Signal and stop-transfer sequences are the known determinants involved in topogenesis of integral membrane proteins. To study the characteristics of stop-transfer sequences, artificial proteins have been created on the DNA level based on the cDNA of the asialoglycoprotein receptor H1. Its internal signal/anchor domain initiates translocation of the downstream sequence across the endoplasmic reticulum membrane. The ability of several hydrophobic sequences inserted into the translocating polypeptide to stop further transfer was analyzed by translation of the fusion proteins using the wheat germ extract and rabbit reticulocyte lysate systems with dog pancreas microsomes. We discovered that some of the sequences behave differently with respect to translocation across the membrane depending on the translation system. Expression of one of the fusion proteins in fibroblasts showed that the reticulocyte lysate system reflects more closely the in vivo situation than the wheat germ system. Our results suggest that in a homologous system the translocating ribosomes interact with the translocation machinery and influence the termination of polypeptide transfer by hydrophobic sequences.

In proteins inserted into the endoplasmic reticulum (ER) membrane, two topogenic sequences have been characterized: the signal sequence required for targeting protein synthesis to the ER and for initiating translocation of the protein across the membrane, and the stop-transfer sequence which halts the further translocation of the polypeptide chain (Blobel, 1980; Sabatini et al., 1982; Walter et al., 1984; Wickner and Lodish, 1985). The main characteristic of both elements is a stretch of apolar amino acids, typically 7–15 in cleaved amino-terminal signals (Watson, 1984; von Heijne, 1985), and around 20 in uncleaved signals and stop-transfer sequences spanning the lipid bilayer. Mutational analyses indicate that an uninterrupted sequence of 8–12 hydrophobic residues is necessary to retain signal activity (Garoff, 1985; Kaiser et al., 1987; Spiess and Handschin, 1987) and, similarly, of 8–16 residues for stop-transfer activity (in eukaryotes: Adams and Rose, 1985; in bacteria: Davis et al., 1985; Davis and Model, 1985).

Are there distinctive properties specific for signal or stop-transfer sequences? In several cases, signal and stop-transfer sequences were shown to be interchangeable: the anchor sequence of Semliki Forest virus p62 and that of influenza virus hemagglutinin can functionally replace the internal signal sequence of the transferrin receptor (Zerial et al., 1987); several of the presumed stop-transfer segments of opsin can function as signal sequences (Audigier et al., 1987) as well as the stop-transfer sequence of the IgM μ-heavy chain (Rothman et al., 1988); and the signal domain of cytochrome P-450 functions as a stop-transfer sequence when made part of a translocated polypeptide (Finideri et al., 1987). These findings suggest that the function of hydrophobic domains in topogenesis may be determined by their relative position within the polypeptide chain. This concept is supported by a series of constructs we have made in which a portion of the asialoglycoprotein (ASGP) receptor H1 including its internal signal/anchor domain was tandemly repeated up to four times (Wessels and Spiess, 1988). In these proteins, the first copy of the hydrophobic domain functions as a signal, the second as a stop-transfer sequence, and the third initiates a second translocation process, which is stopped by the fourth.

However, signal and stop-transfer sequences are not always equivalent; some sequences were found not to perform both topogenic functions. For example, not all stop-transfer segments tested functioned as targeting signals (Audigier et al., 1987), and several signal sequences placed in an internal position did not stop translocation (Finideri et al., 1987; Rothman et al., 1988). What the distinguishing features are is still poorly understood.

To study the properties of potential stop-transfer sequences, we started out with a plasmid encoding an artificial 2-fold membrane-spanning protein, a derivative of the ASGP receptor H1, containing two copies of the internal signal/anchor domain (pSAA, Wessels and Spiess, 1988). The second copy of this hydrophobic domain, which is in a stop-transfer position, was exchanged by other sequences to be tested for their ability to arrest translocation across microsomal membranes in vitro. We discovered that some sequences behave differently with respect to translocation across the membrane depending on the translation system used (wheat germ extract, rabbit reticulocyte lysate, or in vivo in mouse fibroblast cells). Our results suggest that the interaction between the ribosomes and the ER membrane influences the termination of protein translocation by hydrophobic sequences.

