Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 738–742, February 1988
Biochemistry

Positive charges at the NH_2 terminus convert the membrane-anchor signal peptide of cytochrome P-450 to a secretory signal peptide

(*in vitro* transcription/site-directed mutagenesis/fusion proteins)

ELZBIETA SZCZESNA-SKORUPA, NANCY BROWNE, DAVID MEAD*, AND BYRON KEMPER

Department of Physiology and Biophysics and the College of Medicine at Urbana–Champaign, University of Illinois, Urbana, IL 61801

Communicated by I. C. Gunsalus, October 9, 1987

ABSTRACT **The NH_2-terminal sequences of cytochromes P-450 resemble signal peptides, but these sequences are not cleaved during the insertion of these integral membrane proteins into the microsomes. To examine whether these putative signal peptides are functionally equivalent to signal peptides of secretory proteins, cDNA coding for a fusion protein was produced, in which the signal peptide for preproparathyroid hormone was replaced with the putative signal peptide of cytochrome P450IIC2. The translational product of RNA synthesized *in vitro* from the cDNA was neither processed nor translocated by chicken oviduct microsomal membranes in a reticulocyte cell-free system but was resistant to extraction from the membranes by alkaline solutions. In addition, the translation of the hybrid RNA was arrested by signal recognition particle. Unlike most signal peptides, the cytochrome P450IIC2 NH_2-terminal sequence does not contain basic amino acids preceding the hydrophobic core. Introduction by oligonucleotide-directed mutagenesis of lysine and arginine at the NH_2 terminus resulted in a fusion protein that was partially processed by the microsomal membranes, with translocation across the membrane of both the processed and unprocessed proteins. The positive charges convert the cytochrome P450IIC2 NH_2 terminus from a combination membrane insertion-halt transfer signal to a more classical secretory membrane-insertion signal, possibly by altering the orientation of the signal peptide in the membrane.**

The integration of proteins into cellular membranes may occur by either a cotranslational signal recognition particle (SRP)-dependent insertion, or by a posttranslational mechanism that does not require SRP (1, 2). The SRP-dependent mechanism is similar to that proposed for the initial steps of protein secretion. In contrast to secreted proteins, the hydrophobic signal sequences of some membrane proteins are not cleaved during the translocation process. The uncleaved signal peptides then serve to anchor the protein to the membrane (2). Because the uncleaved signal peptides share common structural features with those that are cleaved, it is not clear what property prevents recognition of the signal peptide by the signal peptidase.

Cytochromes P-450 are integral membrane proteins that are the terminal oxidases in the microsomal mixed-function monooxygenase (3). Cytochromes P-450 are synthesized exclusively in the rough endoplasmic reticulum, and the mature protein in the membrane is the same size as the initial translational product (4). Nevertheless, the insertion of the protein into the membrane is dependent on the presence of SRP (5). These data suggest that cytochromes P-450 contain an uncleavable signal peptide that may also serve as a membrane anchor, but the region of cytochrome P-450 with this function has not been identified. The NH_2-terminal sequences of all known microsomal cytochromes P-450 are strongly hydrophobic and resemble signal peptides. Haugen *et al.* (6) initially suggested that the NH_2 terminus is the signal peptide for these proteins.

To test this hypothesis, we constructed a hybrid protein in which 25 NH_2-terminal amino acids of rabbit P-450PBc2 (P450IIC2)† were substituted for 21 amino acids of the signal peptide of bovine preproparathyroid hormone (pre-pro-PTH). We now report that the NH_2-terminal sequence of cytochrome P-450 directs the integration but not the translocation of the hybrid protein through microsomal membrane. However, if two basic amino acids are introduced at the NH_2 terminus, the modified sequence directs the translocation of the hybrid protein and is partially cleaved.

MATERIALS AND METHODS

Enzymes and Chemicals. DNA restriction and modifying enzymes were obtained from Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs, and Pharmacia P-L Biochemicals. SP6 RNA polymerase and RNasin were from Promega Biotec. The *Nco* I linker CCCATGGG, m^7GpppG, and radioactive compounds were obtained from New England Biolabs, Pharmacia P-L Biochemicals, and Amersham, respectively.

