Construction of Defined Polytopic Integral Transmembrane Proteins

THE ROLE OF SIGNAL AND STOP TRANSFER SEQUENCE PERMUTATIONS*

(Received for publication, March 2, 1988)

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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. We have investigated the role of these topogenic sequences in the biogenesis of polytopic transmembrane proteins. Plasmids encoding various patterns of well-characterized signal and stop transfer sequences fused to a set of topologically 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 observed orientation with respect to the 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.

Termination of chain translocation is an event unique to TMP biogenesis and is responsible for conferring transmembrane orientation. Gene fusion and deletion studies have suggested that translocation termination is directed by another class of topogenic sequences termed stop transfer sequences (Boeke and Model, 1982; Yost et al., 1983). The mechanism by which stop transfer sequences act to terminate translocation is controversial. In one view, the thermodynamics of interactions between the hydrophobic amino acid side chains and the hydrophobic core of the lipid bilayer are sufficient to terminate translocation (Engelman and Steitz, 1981; Davis and Model, 1985). According to another view, termination of translocation is a receptor-mediated event, involving interactions with proteins in the ER membrane, and is intimately related to the events of translocation initiation (Mize et al., 1986; Szczesna-Skorupa et al., 1988).

The next level of transmembrane complexity is displayed by those proteins, termed polytopic TMPs, which span the membrane more than once. Some of these TMPs span the membrane twice and, as a result, have both carboxyl and amino termini on the same side of the membrane (e.g., Eble et al., 1987). Proteins which span the membrane more than twice can adopt very complex orientations, with amino and carboxyl termini on the same or opposite sides of the membrane and multiple transmembrane domains. For example, the acetylcholine receptor spans the membrane five times (Young et al., 1985).

The mechanism by which polytopic TMPs achieve their unique transmembrane disposition is unknown. One hypothesis has been that proteins with multiple membrane-spanning domains are "stitched" into the bilayer by the sequential action of signal and stop transfer sequences (Lingappa et al., 1979; Blobel, 1980). The discovery of internal uncleaved signal sequences in TMPs (Bos et al., 1984; Friedlander and Blobel, 1985) makes this notion plausible. Nevertheless, very little work has been done thus far to test this hypothesis directly.

One approach toward understanding TMP biogenesis has been to use gene deletions and fusions to demonstrate that more than one region within bovine opsin can direct translocation across the ER membrane in a signal recognition particle-dependent fashion (Friedlander and Blobel, 1985). An extension of that work localized additional transmembrane regions with signal activity, although each of these segments was also found to express varying degrees of stop transfer function as well (Audigier et al., 1987). These studies strongly suggest (but do not prove) that multiple topogenic sequences are involved in polytopic TMP biogenesis. Moreover, they do not allow these sequences to be well categorized. The recent finding that bopside stop transfer sequences can, in certain contexts, display signal recognition particle-dependent initiation of chain translocation (Mize et al., 1986; Zerial et al., 1987) demonstrates that signal and stop transfer sequences share properties. For this reason, categorization and elucidation of the role of signal and stop transfer sequences in the biogenesis of polytopic transmembrane proteins is important for understanding how proteins achieve their unique dispositions in the membrane.
Polypotic Membrane Protein Biogenesis

EXPERIMENTAL PROCEDURES

Materials

All restriction endonucleases and nucleic acid-modifying enzymes were obtained from either Boehringer Mannheim or New England BioLabs (Beverly, MA). Placental RNase inhibitor was from Promega Biotech (Madison, WI); Affi-Gel-protein A-agarose was from Bio-Rad; rabbit anti-human hemoglobin serum was from Cappell Laboratories (Cochranville, PA); rabbit anti-ovine prolactin serum was from Chung Nan Chang; proteinase K was from Worthington, NJ; BglII was from New England Biolabs; [35S]methionine was from Du Pont-New England Nuclear; and Nikkolase H and Triton X-100 were from Boehringer Mannheim; endoglycosidase H and Triton X-100 were from Boehringer Mannheim; [35S]methionine was from Du Pont-New England Nuclear; and Nikkolase H (octaethylene glycomono-n-dodecyl ether, a nonionic detergent) was from Nihon Kayaku (Tokyo, Japan).

