Molecular cloning and structural analysis of cDNAs encoding cytochrome P-450s from phenobarbital-treated rabbit liver microsomes

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ABBREVIATIONS

PB	: phenobarbital
MC	: 3-methylcholanthrene
β-NF	: β-naphthoflavone
ISF	: isosafrole
SDS	: sodium dodecyl sulphate
kb	: kilobase(s)
bp	: base pair(s)
1 x SSC	: 0.15 M NaCl, 15 mM sodium citrate (pH 7.0)
1 x Denhart's	: 0.02 % Ficoll, polyvinylpyrrolidone, BSA
$P\text{-}450_1$(LM2)	: main PB-inducible form of rabbit liver microsomal cytochrome P-450
LM3b	: constitutive form of rabbit liver microsomal P-450
21-OH	: rabbit hepatic progesterone 21-hydroxylase

SUMMARY

mRNA encoding cytochrome P-450 was extracted from liver microsomes of phenobarbital (PB)-treated rabbits and partially purified by oligo(dT)-cellulose chromatography and subsequent sucrose density gradient centrifugation. The mRNA activity was located in the 18-21 S fraction by their translation *in vitro* and Northern blot analysis. Plasmids containing ds-cDNA complementary to the partially purified mRNA were constructed by using a repair system and used to transfrom *E. coli*. By appropriate screening techniques, eight cDNA clones, including three full-length ones, were isolated. The nucleotide sequences of these cDNAs were determined mainly by the M13 dideoxy termination method. The inserts of pHP-1, pHP-2, and pHP-3 were 1,802, 1,948 and about 1,700 bp long and contained open reading frames coding for polypeptides consisting of 491, 490, and 490 amino acid residues, respectively. Only pHP-2 possessed a poly(A) tail and two possible poly(A) addition signals. pHP-2 was found to be identical with pP-450PBc2, a clone encoding a PB-inducible rabbit cytochrome P-450, described by Leighton *et al.* except that the latter lacks the sequence coding for the NH_2-terminal 11 amino acids as well as the poly(A) sequence and there are two nucleotide substitutions in their overlapping region. The molecular weights calculated for the deduced primary structures of the three clones were 55,700 to 55,800, and pHP-2 and pHP-3 contained 2-3 times more cysteine and asparagine residues than pHP-1. The hydropathy profiles are very similar to one another, in spite of the low homology in primary structure (about 50 %

between pHP-1 and pHP-2 or pHP-3). Among the incomplete length cDNA clones, three (tentatively called M046, B14 and B52) were more than 95 % homologous with pHP-1. Thus, these four cDNAs belong to the same group that displays microheterogeneity. The primary structure reported for P-450_{LM2}, the major PB-inducible form of rabbit liver cytochrome P-450, is also highly homologous with those of the pHP-1 group. Unlike the case for the microheterogeneity in PB-inducible rat liver cytochrome P-450s, the amino acid substitutions in the rabbit pHP-1 group are seen rather randomly over the whole sequence. The cDNA in aclone provisionally called M12 shows a very high homology (91 %) with rabbit liver progesterone 21-hydroxylase. The overlapping clones, b43 and b43FP1, together encode a unique form of cytochrome P-450 which is clearly different from any other forms so far sequenced. The primary structure encoded bythese two clones seems to lack the NH_2-terminal three amino acid residues. Homology comparisons among the eight cDNAs examined here and those studied by other investigators indicate that at least five groups of cytochrome P-450 are expressed in the liver of PB-treated rabbits and it is likely that each group displays varying degrees of microheterogeneity. Northern blot analysis showed that expression of cytochrome P-450s encoded by pHP-1 and related clones is strongly induced by PB and moderately by isosafrole (ISF), whereas the induction of pHP-2 (pHP-3 and M12) type cytochrome P-450s by PB and ISF is very weak. On the other hand, cytochrome P-450 encoded by b43 is weakly inducible by 3-methylcholanthrene (MC), β-naphthoflavone (β-NF), and ISF, but not by PB. Southern blot analysis of genomic DNA suggested that

genes corresponding to pHP-1 and pHP-2 belong to separate multi-gene families.

INTRODUCTION

Hepatic cytochrome P-450s are the terminal enzymes of the microsomal monooxygenase system that catalyze oxidative transformation of a large variety of xenobiotics as well as endogenous substrates such as steroids and fatty acids (1-3). The very broad susbstrate specificity displayed by this monooxygenase system can be at least in part accounted for by the occurrence of multiple forms of cytochrome P-450 in liver microsomes. A number of different forms of cytochrome P-450 have been purified from liver microsomes of drug-treated and untreated mammals(4-16). An important feature of liver microsomal cytochrome P-450s is that administration of various drugs to animals results in specific induction of a form or forms of cytochromeP-450 depending on the drug given (1). Although the mechanism of induction is not yet well understood, the induction by polycyclic aromatic hydrocarbons involves a cytosolic receptor thatbinds the inducer and enters the nucleus (17). In order to elucidate the biological significance of the multiplicity of hepatic cytochrome P-450 at the DNA level, several cDNA and genomic clones encoding different forms of cytochrome P-450 have been isolated and their structures have been analyzed (18-31). Based on these studies, it now seems that there are at least three types of cytochrome P-450 gene family in the rat, namely phenobarbital (PB)-inducible, 3-methylcholanthrene (MC)-inducible, and 16α-pregnenolone carbonitrile (PCN)-inducible cytochrome P-450 gene families.

Two closely similar forms of cytochrome P-450, called P-450b

and P-450e, have been purified from liver microsomes of PB-treated rats (12,13). That these two structurally related forms are the products of different genes has been confirmed by isolation and structural analysis of the corresponding genomic clones (19,20). These findings suggested that there is microheterogeneity at least among PB-inducible forms of cytochrome P-450 in rat liver microsomes. However, such microheterogeneity has not yet been extensively studied. Many forms of PB-inducible cytochrome P-450 have been purified from rabbit liver microsomes in this laboratory (4,5,7). It is, therefore, highly likely that microheterogeneity also exists in PB-inducible rabbit liver cytochrome P-450s, as in the case of the rat counterparts.

We report here the isolation of eight cDNA clones, including three full-length ones, that encode PB-treated rabbit liver cytochrome P-450s. Their nucleotide sequences and primary structures deduced therefrom are also presented and compared with sequencing data reported by other investigators. Some of the cDNA clones isolated were used for Northern blot analysis of cytochrome P-450 expression as well as for Southern blot analysis of their genes.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (1,000 Ci/mmol), [α-^{35}S]dATPαS (650 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), [γ-^{32}P]ATP (3,000 Ci/mmol), and [^{3}H]dCTP (50-85 Ci/mmol) were purchased from Radiochemical Centre, Amersham. The following chemicals and biochemicals were purchased from the sources indicated in parentheses: avian myeloblastosis virus reverse transcriptase (Life Science Inc.); Klenow fragment of *E. coli* DNA polymerase I, terminal deoxynucleotidyl transferase, and *Pst*I-cut, oligo (dG)-tailed pBR322 (Bethesda Research Laboratories); S_1 nuclease (Sankyo Co.); oligo $(dT)_{12-18}$ and oligo (dT)-cellulose (P-L Biochemicals); nick translation kits, M13 cloning and sequencing kits (Amersham International, Takara Shuzo), restriction enzymes (New England Biolabs, Nippon Gene, and Bethesda Research Laboratories); and nitrocellulose filters (S&S). Wheat-germ S-23 extracts were prepared as described (32). Plasmid pcP-450pb4 was a generous gift from Dr. Y. Fujii-Kuriyama of Cancer Institute, Japanese Foundation for Cancer Research (18).

Animals and Phenobarbital Treatment

Male, white rabbits, weighing 2-2.5 kg, were maintained on a standard laboratory chow and injected intraperitoneally with PB 16 h before sacrifice (for RNA preparation) or daily for 7 days (for P-450 proteins preparation) at a dose of 100 mg per capita each time.

Preparation of Anti-P-450 Antibodies

Cytochrome $P\text{-}450_1$ (the major form), $P\text{-}450_2$ and $P\text{-}450^*$ (the minor forms) were purified from PB-treated rabbit liver by the method described previously (33). Monospecific anti-P-450 antibodies were produced in guinea pigs and isolated as described (34). Their specificities were checked by an immunoblotting method (35).

RNA Preparations

Preparation of mRNA for cDNA synthesis was carried out as schematically shown in Fig 1. Total RNA was extracted with a sodium dodecylsulfate(SDS)-phenol-chloroform mixture from liver microsomes of PB-treated rabbit as described (36). The extracted RNA was then precipitated with 2 M LiCl as described by Schimke et al. (37). $Poly(A)^+$ RNA was enriched from the total RNA by two cycles of oligo(dT)-cellulose column chromatography (38) and size-fractionated by centrifugation through a sucrose concentration gradient from 5 to 25 % (w/v) in 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.2 % SDS at 30,000 rpm for 15 h at 15°C. After centrifugation, 0.4-ml fractions were collected by means of an ISCO model 640 fractionator. RNA in each fraction was precipitated with ethanol, dissolved in water, and an aliquot of each fraction was translated in wheat-germ S-23 extracts at 29°C for 60 min. The translates were then subjected to immunoprecipitation with antibodies raised against the three purified cytochrome P-450s. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis as described by Laemmli (39) and subsequently subjected to fluorography (40).

Each fractions was also analyzed by Northern blot hybridization using a fragment of pcP-450pb4 as a probe. Fractions in which mRNAs encoding cytochrome P-450s (sedimentation coefficient, 18-21 S) were pooled and used for cDNA synthesis. The guanidine thiocyanate/CsCl method (41) was used for small-scale preparation of mRNA that was used for Northern blot analysis. In this case, livers from variously drug-treated rabbits (which were generous gifts from Mr. N. Kagawa in this laboratory) were cut into small pieces and frozen in liquid nitrogen as soon as they were removed.

Construction of cDNA Library and Screening

cDNA synthesis was first performed according to the S_1 nuclease method described by Maniatis et al. (42), then by two methods as shown in Fig. 2 using the repair system described by Okayama and Berg (43). In Method I, cDNA was synthesized by reverse transcriptase using a "small primer" as a primer. This primer was prepared from pSV7186 as a by-product of preparation of the vector primer of Okayama and Berg as shown in Fig. 3. The DNA-RNA hybrid thus synthesized was oligo(dC)-tailed with terminal deoxynucleotidyl transferase and annealed with PstI-cut, oligo(dG)-tailed pBR322. Then the RNA strand was replaced by DNA with the aid of the repair system containing ribonuclease H, DNA polymerase I, and NAD^+-dependent DNA ligase (43). The ds-cDNA thus obtained was used to transform E. coli DH1. This method resulted in selection of a clone called pHP-2. In Method II, cDNA was synthesized by reverse transcriptase using oligo$(dT)_{12-18}$ as a primer. Then the RNA strand of the DNA-RNA hybrid was

replaced by DNA as described above in the linear state. After size fractionation by Sepharose CL-4B gel filtration, the cDNA fractions which were larger than 500 bp were pooled, oligo(dC)-tailed, annealed with PstI-cut, oligo(dG)-tailed pBR322, and used for transformation of E. coli DH1 or HB101. Two full-sized clones, pHP-1 and pHP-3, and other short clones were selected by this method. All the cloning procedures were conducted in a P-2 biocontainment facility in accordance with the Guidelines for Reseach Involving Recombinant DNA Molecules issued by the Ministry of Education, Science and Culture of Japan.

Colony and Southern Blot Hybridization

Approximately 30,000 tetracycline-resistant transformants were screened by in situ colony hybridization on nitrocellulose filters as described (44) using a fragment of pcP-450pb4 as a probe. Plasmid pcP-450pb4, whose insert encodes a PB-inducible form of rat liver microsomal cytochrome P-450 (18), was digested with BglII. A 390 bp BglII fragment thus obtained (see Fig. 9) was labeled with [α-^{32}P]dCTP by nick-translation, and used as a probe in this colony hybridization. A 180 bp BglII-HindIII fragment was also used as a probe. The colony hybridization was carried out at 55°C for 18 h in a sealed bag containing a hybridization buffer (5 x SSC-10 x Denhart's-0.1 % SDS-100 µg/ml of sheared and heat denatured salmon sperm DNA). After hybridization, the filters were washed twice with 2 x SSC-0.2 % SDS at 55°C, dried, and autoradiographed at -80°C. Rabbit liver DNA was isolated as described (45), digested with various restriction enzymes, and subjected to agarose (1 %) gel

electrophoresis. The DNA fragments were then subjected to Southern blot analysis (46). PstI digests of positive cDNA clones were also subjected to Southern blot analysis. Southern hybridization was carried out as described above (see colony hybridization) except that the hybridization temperature was performed 65°C.

Restriction Enzyme Mapping and DNA Sequencing

DNAs of hybrid plasmids were prepared by lysozyme-alkali SDS lysis of cells and polyethylene glycol precipitation or CsCl gradient centrifugation as described (42,47). Restriction enzyme mapping of hybrid plasmid was carried out using various restriction endonucleases, and the digested DNA fragments were analyzed by 1-1.5 % agarose gel electrophoresis. Sequence determination of the DNA fragments was mainly carried out by the dideoxy termination method (48) using [α-^{35}S]dATPαS as a radioactive label, essentially according to the instructions provided by the kit supplier. In some cases the sequences were determined by the chemical cleavage method (49).

Northern Blot Hybridization

Total RNA (about 10 µg) isolated from livers of drug-treated and untreated rabbits was denatured in 2.2 M formaldehyde and 50 % formamide for 5 min at 55°C and subjected to electrophoresis on 1 % agarose gel containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred to a nitrocellulose filter and baked at 80°C for 2 h. Hybridization was carried out first with sheared and heat denatured salmon sperm DNA, then with ^{32}P-

labeled probes at 42°C for 18 h in a hybridization buffer (50% formamide-50 mM sodium phosphate (pH 6.5)-5 x Denhart's solution-5 x SSC-250 µg/ml of sheared and heat denatured salmon sperm DNA). Washing procedures were carried out as described in "Colony and Southern Blot Hybridization".

Other Methods

Purified P-450 was digested by lysyl-endopeptidase and the resulting peptide fragments were purified by HPLC (50). The NH_2-terminal sequences of these peptide fragments and some purified P-450s were determined with a Gas Phase Sequenator (Applied Biosystems, 470A) (51). Evaluation of hydropathy were performed by the published procedures (52).

RESULTS

Partial Purification of Cytochrome P-450 mRNA

We purified three forms of cytochrome P-450, P-450_1, P-450_2, and P-450^*, from liver microsomes of PB-treated rabbits (4,7,32) and prepared antibodies in guinea pigs against these forms of cytochrome P-450. P-450_1 is the major PB-inducible form, P-450_2 is a minor form having high affinity with coumarin derivatives (7), and P-450^* is a by-product in the purification of P-450_2 (33). The NH_2-terminal sequence of P-450^* was quite identical with that of rabbit liver cytochrome P-450 (isozyme 3c; LM3c) (53). As a first step of partial purification of mRNA encoding PB-inducible forms of cytochrome P-450, we examined the levels of these forms of cytochrome P-450 in liver microsomes from variously treated rabbits by Western blot hybridization. Fig. 4 shows the results obtained with anti-P-450_2 antibodies. As can be seen, the antibodies reacted strongly with microsomes from untreated (lane 1) and PB-treated (lane 2) rabbits. They cross-reacted with purified P-450_1 (lane 6) but not with P-450^* (lane 8). The content of cytochrome P-450 reacting with anti-P-450_2 antibodies was highest in microsomes from untreated and PB-treated rabbits, followed by those from ISF-treated animals (lane 5). The contents in liver microsomes from MC- and β-NF-treated rabbits were low (lane 3 and 4). Although data are not shown, anti-P-450_1 antibodies reacted strongly with microsomes from PB-treated rabbits, but did not practically react with those from untreated animals. It therefore seemed desirable to use microsomes from PB-treated rabbits for preparation of RNA.

