Signals for the Incorporation and Orientation of Cytochrome P450 in the Endoplasmic Reticulum Membrane

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Abstract. Cytochrome P450b is an integral membrane protein of the rat hepatocyte endoplasmic reticulum (ER) which is cotranslationally inserted into the membrane but remains largely exposed on its cytoplasmic surface. The extreme hydrophobicity of the amino-terminal portion of P450b suggests that it not only serves to initiate the cotranslational insertion of the nascent polypeptide but that it also halts translocation of downstream portions into the lumen of the ER and anchors the mature protein in the membrane. In an in vitro system, we studied the cotranslational insertion into ER membranes of the normal P450b polypeptide and of various deletion variants and chimeric proteins that contain portions of P450b linked to segments of pregrowth hormone or bovine opsin. The results directly established that the amino-terminal 20 residues of P450b function as a combined insertion–halt-transfer signal. Evidence was also obtained that suggests that during the early stages of insertion, this signal enters the membrane in a loop configuration since, when the amino-terminal hydrophobic segment was placed immediately before a signal peptide cleavage site, cleavage by the luminally located signal peptidase took place. After entering the membrane, the P450b signal, however, appeared to be capable of reorienting within the membrane since a bovine opsin peptide segment linked to the amino terminus of the signal became translocated into the microsomal lumen. It was also found that, in addition to the amino-terminal combined insertion–halt-transfer signal, only one other segment within the P450b polypeptide, located between residues 167 and 185, could serve as a halt-transfer signal and membrane-anchoring domain. This segment was shown to prevent translocation of downstream sequences when the amino-terminal combined signal was replaced by the conventional cleavable insertion signal of a secretory protein.

THE endoplasmic reticulum (ER)[1] is a membrane-bound organelle richly endowed with a variety of enzymatic systems that operate on cytoplasmic substrates or on molecules segregated in its lumen. Some of these systems carry out general biosynthetic functions whereas others participate in metabolic reactions that may be characteristic of specific cell types. Several different mechanisms ensure that proteins incorporated into the ER acquire the disposition, with respect to the membrane, that is necessary for their function. Those polypeptides that reside in the ER lumen or are peripherally attached to the luminal surface of the membrane, such as the hepatocyte microsomal esterases (36), the disulfide exchange enzyme (15), and a recently described protein that binds incompletely assembled secretory polypeptides (7, 40), must use the same mechanism that is used to segregate newly synthesized secretory proteins and lysosomal hydrolases into the lumen. These nascent chains contain amino-terminal transient signal sequences that determine their cotranslational insertion into the membranes of the rough ER and mediate their complete translocation into the lumen of the organelle.

Those ER proteins, such as the ribophorins (24, 51) and UDP glucuronyl-transferase (35), that have single membrane-spanning segments and large glycosylated amino-terminal domains free in the lumen of the ER are topologically similar to a class of well-studied plasma membrane glycoproteins (type I proteins), which includes the G and HA viral envelope glycoproteins and the low density lipoprotein and epidermal growth factor receptors (21, 22, 48, 53, 64). These proteins are thought to acquire their transmembrane disposition during their synthesis as a result of the action of two different types of signals that are present within the same polypeptide chain (5, 53): a cleavable amino-terminal insertion signal which is equivalent to that in secretory proteins, and a halt-transfer signal which interrupts cotranslational translocation and contains a hydrophobic segment that serves to permanently anchor the mature protein in the membrane. The relative lengths of the luminal and cytoplasmic domains of these proteins are determined by the location of the halt-transfer signal within the polypeptide.

Portions of this work have appeared in abstract form (1986. *J. Cell Biol.* 103 [No. 5, Pt. 2]:290*a*).

1. *Abbreviation used in this paper*: ER, endoplasmic reticulum.

The ER also contains integral membrane proteins that are not glycosylated and are almost completely exposed on the cytoplasmic surface of the ER membrane (see reference 30). Some polypeptides of this type, like cytochrome b_5 and NADH-cytochrome b_5 reductase, are synthesized on free polyribosomes and are inserted in the membranes only after they are discharged into the cytosol (8, 44, 47). Others, such as NADPH-cytochrome P450 reductase, are synthesized on membrane-bound ribosomes but do not undergo extensive translocation across the membrane (4, 44). The P450 reductase, for example, is anchored in the membrane solely by a short hydrophobic segment that is located near the amino-terminus (4, 46) and is likely to serve both as an uncleaved insertion signal and as a membrane-anchoring domain. The fact that the regions of the polypeptide that follow the membrane-embedded segment are not translocated across the ER membrane suggests that this segment also serves as a halt-transfer signal which, after insertion into the membrane, interrupts translocation. In this regard, the permanent insertion-signal membrane-anchoring domain of such proteins differs fundamentally from the permanent insertion-signal membrane-anchoring domains identified in plasma membrane glycoproteins that cross the membrane only once but have large luminally exposed carboxy-terminal portions (type II proteins), such as the influenza neuroaminidase (6), the sucrase isomaltase (26), and the asialoglycoprotein (57) and transferrin receptors (56). In these cases, the insertion signals, although remaining membrane associated, are able to mediate the translocation of later portions of the polypeptides across the membrane.

Like its reductase, hepatic cytochrome P450, the mixed function oxidase responsible for the metabolism of a wide variety of endogenous and exogenous substrates, is also synthesized on membrane-bound ribosomes and does not undergo cleavage of a signal sequence during its insertion into the membrane (3, 45). Although it is now clear that there are at least a dozen distinct species of cytochrome P450 in rat liver (1), most studies on the biosynthesis and membrane disposition of these polypeptides (3, 13, 34, 60) have been carried out with two closely related (~97% sequence identity) phenobarbital-inducible forms, P450b and P450e, now designated P450IIB1 and P450IIB2 (42). The use of macromolecular probes such as proteases (10, 43), antibodies (13, 52, 60), and enzymatic labeling reagents (50, 63) has shown that large portions of these P450 polypeptides are exposed on the cytoplasmic surface of the ER membrane, although it has not been possible to recover after proteolytic treatment of intact microsomal vesicles sizable solubilized fragments of these molecules. These findings, together with the extremely hydrophobic character of the amino-terminal segment of P450b (Fig. 2), have suggested (3) that this segment also serves as a combined insertion-halt-transfer signal that determines the cytoplasmic exposure of the later portions of the polypeptide.

In this work we have analyzed the role of the amino-terminal portion of cytochrome P450b in determining the incorporation of this polypeptide into the ER membrane and its disposition with respect to the phospholipid bilayer. The results demonstrate that the amino-terminal 20 residues of P450 display both insertion and halt-transfer functions and suggest that this portion of the polypeptide first enters the membrane in a loop configuration and soon thereafter reorients so that the amino terminus is transferred from the cytoplasmic to the luminal side of the membrane. It is proposed that this reorientation leads to the halt in translocation.

Materials and Methods

Reagents

SP6 RNA polymerase, RNase inhibitor (RNasin), and the pSP64 and pSP65 vector plasmids were purchased from Promega Biotec (Madison, WI) and other polymerases, restriction endonucleases, and mung bean exonuclease from New England Biolabs (Beverly, MA) and Bethesda Research Laboratories (Gaithersburg, MD). 7mGpppG was purchased from Pharmacia Fine Chemicals Inc. (Piscataway, NJ), and other nucleotides were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). All radiolabeled compounds were purchased from New England Nuclear (Boston, MA).

Oligonucleotides

Three synthetic double-stranded oligonucleotides were used to insert specific coding sequences into gene constructs or to link two pieces of DNA. Each was prepared by annealing as described below. Two complementary single-stranded oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems Inc. (Foster City, CA) model 380A DNA synthesizer. After the deblocking reaction in presence of NH_4OH, the oligonucleotides were purified by gel filtration through Sephadex G50, mixed, and annealed by boiling for 5 min in 0.5 M NaCl, 6.5 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 1 mM dithiothreitol, followed by rapid cooling to 65°C, and then slow cooling to room temperature. After annealing, the solution was desalted by gel filtration on a Sephadex G50 column, and the double-stranded product was purified by electrophoresis on a 10% polyacrylamide gel, followed by electroelution.

Plasmid Construction

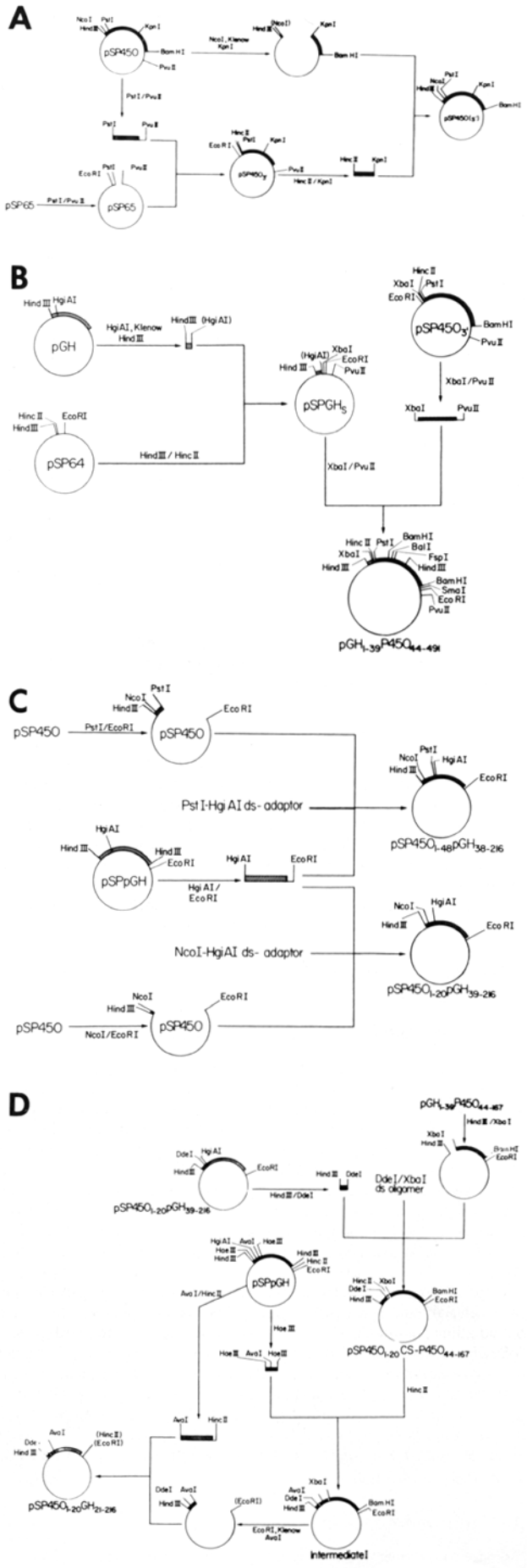
All the cDNAs were cloned into the plasmid vector pSP64 downstream from the SP6 bacteriophage promoter. The construction from two overlapping cDNA clones and a segment of a genomic clone of pSP450, a recombinant plasmid that contains the complete coding region of P450b cDNA in the plasmid vector pSP64, is described by Dohmer, J., S. Dogra, T. Friedberg, S. Monier, M. Adesnik, H. Glatt, and F. Oeschi (manuscript submitted for publication). The procedures used in constructing pSP450(s^-), $pSpGH_{1\text{-}39}P450_{44\text{-}491}$, $pSP450_{1\text{-}48}$, $pGH_{38\text{-}216}$, and $pSP450_{1\text{-}20}pGH_{21\text{-}216}$ are described in Fig. 1. The construction of the other plasmids used is described below. A list of the hybrid and modified polypeptides encoded in all these genes and relevant portions of their amino acid sequences are shown in Fig. 2.