**EXPERIMENTAL PROCEDURES**

**Materials**

SP6 RNA polymerase, soybean trypsin inhibitor, and endo-β-N-acetylgalactosaminidase (Endo H) were from Boehringer Mannheim. Other modifying and restriction enzymes, linkers, and 5'-[³²P]-methylguanosine 5'-adenosine triphosphate were purchased from Boehringer Mannheim.

The abbre-iations used are: ER, endoplasmic reticulum; ASGP, asialoglycoprotein; PBS, phosphate-buffered saline; RL, rabbit reticulocyte lysate; SDS, sodium dodecyl sulfate; SRP, signal recognition particle; WG, wheat germ extract; Endo H, endo-β-N-acetylgalactosaminidase; H; bp, base pair(s); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TPCK, tosylphenylalanyl chloromethyl ketone.

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was from Du Pont-New England Nuclear. Wheat germ was provided by General Mills. TPCK-treated trypsin was purchased from Worthington Biochemicals, RNase inhibitor from Promega Biotech.

cDNA Constructs

The techniques and conditions used for DNA manipulations were essentially as described by Maniatis et al. (1982). All the constructs were made in the plasmid vector pSP64, which contains the SP6 RNA polymerase promoter. In Fig. 1, the pSAl plasmid is described by the plasmids used in this work are summarized, and the sequences at the segment junctions are presented. pSA1 (Spiess and Lodish, 1986) carries the wild-type cDNA of the ASGP receptor HI from the HindIII site (100 bp upstream of the initiation codon) to the 3' EcoRI site. The resulting pSAl and pSAX has been described before (Wessels and Spiess, 1988).

pSA0 — The Clal-EcoRI fragment of pBal26 (a Bal26 deletion construct of pSA1 described in Spiess and Handchitz, 1987) was ligated into the Clal-EcoRI vector fragment of pSAAA. From the resulting plasmid pSA00, the internal 360-bp BamHI fragment was removed to yield pSAA.

pSAH — pSHa is the hemagglutinin cDNA (strain A/Jap/305/87 (H2N2)) from the HindIII to the BamHI site of the plasmid pJHB16 (a gift from Dr. M.-J. Getting; Getting and Sambrook, 1981) cloned into pSP64. A Clal linker (CATGAGT) was ligated into the BalI site, resulting in the coding sequence, and the HindIII site was filled in with Klenow, thus generating a unique NruI site. From the resulting plasmid pSAAHa, the internal 360-bp BamHI fragment was removed to yield pSAAH. In parallel, a BamHI linker (CCCGATGCCCCG) was ligated into the blunt NruI site of pSHa (at the end of the segment encoding the signal sequence of hemagglutinin). The sequence encoding the mature part of hemagglutinin from this BamHI to the last EcoRI site was replaced by the BamHI-EcoRI fragment of pSAAH. The Clal-EcoRI insert of pSAAH was then ligated into the pSAAH-EcoRI site which was used to replace the hemagglutinin sequence of pSAAHa with the same enzymes, resulting in pSAH.

pSAP — The entire cDNA of the ASGP receptor HI was isolated as the EcoRI insert fragment of pSA1 (Spiess et al., 1985) and digested with NruI. The fragments were blunted with T4 DNA polymerase and then digested with HindIII. The resulting 360-bp HindIII-HeyAI (blunted) fragment was ligated into a HindIII-HindII-digested pSP64 vector whose BamHI site has been destroyed by filling it in. The HindIII-BamHI fragment was repeated by the 600-bp fragment of pSAPH produced by complete digestion with HindIII and partial digestion with Kpnl. Finally, the XclI site was filled in with Klenow to create a stop codon.

pSA7 and pSAC — The plasmid p126* encodes the first 5' HindIII-BamHI segment of pSAl, resulting in the plasmids pSA7 and pSAC, respectively. In Fig. 1, the HindIII fragment was replaced by the HindIII-EcoRI fragments of pSA1 and pSA1/C to create pSA7 and pSAC, respectively.

