INSERTION OF CYTOCHROME P-450 INTO MICROSOMAL MEMBRANES

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Molecular Mechanism for Insertion of Newly Synthesized Cytochrome P-450 into Microsomal Membranes.

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PART I

Signal Recognition Particle Is Required for Co-translational Insertion of Cytochrome P-450 into Microsomal Membranes*

* A large portion of this study has been published (39).

SUMMARY

Insertion of newly synthesized P-450_1, the major phenobarbital-inducible form of rabbit liver microsomal cytochrome P-450, into microsomal membranes was studied in a wheat germ cell free translation system programmed with total RNA from the liver of a phenobarbital-treated rabbit. P-450_1 synthesized *in vitro* had the same molecular weight as the mature molecule and was co-translationally inserted into dog pancreas rough microsomal membranes. In the presence of salt-washed microsomes, instead of unwashed ones, the insertion was greatly diminished. It could, however, be restored by supplementation of the system with purified signal recognition particle (SRP), a known component of the membrane translocation machinery for secretory proteins. In the absence of microsomes, SRP inhibited the translation of mRNA encoding P-450_1 and this translation arrest was released by the addition of salt-washed microsomes. This translation arrest occured only when SRP was added within 30 sec after the initiation. On the other hand, SRP did not affect the translation of mRNAs encoding yeast porin and reticulocyte globin, which are mitochondrial and cytosolic proteins, respectively. We conclude that co-translational insertion of P-450_1 into microsomal membranes requires SRP at an early stage of the translation and postulate that P-450_1 possesses an uncleavable signal sequence that can be recognized by SRP.

INTRODUCTION

Secretory proteins are synthesized by membrane-bound polysomes and translocated to the cisternal space of the endoplasmic reticurum (1). This translocation depends on the presence in nascent secretoy proteins of a transient signal sequence that is rich in hydrophobic amino acid residues and located usually at the NH_2-terminal end (2). Walter and Blobel (3,4) have purified a ribonucleoprotein complex called signal recognition particle (SRP) from dog pancreas rough microsomes (RM) and shown that it is required for co-translational translocation of secretory proteins into salt-washed RM [RM washed with 0.5 M potassium acetate/5 mM magnesium acetate (KRM)]. Further studies have shown that SRP mediates the binding of polysomes synthesizing secretory proteins to microsomal membanes (5,6) and that in the absence of microsomes, but not in their presence, it specifically inhibits the translation of mRNAs encoding secretory proteins (7).

Recent studies have indicated that SRP is also required for co-translational insertion of the δ-subunit of Torpedo acetylcholin receptor, a plasma membrane protein, into the microsomes (8) and translocation of cathepsin D, a lysosomal protease, across microsomal membranes (9). Therefore, the function of SRP is not confined to membrane translocation of secretory proteins. Since membrane-bound polysomes synthesize not only secretory, lysosomal, and plasma membrane proteins but also several integral proteins that remain bound to microsomal membranes (10), it was

of interest to see if SRP is also required for the insertion of such microsomal proteins into membranes.

Multiple forms of cytochrome P-450 exist in liver microsomes (11) and they are synthesized by membrane-bound polysomes (12,13). Bar-Nun et al. (13) have reported that a form of cytochrome P-450 synthesized in a cell-free translation system can by inserted into liver microsomal membranes in a way hardly distinguishable from that synthesized in vivo and that the cytochrome synthesized in vitro has the same NH_2-terminal sequence as the mature molecule.

It is reported here that SRP is actually required for co-translational insertion of $P\text{-}450_1$, the major phenobarbital-inducible form of rabbit liver microsomal cytochrome P-450 (14), into heterologous (dog pancreas) microsomal membranes. It is also reported that in the absence of microsomes SRP specifically inhibits the translation of mRNA encoding $P\text{-}450_1$.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (1110 Ci/mmol; 1 Ci=37 GBq) was purchased from Amersham. RM and KRM were prepared as described (3). SRP was isolated from RM as described Walter and Blobel (3) and was further purified by sucrose gradient centrifugation (5,15). One unit of SRP was defined as described (3). The guanidine thiocyanate/CsCl method (16) was used to prepare total RNA from bovine anterior pituitary gland and the liver of a rabbit that had been injected twice (36 and 12 h before sacrifice) with

phenobarbital (200 mg/kg of body weight). Total RNA from yeast cells (17) and antibodies to yeast mitochondrial porin (18) were prepared as described. Total RNA from rabbit reticulocytes was obtained by the proteinase K/phenol/chloroform method (19). A nuclease treated wheat germ S-23 extract was prepared as described (3).

Preparation of Anti-$P450_1$ Antibodies.

About 1 mg of $P-450_1$, purified by the method of Imai et al (14), was subjected to SDS-polyacrylamide slab gel electrophoresis in 20 gel slots (1 mm thick, 12.5 % cross-linked). After staining the gel with Coomassie brilliant blue, the bands corresponding to $P-450_1$ were cut out of the gel and injected into a guinea pig as described (17). Production of antibodies and their specificity were checked by an immunoblotting method (20). Anti-$P-450_1$ antibodies were enriched by chromatography on Sepharose 4B covalently coupled to SDS-denatured $P-450_1$ essentially as described (18).

Immunoblotting analysis

Transfer of protein bands from a gel slab to a nitrocellulose paper was performed electrophoretically as described by Howe and Hershey (20), except that the transfer buffer containd 348 mM glycine, 50 mM Tris base, and 20 % (v/v) methanol. The transfer was carried out at 220 mA (8-10 V) for 8 h. The step of Amido Schwartz staining was omitted. Antibody-antigen reaction on the nitrocellulose paper was performed by the method of Fisher et al.

(21); 1-2 μg of anti-$P450_1$ immunoglobulin G was used for each slot (1.5 x 20 cm). After incubation at room temperature for 8 h, ^{125}I-labeled protein A (ca. 1 x 10^6 cpm/slot) was added and the position of antigen-antibodies complex was localized by autoradiography.

In Vitro Protein Synthesis And Immunoprecipitation

Total RNA from the liver of a phenobarbital-treated rabbit (final concentration, 5 A_{260} units/ml) was translated at 26 °C for 60 min in a wheat-germ (S-23) extract (total volume, 50 μl) containing [^{35}S]methionine (800 μCi/ml), as described (5), except that the salt concentrations were varied to compensate the "carry-over" of the salts from the SRP preparation. The final concentrations of potassium acetate and magnesium acetate were always kept to 110 mM and 1.1 mM, respectively. After translation, $P-450_1$ synthesized was immunoprecipitated with anti-$P-450_1$ antibodies in the presence of 0.4 % (wt/vol) SDS and 2% Triton X-100 as described (22), except that protein A-Sepharose (Pharmacia) was used as immunoadsorbent instead of formaldehyde fixed Staphylococcus aureus cells. The immunoprecipitate was analyzed by SDS-polyacrylamide slab gel (12.5 %) electrophoresis (2) followed by fluorography (23). Total RNAs from yeast cells and rabbit reticulocytes were similarly translated in the in vitro system. Yeast porin synthesized was immunoprecipitated with anti-yeast porin antibodies and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. The total translates obtained in the presence of total reticulocyte RNA or

total anterior pituitary gland RNA were directly applied to SDS-polyacrylamide gel electrophoresis without immunoprecipitation.

Assay for $P\text{-}450_1$ Insertion into Microsomal Membranes

Cell free translation of the rabbit liver RNA was performed as above in the presence of RM or KRM. After translation, 15 µl of 3M NaCl and 7 µl of 0.2 M EDTA were added to the translation system to minimize nonspecific adsorption of $P\text{-}450_1$ to the membranes. After incubation at 4°C for 15 min, the mixture (72 µl) was layered over a discontinuous gradient consisting of 390 µl of 2.5 M sucrose and 200 µl of 0.5 M sucrose, both containing 0.5 M NaCl and 50 mM triethanolamine acetate (pH 7.5) in a 800 µl nitrocellulose tube. The tube was centrifuged at 50,000 rpm (227,800 x g_{av}) for 8 min in a Hitachi RPS-50 II rotor equipped with a Teflon adapter. After centrifugation, 250 µl of the solution was carefully removed from the top of the tube and used as the supernatant fraction (containing unbound P-450). To the 0.5 M/2.5M interface where microsomes were located was added 50 µl of 2.5 % (wt/vol) SDS to solubilize the membranes. After complete solubilization, 100 µl of the solution was withdrawn and used as the membrane fraction. These two fractions were made 2.5 % (wt/vol) with respect to SDS, boiled for 5 min, diluted, and subjected to immunoprecipitation with anti-$P450_1$ antibodies as described above. The immunoprecipitates were analyzed by SDS-polyacrylamide slab gel electrophoresis and subsequent fluorography. The extent of $P\text{-}450_1$ binding to the membranes was obtained by determining the radioactivities of $P\text{-}450_1$ bands from

the supernatant and membrane fractions by using a toluene/diphenyloxazole scintillation cocktail.

RESULTS

Characterization of Anti-P-450$_1$ Antibodies

Purified P-450$_1$ and total liver microsomes from a phenobarbital-treated rabbit were subjected to SDS-polyacrylamide slab gel electrophoresis. Coomassie blue-stained gels thus obtained are shown in lanes 1 and 3, respectively, of Fig. 1. As can be seen, the P-450$_1$ preparation contained several minor contaminant bands. These gels were then subjected to immunoblottig analysis by using the P-450$_1$ antibodies prepared in this study. As shown in Fig. 1 (lanes 4 and 6), the antibodies reacted strongly with the P-450$_1$ band but did not react with any other microsomal proteins, indicating that the antibodies were monospecific to P-450$_1$.

In vitro synthesis of cytochrome P-450$_1$

Total RNA from the liver of a phenobarbital-treated rabbit was translated in the wheat germ cell-free system containing [^{35}S]methionine and P-450$_1$ synthesized was immunoprecipitated. Upon SDS-polyacrylamide gel electrophoresis and subsequent fluorography, the immunoprecipitate gave a single radioactive band (Fig. 2, lane 2), which had the same apparent molecular weight (Mr 48,000) as purified P-450$_1$ (Fig. 2, lane 1). This indicated that P-450$_1$ is synthesized in vitro in the mature size and is consistent with the observation that the cytochrome synthesized

in vitro had the same NH_2-terminal sequence as the mature molecule (13). No radioactive band was detectable when 5 μg of purified, unlabeled P-450_1 was added before immunoprecipitation (Fig. 2, lane 3), preimmune control serum was used for immunoprecipitation (Fig. 2, lane 4), and the rabbit liver RNA was omitted from the translation system (Fig. 2, lane 5). These observation confirmed that the radioactive band precipitated by the antibodies was actually P-450_1 synthesized *in vitro*.

Characterization of Purified SRP

Before studying the effect of SRP on the insertion of P-450_1 into microsomal membranes, the properties of the SRP preparation purified in this study were checked. Upon SDS-polyacrylamide slab gel electrophoresis, the SRP prepared in this study gave 6 polypeptide bands and no other bands were observed (Fig. 3A). The molecular weights of these polypeptides were estimated to be 72,000, 68,000, 54,000, 19,000, 14,000 and 9,000 in excellent agreement with those reported by Walter and Blobel (3) for their SRP preparation. However, the Mr 72,000 band in our preparation was somewhat less intense than that in theirs for unknown reasons. Our SRP preparation could restore the pre-prolactin processing activity of salt-extracted dog pancreas rough microsomes (KRM). As shown in Fig. 3B, in the presence of dog pancreas rough microsomes (RM) 58% of preprolactin synthesized in the wheat germ cell free system programmed with total anterior pituitary gland RNA was processed to prolactin (Fig. 3B, lane 1). This processing activity was very low in the presence of KRM,

instead of RM (Fig. 3B, lane 2). However, the addition of 5 units of SRP back to the system could clearly restore the processing activity (Fig. 3B, lane 3). Another important feature of SRP is its activity to inhibit the translation of mRNA's coding for secretory proteins in the absent of microsomal membranes (7). As shown in Fig. 3B (lanes 4 through 7), translation of pre-prolactin mRNA was strongly inhibited by SRP in the absence of microsomes. In the presence of 20 units of SRP the inhibition was high as 79 % (Fig. 3B, compare lane 7 with lane 4). This inhibition was accompanied by the appearance of an arrested peptide segment having an apparent molecular weight of 12,700. This arrested product has been reported to have a molecular weight of 8,000 when examined in a 22.5 % cross-linked poly-acrylamide system containing 6 M urea (7). The apparent discrepancy in the molecular size of the arrested product seemed to be due to the difference in the gel systems used in the two studies. In any case, the translation arrest was released by the addition of KRM (Fig. 3B, lane 3), which also restored the processing of pre-prolactin. The presence of a 7 S RNA in our SRP preparation could also be confirmed (data not shown) as reported by Walter and Blobel (4). These observations led to the conclusion that our SRP preparation was reasonably pure and functionally active.

SRP Requirment for Insertion of P-450$_1$ into Microsomal Membranes

In the next series of experiments, the rabbit liver RNA was translated in the cell-free system in the presence of RM or

KRM, and then membrane-bound and uninserted P-450_1s were separated from each other by discontinuous density gradient centrifugation. In the absence of membranes in the translation system, most P-450_1 synthesized was recovered in the supernatant fraction (Fig. 4, lane 1). Radioactivity measurement, however, indicated that about 10 % of the P-450_1 was present in the "membrane-bound" fraction even though no membranes were added in this experiment. This apparent sedimentation of unbound P-450_1 was thought to be due to incomplete withdrawal of the supernatant fraction. However, we adhered to the separation procedure described under Materials and Methods to minimize contamination of the supernatant fraction by membranes. In any case, when RM were added to the system, a substantial portion of P-450_1 synthesized (55%) bound to the membranes (Fig. 4, lane 2). The reason for the incomplete insertion of P-450_1 is unclear, but a likely possibility is that mRNAs coding for serum albumin and other secretory proteins (contained in the RNA used) competed with P-450_1 mRNA for SRP. In the presence of KRM, on the other hand, virtually no insretion of P-450_1 into the membranes was observed (Fig. 4, lane 3). This insertion in this system, however, dramatically increased to 87 % when purified SRP (20 units/50 µl) was included in the translation mixture (Fig. 4, lane 4).

As shown in Fig. 5, the extent of P-450_1 binding to KRM was roughly dependent on the amount of SRP added. It is to be noted that even in the absence of added SRP a significant portion of P-450_1 was recovered in the membrane-bound fraction, because of the apparent sedimentation of unbound P-450_1 (see above) and of a

small amount of SRP remaining in KRM. When SRP (20 units/50μl) was added to the system after completion of translation, practically no enhancement of P-450_1 binding occurred (Fig. 5, open square). This indicated that SRP assists the insertion of P-450_1 only co-translationally.

Inhibition of P-450_1 Translation by SRP

The finding that co-translational insertion of P-450_1 into microsomal membranes is mediated by SRP suggests that, like secretory proteins, P-450_1 also possesses a signal-equivalent sequence which can be recognized by SRP. This possibility was explored by taking advantage of the fact that SRP causes a signal-induced arrest of translation of mRNAs encoding secretory proteins in the absence of microsomal membranes (7). As expected, SRP was found to inhibit strongly (78 % inhibition) the translation of P-450_1 mRNA (Fig. 6A, lanes 1 and 2). Moreover, as in the case of secretory protein mRNAs (7,24,25), this inhibition was largely reversed when KRM were included in the translation mixture (Fig. 6A, lane 3). However, no arrested peptide fragment of P-450_1 was detectable in the absence of KRM (Fig. 6A, lane 2), contrary to the observations reported for the translation of mRNAs encoding secretory proteins (7,25). The arrested fragment could not be detected even when the translation was synchronized by adding 2 mM 7-methylguanosine monophosphate 2 min after the start of translation. The arrest of translation of both preprolactin mRNA (Fig. 7A) and P-450_1 mRNA (Fig. 7B) in the synchronized system took place only when SRP was added within about

20 sec after the initiation of translation. SRP added 60 sec after the initiation could no longer inhibit the translation to significant extents (Fig. 7A, lane 4; Fig. 7B, lane 4). In this particular experiment no arrested peptide could be detected in the SRP-induced arrest of preprolactin synthesis for unknown reasons. In any case, these observations indicate that the translation arrest occurs only when SRP binds to a signal sequence at an early stage of translation and suggest that the putative signal sequence of P-450_1, like that of pre-prolactin, is located near the NH_2-terminal end. On the other hand, this inhibition in the absense of microsomal membranes was not observed in the translation of mRNAs encoding yeast porin (Fig. 6B) and rabbit reticulocyte globin (Fig. 6C), proteins that are destined for the outer mitochondrial membrane (18) and cytosol, respectively.

DISCUSSION

SRP has so far been shown to be involved in membrane translocation of secretory proteins (3,24-26), insertion of the δ-subunit of Torpedo acetylcholine receptor, a plasma membrane protein, into microsomal membranes (8), and translocation of the lysosomal protease cathepsin D across microsomal membranes (9). The results described above indicate that co-translational insertion of P-450_1 (and most likely other forms of liver microsomal cytochrome P-450) into microsomes is also mediated by SRP. Therefore, it seems that all proteins synthesized by membrane-bound polysomes interact (translocation or insertion) co-trans-

lationally with the aid of SRP. However, it should be noted that P-450_1 remains tightly bound to the endoplasmic reticulum membranes and does not exist even in Golgi membranes (27,28). Therefore it differs from secretory proteins that are released into the lumen of endoplasmic reticulum (1), from the acetylcholine receptor subunit that is destined for the plasma membrane (29), and from cathepsin D that is to be finally transferred to lysosomes. Another difference is that P-450_1, like such microsomal membrane proteins as NADPH-cytochrome P-450 reductase and epoxide hydrolase (30), is synthesized in vitro in the mature size, whereas secretory proteins and the receptor subunit are synthesized in larger size carrying transient (cleavable) signal sequence (2,8). Recently, 3-hydroxy-3-methylglutaryl-coenzyme A reductase of the endoplasmic reticulum and Ca^{2+}-ATPase of the sarcoplasmic reticulum have been shown to be inserted into RM in a SRP-mediated fasion (31,32). However, little is as yet known of the mechanism by which these four types of proteins are sorted for their respective destinations.

In the absence of microsomes, SRP inhibits the translation of P-450_1 mRNA and this inhibition is reversed by the addition of KRM, as has been observed for the translation of mRNAs encoding secretory proteins (7,24-26). These findings suggest that P-450_1, like nascent secretory proteins, possesses a signal sequence that can be recognized by SRP. Furthermore, the finding that SRP inhibits the translation of P-450_1 only when added at an early stage of translation suggests that the signal sequence of the cytochrome is located near the NH_2-terminus, as in the case

of secretory proteins. Since in all of the forms of liver microsomal cytochrome P-450 so far studied, the NH_2-terminal portion is quite rich in hydrophobic amino acid residues (33-36), it is reasonable to assume that this portion constitutes the signal sequence. It is to be noted that the signal sequence of $P\text{-}450_1$ is uncleavable and remains in the mature molecule.

An unexpected finding was our failure to detect any "arrested peptide fragment" during the SRP-induced arrest of $P\text{-}450_1$ translation. Since the NH_2-terminal residue of $P\text{-}450_1$ is methionine (33-36), it is expected that the arrested fragment if formed, should be radioactive. Therefore, a likely explanation for the above failure is to assume that the anti-$P\text{-}450_1$ antibodies used in this study were unable to recognize the arrested peptide. However, no arrested peptide fragment has been detected even in the total translation products of mRNAs encoding two membrane proteins, i.e. coronavirus E1 protein and bovine opsin (37,38).

