an alpha helix (20 amino acids are sufficient to form a helix which crosses the membrane) (Tanford, 1978), with the basic residues serving to stablize integration, via their interaction with the negatively charged phospholipid head groups at the cytoplasmic face of the membrane (Sabatini et al., 1982).

In order to determine the precise structural requirements for stop transfer function, defined deletions or mutations in specific regions of the transmembrane domain of a variety of Type I TMPs were made, and their effect on halt transfer activity was assessed. Deletion experiments suggested that an uninterruped stretch of 20 amino acids is not an inviolable structural requirement for stop transfer activity. When the transmembrane region of VSV G protein was truncated to 8 amino acids, termination of translocation still occurred (Adams and Rose, 1985). Fine structural analysis of another transmembrane domain (the 23 amino acid uncharged transmembrane segment

of bacteriophage f1 gene III protein, pIII) also demonstrated that a shortened hydrophobic stretch (in this case, a 12 amino acid core) was still functional (Davis, Boeke and Model, 1985). Finally, introduction of a charged residue into the middle of the uncharged hydrophobic 24 amino acid membrane spanning region of the surface protein of Semliki Forest Virus (SFV), did not disrupt the ability of that transmembrane domain to confer a transmembrane topology to the protein, again suggesting that hydrophobic regions shorter than 20 amino acids are sufficient to terminate translocation (Cutler and Garoff, 1986).

Although the altered transmembrane regions were demonstrated to terminate translocation, biochemical characterization of the mutant TMPs revealed that their stability in the membrane was weakened, relative to that of wild-type TMPs. A gradual diminution in membrane stability, as assessed by alkali extractability, was observed in the pIII mutants with transmembrane regions shorter than 17 amino acids (Davis, Boeke and Model, 1985). The thermodynamic stability of the SFV mutant (in which a charged residue was inserted into the hydrophobic stretch) in the membrane was also found to be significantly reduced (Cutler et al., 1986). These findings suggested that although shortened transmembrane regions can halt translocation, a minimum hydrophobic core may be required for stable anchoring.

To directly assess whether the only structural requirement for halting translocation and establishing a stable protein-membrane association is a region of sufficient hydrophobicity, artificial hydrophobic segments of various lengths were tested for stop transfer activity. Gene fusion studies in *E. coli* were carried out in which oligonucleotide coding repeats of hydrophobic amino acids were inserted into genes encoding a secretory protein. Hydrophobic inserts of sixteen or more residues were found to cause conversion from the secreted form to a membrane anchored form of

predictable topology, thereby defining a minimum threshold of hydrophobicity (in bacteria) required for the termination of translocation (Davis and Model, 1985). Although these experiments might suggest that any sufficiently hydrophobic region can act as a stop transfer sequence, several experimental findings contradict an argument for hydrophobicity as the sole determinant of stop transfer function.

Sequence analysis of fusion proteins generated either by deletion of a wild-type transmembrane sequence (Adams and Rose, 1985), or by insertion of an artificial hydrophobic insert (Davis and Model, 1985), reveal that neighboring domains can exert significant influence on the abilility of a sequence to terminate translocation. Thus, an inherent problem of testing a cassette for function in a fusion protein is that the context in which it is assayed may influence its activity. The existence of hydrophobic residues adjacent to the transmembrane region of VSV G may have been responsible for some of the stop transfer activity which was observed in the deletion mutants (Adams and Rose, 1985). Additionally, fusions in which an artificial hydrophobic insert was localized closer to the carboxy terminus of a secretory protein, were found to be much less stably integrated than those fusion in which the identical cassette was inserted in a more internal location (Davis and Model, 1985).

In several cases the topology of a protein based on an analysis of hydrophobicity resulted in an incorrect prediction of its transmembrane disposition, making it difficult to reconcile a model in which hydrophobicity alone dictates stop transfer activity. In one example, a region of 26 hydrophobic residues was found to be entirely translocated, both in its natural context and in a fusion construct (Davis and Hsu, 1986). These findings have led even the proponents of a thermodynamic model for stop transfer to concede that stop transfer recognition may also require receptor proteins in the ER (Wickner and Lodish, 1985). Furthermore, regions that are by hydrophobicity

analysis insufficient to terminate translocation, appear experimentally to span the membrane (Young et al., 1983; Devillers-Thierry et al., 1983; Noda et al., 1983; Hay et al., 1987 a,b). Thus, although a hydrophobic core is clearly essential for termination of chain translocation, considerable variation is tolerable, and more subtle features as yet undefined, may be critical for stop transfer activity.

In addition to examining the requirements for a hydrophobic region, structural studies have also assessed the importance of the carboxy terminal basic residues found in most TMPs, for stop transfer function. Studies in which specific deletions or mutations were made in the charged domain of either pIII (Davis, Boeke and Model, 1985) or a class I histocompatibility antigen (Zuniga and Hood, 1986) demonstrated that the basic residues are not absolutely required for transmembrane expression. Similarly, alteration of the two basic amino acids in the cytoplasmic region next to the hydrophobic stretch of SFV was found not to interfere with the protein achieving its normal transmembrane orientation (Cutler and Garoff, 1986). However, the finding that the stability of the membrane association was compromised led to the suggestion that the charge cluster might be important not as a stop transfer signal, but in stabilization of the TMP in the bilayer (Cutler and Garoff, 1986).

The structural-functional studies of stop transfer sequences do not allow a precise definition of the requirements for stop transfer activity. Instead, these findings demonstrate that the sequence information in a stop transfer sequence is highly degenerate, allowing only a definition of the minimal information required for function. In terms of addressing molecular mechanisms for arresting translocation, these findings are consistent with either model described above: a transmembrane region might halt translocation through direct interaction with the bilayer, or through interaction with a protein receptor (perhaps a component of the proposed ER channel) (Garoff,1985). In view of the lack of consensus information seen in a signal sequence, which has been

shown to interact with proteinaceous receptors, it seems reasonable to suggest that a similar situation may exist for receptor recognition of a stop transfer sequence, perhaps reflecting the unusual nature of topogenic sequence-receptor recognition rather than the lack of receptor involvement (Lingappa, 1989; Cutler and Garoff, 1986; Adams and Rose, 1985). Recent in vitro experiments have demonstrated that at least in certain contexts a stop transfer sequence interacts with receptors required for translocation (Mize et al., 1986, see V: c2)

e. Signal Sequence Subtypes

1. Internal Signal Sequences

Two types of topogenic sequences, an amino terminal cleaved signal sequence, and a stop transfer sequence, have so far been described. The observation that the secretory protein chicken ovalbumin, lacks an amino terminal cleaved signal sequence (Palmiter et al., 1978), led to the suggestion that the signal sequence for this polypeptide was located internally. The existence of a functional signal sequence in this molecule was supported by the finding that nascent ovalbumin competed with nascent prolactin for translocation across microsomal membranes in a cell-free translation system (Lingappa et al., 1979). Several groups have attempted to define the precise location of the ovalbumin internal signal sequence (Lingappa et al., 1979; Braell and Lodish, 1982; Meek, et al., 1982), but have provided conflicting results. Nevertheless, experimental evidence indicates that a unique topogenic element is encoded by ovalbumin; this internal uncleaved signal sequence directs its own translocation as well as that of both the amino and carboxy flanking domains.

2. Signal/Stop Transfer Sequences

Since Type II membrane proteins (those proteins which are oriented with their carboxy terminus in the extracytoplasmic space, and their amino terminus in the

cytoplasm) are synthesized without amino terminal cleaved signal sequences, it was suggested that the insertion of this class of TMPs might also be directed by an internal signal sequence (Blobel, 1980). By deleting internal segments from the Type II TMP influenza neuraminidase (Bos et al., 1984), and analyzing the topology of the resulting mutants in an SV40 expression system, the information responsible for directing both translocation and integration was localized to the transmembrane sequence. In vitro analysis of the disposition of fusion proteins with defined internal deletions have shown that the transmembrane domain of several other Type II TMPs (e.g. ASGP R [Spiess and Lodish, 1986], TR [Zerial et al., 1986] and the invariant chain [Lipp and Dobberstein, 1986]) also possess the necessary information for directing translocation and membrane insertion. Furthermore, when a transmembrane cassette taken from the ASGP R or TR was engineered between two passenger encoding domains, it directed translocation of the carboxy domain, and its own integration into the bilayer, leaving the amino terminus in the cytosol (Spiess and Lodish, 1985; Zerial et al., 1986). This sequence has therefore been termed a signal/stop transfer element. Similar to an amino terminal signal sequence, the signal/stop transfer sequence of ASGP R requires SRP and SRP R for translocation, which is obligately co-translational (Holland and Drickmar, 1986).

Sequence analysis of the signal/stop transfer element of ASGP R revealed a positively charged amino terminal domain, similar to that seen at the amino terminus of a cleaved signal sequence, or at the carboxy terminus of a stop transfer sequence. This region is followed by a long hydrophobic stretch, with no apparent site for signal cleavage recognition at the carboxy terminal end (Spiess and Handschin, 1987). Fine structural analysis, carried out by examining the in vitro translocation of mutants in which precise deletions have been made in the signal/stop transfer domain, suggested

that a positively charged domain immediately preceding the hydrophobic sequence is not an absolute requirement for function. Furthermore, truncated domains (as short as 12 amino acids) were found to still interact with SRP and direct the correct transmembrane topology. However, the stability of integration of mutants encoding hydrophobic domains shorter than 19 amino acids was severely compromised (Spiess and Handschin, 1987).

The TMP cytochrome P450 is oriented with its amino terminus anchored in the membrane and the carboxy terminus cytoplasmically disposed. In a fusion protein, the amino terminus of cytochrome P450 was demonstrated to function as both a translocation signal and a membrane anchor, similar to the transmembrane region of Type II TMPs (Sakaguchi et al., 1987). However, this element differed from the signal/stop transfer sequence described above in that it halted translocation of carboxy terminal domains (Sakaguchi et al., 1987; Monier et al, 1988). The behavior of the first transmembrane domain of the polytopic TMP opsin is similar to that of the uncleaved P450 signal/stop transfer element, in that it inserts into the membrane and leaves the carboxy terminal domain in the cytoplasm (Audigier et al., 1987).

As yet, it is not well understood what causes the differences in polarity of TMPs generated by the various classes of uncleaved signal sequences. It has been suggested that the final disposition of TMPs might be a result of the unique properties of the topogenic sequences themselves. One model to account for the disposition of Type II TMPs versus those like cytochrome P 450 is that the internal uncleaved signal sequence of Type II TMPs enters the membrane in a loop configuration and anchors to a signal binding site. This orientation results in the amino terminal domain remaining on the cytoplasmic side of the membrane, and allows for translocation of downstream sequences (Monier et al., 1987; Audigier et al., 1987). The reverse polarity of P450 or the

first transmembrane domain of opsin has been suggested to be accounted for by the flipping or reorienting of a signal/stop sequence in the membrane after first entering in the normal loop configuration (Monier et al., 1988; Audigier et al., 1987). Evidence supporting this model is indirect however, and other factors (e.g. passenger domain influences) might be important in determining the polarity of TMPs.

f. Insertion Sequences

An internal signal sequence which directs translocation in an SRP-independent fashion has also been described. The TMP cytochrome b5 is synthesized on free polysomes without an amino terminal cleaved signal sequence. Insertion into the ER takes place *post*translationally in an SRP-independent fashion. The mature product is found to be anchored to the membrane by apolar residues at the carboxy terminus (Anderson et al., 1983). This SRP-independent mechanism of insertion may be limited to translocation of a subclass of very small proteins (e.g. Watts et al., 1983). However, it has also been suggested that such insertion sequences might be involved in specifying the translocation of short loops of polytopic TMPs (Blobel, 1980). It is important to consider that these sequences, in addition to the other topogenic elements described above are all candidate substrates for the programming of the many different classes of polytopic TMPs observed in nature.

V. Polytopic Transmembrane Proteins

a. Theories of Polytopic TMP Biogenesis

The next level of transmembrane complexity is displayed by proteins termed polytopic TMPs, proteins which span the membrane more than once. The analysis of bitopic TMPs, which led to the identification and characterization of various topogenic sequences provided a conceptual framework for proposing models and devising experiments directed towards understanding polytopic TMP biogenesis.

As has been described for both secretory and transmembrane proteins, different theories regarding the mechanism by which polytopic TMPs achieve their final topology have been suggested. Proponents of a non-receptor mediated model of translocation argue that it is difficult to imagine a protein transport system which could catalyze the ordered weaving back and forth of complex TMPs (von Heijne and Blomberg, 1979).

One alternative mechanism that has been suggested is that the interaction of several hairpins with the ER membrane mediates the insertion of a polytopic TMP (Engelman and Steitz, 1981). According to another view, interaction of a nascent polytopic TMP with the membrane triggers conformational changes in the protein which result in exposure of hydrophobic residues to the fatty acyl core of the bilayer and determines the protein's final transmembrane disposition (Wickner, 1979).

The signal hypothesis on the other hand, proposes that multispanning membrane domains are 'stitched' into the bilayer by the sequential action of topogenic sequences (Lingappa et al., 1979; Bonatti et al., 1979). The information for the orientation of each membrane protein is thus suggested to be contained in discrete segments of the polypeptide that act independently to program topology via their interactions with receptors in the ER membrane (Blobel, 1980; Singer et al., 1987).

b. Predicting Membrane Spanning Regions by Sequence Analysis and Structural Information

The deduced primary amino acid sequence of polytopic TMPs has been widely used to predict their transmembrane topology (Nathans and Hogness, 1982; Devillers-Thierry, 1983; Noda et al., 1983). By assessing the hydrophobicity or membrane propensity of strings of amino acid sequences, the regions which span the membrane are predicted, allowing the formulation of models which describe the topology of multispanning transmembrane proteins. Several different scales have been used to predict transmembrane spanning domains by measuring thermodynamic properties like the

solubility of amino acid sides chains in organic solvents and water, or vapor pressures of side chain analogs (e.g. von Heijne, 1981 & 1984; Engelman and Steitz, 1981; Kyte and Doolittle, 1982; Eisenberg, 1984) .

A detailed structural analysis of one polytopic TMP demonstrated that predictions of topology based on amino acid sequence data alone may give an inaccurate prediction of topology (Michel et al, 1986). Based on the amino acid sequence of one subunit of the AchR it had been proposed that this TMP contains four transmembrane segments, because four regions each composed of approximately 20 uncharged and mostly hydrophobic amino acids, flanked by charged residues were detected (Noda et al., 1983; Devillers-Thierry et al., 1983). However, using antibodies directed against specific regions of the AchR subunit, immunoprecipitation and immunocytochemical analysis provided data which supported a model in which the homologous receptor subunits cross the membrane five times (Young et al., 1985). The putative fifth transmembrane domain which was overlooked by hydrophobicity analysis was amphipathic, and is predicted to form an alpha-helix with the charged or polar residues confined to one face of the helix and the hydrophobic residues on the other side. The polar faces of several adjacent sequences (making up by the homologous amphipathic helices of the five subunits of the Ach R) are predicted to from a polar pore or ion channel through the membrane (Young et al., 1985; for review see Wickner and Lodish, 1985). These findings have led investigators to question predictions of topology based on sequence analysis and to pursue alternative approaches to study polytopic TMP biogenesis.

c. Application of Recombinant DNA Technology for Studying Polytopic TMP Biogenesis

1. Identification of Transmembrane Domains in Native Polytopic TMPs

An indirect test of the model that polytopic TMPs are programmed by alternating

topogenic sequences has been carried out. The gene fusion approach was used to study the nature of the topogenic information encoded by bovine opsin, a polytopic TMP believed to contain seven transmembrane segments (Ovchinnikov, 1982; Nathans and Hogness, 1982). Construction of plasmid fusions encoding transmembrane domains plus a variable number of flanking residues (either by truncating a full length complimentary DNA clone of bovine opsin, or by making precise deletions in the cDNA and ligating various segments of interest), allowed the function of individual domains to be tested separately (Friedlander and Blobel, 1985). The translocation and stop transfer activity of each domain was studied by assessing their effect on the topology of passenger domains. Preliminary analysis demonstrated that at least two of the predicted transmembrane regions of bovine opsin can direct translocation in an SRP-dependent fashion. It was therefore concluded that this polytopic TMP contains at least two functional signal sequences.

More detailed analysis of bovine opsin (Audigier et al., 1987) showed that five of the six transmembrane domains which were analyzed can mediate SRP-dependent translocation, as determined by their ability to confer proteolytic protection and glycosylation to passenger domains in a cell-free translation system complemented with microsomal membranes. Furthermore, each of these transmembrane segments expressed varying degrees of integration function, as assessed by the alkali extractibility of the encoded products. These findings lend support for a model which describes polytopic TMP biogenesis to proceed via a cascade of interactions between multiple topogenic elements in the nascent chain and known receptors in the ER membrane. However, the observed topology of several of the fusion proteins was at variance with the proposed model. Testing topogenic sequences separately and out of context from one another therefore allowed only limited interpretation regarding the

role of individual elements in the biogenesis of a polytopic TMP. This deletion and fusion approach was nevertheless significant in that it permitted a preliminary localization and identification of multiple elements which encode topogenic information in a polytopic TMP.

In prokaryotic systems another approach has been used to study polytopic membrane protein topology (Manoil and Beckwith, 1986; Boyd et al., 1986). In these studies, successive deletions from the carboxy terminus of a polytopic TMP were replaced by a reporter domain, allowing changes in transmembrane disposition to be monitored. Positioning the reporter domain at different sites in the coding region resulted in localization of the reporter to different sides of the membrane, suggesting that topological information is decoded linearly as the chain is synthesized. However, whereas the reporter domain was well-defined in these studies, the precise number, order and nature of individual topogenic elements within the TMP and its deletion mutants was difficult to determine. Thus it was problematic to understand exactly how a particular topology was achieved and what the role of an individual topogenic sequence was in bringing about a transmembrane orientation (Boyd et al, 1986). Further limitations of this fusion approach include: the reporter domain itself may affect the transmembrane disposition of the fusion construct; the activity of the reporter might be hindered by the sublocal environment in which it is engineered; and, attaching a reporter to a truncated version of the wild-type TMP neglects effects that regions carboxy to the fusion may have in determining transmembrane topology. The principal utility of these studies therefore was that they were able to localize potentially functional topogenic elements.

2. Effect of Position on Topogenic Sequence Activity

The fusion studies described suggested that the context in which a topogenic element

is positioned might influence its activity. Therefore studies which have attempted to address the question of how topogenic sequences influence the activity of one another are critical for developing an understanding of polytopic TMP biogenesis. In some cases, these studies have revealed unique properties of a topogenic element which were not apparent in other contexts. In other cases, the findings highlight the importance of assessing the effects of passenger domains on translocation activity, in fusion studies designed to test the role of individual topogenic sequences in bringing about complex transmembrane phenotypes.

The classical stop transfer sequence was determined to cause termination of translocation in the context of a preceding amino terminal signal sequence (Yost et al., 1983). In vitro analysis of the activity of this sequence in a fusion protein lacking an amino terminal signal sequence, demonstrated that a stop transfer element also has intrinsic domain translocation activity (Mize et al., 1986; Zerial et al., 1987). The observation that stop transfer-directed translocation activity is mediated by SRP and SRP R provides strong support for a receptor-mediated model for both initiation and *termination* of translocation (see Chapter 2 and 3). Regarding the influence of position on the activity of a topogenic element, these results led to speculation that the function of a topogenic sequence is determined only by its position relative to other topogenic elements in the protein (Zerial et al, 1987).

Studies which have tested the effects of one topogenic element on another, have provided conflicting results. In a prokaryotic system, Coleman et al (1985) reported that when a normally disposed amino terminal signal sequence is positioned behind a previous amino terminal signal sequence, it direct either a transmembrane or secretory phenotype, depending on the length of the passenger domain between the two signal sequences. On the other hand, results from two different laboratories have observed that in fusion proteins encoding a pair of signal sequences, the second signal sequence

does not terminate translocation (Strizacker et al, 1987; Finidori et al., 1987). These paradoxical findings may reflect differences between prokaryotic and eukaryotic translocation machinery, or could be a consequence of steric or other nonspecific effects of protein folding in these particular constructs.

3. Passenger Influences on Translocation Activity

The influence of neighboring sequences on the function of a topogenic element has been well-documented. For example, in prokaryotes, a single amino acid substitution 283 residues from the carboxy terminus of a signal sequence abolished translocation (Randall and Hardy, 1986). Similarly, in eukaryotes, deletion in the domain carboxy to the prolactin signal sequence dramatically reduced translocation efficiency (Andrews et al., 1988). The protein domain preceding a signal sequence also affects translocation. A deletion in the ASGP R resulting in the juxtaposition of a proline-rich region with the amino terminus of an internal signal sequence completely abolished translocation in a eukaryotic cell-free assay (Spiess and Handschin, 1987). These findings highlight the need for a systematic approach to the study of polytopic TMP biogenesis.

III. Purpose, and Experimental Approach

The use of engineered fusion proteins for studying translocation has resulted in validation of many of the principles of the signal hypothesis. Topogenic elements have been isolated, and defined as discrete sequences in a nascent polypeptide which encode information for the proper localization of the mature protein. Manipulation of these discrete movable cassettes of information into different contexts has proved to be an invaluable tool for characterizing the properties of these substrates, and along with biochemical fractionation has allowed identification of the receptors with which they interact. The gene fusion approach has also led to insight into the mechanism of

translocation.

Although significant progress has been made towards understanding the activity of topogenic elements, recent studies demonstrate that signal and stop transfer sequences share some functional properties, raising questions regarding the information content of the different elements. If both signal and stop transfer sequences can initiate translocation, might their activity be determined solely by their position relative to one another? While some work has been done to address this question the findings are inconsistent and emphasize the potential pitfalls of using gene fusions to develop an understanding of the precise properties of the substrates involved in programming complex processes, such as polytopic TMP biogenesis. Two recent studies demonstrate the inherent difficulties of a gene fusion analysis: (1) a sequence within the cytoplasmic protein dihydrofolate reductase was found to be capable of acting as a signal sequence when attached to the amino terminus of a passenger (Hurt and Schatz, 1987), and; (2) in a study designed to test the influence of one topogenic element on another, the results were confounded by an apparent masking of an internally engineered signal sequence (Finidori et al.,1987).

Since an understanding of the precise function of topogenic sequences is of critical importance for clarifying the mechanism by which different TMPs are correctly disposed across the membrane of the ER, I first set out to define the activity of signal and stop transfer sequences, both alone and in the context of one another. Special attention was paid to characterize function independent of the effect of passenger sequences. Since a definition of the topogenic element's activity may be obscured or altered by the passenger and the particular context in which it was being studied; it was of utmost importance that the engineering experiments be carried out as precisely as possible, with identical passenger domains and topogenic elements employed. The results of a

systematic characterization of the function of a 'classical' signal and stop transfer sequence are presented in chapters 2 and 3. My in vitro analysis of a series of fusion constructs shows that while signal and stop transfer sequences share some activities, they are functionally distinct and non-interchangable elements.

These findings had important implications for understanding polytopic TMP biogenesis. Exploiting the 'rules' for topogenic sequence activity elucidated in Chapter 3, I constructed a hybrid protein which employed simple, well-characterized signal and stop transfer sequences in register to program the biogenesis of a complex polytopic TMP. These results are presented in chapter 4.

An approach employing fusion constructs was also applied to address questions regarding the mechanism by which signal and stop transfer sequences direct translocation. Based on the observation that an internally located signal or stop transfer sequence can direct translocation of the previous amino terminal domain, it was proposed that translocation can occur without ongoing protein synthesis. The experimental dissociation of translocation from translation is presented in Chapter 5; the demonstration that nucleoside triphosphate hydrolysis is required for translocation is discussed. My findings are summarized in Chapter 6 and proposals for future studies are presented.

Chapter 2:

Characterization of the Activity of Signal and Stop Transfer Sequences in the Context of Defined Passenger Domains

INTRODUCTION

The use of molecular genetic techniques to construct plasmids encoding hybrid fusion proteins has allowed for the localization of translocation ligands and an analysis of their functional properties. The power of this approach was dramatically demonstrated by showing that a cassette encoding a signal sequence, fused to the amino terminus of a normally cytoplasmic protein resulted in that protein being targeted to and translocated across the membrane of the ER (Lingappa et al., 1984). A similar fusion approach was used to define a stop transfer sequence as one that serves to terminate translocation (Yost et al., 1983). More recently, application of the gene fusion technique demonstrated that in certain contexts a stop transfer element can initiate translocation, thus obscuring the distinction between signal and stop transfer sequences.

The purpose of the following study was two-fold. First, we wished to clarify the functional similarities and differences between signal and stop transfer elements. Secondly, we sought to provide a conceptual and technical framework for pursuing our ultimate aim of building a polytopic transmembrane protein. Both of these goals could be met by carrying out systematic and controlled gene fusion studies.

Despite the appeal of applying the gene fusion approach to the problem of protein trafficking, there are inherent pitfalls which must be systematically controlled for when using fusion proteins as experimental tools. Localization and characterization of a topogenic element requires: (1) that the element be defined autonomously from the surrounding sequences; (2) that the passenger sequence which is being used as a reporter domain contain no cryptic signal or stop transfer information (i.e. it is topogenically inert); and, (3) that the conclusions from the study of one set of fusions be confirmed by the study of fusions to a structurally different passenger. Furthermore, interpretation of results is most meaningful when experiments are

carried out, in both in vitro systems and living cells, employing either eukaryotic or prokaryotic systems.

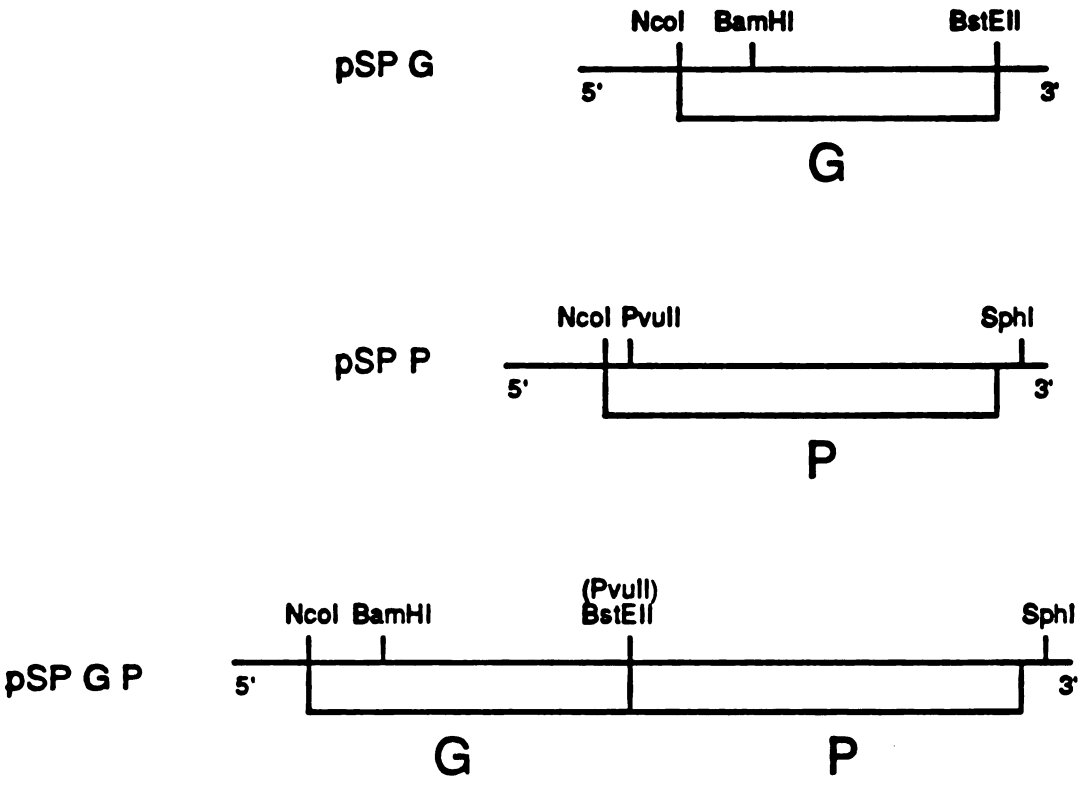
We have pursued such an exhaustive approach here. Simple signal and stop transfer sequences were defined in the context of fixed passenger domains, at both amino and internal locations. The results of these studies demonstrate that although signal and stop transfer sequences share some activities, they are functionally distinct elements.