In Vitro Transcription and Translation

Plasmids were transcribed in vitro by SP6 RNA polymerase according to the manufacturer's protocol using 0.5 mM 5'-(7-

methyl)guanosine 5'-adenosine triphosphate. Wheat germ extract (WGM) served as the lysate for translation. To test for surface exposure of the fusion protein, cells were grown on 2-well plates by rinsing the cells with cold PBS, scraping them into 5 ml of cold PBS containing 2 mM phenylmethylsulfonyl fluoride, and homogenizing them with 5 strokes of a Teflon potter on ice. The membrane fractions were then pelleted by centrifugation for 10 min with 20,000 × g at 4 °C, resuspended in PBS with phenylmethylsulfonyl fluoride, and pelleted again. Aliquots of 5 µg of protein of the resuspended membrane preparation were analyzed by immunoblot analysis either directly or after trypsin or Endo H digestion under...
the same conditions as described for the in vitro translation products or as indicated in the text.

RESULTS

A cDNA Construct to Test Potential Stop-Transfer Sequences—The human asialoglycoprotein (ASGP) receptor H1 is a single-spanning membrane protein with an uncleaved internal signal sequence. Mediated by signal recognition particle (SRP) and SRP receptor, the signal sequence targets protein synthesis to the ER membrane, initiates translocation of the downstream sequence, and anchors the final protein in the bilayer with an N-in/C-out orientation (Spiess et al., 1985; Spiess and Lodish, 1986). By duplicating the signal/anchor domain as well as an adjacent segment including the two sites for N-linked glycosylation, a potentially 2-fold membrane-spanning protein encoded by the plasmid pSAA had been created (Wessels and Spiess, 1988), as is illustrated in Fig. 1A. The possible topologies this protein (and the other constructs described here) could assume in the membrane are schematically shown in Fig. 2. If the second hydrophobic domain stops translocation, only two glycosylation sites will be translocated and a loop fragment of only approximately 150 amino acids will be protected from exogenous protease (Fig. 2A). Failure to stop polypeptide transport across the membrane would result in a single-spanning protein with all four glycosylation sites located in the ER lumen and with only the amino-terminal 40 residues exposed to externally added protease (Fig. 2B). The topology of the protein in the microsomal membrane can be derived from the glycosylation pattern (each oligosaccharide added contributes approximately 3 kDa to the apparent molecular mass) and the protease-resistant fragment produced by exogenously added trypsin.

Translation of pSAA mRNA in wheat germ extract (WG, lanes 1–3) or reticulocyte lysate (RL, lanes 4–10) and membrane insertion of the products is shown in Fig. 3A (see also Wessels and Spiess, 1988). Without membranes, a polypeptide of 42 kDa is synthesized (lanes 1 and 4) which is entirely sensitive to exogenous trypsin (lane 5). If microsomes are

\[
\begin{align*}
\text{pSAA} & \ldots \text{NGSERTCCPV} + + \text{RCGPRLLLSSQLSLLLYLVCCV}I \ldots \text{GSQNSQLQELRGG} \ldots + + \\
\text{pSAX} & \ldots \text{NGSERTCCPV} + + \text{HRPSCRTLCSGPRLLLSSQLSMVVCCV}I \ldots \text{GSQNSQLQELRGG} \ldots + + \\
\text{pSAC} & \ldots \text{NGSERTCCPV} + + \text{PKLTQKLVTAYAAGTASTLLYADSLTAEMTA} \ldots \text{GSQNSQLQELRGG} \ldots + + \\
\text{pSA7} & \ldots \text{NGSERTCCPV} + + \text{PRIVAVTAATGIAAYYYNYQQQQQRGKXSTIR} \ldots \text{GSQNSQLQELRGG} \ldots + + \\
\text{pSAH} & \ldots \text{NGSERTCCPV} + + \text{HRDAAATLLLLTTAVRGDQIR} \ldots \text{GSQNSQLQELRGG} \ldots + + \\
\text{pSA0} & \ldots \text{NGSERTCCPV} + + \text{HRW} \ldots \text{GSQNSQLQELRGG} \ldots + + \\
\text{pSAl} & \ldots \text{NGSERTCCPV} + + \text{AIYLLLITAVRDQIR} \ldots \text{GSQNSQLQELRGG} \ldots + + \\
\text{pSAH} & \ldots \text{NGSERTCCPV} + + \text{HRW} \ldots \text{GSQNSQLQELRGG} \ldots + + \\
\end{align*}
\]