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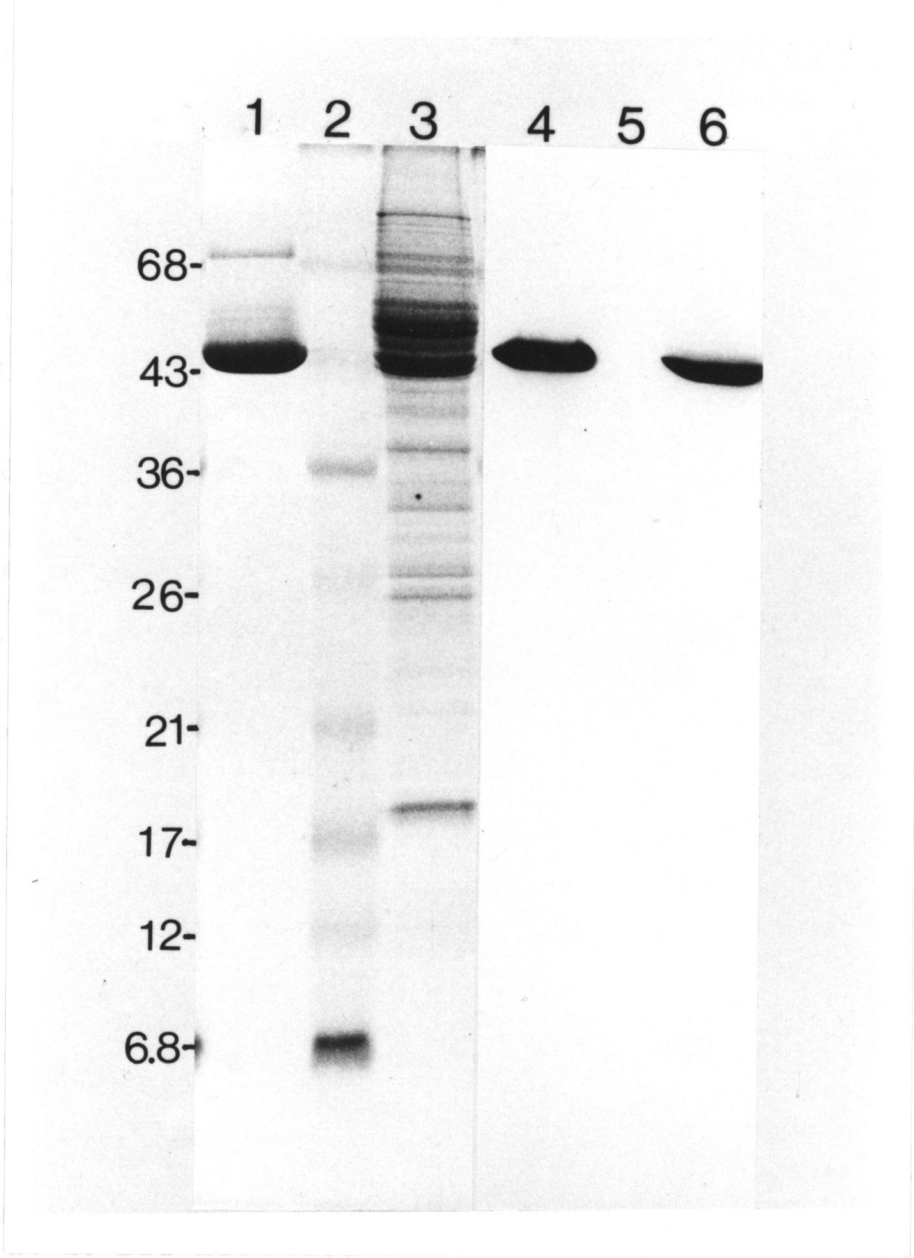

Fig. 1 - Immunoblotting characterization of immuno-selected anti-P-450$_1$ antibodies. Purified P-450$_1$ (15 µg, lane 1), molecular weight marker proteins (lane 2; rabbit phosphorylase a, bovine serum albumin, chicken ovalbumin, rabbit lactate dehydrogenase, bovine α-chymotrypsinogen, soybean trypsin inhibitor, sperm whale myoglobin, horse cytochrome c and aprotinin) and total protein of phenobarbital-induced rabbit liver microsomes (50 µg, lane 3) were subjected to SDS-polyacrylamide gel electrophoresis (15 % gel) and stained with Coomassie brilliant blue. P-450$_1$ was overloaded to visualize minor contaminants. The electrophoretograms corresponding to lanes 1-3 were blotted to nitrocellulose paper. The blotted paper was incubated with anti-P-450$_1$ antibodies and detected by means of ^{125}I-labeled protein A as described under Materials and Methods in methods. Lanes 4-6 correspond to lanes 1-3 respectively. Numbers on the left of the lane 1 indicated the Mr x 10^{-3} of marker proteins.

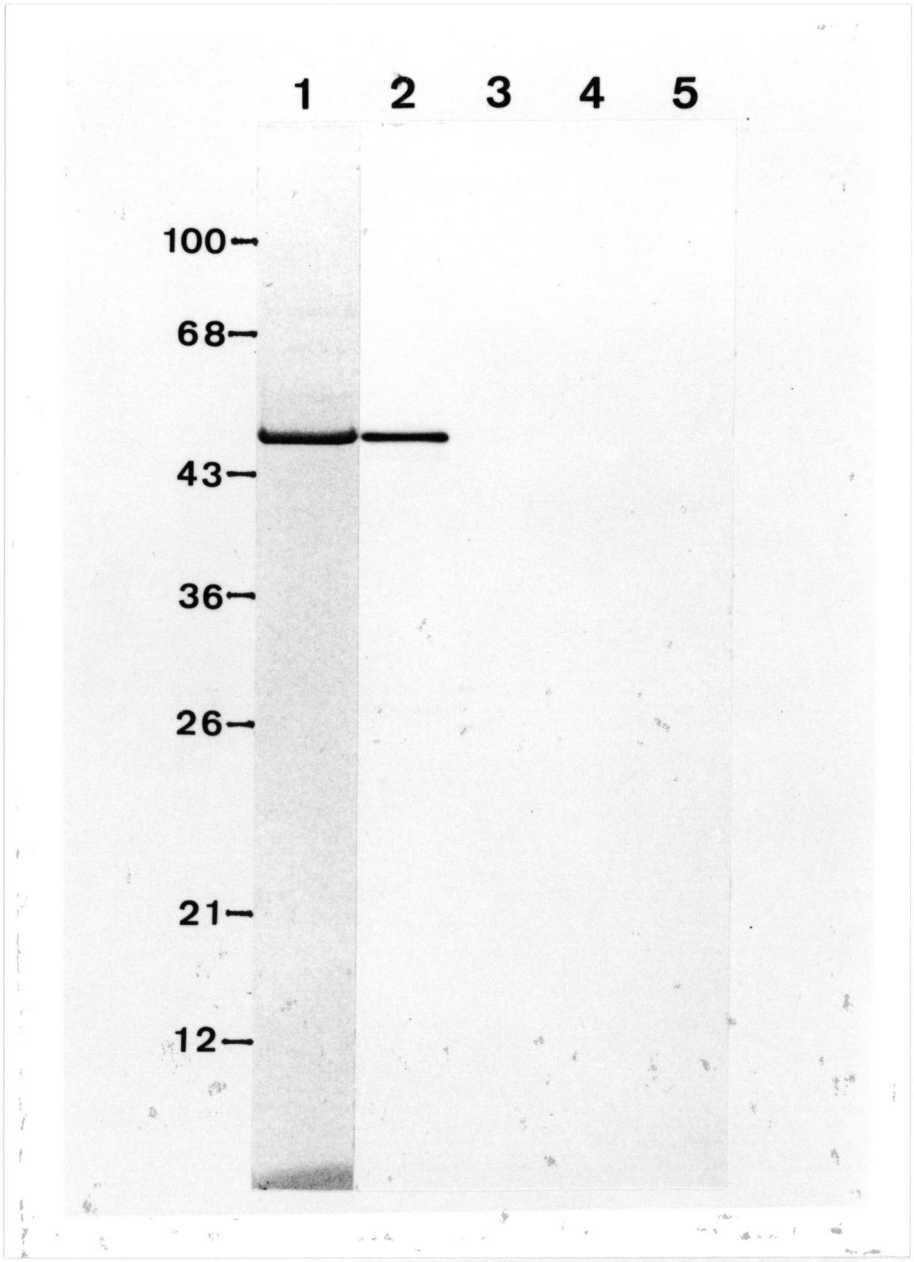

Fig. 2. - Synthesis of P-450$_1$ *in vitro*. Protein synthesis was allowed to proceed in the wheat germ cell-free extract (50 µl) containing [^{35}S]methionine in the presence (lanes 2-4) and absence (lane 5) of total RNA from the liver of a phenobarbital-treated rabbit, and a 25 µl aliquot of the translation mixture (1 x 10^5 cpm) was subjected to immunoprecipitation with anti-P-450$_1$ antibodies (lanes 2,3 and 5) or with preimmune control serum (lane 4). In the experiment shown in lane 3, 5 µg of purified, unlabeled P-450$_1$ was added before immunoprecipitation. The immunoprecipitates were subjected to SDS-polyacrylamide slab gel electrophoresis and detected by fluorography. In the experiment shown in lane 1, purified P-450$_1$ (5 µg) was subjected to gel electrophoresis under the same conditions as above and stained with Coomassie brilliant blue. Molecular weight marker proteins (shown as Mr x 10^{-3} from top to bottom; rabbit phosphorylase a, bovine serum albumin, rabbit lactate dehydrogenase, bovine α-chymotrypsinogen, soybean trypsin inhibitor, and horse cytochrome *c*) were radiolabeled with [^{14}C]formaldehyde (21) and subjected to SDS-polyacrylamide slab gel electrophoresis. The location of these marker proteins are shown on the left-hand side of lane 1.

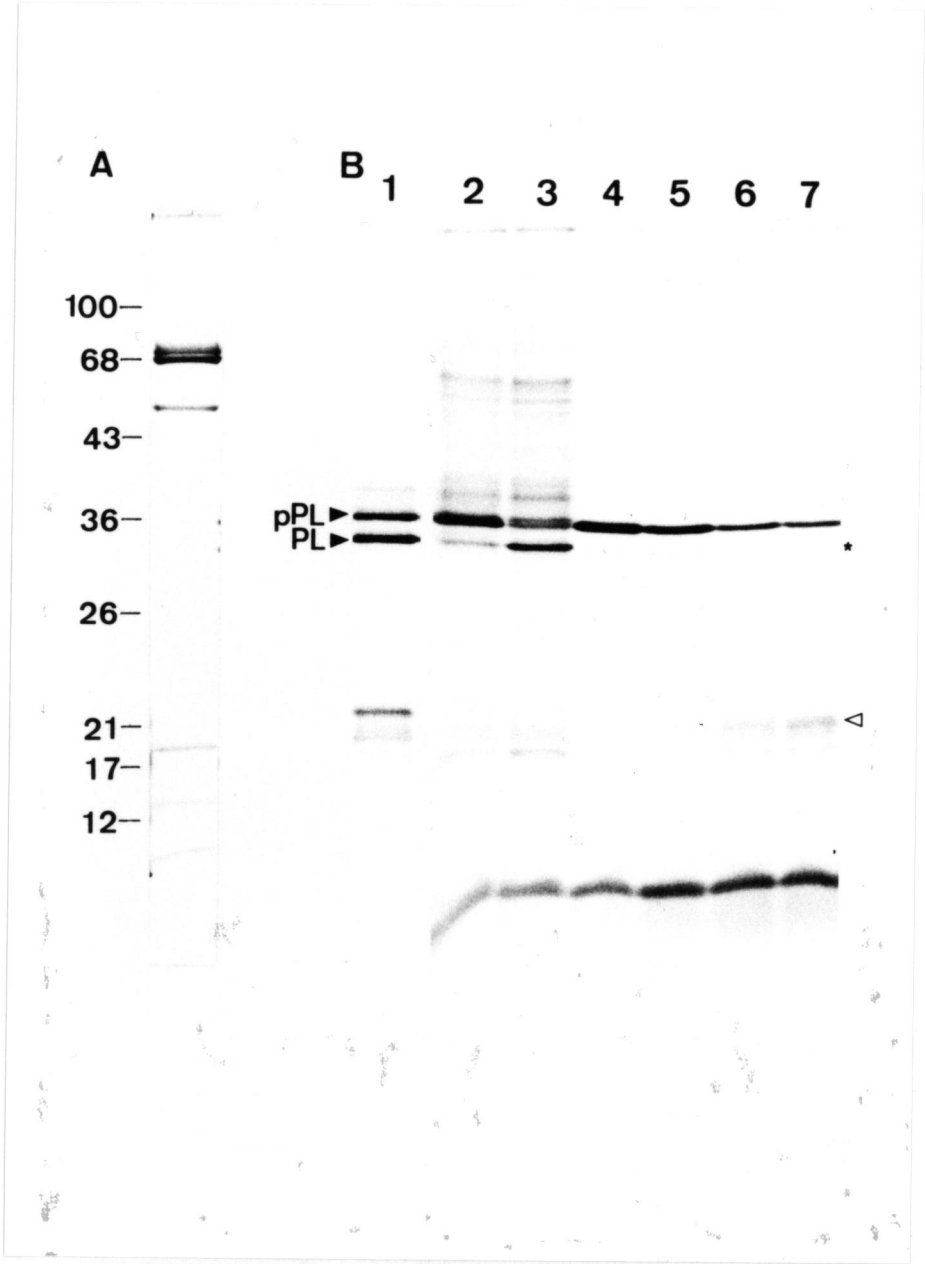

Fig. 3. - Characterization of purified SRP. (A) SDS-polyacrylamide gel electrophoresis analysis of SRP. Purified SRP (12 µg) was subjected to SDS-polyacrylamide slab gel electrophoresis (12.5 % gel) and the gel was stained with Coomassie brilliant blue. (B) Restoration of preprolactin processing activity of KRM and inhibition of preprolactin translation by SRP. Total RNA from bovine anterior pituitary gland (4 A_{260} units/ml) was translated in a wheat germ cell free system (total volume, 25 µl) at 26 °C for 60 min. Translation was synchronized by adding 7-methylguanosine monophosphate (final concentration, 1.5 mM) 3 min after the initiation of incubation (lanes, 4-7). Translation products were analyzed by SDS-polyacrylamide gel slab electrophoresis (15 % gel) and subsequent fluorography. Lane 1, translate obtained in the presence of RM (2 A_{280}/ml); lane 2, translate obtained in the presence of KRM (1 A_{280}/ml); lane 3, same as lane 2 except that 5 units of SRP was added to the translation system; lane 4 through 7, translate obtained in the absence of membranes but in the presence of 0, 5, 10, and 20 units of SRP, respectively. Arrested product is indicated by a white triangle. Numbers on the left indicate the Mr x 10^{-3} of marker proteins.

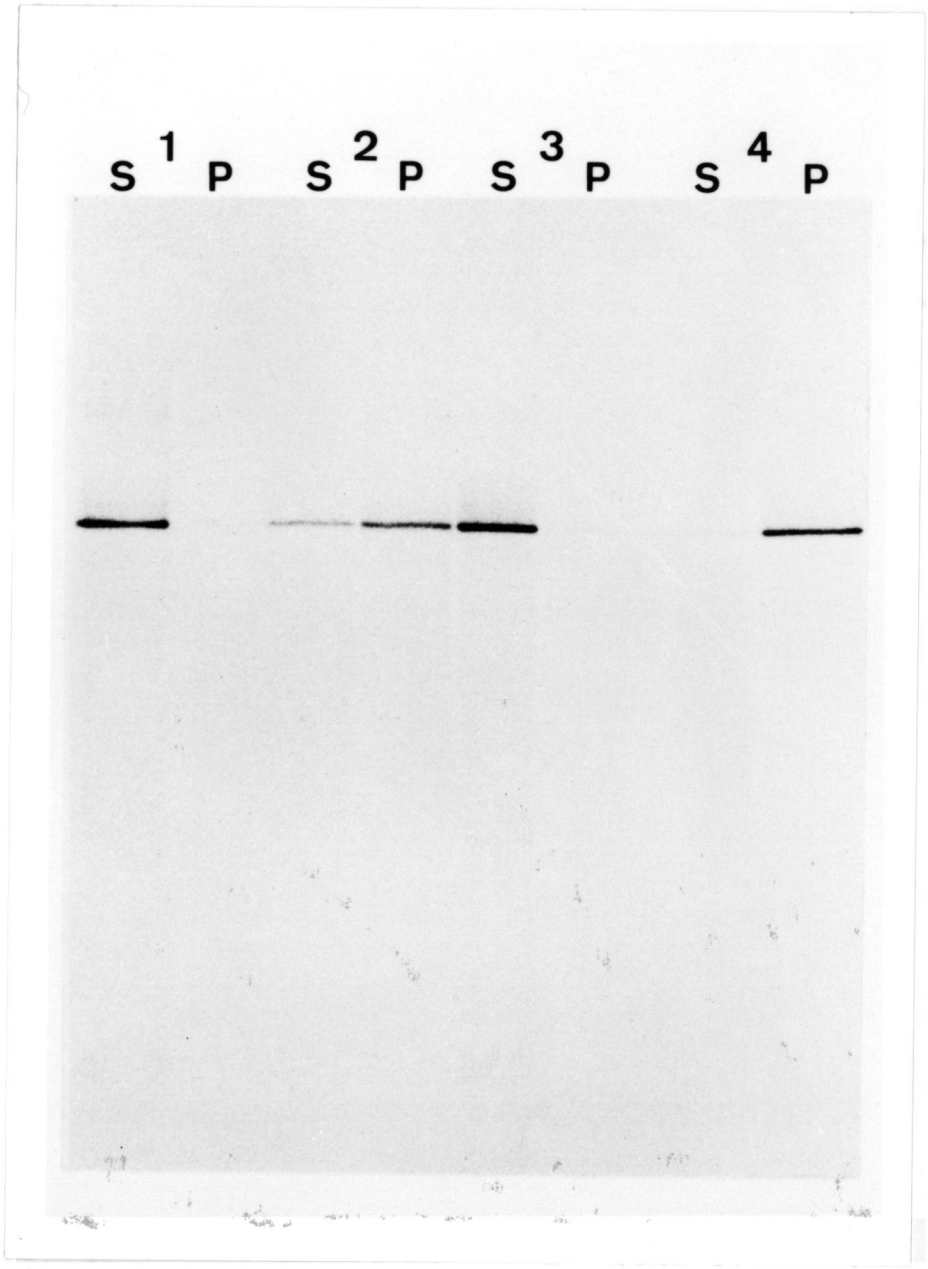

Fig. 4. - SRP requirement for insertion of P-450_1 into KRM. Cell-free translation of the rabbit liver RNA was carried out as described in the legend to Fig. 1 in the presence or absence of either RM (5 A_{280} units/ml) or KRM (5 A_{280} units/ml). After translation, the mixture was separated into the supernatant and membrane fractions and P-450_1 in both fractions was immunoprecipitated with anti-P-450_1 antibodies. The immunoprecipitats were subjected to SDS-polyacrylamide slab gel electrophoresis and detected by fluorography. Lane 1, translate obtained in the absence of microsomes; lane 2, translate obtained in the presence of RM; lane 3, translate obtained in the presence of KRM; lane 4, translate obtained in the presence of both KRM and SRP (20 units/50 µl). S and P stand for the supernatant and membrane fractions, respectively.

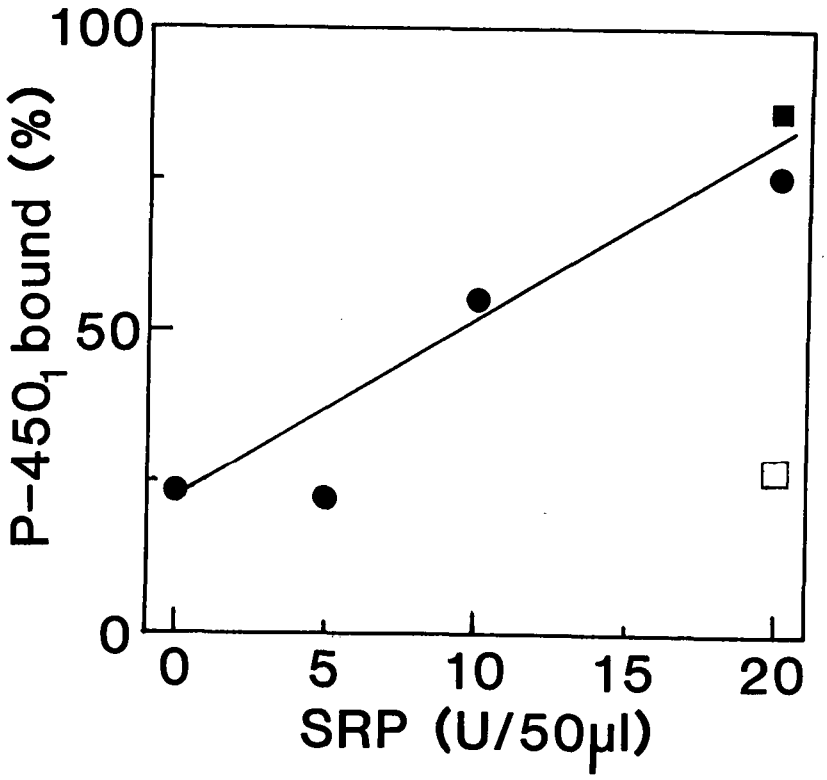

Fig. 5. - Effect of SRP concentration on P-450$_1$ binding to KRM. Co-translational binding of P-450$_1$ to KRM was determined as described in the legend to Fig. 4, except that the amount of SRP added to the translation system was varied as indicated. The percentage of P-450$_1$ bound to KRM was calculated from the radioactivities associated with P-450$_1$ bands excised from the slab gels. The result obtained are indicated with closed circles (●). The closed square (■) shows the result obtained in Fig. 4 (lane 4). In one experiment, translation was performed in the absence of SRP (but in the presence of KRM) for 60 min and then it was inhibited by adding 2 mM cycloheximide. Desalted SRP (20 units/50 µl) was then added to the system and the mixture was further incubated for 60 min. The open square (□) indicates the level of P-450$_1$ bound in this experiment.