Methods

Plasmids

The globin coding region was derived from a full-length chimpanzee α-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 β-lactamase signal sequence, and the resulting SP6 plasmid was called pSP SG1 (Perara and Lingappa, 1985). This plasmid was also the source of the β-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 of the prolactin signal sequence, which was modified by the insertion of a SalI site, one codon beyond the cleavage site of bovine prolactin, as described.1 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.G—Plasmid pSP SG1 was cut with restriction endonucleases BstEII and XbaI, treated with the Klenow fragment of DNA polymerase I, religated, and digested with XbaI.

pSP G—This was generated from pSP S.G by digestion with Ncol and BglII, followed by treatment with the Klenow fragment, religation, and digestion with BglII.

pSP ST—pSP ST was cloned into pSP T.SST.P by digestion of both with BstEII and SalI, followed by treatment of the former with calf intestinal alkaline phosphatase, purification of the fragment from the latter, and its ligation into the phosphatase-treated vector.

pSP ST. P—A HindIII fragment was isolated from pSP ST. P, digested with BstEII and SalI, followed by religation and digestion with BglII.

pSP ST. G—A HindIII fragment was isolated from pSP ST. G and ligated with pSP gg ST. P (Mize et al., 1986) that had been cut with HindIII and treated with calf intestinal alkaline phosphatase.

Transformants were screened for inserts with the correct orientation.

pSP ST. G ST. P—This was generated as described above for pSP ST. G ST. P, except that the HindIII fragment was from pSP ST. G, and the HindIII-cut, calf intestinal alkaline phosphatase-treated vector was from pSP gGSPK1 (Mize et al., 1986). The resulting plasmid (pSP ST. gg ST. P) was digested with SalI and BstEII, treated with calf intestinal alkaline phosphatase, and ligated with a SalI/BstEII fragment isolated from pSP gG SPK1.

pSP ST. G ST. P—This was generated as described above, except that the HindIII fragment was from pSP SG1, and the resulting plasmid was digested with SalI and BstEII, treated with calf intestinal alkaline phosphatase, and ligated with the SalI/BstEII fragment prepared as described above.

pSP ST. G ST. P—This was generated from pSP ST. G ST. P by digestion with Ncol and religation.

pSP S.L—S.L.—ST. G—This was generated from pSP ST. L—S.L.—G and pSP G P by digestion of both with BstEII and SalI, followed by treatment of the former with calf intestinal alkaline phosphatase, purification of the fragment from the latter, and its ligation into the phosphatase-treated vector.

pSP S.L—L.—ST. G—P and pSP S.L—L.—ST. G—P—These were generated as described above, except that pSP G S-P and pSP G—P were used as source of BstEII/Sall 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, and β-lactamase antisera (Perara and Lingappa, 1985). Sizes of the encoded total and immunoreactive products were compared to markers and each other on SDS-PAGE.

Transcription-linked Translation-coupled Translocation

Transcription-linked translation of SP6 plasmids in the RRL was performed as described (Perara and Lingappa, 1985). Dog pancreas microsomal membranes were prepared as described (Walter and Blobel, 1983). Translation across microsomal membranes was assayed by several criteria: signal cleavage, detergent-sensitive protection from protease K, and sensitivity to digestion with endoglycosidase H (see below).

Proteolysis and Immunoprecipitation of Cell-free Translation Products

Proteolysis protection experiments, immunoprecipitation, and sample preparation for SDS-PAGE were as described (Blobel and Dobberstein, 1975; Perara and Lingappa, 1985). Bands were visualized by autoradiography following fluorography and quantified by densitometry of the autoradiograms (Mize et al., 1986).

