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A short amino-terminal segment of microsomal cytochrome P-450 functions both as an insertion signal and as a stop–transfer sequence

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Co-translational insertion of liver microsomal cytochrome P-450 into the endoplasmic reticulum membrane is mediated by the signal recognition particle (SRP) and the presence in the cytochrome molecule of a signal sequence that can be recognized by SRP has been postulated. To locate this signal sequence, six hybrid cDNAs were constructed in which various segments of a cDNA for a rabbit liver cytochrome P-450 are fused with a cDNA or its fragment encoding yeast porin (an outer mitochondrial membrane protein) or with a cDNA for pre-interleukin 2 (a secretory protein) from which the 5′-terminal portion encoding most of its signal sequence had been removed. These hybrid cDNAs were inserted into an SP-6 transcription vector and transcribed *in vitro*. The mRNAs thus synthesized were translated in a cell-free system in the presence of rough microsomes. It was thus found that only those chimeric proteins containing (at their amino-terminal end) the amino-terminal cytochrome P-450 segments consisting of ≥29 amino acid residues were co-translationally inserted into the membrane in an SRP-dependent fashion. These proteins were, however, neither processed nor translocated across the membrane. These findings, coupled with the observation that the major portion of these proteins, when inserted into the membrane, was degraded by trypsin, led to the conclusion that a short amino-terminal segment (<29 residues) of the cytochrome P-450 functions not only as an insertion signal but also as a stop-transfer sequence. This segment is, therefore, similar to the internal signal of type II plasma membrane proteins, but differs from the latter in the topogenic function.

Key words: cytochrome P-450/membrane biogenesis/membrane insertion/signal sequence/stop-transfer sequence

Introduction

Hepatic microsomal cytochrome P-450 is synthesized on membrane-bound polysomes and inserted co-translationally into the endoplasmic reticulum (ER) membrane (Negishi *et al.*, 1976; Bar-Nun *et al.*, 1980). Our previous studies have further shown that this insertion requires the signal recognition particle (SRP) (Sakaguchi *et al.*, 1984). It is, therefore, evident that the cytochrome P-450 molecule contains a signal sequence that can be recognized by SRP, although this signal sequence is not cleavable (Sakaguchi *et al.*, 1984). In this respect, the putative signal in cytochrome P-450 is different from cleavable signals of most secretory proteins (Blobel and Dobberstein, 1975; von Heijne, 1985a) and type I plasma membrane proteins (Yost *et al.*, 1983; Guan and Rose, 1984) and similar to the internal signals (transmembrane segments) of type II plasma membrane proteins, which have been shown to function both as a translocation signal and as a membrane anchor (Lipp and Dobberstein, 1986; Spiess and Lodish, 1986; Zerial *et al.*, 1986). (For topological classification of plasma membrane proteins into type I, II, etc., see Garoff, 1985; Wickner and Lodish, 1985.)

This study was undertaken to locate the putative signal sequence in the cytochrome P-450 polypeptide chain and to elucidate its topogenic function. For this purpose, we constructed hybrid cDNAs in which fragments of a cDNA encoding a polycyclic aromatic hydrocarbon-inducible form of rabbit liver cytochrome P-450 are fused with a cDNA or its fragment coding for yeast porin (Mihara and Sato, 1985), which is an outer mitochondrial membrane protein and incorporated into that membrane post-translationally without the aid of SRP (Mihara *et al.*, 1982). We also constructed another set of hybrid cDNAs in which the 5′-terminal portion (encoding most of the transient signal) of murine pre-interleukin 2 (Kashima *et al.*, 1985) is replaced by cDNA fragments encoding the amino-terminal segments of the cytochrome P-450. These cDNAs were transcribed with the aid of SP-6 promoter and translated *in vitro*; the capacity of the protein products to be inserted co-translationally into dog pancreas rough microsomes was examined.

The results thus obtained have provided evidence that a short amino-terminal segment (<29 residues) of the cytochrome P-450 serves not only as a signal required for the membrane insertion but also as a stop-transfer sequence. This segment, therefore, resembles the internal signal (transmembrane segment) of type II plasma membrane proteins. In contrast to the transmembrane segment of type II proteins, however, the short amino-terminal segment of the cytochrome leads to the disposition of the inserted protein in which the carboxy terminus is exposed on the outer (cytoplasmic) surface of the ER membrane. Therefore, this segment is functionally different from the transmembrane segment of type II membrane proteins. Another conclusion reached in this study was that the cytochrome molecule is anchored to the membrane only by inserting its short amino-terminal segment into the lipid bilayer.

Results

Hybrid cDNAs constructed

Figure 1 shows schematically the six hybrid cDNAs constructed in this study. All these cDNAs are inserted into the transcription vector pSP-64 or pSP-65 so that they are placed just downstream of the SP-6 promoter that is present in the plasmids. In pSP-N39 and pSP-N79, cDNA fragments encoding the amino-terminal 39 and 79 residues respectively of the rabbit liver cytochrome P-450 are ligated to a cDNA fragment coding for the carboxy-terminal 184 residues of yeast porin. In pSP-Δ98, on the other hand, a cDNA fragment specifying the cytochrome P-450 segment spanning residues 99–517 (the cytochrome is composed of 518 residues) is inserted into the middle of the porin cDNA. The other three constructs are hybrids of cDNAs for the cytochrome and