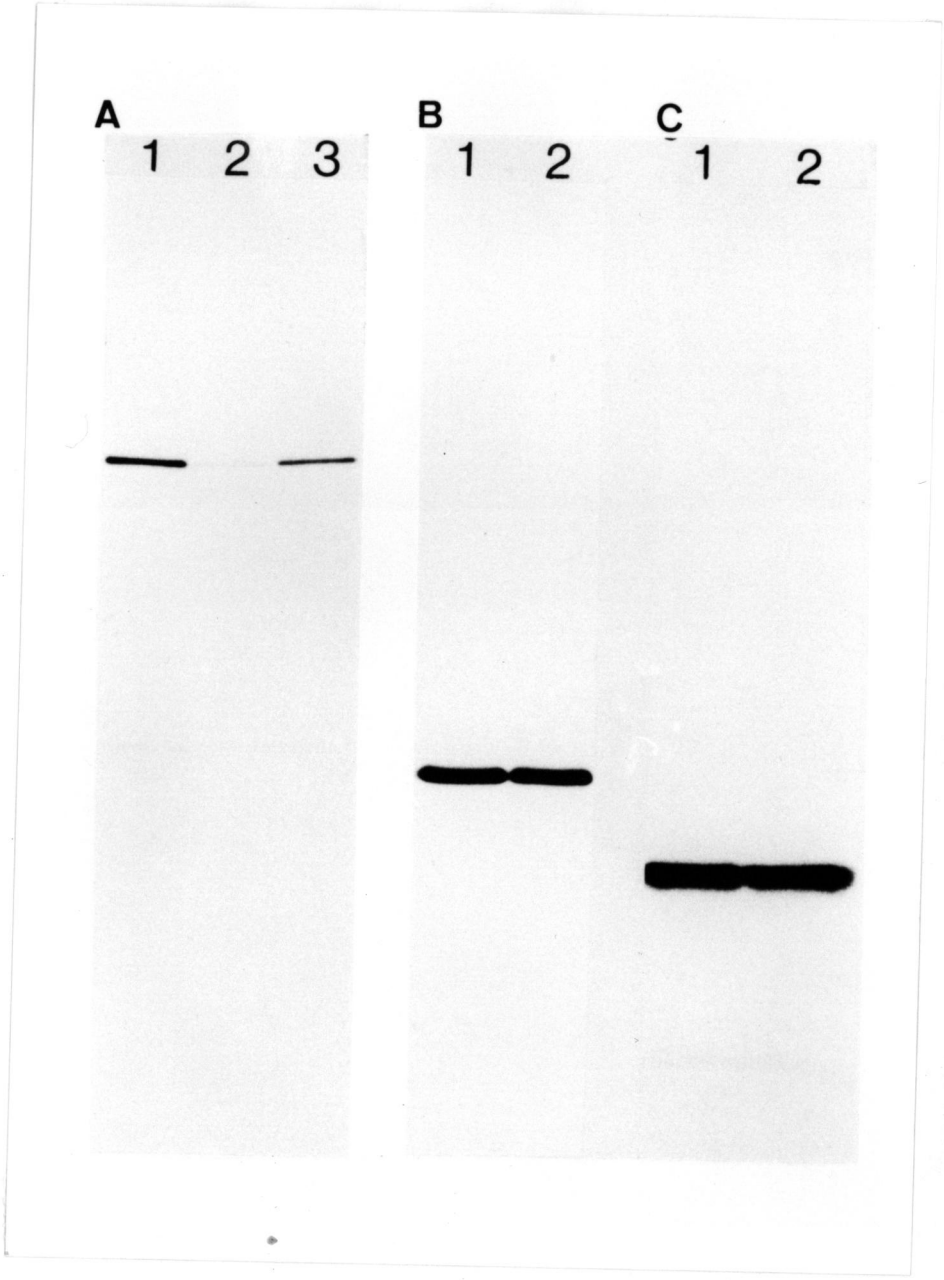

Fig. 6. - Effect of SRP on the translation of mRNAs coding for P-450$_1$, yeast mitochondrial porin, and rabbit reticulocyte globin. (A) The rabbit liver RNA was translated, P-450$_1$ synthesized was immunoprecipitated, and the immunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described in the legend to Fig. 2. Lane 1, translate obtained in the absence of both SRP and KRM; lane 2, translate obtained in the presence of SRP (25 units/50 µl); lane 3, translate obtained in the presence of both SRP (25 units/50 µl) and KRM (5 A_{280} unit/ml). (B) Total RNA from yeast cells (12 A_{260} units/ml) was translated in the wheat germ cell-free system (25 µl). Yeast porin synthesized was immunoprecipitated with anti-yeast porin antibodies and analyzed by SDS-polyacrylamide slab gel electrophoresis, followed by fluorography. Lane 1, translate obtained in the absence of SRP; lane 2, translate obtained in the presence of SRP (12.5 units/25 µl). (C) Total RNA from rabbit reticulocytes (4 A_{260} units/ml) was translated in the cell-free translation system (25 µl). The translation mixture was directly analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane 1, translate obtained in the absence of SRP; lane 2, translate obtained in the presence of SRP (12.5 units).

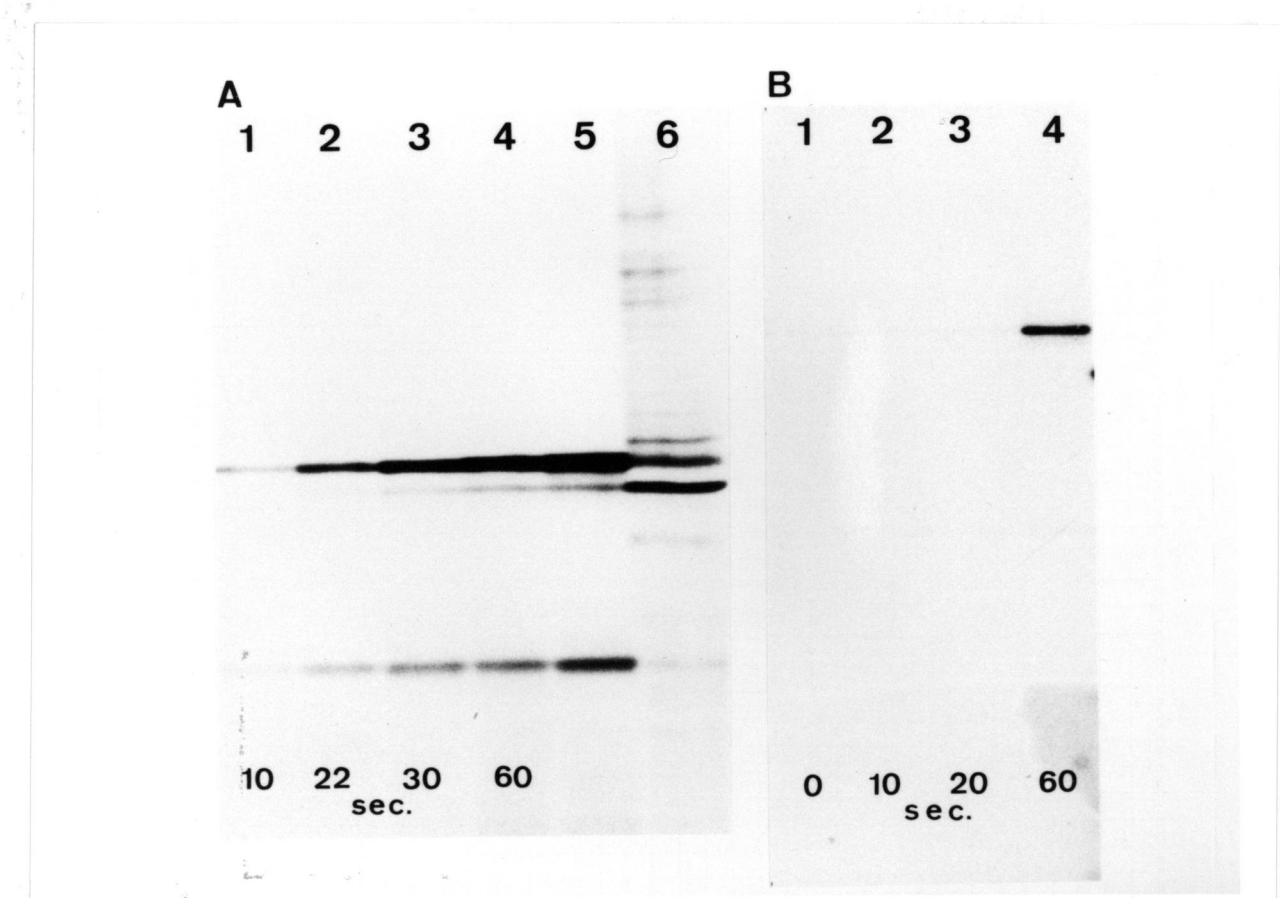

Fig. 7 - Timing of translation arrest by SRP. Translation of the rabbit liver RNA(B) and bovine anterior pituitaty gland (A) RNA was carried out as described. After preincubation at 26 °C for 30 second, the translation was started by adding RNA and 2 min later 7-methylguanosine monophosphate was added (2 mM final). During translation, desalted SRP was added at indicated timing. (A) Translation of preprolactin mRNA. lanes 1-4, translate obtained in the presence of SRP (12.5 units/25 µl) added at indicated timing; 5, translate obtained in the absence of SRP and KRM; 6, translate obtained in the presence of SRP and KRM. (B) Translation of P-450_1 mRNA lanes; 1-4, translate obtained in the presence of SRP (25 units/ 50 µl) added at indicated timing.

PART II

Signal Sequence of Microsomal Cytochrome P-450 Is Located at the NH_2-terminal Portion

SUMMARY

Co-translational insertion of hepatic microsomal cytochrome P-450 into microsomal membranes is mediated by signal recognition particle (SRP) and the presence in the cytochrome molecule of a signal sequence that can be recognized by SRP has been postulated (Part I of this thesis). To identify this signal sequence, three chimeraic cDNA clones were constructed. In two of them cDNA fragments encoding the NH_2-terminal 39 and 79 amino acid residues of a form of rabbit liver microsomal cytochrome P-450 were fused with a cDNA fragment encoding the COOH-terminal 184 residues of yeast porin. In the third one a cDNA fragment encoding the segment spanning the 99th through 517th residues of cytochrome P-450 was inserted into the BclI site of yeast porin cDNA. In all the clones the chimeraic cDNAs were placed just downstream of SP-6 promoter and, therefore, these chimeraic cDNAs could be efficiently transcribed with the aid of SP-6 RNA polymerase. When the chimeraic mRNAs thus synthesized were translated in a wheat-germ cell-free extract and the products were examined for their capacity to be co-translationally inserted into dog pancreas rough microsomal membranes, only the chimeraic proteins encoded by the first two cDNA clones were found to be inserted. It is concluded that the signal sequence that is required for co-translational insertion of cytochrome P-450 is located in the peptide segment consisting of the NH_2-terminal 39 amino acid residues.

INTRODUCTION

Like secretory proteins, hepatic microsomal cytochrome P-450 is synthesized by membrane-bound polysomes and co-translationally inserted into microsomal membranes (1,2). We have recently shown that this insertion is dependent on signal recognition particle (SRP) and postulated the presence in the cytochrome molecule of a signal sequence that can be recognized by SRP (1,2). As in the case of secretory protein mRNAs, translation of mRNA coding for cytochrome P-450 is arrested by SRP in the absence of microsomal membranes (1,2). We have found that this arrest of cytochrome P-450 synthesis took place only when SRP was added within a very short period after the initiation of translation (1,2), a finding suggesting that the signal sequence is located near the NH_2-terminal end of the cytochrome molecule (2). However, the precise location of the signal sequences is not yet known. The purpose of this study was to identify the signal sequence by means of recombinant DNA techniques.

For this purpose, we constructed a full-length cDNA encoding a β-naphthoflavone-inducible form of rabbit liver microsomal cytochrome P-450 from two overlapping cDNA clones, pd-37 and pd-38, which lack the 5'- and 3'-portions, respectively, of the coding sequence of the cytochrome (3). Several segments of the cytochrome P-450 cDNA, thus constructed, were fused to a cDNA encoding yeast porin (4), which is an outer mitochondrial membrane protein and incorporated into the membrane post-translationally without the aid of SRP (5). These fused DNAs were then

transcribed and translated *in vitro* and the capacities of the chimeraic proteins thus synthesized to be inserted into dog pancreas rough microsomes were examined. The results obtained suggest that the signal sequence of this cytochrome is contained in the stretch consisting of the NH_2-terminal 39 amino acid residues.

MATERIALS AND METHODS

Materials

The following chemicals and biochemicals were purchased from the sources indicated in parentheses: restriction enzymes (Nippon Gene, New England Biolabs and Toyobo Biochemicals); M-13 cloning and sequencing kit (Amersham International); Klenow fragment of DNA polymerase, T_4 DNA polymerase, T_4 polynucleotide kinase (Takara Shuzo); SP-6 RNA polymerase (Boehringer Mannheim); and pSP-64, pSP-65 and RNasin (Promega Biotech). [^{35}S]methionine (1160 Ci/mmole) and [^{35}S]dATPαS (>600 Ci/mmole) were obtained from Amersham International. Dog pancreas rough microsomes (RM), high-salt washed RM (KRM), SRP and wheat-germ S-23 cell-free extract were prepared as described previously (6,7). Plasmids pPor-1,117 (4), pd-37 and pd-38 (3) were kindly supplied by Dr. K. Mihara. Guinea pig polyclonal antibodies to the β-naphthoflavone-inducible cytochrome P-450 ("anti-P448 antibodies") and rabbit polyclonal antibodies to yeast porin ("anti-porin antibodies") were generous gifts from Mr. N. Kagawa and Dr. K. Mihara, respectively.

DNA Construction

Strategies adopted for DNA construction are outlined in Fig. 1 for pSP-d450, Fig. 2 for pSP-N39 and pSP-N79, and Fig. 3 for pSP-Δ98. After recombination, all the recombinants described below were screened by restriction endonuclease mapping.

pSP-d450 (see Fig. 1). Two cDNA clones, pd-37 and pd-38, both inserted into the PstI site of pBR322, lack the 5'- and 3'-portions, respectively, of the coding sequence of cytochrome P-450. However, the entire coding region is covered by these two clones (3). To construct a full-length cDNA encoding the cytochrome, pd-37 was digested with HindIII and EcoRV whereas pd-38 with HindIII alone. The two digests were mixed, ligated, and used to transform E. coli strain HB 101. The transformants were screened for clones carrying a full-length cytochrome P-450 cDNA, leading to the isolation of pd-450. This clone was digested with PvuI and the cohesive ends produced were converted to blunt ends by T_4 DNA polymerase. A SacI linker was then ligated to the newly produced blunt ends. This was then digested with SacI and the fragment containing the full-length cDNA was isolated by agarose gel electrophoresis followed by electroelution. The isolated cDNA containing fragment was inserted into the SacI site of pSP-65. This construct was termed pSP-d450.

pSP-Por. A cDNA encoding the entire yeast porin sequence was excised by PstI from pPor-1,117 (4) and isolated by agarose gel electrophoresis and subsequent electroelution. The cDNA thus isolated was inserted into the PstI site of pSP-64. This construct was called pSP-Por.

pSP-N39 (see Fig. 2). pSP-d450 prepared as described above was cut with AatI and BamHI and the resultant 5'-protruding ends at the BamHI site were filled in with the aid of Klenow fragment. This was then circularized by T_4 ligase to produce a BamHI site (pSP-T39). pSP-T39 was digested with BamHI and BglI. pSP-Por, on the other hand, was digested with BclI and BglI. The two digests were mixed and ligated. The transformants were then screened for clones carrying both cytochrome P-450 and yeast porin sequences. The clone thus obtained was termed pSP-N39.

pSP-N79 (see Fig. 2). pSP-d450 was digested with Sau3A and the 550 bp fragment encoding the NH_2-terminal 79 amino acid residues of cytochrome P-450 was isolated. This fragment was inserted into the BclI site of pSP-Por. This construct was called pSP-N79.

pSP-Δ98 (see Fig. 3). pSP-d450 was digested with AatI and the 1254 bp fragment encoding the cytochrome P-450 fragment consisting of the 99th through 517th residues was isolated. This fragment was then ligated with a BamHI linker (12mer) in order to maintain the correct reading frame. This was cut with BamHI and inserted into the BclI site of pSP-Por. This was termed pSP-Δ98.

In all the constructs described above, cytochrome P-450 cDNA, yeast porin cDNA or their fusion products are inserted downstream of SP-6 promoter and, therefore, the inserted cDNAs can be transcribed *in vitro* with the aid of SP-6 RNA polymerase. The constructions of the hybrid inserts in pSP-N39, pSP-N79 and pSP-Δ98 are illustrated in Fig. 4.

In Vitro Transcription and Translation

The cDNAs inserted in the above constructs were transcribed in vitro by the action of SP-6 RNA polymerase essentially as described previously (8). The transcription mixture contained 10 μg of template DNA (linearized with an appropriate restriction enzyme), 40 mM Tris-HCl buffer (pH 7.5), 7 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, 2 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 5-20 units of RNasin, and 15 units of SP-6 RNA polymerase in a final volume of 50 μl. After incubation at 39°C for 60 min, the transcribed products were extracted with phenol/chloroform and precipitated with ethanol. The precipitates were washed twice with 80 % ethanol, dried, and dissolved in 50 μl of water. This was used for translation without further purification.

The mRNAs thus synthesized were translated in a wheat-germ S-23 cell-free extract in the presence of [^{35}S]methionine as described previously (2). In experiments in which the capacity of translation products to be inserted co-translationally into microsomal membranes was examined, RM or KRM plus SRP were included in the translation mixture as described (1,2). The reaction was conducted at 26°C for 60 min and terminated by the addition of an equal volume of 20 % trichloroacetic acid. The protein products were collected by centrifugation and analyzed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis followed by fluorography.

Assay for Insertion of Translation Products into Microsomal Mem-

branes

In the preceding paper (1,2) co-translational insertion of *in vitro* synthesized cytochrome P-450 into microsomal membranes was assayed by separating membrane-bound and unbound cytochrome P-450s by discontinuous sucrose density gradient centrifugation. To avoid errors arising from nonspecific adsorption of translation products to membranes, in this study we used an alkali treatment method first developed by Fujiki *et al.* (13). To 10 μl of the translation mixture incubated in the presence of RM or KRM *plus* SRP was added 100 μl of ice-cold 50 mM Na_2CO_3 (pH 11.2) and 5 μl of RM (50 A_{280}/ml) as a carrier. After incubation at 4°C for 20 min, the mixture was centrifuged in a Beckman Airfuge for 110 sec at 30 psi to precipitate the membranes. The supernatant was removed and mixed with an equal volume of 20 % trichloroacetic acid to precipitate unbound proteins. The membranes were solubilized with loading buffer described by Goldman (14). These two fractions were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

Other Methods

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis were carried out as described in the preceding paper (1,2). DNA manipulations were performed according to the methods described by Maniatis *et al.* (9). Nucleotide sequencing by the chain termination method was conducted with the use of M-13 cloning and sequencing kit according to the manual provided by the supplier. Fluorography was performed by using an "Amplify"

purchased from Amersham International.