Results

The choice of the passenger coding regions used in these studies was subject to several restrictions. Since we were interested in comparing the activity of different topogenic sequences in the same location it was important that the passenger coding regions be identical from one permutation to the next. Additionally, for the studies in which either internal or multiple topogenic sequences were being analyzed, the passenger domains preceding and flanking the topogenic sequence(s) must be antigenically distinct to permit unambiguous assignment of the topology of the encoded product. Finally, since it has been shown that coding sequences may be more or less hindered in their ability to be translocated by a signal sequence (Kadanoga et al., 1984), not all protein domains lacking a topogenic sequence were suitable to serve as passengers. Therefore, for the purposes of this study, a 'passenger domain' was defined as one that was easily movable, immunologically distinct, contained no demonstratable topogenic sequences itself, and was permissive to the action of either signal or stop transfer sequences.

Two passenger domains were chosen, and served as invariant cassettes throughout this study. Fig. 2-1 shows a restriction map and coding region of these domains, which were taken from the cytoplasmic protein alpha-globin and the secretory protein,

Figure 2-1. **Restriction maps and coding regions of passenger domains.** Restriction sites are above, and defined coding regions are below. Coding regions are represented as follows: *G*, chimpanzee alpha-globin passenger containing the first 110 amino acids of globin and a glycosylation site; *P*, prolactin passenger coding region containing amino acids 57-199 of mature prolactin.


pSP G
NcoI
BamHI
BstEII
5'
3'
G
pSP P
NcoI PvuII
SphI
5'
3'
P
pSP G P
NcoI BamHI
(PvuII)
BstEII
SphI
5'
3'
G
P

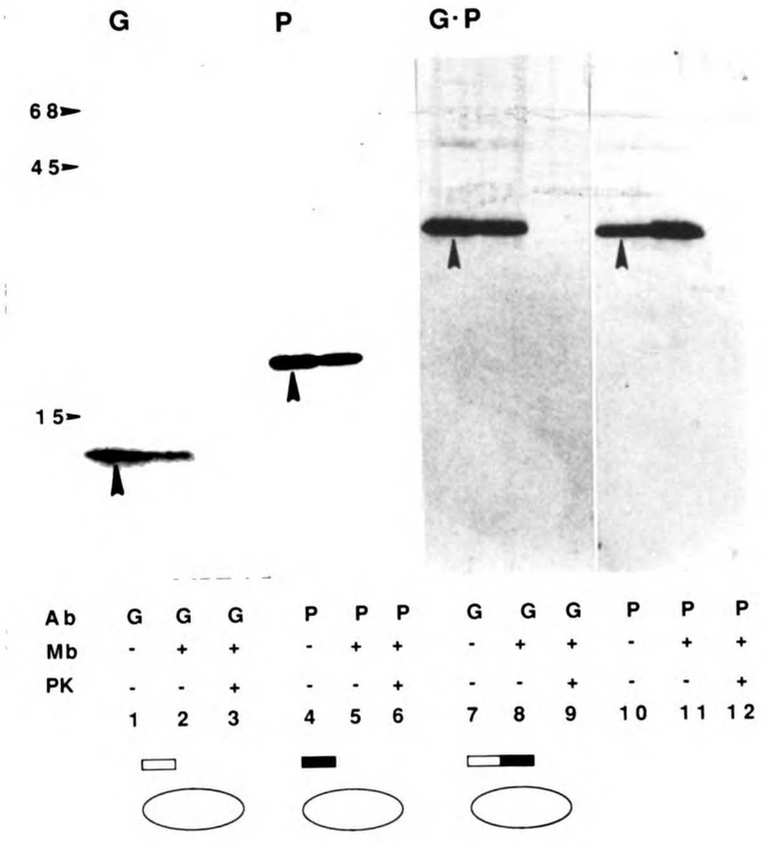
prolactin. The globin-derived passenger chain (G) consisted of the first 110 amino acids of chimpanzee alpha-globin containing an additional 8 codon segment encoding a functional N-linked glycosylation site inserted at codon 20 in the globin sequence (see Methods). The prolactin-derived passenger (P) encoded amino acid 57-199 of bovine prolactin. G-P is a precise fusion polypeptide of these two domains.

Each of these passengers was transcribed in a cell-free system using SP6 polymerase. Transcription products were translated in a rabbit reticulocyte lysate (RRL) protein synthesizing system in the absence or presence of microsomal membranes. All results described below for the in vitro expression of passenger domains, with and without single topogenic elements, were confirmed by analysis in Xenopus oocytes (data not shown).

Expression of the G and P passengers encoded by pSP G and pSP P resulted in synthesis of polypeptides of predicted molecular weights designated G and P (Fig. 2-2, lanes 1 and 4, respectively). As expected for a passenger domain lacking a topogenic element, no shift in molecular weight was observed when translation was carried out in the presence of microsomal membranes (lanes 2 and 5).

Post-translational proteolysis with proteinase K was used to determine the transmembrane disposition of these products and those in subsequent experiments. Any polypeptide that is localized completely outside of the microsomal vessicle will be totally digested by the addition of protease (Blobel and Dobberstein, 1975a). On the other hand, a polypeptide that is completely translocated across the bilayer will be resistant to proteolysis unless the integrity of the membrane is disturbed by the addition of nonionic detergent (Blobel and Dobberstein, 1975b). A protein spanning the microsomal membrane will be reduced in size by digestion of cytoplasmically disposed domain(s) (Katz et al., 1977; Lingappa et al., 1978).

Figure 2-2. **Expression of passenger domains.** Plasmids are identified by name above the first lane of each series. SP6 polymerase transcription products were translated in the RRL in the absence or presence of microsomal membranes (*Mb*). Products were subjected to post-translational proteolysis with proteinase K (*PK*) in the absence or presence of Nikkol detergent (*Det*). Immunoprecipitates were analyzed by SDS-PAGE and visualized by autoradiography. *Ab*, antisera: G, rabbit anti-human hemoglobin; P, rabbit anti-ovine prolactin. Components added to the translation reaction are indicated by (+) below the lanes. *Arrowheads pointing upward* indicate full-length products. The diagrams below the gels indicate inferred orientation of products with respect to microsomal membranes. *White and black bars* designate globin and prolactin domains, respectively. The positions of molecular weight ($X10^3$) markers are shown.


G
P
G·P
68
45
15
Ab G G G P P P G G G P P P
Mb - + + - + + - + + - + +
PK - - + - - + - - + - - +
1 2 3 4 5 6 7 8 9 10 11 12

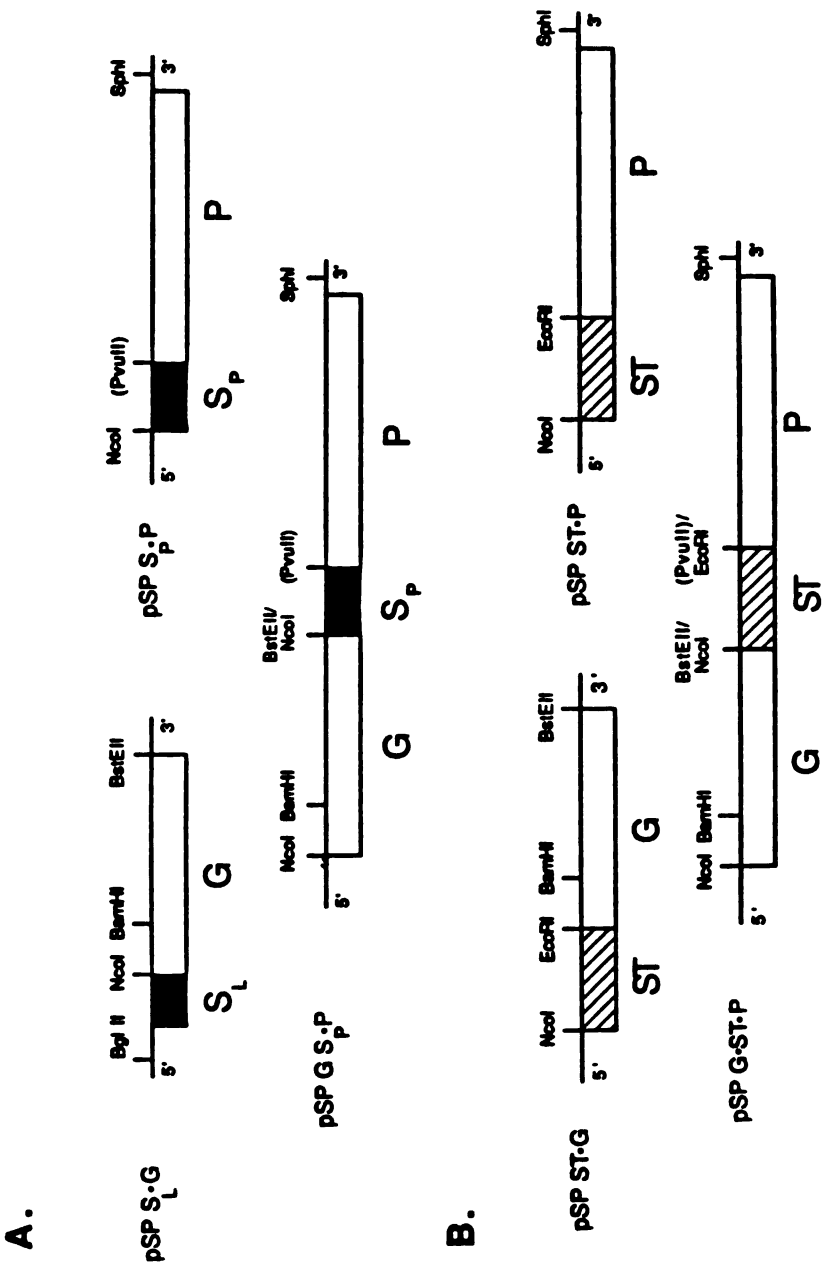
Both globin and prolactin were demonstrated to have cytoplasmic dispositions since posttranslational addition of proteinase K in the absence of detergent, resulted in their being completely degraded (Fig. 2-2, lanes 3 and 6). Similarly, when pSP G·P was transcribed and translated in the presence of microsomal membranes, proteolysis revealed that the product was cytoplasmically localized (Fig. 2-2, lanes 9 and 12). Thus, the passenger domains either alone, or in tandem were not translocated, but were retained in the cytoplasm, as illustrated in the diagrams below the gels.

We next engineered these passengers 3' to an in frame with the precise coding region of either the beta-lactamase signal sequence (S_L), or the prolactin signal sequence (S_P) (see Fig. 2-3A). The beta-lactamase signal sequence cassette encoded precisely the amino terminal extension of beta-lactamase, ending at an Nco I site which included the codon for the first amino acid of the globin passenger. In the case of pSP S_P·P, the prolactin signal sequence cassette included the entire 30 amino acid coding region of the amino terminal cleaved signal sequence, as well as the first amino acid after the cleavage site. S_P begins with an Nco I site encoding the initial methionine of the prolactin signal sequence and ending in a glycine codon, joined to residue 57 of authentic prolactin (see Methods). In pSP G·S_P·P the region encoding the prolactin signal sequence cassette was engineered in frame between the globin and prolactin encoding passenger domains.

A homologous family of molecules was constructed in which a cassette encoding the stop transfer region of IgM heavy chain (ST) was engineered in the identical context as that described for the signal sequence containing constructs above (i.e. at the amino terminus of both G and P as well as in an internal location between G and P). The restriction maps and coding domains of these ST encoding fusions are illustrated in Fig. 2-3B.

Two standard techniques, in addition to protease protection were employed for assessing translocation of these molecules (and those in subsequent studies): (1)

Figure 2-3. **Restriction maps and coding regions of passenger domains with signal (A) and stop transfer (B) sequences.** Restriction sites are above, and defined coding regions below. Coding regions are represented as described in Fig. 2-1, with the following additions: S_L, signal sequence derived from beta-lactamase; S_P, signal sequence derived from bovine prolactin, including one additional amino acid beyond the cleavage site; *ST*, stop transfer sequence from the transmembrane segment of immunoglobulin M heavy-chain. *Blackened rectangle*, signal sequences; *stripped rectangle*, stop transfer sequence; *unfilled rectangle*, passenger domains.


A.
pSP S·G L
Bgl II
NcoI
BamHI
BstEII
5'
3'
S L
G
pSP S·P P
NcoI
(PvuII)
SphI
S P
P
pSP G S·P P
BstEII/ NcoI
(PvuII)
G
S P
B.
pSP ST·G
EcoRI
ST
G
pSP ST·P
ST
P
pSP G·ST·P
(PvuII)/ EcoRI
ST
G
P

cleavage of signal sequences, since signal peptidase of intact membranes appears to be accessible only to translocated chains (Jackson and Blobel, 1977); and (2) glycosylation of newly synthesized chains (since oligosaccharidyltransferases are exclusively lumenally disposed enzymes (Katz, et al., 1978; Lingappa et al., 1978).

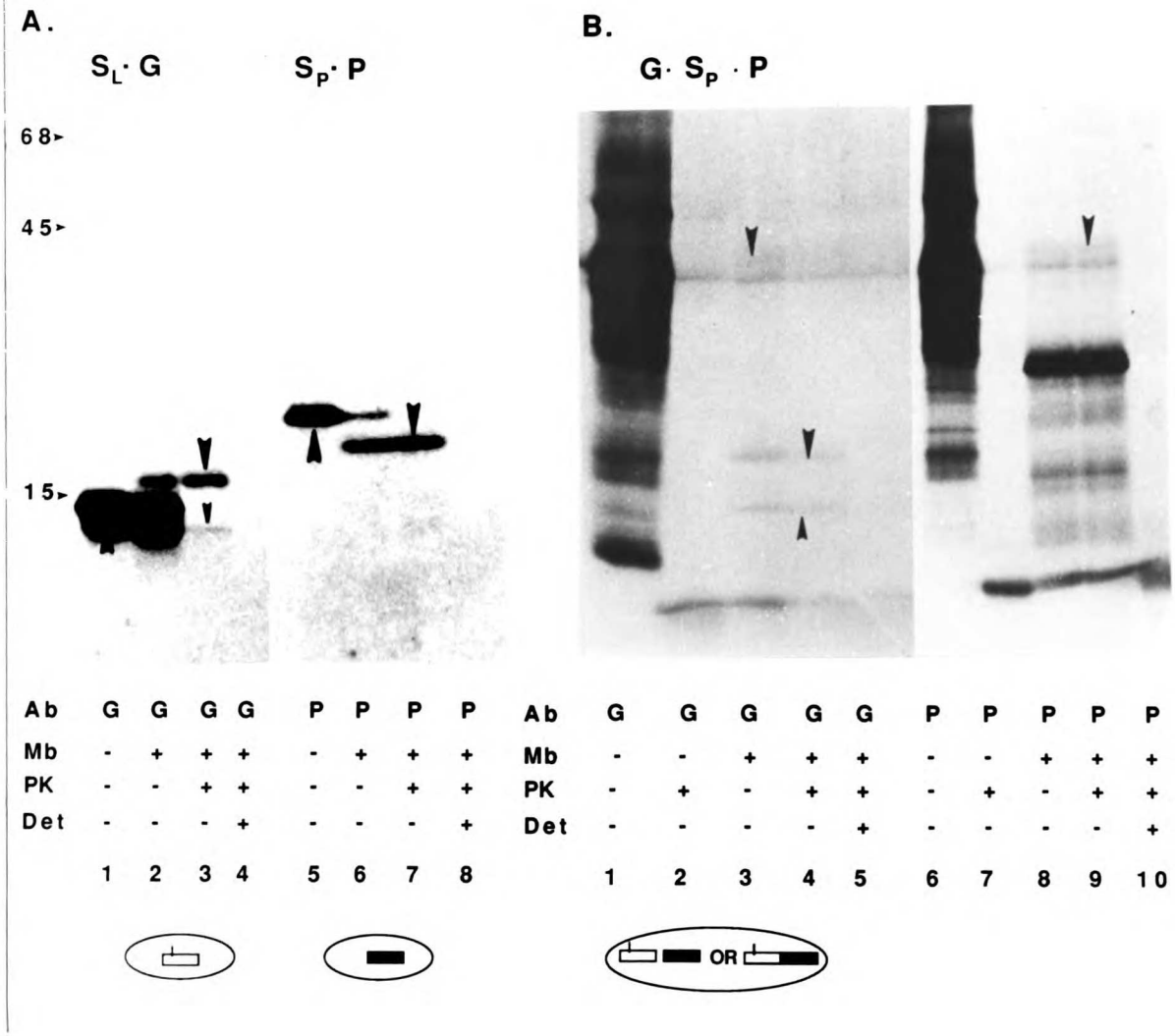
Translation of the transcription product of pSP S_L·G in the absence of microsomal membranes gave a full-length prescursor of the appropriate size (Fig. 2-4A, lane 1). In the cotranslational presence of microsomal membranes two new bands were seen. A product which migrated slightly faster than the precursor and was resistant to proteinase K digestion represents a cleaved, nonglycosylated species (lane 3, small downward pointing arrowhead). In addition, a slower migrating species which was protected from proteinase K (lane 3, large downward pointing arrowhead) is apparent, and represents a cleaved, glycosylated product. The identity of this species was confirmed by its downward shift to the position of the cleaved nonglycosylated product upon addition of endoglycosidase H (or endo H), an enzyme which removes N-linked sugars (data not shown).

Expression of pSP S_P·P also gave a secretory phenotype, as demonstated by the protection of a population of signal-cleaved molecules (Fig. 2-4A, lane 7). In both S_L·G and S_P·P protease protection was abolished by the addition of nonionic detergent which solublizes the bilayer (lanes 4 and 8).

These data show that S_L and S_P direct translocation of their passenger domains (G and P, respectively) across the ER membrane. The corresponding experiment, for each passenger domain with the other signal sequence (i.e. pSP S_L·P and pSP S_P·G) gave similar results (data not shown). The lumenal disposition of the expression products, S_L·G and S_P·P are represented schematically by the diagrams below the gel in Fig. 2-4.

Our laboratory has previously demonstrated that an amino terminal signal sequence

Figure 2-4. **Expression of passenger domains with a signal sequence at the amino terminus (A) or at an internal location (B).** Plasmids are identified by name above the first lane of each series. SP6 polymerase transcription products were translated in the RRL in the absence or presence of microsomal membranes (*Mb*). Products were subjected to post-translational proteolysis with proteinase K (*PK*) in the absence or presence of Nikkol detergent (*Det*). Immunoprecipitates were analyzed by SDS-PAGE and visualized by autoradiography. *Ab*, antisera: *G*, rabbit anti-human hemoglobin; *P*, rabbit anti-ovine prolactin. Components added to the translation reaction are indicated (+) below the lanes. In Panel **A**: *arrowheads pointing upward* indicate full-length products; *small downward pointing arrowhead* in lane 3, and *downward pointing arrowhead* in lane 7, indicate processed protected products; *large downward pointing arrowhead* in *lane 3*, designates a cleaved glycosylated species. In Panel **B**: *downward pointing arrowheads* at approximately 40 kDa indicate uncleaved, glycosylated species; *downward*,and *upward pointing arrowhead* in lane 4 designate the glycosylated and nonglycosylated globin fragments, respectively, generated from signal cleavage. *This gel analyzes the behavior of pSP gGP, which is identical to pSP G·S_P·P, except that it includes the entire globin coding region at the 3' end (instead of a variant which is truncated at the BstE II site in globin). When pSP G·S_P·P was analyzed, its topology was the same as that shown here. The diagrams indicate inferred orientation of products with respect to microsomal membranes. *White and black bars* represent globin and prolactin domains, respectively. The *solid line* in the globin domain designates glycosylation. The positions of molecular weight ($X10^3$) markers are indicated.


A.
S_L· G
S_P· P
68
45
15
Ab G G G G P P P P
Mb - + + + - + + +
PK - - + + - - + +
Det - - - + - - - +
1 2 3 4 5 6 7 8
B.
G· S_P · P
Ab G G G G G P P P P P
Mb - - + + + - - + + +
PK - + - + + - + - + +
Det - - - - + - - - - +
1 2 3 4 5 6 7 8 9 10
OR

can mediate transocation from an internal postion in a fusion protein (Perara and Lingappa, 1985). Because we wished to characterize the activities of topogenic elements by analyzing their behavior in identical contexts, the fusion pSP G·S_P·P was studied. Analysis of the transmembrane topology of the expression product of this plasmid shows that S_P directs translocation in this context as well. Two populations of lumenally disposed species were generated; one in which signal sequence cleavage did not occur, and the other in which the signal sequence was cleaved. .

Expression of pSP G·S_P·P, resulted in the synthesis of a product of the predicted molecular weight which was both globin and prolactin immunoreactive (Fig. 2-4B, lane 1 and 6). Cotranslational addition of microsomal membranes resulted in the appearance of several additional products. A species of slightly higher molecular weight than the precursor, which was both globin and prolactin immunoreactive (lane 3 and 9, respectively) represents a population of translocated molecules which were glycosylated but uncleaved. The identity and disposition of this product was demonstrated by its shift down to the position of the precursor upon addition of endo H (data not shown), and its resistance to posttranslational proteolysis (lane 9). The existence of such an uncleaved fully protected product indicates that the internal signal sequence (S_P), served to direct translocation of itself, as well as *both* amino and carboxy flanking domains.

Two unique globin immunoreactive bands, and a prolactin immunoreactive species were also generated upon addition of microsomal membranes to a translation reaction encoding G·S_P·P (lanes 3 and 8, respectively). These products represent molecules in which signal cleavage occured. The two globin immunoreactive species were protected from protease digestion (lane 4). The larger fragment represents the glycosylated variant of the smaller species as demonstrated by endo H analysis (data not shown). The major prolactin immunoreactive product generated upon addition of microsomal

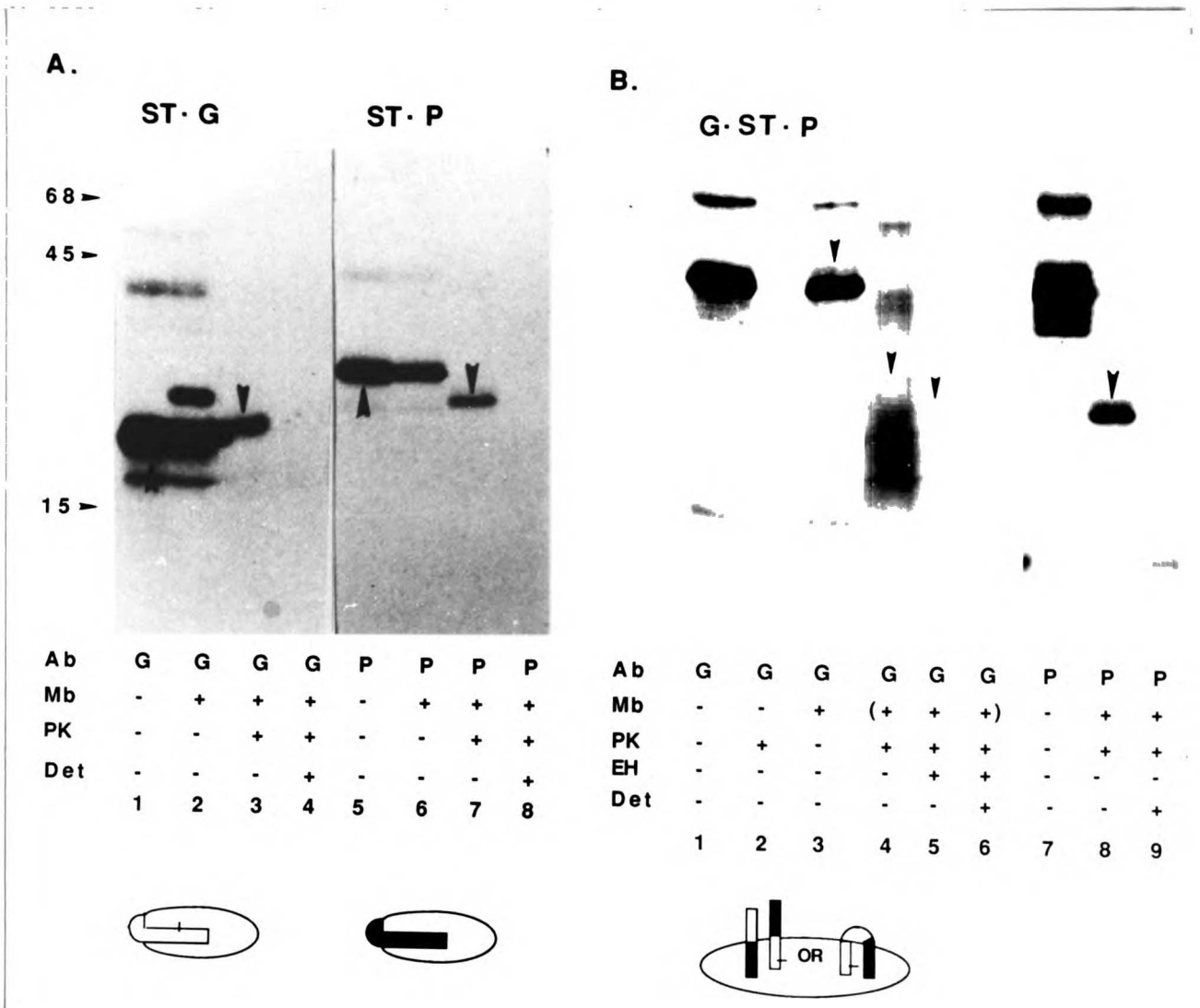
membranes was also protected from proteinase K digestion, and taken to be derived from cleavage of the full-length precursor (Fig. 2-4 B, lane 9). Both the full-length protected glycosylated product, as well as the cleaved protected fragments were degraded upon addition of proteinase K in the presence of nonionic detergent (lanes 5 and 10). The disposition of both the cleaved and uncleaved products are depicted in the diagram below the gel. From this analysis, we conclude that the internal signal sequence (S_P) mediates translocation of both G and P passenger domains, and may or may not be cleaved.

The stop transfer sequence from the heavy chain of IgM (ST) has 'signal-like activity' which was unmasked when ST was displayed in a fusion protein lacking a previous amino terminal signal sequence (Mize et al., 1986); when ST was positioned between two passenger domains, and was the only topogenic element encoded in a polypeptide it served to initiate translocation. In the context of our defined passengers, the translocation initiation activity of ST was also observed.

Expression of the plasmid pSP G·ST·P gave a product of the correct size which was both globin and prolactin immunoreactive (Fig. 2-5B, lanes 1 and 7). Addition of microsomal membranes resulted in the appearance of a higher molecular weight species (arrowhead, lane 3); this product was determined to be the glycosylated derivative of the precursor since digestion with endo H caused a shift in its position down to that of the precursor (data not shown).

Posttranslational digestion of the expression products of pSP G·ST·P with proteinase K generated both globin (lane 4), and prolactin (lane 8) immunoreactive protected fragments. The protected globin immunoreative fragment was glycosylated, as evidenced by its shift down to lower molecular weight upon addition of endo H (arrowhead, lane 5). When proteolysis was carried out in the presence of detergent, both the prolactin and

Figure 2-5. **Expression of passenger domains with ST at the amino terminus (A) or at an internal location (B).** Plasmids are identified by name above the first lane of each series. SP6 polymerase transcription products were translated in the RRL in the absence or presence of microsomal membranes (*Mb*). Products were subjected to post-translational proteolysis with proteinase K (*PK*) and endoglycosidase H (*EH*) in the absence or presence of Nikkol detergent (*Det*). Immunonoprecipitates were analyzed by SDS-PAGE and visualized by autoradiography. *Ab*, antisera: G, rabbit anti-human hemoglobin; P, rabbit anti-ovine prolactin. Components added to the translation reaction are indicated (+) below the lanes. In Panel A: *upward pointing arrowheads*, full-length product; downward pointing arrowheads, partially protected fragment *(lane 7)* and partially protected glycosylated fragment (lane 3). Panel B: *downward pointing arrowhead (lane 3)*, full-length glycosylated species; *downward pointing arrowheads (lanes 4 & 5)*, protected glycosylated and nonglycosylated fragments; (*Lanes 4-6* were taken from an identical experiment in Xenopus occytes); *downward pointing arrowhead (lane 8)*, protease protected prolactin fragment. The diagrams indicate inferred orientation of products with respect to microsomal membranes. *White and black bars* indicate globin and prolactin domains, respectively. The *solid line* in the globin domain designates glycosylation. The positions of molecular weight ($X10^3$) markers are indicated.


