The Influenza Hemagglutinin Insertion Signal Is Not Cleaved and Does Not Halt Translocation When Presented to the Endoplasmic Reticulum Membrane as Part of a Translocating Polypeptide

Joelle Finidori, Lawrence Rizzolo, Alfonso Gonzalez, Gert Kreibich, Milton Adesnik, and David D. Sabatini
Department of Cell Biology and The Kaplan Cancer Center, New York University School of Medicine, New York 10016

Abstract. The co-translational insertion of polypeptides into endoplasmic reticulum membranes may be initiated by cleavable amino-terminal insertion signals, as well as by permanent insertion signals located at the amino-terminus or in the interior of a polypeptide. To determine whether the location of an insertion signal within a polypeptide affects its function, possibly by affecting its capacity to achieve a loop disposition during its insertion into the membrane, we have investigated the functional properties of relocated insertion signals within chimeric polypeptides.

An artificial gene encoding a polypeptide (THA-HA), consisting of the luminal domain of the influenza hemagglutinin preceded by its amino-terminal signal sequence and linked at its carboxy-terminus to an intact prehemagglutinin polypeptide, was constructed and expressed in in vitro translation systems containing microsomal membranes. As expected, the amino-terminal signal initiated co-translational insertion of the hybrid polypeptide into the membranes. The second, identical, interiorized signal, however, was not recognized by the signal peptidase and was translocated across the membrane. The failure of the interiorized signal to be cleaved may be attributed to the fact that it enters the membrane as part of a translocating polypeptide and therefore cannot achieve the loop configuration that is thought to be adopted by signals that initiate insertion. The finding that the interiorized signal did not halt translocation of downstream sequences, even though it contains a hydrophobic region and must enter the membrane in the same configuration as natural stop-transfer signals, indicates that the HA insertion signal lacks essential elements of halt transfer signals that makes the latter effective membrane-anchoring domains.

When the amino-terminal insertion signal of the THA-HA chimera was deleted, the interior signal was incapable of mediating insertion, probably because of steric hindrance by the folded preceding portions of the chimera. Several chimeras were constructed in which the interiorized signal was preceded by polypeptide segments of various lengths. A signal preceded by a segment of 111 amino acids was also incapable of initiating insertion, but insertion took place normally when the segment preceding the signal was only 11-amino acids long. In contrast to the behavior of the interiorized insertion signal of HA, the interiorized insertion signal of cytochrome P-450 served as a haltertransfer signal in a chimeric protein (THA-P-450t65) consisting of the luminal segment of HA, preceded by its signal, linked at its carboxy-terminus to a polypeptide segment corresponding to the first 165 residues of cytochrome P-450. When synthesized in the presence of membranes, the hemagglutinin portion of the polypeptide was translocated across the membrane, but the P-450 segment remained exposed on the surface of the microsomes where it was accessible to proteases.

These observations verify the capacity of the amino-terminal insertion signal of P-450, which normally initiates insertion, to halt translocation of downstream segments of P-450 across the membrane.

Secretory, lysosomal, and many integral membrane proteins are synthesized in ribosomes bound to the endoplasmic reticulum (ER) and are co-translationally inserted into the ER membrane (see Blobel, 1980; Sabatini et al., 1982; Walter et al., 1984; Wickner and Lodish, 1985). The insertion of these proteins into the membrane is initiated by signal sequences present in the nascent polypeptides. It is now apparent, however, that there are many types of inser-
The co-translational insertion of proteins into the ER may also be mediated by permanent (noncleavable) insertion signals. Such signals may traverse the membrane with the translocated polypeptide, as in the case of the secretory protein ovalbumin (Tabe et al., 1984), and the E3 envelope glycoproteins of Sindbis (Bonatti and Blobel, 1979; Bonatti et al., 1979), and Semliki Forest virus (Garoff et al., 1980), or may remain in the membrane and contribute to the anchoring of the mature polypeptide (Sabatini et al., 1982; Wickner and Lodish, 1985).