Truncated Derivatives of $pSpGH_{1\text{-}39}P450_{44\text{-}491}$

Plasmids encoding a series of hybrid polypeptides similar to that encoded by $pSpGH_{1\text{-}39}P450_{44\text{-}491}$ but lacking carboxy-terminal portions of P450, were obtained by digestion of that plasmid with the appropriate restriction enzyme(s) and religation in the same vector, the termination codon being provided by the vector sequence. The P450 portions encoded in these derivatives terminated at amino acid residues 167, 246, 271, or 425 as a result of cleavages at the unique Bam HI, Bal I, Fsp I, and Hind III sites, respectively. For the first construct, the original plasmid was simply cut with Bam HI to remove the 3′ portion of the P450 cDNA and religated. The resulting plasmid ($pSpGH_{1\text{-}39}P450_{44\text{-}167}$) encodes a polypeptide that contains 6 (R A S S N S) additional COOH-terminal amino acids. The second plasmid ($pSpGH_{1\text{-}39}P450_{44\text{-}246}$) was obtained by digestion of the parental plasmid with Bal I and Sma I and religation. The encoded polypeptide contains 33 additional amino acids at its COOH terminus. The third plasmid ($pSpGH_{1\text{-}39}P450_{44\text{-}271}$) was obtained by cloning the Hind III-Fsp I fragment of the parental plasmid back into pSP64 cleaved with Hind III and Hinc II. The encoded polypeptide contained one extra amino acid, leucine, at its COOH terminus. The plasmid $pSpGH_{1\text{-}39}P450_{44\text{-}425}$ was obtained by cloning the Hind III fragment of the parental plasmid into pSP64. Six amino acids (W A A G R L) were added at the COOH terminus of the encoded polypeptide.

$pSpGH_{1\text{-}39}P450_{\Delta(166\text{-}203)}$

This plasmid encodes a hybrid polypeptide similar to $pGH_{1\text{-}39}P450_{44\text{-}491}$



from which a 39-amino acid segment extending from residues 166 to 203 of P450 was deleted. To effect this deletion, an Hpa II P450 cDNA fragment (bp 639 to 1035) was isolated from $pSpGH_{1\text{-}39}P450_{44\text{-}491}$ and, after its protruding ends were filled in with the Klenow fragment of DNA polymerase, it was ligated to $pSpGH_{1\text{-}39}P450_{44\text{-}491}$ from which the Bam HI P450 3′ cDNA fragment had been removed and the ends had been made blunt with mung bean nuclease. The Hind III–Fsp I fragment of this intermediate, carrying the required deletion, was rejoined to the 3′ end of the P450 cDNA in a three-way ligation with the Fsp I–Bam HI fragment of $pSP450_3$ (shown in Fig. 1 *A*) and the vector pSP64 cleaved with Hind III and $pSP450_{3'}$ Bam HI.

$pSpGH_{1\text{-}39}P450_{204\text{-}491}$

This plasmid encodes a hybrid polypeptide in which the first 39 residues of pregrowth hormone are linked to the carboxy-terminal 288 residues of

Figure 1. (*A*) Construction of pSP450(s⁻). This plasmid encodes a P450b polypeptide from which amino acids 2–43 were deleted. The manipulations involved in this construction were designed to fuse the filled-in Nco I site which contains the initiation codon with the Pst I site, without altering the reading frame. Nucleotides linking the two sites were derived from the multiple cloning site of pSP65. This resulted in one extra amino acid (Asp) being added at position 2, which was followed directly by Leu 44 of P450. (*B*) Construction of $pSpGH_{1\text{-}39}P450_{44\text{-}491}$. This plasmid encodes a chimeric protein in which the first 39 amino acids of rat pregrowth hormone were linked to residues 44–491 (COOH terminus) of P450 via a short extraneous peptide (DSRVD) encoded by a portion of the multiple cloning site in the vector pSP65. The first 117-bp fragment of pregrowth hormone cDNA, coding for the signal peptide plus 13 amino acids of the mature growth hormone, was linked to a 3′ portion of the P450 cDNA located downstream from the Pst I site (contained in plasmid $pSP450_{3'}$, shown in *A*) by a small DNA segment derived from the multiple cloning sites of pSP64 and pSP65. (*C*) The plasmid $pSP450_{1\text{-}48}pGH_{38\text{-}216}$ encodes a hybrid polypeptide consisting of the amino-terminal 48 residues of P450 followed by the growth hormone sequence, beginning at residue 38 of pregrowth hormone (i.e., residue 12 of the mature protein) and extending to its COOH-terminal end. In this construction, the 5′ Nco I–Pst I fragment of the P450 cDNA was linked to a 3′ fragment of the rat growth hormone cDNA beginning at its Hgi AI site. This was achieved using a double-stranded oligonucleotide adaptor (21-mer) that encodes three amino acids of rat pregrowth hormone (Asn 38 to Val 40) and two amino acids of P450 (Leu 46 and Arg 48) but introduces an alanine residue in place of an aspartic acid at position 47 of P450 to create a convenient restriction site (Bal I) useful in subsequent cloning procedures. The plasmid $pSP450_{1\text{-}20}pGH_{39\text{-}216}$ encodes a hybrid polypeptide that contains the first 20 amino acids of P450 linked to growth hormone beginning at residue 39 of the pregrowth hormone sequence; i.e., residue 13 of the mature protein. In this construction, the Nco I site of P450 was linked to the Hgi AI site via a synthetic adaptor that restores the first 20 codons of P450 (Met 1 to Val 20) and residues 39 and 40 (Ala 39 Val 40) of rat pregrowth hormone. (*D*) Construction of $pSP450_{1\text{-}20}pGH_{21\text{-}216}$. This plasmid encodes a hybrid polypeptide consisting of the first 20 amino acids of P450 linked to residue 21 of the pregrowth hormone signal. The polypeptide contains the signal peptidase cleavage site (after residue 26) in pregrowth hormone and the entire growth hormone sequence. First, an intermediate, $pSP450_{1\text{-}20}$-CS-$P450_{44\text{-}167}$ was constructed that encodes the first 20 amino acids of P450, an artificial signal peptidase cleavage site (DAGAL) designed according to the rules of von Heijne (61), four extraneous residues (SRVD) encoded by the vector sequence, and residues 44–167 of P450. The synthetic adaptor (Dde I/Xba I double-stranded oligomer) encoding the cleavage site contained a Hinc II site at the codon for Val 20 of P450. This permitted direct linkage of this codon to codon 21 of the pregrowth hormone cDNA via the Hae III site in this DNA.

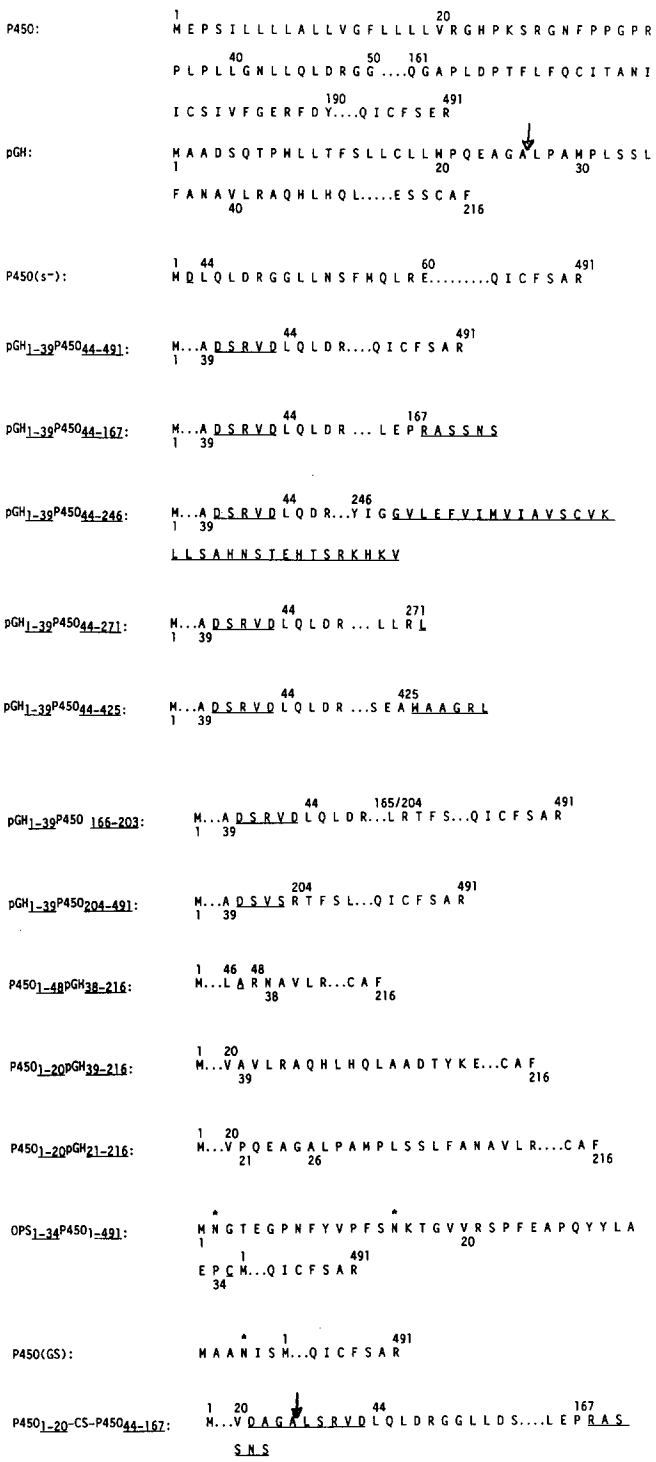


Figure 2. Amino acid sequence of natural, mutated, and chimeric polypeptides used in membrane-insertion studies. The single letter amino acid abbreviations are used. Numbers over residues indicate their position within rat cytochrome P450b (20, 65). Numbers below residues correspond to position within rat pregrowth hormone or bovine opsin (43). Residues that are not present in the natural polypeptides but are encoded by nucleotide sequences introduced by genetic manipulations are underlined. *Asterisks*, potential glycosylation sites. *Arrows*, potential cleavage sites for the signal peptidase.