Expression and Characterization of Products in Xenopus Oocytes

Groups of 30 Xenopus oocytes were injected with approximately 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 h to allow transcripts to equilibrate, after which 50 nl of [35S]methionine at a concentration of 100 μCi/ml was injected. Xenopus oocytes were then incubated for an additional 6 h in modified Barth’s saline solution with 10%...
fetal calf serum. Those *Xenopus* oocytes which were injected with transcript derived from the plasmids encoding the three passenger domains were incubated for only 2 h after injection of label, as degradation of products was observed with longer incubation times. *Xenopus* oocytes were harvested, homogenized, proteolyzed, and immunoprecipitated as described previously (Simon *et al.*, 1987). The presence of core carbohydrates was confirmed by digestion with endoglycosidase H (Tarentino *et al.*, 1974; Perara *et al.*, 1986).

**RESULTS**

**Definition of Passenger Domains and Simple Topogenic Elements.**—As a first step toward building defined proteins encoded by gene fusions, we selected prototypic signal, stop transfer, and passenger coding regions. The signal sequence of β-lactamase and of bovine prolactin and the stop transfer sequence of IgM μ-heavy chain were chosen because they have previously been shown to initiate and terminate translocation, respectively, when engineered in a variety of simple fusion proteins (Yost *et al.*, 1983; Lingappa *et al.*, 1984; Perara and Lingappa, 1985; Simon *et al.*, 1987).

The choice of the passenger coding regions was subject to several restrictions. First, because we wished to compare 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, the preceding and subsequent passengers flanking a topogenic sequence must be antigenically distinct to permit unambiguous assignment of the topology of the encoded product. Not all protein domains lacking a topogenic sequence are suitable to serve as passengers. Studies suggest that some regions are more or less

![Restriction maps and coding regions of passenger domains without (A) and with (B) topogenic sequences](image-url)
The secretory protein prolactin as passengers because they hindered in their ability to be translocated by signal sequences (Kadanoga et al., 1984). Thus, for the purposes of this study, a passenger domain was defined as one that contained no demonstrable topogenic sequences itself and was permissive to the action of either signal or stop transfer sequences.

We chose domains from the cytoplasmic protein globin and the secretary protein prolactin as passengers because they met the above criteria (Lingappa et al., 1984). Fig. 1 diagrams these passengers alone and with signal or stop transfer sequences engineered at the 5' end in SP6 expression plasmids. The globin-derived passenger domain (G) consists of the first 110 amino acids of chimpanzee α-globin containing an eight-codon insert encoding a functional N-linked glycosylation site inserted at codon 20 in the globin sequence; the prolactin-derived passenger (P) encodes amino acids 57-199 of bovine prolactin; G·P is a precise fusion polypeptide of the two domains (Fig. 1A). These passengers were then engineered 3' to and in-frame with the precise coding regions of either the α-globin-derived passenger (G) or prolactin (Sp) signal sequence or the IgM μ-heavy chain stop transfer sequence (ST) (Fig. 1B). As can be seen in Fig. 2, each passenger and each topogenic sequence in the presence of a passenger showed properties expected: G and P alone and together were completely digested in the presence of membranes by added proteases, with no protease-protected products observed (Fig. 2, lanes 1-3, 12-14, and 23-28). This indicated cytosolic disposition of the passenger domains, as diagramed below the gels. The corresponding signal sequence fusion proteins displayed a secretory phenotype (lanes 4-7 and 15-18). Expression of both pSP ST·G and pSP ST·P resulted in populations of signal-cleaved products that were fully protected from protease digestion (lanes 6 and 17). Most of the former were glycosylated as expected (lane 6, large downward pointing arrowhead) with a few molecules nonglycosylated and cleaved (small downward pointing arrowhead). The corresponding experiments for each passenger domain with the other signal sequence (i.e. pSP ST·G and pSP ST·P) gave similar results (data not shown).