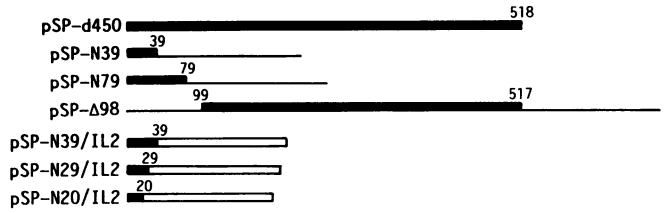

Fig. 1. Schematic illustrations of cDNAs in the hybrid plasmids constructed. Solid boxes, lines and open boxes represent the coding sequences for cytochrome P-450, yeast porin and pre-interleukin 2 respectively. Numbers are amino acid residue numbers in the primary structure of cytochrome P-450.

murine pre-interleukin 2, a secretory protein. Pre-interleukin 2 possesses a 20-residue, cleavable signal sequence at its amino-terminal end (Kashima *et al.*, 1985). In one of these constructs, pSP-N39/IL2, the 5'-terminal portion of pre-interleukin 2 cDNA, encoding the amino-terminal 18 residues of the transient signal, is replaced by a cDNA fragment specifying the amino-terminal 39 residues of the cytochrome P-450. The chimeric protein encoded by this plasmid, therefore retains the site of signal peptidase attack in the pre-interleukin 2 sequence. We also prepared, by oligonucleotide-directed, site-specific mutagenesis, two deletion mutants of pSP-N39/IL2, in which DNA stretches encoding residues 30−39 (pSP/N29/IL2) and residues 21−39 (pSP-N20/IL2) are missing. Nucleotide sequencing of the joining regions of all the hybrid cDNAs confirmed that the ligation has been made as designed and the correct reading frame is maintained (see Figure 8E). Finally, we also used plasmids in which the complete cDNAs for the cytochrome P-450 (pSP-d450) and yeast porin (pSP-Por) are placed just downstream of the SP-6 promoter in pSP-65.

Insertion of in vitro synthesized cytochrome P-450 into microsomes

To examine whether the cytochrome P-450 and yeast porin − synthesized *in vitro* with pSP-d450 and pSP-Por respectively as templates − can be inserted co-translationally into dog pancreas rough microsomes (RM), these plasmids were appropriately linearized and transcribed with the aid of SP-6 RNA polymerase. The mRNAs thus synthesized were then translated in a wheat-germ cell-free extract in the presence of [^{35}S]methionine and RM. The reaction mixture was treated with 50 mM Na_2CO_3, which extracts all peripheral membrane proteins (Fujiki *et al.*, 1982), and the membrane fraction was recovered by centrifugation. The supernatant and membrane fractions obtained were analyzed by SDS−PAGE and subsequent fluorography. As shown in Figure 2, a substantial amount of the cytochrome synthesized (the band having the lowest mobility in lane 1) was recovered in the membrane fraction (lane 1), whereas all the porin synthesized remained in the supernatant fraction (lane 2). The cytochrome and porin could be specifically immunoprecipitated by antibodies raised against P-448$_1$, a polycyclic hydrocarbon-inducible form of rabbit liver cytochrome P-450 (Imai *et al.*, 1980) and those against yeast porin (Mihara *et al.*, 1982) respectively (data not shown). When high salt-washed RM (KRM), which is deficient in SRP (Walter and Blobel, 1980), was included in the translation mixture instead of RM, no insertion of the cytochrome into the membrane was detected (lane 3). However, supplementation of the KRM-containing system with purified SRP restored the insertion (lane 4). These findings thus confirmed our previous results that SRP is required for the membrane insertion of cytochrome P-450 (Sakaguchi *et al.*, 1984).

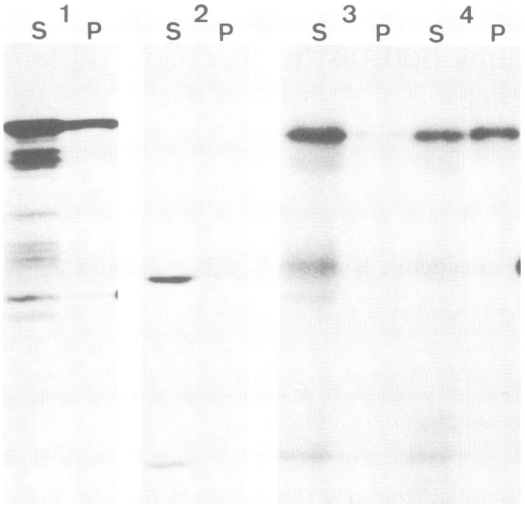

Fig. 2. Insertion of cytochrome P-450 synthesized *in vitro* into microsomes. The mRNAs transcribed from pSP-d450 and pSP-Por were translated in a wheat-germ cell-free extract in the presence of [^{35}S]methionine and RM (5 A_{280} units/ml) or KRM (5 A_{280} units/ml). After translation, the mixtures were treated with Na_2CO_3 and then separated into the membrane ('P') and supernatant ('S') fractions. These fractions were analyzed by SDS−PAGE (12.5% gel) followed by fluorography. **Lane 1**, translate of pSP-d450 mRNA in the presence of RM; **lane 2**, translate of pSP-Por mRNA in the presence of RM; **lane 3**, translate of pSP-d450 mRNA in the presence of KRM; **lane 4**, same as in **lane 3** except that SRP (10 units/50 μl) was included in the translation mixture.

The failure to insert yeast porin into RM warranted the use of this protein as a negative control in the study of membrane insertion of cytochrome P-450.