The co-translational insertion of proteins into the ER may also be mediated by permanent (noncleavable) insertion signals. Such signals may traverse the membrane with the translocated polypeptide, as in the case of the secretory protein ovalbumin (Tabe et al., 1984), and the E3 envelope glycoproteins of Sindbis (Bonatti and Blobel, 1979; Bonatti et al., 1979), and Semliki Forest virus (Garoff et al., 1980), or may remain in the membrane and contribute to the anchoring of the mature polypeptide (Sabatini et al., 1982; Wickner and Lodish, 1985; Walter et al., 1984). Permanent signals of the latter type may be located at the amino-terminus, as in the case of the neuraminidase of influenza virus (Ros et al., 1984) and the HN protein of Sendai virus (Blumberg et al., 1985), or in the interior of the polypeptide, as in the erythrocyte anion channel, Band III (Kopito and Lodish, 1985), and the hepatocyte asialoglycoprotein receptor (Spieß et al., 1985; Spieß and Lodish, 1986). In the latter cases, amino-terminal segments of the proteins preceding the signals remain exposed on the cytoplasmic side of the membrane.

Yet other types of noncleavable insertion signals exist that mediate cotranslational insertion and remain associated with the membrane but do not promote translocation of downstream sequences. Such signals effectively function as combined signals for insertion and halt transfer. The amino-terminal segment in cytochrome P-450 (Bar-Nun et al., 1980) and the first insertion signal in rhodopsin (Friedlander and Blobel, 1985) appear to function in this manner. The signal in rhodopsin, however, is not. The amino-terminal segment and, in some manner, it also mediates translocation across the membrane of the 35–40 amino acid segment that precedes it (Friedlander and Blobel, 1985).

The nature of the insertion signals within a membrane polypeptide and their relationship to halt transfer signals within the same molecule can therefore be an important factor in determining the transmembrane disposition of a protein.

Amino-terminal insertion signals generally contain a hydrophobic core region of eight or more residues that is preceded by a short segment bearing one or more positive charges and is followed by a non–alpha-helical region that leads to the peptidase cleavage site (von Heijne, 1981; Perlman and Halvorson, 1983). It has been suggested that insertion signals adopt a loop configuration during their entry into the ER membrane (Inouye et al., 1977; Inouye and Halegoua, 1980; Steiner et al., 1980; Sabatini et al., 1982). In this "loop" model, the charged amino terminus of the signal remains exposed on the cytoplasmic side of the membrane during the early stages of translocation. The existence of permanent insertion signals that leave the amino-terminal portion of the polypeptide on the cytoplasmic side of the membrane, in fact, requires that these signals enter the membrane in a loop configuration. A loop configuration of the signal was also assumed in a model for translocation across membranes that was based on thermodynamic considerations and postulated that the formation of a helical hairpin in the nascent polypeptide provides the energy for translocation (Engelman and Steitz, 1981).

In this paper we studied the incorporation into membranes of a hybrid polypeptide that contains two potentially cleavable identical insertion signals, without an intervening halt-transfer signal between them. Of the two insertion signals, one was located at its normal amino-terminal position and the other in the interior of the polypeptide, ~500 amino acids from the first. This interior insertion signal presumably was unable to assume the loop configuration, because it entered the membrane as part of a translocating polypeptide whose insertion was initiated by the amino-terminal signal. An analysis of the products generated when the polypeptide was synthetized in the presence of membranes showed that when presented in this mode, the interior signal was neither cleaved nor functioned as a halt-transfer signal to block translocation of downstream sequences. In addition, we found that, in the absence of the amino-terminal insertion signal, the capacity of the interior signal to mediate insertion was blocked by the preceding THA even when this was shortened to 31 amino acids. In contrast to the HA-insertion signal, the amino-terminal segment of P-450, when placed internally, was able to halt the transfer of downstream portions of a hybrid polypeptide across the membrane.

Materials and Methods

Construction of Plasmids

The vectors pSP64 and pSP65 were obtained from Promega Biotec (Madison, WI). The various cDNAs, constructed as described below, were cloned into these vectors for in vitro transcription–translation experiments. pSP64THA. A cDNA for the influenza hemagglutinin (HA), strain A/PR/8/34, originally obtained from Dr. Peter Palese (Mount Sinai School of Medicine, New York), had previously been subcloned into the pSV2 vector (Gottlieb et al., 1986). A fragment containing the HA sequences, together with the SV40 polyadenylation signal, was excised from this vector by digestion with Hind III and Bam HI endonucleases. This fragment, containing a very short 5' untranslated region (6 bp), the coding region for HA, the 3' untranslated region of the HA mRNA, and an SV40 DNA segment containing both the early and late polyadenylation signals, was inserted between the Hind III and Bam HI sites of pSP64 to yield the plasmid pSP64HA.