The stop transfer fusion proteins displayed the character-
Phenotype generated from the expression of fusions encoding the cell-free translocation system posed a significant problem. These plasmids were next used to construct the ultimate goal of achieving a protein with polytopic orientation.

Lingappa, unpublished observations.

whose first topogenic sequence fails to engage a receptor may be passenger domains. However, the relative inefficiency of a first step toward this goal, we wished to compare systematically the topology of fusion proteins containing various arrangements of signal and stop transfer sequences separated by 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 for the analysis of plasmids encoding multiple topogenic sequences, with the ultimate goal of achieving a protein with polytopic orientation.

Fusion Protein Containing Two Topogenic Sequences—As a first step toward this goal, we wished to compare systematically the topology of fusion proteins containing various arrangements of signal and stop transfer sequences separated by 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. Analysis of molecules containing two topogenic sequences is problematic in cell-free systems such as the RRL because those molecules whose first topogenic sequence fails to engage a receptor may now present their second topogenic sequence as if it were the first. This phenomenon could obscure interpretation of the phenotype generated from the expression of fusions encoding multiple topogenic sequences. To avoid these problems inherent in cell-free systems, we decided to study the expression of plasmid encoding molecules containing multiple topogenic sequences in Xenopus oocytes, where nascent polypeptides have been shown to be translocated with a significantly higher efficiency but with congruent topology to that observed in the RRL (Simon et al., 1987).

Based on previous work in cell-free systems (Yost et al., 1985), 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 unpredictable. Since the nature of stop transfer-mediated translocation remains poorly understood, it was important to determine if a subsequent stop transfer sequence would terminate translocation initiated by an amino-terminal stop transfer sequence. Two plasmids, diagramed in Fig. 3, were constructed. The first (pSP SLG-ST-P) was designed to confirm the classical function of a stop transfer sequence in the presence of the passengers used here. The second (pSP STG-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 SLG-ST-P was observed to be a glycosylated molecule of the expected size (~40 kDa) that was reactive to both globin and prolactin antisera (Fig. 4A, lanes 1–3). Upon proteolysis, a glycosylated globin domain of the expected size (lanes 5 and 6) was observed (lanes 4), but no prolactin-reactive domain (lane 4), was seen. The globin-reactive protease-protected fragment was abolished by proteolysis in the presence of a 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).

When the product encoded by pSP STG-ST-P was similarly analyzed, a globin- and prolactin-reactive molecule of predicted size (approximately 42 kDa) was observed (Fig. 4B, 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 the gels). 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 STG-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) occurs 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).

Next, we sought to test whether a signal sequence would terminate translocation following translocation initiated by either a signal or stop transfer sequence. Previous studies have addressed this question, but have provided conflicting results (Coleman et al., 1985; Finidori et al., 1987; Stirzaker et al., 1987). We engineered three plasmids as companion constructions (each encoding a signal sequence in the second position) (see Fig. 5). Plasmid pSP SLG-ST-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. 1B. In a second construction (pSP STG-S-P), the initial topogenic sequence is ST rather than SL. These permutations were made so that the ability of an internal signal sequence to terminate translocation (which had been initiated by either a signal or stop

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transfer sequence at the amino terminus) could be assessed. In a third construction (pSP Sl-G.Sp-P), the globin coding region was removed by an in-frame deletion from pSP Sl.G.Sp.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).

The results obtained when these three constructions were expressed in Xenopus oocytes are illustrated in Fig. 6. Expression of pSP Sl.G.Sp.P in Xenopus oocytes gave a product of predicted molecular weight, which was both globin- and prolactin-immunoreactive (Fig. 6A, lanes 2 and 3). The product was a glycosylated, fully translocated molecule, as evidenced by sensitivity to endoglycosidase H both before (lane 1) and after (lane 6) proteolysis with proteinase K. The presence of 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).