Fig. 1. Summary of the fusion proteins. A, the general structure of the wild-type ASGP receptor H1 (pSAl) and of the fusion proteins are schematically represented. The signal/anchor sequence is stippled, and the second hydrophobic domain is indicated as a striped area. Diamonds indicate the position of potential sites for N-linked glycosylation. The protein sequences of the potential stop-transfer segments are shown below. The apolar sequences are shaded, charged residues are indicated, and potential glycosylation sites are marked by asterisks. Bold letters indicate the sequence derived from the respective source protein, while the other amino acids are generated by linker sequences inserted during cDNA cloning (inside the box) or are derived from ASGP receptor sequence. The ability of the sequences to stop translocation across the microsomal membrane in the wheat germ (WG) and the reticulocyte lysate (RL) translation system is summarized on the right. B, the general structure and carboxyl-terminal amino acid sequence of the truncated protein encoded by pSAH* is shown. The carboxyl terminus is indicated as a dot. All other features are as in panel A.
FIG. 2. Possible topologies of the fusion proteins inserted into microsomes. Membrane insertion is initiated by the internal signal/anchor sequence of the ASGP receptor (white box). The second hydrophobic domain (striped box) either stops translocation (panel A) or is transported across the membrane (panel B). The expected protein fragment resistant to exogenous trypsin is shown on the right of each panel. The cytoplasmic and the luminal/exoplasmic sides of the membrane are specified by cis and trans, respectively. Diamonds indicate the positions of potential glycosylation sites. The ones that are used are filled. The site closest to the cell surface is shown in gray, since it is not equally accessible in all constructs analyzed.

present during translation, a significant fraction of the protein is made with an increased apparent molecular mass of 45 kDa (lanes 2 and 6). This 3-kDa increase is reversed by treatment with Endo H (lane 7), which removes all but the first sugar residue of N-linked oligosaccharides added in the ER lumen. Upon posttranslational digestion with trypsin, a fragment of only 23 kDa is resistant (lanes 3 and 8) which can be deglycosylated to a 20-kDa polypeptide by Endo H treatment (lane 9). When the microsomal membrane is disrupted by detergent, however, even the glycosylated protein is completely digested by trypsin (lane 10). These results agree with what is expected if the protein is inserted with the 2-fold membrane-spanning topology illustrated in Fig. 2A, except that only one of the two translocated glycosylation sites is modified (resulting in an increase of the apparent molecular mass of approximately 3 kDa). The second potential glycosylation site in the translocated loop segment (indicated with an asterisk in Fig. 1A) is very close to the surface of the membrane in this fusion protein and not accessible to the oligosaccharide transferase. A similar observation has been made by Audigier et al. (1987).

In other constructs in which the distance between the second site and the hydrophobic domain is larger, the translocated loop fragment is in fact glycosylated twice (see below pSAX, pSAC, and Wessels and Spiess, 1988). Fig. 2A shows that the second copy of the signal/anchor domain of the ASGP receptor functions as a stop-transfer sequence.

As a control, the plasmid pSAO was constructed encoding a protein similar to that of pSAA but lacking the second hydrophobic domain (Fig. 1A). Upon membrane insertion, this protein is glycosylated at all four sites (Fig. 3B, lanes 3 and 9) and is almost entirely protected from exogenous protease by the microsomal membrane (lanes 5 and 10); only approximately 3 kDa are digested, corresponding to the amino-terminal domain preceding the signal sequence. The protein is clearly inserted with a single-spanning topology as shown in Fig. 2B. In the following, we have tested other potential stop-transfer sequences by inserting the encoding cDNA segments into the plasmid pSAA in place of the second hydrophobic domain.