A.
ST · G
ST · P
68
45
15
Ab G G G G P P P P
Mb - + + + - + + +
PK - - + + - - + +
Det - - - + - - - +
1 2 3 4 5 6 7 8
B.
G · ST · P
Ab G G G G G G P P P
Mb - - + (+ + +) - + +
PK - + - + + + - + +
EH - - - - + + - - -
Det - - - - - + - - +
1 2 3 4 5 6 7 8 9
OR

globin fragments were degraded (lanes 6 & 9).

Although it is clear that the stop transfer sequence can direct translocation of either the amino or carboxy flanking domains, it is not apparent from this analysis whether ST allowed translocation of *both* flanking domains in a single molecule (as was evident in the case of G·S_P·P), or whether only one domain or the other was translocated in any particular molecule (see diagram below gel). We carried out preliminary experiments designed to address this question.

We engineered a molecule, pSP gG·ST·gG, to encode a glycosylation site in both the amino and carboxy passenger regions. When the transcription products of this plasmid were expressed either in a cell-free system or Xenopus oocytes, we were unable to detect any products in which both domains were glycosylated (data not shown). This result provides indirect evidence that at least in certain contexts, ST only mediates translocation of one or the other, but not both flanking domains. Resolution of this question however, awaits further analysis.

In order to further characterize the translocation initiation activity of ST, we next analyzed the behavior of fusions in which ST was positioned at the amino terminus of a passenger domain. ST was found to have translocation initiation activity in this context as well. The passenger domains of both ST·G and ST·P were translocated, as evidenced by protection from protease, and both protection and glycosylation of the former (Fig, 2-5A, lane 7 and lanes 2-3, respectively). In both cases, a downward shift in mobility following proteolysis was observed, indicating cleavage of an exposed domain corresponding to approximately 30 amino acids (by mobility shift on SDS-PAGE [lanes 2, 3, 6 and 7]). Since the shift down to lower apparent molecular weight upon proteolysis was observed for both ST·G and ST·P, we presume it to represent cleavage of a cytoplasmically accessible region of the stop transfer sequence (Fig. 2-5 A, diagrams

below the gels).

To summarize the findings thus far, both the signal and stop transfer sequences studied here were found to be capable of directing translocation when expressed either at the amino terminus or in an internal position (as the sole topogenic sequence) in a nascent polypeptide chain. The only apparent difference in the behavior of these elements are that while the signal sequence is cleaved and translocated (as evidenced by the fact that signal peptidase is found only on the lumenal face of microsomal vessicles), the stop transfer sequence is NOT cleaved by signal peptidase and remains exposed on the cytoplasmic face of the membrane (as revealed by protease protection experiments). These results are in agreement with previous findings from studies with individual topogenic elements and confirm the fidelity of the simplest set of topogenic sequence-passenger fusion proteins.

In its *native* context, that is, after a preceding signal sequence, ST causes both termination of translocation and *integration* into the bilayer (Yost et al., 1983). Since the translocation initiation activity of a stop transfer sequence was an unanticipated activity, we were interested in investigating what the nature of the association of the stop transfer sequence was with membrane when it functioned to initiate translocation.

Secretory protein are characteristically soluble in the lumen of the microsomal vessicle, while transmembrane proteins are by definition, integrated into the bilayer. The disposition of proteins with respect to the ER membrane can be analyzed by the following procedure: subsequent to translation of the molecule of interest in the presence of microsomal membranes, the reaction is incubated in either isotonic sucrose buffer or sodium carbonate, pH 11.5. Such alkali treatment has been demonstrated to convert membrane vessicles into sheets, so that peripheral membrane proteins and content proteins can then be separated from integral membrane proteins by sedimentation. Only

those proteins that are integrated into the bilayer are associated with the membrane in a fashion that is not disrupted by carbonate extraction. Therefore, upon carbonate treatment and subsequent sedimentation by centrifugation, integral membrane proteins separate with the membrane fraction (i.e. the pellet), while all other proteins are left in the supernatant (Fujiki et al., 1982).

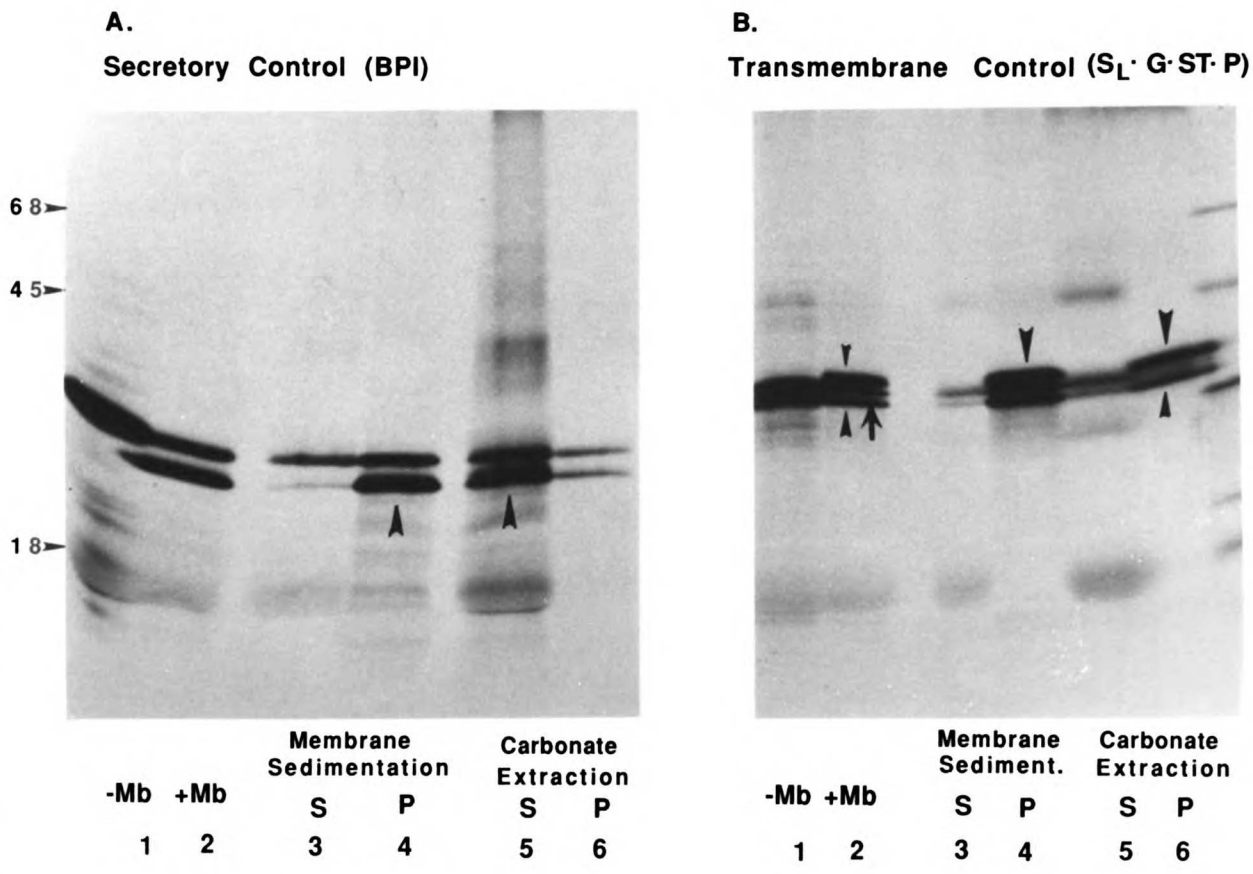
A standard secretory and transmembrane protein were first subjected to this procedure in order to establish experimental conditions in our hands, and to generate controls with which to compare the molecules of interest. The plasmid pSP BPI, encodes the secretory protein prolactin (see Andrews et al., 1988), while pSP S_L·G·ST·P encodes an integral transmembrane protein (see Methods). Fig. 2-6 presents the results of a sedimentation analysis for these control molecules.

Upon sedimentation in isotonic sucrose, processed prolactin, which is lumenally disposed, separates with the membrane fraction or pellet (Fig. 2-6A, arrowhead, lane 4). Extraction with carbonate releases this translocated species from vessicles, resulting in the quantitative appearance of this product in the supernatant following centrifugation (arrowhead, lane 5). This behavior is typical of a secretory protein and indicative of its soluble, lumenal disposition.

In panel B, the products generated upon expression of pSP S_L·G·ST·P, in the presence of microsomal membranes are shown. Both cleaved glycosylated (downward pointing arrowhead, lane 2), and cleaved, nonglycosylated (upward pointing arrowhead, lane 2) species are evident; proteolysis analysis confirmed the bitopic transmembrane disposition of this molecule (data not shown).

Similar to the behavior of BPI, upon sedimentation in isotonic sucrose buffer, the translocated products of S_L·G·ST·P quantitatively pelleted (Fig. 2-6B, lane 4). However, following carbonate extraction, both translocated species (i.e. the cleaved

Figure 2-6. **Membrane sedimentation and carbonate extraction of translation products encoded by pSP BPI and PSP S_L·G·ST·P.** Plasmids pSP BPI and PSP S_L·G·ST·P were transcribed and translated in vitro, in the absence, or presence of dog pancreas microsomal membranes (*Mb*). The (+Mb) translation reaction was split into two equal aliquots, one of which was sedimented in isotonic sucrose buffer and the other extracted with carbonate (see Methods for details). Lanes 1 and 2 are aliquots of the original translation reaction and mark the positions of the precursor and translocated forms of each product. Equivalent volumes of total products from the membrane sedimentation and carbonate extraction reactions are shown in lanes 3 - 6. *S*, supernatant; *P*, pellet. In Panel **A**: *Upward pointing arrowhead*, indicates the position of the processed prolactin species. In Panel **B**: *Upward pointing arrow* designates the full-length precuror; *upward pointing arrowhead* indicates a processed-nonglycosylated product; *downward pointing arrowhead*, indicated a processed glycosylated species. The positions of molecular weight ($X10^3$) markers are indicated.


A.
Secretory Control (BPI)
B.
Transmembrane Control (S_L· G·ST· P)
68
45
18
-Mb
+Mb
Membrane Sedimentation
S
P
Carbonate Extraction
S
P
1
2
3
4
5
6
-Mb
+Mb
Membrane Sediment.
S
P
Carbonate Extraction
S
P
1
2
3
4
5
6

glycosylated and nonglycosylated products) remained in the pellet (lane 6). This is the expected result for an integral membrane protein. Densitometric analysis of these autoradiograms illustrates the fildelity of the carbonate extraction technique in our hands (Fig. 2-8).

ST·G and S_L·G were next subjected to carbonate extraction, the only difference between the two molecules being the topogenic element employed for initiating translocation. As with the secretory control (Fig. 2-6A), the translocated products generated from expression of pSP S_L·G were extracted upon treatment of microsomal vessicles with carbonate (Fig. 2-7A). The downward pointing arrowhead in lane 4 designates the cleaved glycosylated product, which was released from the membrane fraction by carbonate treatment (arrowhead, lane 5).

Translation of pSP ST·G in the presence of membranes generated a glycosylated product (Fig. 2-7B, lane 2). When subjected to sedimentation, this species quantitatively pelleted (lane 4). However, unlike S_L·G, the translocated form of ST·G was not released from microsomal vessicles by carbonate extraction but was instead retained in the membrane fraction (lane 6). Thus, even when the stop transfer sequence serves to *initiate* translocation, the final translocated product is an integral membrane protein. Fig. 2-8 shows the results of a densitometric analysis of the distribution of the products, S_L·G and ST·G.

DISCUSSION

The studies presented here represent the first step of a systematic analysis designed to clarify functional similarities and differences between signal and stop transfer sequences. In this chapter, the topology conferred on well-defined passenger domains by either signal or stop transfer sequences was assessed by several assays. From these

Figure 2-7. **Membrane sedimentation and carbonate extraction of translation products encoded by pSP S_L·G and pSP ST·G.** Plasmids pSP S_L·G and PSP ST·G were transcribed in vitro and products were translated for 1 hour at 25°C in the absence or presence of dog pancreas microsomal membranes (*Mb*). The (+Mb) translation reaction was then split into two equal aliquots, one of which was sedimented in isotonic sucrose buffer and the other was extracted with carbonate (see Methods for details). Lanes 1 and 2 are aliquots of the original translation reaction and mark the positions of the precursor and translocated forms of each product. Equivalent volumes of total products from the membrane sedimentation and carbonate extraction fractions are shown in lanes 3-6. *S*, supernatant; *P*, pellet. In Panel **A**: *Downward pointing arrowhead*, indicates the position of the processed *glyosylated* species; In Panel **B**: *Sideward pointing arrowhead* designates the full-length precursor; *downward pointing arrowhead (lane 2),* indicates the translocated, glycosylated product. The additional background bands seen in the autoradiogram are endogenous RRL products. The positions of molecular weight ($X10^3$) markers are indicated.

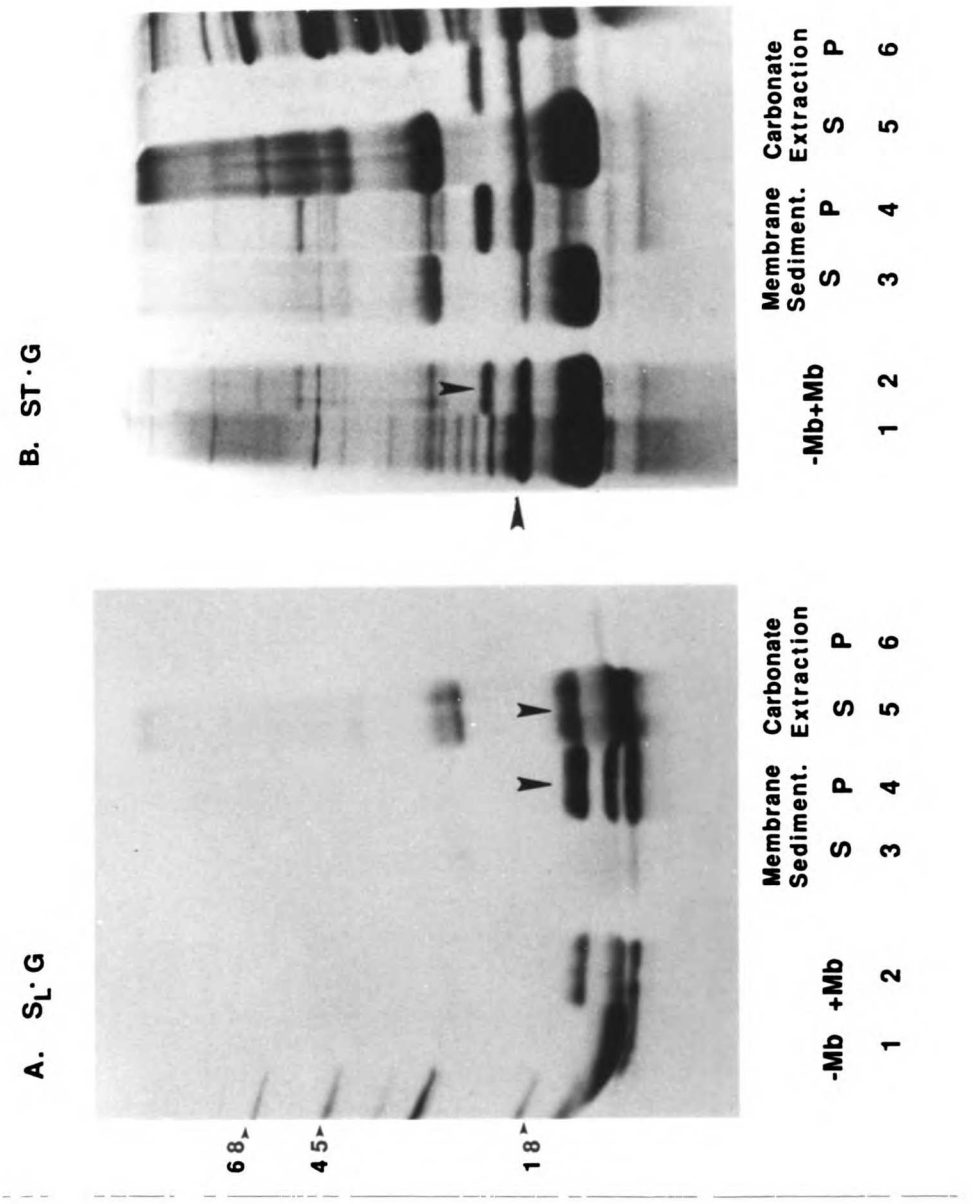
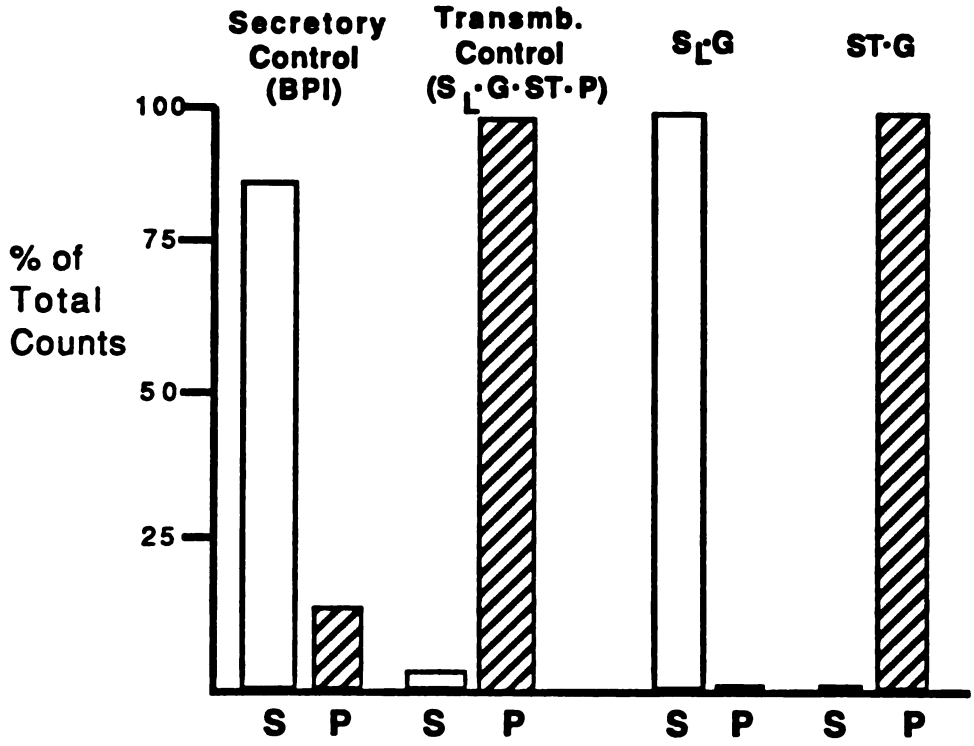

A. S_L·G
B. ST·G
-Mb +Mb
1 2
Membrane Sediment.
S P
3 4
Carbonate Extraction
S P
5 6
-Mb+Mb
1 2
Membrane Sediment.
S P
3 4
Carbonate Extraction
S P
5 6
68
45
18

Fig. 2-8. **Percentage of translocated products which are extracted versus integrated from microsomal membranes.** The ratio of extracted as compared to integrated products was calculated for each of the four molecule described in Figs. 2-6 and 2-7. Percentage of total counts was calculated by adding the absolute number of densitometric units measured for the bands of interest in the carbonate extraction experiment (i.e. the translocated species, supernatant + pellet fractions) and then dividng the number of counts for either the supernatant or pellet, by the total number of counts recovered in that experiment. *S*, supernatant (extracted product), represented by unfilled bar; *P*, pellet (integrated product), designated by stripped bar.


Secretory Control (BPI)
Transmb. Control (S_L·G·ST·P)
S_LG
ST·G
% of Total Counts
100
75
50
25
S P S P S P S P

studies it appears that while signal and stop transfer elements elements share some recognition features and activities, they are functionally distinct.

A classical amino terminal signal sequence was shown to function at the amino terminus to mediate translocation of a passenger. In addition, when displaced to an internal location (i.e. between two flanking domains) the normally amino terminal signal sequence was shown to direct its own translocation as well as that of *both* its flanking domains.

The stop transfer sequence tested was also found to initiate passenger domain translocation. When positioned either at the amino terminus, or internally, as the sole topogenic element, ST directed domain translocation, but its behavior is distinguished from that of a signal sequence in that it is not itself translocated. Furthermore, from the analysis of pSP gG·ST·gG, it appears that when ST is flanked by two passenger domains, it can mediate translocation of only one passenger domain or the other, but not both, across the ER membrane. Whether this finding is a general characteristic of stop transfer directed translocation requires further analysis.

Analysis of the transmembrane disposition of these fusions by carbonate extraction further highlights the differences between signal and stop transfer directed translocation. When translocation was directed by a signal sequence, the product was completely lumenally disposed. In contrast, stop transfer mediated translocation produced an integral membrane protein. Thus, although signal and stop transfer sequences share the property of being able to initiate translocation of a passenger, a stop transfer sequence is neither cleaved nor translocated, but is instead integrated into the bilayer.

The mechanistic basis for the difference between signal and stop transfer directed translocation remains to be resolved. One possibility is that the ability of a stop

transfer sequence to mimic the passenger domain translocation activity of a signal sequence is related to recognition by both signal and stop transfer sequences of a common cytoplasmically disposed receptor. Several lines of evidence provide support for the involvement of receptors in stop transfer mediated translocation. Most compelling is the finding that ST mediated domain translocation activity is dependent upon the presence of SRP as well as at least one receptor in the ER membrane (Mize et al., 1986; Zerial et al., 1987).

According to a receptor-mediated model of stop transfer function the following scenario can be envisioned. Signal and stop transfer sequences might share a common cytoplasmic receptor. This receptor is suggested to serve as a 'toggle switch' capable of opening or closing a specific channel upon recognition of signal and stop transfer sequences. Normally, a stop transfer seqeunce emerges from the ribosome temporally after translocation has been initiated by a preceding signal sequence (at the amino terminus) and serves to disengage the translocation machinery and terminate translocation (itself being integrated into the bilayer). However, in the case where a stop transfer sequence is positioned at the amino terminus (or is in an internal location, but is the first topogenic element in the protein to be detected by receptors), it engages the translocation machinery in a similar fashion to a signal sequence. Unlike a signal sequence however, the stop transfer sequence is not competent to complete the process of translocation, perhaps because it is not recognized by a lumenally disposed receptor (e.g. signal peptidase). The failure of the stop transfer sequence to recognize the hypothetical lumenal receptor (specific for signal sequences), could account for the topology of the products generated by stop transfer directed translocation. ST might have bound to a cytoplasmic receptor but was unable to complete translocation of itself, therefore remaining exposed on the surface of the ER.

Although carbonate extraction of ST·G revealed a product which was integrated into

the bilayer, it is possible that ST initially interacted with the proposed cytoplasmic receptor (to initiate translocation) and was only subsequently integrated into the bilayer. This hypothesis can be tested experimentally (see Chapter 6).

The possibility that a nonreceptor mediated model might account for the lack of translocation of the stop transfer sequence (in stop transfer initiated translocation) is not ruled out by our results. However, a comparison of the hydrophobicity profiles of the signal and stop transfer sequence employed here make this explanation less likely (see Discussion, Chapter 3).

Regardless of the mechanism, differences between signal and stop transfer sequence activity were clarified using a gene fusion approach in which the influence of passenger domains on topogenic sequence function was well controlled for. (The G and P passengers were shown to be functionally inert and permissive to the action of topogenic sequences.) Since the constructs employed here are exact matches, it is likely that the topological fate of any particular fusion protein is attributable to the effect of the topogenic sequence being tested. This approach was next applied to address the question of how simple topogenic elements function in the context of one another.

CHAPTER 3:

ANALYSIS OF THE ACTIVITY OF SIGNAL AND STOP TRANSFER SEQUENCES IN THE CONTEXT OF ONE ANOTHER:

(ELUCIDATION OF 'RULES' GOVERNING SIGNAL AND STOP TRANSFER SEQUENCE FUNCTION IN DEFINED CONTEXTS)

INTRODUCTION

Having shown that either a signal or stop transfer sequence is an effective, but functionally distinct translocator of flanking passenger domains we next addressed the question of how one defined topogenic sequence functions in the context of another. Analysis of this problem was critical, both for the development of a more complete understanding of the activity of each of these elements, as was well as for our ultimate aim, of constructing a polytopic transmembrane protein using a series of signal and stop transfer sequences.

Since both signal and stop transfer sequences share the property of strong hydrophobicity, some investigators have suggested that they might be functionally interchangeable (Zerial et al., 1987). The hypothesis that the activity of a topogenic element (i.e. initiation or termination of translocation) is determined strictly by the order of its appearance in a polypeptide encoding multiple topogenic elements, has previously been addressed. However, these studies provide conflicting results and are inherently inconclusive.

In one study, which suggested that transmembrane orientation is determined solely by the appearance of multiple signal sequences (Coleman et al., 1985) interpretation of the results was, at least in part, obscured by the occurence by more than one topology upon expression of a given construct. In another recent study, an internal signal sequence failed to terminate the translocation of a protein which had been initiated by an amino terminal signal sequence (Finidori et al, 1987). However, in a control construction, the internal signal sequence was shown to lack the ability to initiate translocation when the amino terminal signal sequence was deleted. Thus, it remained unclear whether the internally engineered signal sequence retained ANY functional activity in this context.

The following section of our study was motivated by the need for a well-controlled analysis of the question whether it is merely the order of hydrophobic sequences, rather than their sequence composition (i.e. inherent properties of a topogenic element beyond hydrophobicity) which determines whether a topogenic element initiates or terminates translocation.

Results

We sought to compare the topology of fusion proteins containing various arrangements of signal and stop transfer sequences separated by defined passenger domains. However, the relative inefficiency of the cell-free translocation system posed a significant problem for the analysis of plasmids encoding multiple topogenic sequences.

Typically, cell-free transcription-linked translation results in approximately 25-75% of peptide chains translocated, as a function of membrane concentration. Attempts to improve the efficiency by increasing the membrane concentration results in a counterproductive inhibition of cell-free protein synthesis. Analysis of molecules containing two topogenic sequences is therefore problematic in cell-free systems such as RRL, because those molecules whose first topogenic sequence failed to engage the translocation machinery may now present their second topogenic sequence as if it were the first. The topologies generated by this subgroup of molecules would confound the analysis of contextual phenotype.

To avoid these problems inherent in cell-free systems, we decided to study the expression of plasmids encoding molecules containing multiple topogenic sequences in Xenopus oocytes (*XO*). We have previously shown that such a system directs the translocation of engineered proteins with significantly higher efficiency but with congruent topology to that observed in RRL (Simon et al., 1987). In all our studies to date we have yet to detect residual precursor chains following the introduction of

molecules displaying an amino terminal signal sequence. In addition, Xenopus oocytes display a significantly lower background level of internally initiated and prematurely terminated polypeptide chains. For these reasons, XO were expected to provide a superior systems for the analysis of polypeptides with multiple topogenic elements.

Based on previous work in cell-free systems (Yost et al., 1983) we expected that a stop transfer sequence, emerging from the ribosome subsequent to a signal sequence, would terminate translocation initiated by the preceding signal sequence. However, in view of the unexpected translocation initiation activity of a stop transfer sequence in the absence of a preceding signal sequence (Mize et al., 1986), the outcome of other topogenic sequence pattern permutations seemed unpredicatable.

Two plasmids, diagramed in Fig. 3-1, were constructed. The first (pSP S_L·G·ST·P) was designed to confirm the classical function of a stop transfer sequence in the presence of the passenger domains used here. The second (pSP ST·G·ST·P) was constructed to test the effect of a second stop transfer sequence on translocation directed by an amino terminal stop transfer sequence.