When translocation was initiated by the stop transfer sequence (pSP ST.G.Sp-P), topology of the molecule was essentially identical to that obtained for both pSP ST.G and pSP ST.P in Fig. 2 (lanes 8-11 and 19-22) in that the ST sequence was not fully translocated. The first topogenic sequence (ST) directed translocation and glycosylation of the passenger, but itself remained accessible to protease (Fig. 6B, lanes 1 and 2 versus 5-7). The second topogenic element (Sp) was completely translocated along with both passengers as in pSP Sl.G.Sp.P and was only rarely cleaved (Fig. 6B, lanes 3, 4, and 8). Similar results were found in the RRL (data not shown).

In Fig. 6C, expression of pSP Sl.Sp-P gave an analogous topology to that of pSP Sl.G.Sp-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 Sl.Sp-P, cleavage of the second signal sequence was highly efficient (lanes 2-7).

The inability of Sp as a second element to terminate translocation in all three cases described for Fig. 6 does not reflect steric or other hindrance of signal sequence recognition spe-
cific to this molecule. The internal signal sequence functioned with high efficiency to initiate translocation in the identical fusion protein lacking the amino-terminal signal sequence. Thus, despite the apparent overlap in signal and stop transfer sequence activity revealed by the ability of a stop transfer sequence to initiate translocation, signal and stop transfer sequences are not functionally interchangeable. The results presented here suggest that the most promising approach for generating a polytopic membrane protein would be to use combinations of both signal and stop transfer sequences.

Polytopic Transmembrane Protein Biogenesis—As demonstrated in Figs. 4 and 6, of those molecules containing two topogenic sequences, only those with ST in the second position were found to have bitopic transmembrane topology. We reasoned that a bitopic TMP could be converted into a polytopic TMP if: (i) translocation could be reinitiated subsequent to termination and (ii) the stop transfer sequence responsible for termination of translocation remained anchored in the membrane in spite of reinitiation of translocation.

To build a molecule which could 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). The need for a defined bitopic fusion protein coding region and for a third passenger to be utilized by pSP St-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 (St and ST) as well as one of the two passenger domains (G). The ability of a third topogenic sequence and passenger to convert an encoded bitopic fusion protein into one of polytopic orientation could be assessed by replacing the globin coding region in pSP StL-ST-G with pSP G-St-P, pSP ST-G-St-P, or pSP G-P and analyzing the topology of the encoded products in Xenopus oocytes. The three resulting molecules (pSP St-L-ST-G-StP, pSP St-L-ST-G-ST-P, and pSP St-L-ST-G-St-P), diagramed in Fig. 7, differ only in the presence of St or no topogenic sequence before the subsequent P domain.

Expression of the transcription products of these plasmids in Xenopus oocytes resulted in products of the expected size that was immunoreactive to β-lactamase, globin, and prolactin antisera. As expected, peproteolysis with protease K generated a distinct 20-kDa β-lactamase-reactive band (which was proteolyzed in the presence of detergent) in the products encoded by all three constructions (A, lanes 4 and 10; B, lanes 4 and 10, downward pointing arrowhead; C, lanes 4 and 10). In the case of pSP StLST-G-P, which lacks a third topogenic sequence between the G and P passenger domains, no globin- or prolactin-reactive protease-protected products were observed (A, lanes 5-9). This is consistent with a bitopic disposition (A, diagram below the gel), as expected.