To examine the contents of mRNA coding for PB-inducible forms of cytochrome P-450 in various types of livers, we isolated total RNAs from livers of variously treated rabbits and analyzed by Northern blot hybridization using a Bg1II fragment (containing highly conserved "HR2" region (54)) of pcP-450pb4, a cDNA clone encoding a PB-inducible rat liver microsomal cytochrome P-450 (P-450b) (18) as a probe. As shown in Fig. 5A, this probe hybridized very strongly with total RNA from PB-treated rats (lane 6). Among rabbit liver microsomes, only those from PB-treated animals hybridized with this probe (lane 2). Poly(A)$^+$ RNA from the same source exhibited a stronger hybridization signal (lane 8). In all positive cases, only a single RNA species showing a mobility corresponding to about 2,000 nucleotides long was detected. We therefore decided to use liver microsomes from PB-treated rabbits as the source for partial purification of cytochrome P-450 mRNA. In case of using rabbit cDNA fragment as a probe, its result was shown in Fig. 5B.

Poly(A)$^+$ RNA was enriched by oligo(dT)-cellulose chromatography from total RNA extracted from liver microsomes of PB-treated rabbits. The poly(A)$^+$ RNA preparation was fractionated by centrifugation through a linear sucrose concentration gradient (from 5 to 25 % (w/v)). The content of mRNA coding for PB-inducible cytochrome P-450 in each fraction collected after centrifugation was analyzed by subjecting it to translation in a wheat-germ cell-free system followed by immunoprecipitation with a mixture of anti-P-450$_2$ and anti-P-450* antibodies. As shown in Fig. 6, mRNA encoding these forms of cytochrome P-450 was found to be concentrated around Fraction 18.

This was further confirmed by Northern blot hybridization analysis with the BglII fragment of pcPB-450pb4 (data not shown). We therefore pooled Fractions 15 through 20 and used for cDNA synthesis. These fractions had sedimentation coefficients from 18 to 21 S. It was estimated by immunoprecipitation that about 5 % of the total RNA in the pooled fractions was mRNA encoding these forms of cytochrome P-450.

Synthesis of Double-stranded cDNA and Selection of Plasmids Carrying PB P-450 cDNA

cDNA was synthesized from the partially purified poly(A)$^+$ RNA by two methods as described in "Materials and Methods". In Method I, approximately 2,500 tetracycline-resistant colonies were obtained. These colonies were screened by in situ colony hybridization under a low stringent condition using the ^{32}P-labeled BglII fragment of pcP-450pb4 (rat PB P-450 clone) as a probe, and 16 positive clones were obtained. Of these clones, one called pHP-2 produced a faint signal in colony hybridization but was found to have the longest insert of 2.1 kbp. Although the other clones exhibited stronger signals, they contained smaller inserts of 1.0-1.7 kbp. The length of mRNA coding rat P-450b was estimated to be approximately 2.0 kbp by Northern blot hybridization, as described above. Clone pHP-2 was, therefore, expected to contain a full-length cDNA encoding a cytochrome P-450. Both the single and double stranded cDNAs prepared by Method II were labeled with [α-^{32}P]dCTP and analyzed by 1 % alkaline agarose gel electrophoresis and subsequent autoradiography. As can be seen in Fig. 7A, the first strand

preparation (lane 2) contained components longer than 2 kbp, whereas the distribution of the double stranded DNA preparation was shifted to the larger side (lane 3). This suggested the possibility that a "runback" reaction using hairpin structures took place partially. In any case, the ds DNA preparation was size-fractionated by CL-Sepharose 4B gel filtration (Fig. 7B). The fractions whose minimum length was larger than 500 bp (lanes 2-5) were pooled, oligo(dC)-tailed, and annealed with PstI-cut, oligo(dG)-tailed pBR 322. This was then used to transform E. coli DH1 or HB101, leading to the production of approximately 30,000 transformants. By screening these transformants by in situ colony hybridization with a mixture of appropriate cDNA fragments as a probe, about 160 positive clones were obtained. The strength of their hybridization signals varied among the individual colonies, probably depending on the difference in homology of their sequences with those of the probe used. Fig. 8 shows part of the results of in situ colony hybridization. DNAs of these positive clones were prepared from overnight cultures (1.5 ml each) and digested with PstI to estimate the size of their respective inserts. Five clones, termed pHP-1, pHP-3, M046, b43FP1 and M12, contained fairly large inserts and were, therefore, further studied. Especially, pHP-1 (indicated by an arrow in Fig. 8B) and pHP-3 were expected to contain full-length cDNA coding for cytochrome P-450s. Other clones, termed B52, B14 and b43 were obtained by the S_1 nuclease method.

Restriction Maps of the Cloned cDNAs

Plasmids DNA of the above eight clones containing large

inserts were prepared at large scale and their restriction maps were made, and the results are shown in Fig. 9. pHP-1, M046, B52, and B14, which produced intense hybridization signals with a fragment of pcP-450pb4, showed similar restriction maps. Especially, the restriction maps of B52 (insert, about 1.2 kbp) and B14 (insert, about 1.2 kbp) were identical with that of pHP-1 (insert, 1.8 kbp) in their overlapping region. These three clones may represent the same cDNA. M046 (insert, 1.65 kbp) differed from pHP-1 in deleting two AvaI sites between the AccI and SacI sites and one of the four internal PstI sites of pHP-1. The other five clones, pHP-2, pHP-3, M12, b43 and b43FP1, gave only faint hybridization signals with the rat cDNA probe. Among them, pHP-2, which was obtained by Method I and assumed to contain a full-length cDNA, exhibited an identical restriction map with pP-450PBc2, a clone reported by Leighton et al. (28) encoding a PB-inducible rabbit liver microsomal cytochrome P-450. The cDNA in the latter clone is not a full-length one lacking the sequence corresponding to the NH_2-terminal 11 amino acid residues, whereas the insert in pHP-2 is elongated by 90 bp at the 5' end and its total length is 1.9 kbp. The restriction map of pHP-3 (insert, 1.7 kbp) was similar but clearly different from that of pHP-2 in that the EcoRI and BamHI sites in pHP-3 were missing in pHP-2. The maps of M12 (insert, 1.2 kbp), b43 and b43FP1 were quite different from those of the clones so far described. b43FP1 was isolated by using the 5'-region of b43 as a probe, and they showed the same restriction map in their overlapping portion. These two clones together covers a cDNA that are 1.5 kbp long. From these results, it appears that the

cDNA clones isolated can be classified into at least four distinct groups as follows: (pHP-1, M046, B52 and B14), (pHP-2 and pHP-3), (M-12) and (b43(b43FP1)). Fig. 10 shows the results of Southern blot hybridization among these cDNA clones. When the XmaI fragment of pHP-1 was used as a probe, pcP-450pb4, pHP-1, and M046 gave strong signals, but pHP-2, pHP-3, b43 and M12 gave only faint signals (Fig. 10A). On the other hand, when the PstI-EcoRI fragment of M12 was used as a probe, pHP-2, pHP-3, and M12 gave strong signals, but b43 gave a medium signal. pcP-450pb4, pHP-1 and M046, on the other hand, gave only faint signals (Fig. 10B). These results suggested that M12 is related to the group comprising pHP-2 and pHP-3, whereas b43 (b43FP1) belongs to a group distinct from the other ones.

Sequence Analysis of the cDNA Clones

Sequence analysis of the cloned cDNAs was conducted by the M13 dideoxy method, but the sequences of some regions, where this method gave ambiguous results, were confirmed by the method of Maxam and Gilbert. Fig. 11 shows the strategy adopted for sequence determination of the inserts of pHP-1, pHP-2 and pHP-3. The sequences of most parts of the cDNAs were determined at least twice. Fig. 12 shows the complete nucleotide sequence of the insert of pHP-1. This sequence contains an open reading frame spanning 491 amino acid residues, and the deduced amino acid sequence is also shown in Fig. 12. The total length of the cDNA is 1,802 nucleotides including a 37 bp 5'-noncoding sequence and a 289 bp 3'-noncoding sequence. However, it lacks any polyadenylation sequence. The deduced primary structure contains

two highly conserved regions among cytochrome P-450s: "HR1" from residues 142 through 158 and "HR2" from residues 429 through 446. The conserved cysteinyl residue in the "HR2" region, which is thought to bind to the heme (54), is positioned at residue 436. A molecular weight of 55,700 was calculated for the deduced primary structure. This value is very higher than the molecular weight (49,000) determined for purified P-450_1, the major PB-inducible rabbit liver cytochrome P-450, by SDS-polyacrylamide gel electrophoresis (4,5). Such discrepancies have also been reported for other microsomal cytochrome P-450s (18). The amino acid sequence predicted from the nucleotide sequence of pHP-1 exhibits an approximately 76 % homology with those of P-450b and P-450e, two major PB-inducible forms of rat liver microsomal cytochrome P-450. This high homology suggests that the pHP-1 protein is a rabbit counterpart of rat P-450b and P-450e, which are closely related to each other (13,20). Coon and associates have recently reported the complete amino acid sequence of the major PB-inducible form of rabbit liver cytochrome P-450 (isozyme 2; LM2) (55). This sequence is more than 95 % homologous with that of pHP-1, but there are 17 amino acid substitutions between them (see Table I). The sequences of the cDNA clones that exhibit similar restriction maps to that of pHP-1, i.e. M046, B52, and B14, were also determined as shown in Fig. 13. None of these sequences are identical with that of P-450 isozyme 2, but they are highly homologous with one another. The sequence of another clone (B54) is practically identical with that of M046 except for one substitution, the cysteinyl (TGC) residue at position 425 in B54 is replaced by a glycyl (GGC) residue in

M046. We also conducted partial amino acid sequencing of purified P-450$_1$ (4) by Gas Phase Autosequenator and found that its sequence is consistent with the partial amino acid sequence deduced for B14. These cytochrome P-450s, therefore, seem to belong to a distint group, in which microheterogeneity exists, as in the case of rat P-450b and P-450e. The amino acid substitutions seen in this group are summarized in Table I. In preliminary experiment, another cDNA clone (M03), which was identical with B52, was isolated and found to have at least 230 bp longer 5'-coding sequence. Its partial sequencing data was also included in Table I. Fig. 14 shows the positions where amino acid substitutions are seen in this group and those between rat P-450b and P-450e. As can be seen, the substitutions in the rat P-450 proteins occur only in the COOH-terminal portion, whereas those in the rabbit group are distributed rather randomly over the entire sequence, although two or three small regions seem to be highly variable.

Fig. 15 shows the complete nucleotide sequence and the deduced amino acid sequence of the cDNA insert of pHP-2. This cDNA contains an open reading frame spanning 490 amino acid residues, in which the "HR1" and "HR2" regions can be detected. The total length of the cDNA is 1,948 nucleotides including a 59 bp 5'-noncoding sequence and a 407 bp 3'-noncoding sequence. The 3'-noncoding region includes a polyadenylation sequence and two possible poly(A) addition signals, i.e. AATAGA at nucleotides 1,857-1,862 and AATTAA at nucleotides 1,924-1,929. The occurrence of two or more poly(A) signals in cytochrome P-450 cDNAs has been reported by other investigators (23,28). As

mentioned above, the restriction map of this clone is identical with that of pP-450PBc2 isolated by Leighton et al. (28). The latter clone, however, lacks the 5'-noncoding region, the sequence encoding the NH_2-terminal 11 amino acid residues, and the polyadenylation sequence. Both sequences are identical with each other except for two nucleotide substitutions, i.e. T to C at nucleotide 1,469 leading to a substitution of leucine to proline, and C to T at nucleotide 1,780 in the 3'-noncoding region. The molecular weight calculated for the deduced primary structure is 55,800.

The complete nucleotide sequence determined and the primary structure deduced for the insert of pHP-3 are shown in Fig. 16. This sequence also contains an open reading frame encoding 490 amino acid residues. The total length of the cDNA is 1,659 nucleotides including a 24 bp 5'-noncoding sequence and a 162 bp 3'-noncoding sequence which lacks poly(A). A molecular weight of 55,700 was calculated for the deduced primary structure. The homology in amino acid sequence between pHP-2 and pHP-3 is as high as 80 %. The deduced amino acid sequences of these two clones exhibit 65 and 67 % homology, respectively, with that of form 3b, a constitutive cytochrome P-450 from rabbit liver microsomes, recently reported by Ozols et al. (56) as shown in Fig. 17. The homology between form 3b and pHP-1, the major PB-inducible form, is less than 50 %. The sequences of form 3, pHP-2 and pHP-3 all have the cysteine residue in the "HR2" region at the position of 435, instead of 436 in pHP-1, and a deletion of residue 22 of pHP-1.

Fig. 18 shows the nucleotide sequence determined and the

amino acid sequence deduced for the insert of M12. This cDNA is 1.8 kbp long and lacks a sequence encoding about 110 amino acid residues at the NH_2-terminus. The primary structure encoded by this cDNA is highly homologous (90 %) with that of a rabbit hepatic progesterone 21-hydroxylase (a cytochrome P-450), which has recently been clone by Tukey et al. (30) (see Fig. 17). The result of Southern hybridization of cDNAs is consistent with that the insert of M12 exhibits 77 % homology with those of pHP-2 and pHP-3. This indicates that progesterone 21-hydroxylase is also related to pHP-2 and pHP-3. However, the sequences of M12 and 21-hydroxylase have a three-residue deletion at the position of 286-288 as compared with those of pHP-2 and pHP-3. Like the latter two cDNAs, those of M12 and 21-hydroxylase has a deletion at residue 22 of pHP-1.

Finally, the nucleotide sequence and the deduced primary structure for the overlapping two clones, b43 and b43FP1 are shown in Fig. 19. In the overlapping region the nucleotide sequences of the two clones are exactly identical. Therefore, the fused sequence of the two clones will hereafter be termed the b43(FP1) sequence. The b43(FP1) sequence is approximately 1.52 kbp long. From homology comparison with other cytochrome P-450 sequences, it seems that the b43(FP1) sequence lacks the NH_2-terminal three residues. As compared with the primary structure of pHP-1, there are deletions at residues 22 and 105 and one more COOH-terminal residue (serine) is added in the b43(FP1) sequence. Another feature of this sequence is that the cysteine residue in the "HR1" region of many other cytochrome P-450s is replaced by phenylalanine, although the cysteine in the "HR2" region is

conserved. As in the case of $P\text{-}450_{scc}$ in bovine adrenal cortex mitochondria (57), this fact provides a support to the notion that the cysteine in the "HR2" region interacts with the heme (54). The homology in amino acid sequence between these clones and others is less than 60 %. No hepatic cytochrome P-450s corresponding to this type of P-450 have so far been reported.

Table II shows the amino acid compositions of three cytochrome P-450s encoded by the three full-length cDNA clones isolated in this study, pHP-1, pHP-2 and pHP-3, together with those determined for P-450 LM2 (form 2) (55), form 3b (56), and progesterone 21-hydroxylase (30), all of which are cytochrome P-450s from rabbit liver microsomes. It is evident that the amino acid composition of the pHP-2 protein is closely similar to that of the pHP-3 protein. The pHP-1 protein, on the other hand, contains much less asparagine, cysteine and lysine residues than the former two. However, the composition of the pHP-1 protein is very similar to that of P-450 LM2. The compositions of form 3b and 21-hydroxylase are not so similar to those of both the pHP-2 and pHP-3 group and the pHP-1 and P-450 LM2 group. All the proteins listed in Table II consist of 490 or 491 amino acid residues, except for the 21-hydroxylase protein which contains only 487 amino acid residues. Fig. 20 shows hydropathy profiles of the pHP-1, pHP-2 and pHP-3 proteins together with that of $P\text{-}450_{cam}$, which is a soluble protein purified from camphor-grown Pseudomonas putida (58). The profiles for the three full-length cDNA clone are rather similar to one another in spite of the relatively poor homology in amino acid sequence between pHP-1 and pHP-2 (pHP-3) (50 % homology). The similarity of hydropathy

profiles for the three clones suggest that they belong to a common gene family, which is probably different from those comprising MC-inducible cytochrome P-450s and pregnenolone 16α-carbonitrile-inducible ones. The NH_2-terminal hydrophobic region (about 20 amino acid residues) in each of the three profiles is thought to be required for the protein to be inserted into microsomal membranes (59). This is supported by the fact that the NH_2-terminal region of $P\text{-}450_{cam}$, which is not a membrane protein, lacks a hydrophobic stretch.