P450 via a short extraneous peptide (DSRV). For its construction, the same blunted Hpa II fragment of P450 cDNA (bp 639 to 1035) used in the construction of $pSpGH_{1\text{-}39}P450_{\Delta(166\text{-}203)}$ was ligated to the plasmid $pSpGH_{1\text{-}39}$-$P450_{44\text{-}491}$ which was linearized with Hinc II, an enzyme that cleaves within the short pSP65-derived segment that is interposed between the growth hormone and P450 cDNA sequences. This intermediate contains duplicate copies of the Hpa II fragment, separated by a segment of the P450 cDNA that extends from its Pst I site up to the Hpa II site at nucleotide 639. The portion of the insert in this plasmid between the Fsp I site within the 5′ Hpa II fragment and the Fsp I site within the 3′ Hpa II fragment was removed by cleavage with Fsp I and religation. This reconstructs a continuous natural P450 cDNA sequence extending from the Hpa II site at nt 635 to the 3′ end.

$pSOPS_{1\text{-}34}P450_{1\text{-}491}$

This plasmid encodes a protein containing the amino-terminal 34 residues of bovine opsin linked to the entire P450 polypeptide sequence. A single extraneous amino acid (cysteine) is inserted between the two sequences. For its construction, an Sph I-Nco I 5′ fragment was removed from pSPOPS, a plasmid that contains the opsin cDNA inserted into the Sac I site of PSP65 (19) and is cloned between the corresponding sites of pSP450. In pSP450 the Nco I site is at the initiation codon and in both plasmids the common Sph I site is located a short distance upstream from the SP6 promoter. The resulting plasmid contains an out-of-frame fusion at the Nco I site. To correct the reading frame, the plasmid was cleaved with Nco I, treated with Klenow DNA polymerase, and religated. This process inserts a codon (TGC) for cysteine between the opsin and P450 coding segments.

pSV2-derived Plasmids

pSVP450. This plasmid contains a full-length P450 cDNA whose transcription is controlled by the SV40 early promoter. It was obtained by a trimolecular ligation involving the pSV2 vector (41) cleaved with Hind III and Bam HI, and the Hind III–Fsp I and Fsp I–Bam HI fragments of pSP450. The Fsp I–Bam HI fragment restores the SV40 polyadenylation signal which is removed in the cleavage of pSV2. It was shown (Dohmer, J., S. Dogra, T. Friedberg, S. Monier, M. Adesnik, H. Glatt, and F. Oesch; manuscript submitted for publication) that Chinese hamster fibroblasts permanently transformed with pSVP450 acquire the capacity to carry out the *O*-dealkylation of 7-pentoxyresorufin, a catalytic activity characteristic of P450b.

pSVP450(s⁻). This plasmid encodes a P450 polypeptide from which residues 2–43 containing the putative insertion-halt-transfer signal were deleted. For its construction, the Hind III fragment of pSP450(s⁻) was cloned into pSV450 after removal of the Hind III cDNA fragment and treatment with bacterial alkaline phosphatase.

Transient Expression and Immunofluorescence Staining

HeLa cells were transfected with pSV450 and pSV450(s⁻) and examined by immunofluorescence as previously described (49). About 50 μg of DNA purified by CsCl gradient centrifugation was used to transfect a confluent culture in a T75 flask. Cells were examined by immunofluorescence 3 d after transfection using affinity-purified polyclonal antibody to rat P450b (3).

Cell-free Transcription and Translation

Plasmid DNAs were prepared by the alkaline lysis procedure (33). The DNA preparations were treated with 20 μg/ml of RNase A for 1 h at 37°C, extracted four times with phenol-chloroform, ethanol precipitated, and desalted by several passages through a Sephadex G50 column. About 2 μg of nonlinearized plasmid DNA was used in a 10-μl transcription reaction (31). Before addition of the ribonucleoside triphosphates, the synthetic cap dinucleotide mGpppG was added to 1 mM and the mixture was incubated for 10 min. The ribonucleotides were then added and incubation was continued for 60 min. For translation (51), one tenth of the transcription mixture, containing ~200 ng of the RNA transcript, was used to program 25 μl of a wheat germ translation mixture which, when required, was supplemented with dog pancreas microsomal membranes. It should be noted that the optimal potassium ion concentration for membrane insertion and translocation was lower than that for translation and that it varied between 60 and 80 mM with different batches of wheat germ extract and microsomal membranes.

Analysis of Translation Products

The extent of insertion or translocation of polypeptides across the membranes was assessed by their resistance to proteolytic digestion effected by a 3-h incubation on ice with a mixture of trypsin and chymotrypsin (0.1 mg/ml each). The protease inhibitor trasylol was added (to 1,000 U/ml) before addition of the buffer for gel electrophoresis.

To determine whether polypeptides were membrane associated, aliquots of the translation mixture were also fractionated by flotation on a sucrose gradient in the presence of a high salt concentration. The samples (10 μl), adjusted to 0.6 M NaCl and 20 mM EDTA, pH 7.5, were incubated for 30 min on ice, mixed with 200 μl of 2.1 M sucrose, 50 mM triethanolamine, pH 7.5, 0.5 M NaCl in a 0.8-ml centrifuge tube and overlaid with 200 μl of 1.3 M sucrose followed by 200 μl of 0.5 M sucrose, both containing 50 mM triethanolamine and 0.5 M NaCl. After centrifugation for 2 h at 45,000 rpm in an SW56 rotor, the membrane fraction was recovered at the 0.5–1.3-M interface and collected separately from soluble components in the 2.1-M sucrose fraction. Both fractions were then precipitated with 10% cold TCA and analyzed by SDS-PAGE. The association of the polypeptides with the membrane was also assessed by their resistance to extraction in media of alkaline pH. To this effect, aliquots (15-μl) of the translation reaction were mixed in SW56 polyallomer tubes (Beckman Instruments, Inc., Fullerton, CA) with 4 ml of 0.1 M Na_2CO_3, pH 11.5, and incubated for 30 min on ice before centrifugation at 45,000 rpm for 2 h. The membrane and supernatant fractions were TCA precipitated and analyzed by SDS-PAGE (12–15%) and autoradiography.

The presence of carbohydrate residues in translation products was assessed by the effect of endoglycosidase H on their electrophoretic mobility. After boiling the sample (20 μl) in 1% SDS, 200 μl of 50 mM citrate, pH 5.5, and 0.05 U of endoglycosidase H were added and the samples were incubated at 37°C overnight before precipitation with 10% cold TCA and analysis by gel electrophoresis.

Results

The Amino-Terminal 43-Amino Acid Segment of Cytochrome P450 Is Necessary for the Cotranslational Insertion of the Protein into Microsomal Membranes

Previous work (3) using in vitro translation systems and natural mRNA from rat liver has shown that P450 is synthesized in membrane-bound ribosomes and that, although its incorporation into the ER membrane takes place without cleavage of a signal sequence (3), it involves the participation of the signal recognition particle (54). Although these studies indicated that nascent P450 contains a functional signal sequence that mediates its cotranslational insertion into the ER membrane, the location of the signal within the polypeptide was not directly established. The hydrophobicity of the amino-terminal portion of the protein (Fig. 2), however, strongly suggests that it may function as an insertion signal. On the other hand, a hydropathy analysis of the P450 sequence (13, 25, 59) reveals the presence of several other segments which, although of significantly lesser hydrophobicity than the amino-terminal segment, could in principle also function as insertion or halt-transfer signals and/or interact with the membrane bilayer.

We have used an in vitro expression system to synthesize and incorporate into membranes natural and modified P450 polypeptides in order to identify and characterize signals responsible for the insertion and anchoring of the natural protein in the ER. A cDNA containing the entire coding region of cytochrome P450b was assembled from portions of two overlapping cDNA clones and a portion of a genomic clone that contained the entire first exon and provided a short 5′ coding segment. The reconstructed cDNA was incorporated into the in vitro expression vector pSP64 and the resulting plasmid (pSP450) was used for in vitro transcription–translation experiments in the presence or absence of dog pancreas microsomal membranes.

The polypeptide encoded in the artificial messenger RNA synthesized in vitro comigrated with the authentic P450b, purified from rat liver microsomes, and was immunoprecipitable with anti-P450 antibodies (not shown). When microsomal membranes were present during translation, ~50% of the in vitro synthesized product became membrane associated, as established by its flotation in a high-salt–containing sucrose step gradient in which membranes were separated from soluble components (Fig. 3 *A*, lanes *d–f*). On the other hand, a negligible proportion of the in vitro product was recovered with the membranes when these were added after protein synthesis was terminated (Fig. 3 *A*, lanes *g–i*). The membrane-associated P450 remained exposed on the cytoplasmic surface of the microsomes since it was completely digested to low molecular mass products when proteases were added to the translation mixture (Fig. 3 *A*, lane *m*). The fact that digestion of the membrane-incorporated P450 proceeded as extensively as with the nonmembrane-associated polypeptide (Fig. 3 *A*, lane *k*) indicates that no significant stretch of contiguous amino acids is normally translocated into the microsomal lumen. From these experiments alone, however, one cannot eliminate the possibility that the P450 polypeptide spans the membrane several times with only short segments reaching the luminal face of the microsomes.