pSP64THA. The mature HA is a transmembrane protein that consists of three distinct domains, an exoplasmic domain containing 526 amino acids, a transmembrane segment of 28 residues, and a 11 amino acid segment exposed on the cytoplasmic side of the membrane (Gething and Sambrook, 1982). The protein is synthesized with a 17 residue cleavable amino-terminal

Dr. Finidori's present address is Institut Pasteur, Laboratoire Dr. Louvard, 75015 Paris, France; Dr. Rizzolo's present address is Emory University, School of Medicine, Department of Biochemistry, Atlanta, Georgia 30322; Dr. Gonzalez's present address is Departamento De Inmunologia Clinica y Reumatologia, Facultad De Medicina, Pontificia Universidad Catolica de Chile, Santiago, Chile.
Genes Encoding a PreHA Polypeptide Preceded by Polypeptide Segments of Various Lengths: pSP65HA<sub>m</sub>, pSP65HA<sub>n</sub>, pSP64HA<sub>n</sub>, and pSP64HA<sub>m</sub>

pSP65HA<sub>m</sub> and pSP65HA<sub>n</sub> are plasmids that encode polypeptides that differ from THA<sub>n</sub> in that the THA component was shortened to include only the last 36 or 39 carboxy-terminal amino acids that precede the natural initiation codon of the HA portion of the chimeric gene. The first plasmid was constructed by inserting a partial Eco RI-Bam HI fragment from pSP64HA<sub>n</sub> (the Eco RI site is located in the THA portion of the chimeric gene) and recloning it into pSP65. The encoded polypeptide is initiated at an ATG that is 18 bp downstream from the Eco RI site. pSP65HA<sub>m</sub> encodes a protein that initiates at methionine 493 within the HA segment. Therefore, a 39-amino-acid segment from the carboxy-terminus of THA precedes the natural initiation signal in HA. The construction of this plasmid involved two sequential deletions that removed from the THA cDNA, first a segment extending from the Hind III linker at the 5' end to an Asu I site at nucleotide 868, and then an Ava III fragment extending from residues 891-1505. The first deletion eliminated the natural initiation codon of THA; the second, two potential out of frame initiators between the Asu I site at 868 and the Ava III site at 1505 that contains the new initiator. Subsequently, the intact HA cDNA was introduced downstream from the remaining portion of the THA to form the chimeric gene pSP65THA<sub>n</sub>.

To construct genes encoding a modified preHA that contained peptide segments of 31 (pSP64HA<sub>m</sub>) and 11 (pSP64HA<sub>n</sub>) amino acids preceding the insertion signal sequence, nucleotide sequences from the polylinker regions of the plasmids pSP64 and pSP65 were introduced upstream from the natural initiation codon of HA in pSP64HA<sub>n</sub>. In all cases, the HA insert in pSP64HA<sub>n</sub> was recloned between the Hind III and Pvu II sites of pSP64, as described in Fig. 2. To accomplish this, pSP64-HA was linearized by digestion with Barn HI and treated with Klenow polymerase to generate a blunt end compatible with the Pvu II site in the pSP65 vector. The HA cDNA was then moved with Hind III into the Hind IH site located just upstream of the initiation codon of the HA sequence.