When expressed in Xenopus oocytes, the product encoded by pSP S_L·G·ST·P was observed to be a glycosylated molecule of the expected size (approximately 40 kDA) that was reactive to both globin and prolactin antisera (Fig. 3-2, lanes 1-3). Upon proteolysis, a glycosylated globin domain of the expected size (lanes 5 and 6, downward pointing arrowhead), but no prolactin-reactive domain (lane 4), was seen. The globin-reactive protease-protected fragment was abolished by proteolysis in the presence of nonionic detergent (lane 7). These findings suggest an asymmetric transmembrane orientation as indicated (diagram below the gels), consistent with previous findings (Yost et al., 1983; Mize et al., 1986). Taken together with the 'signal-like' behavior noted for this same stop transfer cassette (when placed in an identical context but in the

Figure 3-1. **Restriction maps and coding regions of fusions: pSP S_L·G·ST·P and pSP ST·G·ST·P.** Restriction sites are indicated above the lines and defined coding regions below. Coding regions are represented as described in Fig. 2-3.

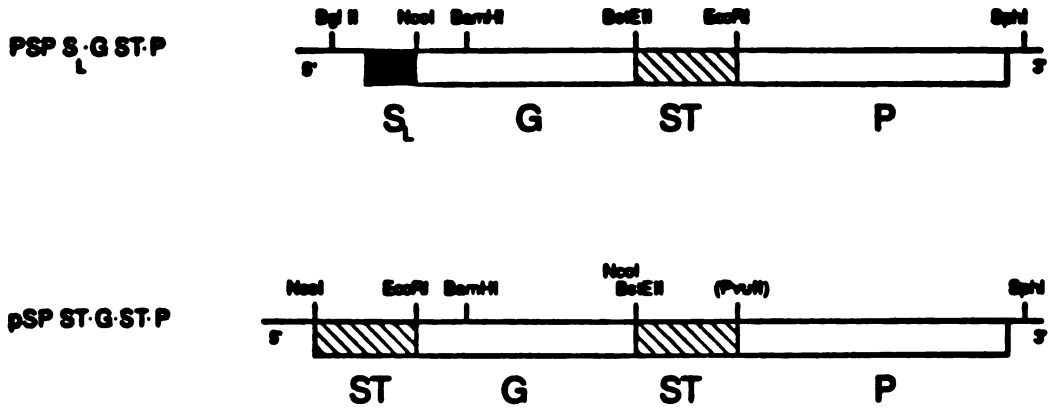
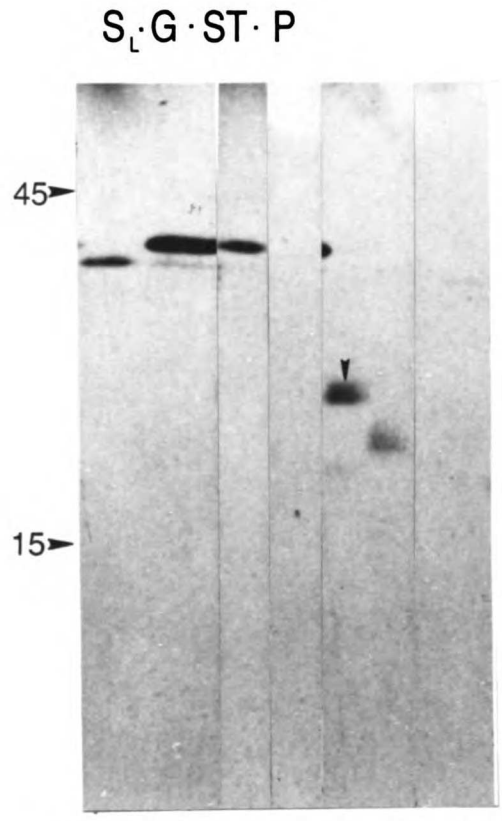
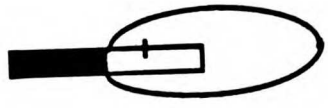
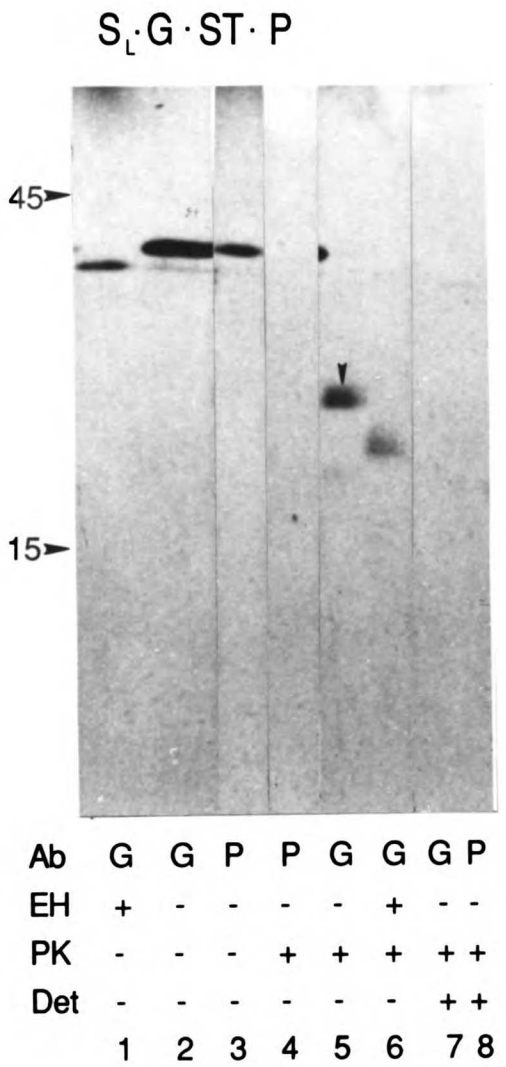
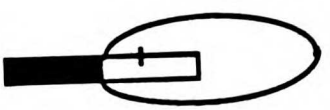

PSP S$_L$·G·ST·P
Bgl II
NcoI
BamHI
BstEII
EcoRI
SphI
5'
3'
S$_L$
G
ST
P
pSP ST·G·ST·P
NcoI
EcoRI
BamHI
NcoI
BstEII
(PvuII)
SphI
5'
3'
ST
G
ST
P

Figure 3-2. **Xenopus oocyte expression of pSP S_L·G·ST·P.** Xenopus occytes were injected with SP6 transcripts synthesized from pSP S_L·G·ST·P and labeled with [^{35}S] methionine. Xenopus occytes were homogenized, aliquoted, and subjected to post-translational proteolysis. Proteolysis products were immunoprecipitated with prolactin or globin antisera, and selected samples were digested with endoglycosidase H, followed by SDS-PAGE. *Ab*, antibody used for immunoprecipitation; *EH*, endoglycosidase H; *PK*, proteinase K; *Det*, Nikkol detergent. *Downward pointing arrowhead in lane 5*, indicates a glycosylated, globin immunoreactive fragment protected from proteinase K. Diagram below the gel indicates the inferred disposition of the product with respect to microsomal membranes. *White and black bars* designate globin and prolactin domains, respectively. The *solid line* drawn in the globin domain indicates glycosylation. The positions of molecular weight (X10^3) markers are indicated.


S_L·G·ST·P
45
15
Ab G G P P G G G P
EH + - - - - + - -
PK - - - + + + + +
Det - - - - - - + +
1 2 3 4 5 6 7 8




S$_L$·G·ST·P
45
15
Ab G G P P G G G P
EH + - - - - + - -
PK - - - + + + + +
Det - - - - - - + +
1 2 3 4 5 6 7 8



absence of a preceding singal sequence, or N-terminal to the identical passenger sequence {see Chapter 2}), these data suggest that the translocation terminating activity of the stop transfer sequence is dependent upon its appearance in the chain distal to a more proximal signal sequence.

Is the termination event mediated by a stop transfer sequence a consequence of its relative position behind ANY preceding topogenic element which is capable of initiating translocation, or is this termination of translocation dependent upon the prior initiation of translocation by a bona fide signal peptide?

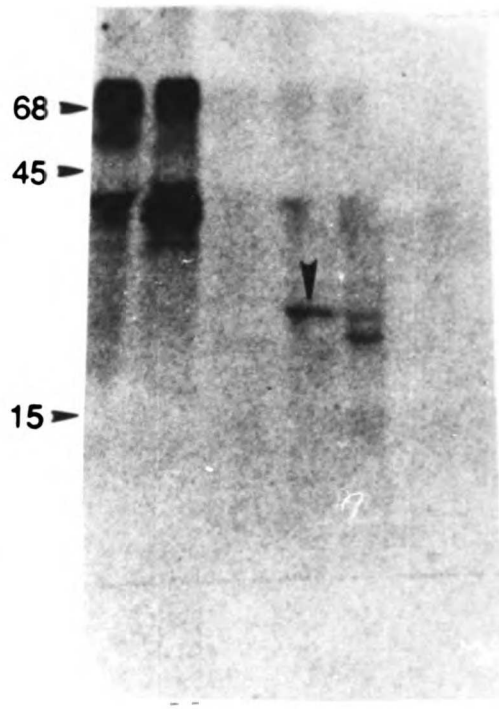
When the product encoded by pSP ST·G·ST·P was analyzed, a globin- and prolactin-immunoreactive molecule of predicted size (approximately 42 kDA) was observed (Fig. 3-3, lanes 1 and 2). Proteolysis resulted in a protected, glycosylated globin, but no prolactin-immunoreactive fragment (lanes 3-7), consistent with the indicated transmembrane orientation (diagram below gel). Although the most likely disposition of the encoded product is given, we can not formally rule out another possible orientation, in which the initial stop transfer sequence is lumenally disposed (see Discussion below).

An internal control (not shown) was used to verify proteolysis conditions. Thus, the relatively low recovery of globin-reactive protease-protected fragments observed for pSP ST·G·ST·P may be due either to lower efficiency of this translocation event or to a lower efficiency of antibody recognition for the fragment generated upon proteolysis of this molecule. In any case, the classical function of a stop transfer sequence (i.e. the ability to terminate previously initiated translocation) occur regardless of whether translocation is initiated by a signal or a stop transfer sequence at the amino terminus, as demonstrated in Xenopus oocytes and also in the RRL (data not shown).

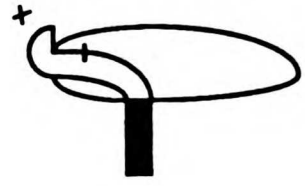
Since a stop transfer sequence displayed some 'signal-like' activity in a position normally occupied by a signal sequence, we wished to determine if the converse were

Figure 3-3. **Xenopus oocyte expression of pSP ST·G·ST·P.** Xenopus occytes were injected with SP6 transcripts synthesized from pSP ST·G·ST·P and labeled with [^{35}S] methionine. Xenopus occytes were homogenized, aliquoted, and subjected to post-translational proteolysis. Proteolysis products were immunoprecipitated with prolactin or globin antisera, and selected samples were digested with endoglycosidase H, followed by SDS-PAGE. *Ab*, antibody used for immunoprecipitation; *EH*, endoglycosidase H; *PK*, proteinase K; *Det*, Nikkol detergent. *Downward pointing arrowhead in lane 5*, indicates a glycosylated, globin immunoreactive fragment protected from proteinase K. Diagram below the gel indicates the inferred disposition of the product with respect to microsomal membranes. *White and black bars* designate globin and prolactin domains, respectively. The *solid line* drawn in the globin domain indicates glycosylation. The *, next to the diagram indicates that the transmembrane disposition of the first ST domain was inferred from the behavior of pSP ST·G (see Fig. 2-5) but could not be directly demonstrated here. The positions of molecular weight (X10^3) markers are indicated.

ST·G·ST·P



Ab	G	P	P	G	G	G	P
EH	-	-	-	-	+	-	-
PK	-	-	+	+	+	+	+
Det	-	-	-	-	-	+	+
	1	2	3	4	5	6	7



true: would the substitution of a signal sequence in the position normally occupied by a stop transfer sequence confer a transmembrane orientation? These studies directly address the issue of whether stop transfer *termination* is determined solely by the appearance of a region of sufficient hydrophobicity in a nascent polypeptide already engaged in translocation.

We engineered three plasmids as companion constructions (each encoding signal sequences in the second position (see Fig. 3-4). Analysis of the expression products of these plasmids would serve to assess the ability of an internal signal sequence to terminate translocation which had been previously initiated (by either a signal or stop transfer sequence at the amino terminus). Plasmid pSP S_L·G·S_P·P encodes a fusion protein in which the two signal sequences are flanked by G and P domains in contexts identical to those of the simple constructions shown in Fig. 2-3. In a second construction (pSP S_L·S_P·P), the globin coding region was removed by an in-frame deletion from pSP S_L·G·S_P·P. The encoded fusion protein thus presents two signal sequences in tandem without an intervening passenger domain. Expression of this plasmid served to investigate the possibility that the distance between two signal sequences may affect the function of the second signal sequence, as suggested previously (Coleman et al., 1985). In a third construction (pSP ST·G·S_P·P), the initial topogenic sequence is ST rather than S_L.

Expression of pSP S_L·G·S_P·P in Xenopus oocytes gave a product of predicted molecular weight, which was both globin- and prolactin- immunoreactive (Fig. 3-5A, lanes 2 and 3). The product was a glycosylated, fully translocated molecule, as evidenced by sensitivity to endo H both before (lane 1) and after (lane 6) proteolysis, and by complete protection from digestion with proteinase K in the absence (lanes 4 and 5), but not in the presence (lanes 7 and 8), of detergent. Analysis in the RRL showed similar topology with respect to the membrane (data not shown).

Figure 3-4. **Restriction maps and coding regions of fusions: pSP S_L·G·S_P·P, pSP S_L·S_P·P and pSP ST·G·S_P·P.** Restriction sites are indicated above the lines, and defined coding regions below. Coding regions are represented as described for Fig. 2-3.

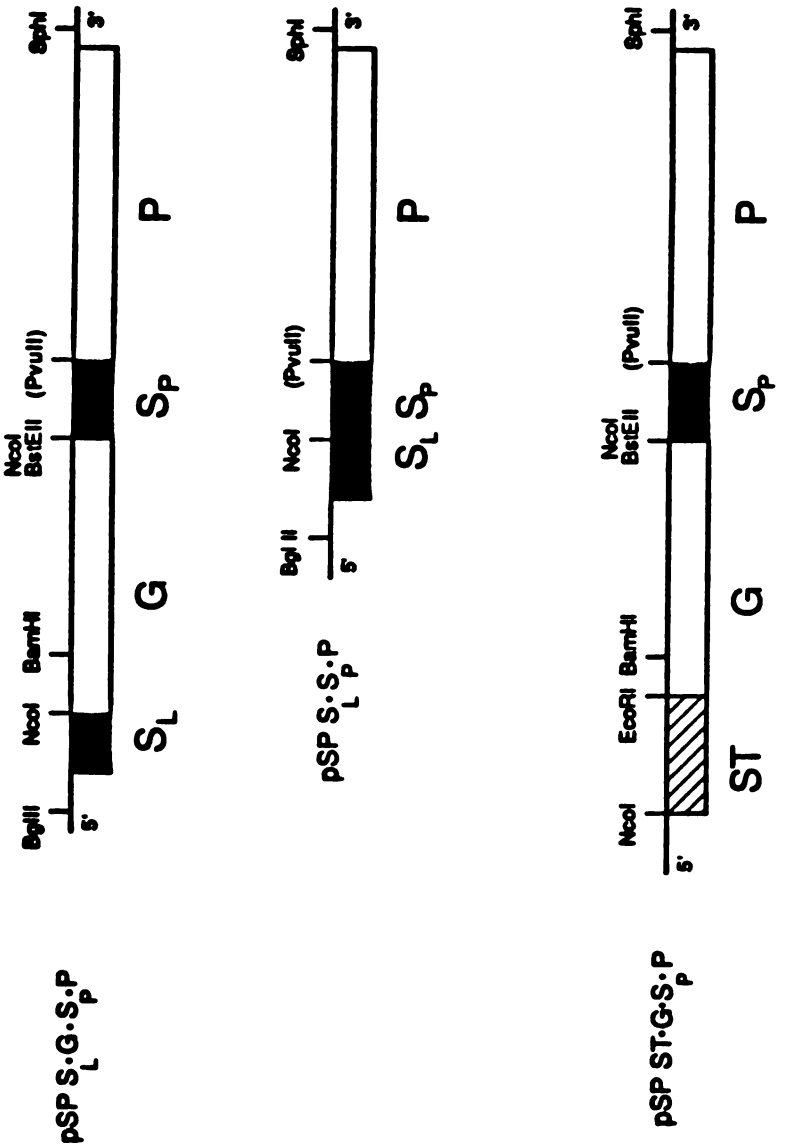
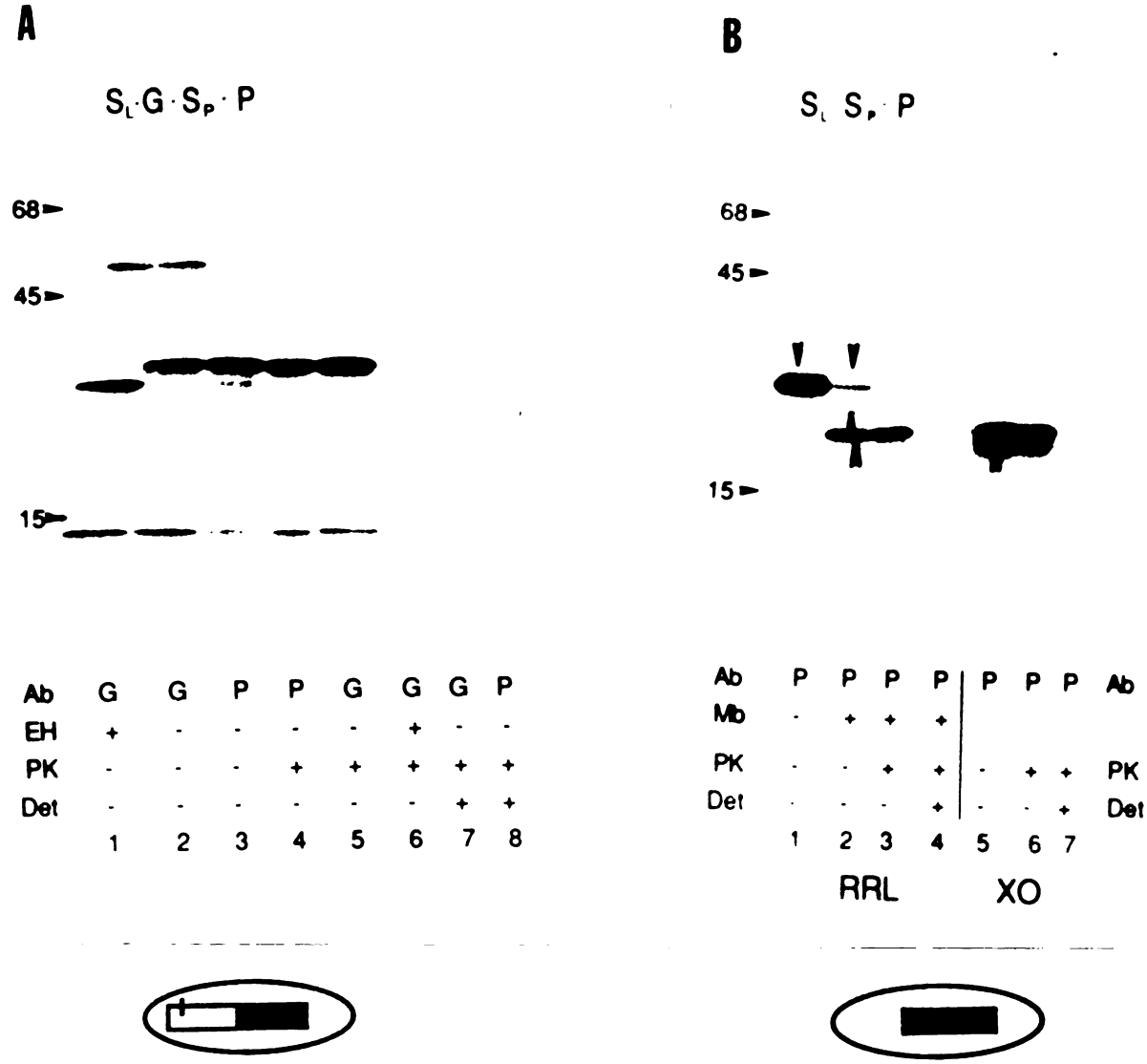

BglII
NcoI
BamHI
NcoI
BstEII
(PvuII)
SphI
5'
3'
S_L
G
S_P
P
pSP S_L·G·S_P·P
BglII
NcoI
(PvuII)
SphI
S_L S_P
P
pSP S_L·S_P·P
NcoI
EcoRI
BamHI
NcoI
BstEII
(PvuII)
SphI
ST
G
S_P
P
pSP ST·G·S_P·P

Figure 3-5. **Xenopus oocytes expression of fusions pSP S_L·G·S_P·P (A) and pSP S_L·S_P·P (B).** Xenopus occytes were injected with SP6 transcripts synthesized from pSP S_L·G·S_P·P or pSP S_L·S_P·P and labeled with [^{35}S] methionine. Xenopus occytes were homogenized, aliquoted, and subjected to post-translational proteolysis. Proteolysis products were immunoprecipitated with prolactin or globin antisera, and selected samples were digested with endoglycosidase H, followed by SDS-PAGE. *Ab*, antibody used for immunoprecipitation; *EH*, endoglycosidase H; *PK*, proteinase K; *Det*, Nikkol detergent. RRL products from pSP S_L.S_P·P (B, *lanes 1-4*) were used as markers. *Downward pointing arrowheads* (B) indicate full-length precursor molecules; *small and large upward pointing arrowheads* designate cleaved products from cleavage of the first or both signal sequences, respectively. Diagrams below the gels indicate inferred topology of products. *White and black bars* designate globin and prolactin domains, respectively. The *solid line* drawn in the globin domain indicates glycosylation. The positions of molecular weight (X10^3) markers are indicated.


A
$S_L \cdot G \cdot S_P \cdot P$
68
45
15
Ab G G P P G G G P
EH + - - - - + - -
PK - - - + + + + +
Det - - - - - - + +
1 2 3 4 5 6 7 8
B
$S_L \; S_P \cdot P$
68
45
15
Ab P P P P P P P Ab
Mb - + + +
PK - - + + - + + PK
Det - - - + - - + Det
1 2 3 4 5 6 7
RRL
XO

In Fig. 3-5B, expression of pSP S_L·S_P·P gave an analogous topology to that of S_L·G·S_P·P. The P passenger was fully protected in a fashion that was abolished by nonionic detergent, in both the RRL (lanes 1-4) and Xenopus oocytes (lanes 5-7). Thus, a second signal sequence did not prevent translocation of P, regardless of the distance between the topogenic sequences. However, in pSP S_L·S_P·P, cleavage of the second signal sequence was highly efficient (lanes 2-7).

When translocation was initiated by the stop transfer sequence (pSP ST·G·S_P·P), topology of the molecule was essentially identical to that obtained for both pSP ST·G and pSP ST·P in Fig. 2-5A. The first topogenic sequence (ST), directed translocation and glycosylation of the passenger, but remained accessible to protease (Fig. 3-6, lanes 1-3 versus 5-7). The second topogenic element (S_P) was completely translocated along with both passengers, as in pSP S_L·G·S_P·P, and was only rarely cleaved (lanes 3, 4 and 8) . Similar results were found in the RRL (data not shown).

The inability of S_p as a second element to terminate translocation in all three cases described does not reflect steric or other hindrance of signal sequence recognition specific to these molecules. The internal signal sequence functioned with high efficiency to initiate translocation in the identical fusion protein lacking the amino-terminal signal sequence (see Fig. 2-4B).

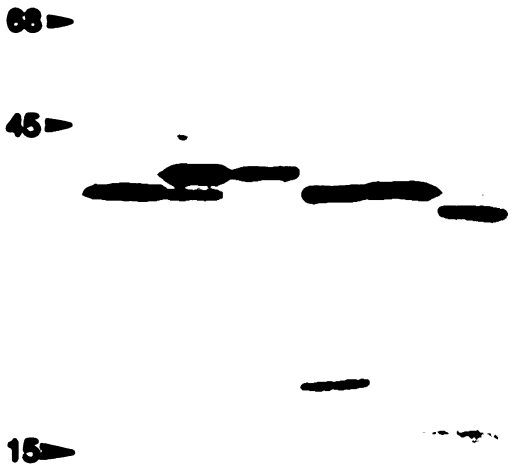
Thus, despite the apparent overlap in signal and stop transfer sequence activity revealed by the ability of a stop transfer sequence to inititate translocation, signal and stop transfer sequences are not functionally interchangeable. The results presented here suggest that the most promising approach for generating a polytopic transmembrane protein would be to use *combinations* of signal and stop transfer sequences.

DISCUSSION

Analysis of the expression products from the matched sets of constructions suggests

Figure 3-6. **Xenopus oocytes expression of the fusion pSP ST·G·S_P·P.** Xenopus occytes were injected with SP6 transcripts synthesized from pSP ST·G·S_P·P and labeled with [^{35}S] methionine. Xenopus oocytes were homogenized, aliquoted, and subjected to post-translational proteolysis. Proteolysis products were immunoprecipitated with prolactin or globin antisera, and selected samples were digested with endoglycosidase H, followed by SDS-PAGE. *Ab*, antibody used for immunoprecipitation; *EH*, endoglycosidase H; *PK*, proteinase K; *Det*, Nikkol detergent. Diagram below the gel indicates inferred topology of products. *White and black bars* designate globin and prolactin domains, respectively. The *solid line* drawn in the globin domain indicates glycosylation. The positions of molecular weight (X10^3) markers are indicated.

ST·G·S_p·P



Ab	G	G	P	P	G	G	G	P
EH	+	-	-	-	-	+	-	-
PK	-	-	-	+	+	+	+	+
Det	-	-	-	-	-	-	+	+
	1	2	3	4	5	6	7	8

that: (1) signal and stop transfer sequence share some, but not all functional properties; and, (2) that the action of a topogenic element is significantly influenced by its order of appearance in the growing chain. These conclusions were drawn from a series of precise fusion studies which employed cassettes encoding topogenic elements in the context of identical well-characterized passenger domains.

Fig. 3-7 summarizes the activities of S_P and ST as determined by an analysis of their effects on passenger domain translocation both alone (Chapter 2) and in the context of one another (Chapter 3). First, either S_P or ST appears to be capable of initiating translocation when presented either at the amino terminus or in an internal location (if the nascent polypeptide was not already being translocated) (see Figs. 2-4 and 2-5). Secondly, although both signal and stop transfer elements can direct translocation initiation, the transmembrane topologies of the resulting products are quite different: the product of signal sequence mediated translocation is entirely lumenal to the ER membrane, while the product of stop transfer mediated translocation spans the membrane, with the initial topogenic element at least partially exposed on the cytoplasmic face of the ER membrane. Thirdly, if translocation is already in progress, a subsequent signal or stop transfer sequence show different activities. While a stop transfer sequence serves to terminate translocation, a bona fide signal sequence appears to be functionally inert, unable to halt translocation across the ER. This 'rule' appears to apply regardless of whether translocation was initiated by the action of a signal or stop transfer element. That is, a subsequent signal sequence always behaved as a passenger, wheareas a stop transfer sequence consistently terminated translocation (compare Fig. 3-5 versus 3-2, and 3-6 versus 3-3).

These conclusions are reinforced by the demonstration that removal of the first topogenic element (either a signal sequence or stop transfer sequence) converts an otherwise inert internal signal sequence into a functional one. Likewise, removal of the

Figure 3-7. **Summary of the activities of S_p and ST as determined by their effect on the topology of defined passenger domains.**

↓ Activity	Topogenic Element: Signal sequence ($S^{\cdot}_{P}$)	Topogenic Element: Stop transfer sequence (ST)
1. Translocation initiation activity	Expressed if translocation has not yet been initiated (at amino terminus or internally)	Expressed if translocation has not yet been initiated (at amino terminus or internally)
2. Topology of product conferred (i.e. disposition of topogenic element)	Lumenal	Membrane-associated
3. Translocation termination activity	Not expressed	Expressed if positioned after a preceding signal or stop transfer sequence

first topogenic element converts an internal stop transfer sequence from one capable of directing translocation termination into one capable of initiating passenger domain translocation. Thus, the action of a topogenic sequence appears to be determined by the topogenic sequence that appeared earlier in the protein. In view of the prototypic nature of the sequences used here, we believe that our conclusions are general. However, these results need to be confirmed for additional examples of each topogenic element.