Bacterial Strains and Bacteriophages. Plasmids were propagated in *Escherichia coli* strain NM522 as described (9) except that strain BW313 (*dut*$^-$, *ung*$^-$, *thi*-1, *rel*A, *spo*T1/F′*lys* A) (10), obtained from T. Kunkel (National Institute of Environmental Health Sciences, Research Triangle Park, NC), was used to prepare a uracil-containing template for oligonucleotide-directed mutagenesis. Growth conditions for this strain were as described (10). The interference resistance phage M13K07 served as the helper phage (11). Plasmid single-stranded DNA for mutagenesis and enzymatic sequencing was prepared as described (12).

Construction of Plasmids. The construction of the plasmid pPNTH contains an insert of pre-pro-PTH cDNA into which a direct internal repeat of 117 nucleotides was introduced (12). The resulting "stretched" pre-pro-PTH [pre-pro-P(N)TH] cDNA codes for a protein 39 amino acids longer than pre-pro-PTH but one which carries an identical signal peptide. P(N)TH thus is Endo-Pro^{18a}-Met^{18b}-Gly^{18c}-Met^{18d} PTH residues 19–52^{18e}-Met^{18f}-parathyroid hormone and has been designated PNTH in earlier publications (12, 13). The nucleotides coding for the 22 NH_2-terminal amino acids of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SRP, signal recognition particle; PTH, parathyroid hormone; P(N)TH, "stretched" PTH, where N represents a cDNA-encoded internal PTH insert of 39 amino acids that includes a repeat of PTH sequence 19–52. [P(N)TH is an endo-parathyroid hormone that allows for clearer gel separation.]

*Present address: Promega Biotec, 2800 South Fish Hatchery Road, Madison, WI 53711.

†The name P450IIC2 for the isozyme previously designated P-450PBc2 (7) is in accordance with the recommended cytochrome P-450 nomenclature (8).

the pre-pro-P(N)TH signal sequence were deleted by exonuclease III digestion and subsequent *Nco* I linker insertion as described (13). The plasmid with this deletion was used to construct a hybrid in which the deleted pre-pro-P(N)TH amino acids were replaced with the NH_2-terminal region of P450IIC2.

To obtain the P-450 sequence coding for the 25 NH_2-terminal amino acids, a 70-bp *Nco* I fragment was isolated from the cloned P450IIC2 gene (7, 14) and inserted into the pre-pro-P(N)TH deletion plasmid at the *Nco* I site. The nucleotide sequence at the fusion site was verified by sequencing. The plasmid encoding the fusion protein is called pc2P.

Oligonucleotide-Directed Mutagenesis. The method used for *in vitro* mutagenesis was modified from Kunkel (10) and Mead *et al.* (11). The synthetic oligonucleotide GGGCCCATGAAACGGGTGGTAG was synthesized on an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer and used as primer in the construction of pc2P1. An *ung*$^-$ *dut*$^-$ strain of *E. coli* (10) was transformed with pc2P, and single-stranded DNA isolated from this strain was used as a template for *in vitro* mutagenesis as described (11). Colonies of *E. coli* NM522 containing plasmid DNA with the appropriate mutations were identified first by hybridization to the 5′ ^{32}P-labeled primer and verified by DNA sequencing.

Transcription and *in Vitro* Translation. Plasmids pPNTH, pc2P, and pc2P1 were linearized with *Eco*RI and transcribed with SP6 polymerase in the presence of m^7GpppG as described (12). Translations in a wheat germ extract and rabbit reticulocyte lysate cell-free systems were done as described (12). Total microsomal membranes, high salt-washed membranes, and SRP were isolated from chicken oviduct (12, 13). SRP purified on omega aminopentylagarose was further concentrated on DEAE-cellulose (15) and, when indicated, 1 μl of this eluate was added per 12.5 μl of translational reaction. Microsomal membranes and salt-washed microsomal membranes were added to the reaction mixture at a final concentration of 2.1 A_{260} units per ml.

For protease protection experiments, tetracaine HCl was added to a concentration of 3 mM after completion of translation. Reactions were incubated with trypsin/chymotrypsin mixture (each at 25 μg/ml) or proteinase K (0.4 mg/ml) at 0°C for 30 min. Triton X-100 at a final concentration of 1% was added to control samples for membrane solubilization. Proteolysis was stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM. Samples were analyzed by electrophoresis on 15% acrylamide gels containing $NaDodSO_4$ followed by autoradiography (12).