Northern Blot Analysis of mRNA in Liver Microsomes

Total RNA was isolated from livers of untreated and variously drug-treated rabbits by the guanidine thiocyanate/CsCl method and subjected to agarose gel electrophoresis in the presene of 2.2 M formaldehyde. After blotting to nitrocellulose membranes, the RNAs were hybridized with the XmaI fragment of pHP-1 cDNA (Fig. 21A), the HindIII-BamHI fragment of pPH-2 cDNA (Fig. 21B), and the PstI-EcoRI fragment of b43 cDNA (Fig. 21C), all of which had been ^{32}P-labeled by nick translation. The hybridization was visualized by autoradiography. As shown in Fig. 21A, the pHP-1 probe hybridized with a single RNA species of about 2,000 nucleotides long in all cases. The hybridization was most extensive with RNA from PB-treated liver (lane 3) followed by that from ISF-treated one (lane 6). The RNAs from livers from untreated (lane 2), MC-treated (lane 4) and β-NF-treated rabbits (lane 5) hybridized with this probe only weakly. When the HindIII-BamHI fragment of pHP-2 cDNA was used as a probe (Fig. 21B), a RNA species of about 2,000 nucleotides long hybridized

strongly with the probe in the RNAs from PB- and ISF-treated livers (lanes 3 and 6), although the intensity of hybridization signals was less pronounced than in the case of pHP-1 probe. In the case of pHP-2 probe, a RNA species larger than 2,000 nucleotides seemed to hybridize with the probe weakly in lanes 3 and 6. It was likely that such double hybridization resulted from altered mRNA splicing. In fact, we isolated another cDNA clone (b32-11) that is identical with pHP-2 but has a much longer 3'-noncoding region. In any case, it could be concluded that PB and ISF induce the expression of the pHP-1 group of genes strongly and that of the pHP-2 group of genes weakly. When the PstI-EcoRI fragment of b43 cDNA (600 bp long) was used as a probe (Fig. 21C), however, hybridization signals were more pronounced with RNAs from MC-, ISF- and β-NF-treated livers than those from PB-treated and untreated livers. These results suggested that the gene group comprising b43 is quite different in inducibility from the above two groups and expressed more strongly under the influence of MC, β-NF and ISF than that of PB.

Southern Blot Analysis of Genomic DNA

To gain preliminary information concerning the diversity of genes encoding PB-inducible forms of cytochrome P-450, rabbit genomic DNA was investigated by Southern blot analysis. Rabbit liver nuclear DNA was isolated, digested with a restriction enzyme (EcoRI, BamHI, HindIII, or PstI), and subjected to agarose gel electrophoresis. After blotting to nitrocellulose membranes, hybridization was carried out under a highly stringent condition with the XmaI fragment of pHP-1 cDNA or the HindIII-BamHI

fragment of pHP-2 cDNA, both ^{32}P-labeled by nick translation. The results obtained with the pHP-1 and pHP-2 probes are shown in Fig. 22A and B, respectively. As can be seen, a number of DNA fragments produced by a restriction enzyme hybridized with the probe and the fragments hybridized with one probe were clearly different from those hybridized with the other probe. It is to be noted that under the hybridization conditions used in this experiment the XmaI fragment of pHP-1 could hybridize with M046, B14 and B52 cDNAs and the HindIII-BamHI fragment of pHP-2 with pHP-3 and M12 cDNAs (data not shown). Although these results provide only preliminary information, genes encoding the pHP-1 and pHP-2 proteins seem to be different from each other and each forms a multigene family as reported previously (19,26,30).

DISCUSSION

In constructing ds-cDNA from partially purified mRNA we first tested several methods: a) ds-cDNA synthesis utilizing the hairpin structure of the first strand and S_1 nuclease digestion, followed by G-C annealing with pBR322 (42); b) G-C annealig of mRNA-ss-DNA hybrids with pBR322 (60); c) the method of Land et al. (61); and d) the method of Okayama and Berg (43). We could isolate several cDNA clones by Method a, but they contained rather short inserts (average size, about 1 kbp). Although method b is very simple, it was disadvantageous because of rather low efficiency of transformation (about 5×10^3 transformants per μg of DNA). Both methods c and d gave higher transformation efficiencies than method b and yielded clones whose inserts were longer than those obtainable by method a. Methods c and d, however, involve tedious procedures. Based on these preliminary experiments, we finally adopted two methods, Method I and Method II, which are described under "Materials and Methods". As reported above, we could isolate many clones and characterize among them three full-length cDNA clones together with five clones containing shorter cDNA, all of which are complementary to mRNAs encoding cytochrome P-450s present in liver microsomes of PB-treated rabbits. Such many cytochrome P-450 cDNA clones have not yet been isolated from one source.

Of the three full-length cDNA clones, pHP-2 contains an insert that is overlapping with that of pP-450PBc2 isolated by Leighton et al. (28) from the same source. pP-450PBc2 is an incomplete clone, which lacks a sequence coding for NH_2-terminal

11 amino acid residues. In any case, in the overlapping region of both cDNAs there are only two nucleotide substitutions. As briefly mentioned above, we isolated another clone, called b32-11, which has also a full-length cDNA and a longer 3'-noncoding region than pHP-2. The nucleotide sequence of the insert of b32-11 is completely identical with that of pHP-2 in their overlapping region. It is likely that the two nucleotide substitutions between pHP-2 (and b32-11) and pP-450PBc2 are due to a strain difference (Japanese domestic and New Zealand white rabbits) or to misreading by reverse transcriptase. Since the insert of pHP-2 contains a 5'-noncoding sequence and a poly(A) sequence at the 3'-terminus, this can be regarded as an almost complete cDNA. As mentioned above , the reason for the longer 3'-noncoding region in b32-11 is to be explored. The deduced primary structure of the polypeptide encoded by another full-length clone, pHP-3, is about 80 % homologous to that of pHP-2, suggesting that they belong to the same group. However, both the pHP-2 and pHP-3 polypepetides are only 50 % homologous to P-450_{LM2}, which is the major PB-inducible cytochrome P-450 in rabbit liver (55).

Among the clones we isolated, pHP-1 (full-length), M046, B14 and B52 encodes polypeptides that exhibit as high as 97 % homology with P-450_{LM2}. It is, therefore, likely that one of these four clones encodes a major PB-inducible cytochrome P-450 in rabbit liver. In fact, the partial amino acid sequence deduced for the B14 polypeptide is consistent with that of P-450_1, the major PB-inducible rabbit liver cytochrome P-450 purified in our laboratory (4). At any rate, it is certain that

genes coding for the pHP-1, MO46, B14 and B52 polypeptides belong to the same multigene family. Southern blot analysis of rabbit genomic DNA with a pHP-1 fragment as a probe actually suggests that there are many genes that hybridize with the pHP-1 probe under stringent conditions. Elucidation of the exact number of genes belonging to this group, however, requires more quantitative assays such as Cot analysis. It is to be noted that the restriction maps of most of the positive clones so far examined are similar or identical with those of the four clones. It has been reported that the rat PB-inducible cytochrome P-450 gene family contains more than six genes (20).

Although the four clones are very similar to one another, some of them can be distinguished on the basis of their restriction maps. For example, the maps of MO46 and pHP-1 are similar but clearly different from each other. Thus, the former lacks two AvaI sites between the AccI and SacI sites. MO3, which is identical with B52 but has a longer 5'-sequence (not mentioned in the Results section), has a XmaI site instead of the AvaI site of pHP-1 and MO46, located between the SacI and BglII sites (see Fig. 9). As mentioned above, we could isolate a number of cDNA clones belonging to this group. Among them so far examined, those having MO46-type restriction maps are rare, suggesting that the MO46 polypeptide is a minor form of cytochrome P-450 in PB-treated rabbit liver. Partial sequence analysis of the "variable region", which has been noticed in rat PB-inducible cytochrome P-450 (19), of the positive clones belonging to this group showed that most of them are identical with pHP-1 and B52. On the other hand, partial amino acid sequencing of purified $P\text{-}450_1$, which is

thought to be the major PB-inducible form of cytochrome P-450 in rabbit liver (4), indicated that the primary structure of P-450_1 protein is encoded by B14 but not by pHP-1 and B52. It is not clear why the most abundant species isolated is different between cDNA and protein levels. Some explanations are, however, possible as follows; i) mRNA was isolated from liver 16 h after an injection of PB to a rabbit, while P-450 protein was purified from liver microsomes after daily treatment of the animals with PB for 7-10 days. There is a possibility that each mRNA belonging to this group shows differently time-dependent response to PB treatment. ii) Assuming the secondary structure of P-450_1 mRNA (B14), extension of cDNA to the nearly full-length is difficult. The sequencing analysis in this work was carried out for the clones having large inserts, so those having small inserts were not detected even if they were present abundantly. Recently Giachelli et al. has reported on synthetic oligonucleotides probes which distinguish P-450b and P-450e mRNAs in rat (62). Moreover Reik et al. has reported on monoclonal antibodies which distinguish P-450b and P-450e (63). Using these techniques may give a clue to elucidate such discrepancy mentioned above.

Microheterogeneity in the structure of PB-inducible forms of liver microsomal cytochrome P-450 has been detected in the rat (13,20). Thus, rat P-450b and P-450e show only 14 amino acid substitutions, which are all located in the COOH-terminal portion consisting of some 200 residues (20). It is clear that microheterogeneity also exists in the four rabbit cytochrome P-450s encoded by the four clones. However, in the case of rabbit

amino acid substitutions are rather randomly distributed over the entire primary structure, though there is a tendency that they are concentrated in the COOH-terminal half (see Fig. 13). It has been shown in the rat that substitutions occur most frequently in exon 7 (20). An explanation for this fact is to assume that extensive gene conversion has occurred within this gene family in the region including exon 7 (64). However, this gene conversion mechanism cannot account for the randomly distributed substitutions in the rabbit. Isolation and structural analysis of genomic clones corresponding to these four cDNA clones are needed to elucidate the evolution of cytochrome P-450 genes in the rabbit.

A homology comparison in the primary structures of the eight cDNA clones investigated here and those sequenced in other laboratories indicates that there are at least five groups of cytochrome P-450 in the liver of PB-treated rabbits as shown in Fig. 23. One group contains pHP-1, M046, B52, B14, and $P\text{-}450_{LM2}$, which show 97 % homology, as mentioned above. pHP-2, pHP-3, and pP-450PBc1 are 80-84 % homologous with one another and thus form another group. A third group includes M12 and hepatic 21-hydroxylase (30), which are 91 % homologous with each other. $P\text{-}450_{LM3b}$ (55) and pP-450PBc3 belong to a fourth group, and b43 is only about 50 % homologous with any of the other groups. It is likely, though not yet proven, that the last four groups, like the first group, exhibit various degrees of microhetrogeneity. It should be mentioned that $P\text{-}450_{LM3b}$ (pP-450PBc3) is a constitutive form of cytochrome P-450 (28,55). Northern hybridization analysis indicates that the cytochrome encoded by

b43 is not inducible by PB but by MC, β-NF and ISF. A likely possibility is that we happened to isolate this clone from the cDNA library containing a very small amount of b43 cDNA. As mentioned above, members of the pHP-1-containing group are approximately 80 % homologous with rat P-450b, whereas homology of members of the other groups with rat P-450b is at most 50 %. Therefore, it is expected that the rat P-450b cDNA probe used in this study for screening could not detect clones other than those belonging to the first group if hybridization conditions were very stringent.

Fig. 24 shows the amino acid residues that are conserved in all the rabbit cytochrome P-450s so far mentioned in this paper. It is evident that a 20-residue stretch containing Cys-436, termed "HR2" region (54), is 75 % conserved (15/20 residues). The conservation of this region is surely related to the role of Cys-436 as the heme-binding site (54). On the other hand, homology of the "HR1" region (residues 142 through 158) (54) is only 47 %. Another highly conserved region, which containing "analogous peptides" reported by Ozols et al. (65), is a stretch extending from residues 341 to 358; this region is 70 % conserved. Two more notable conserved regions are a proline cluster (residues 30 through 38), LPPGP-P-P, and a threonine-serine cluster (residues 298 through 307), AGT-TTS-TL. In addition, there are several regions where four or five continuous amino acid residues are conserved. It is certain that these conserved regions are related to functions common for all the cytochrome P-450s examined, such as the binding to the heme as mentioned above and the interaction with NADPH-cytochrome P-450

reductase or cytochrome b_5. On the other hand, regions that are not conserved are thought to be responsible for functions specific for the individual cyatochrome p-450s, such as substrate binding. A structural comparison of $P\text{-}450_{scc}$ and sterol binding protein (66) and crystallographic analysis of $P\text{-}450_{cam}$ (67) suggest that the region around residue 200 is a substrate binding site. This region is not conserved in the cytochrome P-450s examined in Fig. 24. Further studies are still needed to elucidate the relation between the conserved and variable regions on one hand and the functions of cytochrome P-450s on the other.

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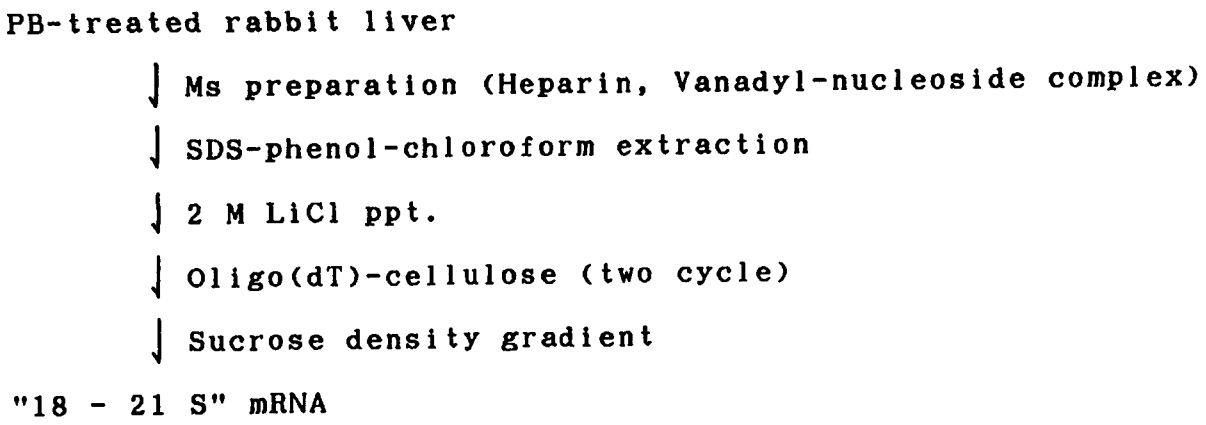

Fig. 1. The scheme of mRNA preparation for cDNA synthesis. See "Materials and Methods" for details. P-450 mRNA activity of each fraction of sucrose density gradient was assayed by the two methods. 1) in vitro translation in wheat-germ extracts followed by immunoprecipitation with anti-P-450 antibodies. 2) Northern blot analysis using rat P-450b cDNA as a probe.