RESULTS

Synthesis of Cytochrome P-450 and Yeast Porin in Cell-free Transcription-Translation System

As described above, we constructed a cDNA encoding a β-naphthoflavone-inducible form of rabbit liver microsomal cytochrome P-450 by joining two overlapping cDNAs, pd-37 and pd-38, at the HindIII site. The nucleotide sequence of the constructed cDNA was found to be identical with that of a full-length cDNA encoding this form of cytochrome P-450, which was recently isolated and sequenced by Mihara and Kagawa (10). As a first step of this study, aiming at the identification of the signal sequence in cytochrome P-450 by recombinant DNA procedures, we attempted to see if this cytochrome P-450 cDNA as well as yeast porin cDNA (3,4) can direct the synthesis of active mRNAs *in vitro*. For this purpose, these two cDNA inserts were excised from the plasmid vectors and inserted into pSP-65 or pSP-64 so that the cDNAs were placed just downstream of SP-6 promoter in these plasmids. SP-6 promoter is a powerful and specific promoter derived from bacteriophage SP-6 infected *Salmonella tryphimurium* (11,12). The DNAs thus constructed, pSP-d450 and pSP-Por, were linearized and transcribed *in vitro* with the aid of SP-6 RNA polymerase. The mRNA transcripts were subjected to agarose gel electrophoresis and detected by ethidium bromide staining. As shown in Fig. 5, a single mRNA species having a size expected for cytochrome P-450 and porin mRNAs were detected

in the transcript of pSP-d450 and pSP-Por (lanes 1 and 2). The fact that the intensities of mRNA bands detected in Fig. 5 were almost the same as those of the linearized DNA templates indicates that the transcription efficiency of the system employed is extremely high. This is especially so in view of the fact that the sensitivity of RNA detection by ethidium bromide staining is much less than that of double-stranded DNA detection. To see if these mRNAs can actually code for the respective proteins, they were translated in the wheat-germ cell-free extract in the presence of [^{35}S]methionine and the translation products were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent fluorography. As can be seen in Fig. 6, each translate gave a major band together with a number of minor bands having lower molecular weights and the major band corresponded to the molecular weight expected for cytochrome P-450 (lane 1) or yeast porin (lane 3). Furthermore, in both cases, the major band was specifically immunoprecipitated by anti-P-448 antibodies or anti-yeast porin antibodies (lane 3). The minor band seen in lane 2 was likely to be a degradation product of cytochrome P-450. These observations indicate that the major bands in lanes 1 and 3 actually represent cytochrome P-450 and yeast porin, respectively. It was concluded that the methods employed in this study permit one to synthesize cytochrome P-450 and yeast porin, *in vitro* from their corresponding cDNAs and detect the proteins without the use of immunoprecipitation.

Insertion of Cytochrome P-450 Synthesized in Cell-free Transcrip

tion-Translation System

The second step of this study was to determine whether the cytochrome P-450 synthesized in the above *in vitro* system can be co-translationally inserted into dog pancreas rough microsomal membranes (RM). To this end, an alkali treatment method was used as a more rigorous criterion for membrane insertion of proteins. As shown in Fig. 7, extraction of RM with Na_2CO_3 at concentrations higher than 5 mM removed about 80 % of protein. It is supposed that under these conditions only integral membrane proteins are recovered in the membrane fraction. This method has recently been used in studies of membrane insertion of several other proteins (15,16). Linearized pSP-d450 and pSP-Por were transcribed and the mRNAs were translated *in vitro* in the presence of RM. The reaction mixtures were treated with 50 mM Na_2CO_3 and the membranes were pelletted by centrifugation. Both the supernatant and membrane fractions were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. As shown in Fig. 8, a substantial amount of cytochrome P-450 synthesized was recovered in the membrane fraction (lane 1). In contrast, all the porin synthesized remained in the supernatant fraction (lane 2). When KRM, which is deficient in SRP, was included in the translation mixture instead of RM, the insertion of cytochrome P-450 into the membranes was greatly diminished (lane 3). However, supplementation of this KRM containing system with purified SRP restored the membrane insertion (lane 4). These findings indicate that the insertion of cytochrome P-450 into microsomal membranes in this transcription-translation sys-

tem also takes place in SRP-dependent fashion, confirming the previous results (1,2). The finding that yeast porin synthesized in this system failed to be inserted into RM is consistent with previous results that it is specifically incorporated into the outer mitochondrial membrane (5) and provides a support to the use of the porin system as a negative control in the study of protein insertion into microsomal membranes.

Construction of Hybrid cDNAs

To identify the signal sequence by which cytochrome P-450 directed to SRP-dependent insertion into microsomal membranes, three recombinant cDNAs were constructed from cDNAs encoding cytochrome P-450 and porin according to the strategies outlined in Fig. 2 and 3. The constructs prepared are pSP-N39, pSP-N79 and pSP-Δ98. pSP-N39 contains a cDNA fragment encoding the NH_2-terminal 39 residues of cytochrome P-450 followed by a cDNA segment corresponding to the COOH-terminal 184 residues of yeast porin. In pSP-N79 the sequence derived from cytochrome P-450 in pSP-N39 was replaced by a cDNA fragment specifying the NH_2-terminal 79 amino acid residues of cytochrome P-450. Finally, pSP-Δ98 is a recombinant in which a cDNA fragment encoding the segment spanning the 99th through 517 amino acid residues of cytochrome P-450 was inserted into the Bcl I site of yeast porin cDNA with the use of a 12mer BamHI linker. After recombination, each recombinant was screened by restriction enzyme mapping. Furthermore, all the junctions in the recombinant cDNAs were sequenced through by the chain termination method as shown in

Fig. 9, which also shows the deduced amino acid sequences of the junction regions. These results indicated that in all the recombinants constructed the expected sequences of cytochrome P-450 and yeast porin were linked without causing any shift of reading frame.

In Vitro Synthesis of Chimeraic Proteins

The hybrid cDNAs constructed above were linearized and transcribed by SP-6 RNA polymerase under the influence of SP-6 promoter and the mRNAs synthesized were examined by agarose gel electrophoresis followed by ethidium bromide staining. As is clear from Fig. 5 (lanes 3, 4 and 5), each transcript gave a single mRNA band, the size of which was in good agreement with that expected from the length of the chimeraic cDNA. These mRNAs were then translated in the wheat germ cell-free system in the presence of [^{35}S]methionine and the translates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. As shown in Fig. 10, each translate gave a number of polypeptide bands (lane 1 for pSP-Δ98; lane 3 for pSP-N39; lane 5 for pSP-N79). In each case, only the band showing the lowest mobility could be immunoprecipitated by anti-porin antibodies (lanes 2, 4 and 6) and by anti-P-448 antibodies (lanes 8 and 9), except that anti-P-448 antibodies precipitated several ploypeptides, in addition to the largest product, from the translate derived from pSP-98 (lane 7). It was concluded that the largest polypeptides obtained in the three experiments represent the chimeraic proteins containing both cytochrome P-450 and yeast porin sequences.

It is also to be noted that the apparent molecular weights of the largest ploypeptides agreed with those expected from the size of chimeraic cDNAs. The nature of the other smaller polypeptides detected is unclear. Partial proteolysis, early termination of translation and degradation of mRNA could cause the appearance of these polypeptides, but they should also be immunoprecipitated by the antibodies.

Insertion of Chimeraic Proteins into Microsomal Membranes

Having confirmed that the chimeraic proteins can be synthesized *in vitro* starting from the chimeraic cDNAs, we next examined whether they can be co-translationally inserted into microsomal membranes. For this purpose, mRNAs synthesized with pSP-d450, pSP-N39, pSP-N79 and pSP-Δ98 as templates were translated in the wheat-germ cell-free system in the presence of KRM *plus* SRP and the translates were separated into the supernatant and membrane fractions by the alkali treatment method. Fig. 11 shows the results of SDS-polyacrylamide gel electrophoresis-fluorography analysis of the separated fractions. It is clear that cytochrome P-450 encoded by pSP-d450 (lane 1) and two chimeraic proteins encoded by pSP-N79 and pSP-N39 (lanes 2 and 3, respectively) were largely recovered in the membrane fraction, indicating that these proteins had been efficiently inserted into the membranes. When RM was added after the termination of translation, the protein encoded by pSP-N39 was recovered only in the supernatant fraction (lane 4). It is, therefore, certain that the insertion of the chimeraic proteins was also a co-trans-

lational event. Taken together, these data strongly suggest that the NH_2-terminal sequence consisting of 39 residues in the cytochrome P-450 molecule is sufficient for the co-translational insertion of proteins into microsomal membranes. The chimeraic protein encoded by pSP-Δ98 could not be inserted into RM (Fig. 11, lane 5). It is, therefore, likely that there is no signal sequence, which is required for membrane insertion of cytochrome P-450 in a SRP-mediated fashion, in the COOH-terminal portion behind the 99th residue. Finally, the effect of SRP on the insertion of the chimeraic protein encoded by pSP-N39 was examined. When the translation was carried out in the presence of KRM (but in the absence of added SRP), about 30 % of the protein synthesized was recovered in the membrane fraction (Fig. 12 A). This insertion was probably due to a small amount of SRP still remaining in this particular KRM preparation. Supplementation of this system with purified SRP resulted in an almost complete recovery of the protein in the membrane fraction (Fig. 12 B). Therefore, it was concluded that the insertion of the pSP-N39 protein, like that of cytochrome P-450, is dependent of SRP.

DISCUSSION

In the present study the location of signal sequence(s) of cytochrome P-450 was examined by three fused cDNA clones containing both cytochrome P-450 and yeast porin sequences. The results obtained indicate clearly that the NH_2-terminal segment of cytochrome P-450 consisting of 39 amino acid residues is sufficient for insertion of the chimeraic proteins into microsomal mem-

branes. Since the insertion is an SRP-dependent event, it is certain that an SRP-recognizable signal sequence of cytochrome P-450 is also contained in this NH_2-terminal 39-residue segment. This conclusion is consistent with the fact that in all forms of liver microsomal cytochrome P-450 so far sequenced the NH_2-terminal portion is rich in hydrophobic residues (17-20). A further refinement of the location of this signal sequence will be reported in the following paper (Part III of this thesis).

Although the signal sequence of most secretory proteins is located in the NH_2-terminal region (21), Perara et al. (15) have reported that a chimeraic protein whose NH_2- and COOH-terminal halves consist of a globin (cytoplasmic protein) sequence and a pre-prolactin (secretory protein) sequence including its signal sequence, respectively, can be translocated across microsomal membranes and processed co-translationally. These observations indicate that a signal sequence located in the middle of the molecule can also be recognized by SRP and thus can function in membrane translocation. A more recent report on the insertion of bovine opsin into microsomal membranes, studied by methods similar to those adopted here, suggests that this protein contains at least one more signal sequence in addition to that located at the NH_2-terminal region (22). It has been postulated that the occurrence of multiple signal sequences in this integral membrane protein provides a basis for its multiple transmembrane topology (22).

Are there any other signal sequences than the NH_2-terminal one in the cytochrome P-450 molecule? The observation that the

protein encoded by pSP-Δ98 can be neither inserted into nor translocated across the membranes excludes the possibility that the segment of cytochrome P-450 spanning the 99th through 517th residues contains a signal sequence(s). Although the presence of a signal sequence in the segment between the 40th and 98th residues is still to be explored, it is likely that the NH_2-terminal signal sequence is the only one occurring in the cytochrome P-450 molecule. If so, it can be tentatively concluded that only the NH_2-terminal region of cytochrome P-450 is tightly associated with the membranes and the rest of the molecule is extruded to the cytoplasm. This point will be discussed in more details in Part III of this thesis.

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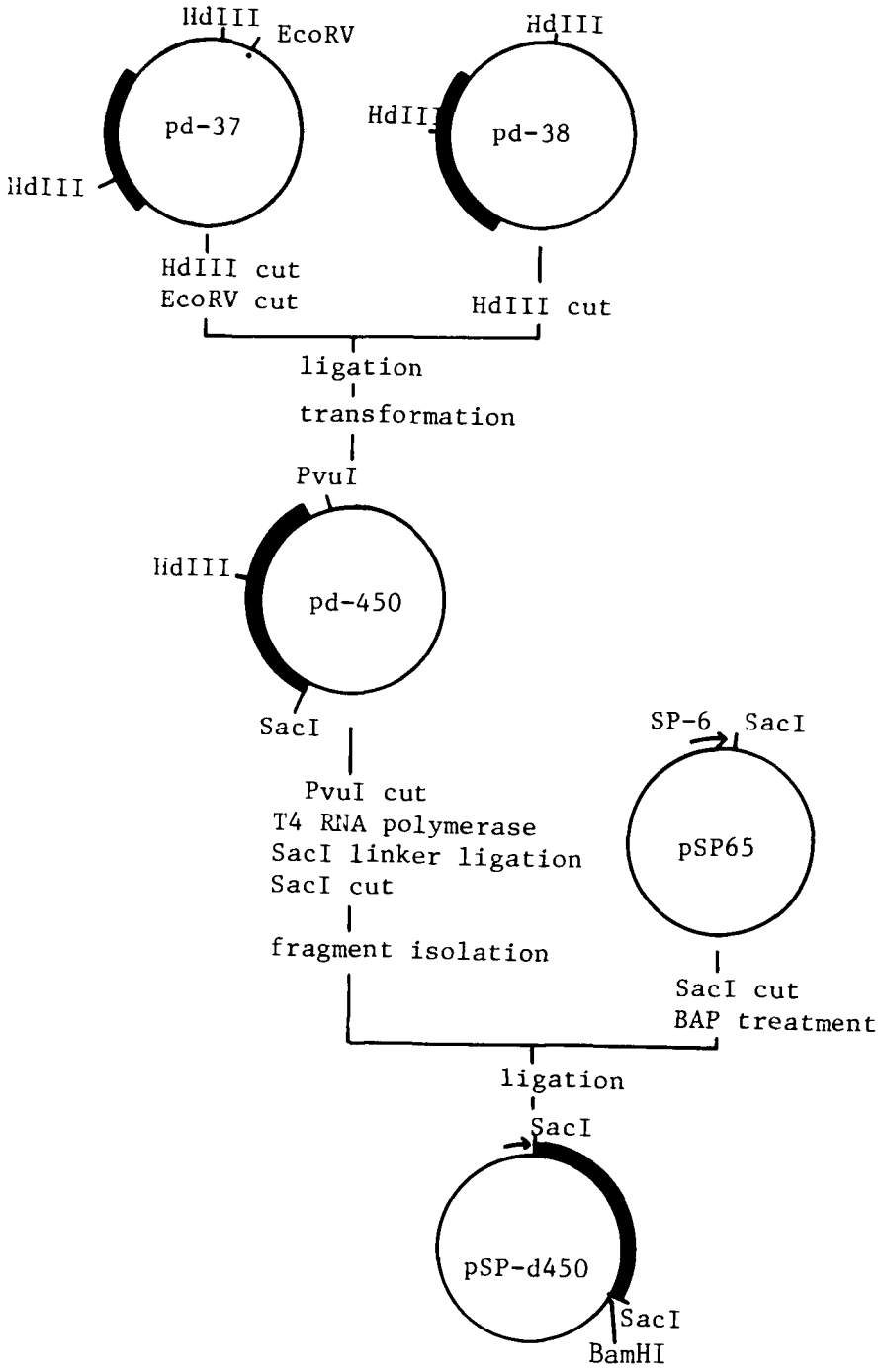

Fig. 1 Construction of pSP-d450. A cDNA clone (pd-450) encoding β-naphthoflavone inducible form of cytochrome P-450 was constructed from two partly overlapping subclones (pd-37 and pd-38). This 1600 bp insert was removed from pBR-322 and inserted into just down stream of SP-6 promoter of pSP-65. See Materials and Methods for details. Solid bars represent cDNA regions coding for P-450. Arrows represent the region of SP-6 promoter and the direction of transcription.

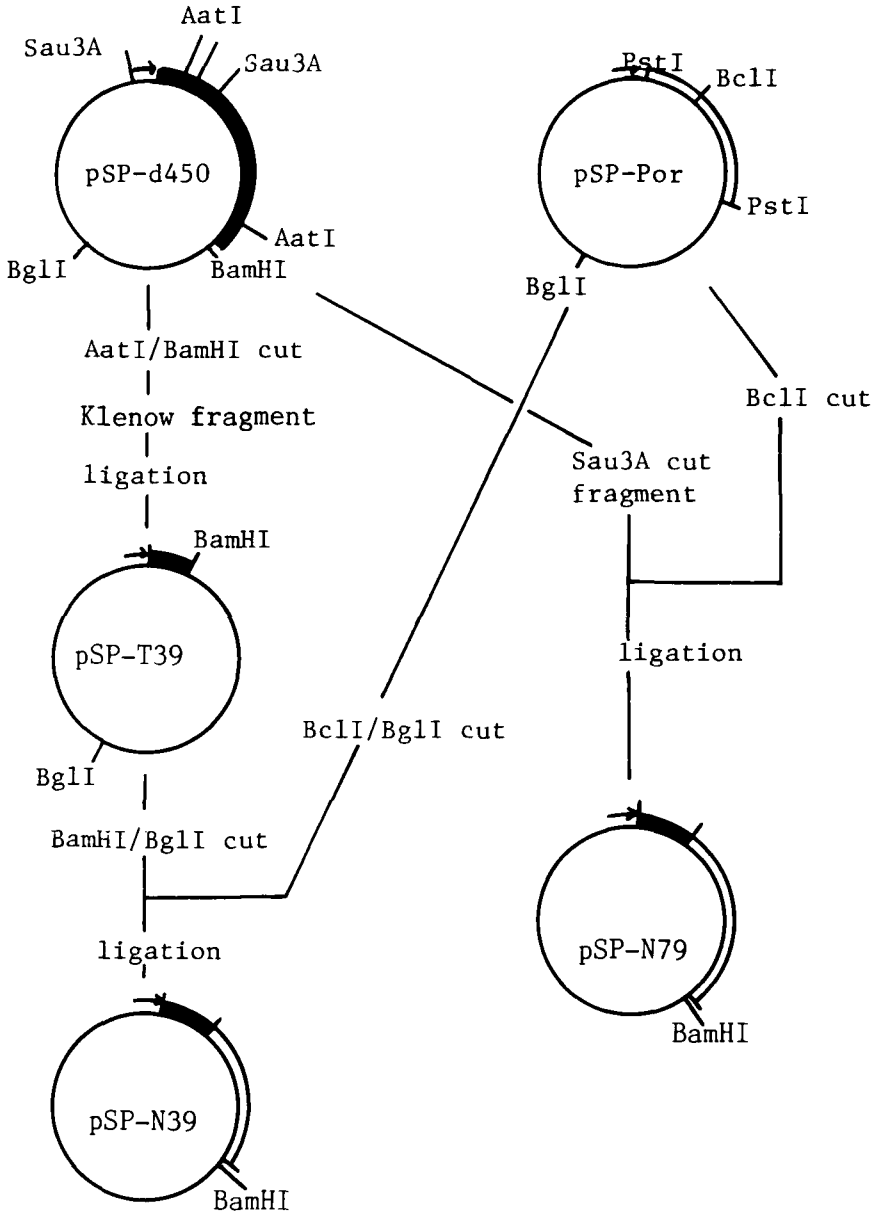

Fig. 2 Construction of pSP-N39 and pSP-N79. cDNA fragments coding for NH_2-terminal region of P-450 were inserted into an SP-6 plasmid carrying a part of yeast porin cDNA such that amino acid resides 1-98 of porin is replaced by NH_2-terminal 39 (pSP-N39) and 79 (pSP-N79) amino acid residues of P-450. In pSP-N39, Gly was newly inserted at COOH-terminal junction of the fusion protein due to the fill-in reaction by Klenow fragment. (see Fig. 9) See Materials and Methods for construction details. Symbols are same as those described in Fig. 1.

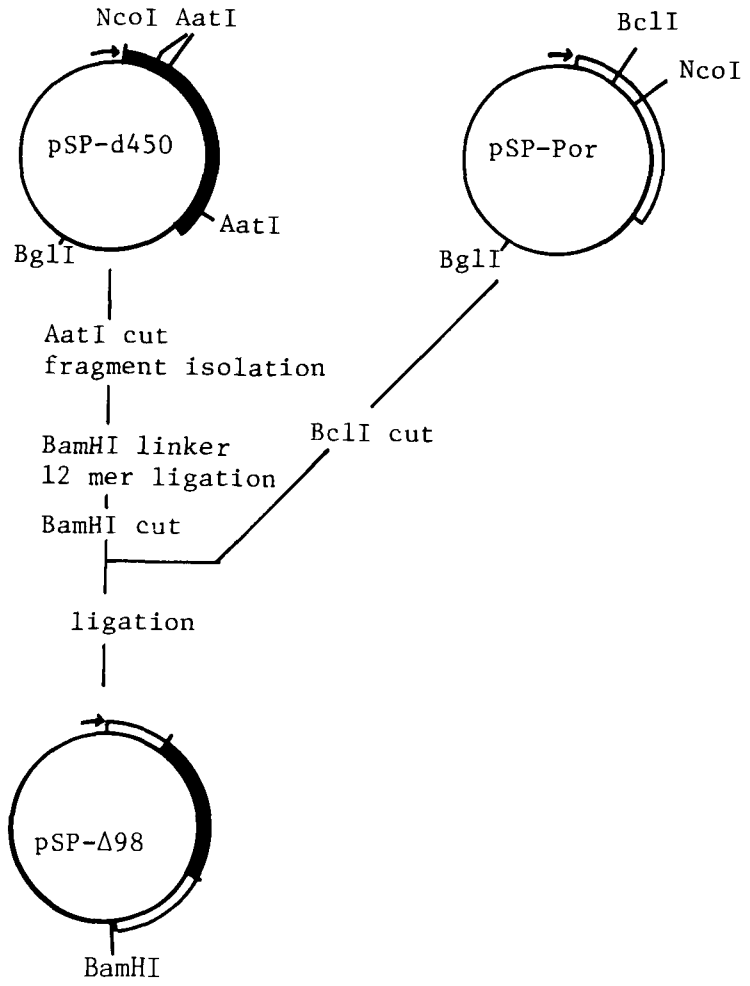

Fig. 3 Construction of pSP-Δ98. 1254 bp cDNA fragment coding for amino acid residues 99 through 517 of P-450 was inserted into BclI site of pSP-Por to create pSP- 98. Due to the addition of BamHI linkers in the construction, Arg (at NH_2-terminal junction) and Arg-Ile (at COOH-terminal junction) were newly inserted. (see Fig. 9) Solid and open bars denote for cDNAs coding for P-450 and porin respectively. Arrows on the plasmid diagrams indicate the site of SP-6 promoter and the direction of transcription by SP-6 RNA polymerase. See Materials and Methods for detailes.