Insertion of cytochrome P-450/porin chimeric proteins into microsomes

Similar experiments as above were next carried out with linearized pSP-N39, pSP-N79 and pSP-Δ98 as templates, and the results obtained are shown in Figure 3. It is clear that the cytochrome encoded by pSP-d450 (lane 1) and two chimeric proteins encoded by pSP-N79 (lane 2) and pSP-N39 (lane 3) were largely recovered in the membrane fraction, indicating that these proteins had been effectively inserted into RM which was present in the translation mixtures. When RM was added after the termination of translation, the protein encoded by pSP-N39 was recovered only in the supernatant fraction (lane 4), confirming that the insertion of this chimeric protein is also a co-translational event. The chimeric protein encoded by pSP-Δ98, on the other hand, was not inserted into RM at all (lane 5). Taken together, these data provide evidence that the amino-terminal sequence of the cytochrome consisting of 39 residues is sufficient for co-translational insertion of proteins into the RM membrane. The inability to insert the pSP-Δ98 protein into the membrane suggested that there is no signal sequence mediating the SRP-dependent membrane insertion in the carboxy-terminal portion of the cytochrome P-450 starting from residue 99. It is to be noted that all the chimeric proteins synthesized *in vitro* could be immunoprecipitated by both anti-P-448$_1$ and anti-porin antibodies (data not shown). We also studied the effect of SRP on the insertion of the protein encoded by pSP-N39. As shown in Figure 4, translation of pSP-N39 mRNA in the presence of KRM (but in the absence of SRP) resulted in a very low recovery of the protein in the membrane fraction (lane 1). The slight insertion observed was probably due to a small amount of SRP remaining

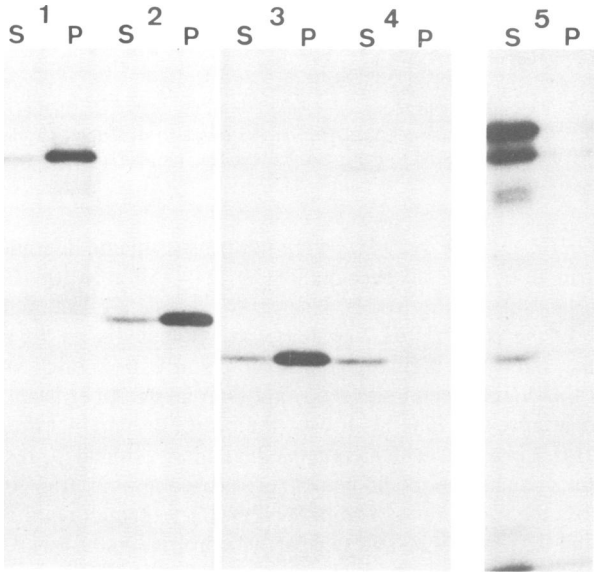

Fig. 3. Insertion of the cytochrome P-450/porin chimeric proteins synthesized *in vitro* into microsomes. The mRNAs transcribed from pSP-d450 (**lane 1**), pSP-N79 (**lane 2**), pSP-N39 (**lanes 3** and **4**) and pSP-Δ98 (**lane 5**) were translated as in Figure 2 in the presence of KRM (5 A_{280} units/ml) plus SRP (10 units/50 μl) (**lanes 1, 2** and **3**) or RM (5 A_{280} units/ml) (**lane 5**). In **lane 4**, RM (5 A_{280} units/ml) was added after the translation was terminated by adding RNase (100 μg/ml) and the mixture was further incubated at 26°C for 45 min. The mixtures were separated into the membrane ('P') and supernatant ('S') fractions after the Na_2CO_3 treatment. These fractions were analyzed by SDS–PAGE (12.5% gel) and subsequent fluorography. The reason for the low translation efficiency in **lane 4** is not known. **Lane 5** is a result obtained in a separate experiment.

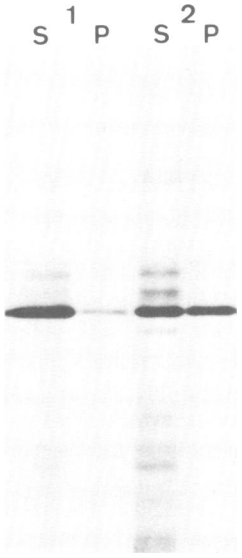

Fig. 4. Effect of SRP on the insertion of the chimeric protein encoded by pSP-N39 into microsomes. The mRNA transcribed from pSP-N39 was translated as in Figure 2 in the presence of KRM (5 A_{280} units/ml) (**lane 1**) or KRM (5 A_{280} units/ml) plus SRP (10 units/50 μl) (**lane 2**). Insertion of the protein into the membrane was assayed as in Figure 2.

in the KRM preparation used. When purified SRP was included in the KRM-containing system, a substantial amount of the protein was recovered in the membrane fraction (lane 2). These observations indicated that insertion of the pSP-N39 protein, like that of the cytochrome P-450, is dependent on SRP and that the