Membrane-Integration Assay. After translation in a wheat germ cell-free system, 50-μl reactions containing microsomal membranes were adjusted to pH 11.5 with 1 M NaOH. After 15-min incubation on ice, reactions were layered over a 50-μl cushion of 0.2 M sucrose solution/150 mM KCl/1 mM $MgCl_2$, pH 11.5, and centrifuged in a Beckman Airfuge at 30 psi (1 psi = 6.895×10^3 Pa) for 15 min. After the supernatants were collected, membrane pellets were washed by resuspending them in 50 μl of 100 mM sodium carbonate, pH 11.5. After another 15-min incubation on ice, membranes were pelleted as before and resuspended in $NaDodSO_4$/gel loading buffer. The supernatants were combined and precipitated with 10% (vol/vol) trichloroacetic acid, and the pellets were resuspended for analysis by $NaDodSO_4$/acrylamide gel electrophoresis (12).

RESULTS

Translocation and Processing of P-450–P(N)TH Fusion Proteins. To study whether the strongly hydrophobic NH_2-terminal region of cytochrome P-450 can functionally replace the cleavable signal sequence of pre-pro-P(N)TH, we fused the NH_2-terminal 25 amino acids of P450IIC2 to a pre-pro-P(N)TH sequence from which the signal peptide had been deleted, except for four amino acids preceding the cleavage site (Fig. 1). In the presence of chicken oviduct microsomal membranes, the translational product of pre-pro-P(N)TH RNA was processed to pro-P(N)TH (Fig. 2). The latter was resistant to proteolysis by either trypsin and chymotrypsin (Fig. 2*A*) or proteinase K (Fig. 2*B*) unless Triton X-100 was added to the reaction, indicating that the pro-P(N)TH had been translocated across the microsomal membrane. A single major translational product of the pc2P RNA was seen that had an electrophoretic mobility similar to that of pre-pro-P(N)TH, as expected. In contrast to the results with P(N)TH, no processing of the pc2P translational product was seen when microsomal membranes were added to the reaction. Furthermore, only a trace amount of the translational product was resistant to protease digestion (Fig. 2). These results indicate that the cytochrome P-450 NH_2 terminus did not mediate the translocation of the hybrid protein across the microsomal membrane and, thus, the cytochrome P-450 NH_2 terminus is not functionally equivalent to the signal peptide of pre-pro-P(N)TH.

A major difference between the cytochrome P-450 NH_2 terminus and typical eukaryotic signal peptides is the lack of positively charged amino acids preceding the P-450 hydrophobic core. Comparisons of the sequences of eukaryotic signal peptides showed that nearly all signal peptides have a net positive charge in the NH_2-terminal portion with an average of two positive charges (16). For example, the pre-pro-PTH signal sequence (MMSAKDMVKVMIVMLAICFLARSDG) has a net charge of +1 at its NH_2 terminus. To analyze the possible effect of positively charged amino acids on the function of the cytochrome P-450 NH_2-terminal sequence, the cDNA was modified so that it coded for lysine and arginine at positions −28 and −27, respectively (Fig. 1). These amino acids substituted for aspartate and leucine residues, thereby changing the net charge at the NH_2 terminus from −1 to +2. Other than these two changes in this new construction, called pc2P1, the sequence was identical to that of pc2P.

When pc2P1 RNA was translated in the presence of microsomal membranes a new lower-molecular weight protein species was seen, indicating that partial cleavage of the pc2P1 translational product had occurred (Fig. 2). In addition, this processed product was protected from protease digestion, indicating that it was translocated across the microsomal membrane. Unexpectedly, a significant fraction

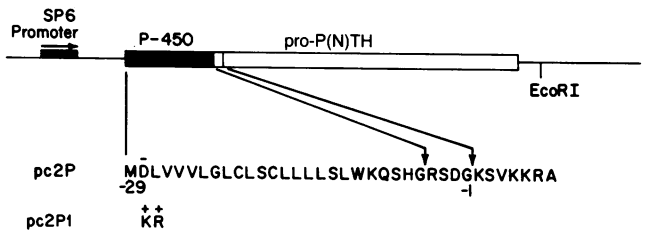

FIG. 1. Schematic representation of the primary structures of the fusion proteins encoded by plasmids pc2P and pc2P1. The diagram (at top) shows the structure of the cDNA insert in the expression vector. P-450 corresponds to the 25 NH_2-terminal amino acids of cytochrome P450IIC2, followed by 4 COOH-terminal amino acids of the pre-pro-P(N)TH signal sequence and the rest of the pro-P(N)TH sequence. The NH_2-terminal sequence of the fusion protein, pc2P, is shown. The normal pre-pro-P(N)TH processing site is at the glycine at position −1. Charges of NH_2-terminal amino acids are shown above the sequence. Differences in the NH_2-terminal amino acid sequence of the protein encoded by pc2P1 RNA are shown below the pc2P sequence.