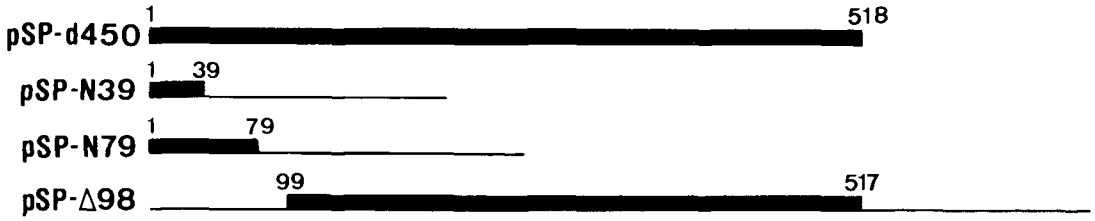

Fig. 4 Summary of the constructions of cytochrome P-450 and its fusion derivatives in SP-6 plasmids. Solid boxes denote for coding regions of cytochrome P-450 and solid lines denote for the regions of yeast porin. Numbers above the solid boxes are those of amino acid residue of cytochrome P-450.

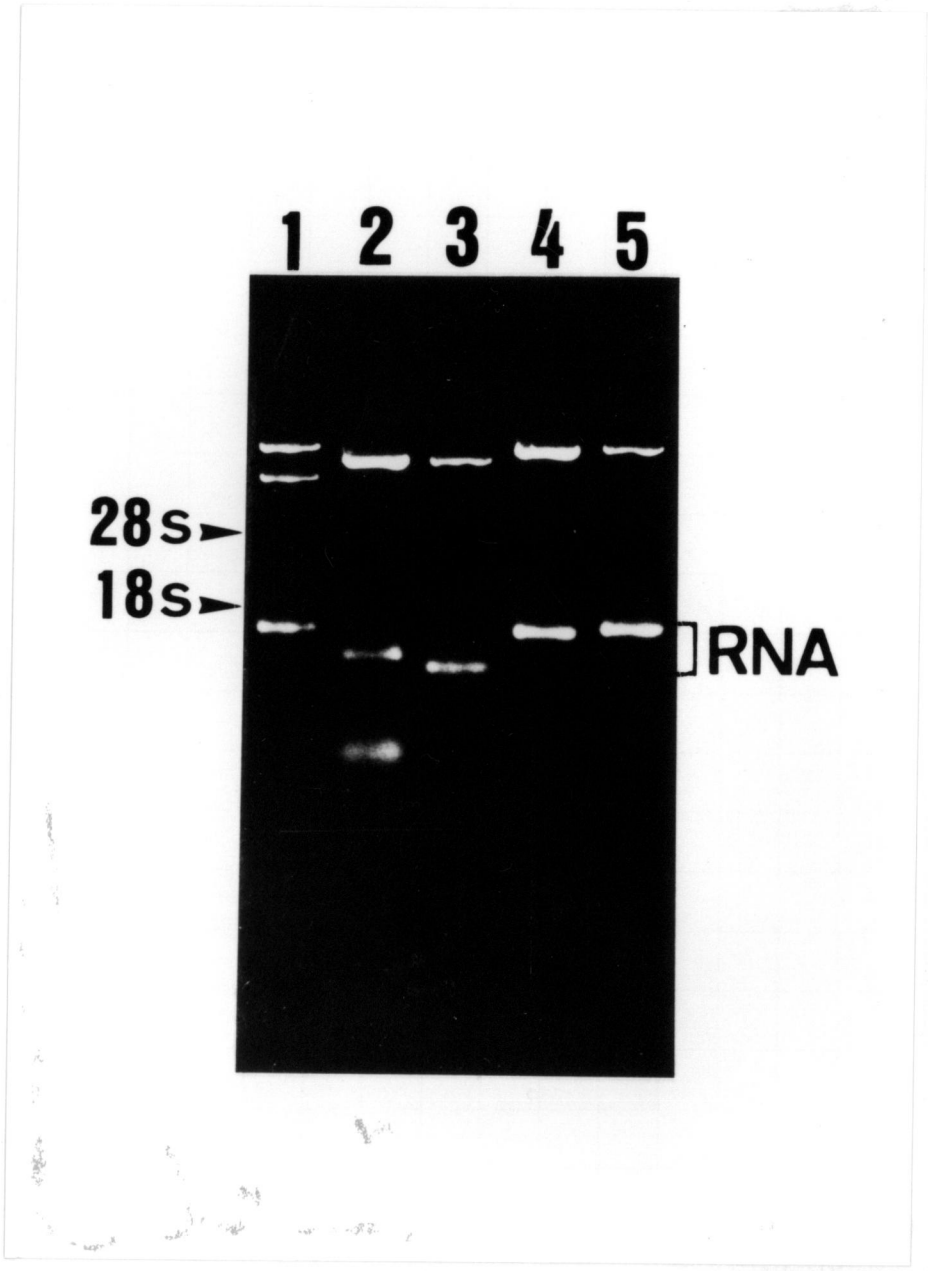

Fig. 5 In vitro transcription of cDNA constructs in SP6-plasmid. cDNA constructs in SP6-plasmid were linearized with BamHI and transcribed in vitro by SP6-RNA polymerase (See Materials and Methods for detailes). 1 µl aliquots of the transcription reaction mixtures were analyzed by 1.5 % agarose gel electrophoresis in 1 x TPE buffer (9) containing ethidium bromide and photographed under long-wave UV irradiation. Lanes 1 - 5 correspond to the transcripts of pSP-d450, pSP-Por, pSP-N39, pSP-N79 and pSP-Δ98, respectively. Positions of calf liver ribosomal RNAs are indicated by arrow-heads at left-hand side of the panel. The band with the highest mobility in lane 2 is a carry-over of bacterial RNA contaminated in the template DNA.

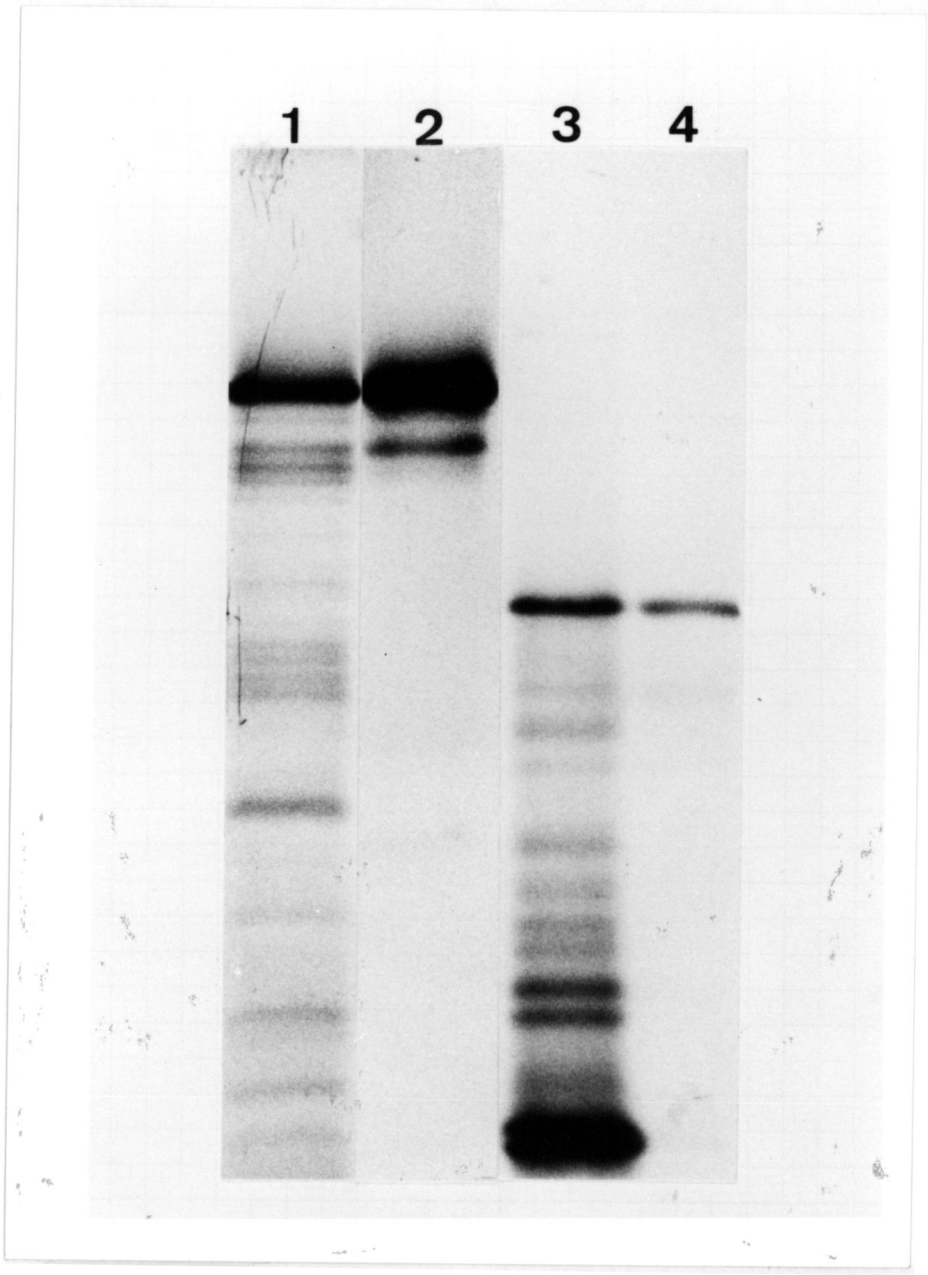

Fig. 6 In vitro synthesis of cytochrome P-450 and yeast porin. SP6-generated mRNAs were translated in a wheat-germ cell-free system in the presence of [^{35}S]methionine and analyzed by SDS-PAGE (15% cross-linked gels) and subsequent fluorography. 5 µl of the translates were used for immunoprecipitations. Details are described in Materials and Methods. Lane 1; total translate of pSP-d450 generated mRNA, Lane 2; immunoprecipitate from the translate in Lane 1 by anti-cytochrome P-450 antibodies, Lane 3; total translate of pSP-Por generated mRNA, Lane 4; immunoprecipitate from the translate in Lane 3 by anti-porin antibodies. Lanes 1,2 & 4 are fluorograms of over night exposure, while Lane 2 is that of 2-days exposure.

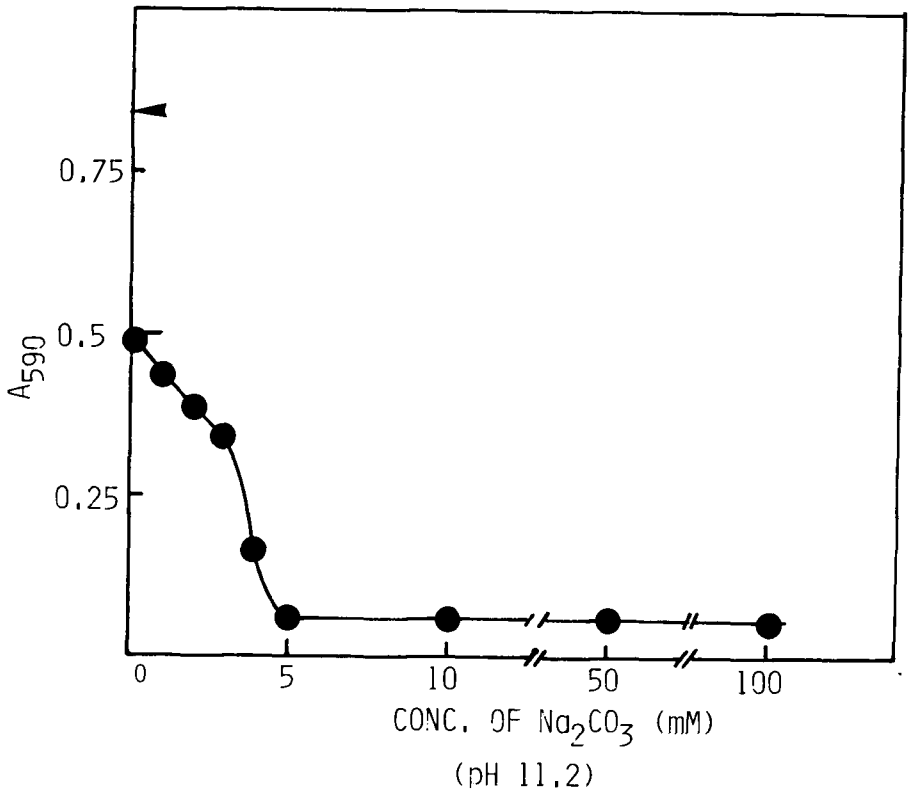

Fig. 7 Effect of alkali concentrations on the protein extraction of dog pancreas microsomal membranes. 2500 A_{280} units of salt-washed dog pancreas microsomal membranes (KRM) were treated with indicated concentrations of Na_2CO_3 for 20 min at 4 °C. The mixtures were then ultra-centrifuged at 30 psi for 110 sec in a Beckman Airfuge. Proteins recovered in membrane fraction were determined by the assay kit of Otsuka Assay Lab. An arrow-head indicates the amount of KRM used for this treatment.

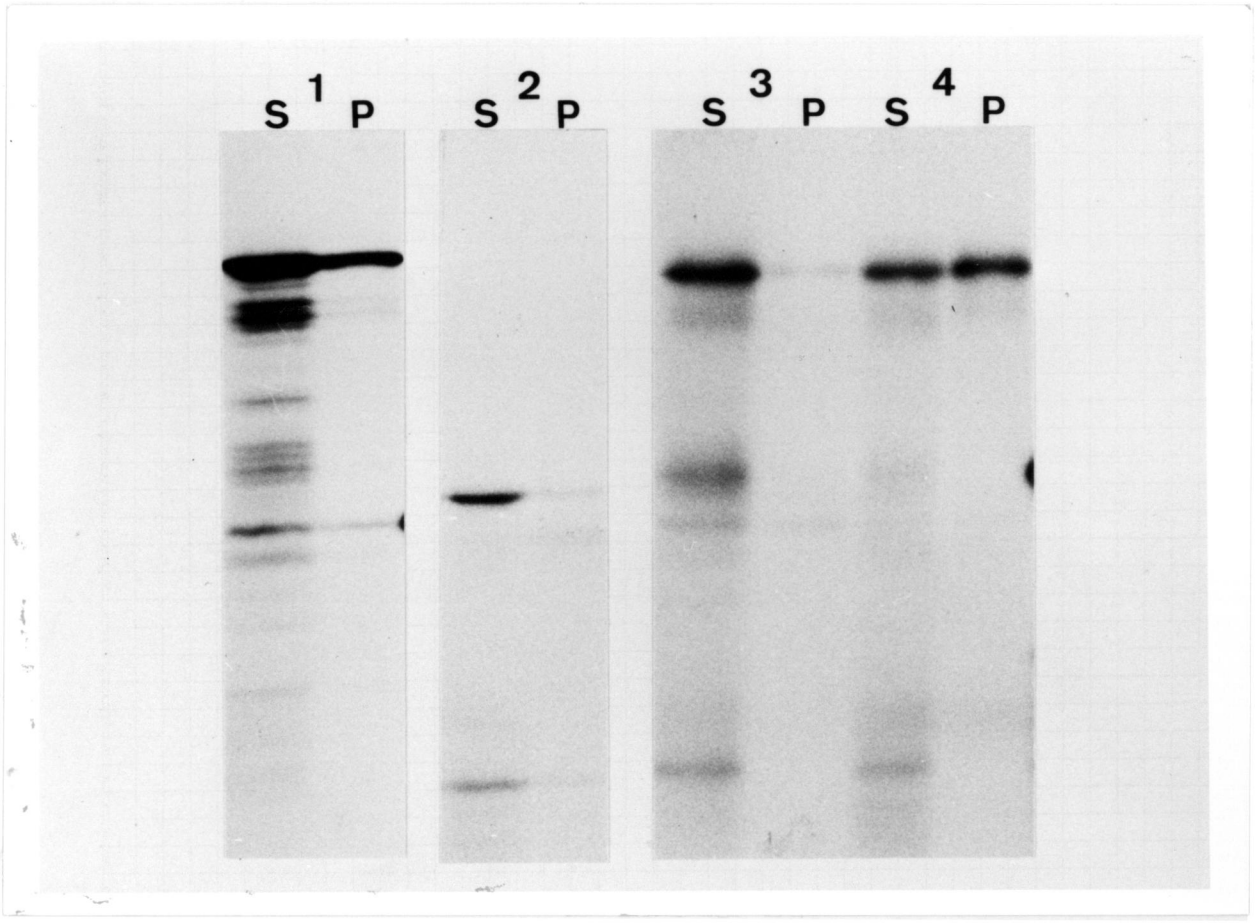

Fig. 8 Cytochrome P-450 is inserted *in vitro* into microsomal membranes in an SRP-dependent fashion. mRNAs transcribed from pSP-d450 and pSP-Por were translated in a wheat-germ cell-free system in the presence of [^{35}S]methionine. The translation system contained either 5 A_{280} units/ml RM, 5 A_{280} units/ml KRM or 5 A_{280} units/ml KRM *plus* 10 units/50 µl of SRP. After translation, the reaction mixtures were treated with 50 mM Na_2CO_3 and were centrifuged at 30 psi for 110 sec in a Beckman Airfuge to separate into supernatant ("S") and membranes fractions ("P"). Each fraction was solubilized with loading buffer and analyzed by SDS-PAGE and subsequent fluorography. Lane 1; translate of pSP-d450 generated mRNA in the presence of RM, Lane 2; transltate of pSP-Por generated mRNA in the presence of RM, Lane 3; translate of pSP-d450 generated mRNA in the presence of KRM, Lane 4; same as in Lane 3 except that SRP was included in the reaction mixture.

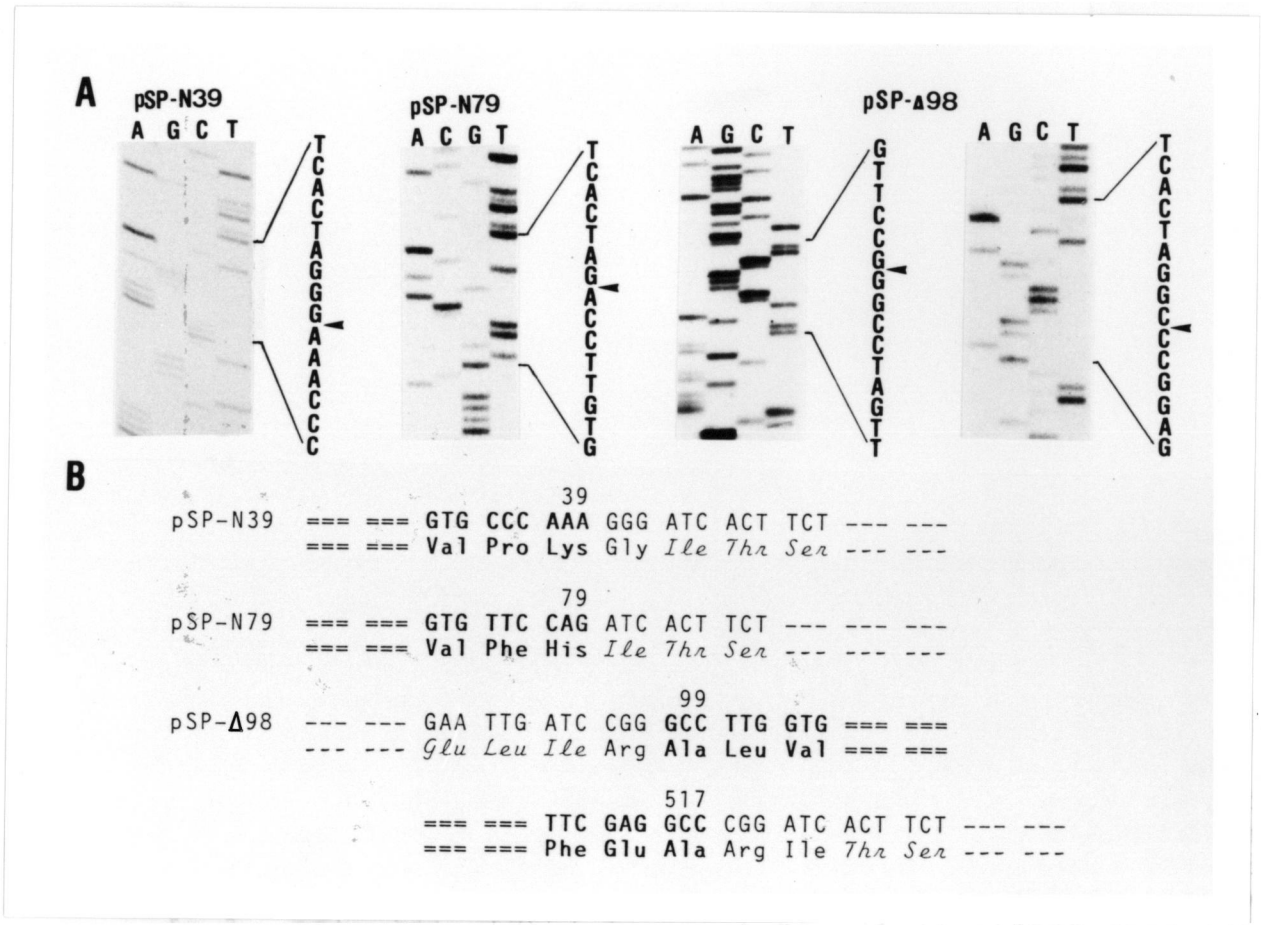

Fig. 9 Nucleotide sequences around the joining region of insert cDNAs in SP6-plasmids and amino acid sequences deduced wherefrom.
(A) Auto radiogram of the sequencing gels. Recombinant plasmid sequenced is shown above each panel.
(B) Amino acid sequences deduced from the nucleotide sequences.