Figure 1. Schemes for the construction of pSP64THA<sub>n</sub> and pSP64THA<sub>m</sub>. The steps outlined here for the construction of these plasmids are described in detail in Materials and Methods. The insert in pSP64THA-encodes a hybrid polypeptide (THA-HA) consisting of the truncated pre-HA, lacking its carboxy-terminal membrane-anchoring segment, linked at its COOH-terminus to an intact pre-HA. The insert in pSP64THA-HA<sub>m</sub> encodes a similar hybrid polypeptide that lacks the amino-terminal insertion signal in the THA moiety.
excised by Hind III digestion, recovered by agarose gel electrophoresis, and ligated to the pSP65 vector that had been linearized by digestion with both Hind III and Pvu II. After the ligation, which yielded the plasmid pSP65HA, the Bam HI site at the 3' end of the HA cDNA was regenerated. The SP6 polymerase transcript from this plasmid contains 45 additional nucleotides upstream from the natural initiation codon but, nevertheless, still encodes the natural HA, since it contains no upstream initiation codon. The first five nucleotides of the coding region of growth hormone cDNA, as well as 31 additional nucleotides from the polylinker region of pSP64, were added upstream from the Eco RI site at the 5' end of the insert in pSP5, by the following sequence of cloning steps that involved recloning the insert back into pSP64. First, the Pst I fragment, containing nearly the entire coding region of growth hormone, was removed from pSP64GH by digestion with Pst I and the resulting vector DNA fragment was made blunt with Klenow polymerase and recircularized with DNA ligase. It should be noted that the sequences that are between the Pst I and Eco RI sites downstream from the coding region of the growth hormone cDNA in pSP64GH are derived from the polylinker region of pSP64 and in the recircularized plasmid are placed just downstream from the growth hormone initiation codon. pSP65HA was digested with Eco RI and Bgl I, and the fragment containing the HA cDNA was recovered by gel electrophoresis. This fragment was ligated to the SP6 promoter–containing Bgl I–Eco RI fragment of the modified pSP64GH plasmid that lacks the GH coding region, thus generating pSP64HA. This plasmid encoded a protein containing a 31-amino acid sequence, M A V D S R G S P A E L E F L A R G S S R V D L E P K L A K, immediately preceding the natural initiator methionine in HA. Digestion of pSP64HA with Sal I removed a small fragment derived from the fused polylinker regions that encoded part of the amino-terminal extension. Recircularization of the vector generated pSP64HAa, which codes for a protein containing the II-amino acid sequence, M A V D L I Q P K L A K, immediately preceding the natural initiator in HA. pSP64THA-P450b. The cDNA insert in this plasmid encodes a hybrid polypeptide consisting of THA linked at its carboxy-terminal end to an amino-terminal segment of cytochrome P450, the major phenobarbital form of rat hepatic cytochrome P-450 (see Adesnik and Atchison, 1986). The starting plasmids for this construction were pSP64THA and pUCP450a, a plasmid that contains a 5′ portion of the \(500 \text{ kb} \) of P450b cDNA that begins at an Acc I site 22 bp upstream from the initiation codon (Monier, S., V. Van Luc, G. Kreibich, D. D. Sabatini, and M. Adesnik, manuscript in preparation). A cDNA fragment containing the first 165 codons of cytochrome P-450 and the 22 bp of the 5′ untranslated region was excised from pUC8P450b by digestion with Acc I and Bam HI and inserted between the Acc I and Bam HI sites of pSP64THA in the polylinker region located downstream from the THA cDNA (Fig. 3). The hybrid protein encoded in the resulting recombinant plasmid, pSP64THA-P450a, contains the first 541 amino acids of preHA linked to the first 165 amino acids of P-450 by an extraneous tri-decapeptide (K L G L Q V D R G Y T R T) encoded by the Hind III linker at the 3′ end of the THA insert (K L), a portion of the pSP64 polylinker region (G L Q), and the 5′ untranslated segment of the P450 cDNA (V D R G Y T R T). The hybrid protein also contains six extraneous amino acids (R A S N S S) at its COOH-terminus encoded by vector sequences linked to the Bam HI site.

Transcription–Translation Assays

pSP64-based recombinant plasmids were transcribed (Krieg and Melton, 1984) in vitro with SP6 RNA polymerase (Promega Biotec, Madison, WI) and aliquots of the transcription mixtures estimated to contain 100 ng of RNA were translated in the rabbit reticulocyte lysate in the presence or absence of dog pancreas microsomal membranes (2 OD280U/ml) (Rizzolo et al., 1985). Accessibility of the translation products to proteases (trypsin and chymotrypsin, 50 \(\mu\)g/ml of each) and analysis of products by gel electrophoresis, with or without prior immune precipitation, were carried out as described (Rosenfeld et al., 1984). Antibodies to hemagglutinin were provided by Dr. P. Palese. Antibodies to cytochrome P-450 were previously described (Adesnik et al., 1981).