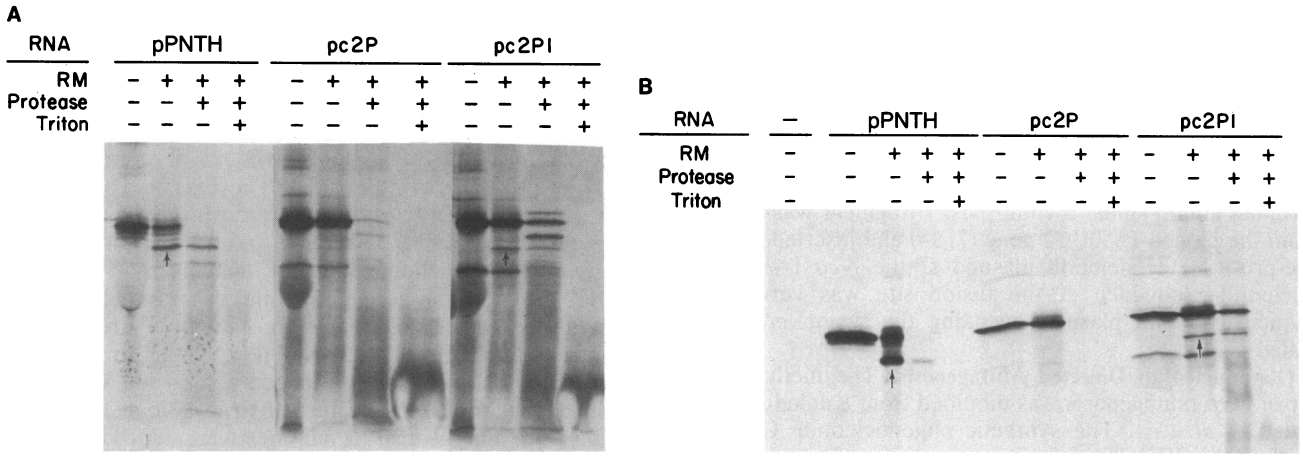

FIG. 2. Processing and protection from proteolytic digestion of translational products of RNAs coding for pre-pro-P(N)TH and the P-450–P(N)TH fusion protein in reticulocyte lysate. pPNTH, pc2P, and pc2P1 RNAs were translated in a reticulocyte lysate cell-free system in the absence or presence of chicken oviduct microsomal membranes (RM). After translation was completed, reactions were treated with trypsin/chymotrypsin (*A*) or proteinase K (*B*) with or without the addition of Triton X-100. Arrows in the pPNTH lanes and the pc2P1 lanes indicate the processed proteins that are formed in the presence of microsomal membranes. The band present between these unprocessed and processed proteins when the pc2P1 reaction is treated with trypsin/chymotrypsin probably results from partial proteolysis of the unprocessed form. The same band is also seen in the pc2P reaction.

of the unprocessed translational product was protected from proteolytic digestion. In the trypsin/chymotrypsin experiment (Fig. 2*A*), but not the proteinase K experiment (Fig. 2*B*), the proteolysis treatment resulted in a major protected band smaller than the unprocessed translational product. Because mild trypsin/chymotrypsin digestion of the translational product in the absence of membranes also produced this band, it probably results from small amounts of protease that penetrate into the lumen of the microsomal membrane. The protection from proteolytic digestion suggests that some of the unprocessed translational product was also translocated entirely across the membrane but was only inefficiently cleaved by signal peptidase. Poor processing of pc2P1 protein may result from fusion of the cytochrome P-450 NH_2-terminal translocation signal with the P(N)TH protein. Inouye *et al.* (17) have shown that structural incompatibility between a signal sequence and a protein attached to it can impair processing by signal peptidase. Because the processed pc2P1 migrated slightly more slowly than pro-P(N)TH, a cryptic cleavage site in the cytochrome P-450 sequence may be utilized that could contribute to the inefficient cleavage.