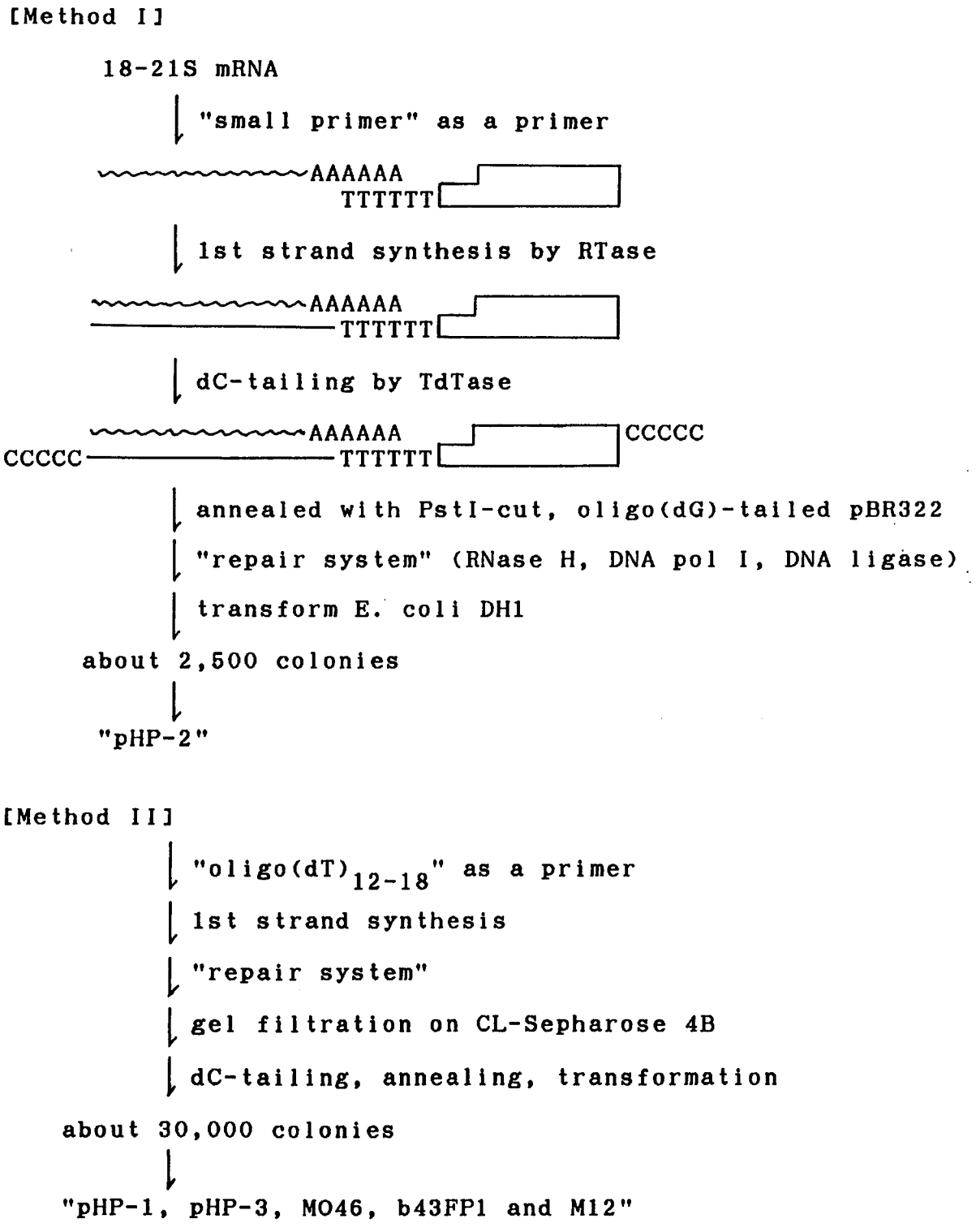

Fig. 2. The scheme of the two methods for cDNA sysnthesis using a repair system. See "Materials and Methods" for details.

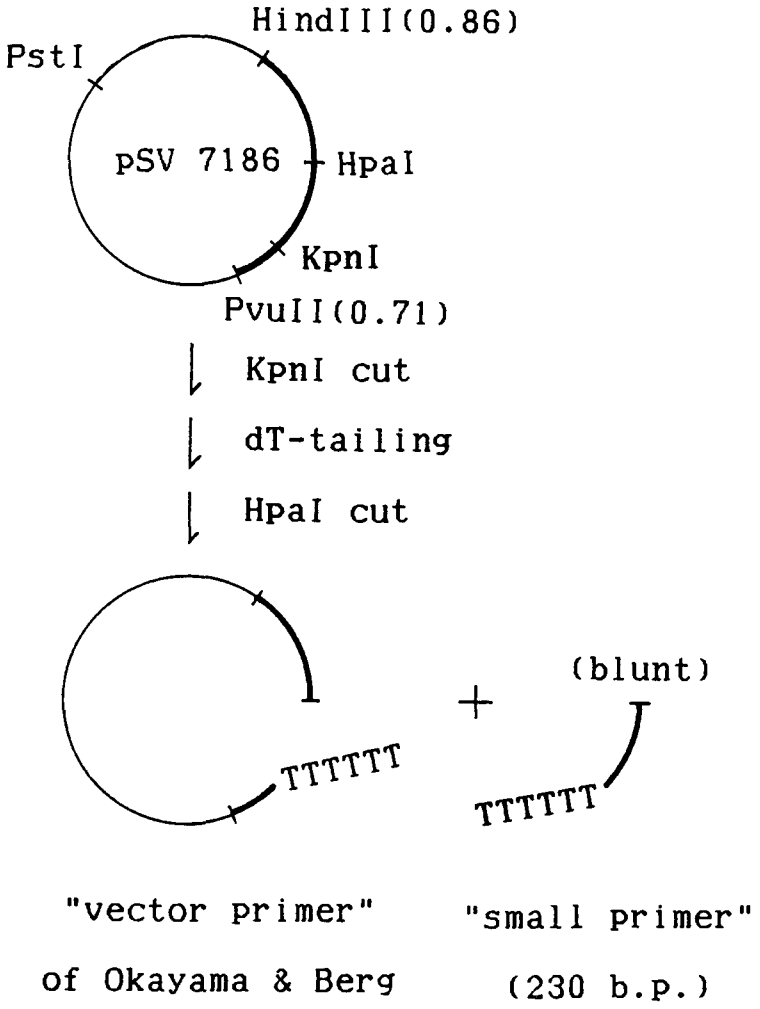

Fig. 3. Preparation of "small primer". This "small primer" (230 bp) prepared from plasmid pSV 7186 as a by-product of the vector primer preparation of Okayama and Berg (42). pSV 7186 was digested with KpnI, d(T)-tailed with terminal deoxynucleotidyl transferase, and digested with HpaI.

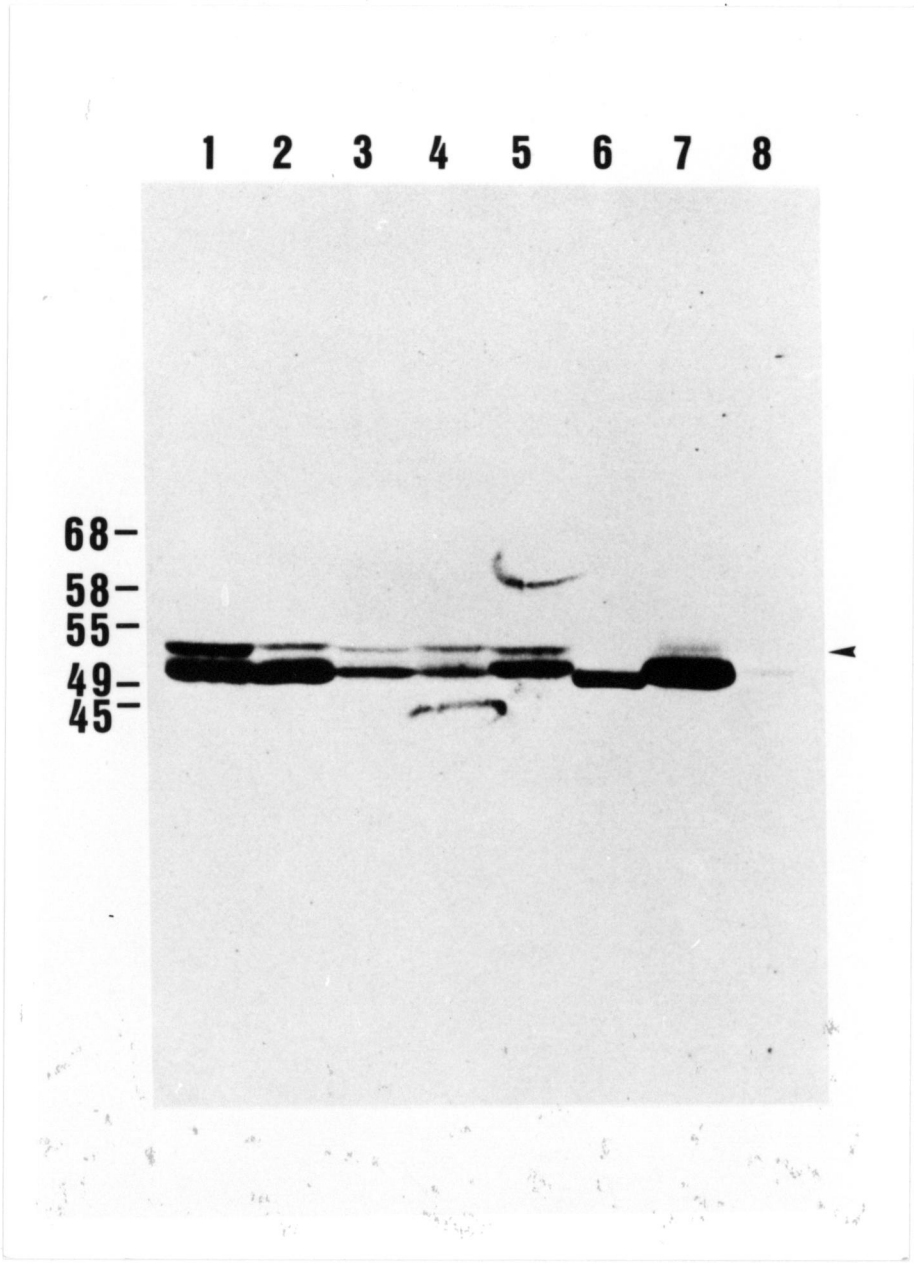

Fig. 4. Western blot hybridization analysis with anti-P-450_2 antibodies. Microsomes (lane 1-5) and purified cytochrome P-450s (lane 6-8) were subjected to SDS-polyacrylamide gel electrophoresis (10 % gel) and then transferred to nitrocellulose membrane. The blotted membrane was incubated with anti-P-450_2 antibodies in 10 mM KPi(pH 7.5)-140 mM NaCl-10 mg/ml BSA-0.1 % Triton X-100 and detected by means of ^{14}C-labeled protein A as described (34). Lanes: 1 to 5, microsomes from untreated, PB-, MC-, β-NF- and ISF-treated rabbit liver, respectively; 6 to 8, purified P-450_1, P-450_2 and P-450*, respectively. Numbers on the left-hand side of lane 1 indicate the Mr x 10^{-3} of marker proteins (bovine serum albumin, 68K; bovine liver catalase, 58K; goat immunoglobulin G heavy chain, 55K; pig heart fumarase, 49K; ovalbumin, 45K). Arrow head on the right-hand side of lane 8 indicates the location of P-450* when stained by Coomassie-blue.

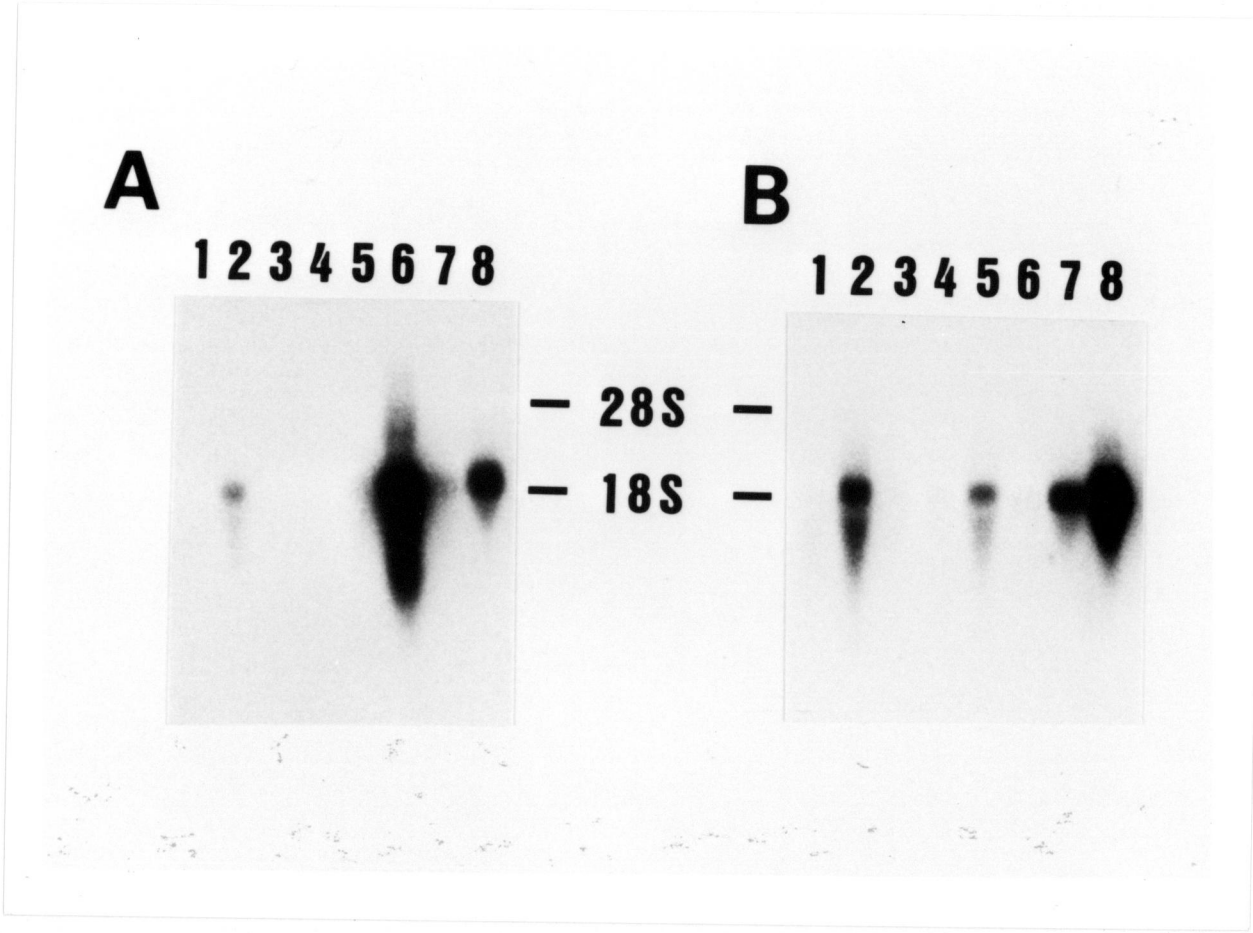

Fig. 5. Northern blot analysis with cDNA fragments containing "HR2" region of rat P-450 (A) and rabbit P-450 (B) as probes. Total RNA and poly(A)$^+$ RNA were prepared as described in "Materials and Methods". Lanes: 1 to 6, total RNA from untreated, PB-, MC-, β-NF-, ISF-treated rabbit liver and PB-treated rat liver, respectively; 7 and 8, poly(A)$^+$ RNA from untreated and PB-treated rabbit liver, respectively. RNA was denatured, electrophoresed on 1 % agarose gel containing 2.2 M formaldehyde, and transferred to nitrocellulose membrane as described in "Materials and Methods" for details. The blotted RNA was hybridized with nick-translated ^{32}P-probe; (A) Bg1II fragment (390 bp) of rat pcP-450pb4, (B) PstI fragment (650 bp) of rabbit pHP-1. Calf liver 18 and 28 S ribosomal RNAs were used as size markers.