It is interesting to note that in the products generated from expression of pSP S_L·G·S_P·P, the initial signal sequence but not the second signal sequence was cleaved. Comparison of the size migration of the full length precursor generated in a cell-free translation system in the absence of microsomal membranes with that of the endo H digestion product (from XO), provides evidence for cleavage of the first signal sequence (data not shown). The lack of cleavage of the second signal might be the result of protein folding rendering the cleavage site inaccessible to signal peptidase. Support for this interpretation comes from study of a related molecule which lacks an amino terminal signal sequence. Expression of that molecule in Xenopus oocytes showed that the second (internal) signal is functional but inefficiently cleaved when in this position (Perara and Lingappa, unpublished observations). Hence in contrast to the conclusion from one recent study (Finidori et al., 1987), lack of cleavage does not imply lack of ability to function as a signal sequence when in an internal position.

In all cases observed here (Fig.s 3-2 and 3-3) and previously (Yost et al., 1983; Mize et al., 1986) a stop transfer sequence in the second postion (i.e. after translocation had been initiated by either a signal or stop transfer sequence) acted to terminate further translocation. The ability of ST to terminate translocation following a translocation initiation event was significant in that S_P was unable to terminate translocation when expressed in the identical context (compare Figs. 3-2 with 3-3 and

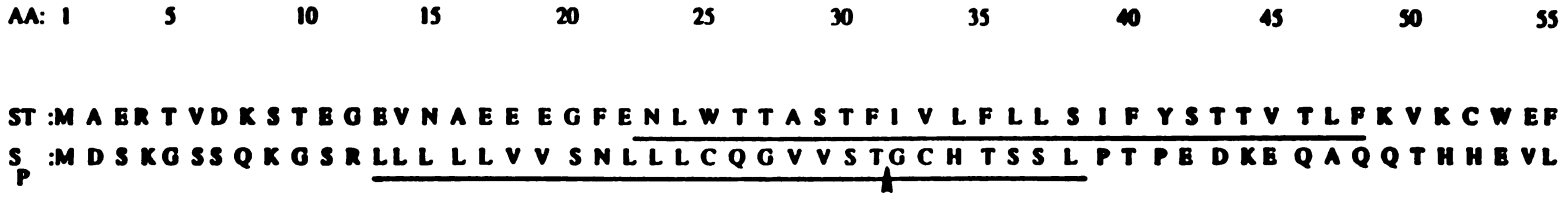
3-5 with 3-6). Although the property peculiar to the stop transfer sequence responsible for translocation termination remains unknown, one possible explanation would be a greater hydrophobicity of ST versus S_P in the identical internal context (Davis and Model, 1985).

To examine this possibility, we compared the hydropathy of the internal signal and stop transfer sequences used in our study (Fig. 3-8). The longest uninterrupted stretch of hydrophobic residues in S_P is 26 and in ST is 25. Hydrophobicity values for the most highly hydrophobic contiguous 25 amino acids in S_P and ST were 12.0 and 14.3, respectively (Eisenberg, 1984). Increasing the length of the sequences compared to 26 increases each index by approximately 1. Thus, the hydrophobicity of ST and S_P in this context is quite similar. It is therefore difficult to ascribe the failure of S_P to terminate translocation solely to insufficient hydrophobicity. Although ST has been reported to be relatively less hydrophobic than other putative ST sequences (Pillai and Baltimore, 1987), it is the only sequence used here which shows translocation termination activity. These observations are consistent with a receptor-mediated event necessary for termination of translocation that is directed by ST, but not by S_P.

The dramatic effect of position of the stop transfer sequence on translocation activity is shown in pSP ST·G·ST·P, where the initial stop transfer sequence *initiates* translocation and the subsequent one *terminates* the process (Fig. 3-3). The physiological significance of the transmembrane orientation generated by the unorthodox signal-like behavior of a stop transfer sequence remains to be determined. This activity may reflect a common receptor-mediated step in the mechanism of both initiation and termination, as described in Chapter 2 (see also Mize et al., 1986). Conversely, the failure of S_P to terminate translocation may reflect a step *not* shared between two related receptor-mediated events. In either case, this molecule is polytopic only by virtue of the approximately 30 cytoplasmically disposed amino acid residues

Figure 3-8. **Sequence comparison of S_P and ST.** One letter code amino acid sequences of the topogenic sequences, S_P and ST studied here. *S_P* refers to the prolactin signal sequence of bovine preprolactin plus the first amino acid of authentic prolactin; *ST* refers to the stop transfer sequence cassette derived from the IgM u heavy chain transmembrane region. *Arrowhead* indicates the end of the signal sequence cassette, not the cleavage site. The sequence shown for ST is the entire cassette. Methionine in position 1 of ST and of S_P is encoded in the Nco 1 site at the 5' end of the respective cassettes. A blunted Sal 1 site encoding glycine 32 in the case of S_P and a filled in Eco R1 site encoding phenylalanine 55 of the ST, represent the 3' ends of the respective cassettes. Additional sequences of the flanking passenger domain are shown in the case of S_P, 31 codons, for purposes of comparison to ST whose cassette is longer (55 codons). Numbers indicate amino acids from the methionine with which each element begins. Underlined residues indicate the 26 most hydrophobic contiguous residues used for hydropathy analysis as discussed in the text.

COMPARISON OF TOPOGENIC ELEMENTS AND PASSENGER SEQUENCE CONTEXTS



presumably derived from the initial stop transfer sequence itself (see Fig. 3-3, diagram below the gel). As mentioned above, proteolysis analysis of this molecule does not allow definitive interpretation of the topology of ST·G·ST·P. Thus, for *this* molecule, polytopic orientation is not definitive, since a transmembrane disposition of the first stop transfer element can only be inferred from the topology of ST·G (Fig. 2-5).

CHAPTER 4:

CONSTRUCTION OF DEFINED POLYTOPIC TRANSMEMBRANE PROTEINS EMPLOYING ALTERNATING SIGNAL AND STOP TRANSFER SEQUENCES

INTRODUCTION

The signal hypothesis predicts that polytopic transmembrane proteins (TMPs) are 'stitched' into the ER membrane by the sequential action of alternating signal and stop transfer sequences (Blobel, 1980). Several lines of experimental evidence support this view, although up to the present time this model of polytopic TMP biogenesis has not been directly tested.

Deletion and fusion experiments employing bovine opsin showed that this polytopic TMP encodes multiple domains with topogenic sequence activity, that is, regions which mediate domain translocation in an SRP-dependent fashion (Audigier et al., 1987). However, the fusion approach employed in these studies did not allow interpretation of the precise role of an individual topogenic sequence in bringing about a transmembrane phenotype. The use of reporter domains to trace the transmembrane disposition of variation deletion mutants of another polytopic TMP (Boyd et al., 1986) also provided limited information since the function of individual topogenic sequences was not well-characterized. In addition, in those studies, investigators were unable to control for the effects of passenger domains, including the reporter domain itself, on the final topology of the encoded product.

The controlled analysis of the activity of topogenic sequence cassettes in the context of fixed 'inert' passenger domains provides a framework for directly testing the hypothesis that a polytopic TMP can be directed into a precise transmembrane disposition by the action of multiple signal and stop transfer sequences. As demonstrated in Figs. 3-2 and 3-3, of those molecules containing two topogenic sequences, only those with ST in the second position were demonstrated to have bitopic transmembrane topologies. We therefore reasoned that a bitopic protein (e.g. S·*x*·ST·*y*, illustrated in Fig. 4-1A) could be converted into a polytopic TMP if: (1)

translocation could be reinitiated subsequent to termination (see Fig. 4-1B); and, (2) the stop transfer sequence responsible for termination of translocation remained anchored in the membrane in spite of reinitiation of translocation.

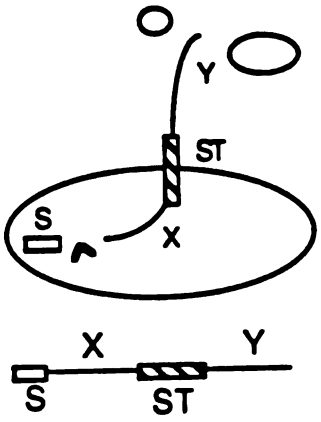
Results

To build a molecule which might generate polytopic topology, we started with a bitopic protein and introduced a third topogenic sequence and another passenger domain (to serve as a marker for reinitiation of translocation). This scheme is illustrated in Fig. 4-1B. The need for a defined bitopic fusion protein coding region and for a third passenger was fulfilled by utilizing pSP S_L·L·ST·G. The topology of this molecule has been well-characterized previously (Yost et al., 1983). It contains two of the three topogenic sequences used in the present work (S_L and ST) as well as one of the passenger domains (G). The ability of a third topogenic sequence and passenger to convert an encoded bitopic fusion into one of polytopic orientation could be assessed by replacing the globin coding region in pSP S_L·L·ST·G with pSP G·S_P·P, pSP G·ST·P, or pSP G·P and analyzing the topology of the encoded products in Xenopus occytes. The three resulting molecules (pSP S_L·L·ST·G·S_P·P, pSP S_L·L·ST·G·ST·P, and pSP S_L·L·ST·G·P), diagrammed in Fig. 4-2, differ only in the presence of S_P, ST, or no topogenic sequence before the subsequent P domain.

Expression of the transcription products of pSP S_L·L·ST·G·P in Xenopus oocytes resulted in a molecule of the expected size that was immunoreactive to beta-lactamase, globin, and prolactin antisera (Fig. 4-3, lanes 1-3). As expected, proteolysis with proteinase K generated a 20 kDa beta-lactamase-reactive band (which was proteolyzed in the presence of detergent) (lanes 4 and 10). In this molecule, which lacks a third topogenic sequence between the G and P passenger domains, no globin- or prolactin

Figure 4-1. **Highly schematic representation of the expermental scheme for construction of a defined polytopic integral transmembrane protein.** **A:** *Line diagram* below cartoon indicates the coding region of a bitopic transmembrane protein. *S*, signal sequence, described by an unfilled rectangle; *ST*, stop transfer sequence, described by a stripped rectangle ; *X* and *Y*, immunologically distinct passenger cassettes, described as solid lines. The topology of such a molecule is designated in the *cartoon above*: the *arrowhead* indicates signal cleavage; the *large oval* represents a microsomal vessicle; the *two smaller ovals* indicate the ribosomal subunits. In Panel **B**: the *line diagram* below includes a third topogenic element (either a signal or a stop transfer sequence), as well as a third passenger domain, *Z*, which serves as a 'reporter' for reinitiation of translocaiton. The cartooon above shows the bitopic transmembrane protein in its transmembrane disposition with the third topogenic element (and the following passenger domain) just emerging from the large ribosomal subunit.

A.


Y
ST
S
X
X
Y
S
ST

B.

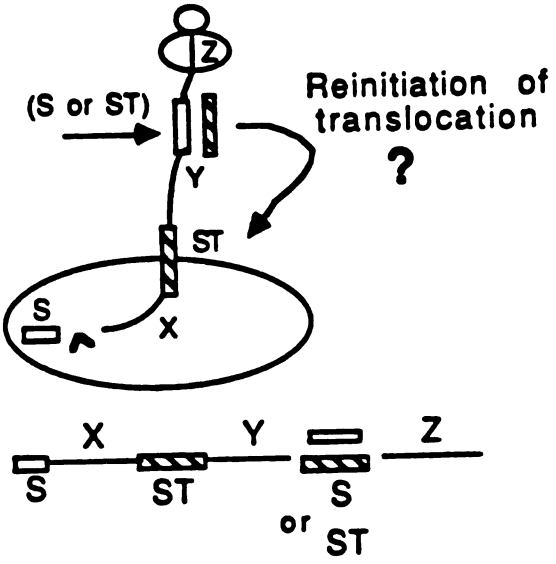

Z
(S or ST)
Reinitiation of translocation
?
Y
ST
S
X
X
Y
Z
S
ST
S
or ST

Figure 4-2. **Restriction maps and coding regions of fusions: pSP S_L·L·ST·G·S_P·P, pSP S_L·L·ST·G·ST·P, and pSP S_L·L·ST·G·P.** Restriction sites are indicated above the lines, and defined coding regions below. Coding regions are represented as described for Fig. 2-3, with the following addition: L, beta-lactamase passenger region from codon 1-163.

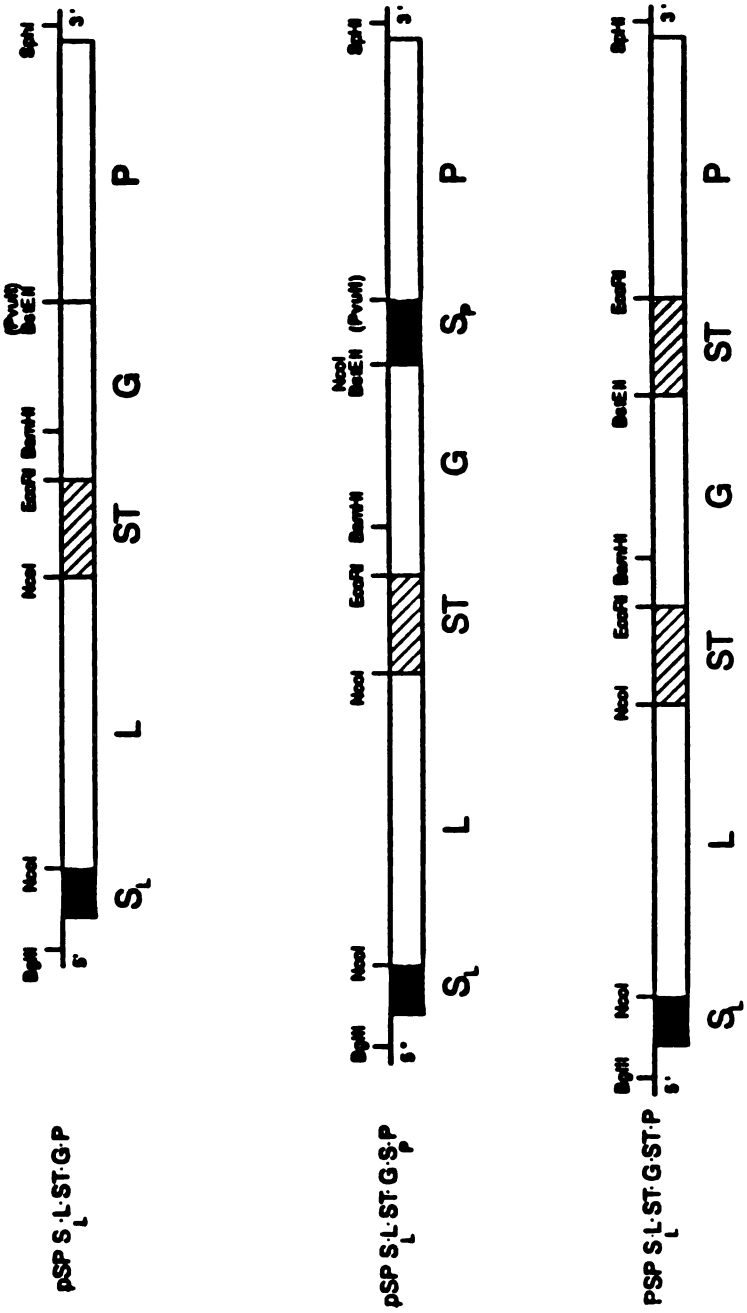
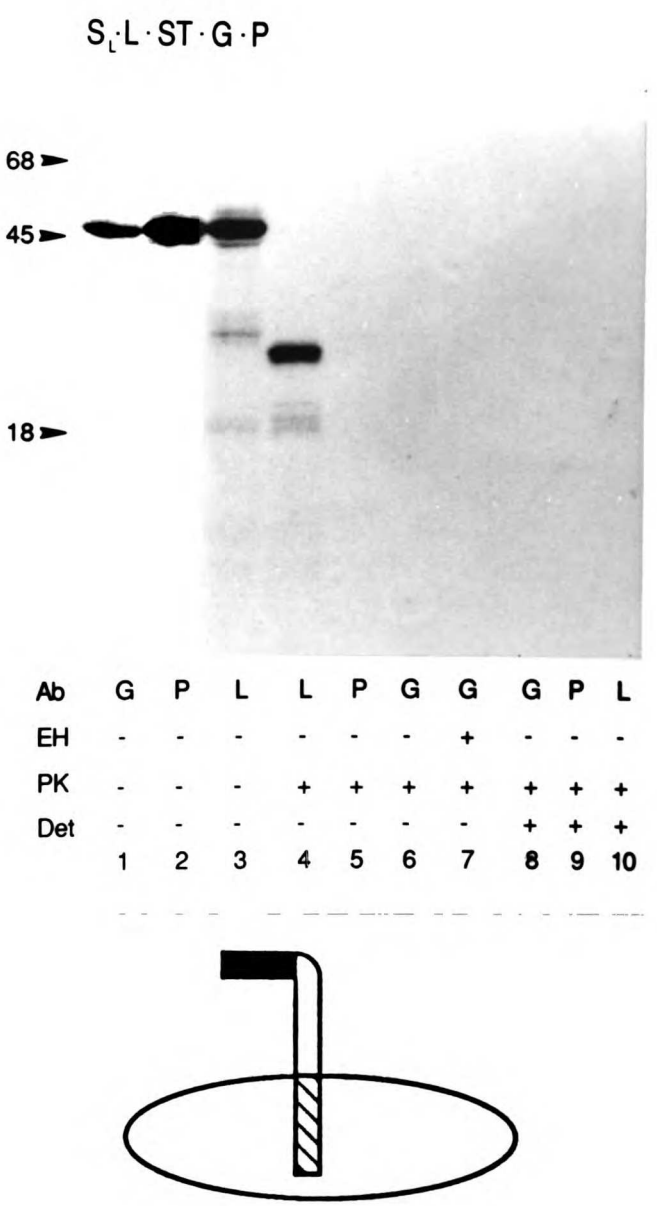

pSP S·L·ST·G·P
L
BglII
NcoI
NcoI
EcoRI
BamHI
BstEII (PvuII)
BglII
5'
3'
S
L
ST
G
P
pSP S·L·ST·G·S·P
P
P

Figure 4-3. **Xenopus occyte expression of pSP S_L·L·ST·G·P (i.e. no third topogenic element).** Xenopus occytes were injected with SP6 transcripts synthesized from pSP S_L·L·ST·G·P, labeled with [^{35}S] methionine, homogenized, aliquoted, and subjected to post-translational proteolysis with proteinase K. Proteolysis products were immunoprecipitated with beta-lactamase (*L*), prolactin (*P*), or globin (*G*) antisera, and selected samples were digested with endoglycosidase H, followed by SDS-PAGE. *Ab*, antibody used for immunoprecipitation; *EH*, endoglycosidase H; *PK*, proteinase K; *Det*, Nikkol detergent. Diagrams below the gels indicate inferred topology of products. *Stripped, white and black bars* designate beta-lactamase, globin and prolactin domains, respectively. Molecular weight (X10^3) markers are indicated.


S_L·L·ST·G·P
68
45
18
Ab G P L L P G G G P L
EH - - - - - - + - - -
PK - - - + + + + + + +
Det - - - - - - - + + +
1 2 3 4 5 6 7 8 9 10

reactive protease-protected products were observed (lanes 5-9). This is consistent with a bitopic transmembrane disposition (diagram below the gel), as expected.

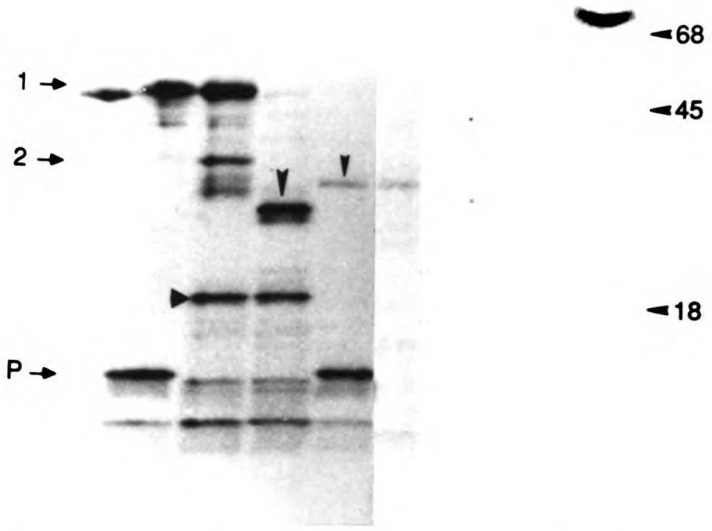
When the product encoded by pSP S_L·L·ST·G·S_P·P was similarly analyzed a slightly larger molecule was observed (as expected due to the presence of the additional S_P domain) (Fig. 4-4, arrow labeled 1). This product was immunoreactive to all three antisera, and generated a 20 kDA beta-lactamase-reactive band upon proteolysis with proteinase K, as observed for pSP S_L·L·ST·G·P (downward pointing arrowhead in lane 4). In addition, a new fragment was generated upon proteolysis. This band (lane 5, downward pointing arrowhead) was approximately 40 kDA in size, both globin- and prolactin-immunoreactive, glycosylated, and digested by protease in the presence of detergent (lanes 5-9). Its size was that predicted for protease protection of the G·S_P·P domain of the full-length molecule.

Another prolactin-reactive fragment was observed before and after proteolysis in the absence of detergent which migrates at the size of the P domain of pSP S_P·P (lanes 2 and 5; see arrow P). This product presumably resulted from signal peptidase activity following reinitiation of translocation by the third topogenic sequence. The relative intensity of the protected bands representing G·S_P·P versus P suggests that approximately 20% of the protected fragments were not processed by signal peptidase.

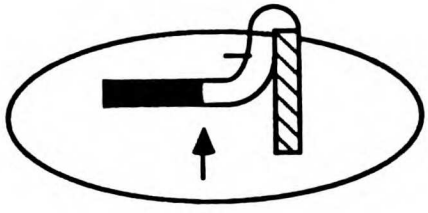
Experiments were carried out to confirm that this prolactin fragment (arrow labeled 'P' in Fig. 4-4) was derived from the full- length precursor (S_L·L·ST·G·S_P·P), and not from an internally initiated product of protein synthesis (i.e., S_P·P). Oocytes were injected with transcript in the presence or absence of hydroxyleucine; incorporation of this leucine analog into nascent chains prevents translocation or signal cleavage from occuring by inhibiting the interaction of the signal sequence with SRP (Hortin and Boime, 1980; Walter and Blobel, 1981b; Simon et al., 1987). If 'P' is derived from the full length precursor, incubation with hydroxyleucine should result in

Figure 4-4. **Xenopus oocyte expression of pSP S_L·L·ST·G·S_P·P (i.e. signal sequence as third topogenic element).** Xenopus occytes were injected with SP6 transcripts synthesized from pSP S_L·L·ST·G·S_P·P, labeled with [^{35}S] methionine, homogenized, aliquoted, and subjected to post-translational proteolysis with proteinase K. Proteolysis products were immunoprecipitated with beta-lactamase (*L*), prolactin (*P*), or globin (*G*) antisera; selected samples were digested with endoglycosidase H, followed by SDS-PAGE. *Ab*, antibody used for immunoprecipitation; *EH*, endoglycosidase H; *PK*, proteinase K; *Det*, Nikkol detergent. *Arrow 1*, full-length product; *arrow 2*, beta-lacatamase- and globin-immunoreactive fragment which co-migrates in *lanes 1 and 3*. A globin-immunoreactive band indicated in *lane 1* is visible only on longer exposure of the gel. It is not visible here because of the relatively poor reactivity of the globin antisera used. *Arrow P*, prolactin-immunoreactive fragment resulting from cleavage after S_P. *Downward pointing arrowhead (lane 4)*, lactamase-protected fragment co-migrating with that seen in A. *Triangle in lane 3*, a lactamase-immunoreactive product presumed to represent a population of molecules that terminated synthesis prematurely. *Downward pointing arrowhead, lane 5*, a prolactin- and globin-immunoreactive fragment that is glycosylated and protected. *Lanes 1-5 and 6-10* were from separate experiments. Diagram below the gel indicates inferred topology of product. *Stripped, white and black bars* designate beta-lactamase, globin and prolactin domains, respectively. The *solid line* drawn in the globin domain indicates glycosylation. The arrow designates cleavage after S_P. Molecular weight (X10^3) markers are indicated.

$S_L \cdot L \cdot ST \cdot G \cdot S_P \cdot P$



Ab	G	P	L	L	P	G	G	G	P	L
EH	-	-	-	-	-	-	+	-	-	-
PK	-	-	-	+	+	+	+	+	+	+
Det	-	-	-	-	-	-	-	+	+	+
	1	2	3	4	5	6	7	8	9	10



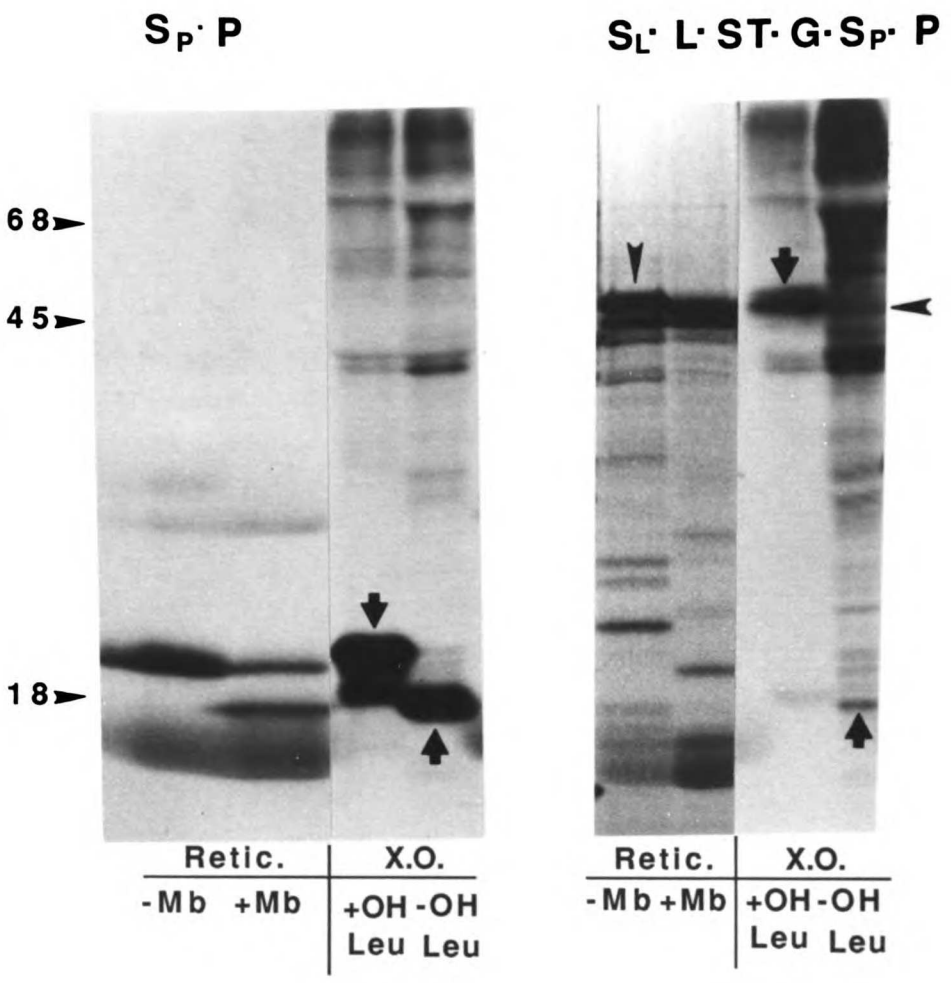
the appearance of S_L·L·ST·G·S_P·P, and not an S_p·P-sized band. Fig. 4-5 shows the results of such an analysis.

The left hand panel describes an experiment in which Xenopus oocytes were injected with the transcript encoding S_P·P, in the presence of hydroxyleucine. The position of the precursor and translocated product are identified from expression of pSP S_P·P in the RRL (Fig. 4-5, lanes 1 and 2, respectively). Injection of transcript into XO in the absence of hydroxyleucine gives only the mature, processed product (upward pointing arrowhead, lane 4). As expected, coinjection of hydroxyleucine results in accumulation of large amounts of the precursor, S_P·P (downward pointing arrowhead, lane 3).