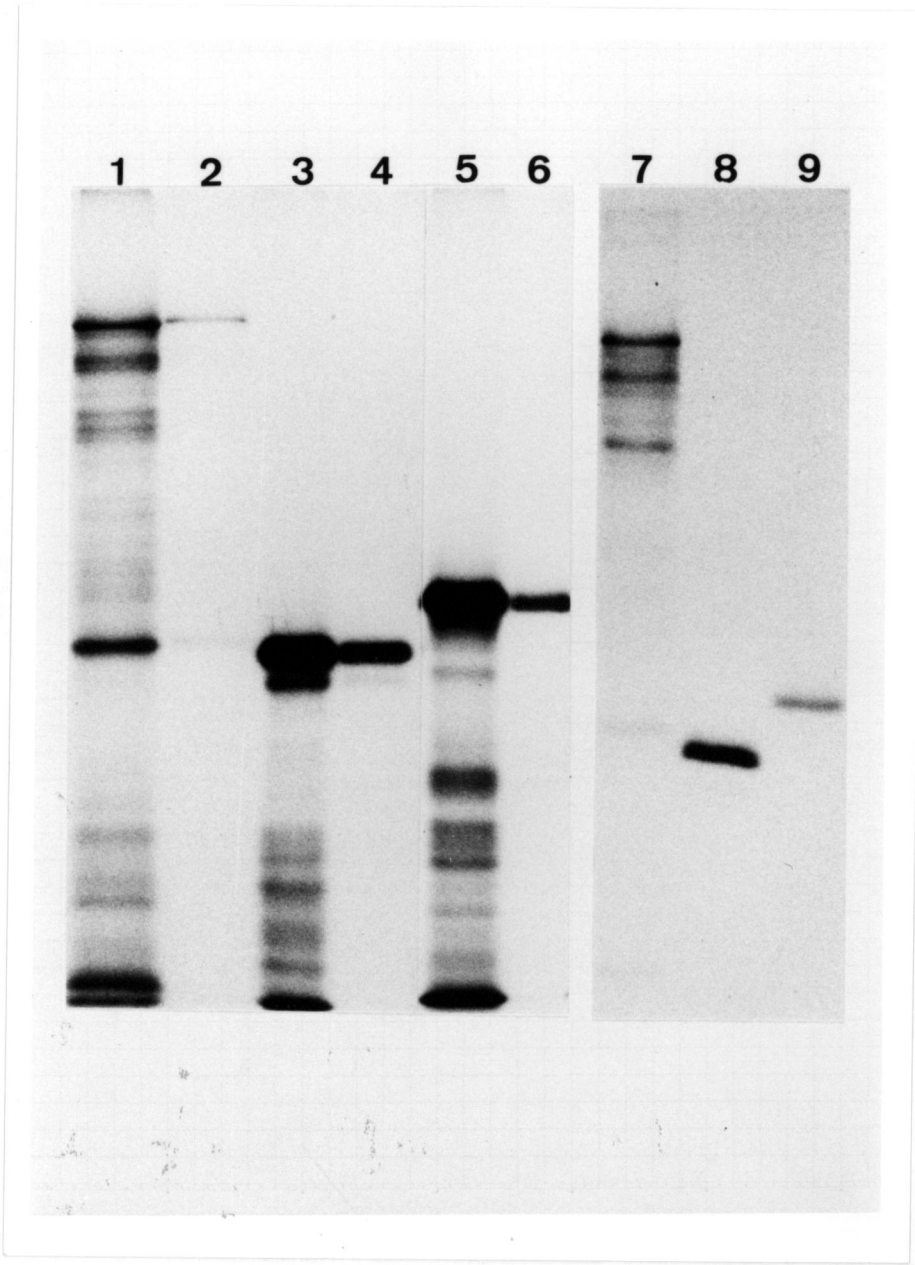

Fig. 10 In vitro translation of mRNAs transcribed chimeric cDNAs in SP6-plasmid. pSP-Δ98 (Lanes 1,2), pSP-N39 (Lanes 3,4) and pSP-N79 (lane 5,6) were transcribed in vitro by SP6-RNA Polymerase. mRNAs thus obtained were translated in a wheat-germ cell-free system in the presence of [^{35}S]methionine. 5 µl-aliquots were subjected to immunoprecipitate by anti-porin (Lanes 2,4 and 6) and anti-cytochrome P-450 (Lanes 7 through 9) antibodies. Total translation products and immunoprecipitates were subjected to SDS-PAGE and subsequent fluorography. See Materials and Methods for details.

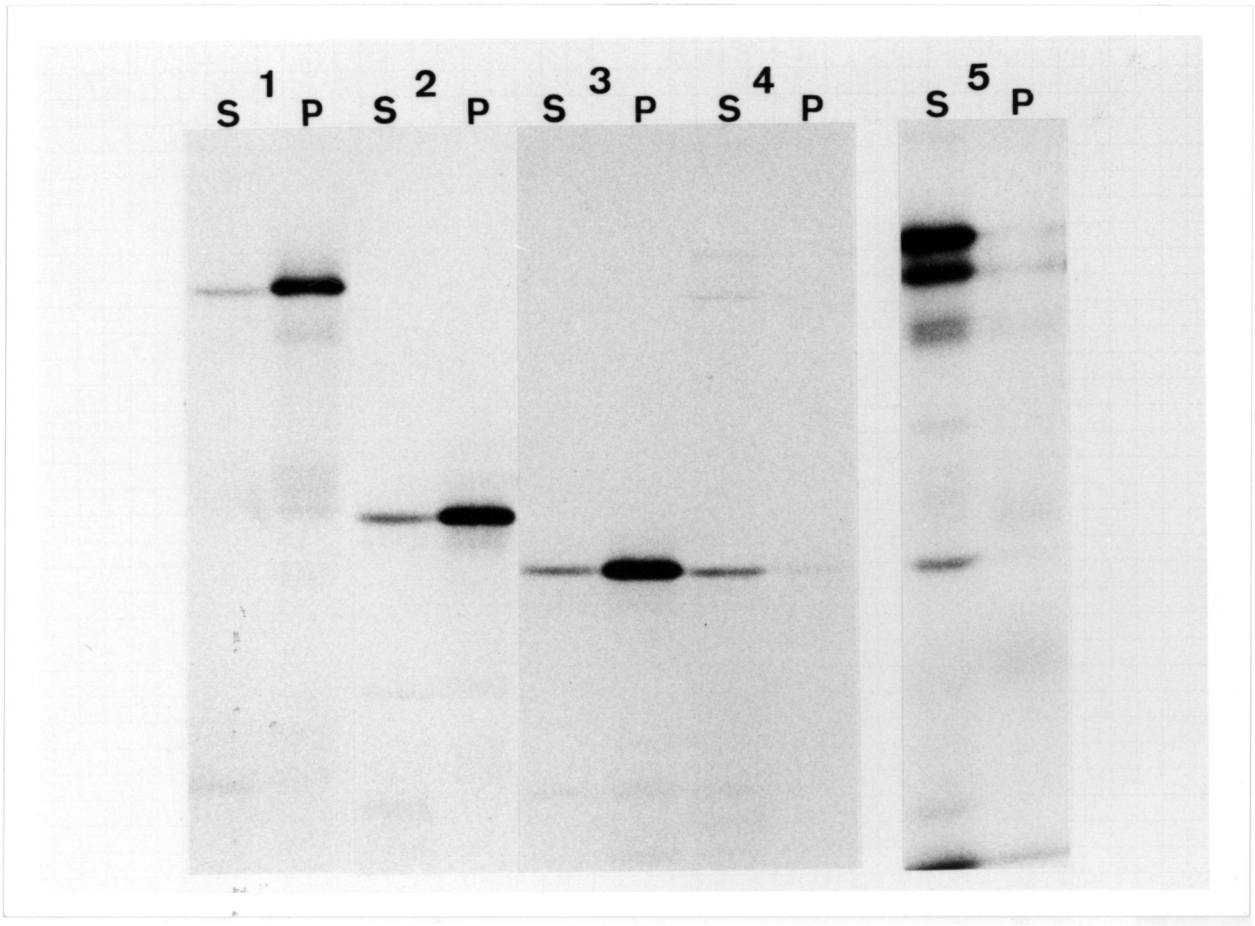

Fig. 11 In vitro insertion of chimeric proteins in dog pancreas microsomal membranes. In vitro synthesized mRNAs coding for cytochrome P-450 and its chimeric proteins were translated in a wheat-germ cell-free system in the presence of [^{35}S]methionene. Either KRM (5 A_{280} units/ml) plus SRP(10 units/50 µl) (Lanes,1,2,3 and 5) or RM (5 A_{280} units/ml)(Lane 5) was included in the reaction system. The reactions were terminated by the addition of RNase (100 µg/ml). In lane 4, however, RM(5 A_{280} units/ml) was added to the reaction mixture after termination of the translation. And incubated for further 45 min at 26 ^{o}C. Reaction mixtures thus obtained were then treated with 50 mM Na_2CO_3 and separated into Supernatant("S") and Membrane ("P") fractions by ultra-centrifugation. These fractions were analyzed by SDS-PAGE and subsequent fluorography. See materials and methods for details. Note that lane 5 is a result of separate experiment.

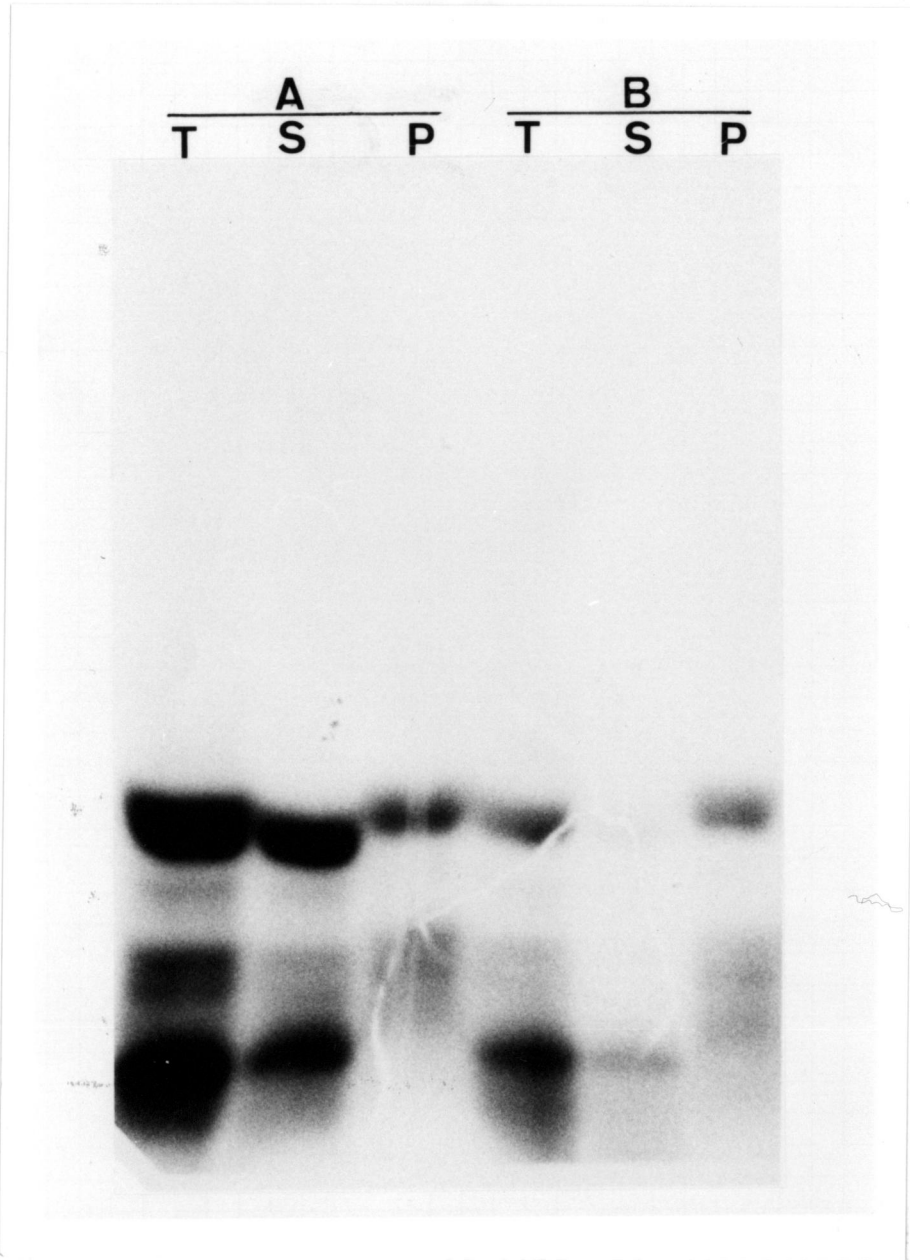

Fig. 12 The first 39 amino-terminal residues of cytochrome P-450 contain a signal sequence. The mRNA transcribed pSP-N39 was translated in a wheat-germ cell-free system in the presence of [^{35}S]methionine. In the translation mixtures were included either KRM (5 A_{280} units/ml)(A), or KRM (5 A_{280} units/ml) plus SRP (20 units/ 50 µl)(B). Cotranslational insertion of the chimeric protein into microsomal membranes was determined as described in legends to Figs, 7 and 8. S, P, and T stand for supernatant, membrane-fraction and translation mix befor ultra-centrifugation, respectively. See Materials and Methods for details.

PART III

Signal and Stop-transfer Sequences of Microsomal Cytochrome P-450 Are Located in a Short NH_2-terminal Segment

SUMMARY

We have shown that the aminoterminal segment consisting of 39 amino acid residues in a form of rabbit liver microsomal cytochrome P-450 contains an uncleavable signal sequence required for co-translational insertion of the cytochrome into microsomal membranes (part II of this thesis). In this study, which attempted to locate the signal more precisely and to examine if it is functionally equivalent to transient signals of secretory proteins, the 5'-portion of murine pre-interleukin 2 cDNA encoding most of the transient signal sequence was replaced by cDNA fragments coding for the amino-terminal 39, 29 and 20 residues of the cytochrome by using recombinant DNA technique and oligonucleotide-directed, site specific mutagenesis. These hybrid cDNAs were transcribed *in vitro* with the aid of SP-6 promoter and SP-6 RNA polymerase. The mRNAs thus synthesized were then translated in a wheat-germ cell-free system in the presence of [^{35}S]methiomine and dog pancreas rough microsomal membranes and the insertion of the protein products into and their translocation across the membranes were examined. It was thus found that the chimeraic proteins containing 39 and 29 residues derived from the amino terminus of P-450 were co-translationally inserted into the membranes but neither translocated across the membranes nor processed by signal peptidase. The protein containing the amino-terminal 20 residues of cytochrome P-450 was neither inserted into nor translocated across the membranes. These findings suggest that a stretch spanning the 21st through 29 residues

of cytochrome P-450 contains not only the signal sequence recognizable by signal recognition particle but also a sequence that stops the transfer of the remaining portion of the cytochrome molecule across microsomal membranes.

INTRODUCTION

Integral proteins in microsomal and plasma membranes are synthesized by membrane-bound polysomes and co-translationally inserted into microsomal membranes (1). Recent evidence suggests that the early stage of this insertion process shares the same mechanism with that of co-translational translocation of secretory proteins across microsomal membranes. Thus both processes require signal recognition particle (SRP) that recognize a signal sequence(s) in the growing polypeptide chain (2-6). Although not explicitly proven, the membrane insertion, like the translocation of secretory proteins (7), seems to be dependent on SRP receptor. However, the two processes differ from each other in later stages. While secretory proteins pass through the membranes and released into the luminal space (8), membrane proteins remain inserted in the membranes. Furthermore, membrane translocation of secretory proteins is accompanied by cleavage of their NH_2-terminal sequences (9), whereas the signal sequences of many, though not all, membrane proteins are uncleavable (3-6). Little is as yet understood how these differences are produced. For instance, it is not known whether the NH_2-terminal signal sequence of membrane proteins is functionally equivalent to that of secretory proteins. Another important problem concerning the membrane insertion is the mechanism by which a membrane protein assumes its own topological disposition in the membranes. It has been proposed that this is accomplished by cooperation of functionally distinct sequences (e.g. signal

sequence, stop-transfer sequence, etc.) in the primary structures of membrane proteins (10). However, no clear-cut evidence has been presented for this mechanism, although it has recently been reported that the multi-transmembrane orientation of bovine opsin is formed by the presence of more than one signal sequence in the molecule (6).

In Parts I and II of this thesis, it has been reported that co-translational insertion of hepatic microsomal cytochrome P-450 into microsomal membranes requires SRP, that the cytochrome possesses an uncleavable signal that can be recognized by SRP, and that this signal is located in the NH_2-terminal segment consisting of 39 amino acid residues. As a continuation of these studies, it was attempted in the present study to locate the signal sequence more precisely and to examine if the signal sequence of the cytochrome can replace the transient signal sequence of interleukin 2, a secretory protein, for membrane translocation. For this purpose, three chimeraic cDNAs were constructed by ligating the cDNA fragments encoding the NH_2-terminal 20, 29 and 39 residues of cytochrome P-450 to a cDNA coding for mature interleukin 2. The proteins encoded by these chimeraic cDNAs were synthesized by in vitro transcription and translation and examined for their capacity to be co-translationally inserted into and translocated across microsomal membranes. The results thus obtained suggest that the cytochrome contains signal sequence and a stop-transfer signal within or near a small segment spanning the 21st through 29th residues. Because of the presence of a stop-transfer sequence, this segment cannot trans-

locate the fused interleukin 2 moiety across the membranes. Based on these observations, the topological orientation of cytochrome P-450 in microsomal membranes is also discussed.

MATERIALS AND METHODS

Materials

pMIL2-45, a plasmid in which murine pre-interleukin 2 cDNA is inserted into the PstI site of pBR322, was a generous gift from Dr. T. Taniguchi of the Institute of Molecular and Cellular Biology, Osaka University (12). Oligodeoxynucleotides (24mer) shown in Fig. 2 were kindly supplied by Nikkaki Co. These oligonucleotides were synthesized by the phosphoamidate method in an Applied Biosystems model A383A DNA synthesizer and supplied in deblocked form. They were concentrated by lyophilization, desalted by gel filtration on a Sephadex G-50 equilibrated with triethylamine carbonate (pH 8.0) and then lyophilized. Bovine trypsin (typeIII) was purchased from Sigma Chemical Co. The other materials used were obtained or purchased as described in part II of this thesis.