The Stop-Transfer Efficiency of a Mutated Anchor Sequence
Depends on the Translation System—Previously, we have analyzed a mutant signal sequence of the ASGP receptor H1 in which the central 6 residues of the hydrophobic domain had been deleted and replaced by the linker sequence Pro-Ser-Met (Spiess and Handschin, 1987; Wessels and Spiess, 1988). In the construct pSAX, the second hydrophobic domain in pSAA was replaced by this mutant sequence (Fig. 1A). In the WG system, pSAX mRNA is translated as a 43-kDa polypeptide (Fig. 4, lane 1) which upon membrane insertion is glycosylated either once or twice to a 46-kDa and a 49-kDa protein, respectively (lane 3; after Endo H digestion, lane 4). Segregated into the microsomes are two corresponding trypsin-resistant fragments of 24 and 27 kDa (lane 5) which can be deglycosylated with Endo H to a 21-kDa polypeptide (lane 6). The glycosylation site close to the second hydrophobic domain appears to be partially accessible to the oligosaccharide transferase in the ER lumen, most probably because its distance to the membrane is larger in pSAX than in pSAA by 8 amino acids. In the WG system, the mutant anchor sequence effectively stops the further translocation of the polypeptide across the membrane.

When pSAX mRNA is translated by the RL system, the same products are found, which are glycosylated once or twice (lane 10) and yield the corresponding fragments of 24 and 27 kDa (lane 12). In addition, however, more than 40% of the protein is glycosylated four times to an apparent molecular mass of approximately 55 kDa and yields a 52-kDa trypsin-resistant fragment (lanes 10 and 12). In this population of the products, the mutant sequence and the carboxyl terminal rest of the polypeptide are transported through the membrane. This particular hydrophobic domain thus behaves differently in translocation into dog pancreas microsomes depending on the translation system used; it is a perfect stop-transfer sequence when translated in the heterologous plant system, but a poor one in the homologous mammalian system.

Hydrophobic Domains of Mitochondrial Targeting Signals Can Halt Translocation across the Microsomal Membrane—Previously, it has been demonstrated that ER stop-transfer sequences are able to halt translocation of mitochondrial precursors across mitochondrial membranes, resulting in novel locations for these mitochondrial fusion proteins (Nguyen and Shore, 1987; Nguyen et al., 1988). Here, we tested the hydrophobic sequences of two mitochondrial precursor proteins for stop-transfer activity in the ER membrane: that of the 70-kDa outer membrane protein and that of cytochrome c. Both proteins are targeted to mitochondria by a typical amphipathic signal sequence at the extreme amino terminus. The signal is immediately followed by a hydrophobic stretch of 20 and 18 residues, respectively. The 70-kDa protein is anchored by this segment in the outer membrane, while the entire carboxyl terminal portion of the protein is exposed on the outside of the mitochondria (Hase et al., 1984). For cytochrome c, on the other hand, it has been proposed that the apolar domain of the presequence functions either as a stop-transfer sequence specific for the second (inner) mitochondrial membrane (van Loon and Schatz, 1987) or, after complete translocation in the matrix and cleavage of the signal sequence, as a re-export signal from the matrix to the intermembrane space (Hartl et al., 1987).

Fig. 5 (lanes 1–8) shows the results of the translation and insertion experiments of the corresponding constructs pSA7 and pSAC (Fig. 1A). Because of the restriction sites available in the cDNA of the 70-kDa protein, the amino-terminal end of the hydrophobic domain was altered from...Lys-Thr-Ala...Leu...to...Lys-Leu...in the process of constructing pSA7. The uncharged segment was thus shortened by 2 residues. Nevertheless, the protein encoded by pSA7 is inserted exclusively in a 2-fold membrane-spanning structure both in the WG and the RL system (Fig. 5, lanes 1–4); it is glycosylated once and yields a 24-kDa trypsin-resistant fragment (in RL, small amounts of a 2-fold glycosylated product can be detected; lanes 3 and 4). The transmembrane domain of the 70-kDa protein thus functions efficiently as a stop-transfer sequence in either translation system.