Results

The Interior Insertion Signal in THA-HA Is Not Cleaved and Does Not Halt Localization of Downstream Sequences

To determine whether the location of the signal within the polypeptide affects its function, we have investigated the in vitro expression of cDNAs (Fig. 1) encoding two related hybrid proteins (THA-HA and THA-HAsp*). THA-HA consists of THA, a truncated derivative of the hemagglutinin of the influenza (HA) that lacks the cytoplasmic and transmembrane domains of this simple transmembrane protein but contains its amino-terminal cleavable natural signal sequence, linked to the amino-terminus of a complete HA. THA-HAsp* is a similar hybrid from which the amino-terminal signal of THA was deleted. Both of these hybrid proteins contain an interior insertion signal and include the stop-transfer signal of HA near the COOH-terminal end of the hybrids. The chimeric cDNAs, and for comparison the cDNAs encoding the natural preHA polypeptide and its truncated derivative, were introduced into the pSP64 vector and transcribed in vitro with SP6 RNA polymerase. The resulting messenger RNAs were translated in a reticulocyte system supplemented with dog pancreas microsomes to assess the capacity of the encoded polypeptides to be co-translationally translocated across microsomal membranes.

When transcripts of the THA and HA genes were translated in the presence of membranes (Fig. 4, a–f), in addition to the primary translation products, the complete HA and the truncated THA (54 and 52 kD, respectively, with their amino-terminal insertion signals), the translation mixtures contained proteins of substantially higher molecular masses (THA*, 64 kD and HA*, 66 kD) that correspond to processed forms of THA and HA that underwent signal cleavage and co-translational glycosylation during their incorporation into the microsomes. Each of these polypeptides contains six potential sites for N-glycosylation (Gething et al., 1980). The electrophoretic mobility and homogeneity of the glycosylated products suggest that all or most of these sites were uniformly utilized. The glycosylated polypeptides THA* and HA* were almost completely resistant to the attack of pro-

Figure 3. Scheme for the construction of pSP64THA-P450a. The insert in this plasmid encodes a hybrid polypeptide containing the luminal domain of pre-HA linked at its carboxy-terminal end to an amino-terminal segment of cytochrome P-450 consisting of its first 165 amino acids. The steps in the construction are detailed in Materials and Methods.
Figure 4. Insertion of THA-HA into microsomal membranes. The interiorized insertion signal in THA-HA is not cleaved by the signal peptidase and does not halt translocation of downstream sequences. In vitro-synthesized mRNA transcripts encoding the intact hemagglutinin (HA), the truncated HA (THA), and the chimeric polypeptides THA-HA and THA-HA\(_{sp}\) were translated in a reticulocyte system in the presence or absence of dog pancreas microsomal membranes, as indicated. After translation, the reaction mixtures were analyzed either directly by SDS gel electrophoresis and autoradiography or were first incubated with a mixture of trypsin and chymotrypsin, as indicated in the top of each lane. Bands labeled with an asterisk superscript correspond to membrane-inserted glycosylated polypeptides. Numbers indicate molecular mass in kilodaltons.

Figure 5. The function of the insertion signal in pre-HA is blocked by a preceding polypeptide segments of 11 amino acids, but not by one containing 11 residues. In vitro-synthesized mRNA transcripts of the plasmids pSP64HA\(_{sp}\) (A, lanes a and b), pSP64HA\(_{II}\) (A, lanes c and d), pSP64HA\(_{III}\) (A, lanes e and f; B, lanes d-f), and pSP64HA (A, lanes g and h; B, lanes a-c) were translated in the presence or absence of dog pancreas microsomal membranes, as indicated at the top of each lane, and the reaction mixtures were analyzed by SDS gel electrophoresis and autoradiography. In B, the electrophoretic analysis was carried out with or without prior protease digestion, also as indicated. Bands labeled with an asterisk superscript correspond to membrane-inserted glycosylated polypeptides.

Long Amino-Terminal Polypeptide Segments Interfere with the Function of an Interiorized HA Insertion Signal

The inability of the interiorized HA signal in THA-HA to initiate insertion in the membrane was confirmed using a THA-HA\(_{sp}\) chimera, from which the amino-terminal signal of
the THA was deleted. When the corresponding mRNA was translated in the presence of membranes (Fig. 4 k) neither the higher molecular mass glycosylated polypeptide (THA-HA*, 135 kD) was produced nor was any protease-resistant polypeptide generated (Fig. 4 i). These findings are consistent with the observation that the putative ectopically initiated products of THA-HA mRNA (Fig. 4, g and h) failed to be translocated despite the fact that they must contain interior insertion signals at varying distances downstream from the utilized initiation codons.