Interaction of the pc2P Translational Product with SRP. The lack of translocation of the pc2P translational product suggested that the cytochrome P-450 NH_2-terminal region might not mediate an SRP-dependent insertion of cytochrome P-450 into the microsomal membrane. To examine the interaction of the NH_2-terminal region with SRP, RNA was translated in the wheat germ cell-free system. In this system SRP arrests the translation of some proteins with membrane-insertion signals (18–20). The addition of SRP to the translation system did not affect the translation of globin, which is a cytoplasmic protein and does not contain a signal peptide (Fig. 3). As shown previously (13), the translation of pre-pro-P(N)TH RNA was inhibited by SRP. The addition of salt-washed microsomal membranes that had been stripped

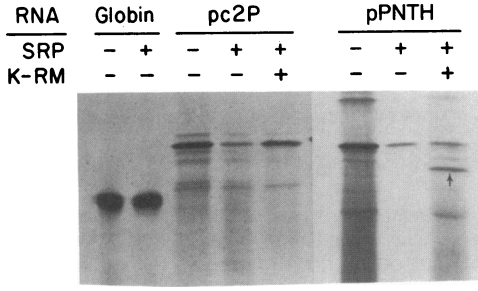

FIG. 3. Effect of SRP on translation in a wheat germ cell-free system. RNA coding for either globin, pc2P, or pre-pro-P(N)TH was translated in a wheat germ cell-free system in the absence or presence of SRP and microsomal membranes extracted with 0.5 M KCl to remove SRP (K-RM). Translational products were analyzed on $NaDodSO_4$/acrylamide gels. The arrow in the pPNTH gel indicates the band corresponding to pro-P(N)TH.

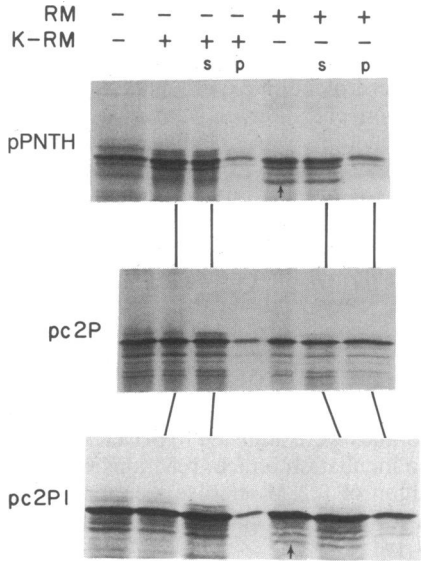

FIG. 4. SRP-dependent insertion of fusion proteins into microsomal membranes. RNAs of pPNTH, pc2P, and pc2P1 were translated in a wheat germ cell-free system in the presence of either complete (RM) or SRP-depleted (K-RM) chicken oviduct microsomal membranes. After translation was completed, the reactions containing membranes were adjusted to pH 11.5, and microsomal membranes were pelleted by centrifugation. An aliquot of the reaction mixture without membranes, the unfractionated reaction mixture with membranes, and the supernatant (s) and pellet (p) containing microsomal membranes were analyzed on $NaDodSO_4$/acrylamide gels. Samples in the supernatant and pellet lanes contain an amount of reaction mixture equivalent to twice that analyzed for the unfractionated samples. Arrows indicate the processed forms of pre-pro-P(N)TH and pc2P1 formed in the presence of membranes.

of SRP resulted in the release of the translational arrest and the processing of pre-pro-P(N)TH to pro-P(N)TH. The translation of pc2P RNA was inhibited by the addition of SRP to about the same extent as that of pre-pro-P(N)TH. When salt-washed microsomal membranes were also added, the inhibition was partially reversed. However, in contrast to the results with pre-pro-P(N)TH, no processed product was seen after addition of membranes, as was expected from the results described above. These results indicate that even though the NH_2-terminal region of P450IIC2 does not mediate translocation of the hybrid protein through the membrane, it does interact with SRP and may function as a membrane-insertion signal.