A new fragment was generated upon peproteolysis of the products of pSP St-L-ST-G-Sp-P. This band (Fig. 8B, lane 5, downward pointing arrow) 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 pSP G-St-P domain of the full-length molecule. In addition, another prolactin reactive fragment was observed before and after peproteolysis in the absence of detergent which migrates at the size of the P domain of pSP Sp-P (lanes 2 and 5; see arrow P). This product presumably results from signal peptidase activity following reinitiation of translocation by the third topogenic sequence. The relative intensity of the protected bands representing G-St-P versus P suggests that approximately 20% of the protected fragments were not processed by signal peptidase. Experiments involving incorporation of β-hydroxyeucine, an amino acid analog that blocks chain translocation (Hortin and Boime, 1980; Walter et al., 1981, Simon et al., 1987), confirm that this P product is derived from the full-length molecule (SpL-ST-G-St-P) and not from internal initiation of protein synthesis (data not shown). Consistent with this interpretation, a protease-sensitive fragment that β-lactamase- and globin-immunoreactive, of predicted size for the remainder of the molecule after peptidase cleavage, is observed (lane 3; see arrow 2). The expected globin reactivity of this fragment is seen on longer exposure (data not shown). 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. 8B.

Analysis of the expression 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. No protease-resistant molecules displaying both globin and prolactin immunoreactivity were evident; however, a faint protected fragment reactive to prolactin antisera alone was observed (Fig. 8B). No protease-resistant globin-immunoreactive fragment which co-migrates in lanes 1 and 3. A globin-immunoreactive fragment which co-migrates 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 indicates the prolactin-immunoreactive fragment resulting from cleavage after S$_P$. In B (lane 4), the downward pointing arrowhead designates the lactamase-protected fragment co-migrating with that in A; the triangle in lane 3 indicates a lactamase-immunoreactive product presumed to represent a population of molecules that terminated synthesis prematurely. The downward pointing arrowhead in lane 5 indicates a prolactin- and globin-immunoreactive fragment which is glycosylated and protected. Lanes 1–5 and 6–10 were from separate experiments. Dots in C (lanes 2 and 4) indicate the positions of ST.-P and P products from RRL translation; these co-migrate with oocyte products in the absence and presence of proteolysis, respectively, and are more easily visible on a longer exposure of the gel. Diagrams below the gels indicate inferred transmembrane orientation of products. Striped, black, and white bars represent β-lactamase, globin, and prolaktin domains, respectively. In B, the line in the globin domain represents glycosylation, and the arrow indicates cleavage after S$_P$. Molecular weights (×10$^3$) are designated.

**Fig. 8. Xenopus oocytes expression of fusions with three topogenic sequences.** Xenopus oocytes were injected with SP6 transcripts synthesized from pSP $S_L$-L-.ST.G.P (A), pSP $S_L$-L-.ST.G-S$_P$-P (B), and pSP $S_L$-L-.ST.G.ST.-P (C), labeled with $[^{35}S]$methionine, homogenized, aliquoted, and subjected to post-translational proteolysis with protein K. Proteolysis products were immunoprecipitated with β-lactamase (L), prolactin (P), or globin (G) antisera, and selected samples were digested with endoglycosidase H, followed by SDS-PAGE. See legend to Fig. 4 for abbreviations. In B, arrow 1 indicates full-length product and arrow 2 the β-lactamase- 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 indicates the prolactin-immunoreactive fragment resulting from cleavage after S$_P$. In B (lane 4), the downward pointing arrowhead designates the lactamase-protected fragment co-migrating with that in A; the triangle in lane 3 indicates a lactamase-immunoreactive product presumed to represent a population of molecules that terminated synthesis prematurely. The downward pointing arrowhead in lane 5 indicates a prolactin- and globin-immunoreactive fragment which is glycosylated and protected. Lanes 1–5 and 6–10 were from separate experiments. Dots in C (lanes 2 and 4) indicate the positions of ST.-P and P products from RRL translation; these co-migrate with oocyte products in the absence and presence of proteolysis, respectively, and are more easily visible on a longer exposure of the gel. Diagrams below the gels indicate inferred transmembrane orientation of products. Striped, black, and white bars represent β-lactamase, globin, and prolaktin domains, respectively. In B, the line in the globin domain represents glycosylation, and the arrow indicates cleavage after S$_P$. Molecular weights (×10$^3$) are designated.