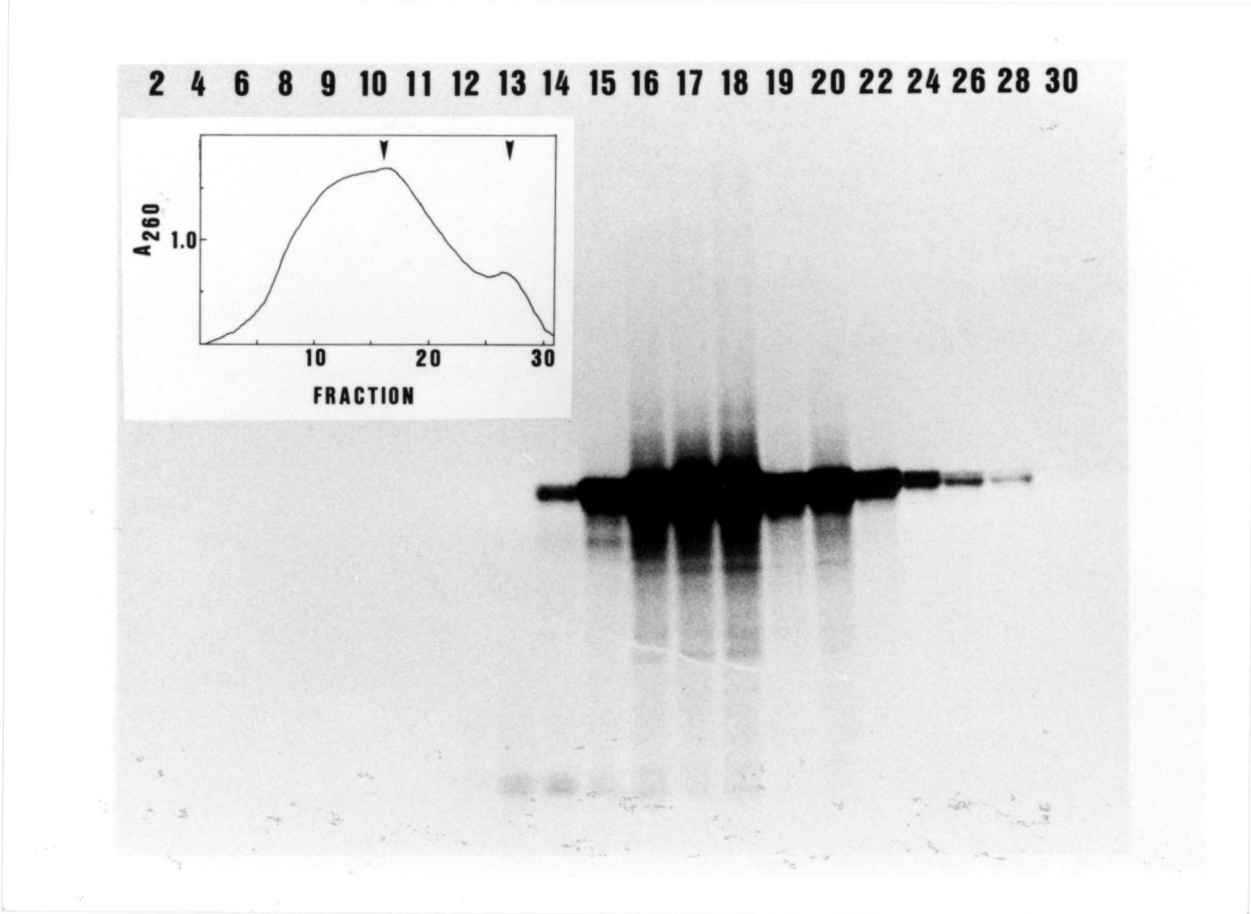

Fig. 6. Sucrose density gradient centrifugation of a poly(A)$^+$ RNA prepared from the livers of PB-treated rabbits. Poly(A)$^+$ RNA was fractionated by centrifugation through a 5 to 25 % sucrose gradient containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA and 0.2 % SDS. After precipitation of RNA in each fraction (0.4 ml) with ethanol, an aliquot (1/20) of each fraction was assayed for mRNA activity in the in vitro translation system and immunoprecipitation with anti-P-450$_2$ and anti-P-450* antibodies as described in "Meterials and Methods". Curve in inset indicates optical density at 260 nm; 18 and 28 S rRNAs were used as size markers. The in vitro translation followed by immunoprecipitation of size-fractionated mRNA by sucrose density gradient. Lane numbers indicate the fractions assayed by the in vitro translation and immunoprecipitation.

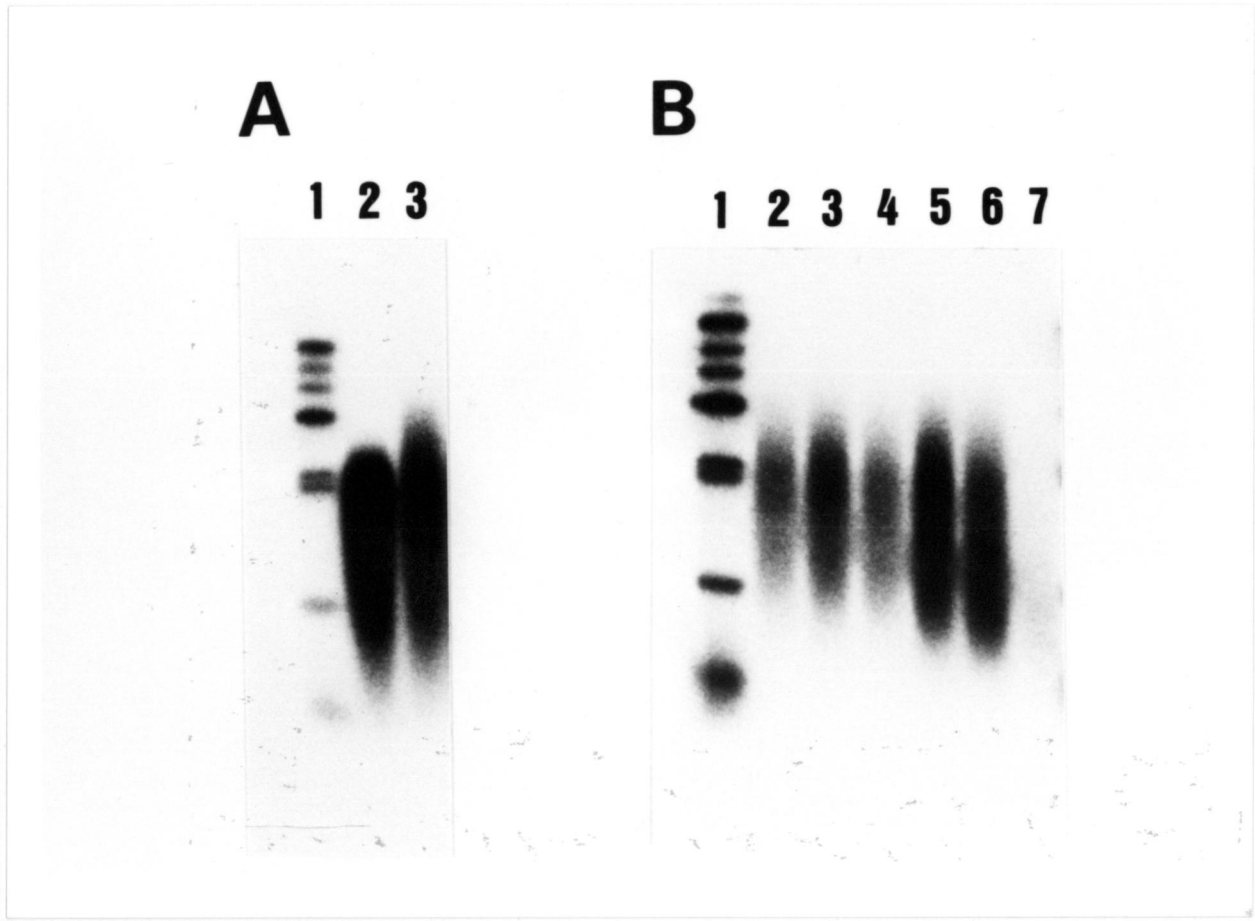

Fig. 7. Synthesis of double-stranded cDNA. (A) cDNA was synthesized by the Method II. First strand cDNA synthesized by reverse transcriptase (lane 2) and ds-cDNA after repairing (lane 3) were analyzed on 1 % alkaline agarose gel electrophoresis. (B) Repaired cDNA was fractionated on Sepharose CL-4B, and an aliquot of each fraction was analyzed on alkali agarose gel (lane 2-7). λDNA-HindIII fragments were used as size markers (lane 1).

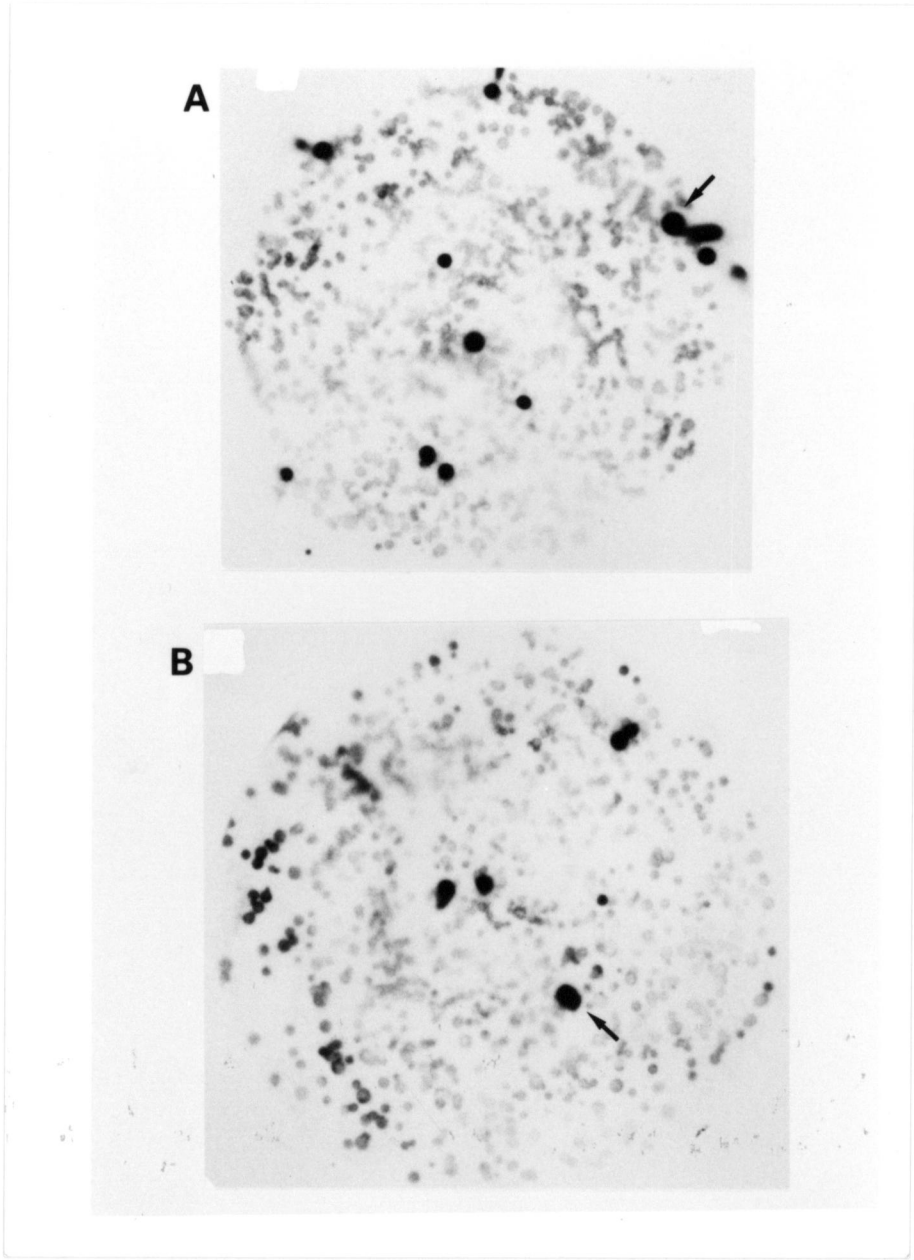

Fig.8. Part of the results of *in situ* colony hybridization. Screening by *in site* colony hybridization was carried out as described in "Materials and Methods" using mixture of ^{32}P-labeled *Bgl*II fragment of pcP-450pb4 and *Hind*III-*Bam*HI fragment of pHP-2 (rabbit cDNA clone) as probes under a condition of low stringency. Arrows indicate examples of strongly positive clones containing large insert; clone M046 (A), clone pHP-1 (B).

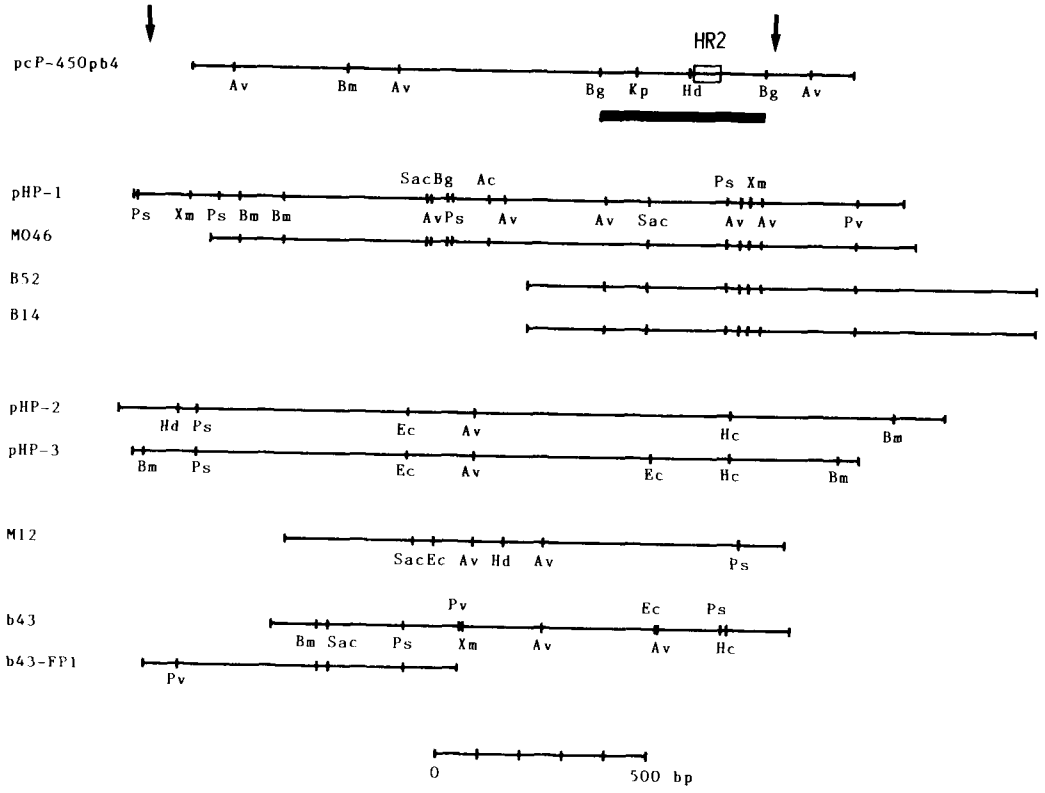

Fig. 9. Restriction endonuclease maps of the various cytochrome P-450 cDNA inserts. Plasmids DNA were digested with a variety of restriction endonucleases under the conditions as described by Maniatis et al. (42), and the resulting DNA fragments were analyzed by 1-1.4 % agarose gel electrophoresis. Restriction enzymes not given in maps are the same as those of the above clones. At the top, the rat P-450 cDNA clone, pcP-450pb4, is indicated. BglII fragment used for colony hybridization is represented by the bold line. HR2, the heme binding region, is also represented by the box. The positions of both initiation and stop codons are indicated by arrows. Ac, AccI; Av, AvaI; Bg, BglII; Bm, BamHI; Ec, EcoRI; Hc, HincII; Hd, HindIII; Kp, KpnI; Ps, PstI; Pv, PvuII; Sac, SacI; Xm, XmaI.