When the products generated from expression of pSP S_L·L·ST·G·S_P·P were similarly analyzed, the precursor which appears when oocytes were coinjected with hydroxyleucine is the full-length molecule S_L·L·ST·G·S_P·P (Fig. 4-5B, downward pointing arrowhead, lane 3), and not S_P·P. The faint band which is slightly shifted-up from the position of 'P' (lane 3) represents molecules in which hydroxyleucine was only partially incorporated and therefore, managed to 'escape' the translocation block. Such a species was similarly observed in the analysis of S_p·P in the presence of hydroxyleucine (Fig. 4-5A, lane 3). Significantly, the absence of any product which migrated at the position of S_P·P (when transcript encoding pSP S_L.L·ST·G·S_P·P was injected in the presence of hydroxyleucine), leads us to conclude that all of the 'P' fragments described in Fig. 4-4 were derived from reinitiation of translocation by the third topogenic element of S_L·L·ST·G·S_P·P .

Consistent with this interpretation, a protease-sensitive fragment which is both beta-lactamase- and globin-immunoreactive, and of predicted size for the remainder of the molecule after peptidase cleavage, is observed (Fig. 4-4, see arrow 2). The expected globin reactivity of this fragment is seen on longer exposure (data not shown).

Figure 4-5. **Expression of pSP S_P·P and pSP S_L·L·ST·G·S_P·P in Xenopus oocytes in the absence or presence of hydroxyleucine.** Xenopus occytes were injected with SP6 transcripts synthesized from pSP S_P·P (**A**) or pSP S_L·L·ST·G·S_P·P (**B**), and incubated for two hours, after which they were injected with [^{35}S] methionine, in the presence or absence of hydroxyleucine, *OH Leu* (see Methods for details). After incubation, oocytes were homogenized and immunoprecipitated with prolactin (P) antisera; products were separated by SDS-PAGE. Transcription products were also synthesized in the RRL (*Retic.*) system in the absence or presence of microsomal membranes (*Mb*). In **A**: *downward pointing arrowhead, (lane 3),* full-length preprolactin precursor; *upward pointing arrowhead (lane 4),* position of processed prolactin; In **B**: *downward pointing arrowheads, (lanes1 and 3),* full-length precursor, S_L·L·ST·G·S_P·P; *upward pointing arrowhead (lane 4),* prolactin-immunoreactive fragment resulting from cleavage after S_P; *sideward pointing arrowhead (lane 4),* processed form of full-length precursor, S_L·L·ST·G·S_P·P. Molecular weight (X10^3) markers are indicated.

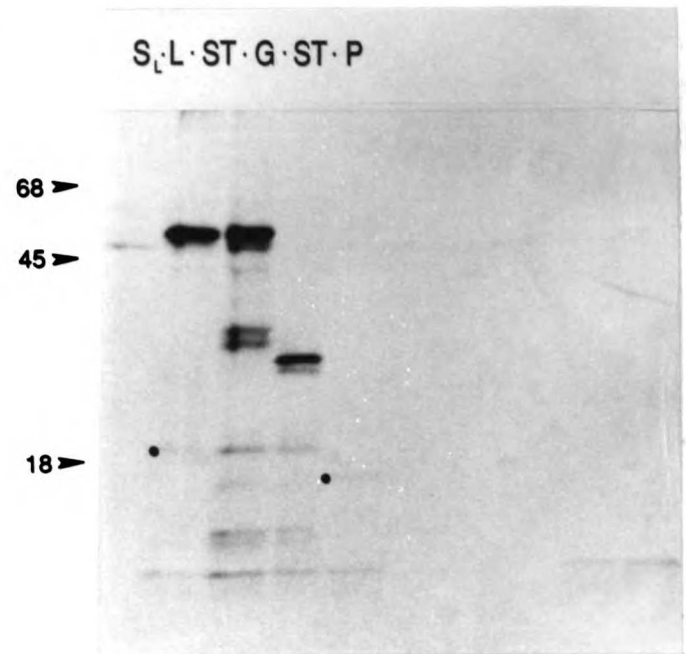

$S_P \cdot P$
$S_L \cdot L \cdot ST \cdot G \cdot S_P \cdot P$
68
45
18
Retic.
X.O.
-Mb +Mb
+OH -OH
Leu Leu
Retic.
X.O.
-Mb +Mb
+OH -OH
Leu Leu

It seems likely that both cleaved and uncleaved P domains represent molecules of a common topology which differ only in having been processed by signal peptidase. The topology of the product encoded by pSP S_L·L·ST·G·S_P·P is thus polytopic, as indicated in the diagram below Fig. 4-4.

Analysis of the expression product of pSP S_L·L·ST·G·ST·P, in which ST is the third topogenic sequence, showed that the majority of chains were not polytopic. As described for the previous two molecules, expression in XO gave a product of the predicted size, which was reactive to beta-lactamase, globin, and prolactin antisera, and which generated a beta-lactamase immunoreactive, protease-resistant fragment (Fig. 4-6, lanes 1-4). No protease-resistant molecules displaying both globin and prolactin immunoreactivity were evident; however, a faint protected fragment reactive to prolactin antisera alone was observed (lanes 5-9). It has not been possible to determine whether this fragment is generated from full-length molecules in which translocation was reinitiated by ST or results from an internal initiation product (lane 2, dot). In any case, the majority of the products encoded by pSP S_L·L·ST·G·ST·P have a bitopic topology (diagram below the gel), indicating molecules in which ST did not reinitiate translocation.

Densitometry of the results, presented in Fig. 4-7, revealed that the presence of a signal sequence as a third topogenic element results in at least a 24-fold increase in reinitiation of passenger domain translocation. The uncleaved G·S_P·P fragment accounted for approximately 16% of the translocated molecules, with the remainder resulting from the smaller, cleaved prolactin fragment (See Fig. 4-4, lanes 5 and 9). Taken together, approximately 50% of S_L·L·ST·G·S_P·P molecules reinitiated translocation resulting in a polytopic orientation. In contrast, the prolactin-reactive protease protected band seen in pSP S_L·L·ST·G·ST·P accounted for approximately 12% or less of

Figure 4-6. **Xenopus oocyte expression of pSP S_L·L·ST·G·ST·P (i.e. ST as third topogenic element).** Xenopus oocytes were injected with SP6 transcripts synthesized from pSP S_L·L·ST·G·ST·P, labeled with [^{35}S] methionine, homogenized, aliquoted, and subjected to post-translational proteolysis with proteinase K. Proteolysis products were immunoprecipitated with beta-lactamase (*L*), prolactin (*P*), or globin (*G*) antisera, and selected samples were digested with endoglycosidase H, followed by SDS-PAGE. *Ab*, antibody used for immunoprecipitation; *EH*, endoglycosidase H; *PK*, proteinase K; *Det*, Nikkol detergent. *Dots (lanes 2 and 4)*, indicate the positions of ST·P and P products from the RRL translation; these co-migrate with oocyte products in the absence and presence of proteolysis, respectively, which are more easily visible on a longer exposure of the gel. Diagram below the gel indicates inferred topology of product. *Stripped, white and black bars* designate beta-lactamase, globin and prolactin domains, respectively. Molecular weight (X10^3) markers are indicated.



Ab	G	P	L	L	P	G	G	G	P	L
EH	-	-	-	-	-	-	+	-	-	-
PK	-	-	-	+	+	+	+	+	+	+
Det	-	-	-	-	-	-	-	+	+	+
	1	2	3	4	5	6	7	8	9	10

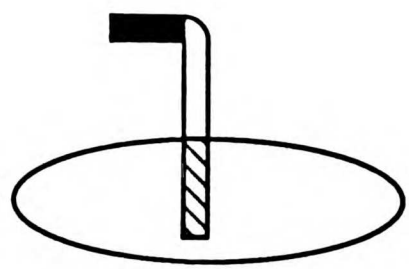
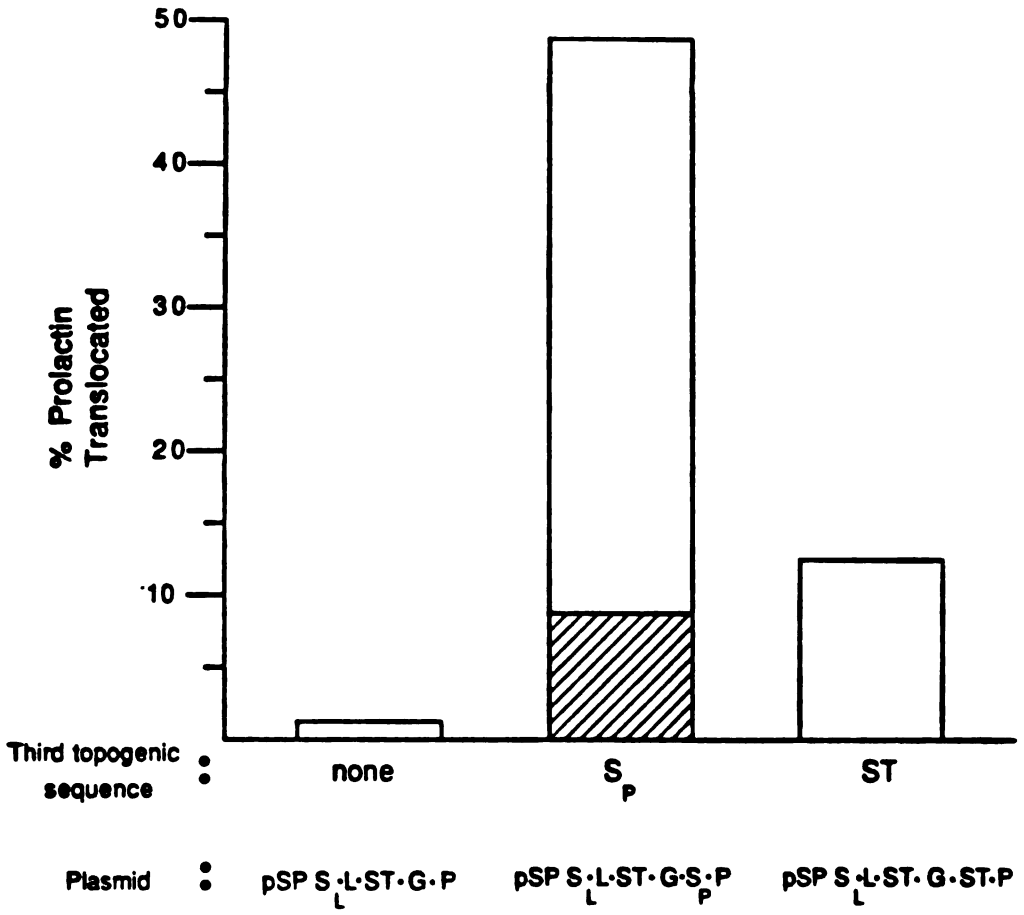


Figure 4-7. **Percentage prolactin protection conferred by third topogenic element.** Prolactin-immunoreactive precursor and protease protected bands in Figs. 4-3, 4-4, and 4-6 were quantified by densitometry. The percentage prolactin translocated is the ratio of protected molecules to precursor molecules, corrected for efficiency of protection in each experiment, defined by the beta-lactamase protection. The absolute value of each band was corrected for methionine distribution by dividing the absolute number of densitometric units measured for the band of interest by the number of methionines present in the protein domain represented. This allowed direct comparison of full-length proteins and proteolytic fragments. To quantify autoradiograms, a series of exposures were analyzed to ensure that all values were from the linear region of the x-ray film. *Bar* S_p, includes both the cleaved (*white bar*) and the uncleaved (*stripped bar*) chains.


50
40
30
20
10
% Prolactin Translocated
Third topogenic sequence
none
S P
ST
Plasmid
pSP S L·L·ST·G·P
pSP S L·L·ST·G·S P·P
pSP S L·L·ST·G·ST·P

all chains. Thus, reinitiation of translocation in Xenopus oocytes appears to be effectively mediated by a signal sequence, but not a stop transfer sequence in this context.

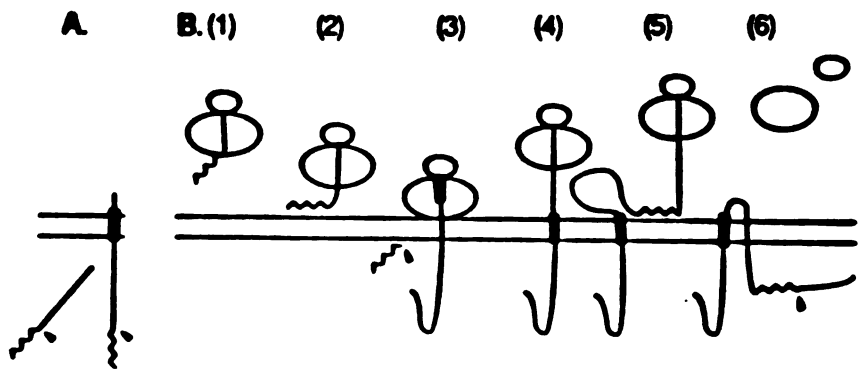
DISCUSSION

We have approached the problem of polytopic TMP biogenesis by attempting to build such a molecule from a series of alternating signal and stop transfer sequences derived from products of simple topology. This involved analysis of the expression of a hierarchy of constructions whose encoded products were progressively more complex. First, individual passenger domains without and then with different individual topogenic sequences were characterized (Chapter 2). Then, the topology of fusion proteins containing various patterns of two topogenic sequences was compared (Chapter 3).

The results described from the expression of plasmids encoding two topogenic sequences suggested to us that positioning the topogenic sequences in either of two patterns (S-ST-S or S-ST-ST) might result in the assembly of a protein with a polytopic transmembrane disposition. As described in Figs. 4-4 and 4-6, the former but not the latter resulted in efficient polytopic disposition of protein passenger domains. Based on the experimentally determined behavior of the signal and stop transfer sequences employed here, a simplified model of likely stages in the biogenesis of this TMP is described in Fig. 4-8.

Our results show that at least one class of polytopic membrane proteins can be assembled using carefully characterized topogenic elements and passenger domains. However, many conceivable topologies were not achieved. Although varying patterns of simple topogenic elements employed here might generate alternate TMP topologies, we suggest a role for topogenic sequences distinct from classical signal and stop transfer

Figure 4-8. **Assembly of a polytopic transmembrane protein employing alternating signal and stop transfer sequences.** Signal sequences taken from the amino terminus of two secretory proteins, and a stop transfer sequence from a bitopic transmembrane protein, were employed in protein fusions to generate a molecule with transmembrane topology. The *long double horizontal lines* represent the membrane of the ER which separates the cytoplasm from the lumen of the ER (above and below the double lines, respectively). In **A**, a secretory and bitopic transmembrane protein (from which topogenic sequences for assembling a polytopic protein were derived) are shown. The *arrowheads* designate peptidase cleavage of the signal sequence, which occurs in the lumenal space, following translocation. Signal sequences are designated by *jagged lines*, and the stop transfer sequence as a *solid rectangle*. A highly schematic illustration of the proposed steps in the assembly of the polytopic protein S_L·L·ST·G·ST·P is shown in **B** as follows: 1, an amino-terminal signal sequence emerges from the ribosome; 2, the signal sequence is recognized by the translocation machinery, resulting in targeting of the nascent polypeptide-ribosome complex to the ER membrane; 3, translocation into the lumenal space is initiated, and the signal sequence is cleaved, as indicated by the *arrowhead* (the ST sequence is shown in this frame as it is being synthesized); 4, the ST sequence terminates translocation, resulting in detachment of the ribosome from the membrane and continued elongation of the nascent polypeptide in the cytoplasmic space; 5 and 6, a second signal sequence is synthesized and targeted to the ER membrane where it directs translocation of both flanking domains. When the termination codon is reached, the completed protein is released from the ribosome, and the ribosomal subunits dissociate. In a subset of molecules, the second signal sequences is cleaved, as indicated by the *arrowhead* in the last frame.


A.
B.(1)
(2)
(3)
(4)
(5)
(6)

varieties.

In nature there exist integral membrane proteins such as influenza neuraminadase (Bos et al., 1984), rhodopsin (Friedlander and Blobel, 1985), asialoglycoprotein receptor (Spiess and Lodish, 1985; Holland and Drickamar, 1986), transferrin receptor (Zerial et al., 1986); Ia antigens (Lipp and Dobberstein, 1986) and Hepatitis B surface antigen (Eble et al., 1986), which contain single topogenic elements with both signal and stop transfer activity. It may be the case that these elements can be dissected into functional components with the properties proposed for signal and stop transfer sequences. It is also possible that these represent novel topogenic sequences distinct from the classes of elements described here.

Experiments involving insertion and permutation of putative topogenic element cassettes in the context of the defined passenger domains studied here could distinguish between the possibilities described above. In addition, application of the present approach also provides a practical point of reference (in the form of well-characterized passenger domains and topogenic sequences in matched sets of constructions) with which to search for novel topogenic sequences in proteins reported to adopt an unusual transmembrane topology (Hay et al., 1987a; Yost et al., submitted).

CHAPTER 5 (APPENDIX):

PROBING THE MECHANISM OF TRANSLOCATION: UNCOUPLING PROTEIN SYNTHESIS FROM TRANSLOCATION

INTRODUCTION

One of the distinguishing mechanistic features of secretory and transmembrane protein translocation in higher eukaryotes is that segregation into the cisternae of the ER appears to be tightly coupled to protein synthesis. This feature was initially recognized when nascent secretory chains were observed to be synthesized on ribosomes attached to the surface of the ER (Palade, 1975). Further analysis revealed that in cell-free systems, microsomal membranes must be present cotranslationally in order for translocation of secretory proteins to occur. Nearly every wild-type and fusion protein containing translocation initiation information (including hybrid proteins encoding an amino terminal stop transfer sequence) have been found to display this strict cotranslational requirement for translocation.

The apparent obligate coupling of synthesis, and translocation across the ER membrane distinguishes this membrane system from that of mitochondria and chloroplasts, where translocation occurs posttranslationally (Dobberstein et al., 1977; Maccechini et al, 1979). Similarly, bacterial proteins (Randall, 1983; Koshland and Botstein, 1982) and at least some proteins in yeast (Hansen et al., 1987) have been shown to be translocated after protein synthesis is complete.

What is the mechanistic significance of cotranslational translocation? Some workers speculated that the driving force for translocation is derived from work done by the ribosome, literally 'pushing' the nascent chain across the membrane (Wickner and Lodish, 1985). However, the apparent dependence of translocation on simultaneous protein synthesis presented a technical obstacle for experimentally testing hypotheses such as this one, concerning the mechanistic requirements for the translocation event itself.

The observation that a protein domain could be translocated after its synthesis was

complete (see Perara and Lingappa, 1985 and Chapter 2, Fig. 2-4B), provided a conceptual insight which led us to attempt to experimentally dissociate translocation across the ER membrane from translation. It occured to us that we might be able to employ an experimental trick using fusion proteins, to investigate the coupling of protein synthesis and translocation in eukaryotes.

Our strategy was to generate a substrate in which translation had been completed, but which retained all the essential information required for carrying out a productive interaction with the ER membrane. Thus, a plasmid DNA encoding a protein with an amino terminal signal sequence joined to a passenger domain, was cleaved at a restriction site 5' to the termination codon of the coding region. Upon in vitro transcription and translation of such a molecule, the initial engaged ribosome should read to the truncated 3' end of the transcript and be unable to release the nascent chain for lack of a termination codon.

This 'dangling' nascent chain (which was still attached to the ribosome) could be assayed for translocation competence, in the absence of protein synthesis. Addition of inhibitors of initiation and elongation would ensure the lack of ongoing translation. In our in vitro reconstitution system, presentation of microsomal membranes to the nascent chain ribosome complex would serve to explore whether translocation could occur independently of translation, and also, provide a means for studying mechanistic requirements for transport across the ER membrane.

At the time this study was initiated we were interested in analyzing the requirements for *signal* sequence directed translocation; these questions have been extended in the present work for *stop transfer* directed translocation as well.

Results

For these studies we chose pSP SG1, an expression plasmid that encodes a fusion

protein consisting of the lactamase signal sequence fused to the amino terminus of chimpanzee alpha-globin, and with a glycosylation site engineered into the globin sequence. A related construction, pSP SG 3, encodes the identical passenger domain, but does not include the initial amino terminal signal sequence. A third construction, pSP ST·G* encodes the same globin coding region as described for pSP SG1 but is fused at its 5' end of the IgM heavy chain stop transfer sequence. (For the remainder of this chapter pSP ST·G* will be referred to simply as pSP ST·G, although it differs from that plasmid described in Chapter 2, in that it includes the entire globin coding region). The restriction maps and coding regions of these three constructions are shown in Fig. 5-1.

When the cell-free transcription products from pSP SG1 were expressed in RRL, a globin immunoreactive product of the correct size was observed (Fig. 5-2A, lane 1). A minor species, generated from internal initiation of protein synthesis was also seen (upward pointing arrowhead, lane1). When membranes were present cotranslationally, nascent preSG1 was converted to a higher molecular weight form (lane 3), which by endo H analysis was demonstrated to be a glycosylated signal-cleaved product of translocation (data not shown). This product was protected from digestion with proteinase K (downward pointing arrowhead, lane 4), as was a small population of signal-cleaved nonglycosylated molecules (upward pointing arrowhead, lane 4), which comigrate with the internally initiated product of protein synthesis (upward pointing arrowhead, lane 1). When detergent was included with proteinase K, both protected products were completely degraded, indicating that protection was conferred by the lipid bilayer and was not a property intrinsic to the translocated molecule (lane 6).

Similar analysis of the expression products of pSP ST·G (Fig. 5-2C), showed that this molecule is also efficiently translocated. In the cotranslational presence of microsomal membranes, a globin immunoreactive product of higher mobility than the

Figure 5-1. **Restriction map and coding region of pSP SG 1, pSP SG 3 and pSP ST·G.** Restriction sites are above, and defined coding regions below. *Stippled and stripped rectangles* represent signal and stop transfer encoding domains, respectively. *Unfilled and filled rectangles* designate globin and glycosylation encoding regions, respectively. The SP6 promotor is indicated by the black bars at the 5' end of the restriction maps. Initiation and termination codons are indicated.

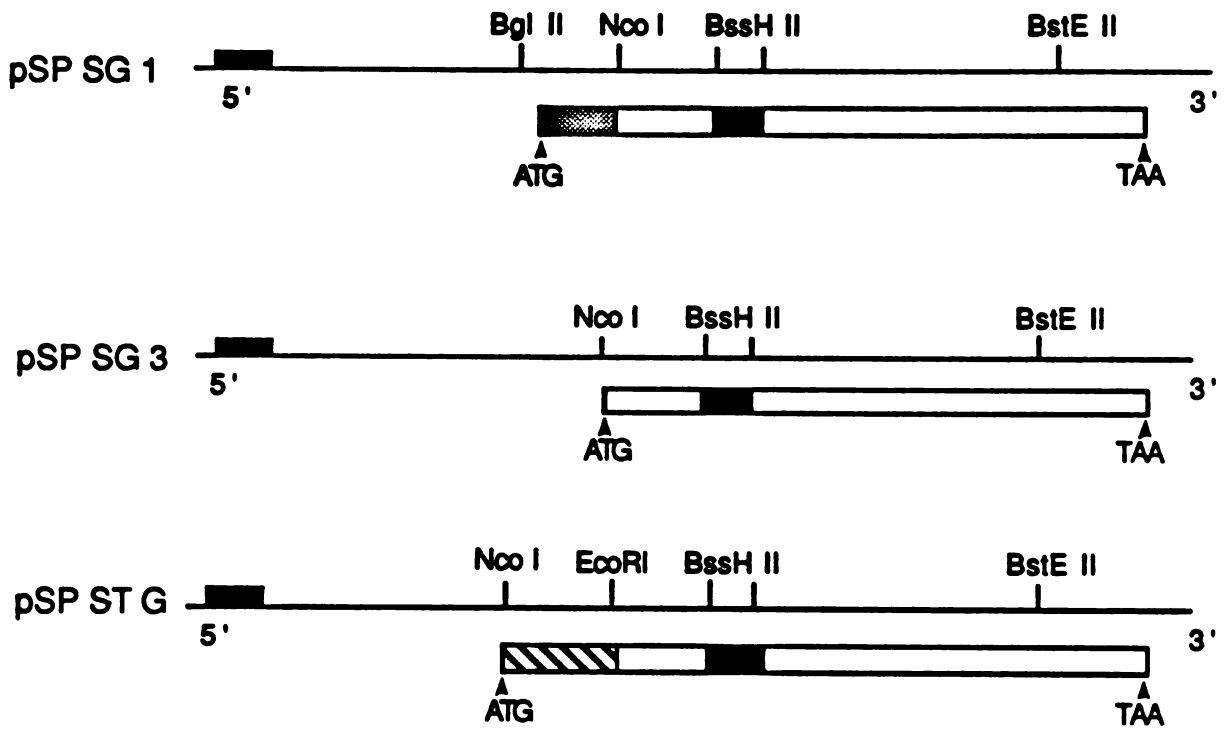
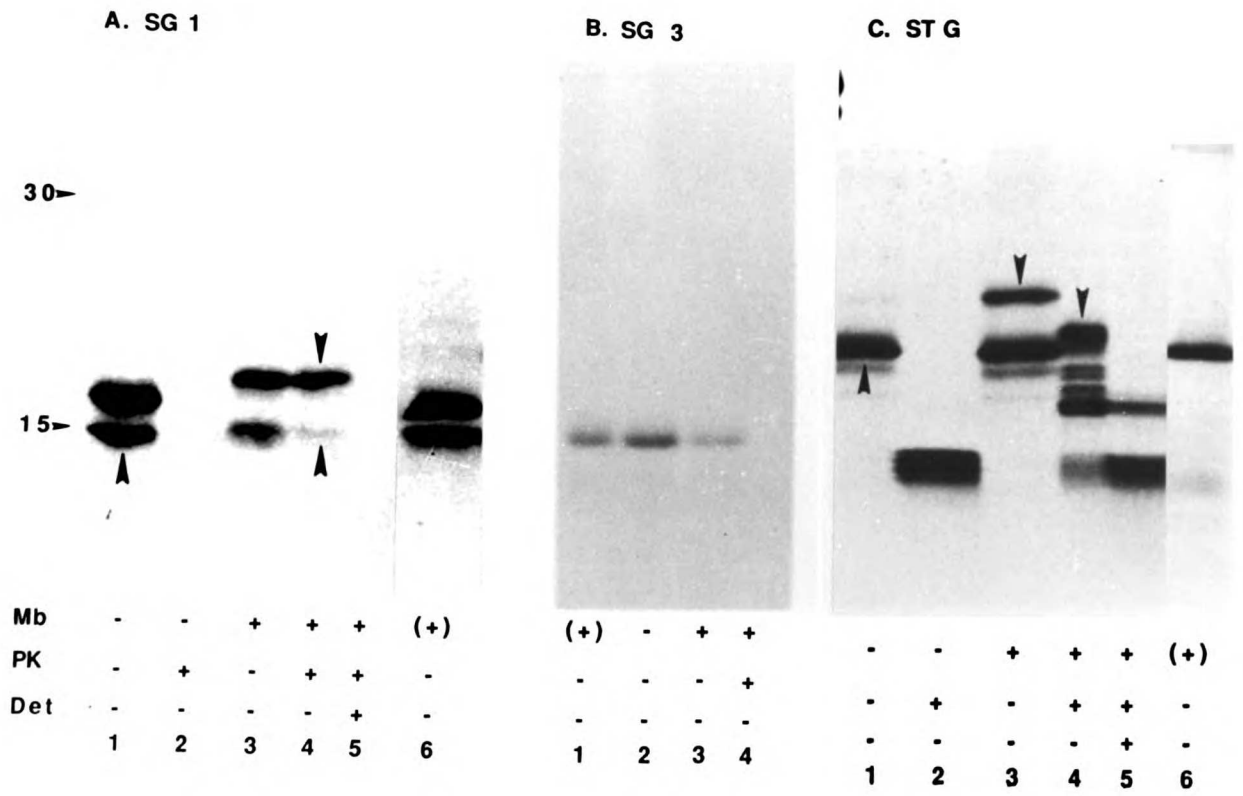

pSP SG 1
Bgl II
Nco I
BssH II
BstE II
5'
3'
ATG
TAA
pSP SG 3
Nco I
BssH II
BstE II
5'
3'
ATG
TAA
pSP ST G
Nco I
EcoRI
BssH II
BstE II
5'
3'
ATG
TAA

Figure 5-2. **Translation of full-length products of pSP SG1 (A), pSP SG3 (B), and pSP ST·G (C) in the co- and posttranslational presence of microsomal membranes.** Transcription-linked translation reactions were carried out in the RRL in the absence or presence of microsomal membrane (*Mb*) added co- or posttranslationally. Components added to the translation reaction are indicated by a, +, below the lanes; *(+)* refers to *post*translational addition of Mb. Products were subjected to posttranslational proteolysis with proteinase K (*PK*) in the absence or presence of Nikkol detergent (*Det*). Immunoprecipitates were analyzed by SDS-PAGE and visualized by autoradiography. In panel **A**: *upward pointing arrowhead (lane 1)*, indicates a product generated from internal initiation of protein synthesis; *upward and downward pointing arrowheads (lane 4)*, designate cleaved non-glycosyolated and glycosylated protected products, respectively. In Panel **C**: *upward pointing arrowhead (lane1)*, indicates the full-length precursor; *downward pointing arrowheads (lanes 3 and 4)*, glycosylated full-length species and protected fragment, respectively. The positions of molecular weight markers ($X10^3$) are indicated.