To determine whether the amino-terminal segment of P450 is indeed necessary for the cotranslational association of the product with the membranes, the behavior of a deletion variant (encoded in plasmid pSP450[s⁻]) from which amino acids residues 2–42 were deleted was examined in a similar experiment (Fig. 3 *B*). In this case, the product did not become associated with the membranes, even when they were present during translation (Fig. 3 *B*, lanes *d–f*). It can therefore be concluded that the amino-terminal segment of P450 is necessary for incorporation of the polypeptide into the membrane, most likely because it contains either the entire or part of the permanent signal that initiates the cotranslational insertion. Moreover, no other segment within the interior of the P450 polypeptide could by itself mediate association with the membrane when the first segment was deleted.

These conclusions were supported by the results of experiments in which the same cDNAs were expressed in transfected HeLa cells using pSV2-based vectors (pSVP450 and pSVP450[s⁻]). Immunofluorescence showed that the intact P450 expressed in vivo was distributed with the characteristic lacelike reticular pattern that corresponds to the ER (Fig. 4 *a*), whereas the P450 lacking the amino-terminal segment was distributed diffusely throughout the cytoplasm (Fig. 4 *b*).

P450 Sequences after the Amino-Terminal Segment Can Be Translocated into the ER Lumen When Preceded by the Cleavable Signal of a Secretory Protein

To determine whether the amino-terminal segment of P450 is also responsible for halting the translocation of downstream sequences, the behavior of a chimera ($pGH_{1-39}P450_{44-491}$) in which the first 43 amino acids of P450 were replaced by a segment of pregrowth hormone containing its 26–amino acid–long, cleavable insertion signal and the amino-terminal

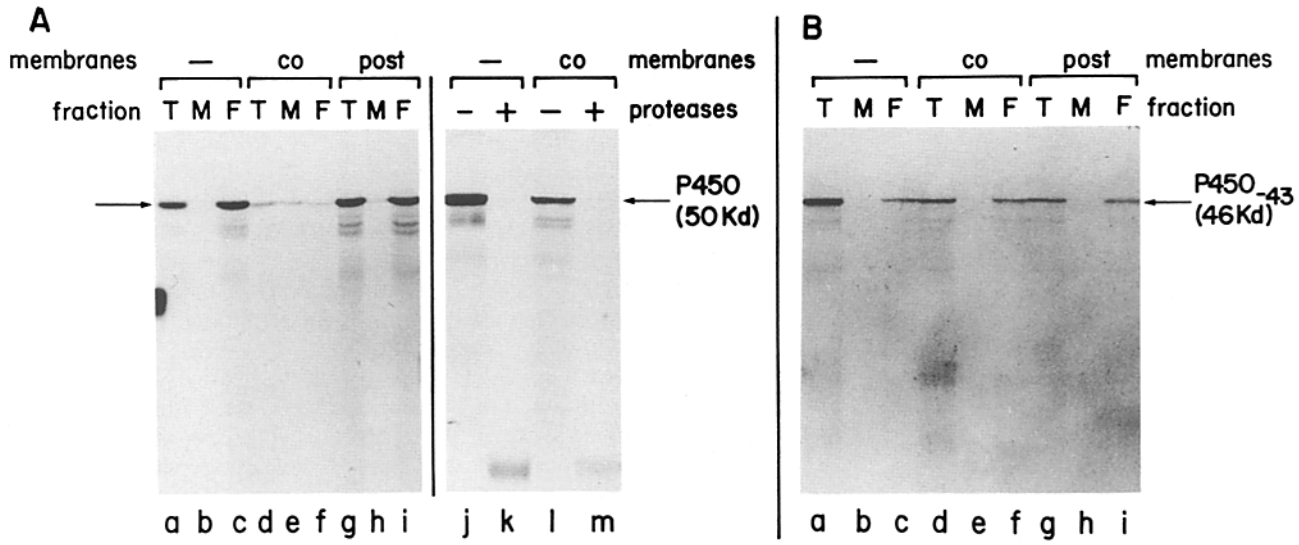


Figure 3. The amino-terminal 43-amino acid segment of cytochrome P450 is necessary for the cotranslational insertion of the protein into microsomal membranes. Full-length cytochrome P450 (*A*) and P450 deleted of amino acid residues 2–43 (*B*) were synthesized in vitro in the absence (−) or presence (*co*) of dog pancreas microsomal membranes, or the membranes were added after translation was completed (*post*). (*A*) After translation, the association of P450 with the membranes was determined by flotation analysis (lanes *a–i*) and its exposure on the microsomal surface by its sensitivity to proteases (lanes *j–m*). In lanes *a–i* total samples were analyzed directly by electrophoresis (*T*; lanes *a*, *d*, and *g*) or were first fractionated into membrane (*M*; lanes *b*, *e*, and *h*) and free (*F*; lanes *c*, *f*, and *i*) fractions. The lower intensity of the bands in lanes *d–f* reflects the inhibition of translation caused by the membranes. (*B*) Samples were subjected to flotation analysis as in *A*.

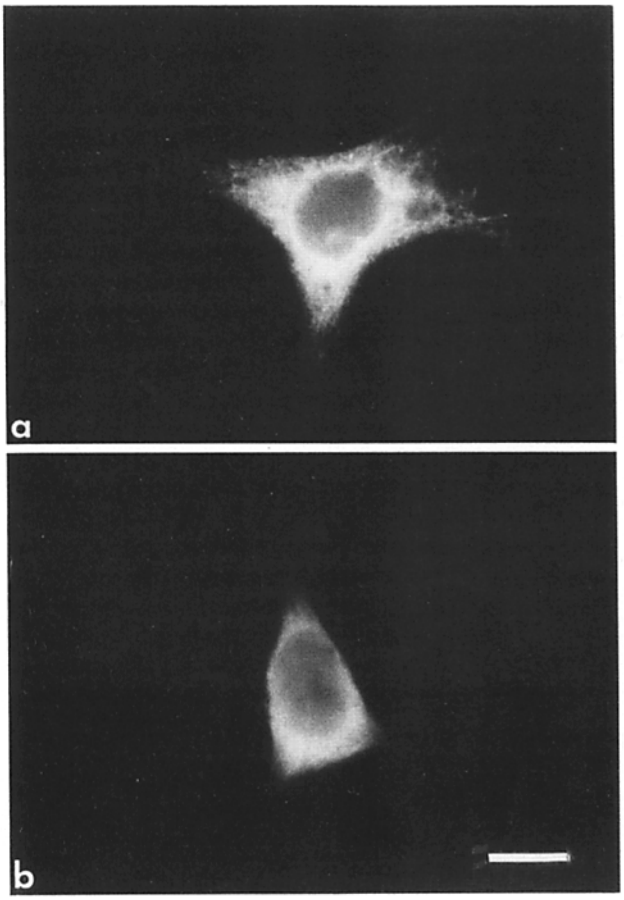


Figure 4. Immunofluorescence patterns of transfected HeLa cells expressing intact P450 (*a*) or the deletion variant lacking residues 2–42 (*b*). The lacelike reticular pattern in *a* is characteristic of proteins localized in the ER, whereas the diffuse cytoplasmic staining in *b* is that expected for a cytosolic protein. Bar, 15 μm.

13 residues of the mature hormone was studied (Fig. 5). As expected, this chimeric protein became membrane associated and underwent cleavage of the pregrowth hormone insertion signal, although in Fig. 5 *A*, lane *c*, the bands corresponding to the intact and cleaved chimeras are not well separated. In contrast to the natural P450, however, the chimeric polypeptides underwent translocation into the microsomal lumen. It was apparent, however, that in most molecules this translocation was only partial, since treatment with proteases converted the polypeptides into a doublet of two closely migrating fragments of ~24 kD (Fig. 5 *A*, lane *d*). Only a very small fraction of the inserted molecules (probably <5%) was completely resistant to proteolysis and therefore appeared to be fully translocated into the microsomal lumen. Similar resistant fragments of the chimeras were not detected when the protease treatment was applied to the translation mixture incubated in the absence of membranes (Fig. 5 *A*, lane *b*). The finding that a segment of P450 that begins at residue 44 can be translocated across the membrane suggests that the deleted amino-terminal portion of P450 is, in the natural protein, not only responsible for initiating insertion but also for halting translocation of the downstream sequences. On the other hand, the incomplete translocation of most of the pregrowth hormone–P450 chimeric molecules indicates that P450 sequences, located approximately between residues 150 and 250, halt translocation of the polypeptide across the membrane.

To more precisely determine the location of the sequences that prevent complete translocation, the behavior of a series of similar pregrowth hormone–P450 chimeric polypeptides in which the P450 sequence was truncated at residues 425, 271, 246, and 167 was studied. With these shorter polypeptides, the change in electrophoretic mobility resulting from cleavage of the pregrowth hormone signal sequence was quite striking (Fig. 5, lanes *g*, *k*, *o*, and *s*). With the polypep-