Integral Association of the Fusion Proteins with the Membrane. To assay for insertion into the membrane, the extraction of the proteins by alkaline solutions (pH 11.5) was analyzed. Under these conditions, only integral membrane proteins remain associated with the membranes and are not extracted (21). As a control for the nonspecific binding of the proteins to membranes, RNA was translated in the presence of salt-washed membranes without SRP added. These salt-washed membranes are inactive for translocation of proteins across membranes. A fraction of the pre-pro-P(N)TH was not extracted with alkaline treatment (Fig. 4), presumably because of nonspecific binding because a similar amount binds to the salt-washed membranes. All translocated and processed pro-P(N)TH was extracted by the alkaline treatment. A small amount of the pc2P translational product remained associated with the salt-washed membranes, similar to that of pPNTH. In contrast, if complete microsomal membranes (RM) were added to the translation reactions, about half of the translational product of pc2P RNA was resistant to extraction from the membranes at pH 11.5. Incomplete binding of pc2P to the membrane is consistent with the relative inefficiency under these conditions of translocation and cleavage of pre-pro-P(N)TH (Fig. 4 *Top*). These results suggest that the P450IIC2 NH_2-terminal region is acting as a membrane-insertion signal and, further, that it serves as an insertion-stop signal and anchors the protein to the membrane. In the presence of total membranes mutant pc2P1 produces a processed protein that is entirely extracted by high pH into the supernatant, whereas the unprocessed protein remains bound to the membrane and is recovered in the pellet (Fig. 4). Thus, translocation of an entire mutated fusion protein (see also Fig. 2) is not followed by its release into the endoplasmic reticulum lumen if the NH_2-terminal sequence is not cleaved off. We conclude that introduction of positive charges at the NH_2 terminus of P-450 signal sequence eliminated its stop-transfer function without changing its role as a membrane anchor.

DISCUSSION

Strong conservation of highly hydrophobic NH_2-terminal segments in microsomal forms of cytochromes P-450 suggests that these regions may play an important role in the assembly of these proteins in the endoplasmic reticulum membrane. Our results show that, if fused to a secretory protein (parathyroid hormone) devoid of its own signal sequence, the NH_2-terminal 25 amino acids of P450IIC2 mediate SRP-dependent translational arrest and initiate translocation of hybrid protein through the membrane. These results indicate that the P-450 NH_2-terminal sequence mediates the SRP-dependent insertion of P-450 into the membrane. However, translocation is apparently halted immediately after the hydrophobic region enters the mem-