A. SG 1
B. SG 3
C. ST G
30►
15►
Mb - - + + + (+)
PK - + - + + -
Det - - - - + -
1 2 3 4 5 6
(+) - + +
- - - +
- - - -
1 2 3 4
- - + + + (+)
- + - + + -
- - - - + -
1 2 3 4 5 6

precursor (lane 1) was generated (downward pointing arrowhead, lane 3). This band represents a glycosylated translocated species, as demonstrated by its downward shift to the position of the precursor upon addition of endo H (data not shown), and by the protection of a fragment of this product in the presence of proteinase K (presumably due to cleavage of part of the exposed stop transfer sequence on the cytoplasmic face of the membrane) (lane 4). Addition of detergent abolished the protease resistance of the remainder of the molecule (lane 5).

When microsomal membranes were added posttranslationally, to translation reactions programmed by transcripts generated from either pSP SG1 or pSP ST·G, no translocation was observed (Fig. 5-2 A, lane 6 and 5-2C, lane 6, respectively). This is in agreement with all previously published data on the cotranslational requirement for translocation in eukaryotic cell-free systems. Analysis of the expression products of pSP SG3 shows that no translocation occured when microsomes were added either co- or posttranslationally (Panel B, lanes 3 and 1, respectively). This is consistent with the established requirement for a topogenic element for directing polypeptide chain translocation.

Following the logic described in the introduction and depicted in Fig. 5-3, we next sought to dissect translocation from translation. A restriction site, BstE II (common to both pSP SG1 and pSG ST·G) was used to create truncated globin coding regions which lacked a termination codon. This site was 34 codons from that of termination. Following cleavage of either plasmid by endonuclease digestion, the truncated DNA molecules were transcribed and translated in vitro.

Translation in RRL gave the products SG1/BstE II (or SG1/B) and ST·G/BstE II (or ST·G/B) (Fig. 5-4A and B, lane 1). These species migrated at predicted molecular weights, approximately 3 kD smaller than their respective full-length products. Co-

Figure 5-3. **Protocol for expression of plasmids pSP SG1, pSP SG3 and pSP ST·G, both with and without termination codons (A).** Messenger RNA lacking a termination codon was prepared by in vitro transcription of plasmids linearized with BstE II, which cleaves the globin coding regions 117 codons downstream from the initiation codon of globin. Full-length transcripts including the termination codon were prepared in separate reactions. Full-length and truncated mRNA's were incubated separately in transcription-linked translation reactions with rabbit reticulocyte lysate. Reactions were carried out at 25^{o}C for 15 minutes; 10^{-4} M ATA (aurin tricarboxylic acid) was added to inhibit initiation, and after another 15 minutes emetine was added to 10^{-4} M to block elongation. **Substrates for posttranslational translocation (B):** Expected translation products of full-length (left) and truncated (right) plasmids are schematically represented. Translation of full-length mRNA yields released full-length polypeptide, free ribosomes, and mRNA, while translation of truncated transcripts produces intact polysomes with arrested nascent polypeptide chains emerging from the ribosome. Polysomes were first incubated with or without 1 mM puromycin before addition of dog pancreas microsomal membranes. Further incubation at 25^{0}C for 20 minutes preceded translocation assays.

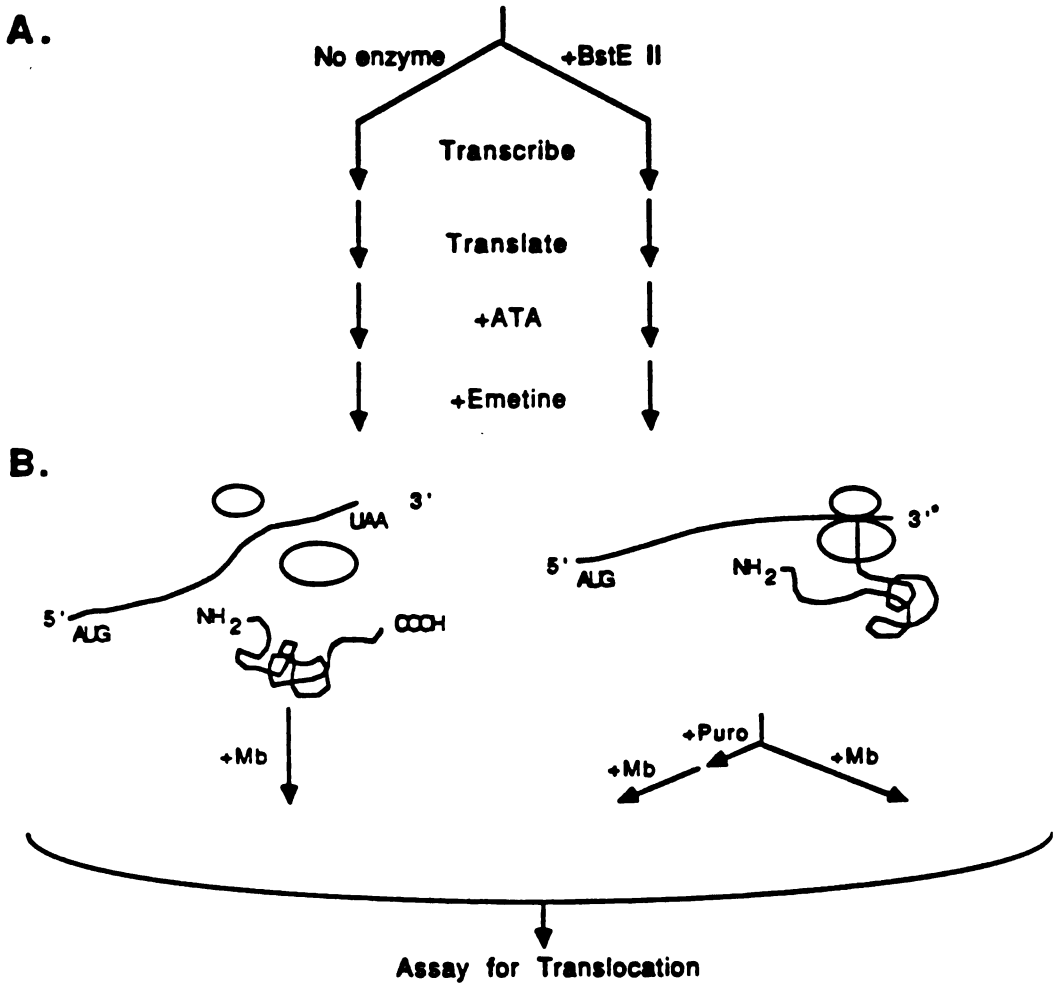
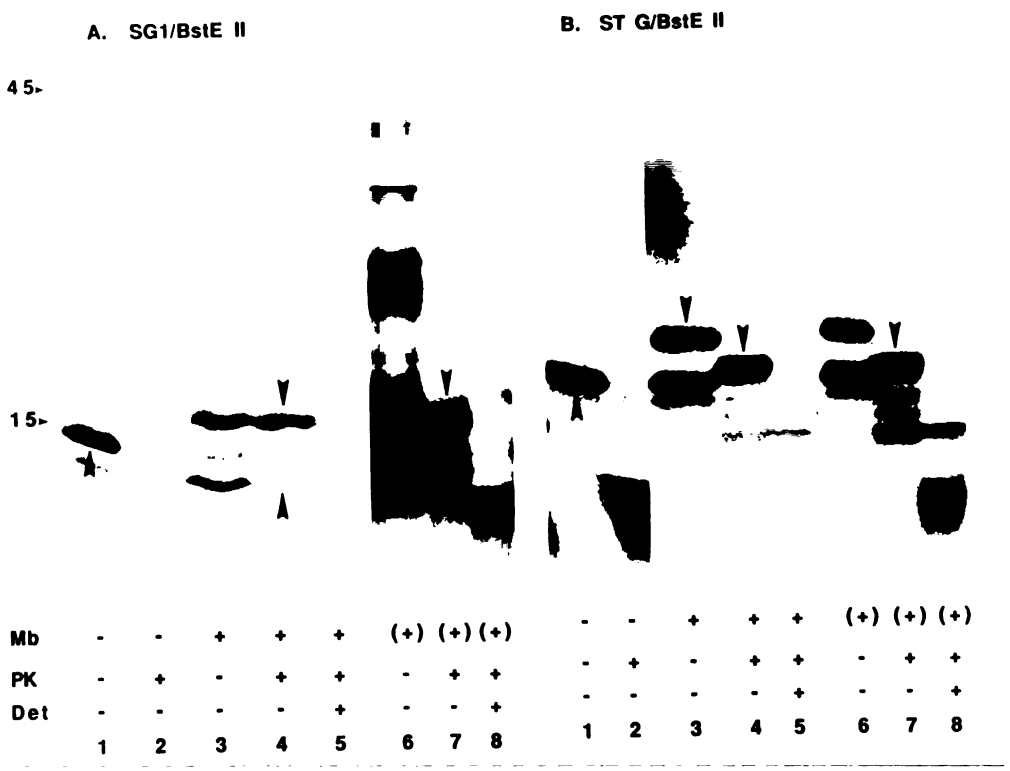

A.
No enzyme
+BstE II
Transcribe
Translate
+ATA
+Emetine
B.
UAA
3'
5'
AUG
NH2
COOH
+Mb
3'
5'
AUG
NH2
+Puro
+Mb
+Mb
Assay for Translocation

Figure 5-4. **Co- and posttranslational translocation of SG1/B (A) and ST·G/B (B) across microsomal membranes.** Plasmids pSP SG1/B or pSP ST·G/B (linearized with BstE II), were transcribed to produce mRNA's lacking a termination codon (see Fig. 5-3). Transcripts were translated in the RRL, in the absence, -, cotranslational presence, +, or posttranslational presence *(+)*, of microsomal membranes (*Mb*). Products were subjected to posttranslational proteolysis with proteinase K (*PK*) in the absence or presence of Nikkol detergent (*Det*). In panel **A**: *upward pointing arrowhead (lane 1)*, designates full-length precursor; *upward and downward pointing arrowheads (lane 4 and 7)*, indicate cleaved non-glycosylated and glycosylated, globin-protected products, respectively. In panel **B**: *upward pointing arrowhead (lane 1)*, indicates full-length precursor; *downward pointing arrowheads (lanes 3, and 4 or 7)* designate glycosylated, and glycosylated-protected products, respectively. All sampes were immunoprecipitated, separated on SDS-PAGE and viewed by autoradiography. Positions of molecular weight markers ($X10^3$) are indicated.


A. SG1/BstE II
B. ST G/BstE II
45
15
Mb - - + + + (+) (+) (+) - - + + + (+) (+) (+)
PK - + - + + - + + - + - + + - + +
Det - - - - + - - + - - - - + - - +
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

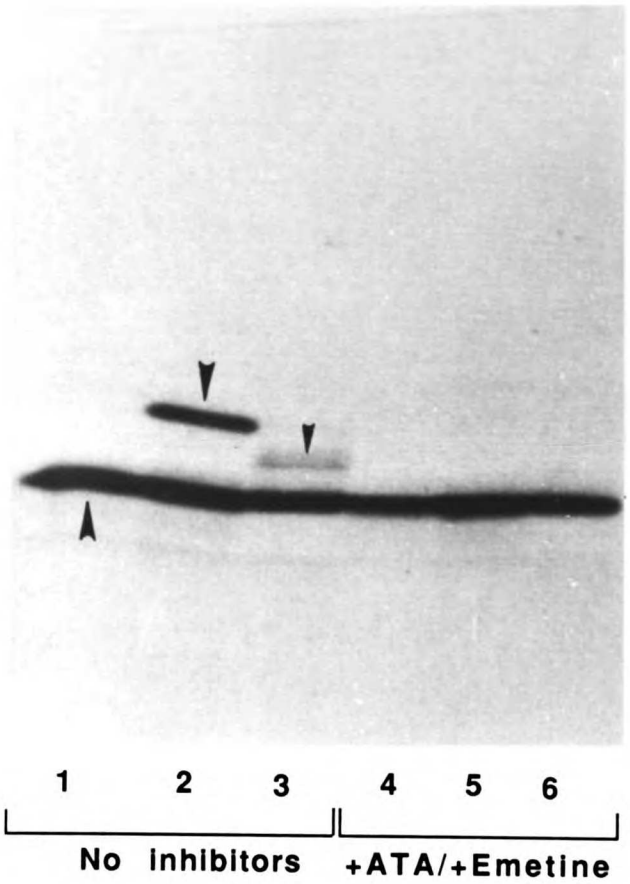
translational addition of microsomal membranes resulted in the efficient translocation of both these products as demonstrated by all criteria described above (Fig. 5-4A and B, lanes 1-5).

As described in the Introduction we wished to assay for translocation in the absence of ongoing protein synthesis. Therefore, following translation of the truncated transcripts, we first added inhibitors of initiation of synthesis, and subsequently inhibitors of elongation, to insure that no protein synthesis was occuring. This condition was critical for establishing an assay in which translocation could be studied independently from translation.

In Fig. 5-5, the absence of ongoing protein synthesis using the method described in Fig. 5-1A is demonstrated. Translation of the transcript generated from pSP ST·G is shown in lane 1 (upward pointing arrowhead). In the absence of inhibitors, and 35 minutes after translation was initiated, addition of a second transcript (generated from pSP BPI, and encoding preprolactin) to the original translation reaction, results in efficient translation of this product as well (downward pointing arrowhead, lane 2). When microsomal membranes were added at the same time as the second transcript, preprolactin was converted to the mature, signal-cleaved product (arrowhead, lane 3). On the other hand, if after 15 minutes of translation of the initial transcript, inhibitors of initiation and translation are added (as described in Methods), the protein synthesizing machinery is shut down, as demonstrated by the lack of translation upon addition of a second transcript (note the absence of any preprolactin products in lane 5). This result indicates that translation is completely inhibited under these conditions.

We were now ready to determine whether translocation could occur in the absence of ongoing protein synthesis. Truncated transcripts (derived from either pSP SG 1/B or pSP ST·G/B) were translated in RRL, after which the reactions were treated under the

Figure 5-5. **Demonstration of the absence of ongoing protein synthesis using the posttranslational protocol described in Fig. 5-3A.** The transcript encoding pSP ST·G was translated in RRL for 15 minutes at 25^0C, after which translation products were separated into two equal aliquots: one aliquot (*lanes 4-6*) was treated with inihibitors of initiation and elongation at time = 15, and 30 minutes as described in Methods, while the other aliquot (*lanes 1-3*) received no inhibitors. After time = 35 minutes the two translation reactions were further aliquoted and incubated with either of the following, for an additional 20 minutes: (*lanes 1 and 3*), no additions; (*lanes 2 and 5*), transcript encoding pSP BPI; (lanes 3 and 6), transcript encoding pSP BPI and microsomal membranes. *Upward pointing arrowhead (lane 1)*, indicates full-length precursor generated from synthesis of the original transcript encoding pSP ST·G; *downward pointing arrowhead (lane 2)*, precursor resulting from synthesis of the second transcript, encoding pSP BPI; *downward pointing arrowhead (lane 3)*, processed version of BPI.


1
2
3
4
5
6
No inhibitors
+ATA/+Emetine

conditions established in Fig. 5-5. Addition of microsomal membranes to these translation products, in which protein synthesis was no longer occuring, resulted in translocation. Translocation of SG1/B is demonstrated by the appearance of a glycosylated, and protease-protected species (Fig. 5-4 A, arrowhead, lane 7); translocation of ST·G/B is evidenced by the partial protection of a glycosylated product (Fig. 5-4B, lane 7). Thus, translation of transcripts lacking 35 codons at the 3' terminus (including the termination codon) permitted transport across the ER membrane to be reconstituted in the absence of ongoing chain elongation; that is, we achieved an uncoupling of translocation from translation.

In order to probe the basis for this uncoupling we first sought to determine whether the polypeptide chains which were translocated posttranslationally had maintained their association with ribosomes. Completed translation reactions (such as those employed in the posttranslational experiment described above), were sedimented in sucrose density gradients, and the distribution of full-length chains was studied. Analysis of gradient fractions by SDS-PAGE revealed that almost all full-length chains were sedimented under conditions which resulted in pelleted ribosomes (data not shown). This suggests that the substrates utilized in the posttranslational translocation reaction described in Fig. 5-4 A and B, were 'dangling' (or ribosome-associated) nascent chains, as depicted in Fig. 5-3B (right side of diagram).

We next treated these translation reactions with puromycin (see Fig. 5-2B), an aminoacyl transfer RNA (tRNA) analog that causes termination and release of nascent chains from ribosomes (Lodish et al., 1971; Blobel and Sabatini, 1971). Treatment with puromycin followed by high speed centrifugation resulted in release of nascent chains to the post-ribosomal supernatant. We found that when these puromycin-released products were supplemented with microsomal membranes (and all other factors

which were present in the original translation reaction) posttranslational translocation no longer occurred (data not shown). These results suggested a role for the ribosome in transport across the ER membrane that is distinct from protein synthesis. This issue has been further investigated in several laboratories including our own (see Discussion).

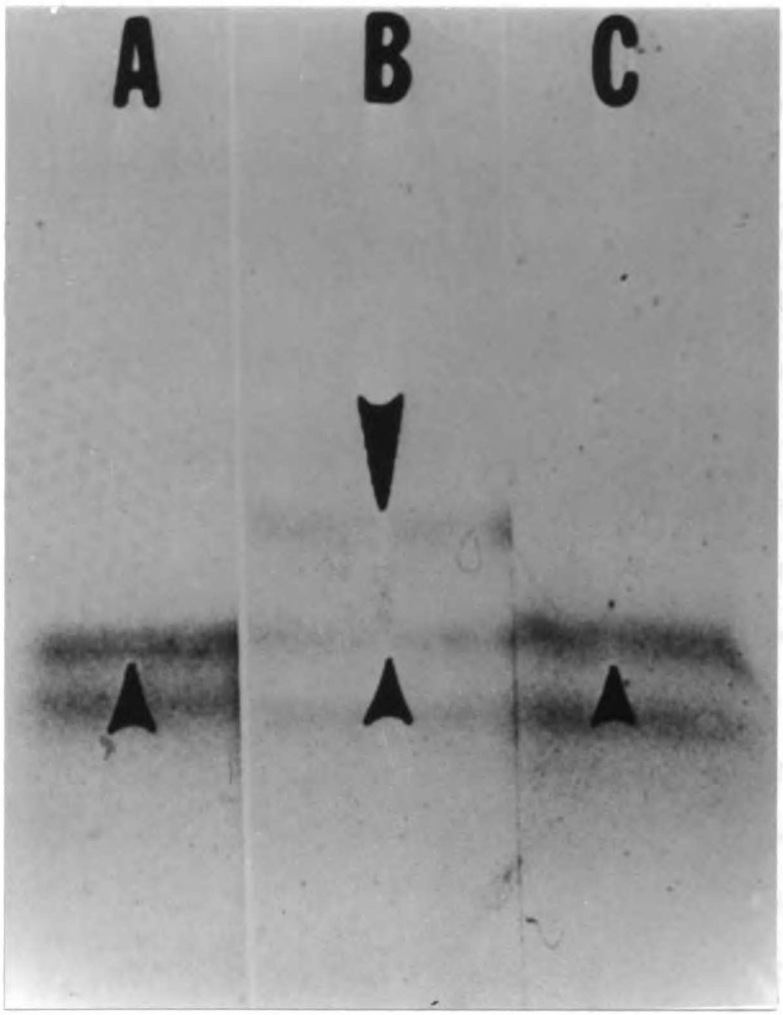
The mechanistic requirements for transport across the ER were further explored by the following experiment. Translation products of SG1/B were filtered on an S-300 Sephacryl gel column, and a void fraction including all polysomes was collected. This fraction was depleted of nucleoside triphosphates and other small molecules. Incubation of the void fraction with microsomal membranes and nucleoside triphosphates (Fig. 5-6, lane B) but not with membranes alone (lane C), resulted in translocation. The requirement for the energy supplement (consisting of adenosine triphosphate [ATP], guanosine triphosphate [GTP], and creatine phosphate with creatine kinase) indicated that translocation was not proceeding spontaneously but rather displayed an energy requirement independent of protein synthesis.

DISCUSSION

We have shown above that expression of certain in vitro transcribed mRNA's that lack termination codons permits the uncoupling of a protein's translocation across microsomal membranes from its synthesis. This result is significant because it demonstrates that translocation does not require protein synthesis per se. Equally important, the novel technique employed here provides the tools for investigating mechanistic requirements for transport across the ER.

In exploring the molecular basis for posttranslational translocation, we observed that for those substrates described, translocation requires the presence of the ribosome

Figure 5-6. **Reconstitution of translocation from S-300 fractionated cytoplasmic components.** Plasmid pSP SG1, linearized with BstE II, was transcribed, translated, and treated with ATA and emetine as before. The translation reaction was applied to a 1-ml S-300 Sephacryl gel filtration column and a void volume was collected. Portions of the void fraction, containing partially purified polysomes, were incubated with microsomal membranes at 25^0C for 20 minutes, either in the presence (**lane B**) or absence (**lane C**) of an Energy cocktail (see Methods). The noncomplemented void fraction is shown in **lane A**. The *upward pointing arrowhead* indicates the unprocessed precursor, pre SG1; the *downward pointing arrowhead* indicates the glycosylated, processed species.


A
B
C

(both co- and posttranslationally). These findings suggest that there may be multiple recognition requirements for the transfer of newly synthesized proteins across the ER. In addition to needing a specific signal for initiating translocation (i.e. either a signal or stop transfer element) it appears that at least in certain cases, the nascent chain must be presented in the context of a ribosome. This might account for the apparent degeneracy of topogenic sequence information, since multiple recognition systems operating in unison could provide the specificity and selectivity required for translocation. The observaton that SRP interacts with signal sequences of nascent secretory proteins only in the context of the synthesizing ribosome (Krieg et al., 1096; Wiedmann et al., 1987a) is consistent with this view. We have recently demonstrated the requirement for SRP in ribosome-dependent post-translational translocation (Rothman, Perara and Hansen, unpublished observations).

In addition to a proposed role in maintaining a translocation competent state, the ribosome may also serve as a ligand for translocation. The requirement for a functional ribosome membrane junction in translocation was discussed above (see Chapter 1, III,3 and Connolly and Gilmore, 1986).

Recent data indicates that the requirement for the ribosome, although general for many proteins, is limited to a particular period of time during chain growth (Perara and Lingappa, 1988; Hansen et al., 1986; Mueckler and Lodish, 1986a; Rothblatt and Meyer, 1986; Water and Blobel, 1986). For example, when truncation is carried out considerably more 5' (i.e. 78 codons after the signal or stop transfer codon) than was done in the case of SG 1/B and ST·G/B described above, translocation was observed to proceed posttranslationally, but in a ribosome *independent* fashion. On the other hand, when truncation was carried out considerably more 3' (175 codons after the signal sequence in SG1/B) posttranslational translocation competence is greatly diminished.

These findings lead to the suggestion that the nascent chain passes through sequential stages of growth in which translocation competence is independent of, and then subsequently maintained by the ribosome. Finally, at a certain point in chain growth translocation competence is lost, presumably because of entanglement of the chain with the signal sequence (Rothman and Lodish, 1977; Perara and Lingappa, unpublished).

The variability in translocation competence of a given polypeptide may reflect the accessibility of the topogenic element to receptors in the cytoplasm and/or the ER membrane (Perara and Lingappa, 1988). This explanation is consistent with the observation that different nascent secretory proteins show variability in the stage of elongation at which translocation competence is lost (Siegel and Walter, 1988c; Ainger and Meyer, 1986; Hansen et al., 1986). It remains unclear whether ribosome-independent translocation is limited to cell-free systems (e.g. reflecting use of partially open translocation sites as a results of damage during cell fractionation), as suggested by some data (Water and Blobel, 1986), or whether the ribosome is involved in events (e.g. steps in unfolding) that may not be required for every newly synthesized translocation substrate.

The demonstration here that translocation across the ER membrane requires a source of energy (in the form of nucleoside triphosphates) independent from that of protein synthesis, has important implications for understanding the mechanism of translocation. These findings argue that translocation is not a spontaneous process, and are consistent with recent data suggesting that translocation proceeds throught a proteinaceous tunnel (Gilmore and Blobel, 1985; Weidmann et al, 1987b).

Several studies have addressed the role of nucleoside triphosphates in translocation. Recent posttranslational translocation experiments have demonstrated that *ATP* hydrolysis is required for transport into the ER (Waters and Blobel, 1986; Hansen et

al., 1986; Schlenstedt and Zimmerman, 1987) In a prokaryotic system, the finding that a non-hydrolyzable ATP analog which has no detectable effect on protein synthesis, was able to block translocation suggests that ATP hydrolysis is required for cotranslational translocation (Chen and Tai, 1987). The requirement for ATP hydrolysis for translocation across yeast microsomal membranes, was recently shown to involve heat shock gene products, which appear to mediate protein unfolding (Deschaies et al., 1988; Chirico et al., 1988). Most recently, GTP has been implicated as a cofactor for SRP R, whose binding causes displacement of SRP from the signal sequence of a nascent secretory polypeptide (Connolly and Gilmore, 1989).

The experimental paradigm described here has allowed, and offers continued promise for, direct experimental analysis of the problem of protein translocation across the ER membrane. Up until the time these experiments were done, progress in defining the mechanism of translocation, as well as in isolating putative membrane components involved in translocation, were hampered by the coupled nature of the assay: ongoing synthesis of the chain whose translocation was to be studied. The novel assay presented here permits discrimination between requirements for these two events by separating the processes of transport from those of synthesis.

As has been seen in the last several years, various applications of this technique has made it possible to distinguish between steps in the transport process (Connolly and Gilmore, 1986 and 1989), and to begin fractionation and reconstitution of translocation components (Weidmann et al., 1987 a and b).

CHAPTER 6:

SUMMARY

Characterization of topogenic sequence activity

We sought to classify the functional properties of signal and stop transfer sequences. Therefore, in chapters 2 and 3, we studied the expression products of various engineered constructs in which these sequences were engineered into different contexts. Such an analysis was of interest because displacement of topogenic elements to different positions in a nascent polypeptide chain had previously revealed unexpected activities of both signal and stop transfer sequences (Perara and Lingappa, 1985; Mize et al, 1986, respectively), and also had provided some insight into the mechanism of translocation. Our results show that although signal and stop transfer sequences share a common function (namely, the ability to mediate passenger domain translocation), each element possesses intrinsic properties which are not shared by the other. While we can not rule out the possibility that other signal and stop transfer sequences display different activities, we believe out findings will hold for a large subset of topogenic elements for the following reasons: the signal and stop transfer sequences studied here were taken from secretory and transmembrane proteins whose topology is representative of large classes of proteins found in nature; and, these elements have been previously tested in numerous fusion proteins, and have exhibited activities consistent with those seen here.

The existence of specialized functions for signal and stop transfer sequences was demonstrated by two findings: (1) translocation of a passenger domain by one or the other class of topogenic element conferred different transmembrane topologies; and, (2) termination of domain translocation is an activity unique to the stop transfer sequence. Since we employed precisely defined passenger domains which were engineered in exactly 'matched' constructions it seems likely that the distinct topologies generated are attributable to intrinsic propertes of the topogenic sequences themselves (i.e. the transmembrane dispositions of the encoded products are passenger

independent). Moreover, the conclusion that signal and stop transfer sequences are *not* functionally interchangeable is directly relevant to the problem of polytopic transmembrane protein biogenesis.