To determine the approximate minimum length of the upstream polypeptide segment that would hinder the function of the internal HA insertion signal, a series of genes (HAIII, HA39, HA31, HAII) was prepared encoding proteins containing segments of III, 39, 31, or 11 amino acids preceding the insertion signal of an intact HA. The III-amino acids long polypeptide segment prevented the function of the immediately adjacent insertion signal (Fig. 5 A, lanes c, and d). Insertion, however, was unimpaired when the segment preceding the signal was only II-amino acids long (Fig. 5 A, lanes e and f). Thus, translation of the HAII transcript in the absence of microsomal membranes gave a product (HAII) of \( \sim56 \text{ kD} \) (Fig. 5 A, lane c; 5 B, lane d). In the presence of membranes, an additional band of \( \sim66 \text{ kD} \) (HA11 II) was observed (Fig. 5 A, lane f; 5 B, lane e), which was resistant to proteolytic digestion (Fig. 5 B, lane f). The fact that this product (HA11 II) had the same electrophoretic mobility as the natural product (HA*, Fig. 5 B, lanes b and e), suggests that the HAII polypeptide underwent signal cleavage at the normal site. When the HA31 and HA39 genes were used, it was impossible to determine whether the internalized signal was functional because there was a high level of ectopic translational initiation (not shown) at what was originally the natural initiation codon of HA.

**The Amino-Terminal Insertion Signal of P-450 Halts Translocation when Placed at the Carboxy-Terminal End of THA**

The preceding experiments with THA-HA demonstrated that the HA insertion signal does not function as a halt transfer signal when located in the interior of a polypeptide containing an amino-terminal insertion signal. Cytochrome P450 is a nonglycosylated ER membrane protein that is synthesized in membrane-bound ribosomes (Bar-Nun et al., 1980) but remains mostly exposed on the cytoplasmic surface of the ER (Matsuura et al., 1983; De Lemos-Chiarandini, et al., 1987). We have previously suggested (Bar-Nun et al., 1980; Kumar et al., 1983) that the amino-terminal portion of this polypeptide behaves as a combined insertion and halt transfer signal that initiates co-translational insertion, but does not mediate the transfer of downstream sequences across the membrane. We, therefore, compared the behavior of the amino-terminal region of P-450, when placed internally in a chimeric polypeptide downstream from THA, with that of the interiorized amino-terminal signal of HA in THA-HA.

A chimeric gene was constructed in which the THA cdNA was linked at its 3’ end to a segment of the P-450 cdNA containing the first 165 codons of P-450 (Fig. 3). Transcripts of this gene yielded a hybrid primary translation product (THA-P450165) of 66 kD that was immunoprecipitable with both anti-HA and anti-P450 antibodies (Fig. 6, a and b). In the presence of microsomal membranes, this polypeptide was co-translationally glycosylated to yield a product, THA-P450165 of \( \sim76 \text{ kD} \) that was also precipitable with both antibodies (Fig. 6, c and d). This product was membrane associated but only its THA portion was translocated across the membrane since after proteolytic digestion it was converted to a polypeptide of \( \sim64 \text{ kD} \), which could be immunoprecipitated with anti-HA (a, c, and e) or anti-P-450 (b, d, and f) antibodies before analysis by SDS gel electrophoresis and autoradiography. Bands labeled with an asterisk superscript correspond to membrane-inserted glycosylated polypeptides. Numbers in parentheses indicate molecular mass in kilodaltons.

**Discussion**

Three main alternative outcomes could be expected from the co-translational insertion of TH-HA into the microsomal membrane (Fig. 7 a). This protein contains two identical insertion signals: one at the amino-terminus and the other far into the interior of the polypeptide sequence. The interiorized HA insertion signal could have, because of its hydrophobicity, functioned as a halt-transfer signal (Fig. 7 b), resulting in the exposure of the HA portion of the chimera on the exterior surface of the microsomes, or could have been recognized and cleaved by the signal peptidase while still facilitating entrance of the THA portion of the chimera into the membrane (Fig. 7 c). The third alternative (Fig. 7 a), supported by the analysis of the products of co-translational insertion, is that the interiorized signal traversed the membrane without being recognized as either an insertion signal.
or as a stop-transfer signal. The fact that the interior signal was not cleaved by the signal peptidase most likely reflects its inability to assume the configuration of normal insertion signals. Natural signals that initiate translocation most likely enter the membrane in a loop configuration (Inouye 1977; Inouye and Halegoua, 1980; Steiner et al., 1980; Sabatini et al., 1982). It is quite possible that the signal recognition particle (Walter et al., 1984) plays a role in establishing this configuration before the insertion signal enters the membrane. An interiorized insertion signal that is part of a translocating polypeptide and is not preceded by a stop-transfer signal could not interact in such a fashion with signal recognition particle since most likely it would enter the membrane as soon as it exits from the ribosome, traversing the membrane in a simple N to C direction. A second, less likely, explanation for the finding that the interiorized signal is not cleaved, could be that a reorganization of the translocation apparatus takes place after cleavage of the first signal, which would make the peptidase inaccessible to the chains undergoing translocation.