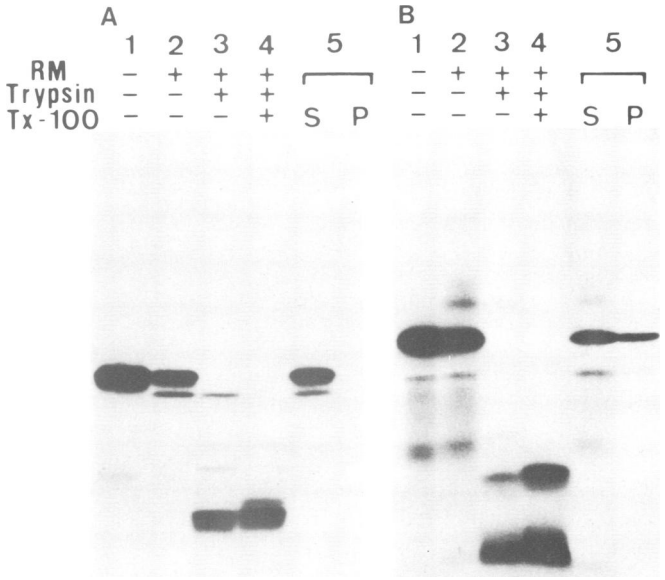

Fig. 5. Synthesis *in vitro* and co-translational interactions with microsomes of pre-interleukin 2 (**A**) and the chimeric protein encoded by pSP-N39/IL2 (**B**). The mRNAs transcribed from pSP-IL2 and pSP-N39/IL2 were translated in the absence (**lane 1**) and presence (**lanes 2–5**) of RM (5 A_{280} units/ml). Aliquots (10 μl) of the translation mixtures were digested with trypsin in the absence (**lane 3**) and presence (**lane 4**) of 1% Triton X-100. In **lane 5**, the same experiment was performed as in **lane 2**, except that after translation the mixture was treated with Na_2CO_3 and then separated into the membrane ('P') and supernatant ('S') fractions. The total reaction mixtures (**lanes 1–4**) and P and S fractions (**lane 5**) were directly subjected to SDS–PAGE (15% gel) and protein bands were visualized by fluorography.

amino-terminal 39 residues of the cytochrome contain a signal sequence that can be recognized by SRP.

Co-translational interactions of pSP-IL2 and pSP-N39/IL2 proteins with microsomes

Since the above results indicated that the signal sequence of the cytochrome P-450 is located in the amino-terminal 39 residues, it was of interest to see whether this signal can functionally replace transient signals of secretory proteins. To test this possibility, we constructed pSP-N39/IL2 in which the cDNA fragment encoding the 39-residue segment of the cytochrome is ligated to a cDNA coding for mature interleukin 2 plus two amino acids (residues 19 and 20) of the interleukin 2 signal sequence. Both this hybrid cDNA and pSP-IL2, which contains the complete cDNA for pre-interleukin 2, were transcribed and translated *in vitro* as described above. When the translates were analyzed by SDS–PAGE and subsequent fluorography, each of the translates gave an intense protein band having a molecular size expected for pre-interleukin 2 or the chimeric protein (Figure 5A, lane 1; Figure 5B, lane 1). When pSP-IL2 mRNA was translated in the presence of RM, pre-interleukin 2 was translocated and processed to the mature form as expected (Figure 5A, lane 2). Although pre-prolactin was efficiently translocated across the RM membrane used in this study (data not shown), the translocation of pre-interleukin 2 was not very marked for unknown reasons. In any case, trypsin degraded the precursor completely leaving the mature interleukin protected (Figure 5A, lane 3). In the presence of Triton X-100, both the precursor and mature protein were susceptible to trypsin digestion (Figure 5A, lane 4). When the translate was treated with 50 mM Na_2CO_3 and

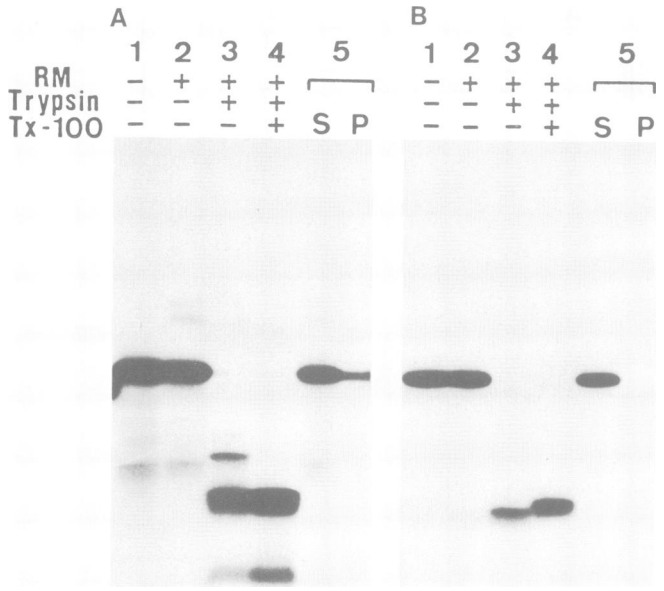

Fig. 6. Synthesis *in vitro* and insertion into microsomes of the chimeric proteins encoded by pSP-N29/IL2 (**A**) and pSP-N20/IL2 (**B**). Experiments were carried out in the same way as in Figure 5, except that the mRNAs transcribed from pSP-N29/IL2 and pSP-N20/IL2 were used as templates for translation. **Lanes 1–5** correspond to those in Figure 5.

separated into the membrane and supernatant fractions, both the precursor and mature interleukin 2 were recovered in the supernatant fraction (Figure 5A, lane 5), indicating that they were not tightly associated with the membrane. It was evident that pre-interleukin 2 was translocated across the membrane, processed by signal peptidase, released into the luminal space, and thus protected from tryptic attack, as has been observed for other secretory proteins (Blobel and Dobberstein, 1975). The chimeric protein encoded by pSP-N39/IL2, on the other hand, was neither processed nor protected from trypsin digestion when synthesized in the presence of RM (Figure 5B, lanes 2–4). After the Na_2CO_3 treatment, however, a substantial portion (~30%) of the protein was recovered in the membrane fraction (Figure 5B, lane 5). These results indicated that this chimeric protein was actually inserted into the membrane, but its major portion was still exposed on the outer surface of the membrane and thus susceptible to trypsin. The failure of the chimeric protein to be processed suggested that the site of cleavge by signal peptidase, which is retained in this protein, did not reach the peptidase present on the inner surface of the membrane. This in turn suggested that the amino-terminal 39-residue segment of the cytochrome contains, in addition to the signal sequence, a sequence that stops the translocation of the rest of the protein across the membrane.