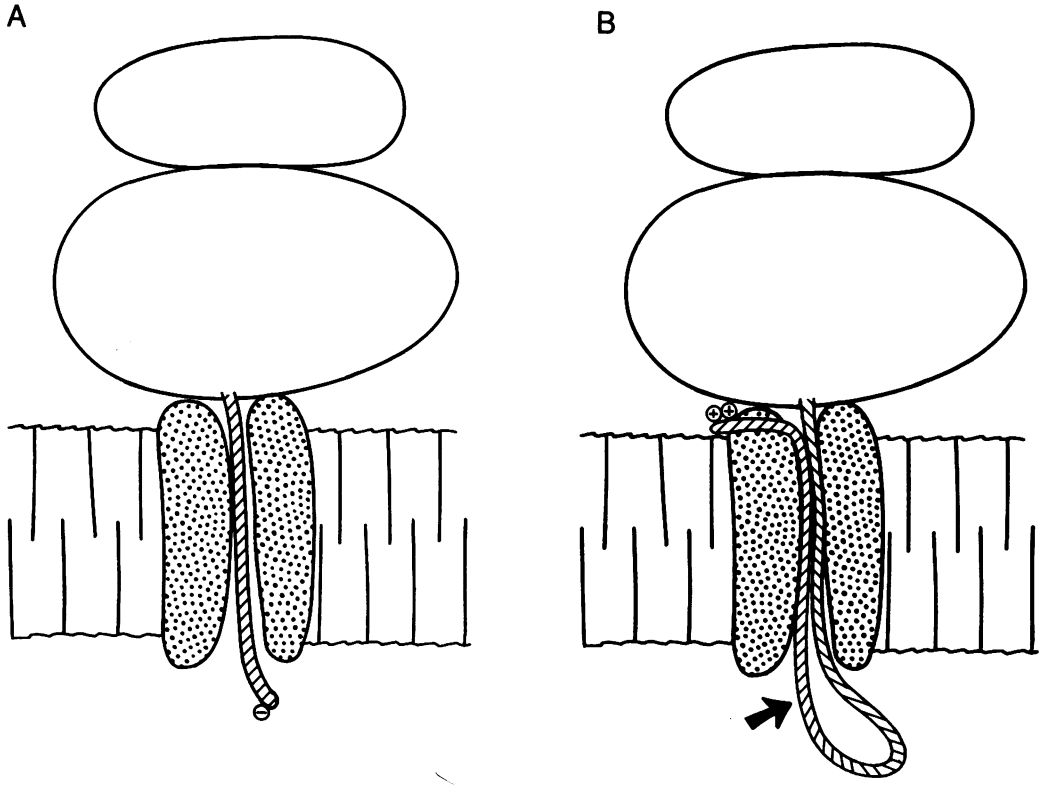

FIG. 5. Model of the orientation of the insertion of the NH_2-terminal sequences of proteins encoded by pc2P and pc2P1 RNAs. A schematic diagram of a ribosome bound to hypothetical proteins in the membrane of the endoplasmic reticulum is shown. In *A* the NH_2-terminal region of pc2P with a negative charge near the NH_2 terminus is shown inserting into the membrane head-inward. We propose that in this orientation the NH_2-terminal region functions as a stop-transfer signal, preventing further translocation across the membrane and anchoring the protein to the membrane. In *B* the NH_2-terminal region of pc2P1 with two positive charges is shown forming a loop structure in the membrane. The altered orientation from that shown in *A* may mediate the translocation of the protein through the membrane and permit cleavage of the NH_2 terminus as indicated by the arrow.

brane because the hybrid protein was sensitive to proteolytic digestion. Therefore, this sequence must function not only as an SRP-dependent signal peptide, but also as a stop-transfer sequence. Similar dual roles have been demonstrated for hydrophobic membrane-spanning regions of class II membrane proteins (18, 19, 22–24). The NH_2 termini of these proteins are on the cytoplasmic side of the membrane, and models in which the hydrophobic region loops through the membrane have been proposed to explain the final membrane conformation (25). The absence of a signal peptidase cleavage site (18, 24) or the length of the polypeptide preceding the hydrophobic core of the signal peptide (19, 22) have been proposed to explain the lack of cleavage of these sequences.

In contrast to the signal peptides of most secretory proteins, the P450IIC2 NH_2-terminal hydrophobic segment is preceded by only one negatively charged amino acid. When the net charge was changed to a +2 by mutagenesis, the hybrid protein was translocated across the membrane and partial cleavage of the NH_2 terminus occurred. This indicates that the lack of cleavage of the original hybrid protein with the negative charge is not due to the absence of a cleavage site. Possibly the orientation in the membrane of the hydrophobic region is dependent on the nature of the charges at the NH_2-terminal end. As shown in the simple model in Fig. 5*A*, the negatively charged NH_2 terminus might insert head-inward into the membrane and then halt translocation so that the potential cleavage site remains on the cytoplasmic side of the membrane and is sequestered from the signal peptidase. This model is supported by recent studies using site-specific antibodies that showed that the cytochrome P-450 region immediately following the NH_2-terminal hydrophobic region (amino acids 24–38) is exposed to the antibodies on the cytoplasmic side of the membrane (26). In contrast, if positive charges are present at the NH_2 terminus, this region may interact with negative charges in the membrane phospholipids, or a hypothetical receptor protein, so that the hydrophobic region loops through the membrane and exposes the cleavage site to the signal peptidase (Fig. 5*B*). This loop structure would also be required to mediate the translocation of the remainder of the protein through the membrane, regardless of whether or not cleavage occurs.

Although our data point to the importance of positively charged NH_2 termini in signal sequence function, this feature is not absolutely required for translocation, as some proteins with acidic or neutral NH_2 termini are also secreted (16, 27, 28, E.S.-S. and B.K., unpublished results). Most probably, the nature of the sequence following the charged NH_2 terminus also plays an important part in the final disposition of the protein.

The molecular basis for the function of stop-transfer sequences is not known. Davis and Model (29) have proposed that stop-transfer activity is dependent only on overall hydrophobicity because many hydrophobic sequences of 16–17 amino acids function as membrane anchors. On the other hand, a sequence in IgM that is normally a stop-transfer signal can also mediate SRP-dependent translocation (30). Experiments on bacterial lipoprotein have suggested that the orientation of the signal peptide may determine whether a sequence is a stop-transfer signal or a translocation signal (31). When the signal peptide of lipoprotein was internalized in a protein containing a normal NH_2-terminal signal peptide, the internalized peptide, which presumably could not loop through the membrane, acted as a stop-transfer signal. As shown in this report, introduction of two positive charges at the NH_2 terminus of a stretch of 16 hydrophobic amino acids in P450IIC2 changed this region from a stop-transfer to a translocation signal, possibly by altering the orientation of the NH_2 terminus in the membrane. These results suggest that the stop-transfer function is not a simple function of hydrophobic interaction with the membrane lipids but may involve interactions with membrane proteins.