On the other hand, the hydrophobic segment of the cytochrome c, presequence in the construct pSAC stops translocation completely in the WG system (Fig. 5, lanes 5 and 6), but not in RL (lanes 7 and 8). Estimated by densitometric quantitation, approximately 42% of the protein is not arrested in translocation when translated in RL, is 4-fold glycosylated, and yields a 57-kDa protease-resistant fragment.

The Hemagglutinin Signal Stops Translocation Efficiently in the Wheat Germ System But Not in the Reticulocyte Lysate—Finidori et al. (1987) reported earlier that the signal sequence of influenza hemagglutinin does not halt translocation when presented to the ER membrane as part of a translocating polypeptide, using the RL translation system. This sequence consists of a hydrophobic domain of 12 or 13 residues (depending on the virus strain), a length that in other model systems in vivo was found to be sufficient to stop translocation (e.g. Adams and Rose, 1985). To test the hemagglutinin signal in our model construct, we created pSAH (Fig. 1A). Translated in the RL, approximately 45% of the polypeptides are indeed translocated across the membrane (Fig. 5, lanes 11 and 12). The rest, however, is stopped in transport, yielding once glycosylated proteins and trypsin-resistant fragments indicative of 2-fold membrane-spanning topology. The difference to the finding by Finidori et al. (1987), who observed complete translocation of the signal domain, is probably due to differences in the signal sequence itself (hemagglutinin of influenza virus strain A/PR/8/34 (HON1) versus strain A/ Japan/305/57 (H2N2), which was used in this work) as well as the flanking sequences in the two constructs. Yet, the sequence of the translocated domain studied by Finidori et al. (1987) (...LESMSGKLADMKANLLLVLCLALAAADTICIGYHAN...) does not appear to be obviously less hydropho-

Fig. 4. Analysis of the stop-transfer activity of a mutant anchor sequence of the ASGP receptor H1 (pSAX). mRNA derived from the plasmid pSAX was translated in the presence and absence of microsomes as described in the legend to Fig. 3. The bands corresponding to the differently glycosylated full-size polypeptides and protease-resistant fragments are indicated with dots in lanes 3, 5, 10, and 12. Abbreviations are the same as in Fig. 3.
Stop-Transfer Activity of Hydrophobic Sequences

Fig. 5. Translocation behavior of internalized hydrophobic sequences. mRNA derived from the plasmids pSA7, pSAC, pSAH, and pSAH* was translated as described in the legend to Fig. 3. The bands corresponding to the different glycosylated full-size peptides and protease-resistant fragments are indicated with dots. Abbreviations are the same as in Fig. 3. The positions of marker proteins (69, 46, 30, and 21 kDa, from top to bottom) are indicated on the left of each panel.

|     | M | M | M | T |
|-----|---|---|---|---|
| **pSA7** |   |   |   |   |
| **pSAC** |   |   |   |   |
| **pSAH** |   |   |   |   |
| **pSAH** |   |   |   |   |

Table: Stop-Transfer Activity of Hydrophobic Sequences

|     | M | M | M | T |
|-----|---|---|---|---|
| **pSA7** |   |   |   |   |
| **pSAC** |   |   |   |   |
| **pSAH** |   |   |   |   |
| **pSAH** |   |   |   |   |

This result was also reproduced with a commercial WG. The translation system is the rate of protein synthesis: in our systems: the protein is glycosylated only once (a small fraction twice), indicative of the 2-fold membrane-spanning structure. Post-translationally, the hemagglutinin signal sequence thus stops translocation efficiently also in the RL system. This result suggests that the translating ribosome influences the interaction of the hydrophobic sequences encoded in pSAH, pSAC, and pSAX with the translation machinery.