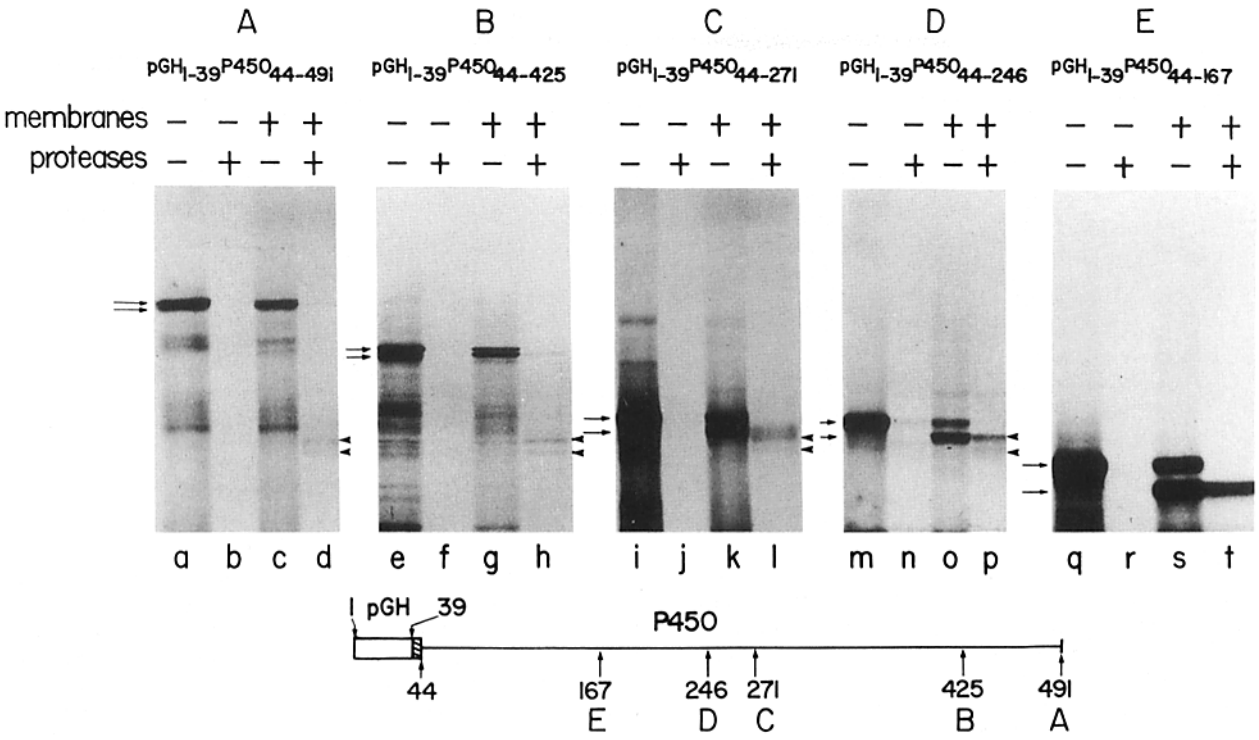


Figure 5. Cytochrome P450 can be partially translocated across microsomal membranes when its amino-terminal segment (43 residues) is replaced by a peptide containing the cleavable insertion signal of growth hormone. A series of hybrid polypeptides containing the first 39 amino acids of pregrowth hormone linked to segments of P450 that extended from residues 44–167 (*E*), 246 (residues, 271 (*C*), 425 (*B*), and 491 (the COOH terminus; *A*) were synthesized in vitro in the presence or absence of membranes, as indicated (*D*), and the accessibility of each product to protease digestion was assessed. The arrows point to the positions of the intact chimeras and of the chimeras processed by removal of the signal peptide. The arrowheads point to the doublets of protease-resistant fragments.

tides extending to residues 425, 271, and 246 (Fig. 5, *B–D*), most molecules were incompletely translocated and protease treatment generated a doublet of protected fragments (Fig. 5, lanes *h*, *l*, and *p*) of the same size as those obtained with the chimera that extends to the COOH-terminal end of P450 (Fig. 5 *A*, lane *d*). With the shortest chimeric polypeptide (Fig. 5 *E*), the product processed for signal removal was completely protected from the exogenous proteases (Fig. 5 *E*, lane *t*). This suggests that, like a secretory protein, residues 44–167 of P450 can be fully translocated into the microsomal lumen. The fact that all the protected fragments generated by proteolytic treatment of the partially translocated polypeptides were of the same size demonstrates that in all cases translocation was interrupted by the same segment within the P450 sequence. The presence of a doublet of protected segments most likely reflects an heterogeneity in the accessibility to proteases of potential tryptic and chymotryptic cleavage sites located near the cytoplasmic surface of the membrane. A further demonstration that the shortest chimera was discharged into the microsomal lumen, whereas the one that extends to residue 246 was incompletely translocated and remained membrane associated, was obtained by analysis of the alkali extractability of both products (Fig. 6). Like natural growth hormone (Fig. 6, lanes *c–f*), the short polypeptide was quantitatively extracted from the microsomes into a soluble fraction by incubation at pH 11.5 (Fig. 6, lanes *i–l*), whereas a significant portion of the longer polypeptide remained membrane associated after alkaline treatment and was recovered with the sedimentable fraction (Fig. 6, lanes *o–r*).

Hydropathy analysis (13) of the P450 amino acid sequence (20) reveals that, after the first amino-terminal 20 residues, the second most hydrophobic segment within the P450 polypeptide lies between residues 167 and 185 (Fig. 2). We therefore considered the possibility that this segment of the protein was responsible for the incomplete translocation of the pregrowth hormone–P450 chimeras and studied the behavior of two additional chimeric variants: one ($pGH_{1-39}P450_{\Delta[166-203]}$) in which residues 166–203 were removed and a second ($pGH_{1-39}P450_{203-491}$) in which the growth hormone–insertion signal was linked directly to the cytoplasmic-terminal 288 residues of P450. With these constructs signal cleavage was also apparent (Fig. 7, *c* and *e*; *h* and *j*) but, in contrast to the behavior of the chimeric polypeptides containing the interior hydrophobic domain, the products processed for signal cleavage were completely resistant to protease digestion. These findings suggest that no other segment downstream of residue 203 was capable of halting the translocation initiated by the cleavable growth hormone signal. It is worth noting that both of these apparently completely translocated polypeptides contain a potential N-glycosylation site, which is present at residue 456 of the P450 sequence. However, glycosylation did not take place, as indicated by the fact that the electrophoretic mobilities of the products were unaffected by digestion with endoglycosidase H (Fig. 7, lanes *d* and *i*).

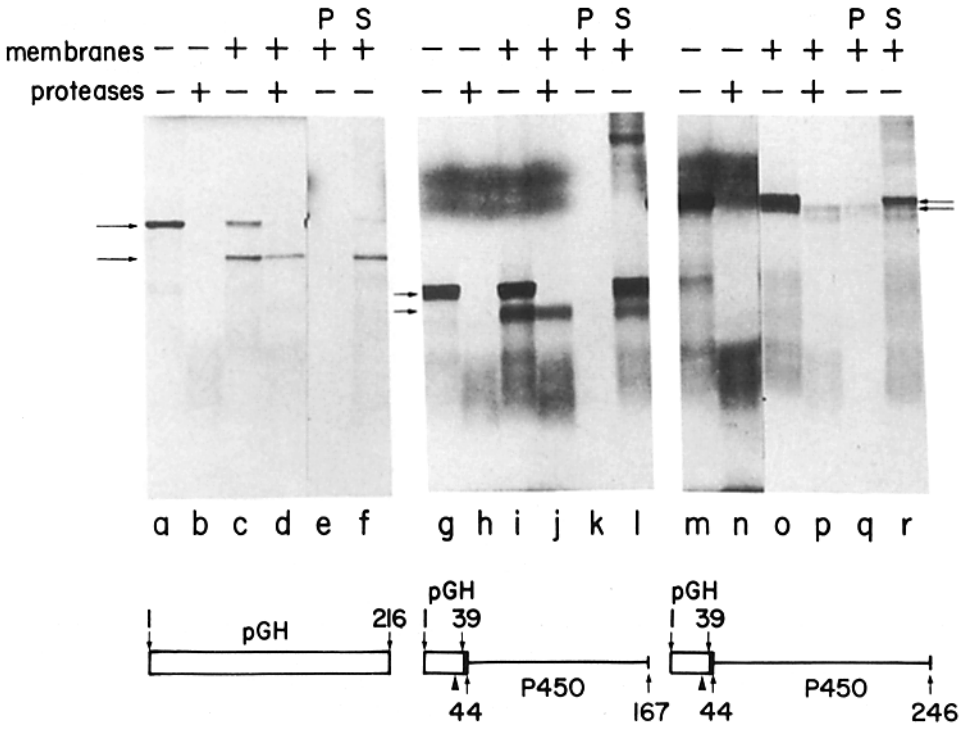


Figure 6. The first 167 residues of cytochrome P450 can be completely translocated into the microsomal lumen when preceded by the growth hormone signal but a segment between residues 167–246 halts translocation and serves as a membrane-anchoring domain. Pregrowth hormone (lanes *a–f*) and the two shortest pregrowth hormone–P450 chimeras described in Fig. 5 (lanes *g–r*) were synthesized in the presence of microsomal membranes. Their exposure on the microsomal surface and integration into the membranes were assessed by protease digestion and resistance to alkaline extraction, respectively. *S* and *P*, supernatant and pellet fractions obtained after alkaline treatment, respectively. The supernatant fraction contains the microsomal luminal content and polypeptides not incorporated into microsomes. The arrows on the side of the autoradiograms point to the primary translation products (*upper*) and to the products which underwent cleavage of the insertion signals (*lower*). In the schemes at the bottom of the figure, the arrows point to the ends of the segments contributed by different polypeptides. The arrowheads point to the cleavage site in the chimeric proteins.

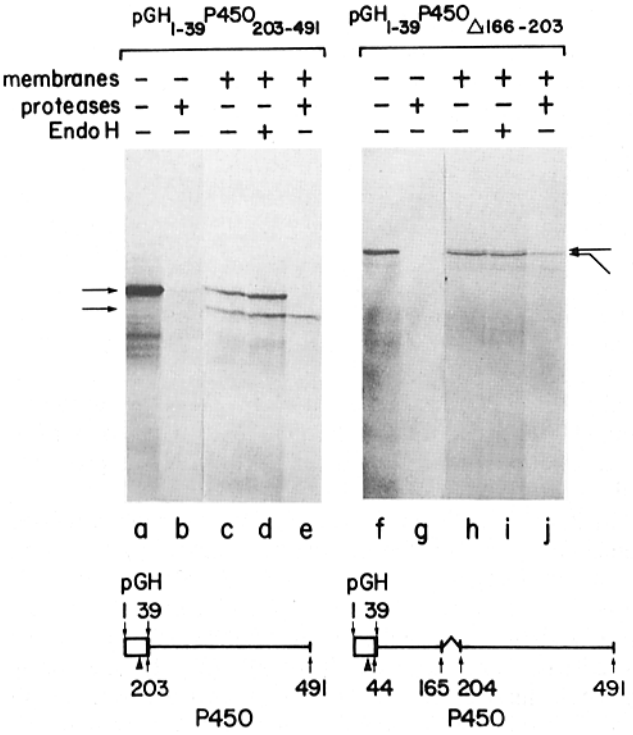


Figure 7. Cytochrome P450 contains only one interior potential halt-transfer signal, which is included in the segment between residues 166 and 203. The amino-terminal segment of rat pregrowth hormone (first 39 residues) was used to mediate the incorporation into membranes of two chimeric polypeptides containing different portions of P450. In one ($pGH_{1-39}P450_{203-491}$, lanes *a–e*), the P450 segment extended from residue 203 to the COOH terminus at residue 491. In the other ($pGH_{1-39}P450_{\Delta(166-203)}$; lanes *f–j*), the P450 segment extended from residue 44 to the COOH terminus, but the portion between residues 166–203 was deleted. The translocation of the polypeptides into microsomes was assessed by their resistance to protease digestion, and the presence of carbohydrate by the effect of endoglycosidase H treatment on the electrophoretic mobility of the product synthesized in the presence of membranes, as indicated. Arrows on the sides of the autoradiograms point to the positions of the primary translation products (*upper*) or of the products which underwent signal cleavage (*lower*). The arrowheads in the schematic diagrams at the bottom of the figure point to the cleavage site for the signal peptidase within the pregrowth hormone segment in each polypeptide.