DNA Construction

pSP-N39/IL2. Murine pre-interleukin 2 cDNA was excised from pMIL2-45 with PstI and inserted into the PstI site of pSP-65 to construct pSP-IL2. The subsequent manipulations performed are outlined in Fig. 1. pSP-IL2 was digested with HincII and PvuI (Digest 1). pSP-T39 containing a cDNA fragment that encodes the

NH_2-terminal 39 residues of a β-naphthoflavone-inducible form of rabbit liver cytochrome P-450 (see Fig. 2 of Part II) was cut with BamHI and the cohesive ends were filled in with Klenow fragment. This was then digested with PvuI (Digest 2). Digest 1 and 2 were mixed, ligated and used to transform E. coli HB-101. Fifty transformants were randomly selected and their plasmid DNAs were prepared by the conventional alkali sodium dodecylsulfate (SDS) method (11). By restriction enzyme mapping of these DNAs, several clones were found to contain a cDNA encoding a fused protein consisting of the NH_2-terminal 39 residues of cytochrome P-450 and mature interleukin 2 plus the last 2 residues of the signal sequence. One of these clones, called pSP-N39/IL2, was used in this study.

pSP-N29/IL2 and pSP-N20/IL2. These two plasmids were constructed by oligonucleotide-directed, site specific mutagenesis essentially as described by Zoller and Smith (13, 14). The 24mer oligodeoxynucleotides used as primers were designed to cause deletion of DNA stretches encoding the 30th through 39th residues (in pSP-N29/IL2) and the 21st through 39th residues (in pSP-N20/IL2) of cytochrome P-450 in the chimeraic protein encoded by pSP-N39/IL2 (see Fig. 2). The cDNA was excised from pSP-N39/IL2 with EcoRI and PstI and ligated to the replicative form of phage M13 mp11 that had been cut with EcoRI and PstI.

The primer oligonucleotide (7 pmoles), which had been 5'-phosphorylated with ATP and T_4 polynucleotide kinase, was annealed with mp11-N39/IL2 (1 pmole) in 10 ul of 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 50 mMNaCl and 1 mM DTT.

After incubation at 65 C for 20 min, primer extension was carried out in 30 ul of 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 0.3 mM dATP, 0.3 mM dGTP, 0.3 mM dCTP, 0.3 mM dTTP, 1.7 mM ATP, 3 units of Klenow fragment and 200 units of T_4 DNA ligase. After incubation at 22 C for 12 h, the reaction mixture was used to transfect E. coli JM 103. Thirty six plaques were randomly picked up and further cultured for phage preparation. The phage DNAs were screened by means of single track sequencing (13) for clones caring inserts having the desired deletion (see Fig. 7). The phages thus screened were isolated by single plaque isolation and the replicative form of their DNAs was prepared. The deleted cDNAs were excised from the phage DNAs with EcoRI and PstI. The excised cDNAs were then isolated by agarose gel electrophoresis and subsequent electro-elution and inserted between the EcoRI and PstI sites of pSP-65. The clones having shorter and longer deletions were designated pSP-N29/IL2 and pSP-N20/IL2, respectively. The structures of chimeraic proteins encoded by the three cDNAs constructed above are illustrated in Fig. 3.

Other Methods

In vitro transcription and translation were carried out as described in partII, except that the transcript was directly used for translation without phenol/chloroform extraction. DNA templates for in vitro transcription were prepared from 500 ml of cultures by alkali-SDS extraction and subsequent CsCl gradient centrifugation (11). DNA manipulations were generally performed

as described by Maniatis et al (11).

Accessibility of trypsin to the translation products synthesized in the presence of dog pancreas rough microsomes (RM) was examined essentially as described by Yost et al (15). After translation in the presence of RM, 1 ul trypsin (2 mg/ml) and 1 ul of 100 mM $CaCl_2$ were added to 10 ul of the translation mixture and the mixture was incubated at 23 C for 30 min. The digestion was then stopped by the addition of 200 ul of 10 % trichloroacetic acid and the precipitate formed was collected by centrifugation. The precipitate was analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. All the other procedures were carried out as described in Part II of this thesis.

RESULTS

Construction of pSP-N39/IL2 and Its Transcription and Translation in vitro

Our first objective in this study was to examine whether the amino-terminal 39 amino acid residues of rabbit liver microsomal cytochrome P-450 is capable of functionally replace transient signal sequences of secretory proteins. For this purpose, a cDNA fragment encoding the NH_2-terminal 39 residues of the cytochrome was ligated to a cDNA specifying mature murine interleukin 2 plus upstream 2 amino acid residues (residues 19 and 20 of the interleukin 2 signal sequence) at the HincII site (see Fig. 2). The fused protein encoded by this cDNA construct should, therefore, retain the site that can be cleaved by signal peptidase (arrow in Fig. 3a). The construction was performed as outlined in Fig. 2

and, therefore, in the construct obtained, pSP-N39/IL2, the chimeraic cDNA is placed just downstream of powerful SP-6 promoter. This should permit transcription of the cDNA with the aid of SP-6 RNA polymerase. As shown in Fig. 4A, nucleotide sequencing of the joining region of the chimeraic cDNA by the chain termination method confirmed that the joining was performed as designed and the correct reading frame is maintained. We therefore attempted to transcribe pSP-N39/IL2 *in vitro*. Transcription of pSP-IL2 encoding the entire sequence of pre-interleukin 2 was also carried out. As can be seen in Fig. 5 (Lane 1 and 2), upon agarose gel electrophoresis and ethidium bromide staining each of the transcripts of these two clones gave two mRNA bands. Because the transcription templates used were linearized at the *Pvu*I site located at 1320 bp downstream of the cDNAs, it was likely that a certain stretch of this vector acted as a transcription terminator for SP-6 RNA polymerase, leading to the formation of two mRNAs for each protein. The mRNAs thus synthesized were efficiently translated in a wheat-germ cell-free system. When the translates obtained in the presence of [^{35}S]methionine were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent fluorography, each of the translates gave an intense protein band having an apparent molecular mass expected for pre-interleukin 2 or the chimeraic protein (Fig. 6A, Lane 1; Fig. 6B, Lane 1). This also confirmed that the two species of mRNA detected in Fig. 5 (Lanes 1 and 2) coded for a single protein.

Membrane Translocation and Insertion of Protein Products Encoded by pSP-IL2 and pSP-N39/IL2

When the translation of pSP-IL2 mRNA was performed in the presence of RM, interleukin 2 precursor was processed to the mature protein, albeit the processing efficiency was very low (Fig. 6A, Lane 2). The reason for the low processing activity for pre-interleukin 2 is not known. However, in view of the fact that the RM used in this study had a high processing activity toward bovine preprolactin (see Fig. 3 of Part I), it seems that the wide spectrum of signal peptidase specificity is reflected in the present results. When this translate containing RM was digested with trypsin, the precursor was completely degraded, whereas mature interleukin 2 was protected from degradation (Fig. 6A, Lane 3). In the presence of 1 % Triton X-100 both precursor and mature forms were susceptible to trypsin digestion (Fig. 6A, Lane 4). Upon alkali treatment (20 mM Na_2CO_3) both pre- and mature interleukin 2 were recovered in the supernatant fraction (Fig. 6A, Lane 5), indicating that both proteins were not tightly associated with the membranes. These observations are consistent with the view that pre-interleukin 2 was actually translocated across the microsomal membranes, processed by signal peptidase, released into the lumen, and thus protected from tryptic digestion, as has been observed for many other secretory proteins (9). On the other hand, the chimeraic protein encoded by pSP-N39/IL2, synthesized in the presence of RM, was neither processed nor protected from trypsin digestion even in the absence of Triton X-100 (Fig. 6B, Lanes 2, 3 and 4). Upon alkali treatment, however,

a substantial amount of the chimeraic protein (30 % of the protein applied) was recovered in the membrane fraction (Fig. 6B, Lane 5). It was, therefore, concluded that the chimeraic protein could actually be inserted into the membranes but could not be translocated into the lumen. Inasmuch as both the precursor and mature forms of interleukin 2 were not associated with microsomes under highly stringent washing conditions (Fig. 6A, Land 5), it is not unreasonable to assume that a segment consisting of the amino-terminal 39 residues of cytochrome P-450 contained, the signal sequence and another sequence that stops the transfer of the chimeraic protein across the membranes ("stop-transfer" sequence).

Membrane Insertion in Vitro of Products of pSP-N29/IL2 and pSP-N20/IL2

To further characterize the signal sequence and putative stop-transfer sequence in the NH_2-terminal portion of cytochrome P-450, two deletion mutants of pSP-N39/IL2 in which stretches encoding the 30th through 39th residues (pSP-N29/IL2) and the 21st through 39th residues (pSP-N20/IL2) are missing, were constructed by oligonucleotide-directed site specific mutagenesis, as described under Materials and Methods. In each case , 36 plaques due to phage M13 mp11 carrying the original and deleted inserts were randomly selected. The DNA inserts in the selected phage clones were examined by single track sequencing for deletion. As shown in Fig. 7, in each case one mutant clone was obtained. That the clones thus isolated had the respective

desired deletions was confirmed by nucleotide sequencing of the joining region of the inserts (see Fig. 3B, C and Fig. 4). These cDNA inserts were excised from the phages and inserted between the EcoRI and PstI sites of pSP-65. These constructs were termed pSP-N29/IL2 and pSP-N20/IL2. When these plasmids were linearized and transcribed *in vitro*, only one mRNA species was produced in each case (Fig. 5, lanes 3 and 4). In these experiments, the template DNAs were linearized with PstI, which cuts the plasmids at the end of their cDNA inserts (see Fig. 1). The mRNA encoded by pSP-N29/IL2 was then translated in the wheat-germ cell-free system in the presence of [^{35}S]methionine and the products were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. As shown in Fig. 8A (lane 1), one major band together with several minor bands were detected and the size of the major band agreed with that expected from the length of template cDNA. When this translation was carried out in the presence of RM, the product was not processed (Fig. 8A, lane 2) and was sensitive to trypsin digestion both in the absence and presence of Triton X-100 (Fig. 8A, lanes 3 and 4). However, a substantial portion of the product was found to be associate with the microsomal membranes after the alkali treatment (Fig. 8A, lane 5). These findings are consistent with the view that the product encoded by pSP-N29/IL2 could be inserted into the membranes but could not be translocated into the lumen of the microsomal vesicles. It was, therefore, suggested that both the signal and stop-transfer sequences of cytochrome P-450 are contained in the segment spanning the NH_2-terminal 29 amino acid

residues. The same translation experiments were also performed with the mRNA encoded by pSP-N20/IL2 as template and the results obtained are shown in Fig. 8B. As can be seen, only one protein product was synthesized in the absence of the membranes (Fig. 8B, lane 1). No processing of this product was observed when the translation was performed in the presence of RM (Fig. 8B, lane 2) and it was degraded with trypsin added post-translationally both in the absence and presence of Triton X-100 (Fig. 8B, lanes 3 and 4). In contrast to the case of the pSP-N29/IL2 product, the product in this case was recovered only in the supernatant fraction after the alkali treatment (Fig. 8B, lane 5). It was, therefore, concluded that the protein encoded by pSP-N20/IL2 could be neither inserted into nor translocated across the membranes.

DISCUSSION

The results described above leave little doubt that the signal sequence of cytochrome P-450, required for its membrane insertion, exists in the NH_2-terminal stretch consisting of 29 amino acid residues. The finding that the chimeraic protein consisting of the NH_2-terminal 20-residue segment and the mature interleukin 2 sequence cannot be inserted into the membrane further suggests that the signal sequence is located within the stretch spanning the 21st through 29th residues or it extends over the 20th residue toward the NH_2-terminus. The available results, however, do not permit one to estimate the actual size and precise location of the signal sequence.

Since the hydropathy profile and secondary structure of the cytochrome P-450 sequence from the 10th to 29th residues, predicted by the methods of Kyte and Doolittle (16) and Chou and Fasman (17), respectively, are closely similar to those predicted for the NH_2-terminal 20-residue signal sequence of interleukin 2 (data not shown), it was though that these two sequences are functionally interchangeable. However, the failure of the chimeraic protein containing the NH_2-terminal 29-residue segment and the mature interleukin 2 sequence to be translocated across the membranes indicates that this is not the case. A highly likely possibility is to assume that the 29-residue segment contains a sequence that halts further translocation of the polypeptide chain across the membranes, in addition to the signal sequence that is recognized by SRP and inserts the nascent chain into the membranes.

Such "stop-transfer" sequences have been detected in several plasma membrane proteins such as vesicular stomatitis virus G protein (18) and immunoglobulin M heavy chain (15). These proteins possess a cleavable signal sequence and by this signal a substantial portion of the molecule is translocated across the membranes and, thereafter, becomes anchored by a hydrophobic region ("transmembrane sequence"). Within or immediately after this hydrophobic region there must be a sequence that halts further translocation of the polypeptide chain and thus generates a transmembrane protein. Such a sequence is called a stop-transfer sequence (10). In a number of transmembrane proteins located in the plasma membrane the hydrophobic (transmembrane)

sequence is immediately followed by a positively charged region and this segment is thought to represent the stop-transfer sequence (1).

What is a candidate for the stop-transfer sequence in cytochrome P-450? Examination of the NH_2-terminal 39-residue sequence of cytochrome P-450 (Fig. 3) reveals that three basic residues (Lys 34, Arg 36, and Lys 39) are located after a relatively long stretch, rich in hydrophobic residues. It is tempting to assume that this cluster of basic amino acid residues constitutes the stop-transfer sequence. However, the behavior of the chimeraic protein containing only 29 residues derived from the NH_2-terminus of cytochrome P-450 indicates that this protein has the stop-transfer sequence even though it lacks the basic amino acid cluster. This leads to the conclusion that both signal and stop-transfer sequences must exist in the hydrophobic stretch extending up to the 29th resideu. Further refinement of the location of stop-transfer sequence as in the case of the signal sequence, requires further investigations. However, there are two possibilities. First, in cytochrome P-450 an identical sequence serves the two functions. In this case the signal sequence of cytochrome P-450 is functionally different from those of secretory proteins. The second possibility is that the signal and stop-transfer sequences are located separately. In any case, it can be concluded that both the signal and stop-transfer sequences of cytochrome P-450 are located within a relatively short NH_2-terminal segment up to the 29th residue.

In Part II of this thesis, it has been pointed out that

there are no signal sequences serving functions in membrane insertion and translocation in the COOH-terminal portion of cytochrome P-450 after the 99th residue. It is, therefore, highly likely that the signal and stop-transfer sequences at the NH_2-terminal portion are the only sequences contributing to the formation of intramembrane topology of cytochrome P-450. If this is actually so, it should be concluded that the NH_2-terminal 29 residues or less of cytochrome P-450 are tightly inserted into microsomal membranes and the rest of the molecule is extruded to the cytoplasmic side. Based solely on the primary structure, several models have been proposed for membrane topology of rabbit liver microsomal cytochrome P-450 (19,20). In these models it is assumed that the cytochrome P-450 polypeptide chain spans the membrane 8 to 10 times. However, the discussion described above shows that these models are very unlikely.

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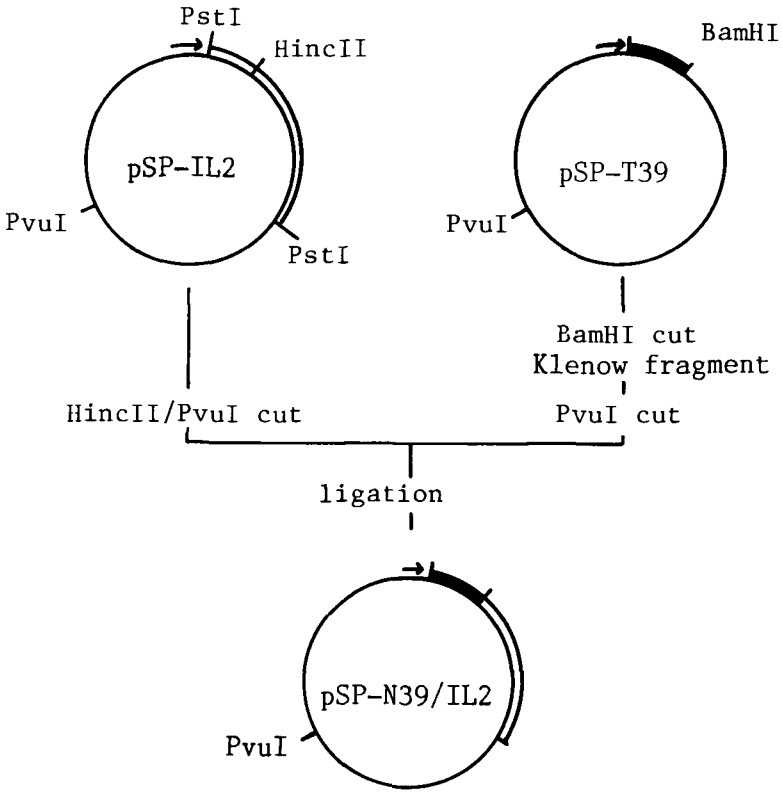

Fig. 1. Construction of pSP-N39/IL2. Two SP-6 plasmids carrying cDNAs for murine pre-IL2 and the NH_2-terminal 39 residues of cytochrome P-450 were manipulated to obtain pPS-N39/IL2, where a cDNA frangment encoding the signal sequence of pre-IL2 is replaced by cDNA fragments encoding the NH_2-terminal regions of cytochrome P-450. Details are described in Materials and Methods. Arrows above plasmid circles indicate both the position of SP6-promotor and the direction of trnascription.

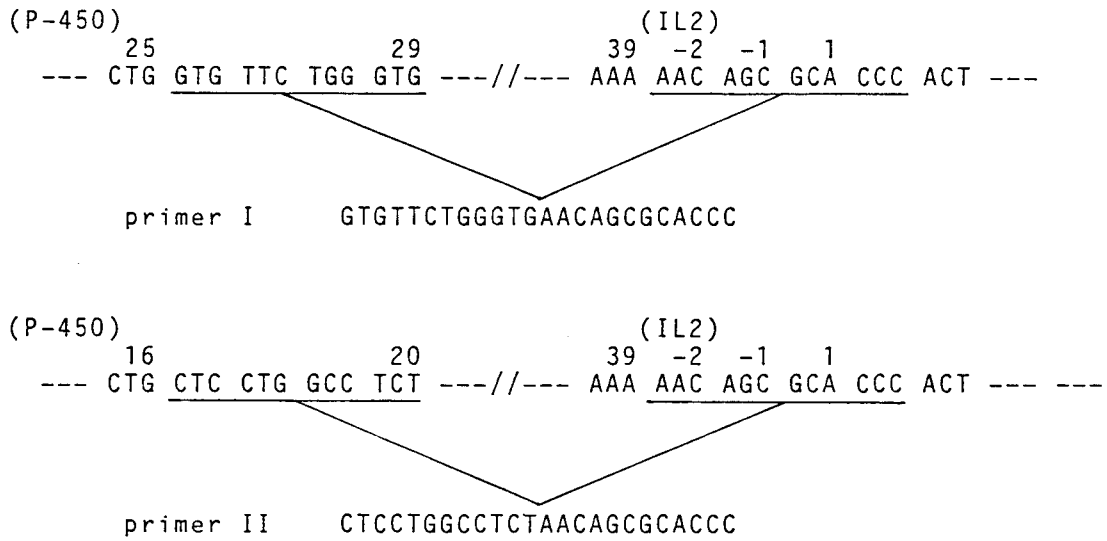

Fig. 2. Oligonucleotide-directed, site-specific mutagenesis of pSP-N39/IL2. Oligodeoxyribonucleotides (24 mer) used as primers are shown (primers I and II). Primers I and II were annealed with mp11-N39/IL2. After primer extension reaction, the reaction mixtures were used for transfection of E. coli strain JM 103. For further details, see Materials and Methods. Only the junctional regions of chimeric cDNA in pSP-N39/IL2 are depicted in the figure. Numbers above the base sequences correspond to those of amino acid sequences of cytochrome P-450 and pre-IL2.

Fig. 3. The NH_2-terminal sequence of chimeric proteins encoded by pSP-N39/IL2 (a), pSP-N29/IL2 (b), and pSP-N20/IL2 (c). The segments derived from the NH_2-terminal portion of cytochrome P-450 are in bold-faced type, whereas those derived from pre-IL2 are in script-faced type. The two-residue segment underlined (GI) represents the Bam HI junction. Residue numbers above the sequence correspond to those of cytochrome P-450, whereas those below the sequence pre-IL2. The site cleavable by signal peptidase is indicated by an arrowhead.