The Hemagglutinin Signal Sequence Does Not Stop Translocation in Vivo—It seems plausible that the more homologous translation/insertion system reflects more closely the situation in vivo. To test this assumption, we cloned the cDNA contained in pSAH into the retroviral shuttle vector pLJ (see “Experimental Procedures”). The resulting plasmid pLH was transfected into mouse fibroblast cells. Cell lines that had stably integrated the transfected DNA were isolated and screened for expression of protein derived from ASGP receptor sequences by immunoblot analysis. In Fig. 6, the protein synthesized by cell line AH1 is analyzed. The immunoreactive material appears as a doublet of approximately 50 kDa (lanes 2, 5, and 11). Upon Endo H digestion, both species are reduced in size to that of the in vitro translation product of pSAH mRNA (compare lanes 9 and 10). The fact that all the protein is sensitive to Endo H and thus carries high mannose-type oligosaccharides suggests that the protein is not transported beyond the ER or the early Golgi. Indeed, the fusion protein is not affected when the surface of AH1 cells is treated with proteinase K (data not shown), indicating that it does not reach the plasma membrane. Digestion with increasing concentrations of Endo H (lanes 5–9) reveals a ladder of peptides that have been deglycosylated to different extents. Five different species can be distinguished, corresponding to the fusion protein carrying from zero to four oligosaccharide moieties. Thus, the protein synthesized from pLJ in fibroblasts is always at least 3-fold, in its majority 4-fold glycosylated, indicating that all four glycosylation sites are translocated across the ER membrane. In addition, both forms of the fusion protein are largely resistant when a membrane preparation of AH1 cells is digested with trypsin; their apparent molecular mass is reduced by only approximately 3 kDa (lanes 2 and 3), corresponding to the size of the amino-terminal domain that is expected to be exposed on the outside of microsomes if the protein is inserted with the single-spanning structure. These results show unambiguously that the internalized copy of the hemagglutinin signal sequence does not stop translocation of the polypeptide in vivo.
Stop-Transfer Activity of Hydrophobic Sequences

From the experimental evidence available (as summarized by Singer et al., 1987a), it can be assumed that polypeptides are translocated through biological membranes inside a proteinaceous structure. As a mechanism by which stop-transfer sequences terminate translocation of the polypeptide chain, it has been proposed that the hydrophobic sequence simply leaves the channel complex by partitioning into the apolar environment of the lipid bilayer (Singer et al., 1987b). Alternatively, it has been suggested that stop-transfer sequences by interacting with a channel subunit might specifically trigger the disassembly of the channel (Blobel, 1980; Audigier et al., 1987).

In this study, we have analyzed the ability of five hydrophobic sequences to stop the transfer of a polypeptide chain across the ER membrane. All five sequences efficiently stop translocation when translated in WG. However, only two of them, the wild-type signal-anchor of the ASGP receptor H1 and the transmembrane segment of the 70-kDa protein of the mitochondrial outer membrane, completely arrest translocation when synthesized by RL, while the others are transported through the membrane to a significant extent (approximately 50% of the polypeptides). Thus, the behavior of hydrophobic sequences in translocation across dog pancreas microsomes depends on the translation system used to synthesize them, but it is not simply a result of different translation rates. Above all, this observation is a caveat for the in vitro analysis of stop-transfer sequences, since the results obtained in one translation system may not be valid in another and may not reflect the situation in vivo.

The WG and RL systems have previously been shown to differ also in another respect; signal recognition particle was found to inhibit translation of secretory proteins in the absence of membranes more strongly in the WG system than in RL (Meyer, 1985). The interaction of canine SRP with the heterologous plant ribosomes thus appears to be different from that with the more homologous rabbit ribosomes. Similarly, differences between the two types of ribosomes, most likely in their interaction with dog pancreas microsomes, seem to be responsible for the observed difference in terminating polypeptide translocation. This is suggested by the analysis of two closely related fusion constructs, pSAH and pSAH*, which only differ in the length of the polypeptide following the internalized hemagglutinin signal sequence. In pSAH, this hydrophobic domain is followed by more than 230 residues and can be assumed to enter the membrane co-translationally. In pSAH*, the carboxyl-terminal segment is truncated to only 32 residues; translation is terminated before the hydrophobic sequence has completely emerged from the ribosome, and interaction with the membrane is post-translational. Only co-translational insertion of the internalized hemagglutinin signal is affected by the translation system (complete stop of translocation in WG but not in RL), while post-translational insertion results in complete translocation arrest in both systems.