Direct Demonstration That the Amino-Terminal Segment of P450 Displays Combined Insertion and Halt-Transfer Activities

The previous experiments demonstrated that the first 43 residues of P450 contain elements required for insertion of the polypeptide into membranes as well as for halting translocation of downstream segments. To determine whether the 43-amino acid segment is sufficient to effect both processes, we examined the behavior of a chimera ($P450_{1-48}pGH_{38-216}$) in which the first 48 residues of P450 replaced a segment comprising the first 37 amino acids of the pregrowth hormone sequence, which includes the cleavable insertion signal (26 amino acids long) and the first 11 residues of the mature hormone. The behavior of this chimera was essentially identical to that of the natural P450: the polypeptide was efficiently integrated into the membranes, as revealed by flotation analysis (Fig. 8 *A*, lanes *d–f*), but remained exposed on the outer surface of the microsomes and was completely digested by the added proteases (Fig. 8 *A*, lanes *c'* and *d'*).

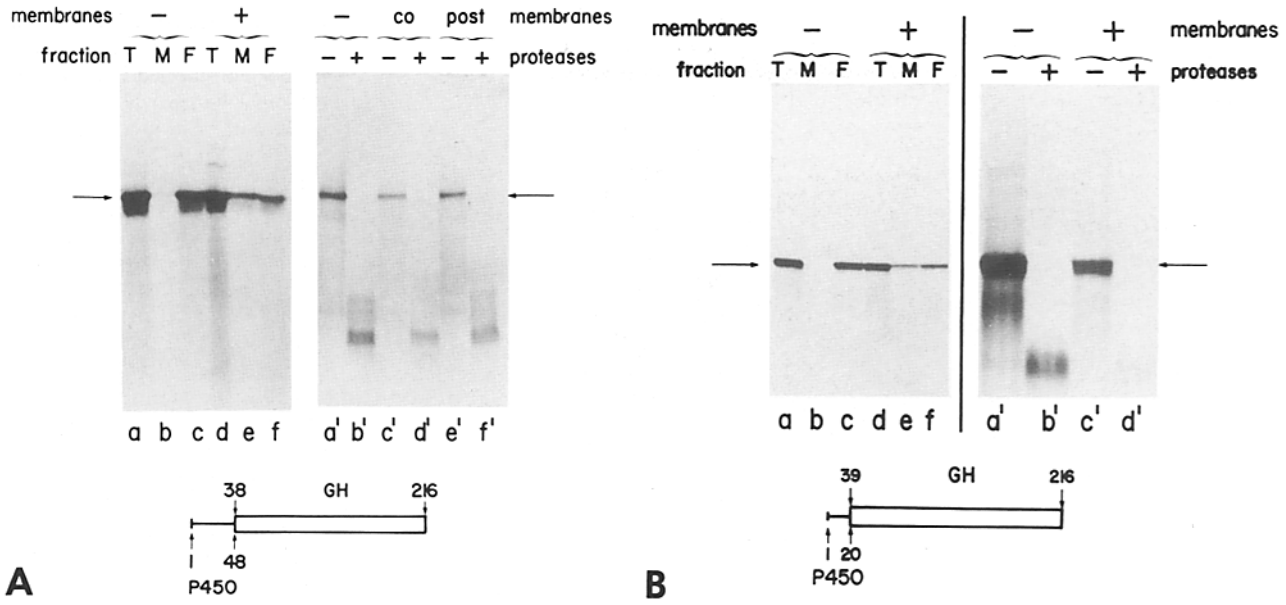


Figure 8. (*A*) An amino-terminal segment containing the first 48 residues of P450 mediates insertion of growth hormone into microsomal membranes but blocks its translocation into the microsomal lumen. The hybrid polypeptide $P450_{1-48}pGH_{38-216}$ (represented schematically at the bottom of the figure) containing the first 48 residues of P450 linked to a portion of pregrowth hormone that lacks the cleavable insertion signal, as well as the following 11 amino acid residues, was synthesized in the presence of dog pancreas microsomes. The association of the translation products with the membranes and their exposure on the surface of the microsomes was assessed by flotation and protease sensitivity, respectively, as in Fig. 3. The protease sensitivity of the hybrid polypeptide is in marked contrast to the resistance of the translocated growth hormone, shown in Fig. 6. (*B*) The first 20 amino acids are sufficient to effect the combined insertion and halt-transfer functions of the terminal segment of P450. The insertion into membranes of $P450_{1-20}pGH_{39-216}$ was studied as in *A*.

A similar experiment with a P450–growth hormone chimera ($p450_{1-20}pGH_{39-216}$) that contains only the first 20 residues of P450 gave identical results (Fig. 8 *B*), indicating that the combined insertion and halt-transfer signal activities of the amino-terminal portion of P450 are in fact wholly contained within this smaller segment, which includes a stretch of 16 contiguous hydrophobic amino acids (Fig. 2).

Disposition of the Amino-Terminal Portion of P450 with Respect to the ER Membrane

It has been suggested (18, 27, 53, 58) that during the early stages of cotranslational insertion amino-terminal signal sequences adopt a loop configuration with the residues that precede the hydrophobic core of the signal remaining on the cytoplasmic surface of the ER membrane. If the hydrophobic segment in P450 achieved this configuration, traversing the membrane in an NH_2 (cytoplasmic) to COOH (luminal) direction, then introduction of a signal peptidase cleavage site immediately after the hydrophobic stretch might be expected to result in signal removal and elimination of the halt-transfer activity. This possibility was examined using the plasmid $pSP450_{1-20}pGH_{21-216}$, which encodes a polypeptide in which the first 20 amino acids of pregrowth hormone are replaced by the corresponding residues of P450. In this hybrid, the P450 signal is followed by the last six residues of the pregrowth hormone signal, which provides the cleavage site, and by the complete growth hormone sequence. In this case, in contrast to the situation with $pSP450_{1-20}pGH_{39-216}$, cleavage of the P450 signal took place in a significant fraction of the molecules (Fig. 9, compare lane *a'* to lanes *a* and *b'*). When cleavage took place, translocation of growth hormone ensued, as shown both by the resistance of the processed polypeptide to digestion by exogenous proteases (Fig. 9, lane *c'*) and by its extraction from the membrane by alkali treatment (Fig. 9, lanes *a–c*). The removal of the P450 signal demonstrates that, at some point during insertion, the residues immediately after the signal reach the luminal side of the ER membrane, as predicted by the loop model. Moreover, it suggests that signal removal after insertion is sufficient to ensure translocation of downstream sequences. It should be noted, however, that some of the molecules in which cleavage did not take place became membrane associated, as indicated by their resistance to alkali extraction (Fig. 9, lane *b*), but these molecules clearly were not translocated since they were digested by the exogenous proteases (Fig. 9, lane *c'*).

To determine whether the extreme amino terminus of P450 remains exposed on the cytoplasmic surface of the membrane, we analyzed the behavior of a chimeric polypeptide in which P450 was preceded by a 34–amino acid segment derived from the amino-terminal region of bovine opsin, which includes two potential glycosylation sites (23, 41). In the opsin molecule this segment is normally glycosylated, which indicates that it is translocated into the ER lumen, even though it precedes the (noncleavable) first opsin insertion signal (19). When $OPS_{1-34}P450_{1-491}$ was synthesized in the presence of membranes, a substantial fraction of the product (Fig. 10, lane *d*) was glycosylated and therefore of higher M_r than the primary translation product (Fig. 10, lane *a*). The presence of carbohydrate in those molecules was demonstrated by the fact that treatment with endoglycosidase

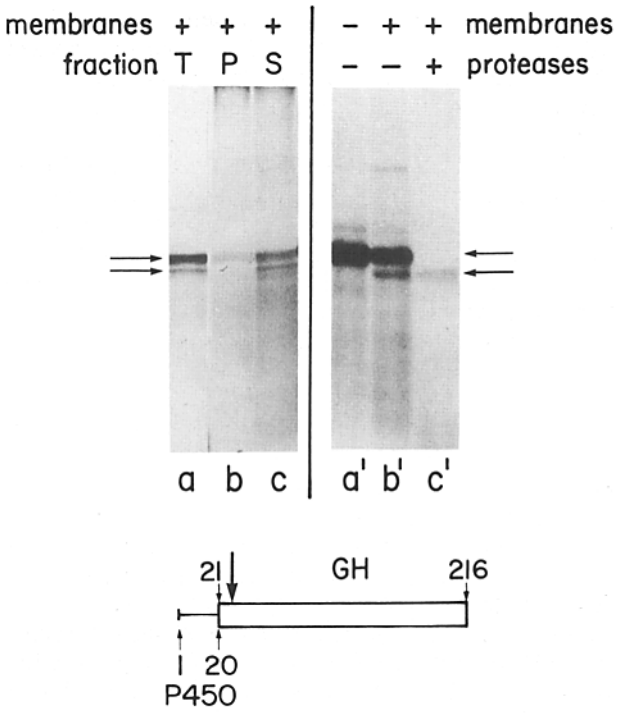


Figure 9. The halt-transfer function of the first 20 amino acids of P450 is partially obliterated when this segment is followed by the last six amino acids of the cleavable insertion signal of pregrowth hormone and the rest of this polypeptide. The $P450_{1-20}pGH_{21-216}$ polypeptide (represented schematically at the bottom of the figure) was synthesized in the absence (*a'*) or presence (*a–c*, *b'*, *c'*) of dog pancreas microsomal membranes. Samples were analyzed directly (*T*, lane *a*; and lanes *a'* and *b'*) or after extraction with alkali (*b* and *c*), which generated supernatant (*S*) and pellet (*P*) fractions. The sensitivity to proteases of products synthesized in the presence of membranes was also assessed (compare lanes *b'* and *c'*). The arrows on sides of the autoradiogram point to the primary translation product (*upper*) and to the processed product that underwent signal cleavage (*lower*).