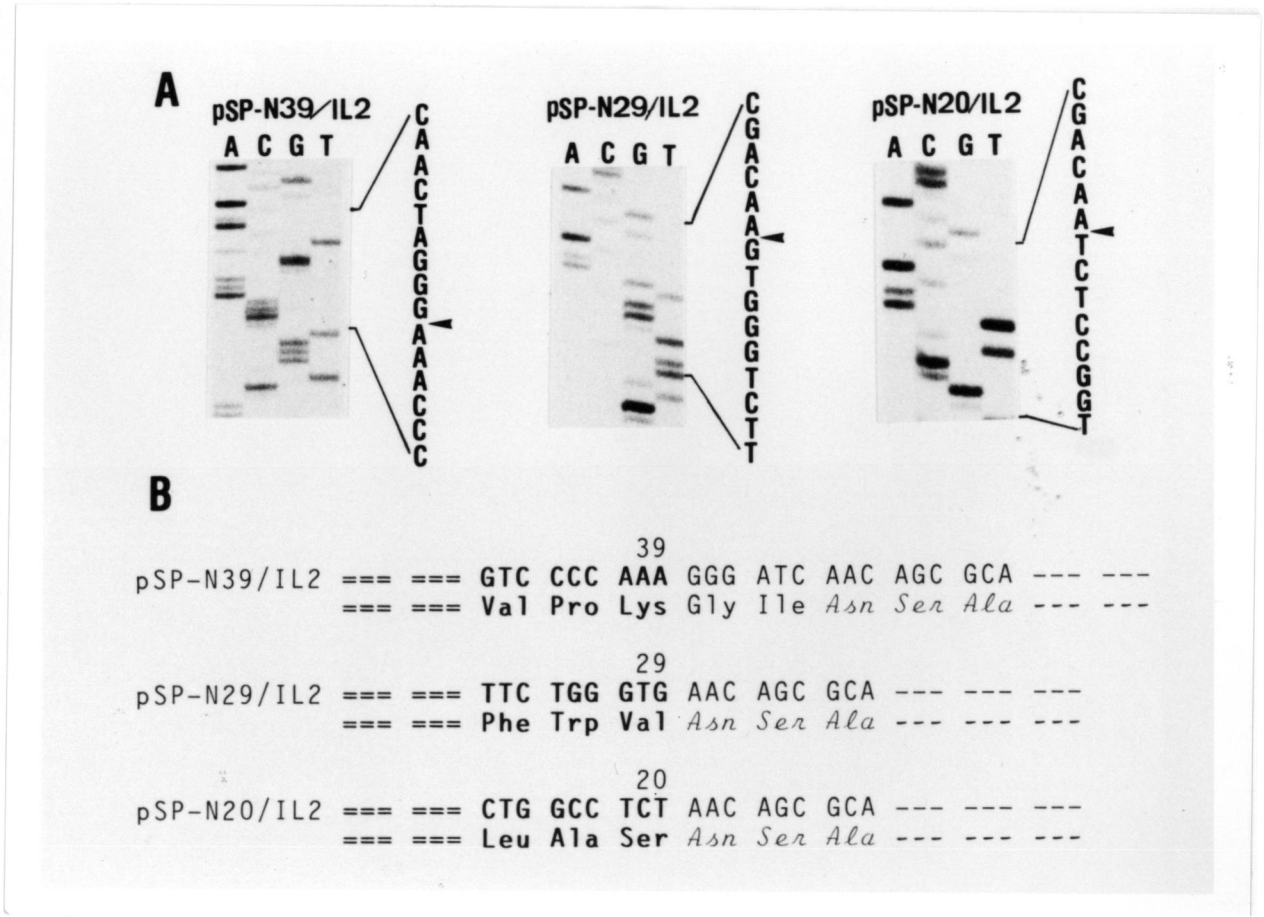

Fig. 4. Structure of the flanking region of the chimeric proteins. (A) DNA sequencing ladders of the flanking region of the chimeric cDNAs in pSP-N39/IL2 (left), pSP-N29/IL2 (centre) and pSP-N20/IL2 (right). Arrowheads indicate the junctions between the sequences for cytochrome P-450 and those for IL2. (B) DNA sequences encoding the chimeric proteins and amino acid sequences deduced therefrom. Numbering is from the NH_2-terminus of cytochrome P-450. The regions derived from cytochrome P-450 and pre-IL2 are in bold- and script-faced types, respectively.

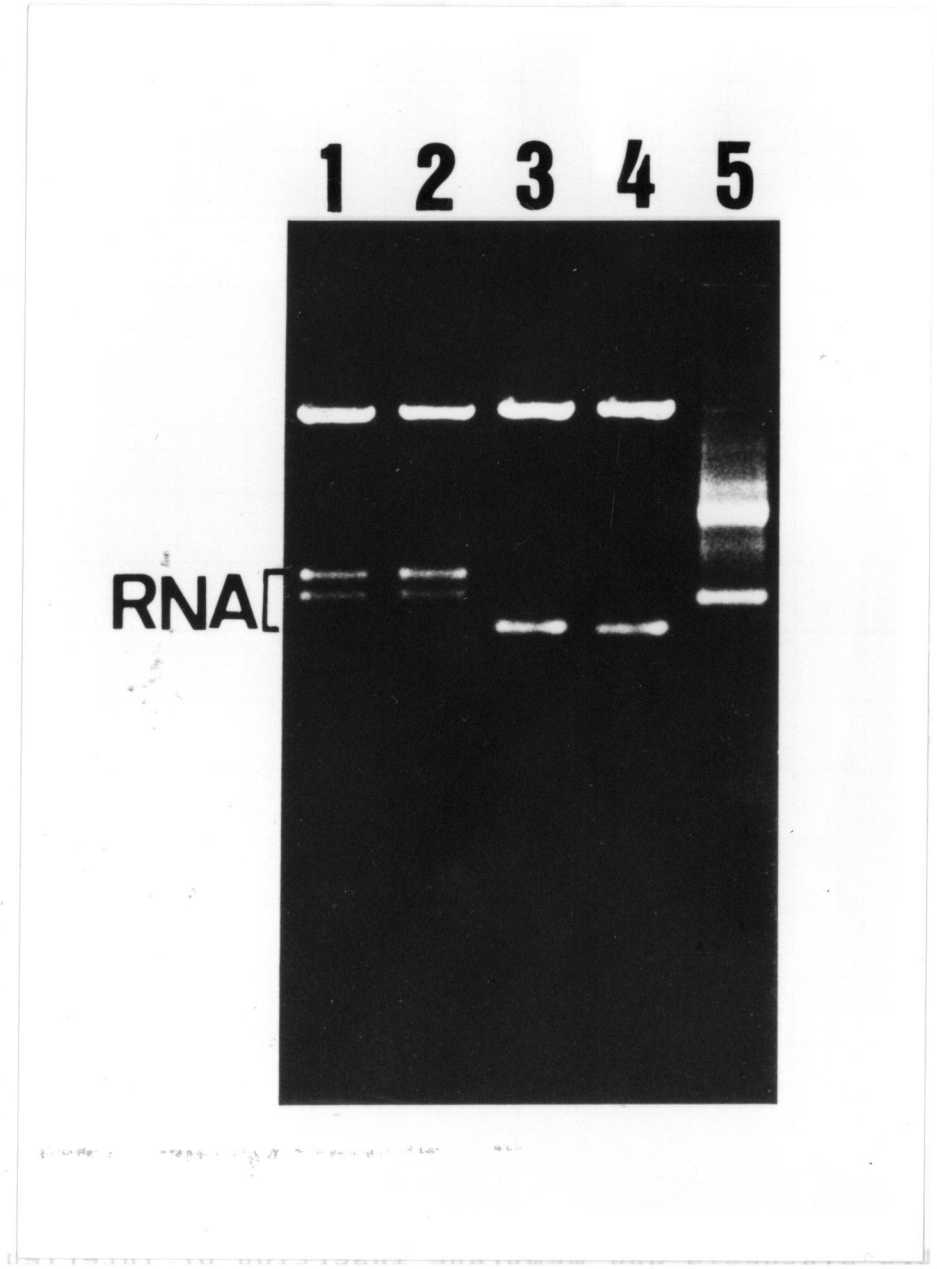

Fig. 5. In vitro transcription of SP6-plasmids carrying cDNAs for pre-IL2 and its chimeric derivatives. pSP-IL2 (Lane 1) and pSP-N39/IL2 (Lane 2) were linearlized with PvuI, while pSP-N29/IL2 (Lane 3) and pSP-N20/IL2 (Lane 4) were linearlized with PstI. The templates were then transcribed in vitro by SP6-RNA polymerase as described under Materials and Methods. Aliquots of the transcripts were analyzed by agarose gel (1.5%) electrophoresis in 1 x TPE containing ethidium bromide and photographed under a long-wave UV lamp. 3.7 ug of calf liver ribosomal RNA was used as a moleculer weight marker (Lane 5).

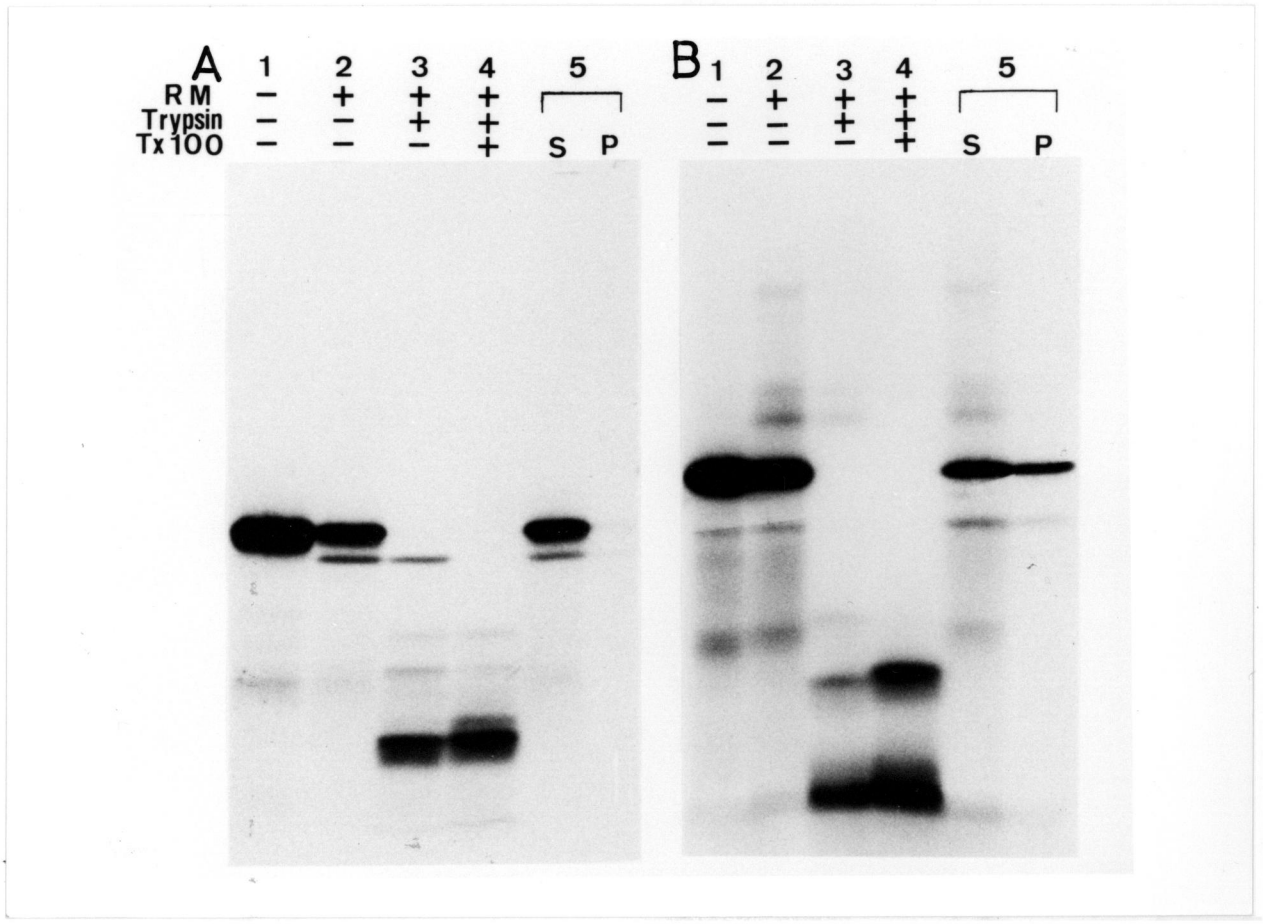

Fig. 6. In vitro synthesis and membrane insertion of interleukin 2 (Panel A) and a chimeric protein encoded by pSP-N39/IL2 (Panel B). MRNAs transcribed from pSP-IL2 and pSP-N39/IL2 were translated in the wheat-germ cell-free system containing [^{35}S]methionine with (+) or without (-) RM. 10 ul aliquots were digested with trypsin in the presence (+) or absence (-) of Triton X-100. In lanes 5, the same experiments were performed as in lanes 2 except that, after translation, the reaction mixtures were treated with 50 mM Na_2CO_3 followed by separation into supernatant (S) and membrane (P) fractions by ultra-centrifugation. The total reaction mixtures (Lanes 1 through 4) as well as fractions S and P (Lanes 5) were directly applied to SDS-polyacrylamide gel electrophoresis and subsequent fluorography.

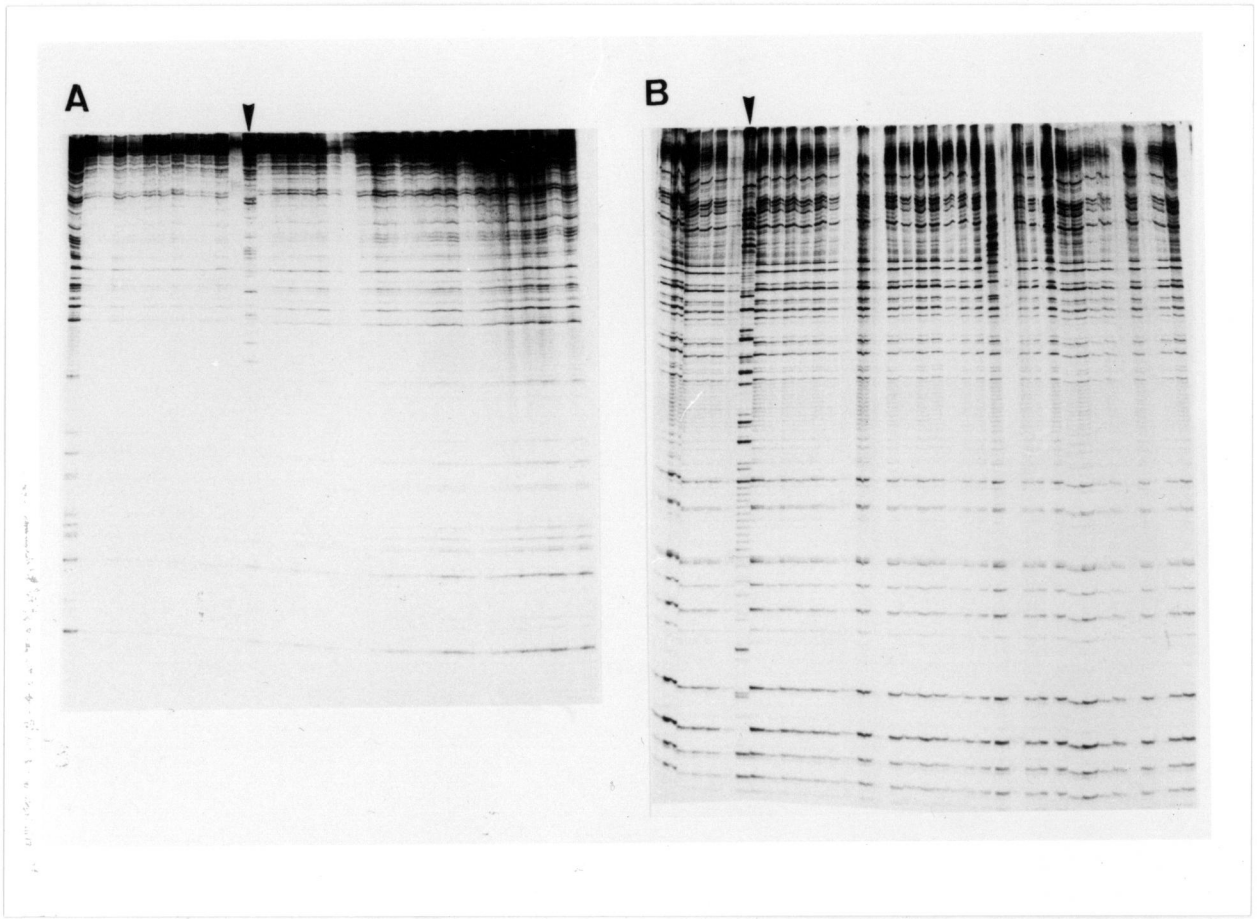

Fig. 7. Screening of mutant phages by single-track sequencing. 36 white plaques were randomly selected and single-stranded DNAs were prepared. The dideoxy chain-terminator sequencing was performed for these DNAs. Shown are sequencing ladders for screening N29/IL2 mutants (Panel A) and for N20/IL2 mutants (Panel B). Dideoxy GTP and dideoxy CTP wer used as a chain-terminator for (A) and (B), respectively. Arrowheads indicate the ladder of the desired mutants.

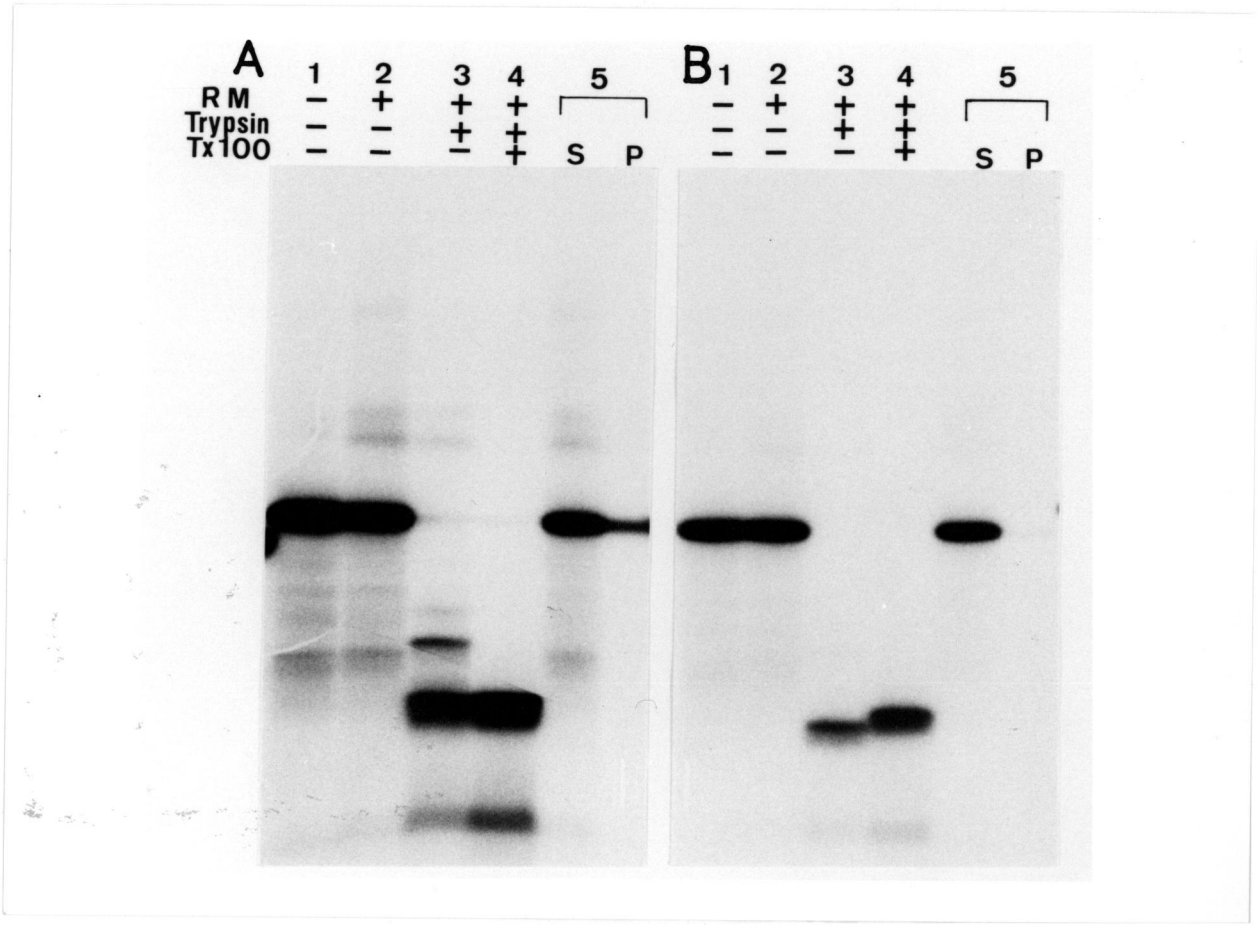

Fig. 8. In vitro synthesis and membrane insertion of chimeric proteins encoded by pSP-N29/IL2 (Panel A) and pSP-N20/IL2 (Panel B). mRNAs transcribed from pSP-N29/IL2 and pSP-N20/IL2 were translated in the wheat-germ cell-free system contaning [^{35}S]methionine with (+) or without (-) RM. All the other conditions are exactly same as described in the legend to Fig. 6.

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