**DISCUSSION**

**FIG. 6. Analysis of the membrane topology of the fusion protein pSAH in transfected fibroblast cells.** The fusion protein encoded by pSAH was expressed in 3T3 fibroblasts and characterized by immunoblot analysis as described under "Experimental Procedures." Abbreviations: -, untreated membranes; T, incubation of isolated membranes with trypsin; D, detergent Nonidet P-40 present during incubation with trypsin; H, Endo H digestion of the membranes using 3, 1/3, or 1/6 milliunit/50 µg of total protein for 1 h at 37 °C. For comparison, the RL in vitro translation products of pSAH mRNA (in the absence of microsomes) are analyzed in lane 10. The position of marker proteins and their molecular masses in kilodaltons are indicated.

**FIG. 7. Hydrophobicity plots of the potential stop-transfer sequences analyzed.** Average hydropathy values (according to Kyte and Doolittle, 1982) for a window of 10 amino acids were plotted for the segments analyzed for stop-transfer activity in the indicated fusion constructs (compare with Fig. 1). The broken lines indicate the limits outside of which the plots are identical for all constructs.
The heterologous WG ribosomes probably interact less strongly with the dog pancreas microsomes than the more homologous RL ribosomes. This could explain why in the WG system the hemagglutinin signal and other less hydrophobic sequences (as in pSAX and pSAC) stop translocation also cotranslationally. Consistent with this hypothesis is the recent observation by Conolly et al. (1989) that in the WG system nascent secretory proteins larger than 100 residues are accessible to proteinase K while they are translocated across dog pancreas microsomes, suggesting that the ribosome might not be bound directly to the membrane during the entire translocation time. Yet, it remains to be determined whether this is not the case in the RL system and in vivo.

In fibroblasts transfected with the fusion construct pSAH, the internalized hemagglutinin signal is efficiently transported across the membrane. This finding indicates that the results obtained with the RL system reflect more closely the in vivo situation than those obtained with WG. The large fraction of polypeptides inserted with a 2-fold membrane-spanning structure in the RL are either due to a partial incompatibility of the rabbit and dog components or simply an insufficiency of the in vitro system. Indeed, Finidori et al. (1987) found complete translocation of a hemagglutinin signal sequence in a similar construct in their RL system.

We propose that ribosomes engaged in the translation of translocating polypeptides interact with the translocation complex and stabilize it. The hydrophobicity of the signal/anchor of the ASGP receptor, but not that of the hemagglutinin signal sequence, is sufficient to stop transport in this situation. After termination of translation and disassembly of the ribosome, the stringency for the recognition of stop-transfer sequences by the translocation machinery is reduced, and a copy of the hemagglutinin signal sequence close to the carboxyl terminus of the polypeptide is sufficient to disrupt further transfer. Previous results are consistent with this model: the signal of preprolactin consisting of 18 uncharged amino acids (among them 2 fairly polar residues, Asn and Gln) inserted 142 residues from the carboxyl terminus was found not to stop translocation when expressed in Xenopus oocytes (Rothman et al., 1988), while a truncated transmembrane sequence of the vesicular stomatitis virus G protein with just eight apolar amino acids only 29 residues from the carboxyl terminus was sufficient to anchor the protein expressed in COS cells (Adams and Rose, 1985).

It has been shown that hydrophobicity suffices to stop protein translocation (Davis and Model, 1985). Yet, it is difficult to predict the properties of the five sequences analyzed here based on their hydrophobicity plots, which are shown in Fig. 7. Of the two sequences that stop translocation efficiently, the signal/anchor sequence of the ASGP receptor (in construct pSAA) is the most hydrophobic one. The transmembrane segment of the 70-kDa protein (in pSA7), however, does not appear to be more hydrophobic than some of the other sequences tested. It is conceivable that the cluster of several positively charged residues following the apolar segment of this sequence is responsible for the higher stop-transfer activity. Furthermore, it cannot be excluded that additional characteristics besides a sufficiently long stretch of apolar residues at least modify the ability of these sequences to terminate translocation.

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