The fact that in THA-HA the interiorized insertion signal was presented to the membrane in the orientation that is normal for halt-transfer signals and yet did not interrupt translocation, indicates that insertion and halt-transfer signals are not interchangeable elements whose functional capacity depends on their location with respect to other signals within the nascent chain. The conclusion that insertion and halt transfer signals are fundamentally different is in agreement with the finding that the halt transfer signal present near the COOH-terminus of the µ-chain of IgM cannot serve as an insertion signal when relocated to the amino-terminus of a translocatable polypeptide (Yost et al., 1983).

Two previous studies have been carried out in bacteria on the membrane insertion of polypeptides that contain interior as well as amino-terminal insertion signals (Zemel-Dreasen and Zamir, 1984; Coleman et al., 1985). One of these studies (Zemel-Dreasen and Zamir, 1984) came to a conclusion similar to that obtained by us; the interiorized signal in a fusion protein consisting of the *Escherichia coli* β-lactamase linked to the amino-terminus of the precursor of a mouse immunoglobulin κ-light chain was not cleaved and the entire hybrid polypeptide was discharged into the periplasmic space. In the other study (Coleman et al., 1985), genes for the prolipoprotein of the *E. coli* outer membrane were constructed that encoded two repeated signal sequences separated by either 27 or 13 amino acids. With the hybrid containing the longer segment between the two sequences, the amino-terminal signal initiated insertion and the interior one behaved as a stop transfer signal that remained membrane associated and failed to be cleaved. On the other hand, when

---

**Figure 7.** Schematic representation of possible fates of the THA-HA polypeptide cotranslationally inserted into the microsomal membrane. The natural and the interiorized insertion signals are represented by the jagged segments and the stop-transfer signal by a rectangle. The oligosaccharide chains in translocated portions of the polypeptides are indicated by the small branched structures. In all cases, insertion is initiated by the cleaved amino-terminal signal, which is depicted, for illustrative purposes only, as remaining in the membrane. In a, the interior signal passes through the membrane into the ER lumen without being cleaved and the THA-HA polypeptide remains anchored in the membrane by the halt-transfer signal. In b, the interior insertion signal functions as a halt-transfer signal blocking translocation of the downstream HA portion of the chimera. In c, the interior signal is cleaved by the peptidase while translocation of the HA portion of the chimera continues. This figure only depicts one possible disposition of the cleaved interior signal, which remains linked to the COOH-terminus of THA portion of the chimera.
The ability of the interiorized prolipoprotein signal to stop translocation in the bacterial system and to serve as an anchoring domain (Coleman et al., 1985) contrasts with our findings with the interiorized HA signal. The different behavior of these signals may reflect a fundamental difference between the prokaryotic and eukaryotic systems, or perhaps between the lipoprotein and other signals. On the other hand, it may simply reflect the different capacity of the interiorized signals to provide a stretch of 18–20 contiguous uncharged (hydrophobic and neutral) amino acids that would be required for a polypeptide segment to span the lipid bilayer in an alpha-helix configuration. Thus, the HA insertion signal contains a core of only 13 uncharged amino acids (Krystal et al., 1982), whereas the prolipoprotein signal contains a stretch of 19 such uncharged residues (Coleman et al., 1985), and it has been suggested (Davis et al., 1985; Davis and Model, 1985) that 16 hydrophobic residues are sufficient for the efficient anchoring of a polypeptide in the bacterial cell membrane. On the other hand, studies of deletions or mutations affecting residues within the membrane-spanning region of the G protein of VSV (Adams and Rose, 1985), suggest that such a long stretch of uncharged residues is not absolutely necessary to anchor a protein in intracellular membranes of eukaryotic cells.