**DISCUSSION**

We have approached the problem of polytopic TMP biogenesis by attempting to build such a molecule from signal and stop transfer sequences derived from products of simple topology. This involved analysis of the expression of a hier-
from the carboxyl terminus of the signal sequence abolished translocation (Randall and Hardy, 1986). Additionally, in eukaryotes, deletions in the domain carboxyl to the prolactin signal sequence dramatically reduced translocation efficiency. The protein domain preceding a signal sequence has also been shown to effect translocation. A deletion in the asialoglycoprotein receptor 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).

In view of these observations, can meaningful conclusions be drawn from the analysis of fusion proteins? This study suggests that they can, provided well-characterized coding regions are used and matched sets of constructions are generated. Using such an approach, we made observations that served as reliable guidelines for the construction of molecules with more complex transmembrane orientations. In every case examined, the stop transfer sequence at the amino terminus initiated peptide chain translocation, irrespective of the nature of topogenic sequences elsewhere in the protein. Conversely, following initiation of translocation by either a signal or stop transfer sequence, a stop transfer sequence served to terminate translocation. By contrast, a bona fide signal sequence was unable to terminate translocation previously initiated by either topogenic sequence. Thus, the action of a topogenic sequence appears to be determined by the topogenic sequences that have appeared earlier in the protein. In view of the prototypic nature of the sequences used here, we believe that our conclusions are general. Nevertheless, these conclusions need to be confirmed for additional examples of each topogenic sequence.

Mechanism of Translocation Termination—In all cases observed here (Fig. 4) and previously (Yost et al., 1985; Mize et al., 1986), a stop transfer sequence in the second position (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. 4 and 6). Thus, signal and stop transfer sequences are functionally distinct and noninterchangeable. 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 hydrophobicity of the internal signal and stop transfer sequences used in our study. The longest uninterrupted sequence of hydrophobic residues in S_p was 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. Therefore, it is 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.

Paradoxical Stop Transfer Functions—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

Fig. 9. Percentage prolactin protection conferred by third topogenic sequence. Prolactin-immunoreactive precursor and protease protected bands in Fig. 8 (A–C) were quantified by densitometry. The percent prolactin translocated is the ratio of protected molecules to precursor molecules, corrected for efficiency of protection in each experiment, defined by the β-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 (striped bar) chains.

Caveats for Analysis of Protein Topogenesis by Gene Fusion—Extensive characterization of individual topogenic sequence and passenger components was necessary in view of several recent studies. Some of these have shown that not all protein domains are translocated when fused to a signal sequence (Moreno et al., 1980; Kadanoga et al., 1984). Other studies have come to contradictory conclusions regarding the action of a signal sequence in an internal position either alone (Perara and Lingappa (1985) versus Kuhn (1987)) or following an amino-terminal signal sequence (Coleman et al. (1985) versus Stirzaker et al. (1987)). Finally, the recent demonstration that stop transfer sequences can, in certain contexts, initiate translocation (Mize et al., 1986; Zerial et al., 1987) placed new urgency on the need for careful definition of fusion protein components. Some of these paradoxical findings may reflect differences in prokaryotic and eukaryotic translocation machinery (e.g. Coleman et al. (1985) versus Stirzaker et al. (1987)). Others may reflect heretofore unrecognized features of the mechanisms of receptor-mediated topogenesis (Mize et al., 1986). Still other differences may be a consequence of steric or other effects of protein folding. For example, in prokaryotes, a single amino acid substitution 283 residues above...
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A series of alternating signal and stop transfer sequences (see Fig. 10B). 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. Perhaps this reflects roles for topogenic sequences distinct from classical signal and stop transfer varieties. Introduction of such novel topogenic sequences (e.g. Zerial et al., 1986; Ebé et al., 1987; Sakaguchi et al., 1987; Audigier et al., 1987) in place of the topogenic sequences used in this study might direct alternate topologies. This study also provides a 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., 1987).

REFERENCES

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