The finding that signal and stop transfer sequences have both shared and specialized functions allows speculation regarding the mechanism of translocation events. Since the translocation initiation activity of ST is dependent upon at least a subset of the same receptors as those utilized by a bona fide signal sequence (Mize et al., 1986; Zerial et al., 1987), we suggest that translocation might be a multi-step process, in which both common and unique receptors are utilized by each class of topogenic element. According to such a scenario, recognition of *shared* receptors might account for the *common* activities observed, while *specialized* receptors would permit the topological *specificity* which is conferred by a signal or stop transfer element.

Consistent with this model, mutants outside the hydrophobic region of the signal/stop transfer domain of cytochrome P-450 (Szczesna-Skorupa et al., 1988, 1989) converted the activity of this element into that of a more 'classical' amino terminal signal sequence. This finding suggests that stop transfer function may not simply be a reflection of hydrophobic interactions with membrane lipids but could involve recognition of a topogenic element by a specialized receptor(s) in the ER membrane, which mediates one, of several alternative steps in the translocation process. However another explanation for these results is that the mutations made in the topogenic sequence (in this case, addition of charged amino acids flanking the hydrophobic core), result in a change in the orientation of the element in the bilayer, such that its reorientation generates a conversion in the phenotype of the encoded product with respect to the ER membrane. The mechanistic basis for topogenic sequence specificity remains unclear, although some studies have attempted to address this question.

According to one line of investigation, if unique structural properties of a topogenic sequence (e.g. charge or secondary structural features) determine the polarity of that domain in the lipid bilayer, it should be possible to generate precise changes in the primary amino acid sequence which would lead to predictable changes in topology. Initial studies pursuing this approach (Haeuptle et al., 1989) have demonstrated that mutations in regions outside of the hydrophobic core of a signal/stop transfer element produce dramatic changes in topology, which could be accounted for by alterations in the disposition of the topogenic element in the bilayer. Interpretation of these results however, was at least partially confounded by failure to characterize the passivity of the passenger domains which were employed as reporters, making it unclear whether the altered phenotypes observed were caused by engineered changes in the topogenic element, or were due to intrinsic properties of the passenger domain. Moreover, such an analysis does not rule out the possibility that altered interactions with receptors in the ER membrane were responsible for observed changes in the orientation of the product.

An alternative approach would be to identify putative components in the cytoplasm or membrane of the ER which might be responsible for alternate topologies. If different translocation phenotypes (e.g. that directed by a signal versus a stop transfer element) are due to blockage, or stimulation, of specific receptor interactions, cross-linking to distinct components of the translocation machinery might be feasible. Photoaffinity cross-linking, employing mutant substrates has already been shown to be a potentially powerful tool for isolating functional receptors (Kurzchalia et al., 1986; Krieg et al., 1986).

Regardless of the mechanism responsible for the variant topologies observed when different topogenic elements are presented to the ER membrane, the systematic approach employed here which resulted in characterization of the activity of a prototypic signal

and stop transfer sequence, would be invaluable for classifying the function of *other* subtypes of topogenic elements (see Chapter 1, iv e & f). It would be of interest to categorize these elements according to several criteria, including: complete or partial translocation function; amino versus carboxy terminal translocation activity; and, effect on the ultimate membrane association of the encoded product (as described in Chapters 2 and 3 above).

Furthermore, the established 'neutrality' of the passenger domains employed here allows determination of whether the behavior of a topogenic element in a native protein is likely to be due to intrinsic properties of that sequence, or whether specific or nonspecific properties of a flanking domain in that molecule render it permissive or resistant to the action of a topogenic element. Finally, application of a controlled gene fusion approach would allow analysis of the determinants of unusual and diverse transmembrane topologies of such native proteins as the scrapie prion protein (Yost et al., submitted) or Hepatitis B surface antigen (Eble et al., 1987).

Polytopic transmembrane protein biogenesis

Once having established that signal and stop transfer sequences are functionally distinct, we went on to demonstrate that the biogenesis of a complex TMP can be directed by employing particular patterns of simple signal and stop transfer sequences. As described in Chapter 4, the positioning of ST behind an amino terminal signal sequence (in S_L·L·ST·G), generates a bitopic transmembrane protein, which can be efficiently 'woven' back into the ER membrane by positioning a third topogenic element (i.e. S_P) between two inert passenger domains, as in S_L·L·ST·G·S_P·P.

The third topogenic sequence employed in the hybrid S_L·L·ST·G·S_P·P functioned with high efficiency to reinitiate translocation, although a significant fraction of those molecules were cleaved after S_P. In order to generate a substrate which might give a

homogenous population of polytopic TMPs, we are presently attempting to engineer a third topogenic element in place of S_P which is not cleaved (e.g the signal sequence of ovalbumin [Lingappa et al., 1979], or the signal sequence from a mutant construct, which initiates translocation with high efficiency but fails to cleave [V.R. Lingappa, unpublished observations]). Additionally, since only one of several possible transmembrane topologies was achieved in our study, it would be of interest to apply the findings from the analysis of topogenic sequence subtype activity (see above), to generate all possible orientations of polytopic membrane proteins. Such an analysis would be useful for understanding how the variety of topologies of TMPs observed in nature is achieved.

Another area of interest which warrants further investigation is the mechanism of the biogenesis of complex TMPs. For example, do transmembrane proteins with multiple signal sequences use SRP for directing the translocation of each extracytoplasmic domain? Since an already targeted chain is in close proximity to the membrane, SRP-SRP receptor interactions might not be necessary for reinitiation of translocation; on the other hand, efficient reinitiation of translocation might require that the nascent chain assume a particular orientation, and SRP might be involved in establishing such a 'translocation competent state'. One recent study, published shortly after the work described in Chapter 4, suggested that when alternating signal/stop transfer elements are employed to direct TMP biogenesis, SRP is *not* utilized for the reinititaiton of translocation (Wessels and Spiess, 1988). However, these conclusions were based on the behavior of a mutant topogenic element (which cannot engage SRP), making it unclear whether this behavior is general for polytopic TMP biogenesis. Moreover, since neither the basis for the mutant signal sequence's failure to bind SRP, nor the nonbinding of SRP in the context of the polytopic TMP were established, it is

possible that when the mutant signal sequence was engineered into a polytopic protein, it now allowed SRP binding.

Our laboratory is presently pursuing studies in which a variant of the molecule S_L·L·ST·G·S_P·P is being employed to explore whether SRP is utilized in the reinitaition of translocation directed by a wild-type signal sequence. By engineering the construct pSP S_L·ST·G·G·G·S_P·P, and expressing it in an in vitro transcription/translation system in the presence of microsomal membranes, a substrate is created in which essentially every chain that has engaged the translocation machinery (as assessed by signal cleavage after S_L), will have also engaged the ST sequence (as demonstrated by protease accessibility of the subsequent protein domain). A relatively homogenous population of 'kinetic intermediates', in which only the amino terminal part of the molecule has been synthesized (i.e., the second signal sequence has not yet been made), could be isolated by carrying out synchronized translation reactions, employing inhibitors of initiation, and 'freezing' the partially translated products by incubation at 0^0 C. This would be followed by high salt treatment, and sedimentation of the vessicles to remove SRP from the reaction. Subsequent incubation of these vessicles (containg the 'frozen' nascent chains) at 25^0 C, with or without the addition of exogenous SRP, would assess the requirement for SRP in translocation reinitiation.

Mechanism of translocation events

In Chapter 5 we directly test whether translocation is an obligately cotranslational event in higher eukaryotes. Truncation of coding regions 5' to the termination codon resulted in RNA transcripts which programmed polypeptide initiation and elongation, but did not allow release of nascent polypeptides from the ribosomes. Translation of such synthetic transcripts allowed us to assay for translocation independently from

translation. Our experiments demonstrate that the transfer of a nascent polypeptide across the ER membrane can occur in the absence of ongoing protein synthesis. Moreover, reconstitution studies resulted in the demonstration that nascent proteins require nucleoside triphosphates for translocation.

These findings have two principal implications. First, they suggest that translocation is not a spontaneous process, but requires an energy source, utilized by components in the cytoplasm and/or the ER membrane. Secondly, since transport across the ER membrane can be reconstituted independently from the confounding influences of protein translation, a detailed investigation of mechanistic questions regarding the translocation process is made possible.

The most recent experments which have exploited the posttranslational translocation assay have resulted in the demonstration that GTP dependent binding of SRP receptor, is required for translocation, mediating SRP displacement from the ribosomes (Connolly and Gilmore, 1989). The exact role of ATP dependent hydrolysis in translocation is as yet undefined. One suggestion is that ATP is required for the action of a specialized cytosolic factor which mediates nascent polypeptide unfolding prior to translocation (Rothman and Kornberg, 1986). Another possible function which has been suggested for ATP is in assembly and disassembly of the putative 'translocon' in the ER membrane (Walter and Lingappa, 1986). Several other mechanistic questions regarding translocation events remain to be resolved, including the exact role of the ribosome in translocation.

Results from our studies characterizing the activity of signal and stop transfer sequences, suggested that these elements might employ common receptors in the ER membrane. Taken together with the demonstration that translocation can be experimentally uncoupled from protein synthesis, we suggest that it may be possible to

experimentally dissect translocation and integration events in the biogenesis of a TMP (see below). This would be done with two principal aims in mind: (1) experimental analysis of the mechanistic requirement for integration, independent from the confounding influences of translocation; and, (2) identification of putative 'common' receptors (other that SRP and SRP receptor) that are shared by signal and stop transfer sequences.

Briefly, such an experiment might be accomplished by employing truncated versions of the molecule pSP S_L·L·ST·G. Several sites in the 'G' domain would be chosen in an attempt to generate a translation product which is tethered to the ribosome (for lack of a termination codon), with the ST sequence just having been synthesized. If ST interacts with a receptor in the ER membrane prior to integrating into the lipid bilayer, one of these truncated products might provide a kinetic intermediate which has engaged its putative receptor but not yet integrated into the bilayer. Isolation of a nonintegrated product would be demonstrated by its carbonate extractibility. Integration of this intermediate into the bilayer might then be achieved by releasing the truncated product from the ribome with puromycin, allowing ST to orient in the ER membrane as it does in the wild-type full-length molecule. Again, carbonate extraction would be employed to assess integration.

Once such an assay is established, the mechanistic requirements for integration could be explored by subjecting the partially translocated, but non-integated intermediate to various protein perturbants and chemical modifying procedures, and then assaying for the ability of such a substrate to integrate into the bilayer upon addition of puromycin. When integration capacity is lost, we would attempt to reconstitute that event by adding back the factor(s) which was inactivated or removed. Additionally, photoaffinity crosslinking could serve to explore the interactions of the kinetic intermediate with its putative receptor(s) in the ER membrane.

Prospectus

Engineered fusion proteins have been shown to serve as powerful tools for the identification and characterization of topogenic elements. In addition, when applied with insight, they have been used to explore the mechanistic requirements for translocation. The challenge for future progress in this field lies in developing novel applications of engineered translocation substrates, with the aim of isolating and purifying molecular components, as well as dissecting and reconstituting steps involved in translocation across, and integration into the ER membrane.

CHAPTER 7:

MATERIALS AND METHODS

MATERIALS

All restriction endonucleases and nucleic acid-modifying enzymes, SP6 RNA polymerase, T4 DNA ligase, E. coli DNA polymerase I, E. coli glycerol kinase, trypsin, trasylol, puromycin, cycloheximide, emetine, aurintricarboxylic acid (ATA), nucleoside triphosphases, calf-liver tRNA, creatine phosphokinase, and creatine phosphate were from Boehringer Mannheim Diagnostics, Inc. (Houston, TX), or from New England Biolabs (Beverly, MA.). Placental RNase inhibitor was from Promega Biotec (Madison, WI); Affi-Gel-Protein A-agarose was from Bio-Rad; rabbit anti-human hemoglobin serum was from Cappel Laboratores (Cochranville, PA); rabbit anti-ovine prolactin serum was from United States Biochemical Corp. (Cleveland, OH); rabbit anit-beta-lactamase was a gift from Chung Nan Chang; proteinase K was from E. Merck AG (Damstadt, Federal Republic of Germany); endoglycosidase H and Triton X-100 were from Boehringer Mannheim; [^{35}S] methionine was from Du Pont-New England Nuclear; and Nikkol (octaethylene glycomono-n-dodecyl ether, a nonionic detergent) was from Nikko Chemical Co., Ltd. (Tokyo, Japan). *Xenopus laevis* were from Nasco, Fort Atkinson, WI. Plasmid pSP BP4 (aka SP BPI) was constructed by William Hansen, Department of Biochemistry and Biophysics, University of California, San Francisco, using bovine preprolactin cDNA (Sasavage, et al., 1982). All globin encoding plasmids were derived from pMC18 (Yost et al., 1983).

METHODS

Plasmids

The globin coding region was derived from a full-length chimpanzee alpha-globin cDNA (Liebhaber and Begley, 1984) into which was inserted a 24-base pair

oligonucleotide, encoding a functional N-linked glycosylation site. This coding region was placed in-frame behind the beta-lactamase signal sequence, and the resulting SP6 plasmid was called pSP SG 1 (Perara and Lingappa, 1985). This plasmid was also the source of the beta-lactamase signal sequence. The prolactin coding region used here was derived from a full-length bovine prolactin cDNA engineered behind the SP6 promoter (a gift from W.B. Hansen). It was also the source for the prolactin signal sequence, which was modified by the insertion of a Sal I site, one codon beyond the cleavage site of bovine prolactin, as described in Andrews et al., 1988. The stop transfer sequence was derived from the transmembrane region of IgM (Early et al., 1980) as described previously (Yost et al., 1983; Mize et al., 1986). Details by which each of the constructions used here were generated follow:

pSP S_L·G - Plasmid pSP SG 1 was cut with restriction endonuclease BstE II and Xba I, treated with Klenow fragment of DNA polymerase I, religated, and digested with Xba I.

pSP G - This was generated from pSP S_L·G by digestion with Nco I and Bgl II, followed by treatment with the Klenow fragment, religation, and digestion with Bgl II.

pSP ST·G* - This plasmid encodes the ST sequence from IgM heavy chain, joined to full-length chimpanzee alpha-globin, with an eight amino acid glycosylation site engineered into the globin coding region at the BssH II site.

pSP ST·G - This was generated from a gene fusion, pSP ST·G*, encoding the ST sequence of IgM heavy chain (5' to full-length chimpanzee alpha-globin, and including the glycosylation insert), by digestion with BstE II and Xba I, followed by treatment with the Klenow fragment, religation, and digestion with Xba I.

pSP P - This was generated from pSP gGP2 (Simon et al., 1987) by digestion with Nco I, followed by religation and digestion with BstE II.

pSP G·P - This was generated from pSP gGP2 by digestion with Pvu II and BstE II, followed by treatment with the Klenow fragment, religation and redigestion with Pvu II.

pSP ST·P - This was generated from pSP ST·G·ST·P (described below) by digestion with Nco I and BstE II, followed by treatment with the Klenow fragment, religation and digestion with BssH II.

pSP S_P·P - This was generated from plasmids pSP gGP2 and pSP CS38 as described by Andrews et al., 1988.

pSP G·S_P·P - Generated as described by Perara and Lingappa,1985.

pSP G·ST·P - Generated as described by Mize et al., 1986.

pSP ST·G·ST·P - A Hind III fragment was isolated from pSP ST·G and ligated with pSP gG·ST·P (Mize et al., 1986) that had been cut with Hind III and treated with calf intestinal alkaline phosphatase. Transformants were screened for inserts with the correct orientation.

pSP S_L·G·ST·P - This was generated as described above for pSP ST·G·ST·P, except that the Hind III fragment was derived from pSP SG 1 (Perara and Lingappa, 1985).

pSP ST·G·S_P·P - This was generated as described above for pSP ST·G·ST·P, except that the Hind III fragment was from pSP ST·G, and the Hind III-cut, calf intestinal alkaline phosphatase treated vector was from pSP gGSTK1 (Mize et al., 1986). The resulting plasmid (pSP ST·gG·S_P·PK1) was digested with Sal I and BstE II, treated with calf intestinal alkaline phosphatase, and ligated with the Sal I/BstE II fragment isolated from pSP gG·S^{+1}·P^T (Andrews et al., 1988).

pSP S_L·G·S_P·P - This was generated as described above, except that the Hind III fragment was from pSP SG 1, and the resulting plasmid was digested with Sal I and BstE II, treated with calf intestinal alkaline phosphatase, and ligated with the Sal I/BstE II fragment prepared as described above.

pSP S_L.S_P·P - This was generated from pSP S_L·G·S_P·P by digestion with Nco I and religation.

pSP gG·ST·gG - This was generated by digestion of pSP G·ST·P with Sph I and EcoR I, gel purification of this insert, and ligation into a gel purified vector, generated from digestion of pSP S_L·ST·gG with SpH I and EcoR I, which encodes the full-length globin coding region with a glycosylation site engineered into the BstE II site of globin (Mize, unpublished construct).

pSP S_L·L·ST·G·P - This was generated from pSP S_L·L·ST·G and pSP G·P by digestion of both with BstE II and Sal I, followed by treatment of the former with calf intestinal alkaline phosphatase, purification of the fragment from the latter, and its ligation into the phosphate-treated vector.

pSP S_L·L·ST·G·S_P·P and pSP S_L·L·ST·G·ST·P - These were generated as described above, except that pSP G·S_P·P and pSP G·ST·P were used as sources of BstE II/Sal I fragments.

All constructions were mapped extensively with restriction endonucleases and characterized by transcription-linked translation in the rabbit reticulocyte lysate cell-free protein-synthesizing system (RRL), followed by immunoprecipitation with globin, prolactin, or beta-lactamase antisera. Sizes of the encoded total and immunoreactive products were compared to markers and each other on SDS-PAGE.

In vitro transcription of SP6 plasmids

SP6 plasmids were cesium-purifed, extracted with phenol/chloroform, ethanol precipitated and redissolved in water. When preparing transcripts for injection into Xenopus oocytes, plasmids were first linearized by restriction endonuclease digestion at sites in the 3' untranslated region. In vitro transcription reactions were carried out in 10 ul-volumes as follows (Krieg and Melton, 1983): 0.1 mg/ml SP6 plasmid was added to a reaction mix containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM

spermidine, 10 mM dithiothreitol, 25 mg/ml calf-liver tRNA, 0.5 mM each ATP, CTP, and UTP, 0.1 mM GTP, 0.5 mM GpppG, 0.9 U/ul RNAsin and 0.9 U/ml SP6 RNA polymerase. Transcription reactions were carried out at 40^0C for 1 hour. Addition of GpppG (the cap reagant), results in increased expression of in vitro transcripts in cell-free translation reactions, and is essential for expression in Xenopus oocytes (Contreras et al., 1982).

In vitro translation of SP6 transcripts in the Rabbit Reticulocyte Lysate transcription-coupled system

Aliquots of the transcription reaction mixture were used directly in transcription-linked translations at a final concentration of 20%. In addition to transcription products, translation reactions included 45% rabbit reticulocyte lysate, 1 mCi/ml [^{35}S] methionine, 0.2 mM each of the other 19 amino acids (minus methionine), 16 mM Tris-HCl (pH 7.5), 100 mM KCl, 2mM $MgCl_2$, 0.44 mM spermidine, 2mM dithiothreitol, 0.9 mM GTP, 1mM ATP, 10 mM creatine phosphate, 0.4 mg/ml creatine phosphokinase and 0.1 mg/ml calf liver tRNA. Reaction mixtures were incubated at 25^0C for 1 hour (unless noted otherwise) in the absence or presence of 2 A_{280} U/ml dog pancreas microsomal membranes (prepared as described by Walter and Blobel, 1983c). In addition to signal cleavage, translocation was assayed by detergent-sensitive protection from proteinase K and sensitivity to digestion with endoglycosidase H, as described below.

Posttranslational analysis of translation products:

Proteolysis protection analysis

Immediately following translation, reaction mixtures were chilled on ice, adjusted to 10 mM $CaCl_2$, and divided into either 2 equal aliquots (for reactions not containing

microsomes) or three equal aliquots (for reactions which included microsomes). For both sets, one aliquot served as a control and received no added protease, and one aliquot was treated with 3 mg/ml proteinase K (dissolved in 10 mM $CaCl_2$, 50 mM Tris [pH 7.5]); for those reactions which included micromal membranes, the third aliquot was supplemented with both proteinase K and 1% Nikkol (a nonionic detergent used to disrupt the lipid bilayer). Proteolysis was carried out for 1 hour at 4^0C, and then stopped by addition of 2 mM phenylmethysulfonyl flouride (PMSF), and immediate transfer to 5X volume, 1% SDS in 0.1 M Tris-HCl (pH 8.9), which had been preheated to 100^0C. Reactions were then boiled for 15 minutes.

Immunoprecipitation

After cooling, proteolysis samples were diluted 20-fold in a solution of 1% Triton, 0.02 M Tris (pH 8.0), 2 mM EDTA, 20 mM NaCl, and supplemented with 2-10 ul of the appropriate antisera. Samples were incubated for 4-6 hours at 4^0C. 15-20 ul of a 50% slurry of protein A-Sepharose in Buffer A was then added to each tube and incubated for 1 hour at 4^0C. Samples were washed three times with Buffer A and subsequently, twice with buffer containing 0.1 M Tris-HCl (pH 7.5) and 0.1 M NaCl, to remove residual Triton. The presence of core carbohydrates was confirmed by digestion of the appropriate samples with endoglycosidase H (as described below).

Samples were eluted from protein A Sepharose with 1% SDS, 0.1 M Tris (pH 8.9), 2mM EDTA, 10 % glycerol, 0.01 % bromophenol blue, 0.5-1.0 M dithiothreitol, incubated for 30 minutes at 37^0C, and boiled for 5 minutes. Products were separated by SDS PAGE and visualized by autoradiography.

Endoglycosidase H analysis

Endoglycosidase H (endo H) removes simple core oligosaccharides from Asn residues, thereby causing a shift to a lower apparent molecular weight upon SDS PAGE. Endo H

digestion was therefore used to determine whether core glycosylation of translation products had occured.

Following immunoprecipitation, translation products were eluted from Protein A-Sepharose by boiling in 100 ul of 0.1 M sodium citrate (pH 5.5), 0.1 % SDS for 3 minutes. Supernatants were then removed and divided into two equal aliquots: Endo H was added to one at a final concentration of 1 ug/ml, and both aliquots were incubated at 37^0C for 12 hours. 10 ug of bovine serum albumin (BSA) was then added, and samples were chilled and adjusted to 15% ice-cold trichloroacetic acid (TCA). Following centrifugation, precipitates were collected, and samples were prepared for SDS PAGE as usual.

Membrane sedimentation and carbonate extraction

Cell-free translation products (40 ul) obtained in the presence of 2 A_{280} U/ml dog pancreas rough microsomes, were diluted in 2 ml of either ice-cold 0.1 M sodium carbonate (pH 11.5) (Fujiki et al., 1982), or ice-cold 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM triethanolamine (pH 7.4), 0.1 M KCl, and incubated on ice for 30 minutes. The samples were then centrifuged at 4^0C for 1 hour at 55,000 rpm in polycarbonate tubes (which had been precoated with 1% bovine serum albumin to prevent sticking) in a Beckman TI70.1 rotor (Beckman Instruments, Inc., Palo Alto, CA.).

Following centrifugation, supernatants were transferred immediately to 4 ml of ice-cold 20 % trichloroacetic acid (TCA) and incubated for 15 minutes on ice, after which precipitates were collected by centrifugation. Supernatant precipitates, and pellets, were dissolved in 1% SDS, 0.1 M Tris (pH 8.9) by vortexing, sonicating and boiling. Equivalent aliquots were then subjected to SDS-PAGE and autoradiography as usual.

Expression and characterization of products in Xenopus Oocytes

Microinjection of Xenopus oocytes

Groups of 30 oocytes were injected with appoximately 50 nl each of capped transcription products transcribed from linearized DNA (Colman et al., 1981; Contreras et al., 1982; Melton et al., 1984). Xenopus oocytes were incubated for 2 hours to allow transcripts to equilibrate, after which 50 nl of [^{35}S] methionine at a concentraiton of 100 mCi/ml was injected. Xenopus occytes were then incubated for an additional 6 hours in modified Barth's saline solution [88mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM Mg SO_4, 0.33 mM Ca $(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM Hepes (pH 7.6)], with 10% fetal calf serum. Those Xenopus occytes which were injected with transcript derived from the plasmids encoding three passenger domains were incubated for only 2 hours after injection of label, as degradation of products was observed with longer incubation times.

When assaying for internal initiation of translation, Xenopus oocytes were injected with transcript as described above, and incubated for 2 hours. Subsequently, oocytes were divided into 2 equal batches, one of which was injected with [^{35}S] methionine and 0.37 M hydroxyleucine, while the other was injected with [^{35}S] methionine and water. Xenopus oocytes were incubated for an additional 2 hours, homogenized and immunoprecipitated as described below.

Proteolysis and immunoprecipitation of Xenopus Oocyte translation products

Following removal of the medium, oocytes were homogenized in 20 ul/oocyte of Buffer A (1% Triton X-100, 0.1 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.01 M EDTA, 1 mM PMSF). Homogenates were then aliquoted for proteolysis.

Each sample was divided into three aliquots: the first aliquot was a control with no added protease, and the other two aliquots were treated with 0.3 mg/ml proteinase K in

the absence or presence of 1% Triton X-100. All samples were incubated for 3 hours at 4^0C. Proteolysis digestion was stopped by the addition of 2mM PMSF followed by immediate transfer to 5X volume boiling solution of 1% SDS in 0.1 M Tris-HCl (pH 8.9) for 15 minutes. Samples were then diluted 20-fold in a solution of 1% Triton, 0.02 M Tris (pH 8.0), 2 mM EDTA, 20 mM NaCl, and subjected to immunoprecipitation as follows.

After cooling to room temperature, proteolysis products were aliquoted, and 2-10 ul of the appropriate antisera was added to each sample; samples were incubated overnight at 4^0C, and centrifuged in an Eppendorf microfuge at 10,000 *g* for 15 minutes to remove aggregates. Supernatants were transferred to a fresh tube containing 15-20 ul of a 50% slurry of protein A-Sepharose in Buffer A and incubated for 1 hour at 4^0C. Samples were then washed three times with Buffer A, and subsequently twice with buffer containing 0.1 M Tris-HCl (pH 7.5) and 0.1 M NaCl, to remove residual Triton.

The presence of core carbohydrates was confirmed by digestion of the appropriate samples with endoglycosidase H (as described above). Samples were then separated by gel electrophoresis and visualized by autoradiography.

Posttranslational translocation assay

Cell-free translation reactions were carried out for 20 minutes at 25^0C; 10^{-4} M ATA was added to inhibit initiation, and incubation was continued for an additional 15 minutes. Translation reactions were then supplemented with emetine at 10^{-4} M to inhibit elongation, and incubated for 5 minutes longer. These reaction mixtures were then supplemented with microsomal membranes and incubated for 30-60 minutes to assay for posttranslational translocation.

Fractionation of polysomes and depletion of energy from a cell-free translation system

Following inhibition of protein synthesis as described above, translation reactions were applied to a 1-ml S-300 Sephacryl gel filtration column equilibrated in RRL buffer (120 mM KCl, 3 mM $MgCl_2$, 20 mM TEA, 1mM dithiothreitol) and a void volume was collected. Void volume fractions containing partially purified polysomes were incubated with microsomal membranes at 25^0C for 20 minutes in the absence or presence of an energy cocktail (1mM ATP, 2 mM GTP, 12 mM creatine phosphate, and creatine phosphokinase at a final concentration of 40 ug/ml). Samples were precipitated with trichloroacetic acid (TCA), subjected to SDS-PAGE and visualized by autoradiography.

High speed centrifugation for preparation of ribosome-depleted supernatants

Translation reactions were performed as described, and some treated with 1 mM puromycin for 15 minutes at 24 ^{0}C, to release nascent polypeptides from the ribosome. Following addition of 10^{-4} M ATA and emetine, reactions were spun at 4 ^{0}C in a Beckman airfuge at 28 psi for 30 minutes. Supernatants were removed and the pellets were resuspended in an equal volume of buffer with the same ionic composition and components as the reticulocyte lysate translation reaction. These samples were supplemented with ATA and emetine to 10^{-4} M, before assaying for posttranslational translocation.

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