The failure of the internalized HA insertion signal to mediate insertion of the THA-HA chimera when the amino-terminal signal was removed, is likely to reflect a steric hindrance by the folded preceding amino-terminal portion of the chimera that prevented interaction of the internalized signal with the signal recognition particle. Thus, when the length of the segment preceding the signal was reduced to 11 residues, the signal worked efficiently. In the light of previous reports in the literature, summarized below, our results indicate that the capacity of polypeptide segments preceding a signal to interfere with signal function depends on the structure of the segment and possibly the nature of the signal itself. The envelope protein of Rous Sarcoma Virus, for example, contains a cleavable amino-terminal segment of 56 residues (Hunter et al., 1983), but the sequence of the segment suggests that the true signal extends only from residues 32–56 and, therefore, that it is preceded by a long stretch of amino acids that does not interfere with signal function. In the case of the asialoglycoprotein receptor, the insertion signal resides between residues 39–58 of the polypeptide (Spiess and Lodish, 1986) and therefore functions internally. This signal is also capable of mediating the insertion of tubulin when placed at the amino-terminus of that protein, however, its function was blocked when it was placed internally within the tubulin polypeptide (Spiess and Lodish, 1986).

Several studies have shown that short amino-terminal polypeptide segments (18–63 amino acids) preceding the signal in products encoded by modified or chimeric genes do not impair signal function in bacterial systems (Talmadge et al., 1981; Coleman et al., 1985; Hayashi et al., 1985). However, in transfected mammalian cells (Kozak, 1983) it was found that the insulin signal was unable to mediate the secretion of insulin when preceded by a 44-amino acid segment, although the signal did function when the preceding segment contained only 22 residues. On the other hand, the signal in bovine preprolactin efficiently mediated translocation, even when the preprolactin sequence was preceded by the first 109 amino acids of the chimpanzee alpha globin (Perara and Lingappa, 1985). In this case, it was found that the signal was cleaved from the nascent prolactin and surprisingly, that both portions of the chimera were translocated into the lumen of the microsomes, where the signal remained linked to the alpha-globin portion of the chimera. In its capacity to mediate translocation of a preceeding sequence, the internalized prolactin insertion signal behaved, therefore, like the first internal insertion signal in bovine opsin (Friedlander and Blobel, 1985).

We found that, in contrast to the failure of the interiorized HA insertion signal to arrest translocation, the amino-terminal insertion signal of cytochrome P450, when placed internally after the truncated HA moiety, effectively halted transfer of the downstream P450 sequences across the membrane. The transmembrane orientation of the hybrid protein, shown schematically in Fig. 8, in which the P450 moiety is shown schematically in Fig. 8, in which the P450 moiety is exposed on the cytoplasmic surface of the membrane was established by the effect of proteases, which reduced the size of the membrane-associated product from a 76-kD polypeptide, immuneprecipitable by both anti-HA and anti-P-450 antibodies, to a 64-kD one, immuneprecipitable only with anti-HA antibodies. The P450-insertion signal (Bar-Nun et al., 1980), therefore, differs from the conventional cleavable insertion signals in secretory, lysosomal, and many membrane polypeptides in that it also functions as a halt-transfer signal that contributes to the anchoring of the polypeptide in the...
membrane. In separate experiments (Monier et al., 1986) we have demonstrated that when the first 43 codons of the pre-growth hormone cDNA are replaced by the first 20 codons of P450 cDNA, the encoded hybrid protein is co-translationally inserted into the membrane but the bulk of the growth hormone polypeptide remains on the cytoplasmic surface and is not translocated into the lumen. Thus, only the first 20 amino acids of P450, including 16 hydrophobic residues, but none of the immediately following positively charged or helix-breaking residues, are required to effect both insertion and halt transfer functions. In a reciprocal experiment (Monier et al., 1986) we have shown that when the P-450 amino-terminal segment is replaced by the signal peptide of growth hormone, the first 165 amino acids of P-450 are completely translocated into the microsomal lumen.

The different behavior of the interiorized HA and P-450 signals highlights the fact that different classes of insertion signals differ in the extent to which they mediate the translocation of downstream sequences. Future studies involving the in vitro expression of genes encoding proteins with modified signals should help in identifying the features that differentiate these various classes of insertion signals.

References