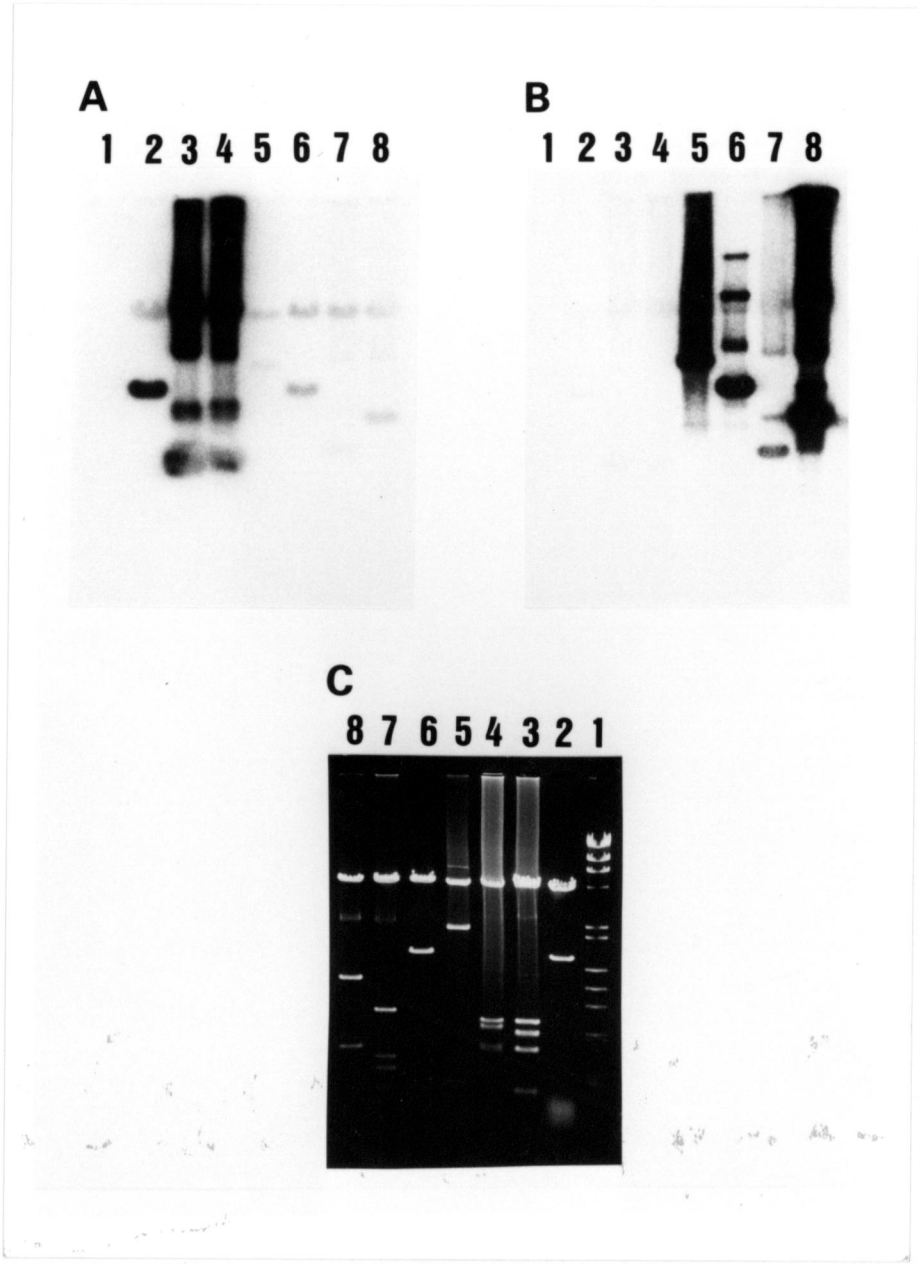

Fig. 10. Southern blot analysis of various cDNA clones. Plasmid DNAs (about 1 g) of pcP-450pb4 (lane 2), pHP-1 (lane 3), M046 (lane 4), pHP-2 (lane 5), pHP-3 (lane 6), b43 (lane 7) and M12 (lane 8) were digested with PstI, subjected to 1 % agarose gel electrophoresis and then stained with ethidium bromide (C). The DNA fragments were transferred to nitrocellulose filter, and then hybridized with ^{32}P-labeled XmaI fragment of pHP-1 (A) and ^{32}P-labeled PstI-EcoRI fragments of M12 (B) under the highly stringent conditions. λDNA-HindIII fragments and ϕX174DNA-HaeIII fragments were used as size markers (lane 1).

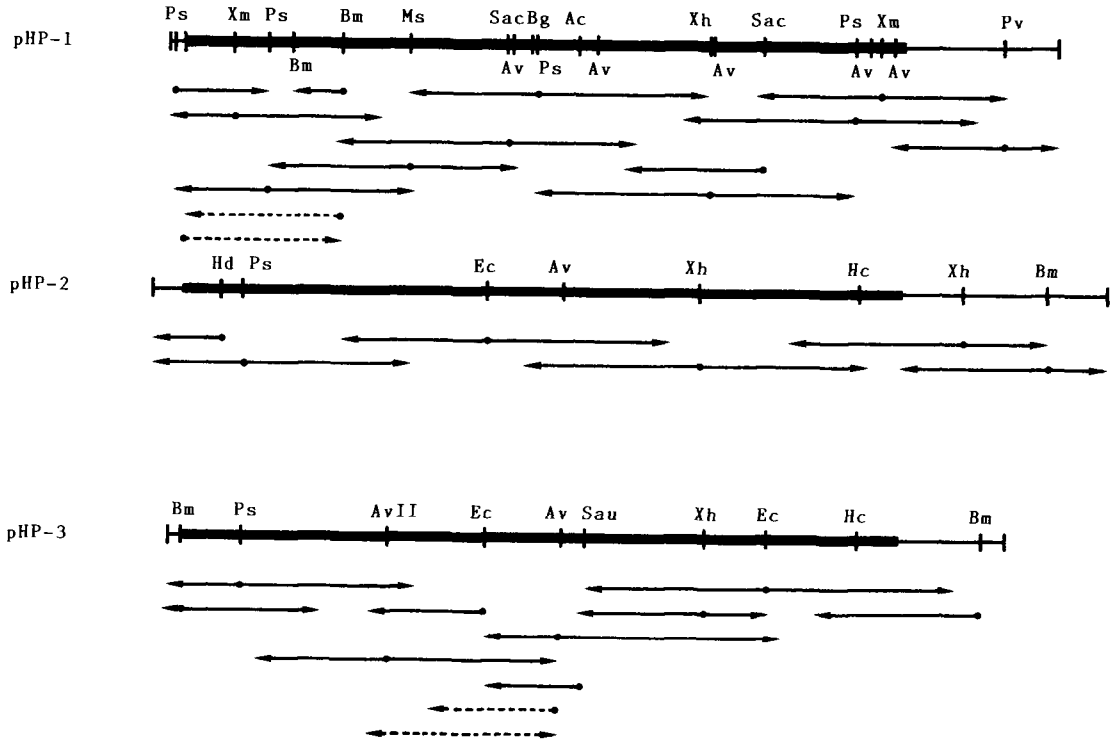

Fig. 11. Sequencing strategy for rabbit cytochrome P-450 cDNA. Restriction enzyme fragments of the insert cDNAs, produced by the indicated restriction enzymes, were subcloned in vectors M13 mp10 and M13 mp11 and sequenced by the dideoxy nucleotide method using [α-^{35}S]dATPαS as a radioactive label (solid line). Arrows indicate the direction and extents of sequencing. Fragments sequenced by the method of Maxam and Gilbert are indicated by dashed lines. AvII, AvaII; Ms, MspI; Sau, Sau3A; Xh, XhoII.

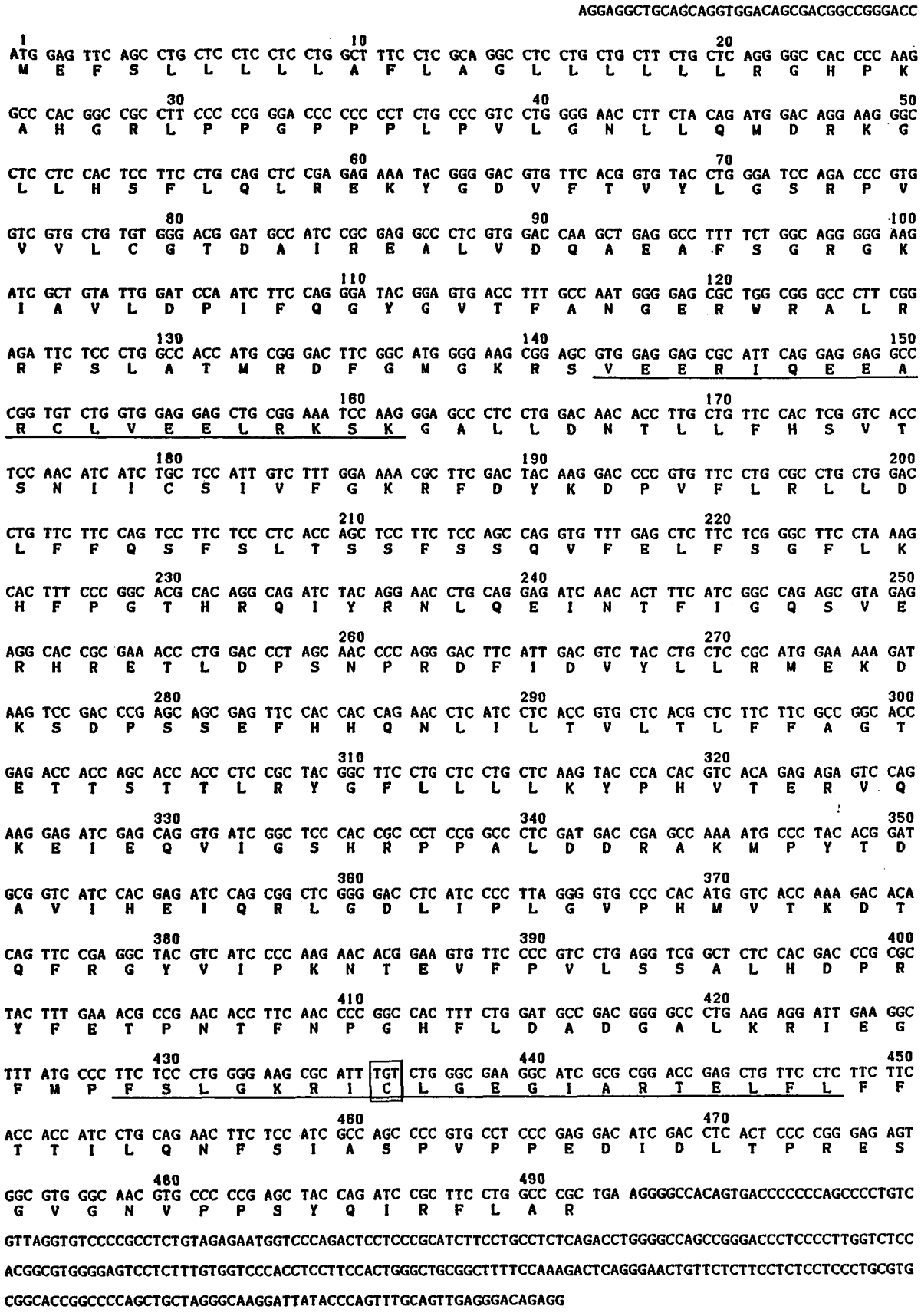

Fig. 12. The nucleotide sequence and deduced amino acid sequence of pHP-1. Deduced amino acid is numbered from initiator methionine. Two highly conserved sequences (54) are underlined: HR1, upper; HR2, lower. The boxed Cys residue is considered as the fifth ligand of the heme iron.

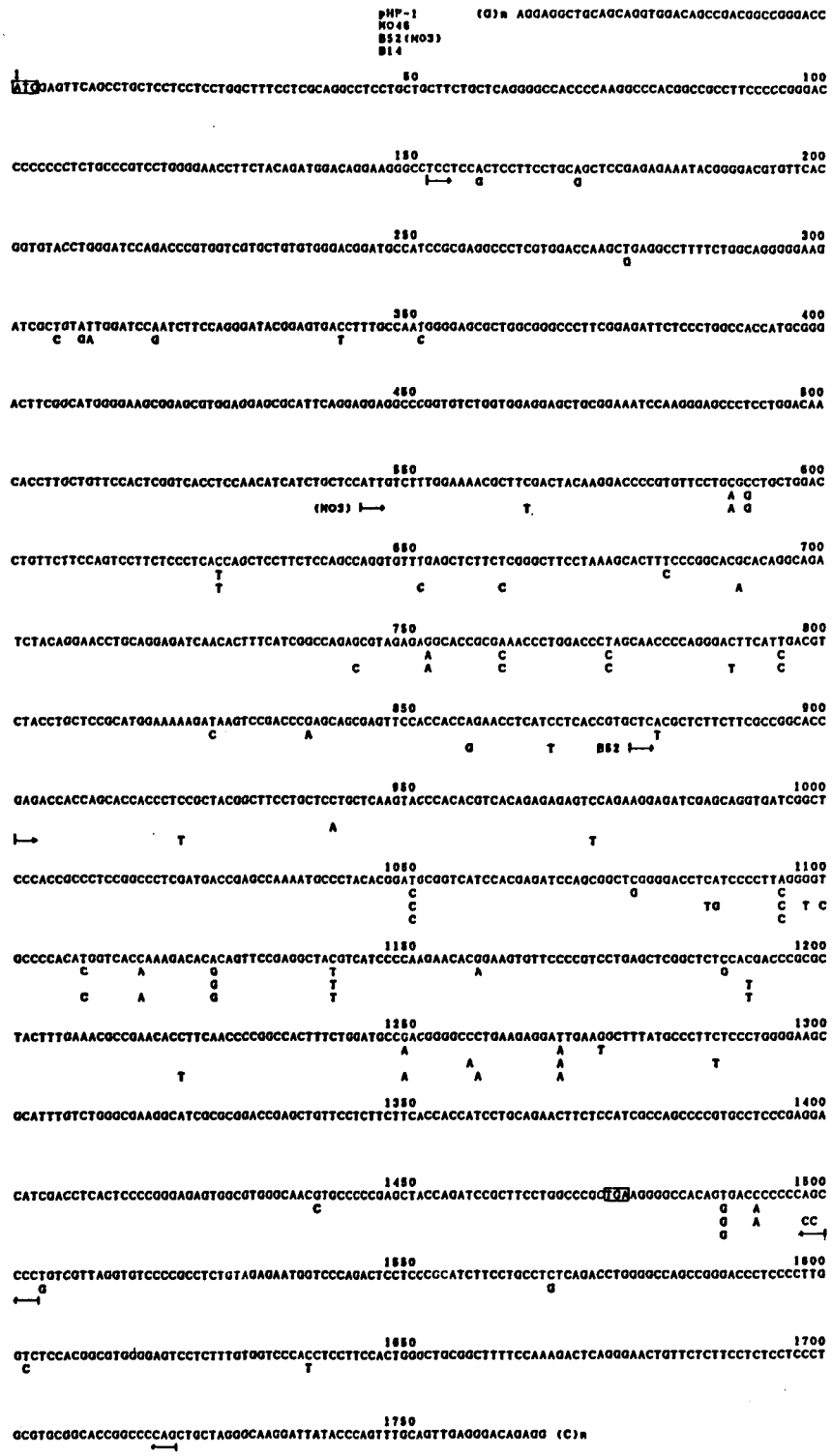

Fig. 13. Comparison of the nucleotide sequences of the three cDNA clones with that of pHP-1. The initiation and termination codons are boxed. Nucleotides are numbered in the 5' to 3' direction. Position 1 corresponds to the first nucleotide of the initiation codon in pHP-1. The nucleotide substitutions in the three clones (M046, B52 (M03) and B14) are shown below the corresponding nucleotides of the sequence of pHP-1. Arrows indicate the ends of the sequences determined so far.