Co-translational insertion of pSP-N29/IL2 and pSP-N20/IL2 proteins into microsomes

To characterize further the signal and putative stop-transfer sequences, two deletion mutants of pSP-N39/IL2 were prepared by site-specific mutagenesis. These mutant cDNAs, pSP-N29/IL2 and pSP-N20/IL2, were transcribed *in vitro*. The mRNAs synthesized were translated in the wheat-germ cell-free extract and the protein products were analyzed as above. In each of the translates one major band was detected and its size was consistent with that expected from the length of template cDNA (Figure 6A, lane 1; Figure 6B, lane 1). When the translation was carried out in the presence of RM, the products encoded by pSP-

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          10        20        30        40
MVSDFGLPTF ISATELLLAS AVFCLVFWVA GASKPRVPKG LKRLP---
-          -                    * *  *   **
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Fig. 7. The amino acid sequence deduced from the cDNA nucleotide sequence for the amino-terminal portion (45 residues) of the cytochrome P-450. Positively and negatively charged residues are marked with * and −, respectively.

N29/IL2 and pSP-N20/IL2 were not processed and degraded by trypsin both in the absence and presence of Triton X-100 (Figure 6A, lanes 2–4; Figure 6B, lanes 2–4). While a substantial portion of the pSP-N29/IL2 protein was associated with the membrane after the Na_2CO_3 treatment (Figure 6A, lane 5), the pSP-N20/IL2 protein was recovered only in the supernatant fraction (Figure 6B, lane 5). It was, therefore, certain that the pSP-N20/IL2 product, which contains the amino-terminal 20 residues of the cytochrome, could be neither inserted into nor translocated across the membrane. The pSP-N29/IL2 protein containing the amino-terminal 29 residues of the cytochrome, on the other hand, behaved identically with the product of pSP-N39/IL2. These observations thus provided evidence that both the insertion signal and stop-transfer sequence are contained in the short amino-terminal 29-residue segment of the cytochrome.

Discussion

The above results leave little doubt that the insertion signal of the cytochrome P-450 is located in the amino-terminal 29-residue segment. The finding that the product of pSP-N20/IL2, which contains only the amino-terminal 20 residues derived from the cytochrome, cannot be inserted into the membrane suggests further that the important portion of the signal is present in the short stretch spanning residues 21–29, though it is highly likely that the signal extends over residue 20 towards the amino terminus. The amino-terminal 29-residue segment of the cytochrome contains a stretch of 14 uncharged residues (Leu-16 through Val-29) including a leucine cluster (Figure 7), as has been invariably detected in microsomal cytochromes P-450 so far sequenced. Since similar uncharged stretches of varying lengths are also seen in amino-terminal, cleavable signal sequences of many secretory proteins, it is tempting to assume that this segment of the cytochrome serves as the translocation signal. However, in contrast to the case for pre-interleukin 2, the chimeric proteins encoded by pSP-N29/IL2 and pSP-N39/IL2 are not translocated across the membrane nor processed, indicating that the amino-terminal segment of the cytochrome is functionally different from the signal sequence of pre-interleukin 2. We conclude that the amino-terminal signal of the cytochrome, unlike those of secretory proteins, functions not only as the insertion signal but also as a stop-transfer sequence that halts further translocation of the polypeptide chain. This conclusion is based mainly on the finding that the chimeric proteins, when inserted into the membrane, are susceptible to trypsin and thus thought to be exposed on the outer surface of the membrane. This view is further supported by a recent study (De Lemos-Chiarandini *et al.*, 1987) in which it has been shown, using site-specific antibodies, that only the amino-terminal short segment of rat liver cytochrome P-450 isozyme PB-4 is embedded in the ER membrane *in vivo*.

The amino-terminal signal/stop-transfer sequence of the cytochrome P-450 is, therefore, functionally similar to the transmembrane segments of type II plasma membrane proteins, which function both as a translocation signal and as a membrane anchor (Lipp and Dobberstein, 1986; Spiess and Lodish, 1986; Zerial *et al.*, 1986), although the latter are not located in the