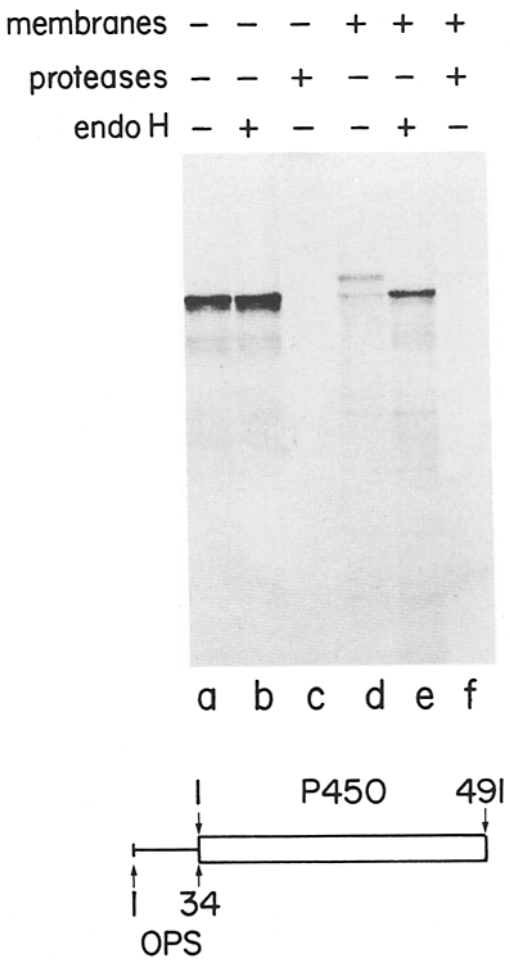


Figure 10. The amino-terminal segment of opsin is translocated into the microsomal lumen when it is linked to the amino terminus of P450. The hybrid polypeptide $OPS_{1-34}P450_{1-491}$ (represented schematically at the bottom of the figure) was synthesized in the absence (lanes *a–c*) or presence (lanes *d–f*) of microsomal membranes. Aliquots of the reaction mixtures were either analyzed directly (*a* and *d*) or after treatment with either endoglycosidase H (lanes *b* and *e*) or with proteases (lanes *c* and *f*).

H increased their electrophoretic mobility (Fig. 10, lane *e*) to that of the polypeptide synthesized in the absence of membranes (Fig. 10, lane *a*). This experiment demonstrates that the opsin segment added to the amino terminus of the P450 was translocated into the ER lumen, which implies that the amino terminus of P450 did not remain on the cytoplasmic surface of the microsomal vesicles. Although most of the glycosylated molecules behaved as P450 and therefore remained largely exposed on the cytoplasmic surface of the membranes where they were accessible to the added proteases (Fig. 10, lane *f*); a small (but variable) fraction of the glycosylated molecules appeared to be protease resistant and therefore to have been completely translocated to the microsomal lumen. This suggests that the addition of the amino-terminal portion of opsin reduces the effectiveness of the halt-transfer signal at the amino terminus of the P450 moiety.

Discussion

The results presented in this paper show that the amino-terminal segment of P450 behaves as a combined insertion–halt-transfer signal, in that it initiates incorporation of the nascent polypeptide into the ER membrane but does not lead to translocation of the following sequences across the membrane. The fact that this segment is required for insertion was demonstrated by in vivo and in vitro experiments in which it was found that a deletion variant of P450, from which residues 2–43 were removed, failed to be incorporated into the membranes. This showed that no other segment of P450 could, independently, initiate the insertion process.

It was also necessary to demonstrate that the amino-terminal segment was responsible for the halt-transfer signal activity since, theoretically, the normal exposure of the bulk of the P450 molecule on the cytoplasmic face of the ER membrane could be due to intrinsic features of the polypeptide that prevent it from traversing the membrane but do not reside in the amino-terminal signal segment itself. We showed, however, that the portion of P450 that follows residue 43 and extends up to residue 167 could be efficiently translocated across the membrane when it was preceded by the conventional cleavable signal of the secretory protein growth hormone. This indicates that the portion that follows the signal does not contain any intrinsic structural barrier to transloca-

tion. Furthermore, the capacity of the first 43-amino acid segment of P450 to effect both insertion and halt-transfer functions was demonstrated directly when this segment was used to mediate the insertion of the growth hormone polypeptide into the ER. In this case, growth hormone became an intrinsic membrane protein that remained exposed on the cytoplasmic surface of the microsomes. The same result was obtained when only the first 20 residues of P450 were linked to growth hormone and this allowed us to conclude that both the insertion and halt-transfer functions are wholly contained in that portion of the P450 molecule.

It should be noted that the P450 sequence (arg-gly-his-pro-lys-ser-arg-gly . . . . .) (Fig. 2) that immediately follows the last hydrophobic residue at position 20 includes several positively charged amino acids which, in principle, could have contributed to the halt-transfer activity (53). In fact, in the $P450_{1-20}pGH_{39-216}$ chimera the P450 hydrophobic sequence was also followed by a sequence (ala-val-leu-arg-ala-gln-his-leu-his-gln-leu-ala-ala . . . . .) (Fig. 2) that contains positive charges. However, the behavior of two other chimeras allows us to discard the possibility that such charges contributed to the halt-transfer activity. Thus, a significant proportion of the $P450_{1-20}pGH_{21-216}$ molecules, which contain the growth hormone insertion signal cleavage site, although incorporated into the membranes, did not undergo cleavage and remained exposed on the cytoplasmic surface (Fig. 9). In this chimera, the sequence (pro-gln-glu-ala-gly-ala-leu-pro-ala-met) (Fig. 2) after the P450 hydrophobic segment was not highly charged and yet, in the uncleaved polypeptides, the amino-terminal segment served as an effective halt-transfer signal. Similarly, a P450 variant ($P450_{1-20}CS$-$P450_{44-167}$), in which the hydrophobic segment was followed by a sequence (asp-ala-gly-ala-leu-ser-arg-val-asp-leu-gln-leu-asp-arg-gly-gly-leu-leu-asp-ser . . . . . .) (Fig. 2) with no net positive charge, was also inserted into the membrane but was not translocated (results not shown).

The observation that the charged residues that follow the amino-terminal hydrophobic segment of P450 are not essential for the halt-transfer and membrane-anchoring activities is consistent with the finding (11, 12, 67) that, in simple transmembrane proteins, the charged residues that follow the halt-transfer signals can be deleted without affecting the transmembrane disposition of the polypeptides.

Recently, Sakaguchi et al. (55) have reported that an amino-terminal segment of a different form of microsomal P450, which is induced in rabbit liver by polycyclic hydrocarbons, also leads to insertion into the ER membrane of linked portions of other polypeptides, such as interleukin-2 and yeast mitochondrial porin, but prevents their translocation into the ER lumen. Moreover, in this work it was demonstrated that positively charged residues that follow the hydrophobic amino-terminal signal could be deleted without altering the capacity of the signal to effect the halt in translocation.

It is worth noting that the capacity of the amino-terminal segment of the rat liver phenobarbital-inducible P450 studied here to act as a halt-transfer signal was also manifested when this segment was placed within a translocatable polypeptide, downstream from a cleavable insertion signal (18). In that case, the first 167 amino acid residues of P450 were linked to a truncated influenza virus hemagglutinin, replacing its transmembrane and cytoplasmic domains. The hybrid protein became anchored in the membrane, with its amino-terminal hemagglutinin portion sequestered in the microsomal lumen and the bulk of the P450 component exposed on the cytoplasmic surface. On the other hand, in a similar experiment (18), a typical cleavable insertion signal, that of the hemagglutinin itself, when relocated to an internal position, failed to halt translocation and traversed the membrane without undergoing cleavage. The incapacity of this signal to halt translocation most likely reflects the fact that, like most conventional insertion signals, it contains a hydrophobic segment shorter than the regular halt-transfer signals that serve as transmembrane domains.

The halt-transfer function of the membrane-anchoring domain of cytochrome P450 distinguishes it from the first membrane-anchoring domain found in proteins such as Band III, which have cytoplasmically exposed amino termini (type II proteins). We have originally proposed (53) that the transmembrane disposition of the latter type of proteins is explained by the action of an interior noncleavable insertion signal that enters the membrane in a loop configuration, leaving its amino-terminal portion on the cytoplasmic surface, and mediates translocation of downstream sequences but remains anchored in the membrane after translocation is completed. Substantial evidence has accumulated supporting this proposal from studies on the sequence of primary translation products of various type II proteins, such as the influenza neuraminidase (6, 17), the transferrin receptor (56), the sucrase isomaltase of the intestinal brush border (26), the hepatocyte asialoglycoprotein receptor (57), and the anion channel (Band III) (29) and glucose transporter (38) of the red blood cell membrane. In some cases, direct evidence for the translocating capacity of membrane-anchoring domains in type II proteins has been obtained from experiments using polypeptides encoded in genetically engineered chimeric genes (9).

There is a class of transmembrane glycoproteins, however, which includes rhodopsin (19) and the β-adrenergic receptor (14), which contain no cleavable signals but have luminally located, glycosylated, amino-terminal domains. In such proteins, the first transmembrane domain spans the membrane in the NH_2 (luminal) to COOH (cytoplasmic) direction, characteristic of halt-transfer signals. In contrast to the situation with type II proteins, this disposition cannot be a direct consequence of the completion of an insertion process which was initiated by a signal that entered the membrane as a loop and remained in that configuration.