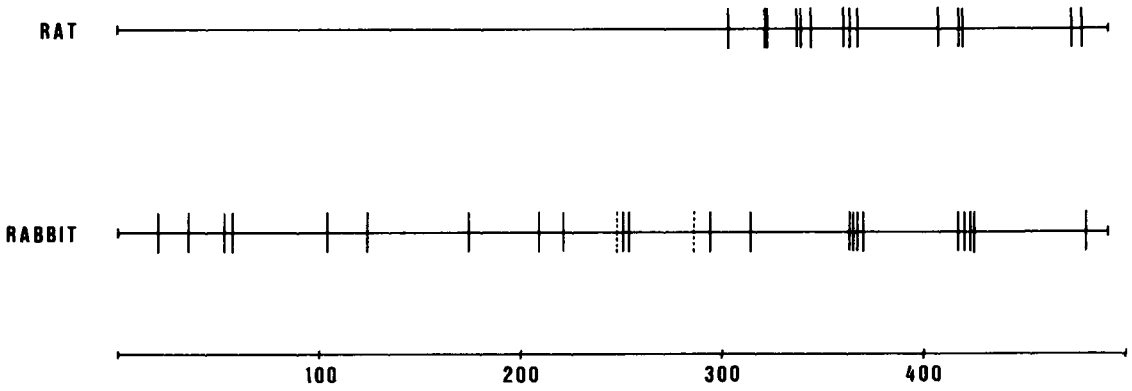

Fig. 14. The position of amino acid substitutions in PB-inducible forms of cytochrome P-450 from rats and rabbits. The vertical solid and dashed (from the data of M03) lines indicate the position of substitution.

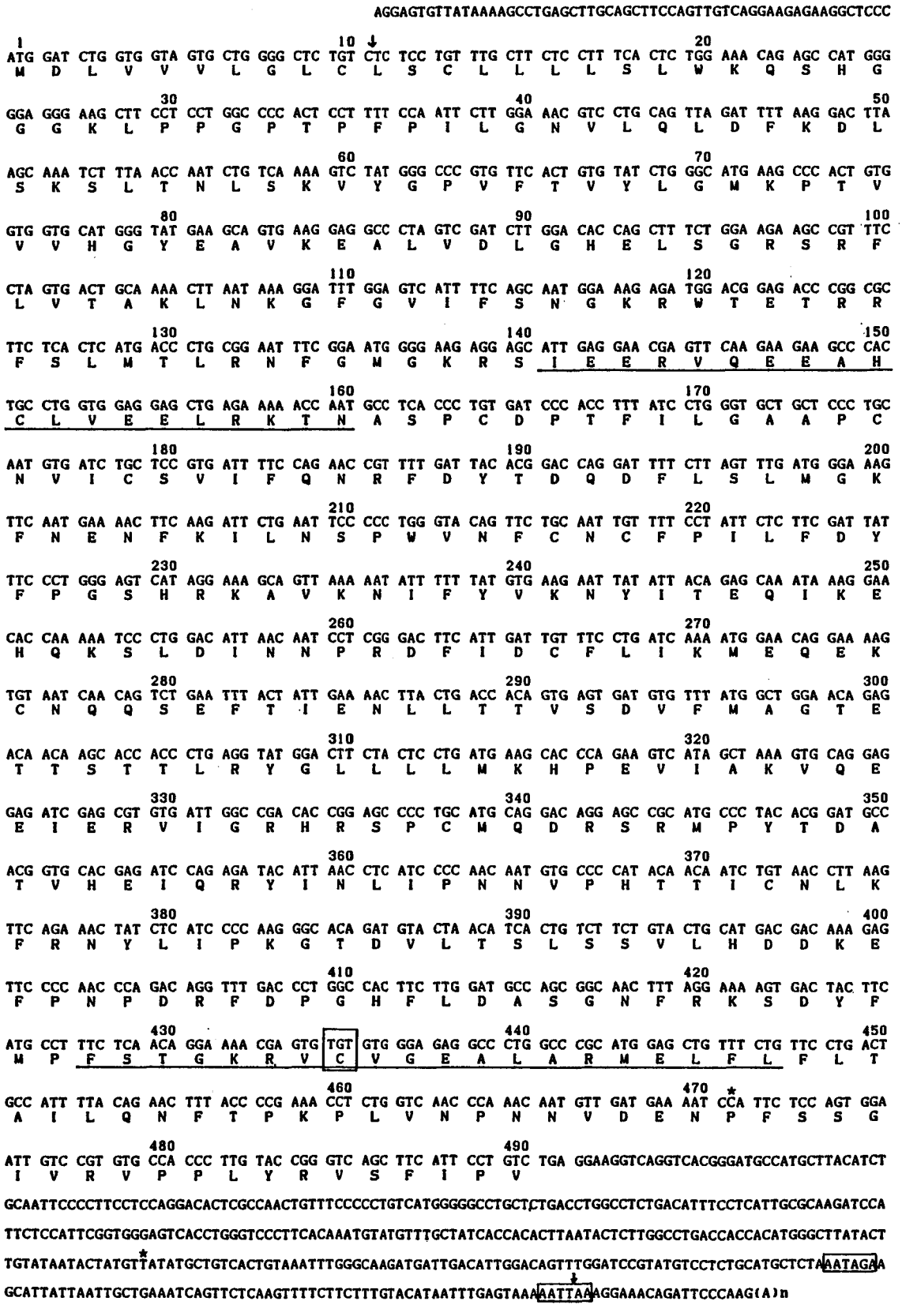

Fig. 15. The nucleotide sequence and deduced amino acid sequence of pHP-2. The underlined sequences and the boxed Cys residue are the same as in Fig. 12. Two boxed sequences in the 3'-noncoding region are the potential polyadenylation signal sequences. The vertical arrows indicate the start and end of the cDNA clone PBc2 reported by Leighton et al. (28). Asterisks indicate the nucleotide substitutions in PBc2.

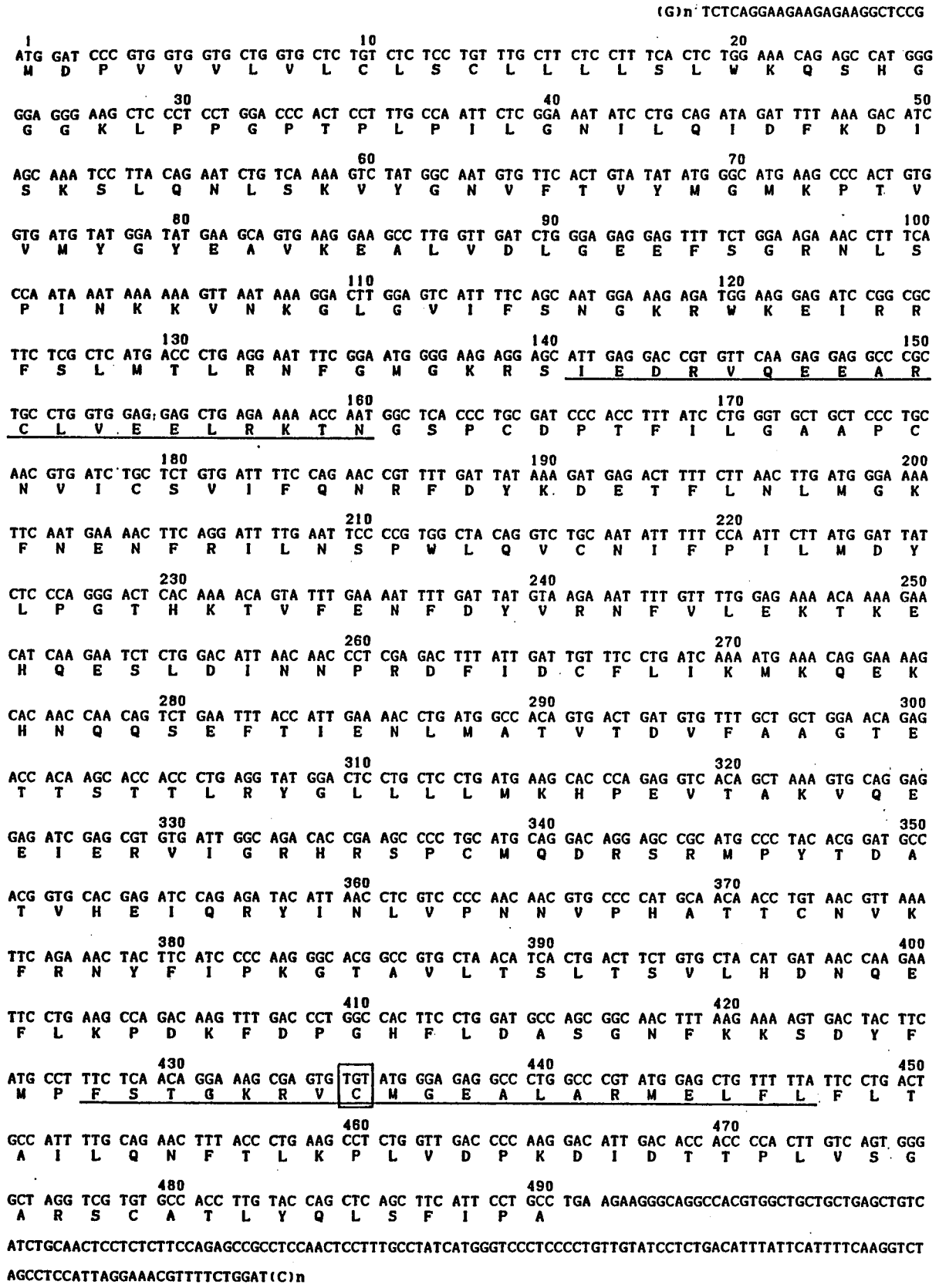

Fig. 16. The nucleotide sequence and deduced amino acid sequence of pHP-3. The underlined sequences and the boxed Cys residue are the same as in Fig. 12.

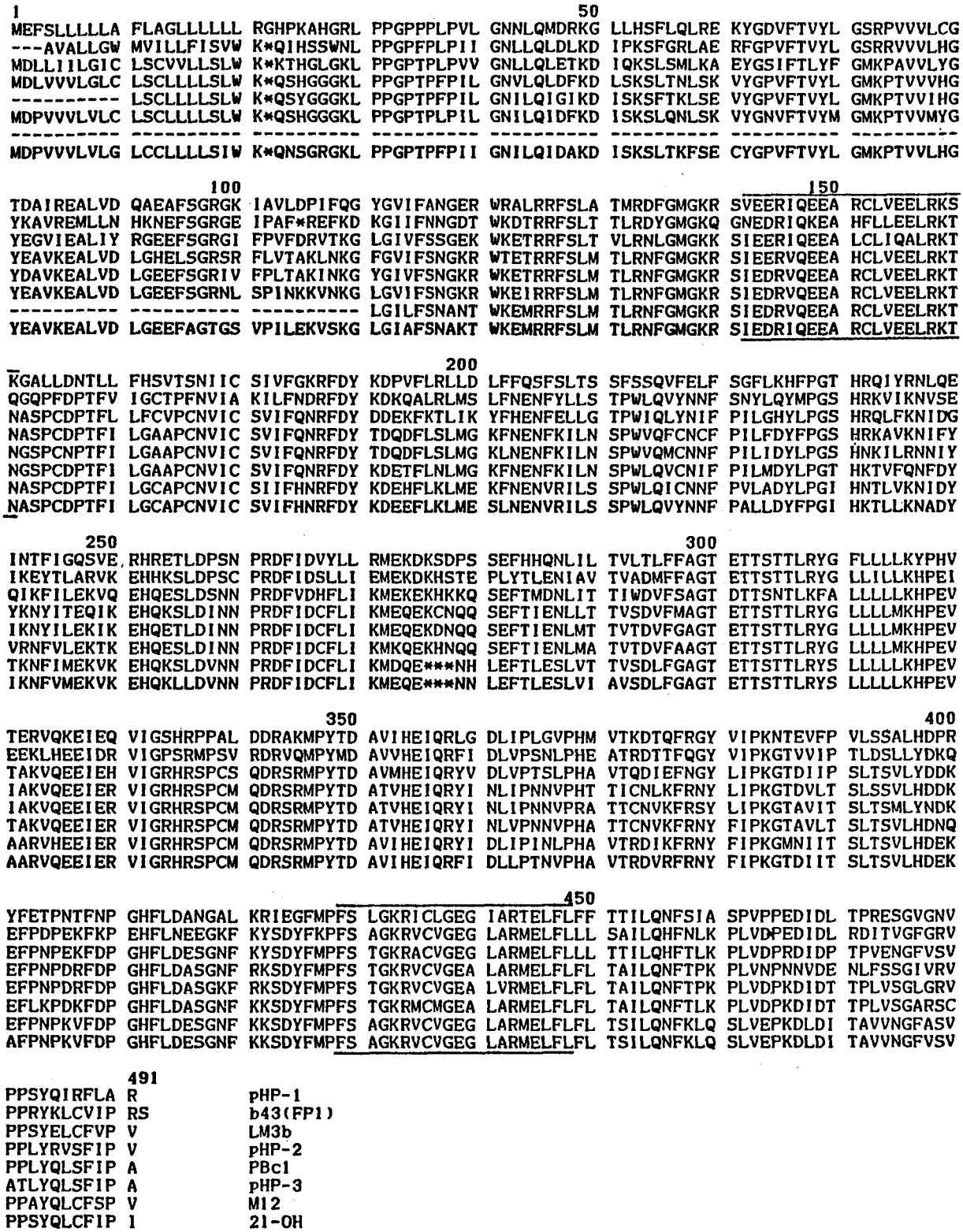

Fig. 17. Comparison of the amino acid sequences of rabbit liver cytochrome P-450s. The sequences of various cytochrome P-450s are compared to the sequence of cytochrome P-450 pHP-1. Asterisks indicate gaps inserted to maximize homology. Dashes indicate the lack of the amino acid residues at amino termini in the cDNA clones. The sequences of LM_{3b}, P-450PBc1, and P450 21-hydroxylase are from Ozols et al. (56), Leighton et al. (28), and Tukey et al. (30), respectively.