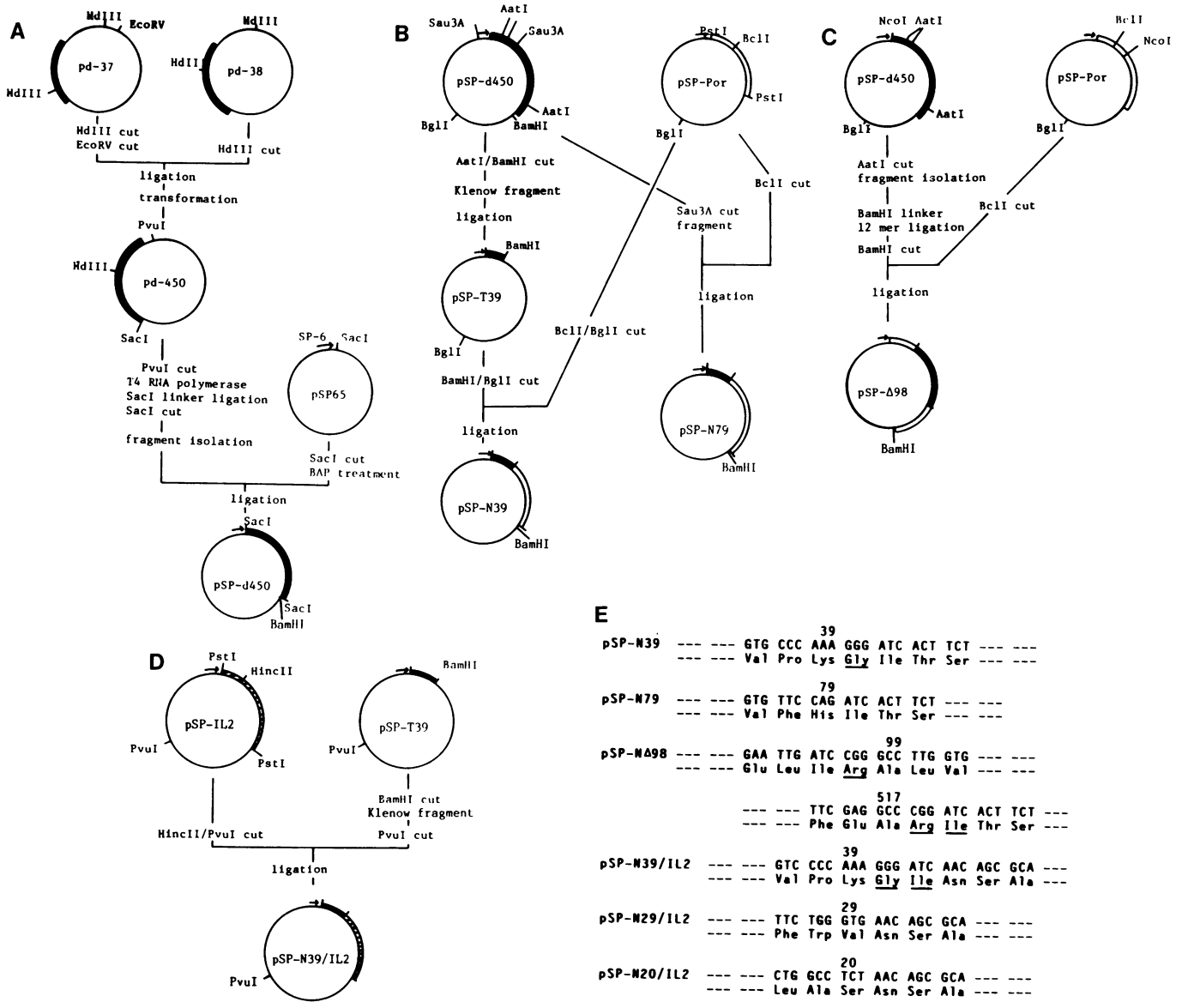

Fig. 8. Outlines of strategies adopted for hybrid plasmid constructions. (**A**) Construction of pSP-d450 from pd-37, pd-38 and pSP-65. (**B**) Construction of pSP-N39 and pSP-N79 from pSP-d450 and pSP-Por. In pSP-N39, Gly was introduced at the joining site due to the fill-in reaction. (**C**) Construction of pSP-Δ98 from pSP-d450 and pSP-Por. Due to the use of *Bam*HI linkers during the construction, Arg (at the amino-terminal joining site) and Arg-Ile (at the carboxy-terminal joining site) were newly introduced. (**D**) Construction of pSP-N39/IL2 from pSP-IL2 and pSP-T39. Due to DNA manipulation during the construction, Gly-Ile was introduced at the joining site. Solid, open and hatched bars represent DNA sequences for cytochrome P-450, yeast porin and pre-interleukin 2 respectively. Arrows on the plasmid diagrams indicate the location of SP-6 promoter and the direction of transcription by SP-6 RNA polymerase. (**E**) Nucleotide and deduced amino acid sequences for the joining regions in the six chimeric constructs. Residues introduced by DNA manipulations are underlined. Numbers are residue numbers in the primary structure of cytochrome P-450.

amino-terminal portion. However, it should be pointed out that the topogenic functions of these two types of sequence are clearly different from each other. In the case of type II membrane proteins, after the anchoring of the transmembrane segment to the ER membrane, translocation of the rest of the polypeptide chain is thought to proceed through its lopping into the lipid bilayer in such a way that the amino-terminal portion is left on the outside and the carboxy-terminal domain is brought into the luminal space. On the other hand, in the case of the chimeric proteins containing the amino-terminal 29 or more residues derived from the cytochrome, the anchoring of the amino-terminal signal into the membrane does not induce the translocation of the remaining polypeptide chain across the membrane. Such a topogenic function of the amino-terminal signal of cytochrome P-450 is a novel one and has not yet been reported.

In both type I and II plasma membrane proteins, basic residues are found at the junction between the hydrophobic transmembrane segment and the domain extruded to the cytoplasm (von Heijne, 1985b, 1986) and this structural feature has been thought to be required for the transmembrane segment to act as a stop-transfer sequence (Sabatini *et al.*, 1982). Zerial *et al.* (1987) have, however, demonstrated that this is not an absolute requirement by showing that the presence of acidic residues, rather than basic ones, at this junction of human transferrin receptor mutants allows the transmembrane segment to function as a membrane anchor. An examination of the amino-terminal 39-residue sequence of the cytochrome P-450 (Figure 7) indicates that three basic residues (Lys-34, Arg-36 and Lys-39) are located at the carboxy-terminal end of a relatively long stretch of uncharged residues. However, this cluster of basic residues has nothing to do with

the stop-transfer function, because the chimeric protein encoded by pSP-N29/IL2, which lacks this cluster, can be inserted into the membrane in the same way as the pSP-N39/IL2 protein containing this cluster. We, therefore, conclude that the short amino-terminal segment of the cytochrome P-450 can function as a stop-transfer sequence even though no charged residues are located at its junction with the cytoplasmic domain. No such transmembrane segments (stop-transfer sequences) have so far been reported in plasma membrane proteins.