Our experiments with P450 provide support for the notion that the amino-terminal portion of this polypeptide first enters the membrane as a loop (Fig. 11 *A*, *a*) but later reorients so that its amino terminus is transferred to the luminal surface (Fig. 11 *A*, *b*). Thus, when the normal cleavage site for pregrowth hormone was placed after the combined insertion-halt-transfer signal of P450 (in $P450_{1-20}GH_{21-216}$), cleavage of the signal occurred in a significant proportion of the molecules which were inserted in the membrane, and these molecules were translocated into the microsomal lumen. This indicated that the cleavage site reaches the luminal side of the membrane (Fig. 11 *B*) where the signal peptidase is known to be located (16, 28). Because the presence of a cleavage site after the hydrophobic segment is not likely to alter the configuration of the signal as it enters the membrane, these experiments support the notion that the natural P-450 signal enters as a loop (Fig. 11 *A*) rather than directly

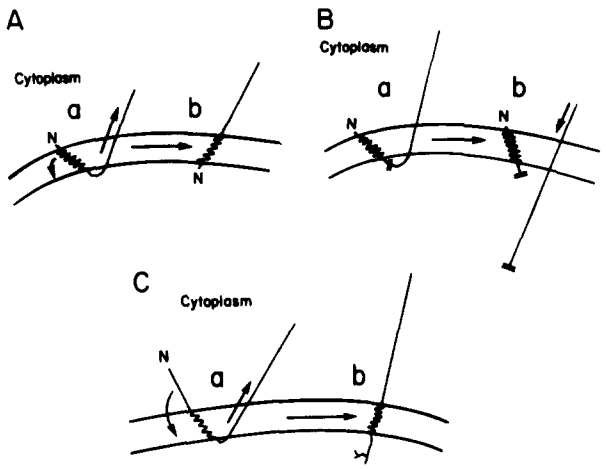


Figure 11. Proposed reorientation of the combined insertion–halt-transfer signal in natural P450 and in the opsin–P450 chimera. (*A* and *B*) Initially, the hydrophobic amino-terminal segment (∿∿) of the nascent P450 molecule would traverse the membrane with its amino-terminus remaining exposed in the cytoplasm (*A*, *a*). The amino acid residues immediately following the hydrophobic stretch would reach the lumen of the ER and cleavage by the signal peptidase would take place, were a cleavage site located at that position, as in $P450_{1-20}pGH_{21-216}$ (*B*, *a*). When cleavage takes place, translocation is not halted and the later portion of the polypeptide is sequestered in the lumen of the ER (*B*, *b*). When cleavage does not take place, as in the native P450 or a fraction of the $P450_{1-20}$-pGH_{21-216} molecules, the signal reorients within the membrane so that its amino terminus is transferred to the luminal face of the membrane and the segment that follows the signal is ejected into the cytoplasm (*A*, *b*). For simplicity, the ribosome in which the nascent chain is contained has been omitted from the schemes. (*C*) The cotranslational insertion into the ER membrane of OPS_{1-34}-$P450_{1-491}$ is depicted. In this protein, the amino terminus of P450 has been extended to include the 34 amino-terminal amino acid residues of opsin, which include two glycosylation sites and in the native opsin are translocated across the ER membrane. When the P450 signal enters the membrane as part of a loop, the preceding opsin portion is initially located on the cytoplasmic surface of the membrane (*a*). It is proposed that soon thereafter, the P450 signal reorients within the membrane and, as a result, the opsin segment is translocated to the ER lumen, where it is glycosylated (*b*). This reorientation brings about the disappearance of the loop, which causes the halt in translocation that is responsible for the predominant exposure of P450 on the cytoplasmic side of the membrane.

in the NH_2 (luminal) to COOH (cytoplasmic) orientation (shown in Fig. 11 *A*, *b*). Evidence for a reorientation of the signal that destroys the initial loop was provided by the finding that when the chimera $OPS_{1-34}P450_{1-491}$ (in which the amino-terminal segment of opsin that precedes the first signal in that protein was linked to the amino terminus of the intact P450) was synthesized in the presence of membranes, the opsin component was glycosylated and hence was translocated to the lumen of the microsomes (Fig. 11 *C*). This leads us to propose that the reorientation of the insertion signal in P450 which causes the disappearance of the intramembrane loop is responsible for the halt in translocation. Such a reorientation would take place soon after insertion begins since, although a large proportion of the chimeric molecules ($P450_{1-20}GH_{21-216}$) that contained a cleavage site immediately after the P450 signal underwent cleavage, a comparable fraction did not undergo cleavage but became inserted and remained anchored in the membranes, with the growth hormone component exposed on the cytoplasmic surface of the microsomes. In these molecules, halt-transfer (and presumably reorientation of the signal) must have taken place before cleavage could occur. The fact that in the mature P450 the membrane-anchoring domain exists in the NH_2 (luminal) to COOH (cytoplasmic) configuration expected from a reorientation is apparent from the finding that the portion of the polypeptide that immediately follows the hydrophobic segment (between residues 29 and 38) has been shown to be accessible to site-specific antibodies applied to the cytoplasmic surface (13). If the capacity of the combined insertion–halt-transfer signal to reorient soon after entering the membrane as a loop is essential to effect the halt in translocation, then it seems possible that the insertion–membrane-anchoring domains of type II proteins do not effect halt-transfer simply because they are incapable of reorienting. This could possibly be due to restrictions imposed by the properties of preceding polypeptide sequences, which would have to be translocated across the membrane, as well as succeeding sequences, which would have to be ejected back into the cytoplasm (Fig. 11, *A* and *C*).

The behavior of the amino-terminal segment of P450 strongly resembles that of the first transmembrane domain in opsin, whose combined insertion–halt-transfer signal function was demonstrated (2, 19) by the analysis of the translocation capacity and transmembrane disposition of truncated and interior deletion variants. This work also led to the recognition that segments of opsin that normally serve as interior insertion signals can undergo a reorientation when engineered to become the sole signals in polypeptides in which they are preceded by the amino-terminal segment of opsin (2).

It has recently been observed that simple interior halt-transfer signals, such as those in the μ heavy chain of the membrane form of IgM (37) or the Semliki Forest Virus envelope glycoprotein (66), which normally have an NH_2 (luminal) to COOH (cytoplasmic) orientation, when relocated at or near the amino terminus of a nascent polypeptide can function as effective insertion signals that facilitate translocation of later portions of the polypeptide and remain membrane anchored but do not halt transfer. The behavior of these relocated signals and their final transmembrane orientation contrasts with that of the natural amino-terminal sequence in P450, which demonstrates that the behavior of the latter is not simply due to its amino-terminal location. Rather, the P450 signal must contain specific features, almost certainly in the regions flanking the hydrophobic domain, that, after it initiates insertion, allow it to reorient and halt translocation.

The analysis of the membrane insertion of a series of chimeric proteins ($pGH_{1-39}P450_{44-491}$, $pGH_{1-39}P450_{44-167}$, pGH_{1-39}-$P450_{44-246}$, $pGH_{1-39}P450_{44-271}$, and $pGH_{1-39}P450_{44-491}$), in which the cleavable insertion signal of growth hormone replaced the amino-terminal portion of P450 indicated that, in addition to the amino-terminal combined insertion–halt-transfer signal, an interior segment of the P450 polypeptide, located approximately between residues 150 and 250, is capable, with high but not complete efficiency, of halting translocation across the membrane. This segment was located more precisely by the analysis of the insertion of pre-growth hormone–P450 chimeras with additional deletions in

the P450 segment. In one case ($pGH_{1-39}P450_{203-491}$) the growth hormone insertion signal was linked directly to a P450 segment extending from residues 203 to 491, and in another ($pGH_{1-39}P450_{166-203}$) it was linked to a P450 portion that extended from residues 44 to 491, but from which residues 165–204 were deleted. With both of these chimeras, translocation was complete. This defined more precisely the position of the segment capable of mediating halt-transfer and demonstrated that no other portion within the molecule had similar properties.

An examination of the P450 sequence (Fig. 2) shows that the interior segment capable of arresting translocation includes the second most highly hydrophobic segment within the molecule, which is located between residues 167 and 185. With an average hydropathy of 1.75 (calculated using the values of Kyte and Doolittle [32]), this sequence is considerably less hydrophobic than most well-characterized membrane-anchoring domains, such as the halt-transfer signals in type I membrane proteins or the insertion-signal membrane-anchoring domains of type II proteins.

The capacity of the interior segment of P450 to arrest translocation in the chimeric proteins raises the possibility that in the natural protein it also contributes to anchoring the polypeptide to the membrane. It should be recalled that when the amino-terminal 43 residues were deleted, P450 did not associate with the membranes, which clearly demonstrated that the interior segment cannot by itself initiate insertion. On the other hand, it is conceivable that when the protein is anchored in the membrane by the amino-terminal insertion–halt-transfer signal, the interior segment can become membrane associated. This could happen cotranslationally, in a process which could involve the signal recognition particle (see reference 62), or posttranslationally, in a process analogous to that which leads to the incorporation in the ER of proteins such as cytochrome b_5 and its reductase (8, 44, 47), which are synthesized in free polysomes and are inserted in the membrane through their COOH-terminal domains. Although in a study using site-specific antibodies raised against synthetic peptides corresponding to different portions of the P450 segment (13) it was not possible to determine whether the segment between residues 168 and 185 is exposed on the microsomal surface or is buried within the membrane, it was shown that preceding (residues 122–131) and later (residues 186–193) peptide segments are exposed on the cytoplasmic surface of the ER. This indicates that if the segment encompassing residues 168–185 contributes to the anchoring of the protein in the membrane, it must only form a shallow loop within the membrane since its length is insufficient for it to traverse the phospholipid bilayer twice. It is of course also possible that the interior segment is not associated with the membrane and that its hydrophobicity simply reflects its capacity to interact with other hydrophobic portions of the molecule or perhaps with hydrophobic substrates that are bound to P450.

We are grateful to Drs. J. Baxter and G. Blobel who provided us with the rat growth hormone and bovine opsin cDNAs, respectively. The excellent work of H. Plesken, B. Zeitlow, and J. Culkin in preparing the illustrations and of M. Cort in typing the manuscript is acknowledged with pleasure.

This work was supported by National Institutes of Health grants GM-20277 to D. D. Sabatini and GM-30701 to M. Adesnik.

Received for publication 11 December 1987, and in revised form 25 March 1988.

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