Note Added in Proof. After submission of this manuscript, Sakaguchi *et al.* (32) also showed that the NH_2-terminal segment of another cytochrome P-450 functions as a combination insertion, stop-transfer signal.

We thank Dr. Shubha Govind for providing the P450IIC2 genomic clone and Dr. Thomas Kunkel for providing *E. coli* strain BW313. This work was supported by National Institutes of Health Grant GM32216.

1. Anderson, D. J., Mostov, K. E. & Blobel, G. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7249–7253.
2. Wickner, W. T. & Lodish, H. F. (1986) *Science* **230**, 400–407.
3. White, R. E. & Coon, M. J. (1980) *Annu. Rev. Biochem.* **49**, 315–356.
4. Bar-Nun, S., Kreibich, G., Adesnik, M., Alterman, L., Negishi, M. & Sabatini, D. D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 965–969.
5. Sakaguchi, M., Mihara, K. & Sato, R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3361–3364.
6. Haugen, D. A., Armes, L. G., Yasunobu, K. T. & Coon, M. J. (1977) *Biochem. Biophys. Res. Commun.* **77**, 967–973.
7. Leighton, J. K., DeBrunner-Vossbrink, B. A. & Kemper, B. (1984) *Biochemistry* **23**, 204–210.
8. Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. & Waterman, M. R. (1987) *DNA* **6**, 1–11.
9. Gough, J. A. & Murray, N. E. (1983) *J. Mol. Biol.* **166**, 1–19.
10. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
11. Mead, D. A., Szczesna-Skorupa, E. & Kemper, B. (1986) *Protein Eng.* **1**, 67–76.
12. Mead, D. A., Szczesna-Skorupa, E. & Kemper, B. (1985) *Nucleic Acids Res.* **4**, 1103–1118.
13. Szczesna-Skorupa, E., Mead, D. A. & Kemper, B. (1987) *J. Biol. Chem.* **262**, 8896–8900.
14. Govind, S., Bell, P. A. & Kemper, B. (1986) *DNA* **5**, 371–382.
15. Walter, P. & Blobel, G. (1983) *Methods Enzymol.* **96**, 682–691.
16. von Heijne, G. (1984) *EMBO J.* **3**, 2315–2318.
17. Inouye, S., Duffaud, G. & Inouye, M. (1986) *J. Biol. Chem.* **261**, 10970–10975.
18. Holland, E. C. & Drickamer, K. (1986) *J. Biol. Chem.* **261**, 1286–1292.
19. Spiess, M & Lodish, H. F. (1986) *Cell* **44**, 177–185.
20. Lipp, J. & Dobberstein, B. (1986) *J. Cell Biol.* **102**, 2169–2175.
21. Mostov, K. E., DeFoor, P., Fleischer, S. & Blobel, G. (1981) *Nature (London)* **292**, 87–88.
22. Lipp, J. & Dobberstein, B. (1986) *Cell* **46**, 1103–1112.
23. Bos, T. J., Davis, A. R. & Nayak, D. P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2327–2331.
24. Zerial, M., Melancon, P., Scheider, D. & Garoff, H. (1986) *EMBO J.* **5**, 1543–1550.
25. Inouye, M. & Halegoua, S. (1980) *CRC Crit. Rev. Biochem.* **7**, 339–371.
26. DeLemos-Chiarandini, C., Frey, A. B., Sabatini, D. A. & Kreibich, G. (1987) *J. Cell Biol.* **104**, 209–219.
27. Kaiser, C. A., Preuss, D., Grisafi, P. & Botstein, D. (1987) *Science* **235**, 312–317.
28. Garcia, P. D., Ghrayeb, J., Inouye, M. & Walter, P. (1987) *J. Biol. Chem.* **262**, 9463–9468.
29. Davis, N. G. & Model, P. (1985) *Cell* **41**, 607–614.
30. Mize, N. K., Andrews, D. W. & Lingappa, V. R. (1986) *Cell* **47**, 711–719.
31. Coleman, J., Inukai, M. & Inouye, M. (1985) *Cell* **43**, 351–360.
32. Sakaguchi, M., Mihara, K. & Sato, R. (1987) *EMBO J.* **6**, 2425-2431.