As mentioned above, the observation that the pSP-Δ98 protein is neither inserted into nor translocated across the membrane indicates that no signal sequences exist in the cytochrome P-450 segment extending from residue 99 through the carboxy terminus. Although the presence of a signal sequence in the stretch spanning residues 40−98 has not been examined, a hydropathy profile of this region (not shown) indicates the absence of any segments sufficiently hydrophobic to be qualified as a signal sequence. It is, therefore, highly likely that the amino-terminal signal/stop-transfer segment is the only sequence contributing to the formation of intramembranous topology of the cytochrome P-450. If this is so, it should be concluded that only the amino-terminal segment (≤29 residues) is tightly inserted in the membrane and the rest of the molecule is extruded to the cytoplasmic side. This orientation is consistent with the susceptibility of the membrane-bound chimeric proteins to trypsin digestion and also with the finding reported by De Lemos-Chiarandini *et al.* (1987) for rat liver cytochrome P-450 isozyme PB-4 *in vivo*. Based solely on the primary structure, two models have been proposed for the intramembranous disposition of a rabbit liver microsomal cytochrome P-450 (Tarr *et al.*, 1983; Ozols *et al.*, 1985). In these models it is assumed that the cytochrome P-450 polypeptide spans the ER membrane 8 or 10 times. In the light of the above discussion, however, these models are very unlikely.

Materials and methods

Materials

The following materials were obtained from the sources indicated: restriction enzymes (New England Biolabs, Nippon Gene and Toyobo Biochemicals): M-13 cloning and sequencing kit, [^{35}S]methionine (1160 Ci/mmol) and [^{35}S]dATPαS (>600 Ci/mmol) (Amersham International); Klenow frament, T_4 DNA ligase and T_4 polynucleotide kinase (Takara Shuzo); SP-6 RNA polymerase (Boehringer-Mannheim); pSP-64, pSP-65 and RNasin (Promega Biotech); and bovine trypsin (Type III) (Sigma). Oligodeoxynucleotides, GTGTTCTGGGTG-AACAGCGCACCC and CTCCTGGCCTCTAACAGCGCACCC, synthesized by the phosphoramidite method, were supplied by Nikkaki Co. in deblocked form. pPor-1,117 (Mihara and Sato, 1985) and rabbit polyclonal antibodies to yeast porin were as described by Mihara *et al.* (1982). Isolation of pd-37 and pd-38 and preparation of guinea-pig polyclonal antibodies to rabbit P-448$_1$, a 3-methylcholanthrene-inducible form of rabbit liver cytochrome P-450 were performed as described by Kagawa *et al.* (1987). pMIL2-45, in which pre-interleukin 2 cDNA is inserted into the *Pst*I site of pBR322 (Kashima *et al.*, 1985), was supplied by Dr T.Taniguchi. Dog pancreas rough microsomes (RM), high salt-washed RM (KRM), SRP purified by sucrose density gradient centrifugation, and wheat-germ S-23 cell-free extract were prepared as described previously (Walter and Blobel, 1980; Walter *et al.*, 1981).

Plasmid constructions

The strategies adopted for plasmid constructions are outlined in Figure 8 (A for pSP-d450, B for pSP-N39 and pSP-N79, C for pSP-Δ98 and D for pSP-N39/IL2).

pSP-d450. For this construction, we used pd-37 and pd-38, in which the cDNAs are inserted into the *Pst*I site of pBR322. The inserts of pd-37 and pd-38 lack the 5′ and 3′ positions respectively of the coding region of a cDNA for a polycyclic hydrocarbon-inducible rabbit liver cytochrome P-450, although the entire coding region is covered by the two clones (Kagawa *et al.*, 1987). To construct a full-length cDNA encoding the cytochrome, pd-37 was digested with *Hin*dIII and *Eco*RI; pd-38 with *Hin*dIII alone. The two digests were mixed, ligated and used to transform *Escherichia coli* HB 101. The transformants were screened for clones carrying the full-length cytochrome P-450 cDNA, leading to the isolation of pd-450. pd-450 was digested with *Pvu*I, ligated with a *Sac*I linker, digested again with *Sac*I and the fragment containing the cDNA was isolated. This fragment was inserted into the *Sac*I site of pSP-65 and the construct was termed pSP-d450.

pSP-Por. A cDNA encoding the entire yeast porin sequence was excised with *Pst*I from pPor-1,117 (Mihara and Sato, 1985) and inserted into the *Pst*I site of pSP-64 to obtain pSP-Por.

pSP-N39. pSP-d450 was cut with *Aat*I and *Bam*HI and the *Bam*HI end of the larger fragment was filled-in with Klenow fragment. The filled-in fragment was circularized with T_4 ligase to produce a *Bam*HI site (pSP-T39) and then digested with *Bam*HI and *Bgl*I. pSP-Por, on the other hand, was cut with *Bcl*I and *Bgl*I. The two digests were mixed, ligated and used to transform *E. coli* HB 101. The transformants were screened for clones carrying the cDNA designed. One of the clones thus isolated was called pSP-N39.

pSP-N79. pSP-d450 was digested with *Sau*3A and the 550-bp fragment containing the sequence encoding the amino-terminal 79 residues of the cytochrome was isolated. This fragment was inserted into the *Bcl*I site of pSP-Por to obtain pSP-N79.

pSP-Δ98. pSP-d450 was cut with *Aat*I and the 1254-bp fragment encoding a cytochrome P-450 sequence spanning residues 99−517 was isolated. This fragment was then ligated with a *Bam*HI linker (12-mer) and inserted into the *Bcl*I site of pSP-Por to obtain pSP-Δ98.

pSP-IL2. This plasmid was constructed by excising the pre-interleukin 2 cDNA from pMIL2-45 (Kashima *et al.*, 1985) and inserting the excised cDNA into the *Pst*I site of pSP-65.

pSP-N39/IL2. pSP-IL2 was cut with *Hin*cII and *Pvu*I (Digest 1). pSP-T39 containing a cDNA encoding the amino-terminal 39 residues of the cytochrome (see above) was digested with *Bam*HI and the cohesive ends were filled-in. This was then digested with *Pvu*I (Digest 2). Digests 1 and 2 were mixed, ligated and used to transform *E. coli* HB 101. The transformants were screened by restriction enzyme mapping for clones carrying a cDNA that encodes a fusion protein consisting of the amino-terminal 39 residues of the cytochrome and mature interleukin 2 plus the last two residues of its signal sequence. One of the clones thus selected was termed pSP-N39/IL2.

pSP-N29/IL2 and pSP-N20/IL2. These two clones were constructed from pSP-N39/IL2 by oligodeoxynucleotide-directed, site-specific mutagenesis essentially as described by Zoller and Smith (1983). The 24-mer oligodeoxynucleotides used as primers were designed to cause deletion of DNA stretches coding for residues 30−39 (in pSP-N29/IL2) and residues 21−39 (in pSP-N20/IL2) of the cytochrome in the chimeric protein encoded by pSP-N39/IL2. The cDNA insert of pSP-N39/IL2 was excised with *Eco*RI and *Pst*I and ligated to the replicative form of phage M13 mp11 that had been cut with *Eco*RI and *Pst*I. The primer oligonucleotide, which had been 5′-phosphorylated with ATP and T_4 polynucleotide kinase, was annealed with mp11-N39/IL2. After incubation, primer extension was carried out with the aid of Klenow fragment and T_4 DNA ligase. The reaction mixture was then used to transform *E. coli* JM 103. Thirty-six plaques were randomly picked up and cultured further for phage preparation. The phage DNAs were screened by single track sequencing (Zoller and Smith, 1983) for clones carrying inserts having the desired deletions. The phages thus selected were isolated by single plaque isolation and the replicative form of their DNAs was prepared. The deleted cDNAs were excised from the phage DNA with *Eco*RI and *Pst*I and isolated by agarose gel electrophoresis and subsequent electro-elution. The cDNAs thus isolated were inserted between the *Eco*RI and *Pst*I sites of pSP-65. The clones having the shorter and longer deletions were termed pSP-N29/IL2 and pSP-N20/IL2 respectively.

All the plasmids constructed above were analyzed by restriction enzyme mapping and the nucleotide sequences of the joining regions were determined by the chain termination method using [^{35}S]dATPαS as a label to confirm that the correct reading frame was maintained. Figure 8E shows the nucleotide sequences and deduced amino acid sequences of the joining regions of all the hybrid cDNA clones constructed.

In vitro transcription and translation

The cDNAs in the above plasmids were transcribed *in vitro* by the action of SP-6 RNA polymerase essentially as described by Krieg and Melton (1984). DNA templates were prepared from 500 ml of culture by alkali-SDS extraction and subsequent CsCl gradient centrifugation (Maniatis *et al.*, 1982). The mRNAs synthesized were translated in wheat-germ S-23 extract in the presence of [^{35}S]-methionine as described by Sakaguchi *et al.* (1984). In experiments in which the capacity of translation products to be co-translationally inserted into the microsomal membrane was examined, RM or KRM plus purified SRP were included in the translation mixture as described (Sakaguchi *et al.*, 1984). The reaction was conducted at 26°C for 60 min and terminated by adding an equal volume of 20%

trichloroacetic acid. The precipitated protein products were collected by centrifugation and analyzed by SDS–PAGE followed by fluorography.

Assay for protein insertion into the membrane

The alkali extraction method of Fujiki *et al.* (1982) was used to assay the insertion of proteins into the microsomal membrane. To 10 μl of translation mixture incubated in the presence of RM or KRM plus SRP, 100 μl of ice-cold 50 mM Na_2CO_3 (pH 11.1) and 5 μl of KRM (50 A_{280} units/ml were added as a carrier. After incubation at 4°C for 20 min, the mixture was centrifuged in a Beckman Airfuge for 110 s at 30 p.s.i. to precipitate the membrane. The supernatant fraction was mixed with an equal volume of 20% trichloroacetic acid to precipitate unbound proteins. The membrane pellet was solubilized with loading buffer as described (Goldman and Blobel, 1978). These two fractions were analyzed by SDS–PAGE and subsequent fluorography.

Other methods

DNA manipulations were generally performed as described by Maniatis *et al.* (1982). The susceptibility to trypsin of the products of translation in the presence of membranes was examined essentially as described by Yost *et al.* (1983). After the translation in the presence of membranes, 1 μl of trypsin (2 mg/ml) and 2 μl of 100 mM $CaCl_2$ were added to 10 μl of the translation mixture and the mixture was incubated at 23°C for 30 min. The digestion was stopped by adding 200 μl of 10% trichloroacetic acid and the precipitate formed was analyzed by SDS–PAGE followed by fluorography. Immunoprecipitation and SDS–PAGE were carried out as described by Sakaguchi *et al.* (1984). Nucleotide sequencing was conducted by the chain termination method (Messing *et al.*, 1981) using M13 cloning and sequencing kit; [^{35}S]dATPαS was used as a radioactive label.

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Note added in proof

After submission of this paper, we became aware of an abstract [Monier,S., Van Luc,P., Kreibich,G., Sabatini,D.D. and Adesnik,M. (1986) *J. Cell Biol.*, **103**, 290A] in which evidence is briefly presented that the amino-terminal segment of rat liver microsomal cytochrome P-450b displays both insertion and halt-transfer functions.