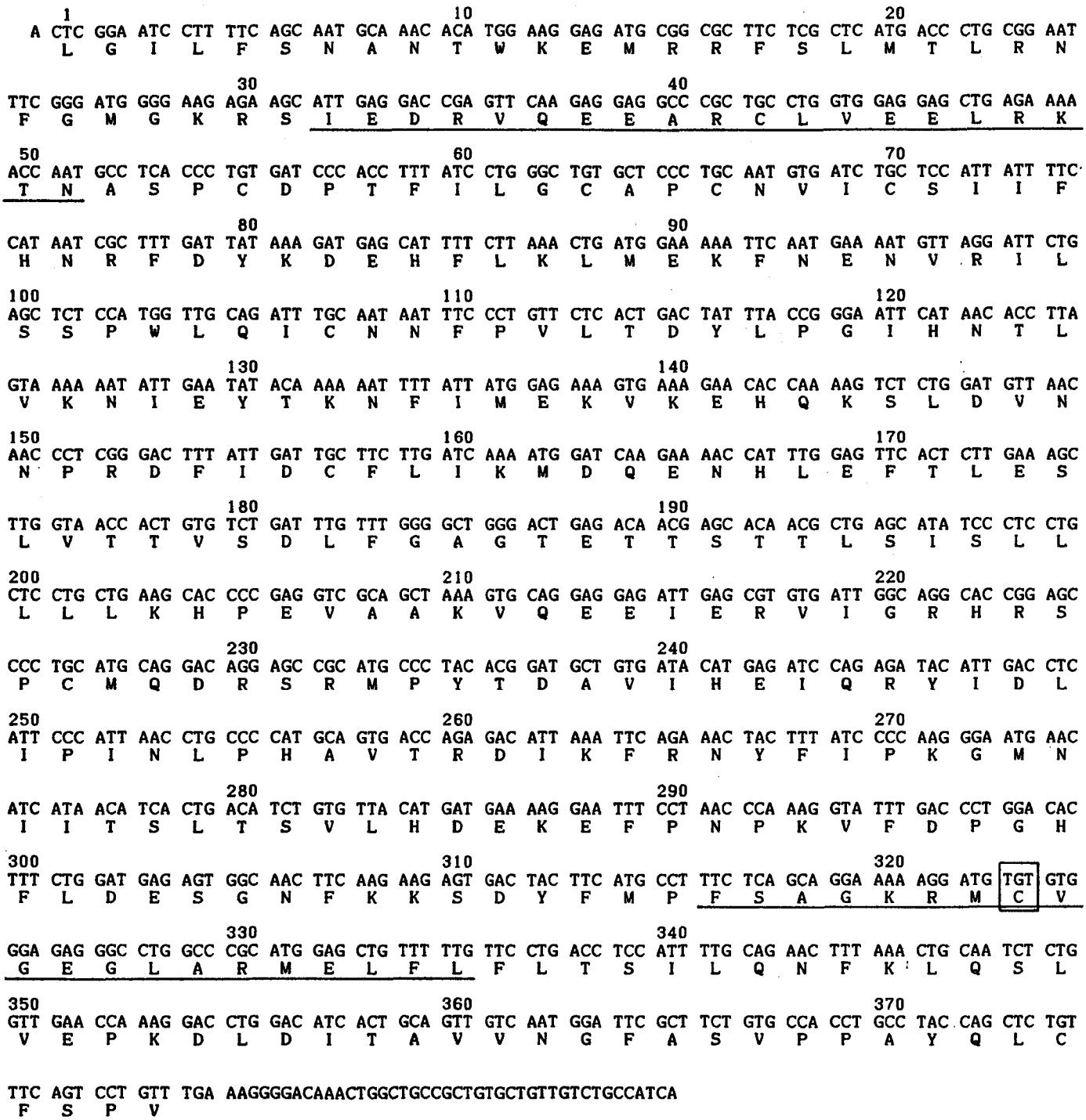

Fig. 18. The nucleotide sequence and deduced amino acid sequence of M12. The underlined sequences and the boxed Cys residue are the same as in Fig. 12.

```
1                                                  10                                                  20
GCC GTC GCC CTG CTG GGG TGG ATG GTC ATC CTC CTG TTC ATA TCC GTC TGG AAG CAG ATC CAC AGC AGC TGG AAC
 A   V   A   L   L   G   W   M   V   I   L   L   F   I   S   V   W   K   Q   I   H   S   S   W   N

                30                                                  40                                                  50
CTG CCC CCA GGA CCT TTC CCA CTG CCC ATC ATC GGG AAT CTT CTC CAG TTG GAT TTG AAG GAT ATT CCC AAG TCC
 L   P   P   G   P   F   P   L   P   I   I   G   N   L   L   Q   L   D   L   K   D   I   P   K   S

                                    60                                                  70
TTT GGC AGG CTG GCA GAG CGC TTT GGG CCG GTG TTC ACT GTG TAC CTG GGC TCC AGG CGT GTT GTG GTT CTG CAC
 F   G   R   L   A   E   R   F   G   P   V   F   T   V   Y   L   G   S   R   R   V   V   V   L   H

                80                                                  90                                                 100
GGC TAC AAG GCG GTG AGG GAG ATG CTG TTG AAC CAC AAG AAC GAG TTC TCT GGG CGT GGC GAG ATC CCT GCT TTC
 G   Y   K   A   V   R   E   M   L   L   N   H   K   N   E   F   S   G   R   G   E   I   P   A   F

                                   110                                                 120
CGG GAG TTT AAG GAC AAG GGG ATC ATT TTC AAC AAT GGA CCC ACC TGG AAG GAC ACT CGG CGG TTC TCC CTG ACC
 R   E   F   K   D   K   G   I   I   F   N   N   G   P   T   W   K   D   T   R   R   F   S   L   T

                130                                                 140                                                 150
ACC CTC CGG GAC TAT GGG ATG GGG AAA CAG GGC AAC GAG GAC CGG ATC CAG AAG GAG GCC CAC TTC CTG CTG GAG
 T   L   R   D   Y   G   M   G   K   Q   G   N   E   D   R   I   Q   K   E   A   H   F   L   L   E

                                   160                                                 170
GAG CTC AGG AAG ACC CAG GGC CAG CCC TTC GAC CCC ACC TTT GTC ATC GGC TGC ACA CCC TTC AAC GTC ATC GCC
 E   L   R   K   T   Q   G   Q   P   F   D   P   T   F   V   I   G   C   T   P   F   N   V   I   A

                180                                                 190                                                 200
GAC CGC TTT GAC TAT AAG GAC AAG CAG GCT CTG AGG CTG ATG AGT TTG TTC AAC GAG AAC AAA ATC CTC TTC AAT
 D   R   F   D   Y   K   D   K   Q   A   L   R   L   M   S   L   F   N   E   N   K   I   L   F   N

                                   210                                                 220
TTC TAC CTG CTC AGT ACT CCT TGG CTG CAG GTT TAC AAT AAT TTT TCA AAC TAT CTA CAG TAC ATG CCT GGA AGT
 F   Y   L   L   S   T   P   W   L   Q   V   Y   N   N   F   S   N   Y   L   Q   Y   M   P   G   S

                230                                                 240                                                 250
CAC AGG AAA GTA ATA AAA AAT GTG TCT GAA ATA AAA GAG TAC ACA CTC GCA AGA GTG AAG GAG CAC CAC AAG TCG
 H   R   K   V   I   K   N   V   S   E   I   K   E   Y   T   L   A   R   V   K   E   H   H   K   S

                                   260                                                 270
CTG GAC CCC AGC TGC CCC CGG GAC TTC ATT GAC AGC CTG CTC ATA GAA ATG GAG AAG GAC AAA CAC AGC ACG GAG
 L   D   P   S   C   P   R   D   F   I   D   S   L   L   I   E   M   E   K   D   K   H   S   T   E

                280                                                 290                                                 300
CCC CTG TAC ACG CTG GAA AAC ATT GCT GTG ACT GTG GCG GAC ATG TTC TTT GCG GGC ACG GAG ACC ACC AGC ACC
 P   L   Y   T   L   E   N   I   A   V   T   V   A   D   M   F   F   A   G   T   E   T   T   S   T

                                   310                                                 320
ACG CTG CGA TAT GGG CTC CTG ATC CTG CTG AAG CAC CCC CAG ATC GAA GAG AAA CTT CAT GAA GAA ATC GAC AGG
 T   L   R   Y   G   L   L   I   L   L   K   H   P   E   I   E   E   K   L   H   E   E   I   D   R

                330                                                 340                                                 350
GTG ATT GGG CCG AGC CGA ATG CCT TCT GTC AGG GAC AGG GTG CAG ATG CCC TAC ATG GAC GCT GTG GTA CAT GAG
 V   I   G   P   S   R   M   P   S   V   R   D   R   V   Q   M   P   Y   M   D   A   V   V   H   E

                                   360                                                 370
ATT CAG CGA TTC ATC GAT CTC GTG CCC TCC AAT CTG CCG CAC GAA GCC ACA CGG GAC ACC ACC TTC CAA GGA TAC
 I   Q   R   F   I   D   L   V   P   S   N   L   P   H   E   A   T   R   D   T   T   F   Q   G   Y

                380                                                 390                                                 400
GTC ATC CCC AAG GGC ACT GTT GTA ATC CCG ACT CTG GAC TCC CTT TTG TAT GAC AAG CAA GAA TTC CCT GAT CCC
 V   I   P   K   G   T   V   V   I   P   T   L   D   S   L   L   Y   D   K   Q   E   F   P   D   P

                                   410                                                 420
GAG AAG TTC AAA CCA GAG CAC TTT CTG AAT GAG GAG GGG AAG TTC AAG TAT AGC GAC TAC TTC AAG CCG TTT TCC
 E   K   F   K   P   E   H   F   L   N   E   E   G   K   F   K   Y   S   D   Y   F   K   P   F   S

                430                                                 440                                                 450
GCA GGA AAA CGC GTG TGT GTT GGA GAA GGC CTG GCT CGC ATG GAG TTG TTT CTG CTC CTG TCC GCC ATT CTG CAG
 A   G   K   R   V   C   V   G   E   G   L   A   R   M   E   L   F   L   L   L   S   A   I   L   Q

                                   460                                                 470
CAT TTT AAC CTC AAG CCT CTC GTT GAC CCA GAG GAC ATT GAC CTT CGC AAT ATT ACG GTG GGC TTT GGC CGT GTC
 H   F   N   L   K   P   L   V   D   P   E   D   I   D   L   R   N   I   T   V   G   F   G   R   V

                480                                                 487
CCA CCA CGC TAC AAA CTC TGT GTC ATT CCC CGC TCG TAA ACCCAAGGGCAGCACCCAGAGGCCACTCTTCTCCTCGAGTGCCCCTG
 P   P   R   Y   K   L   C   V   I   P   R   S

GGGAGGCTCCTGCTGC
```

Fig. 19. The nucleotide sequence and deduced amino acid sequence of the overlapping two clones, b43 and b43FP1. In the overlapping portion, the nucleotide sequences are identical with each other.

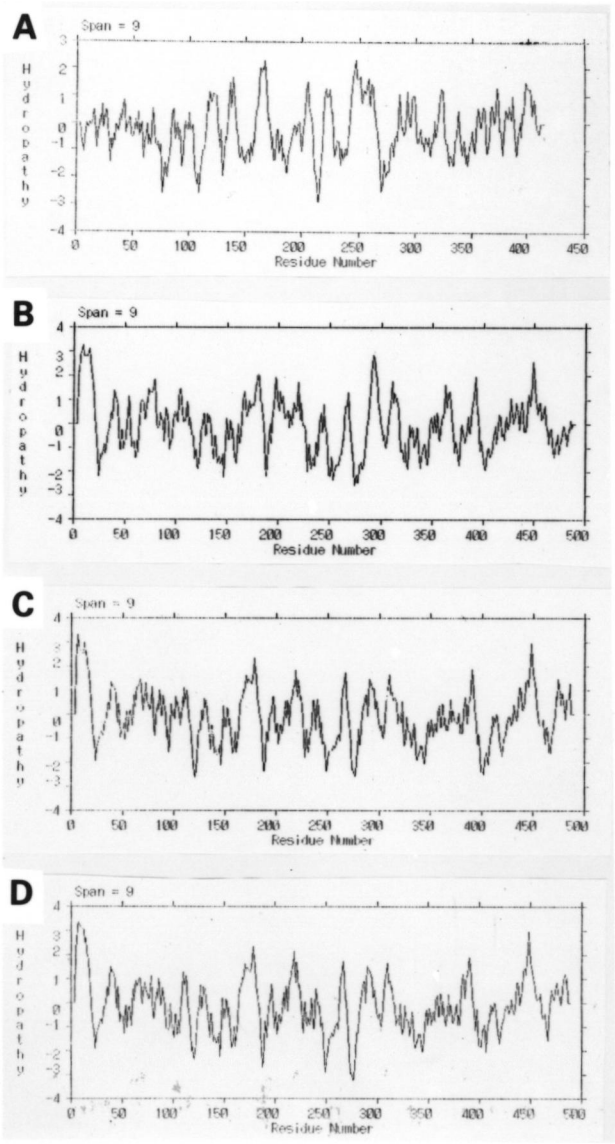

Fig. 20. Hydorpathic profiles of the three chytochrome P-450s (pPH-1, pPH-2, and pPH-3) and P-450cam. Evaluation of hydropathy was performed according to the method of Kyte and Doolittle (52) at a span setting of nine amino acid residues.

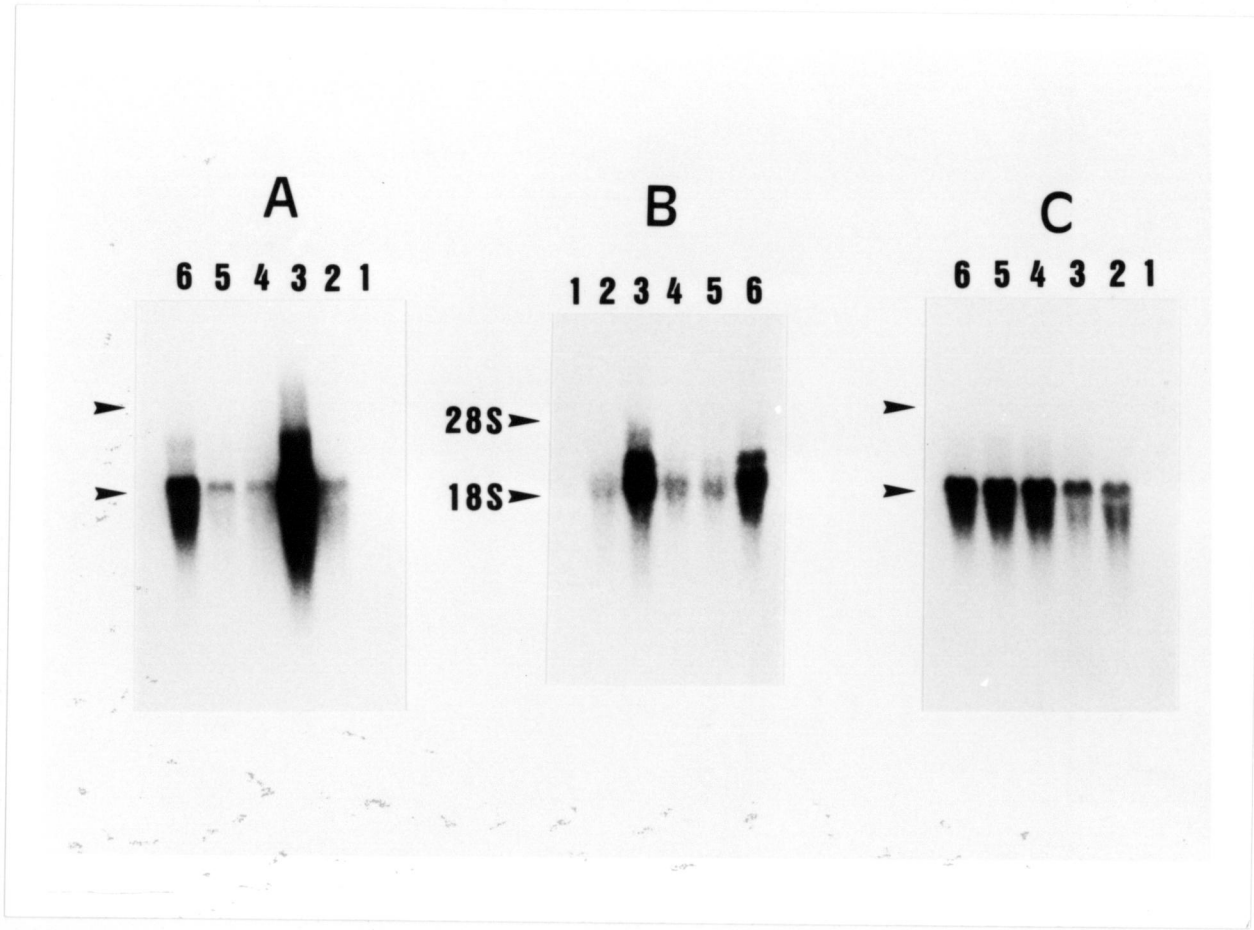

Fig. 21. Northern blot analysis with various cDNA probes. Total liver RNA from differently treated rabbits were denatured, electrophoresed, and transferred to a nitrocellulose filter as described in Fig. 5. Lanes 2 to 6; RNA from untreated, PB-, MC-, βNF-, and ISF-treated rabbits, respectively. The blotted RNA was hybridized with nick-translated cDNA probes under highly stringent conditions as described in Fig. 5. A, XmaI-fragment of pPH-1; B, HindIII-BamHI fragment of pPH-2; C, PstI-EcoRI fragment of b43. Calf liver ribosomal RNA was used as a size marker (lane 1).

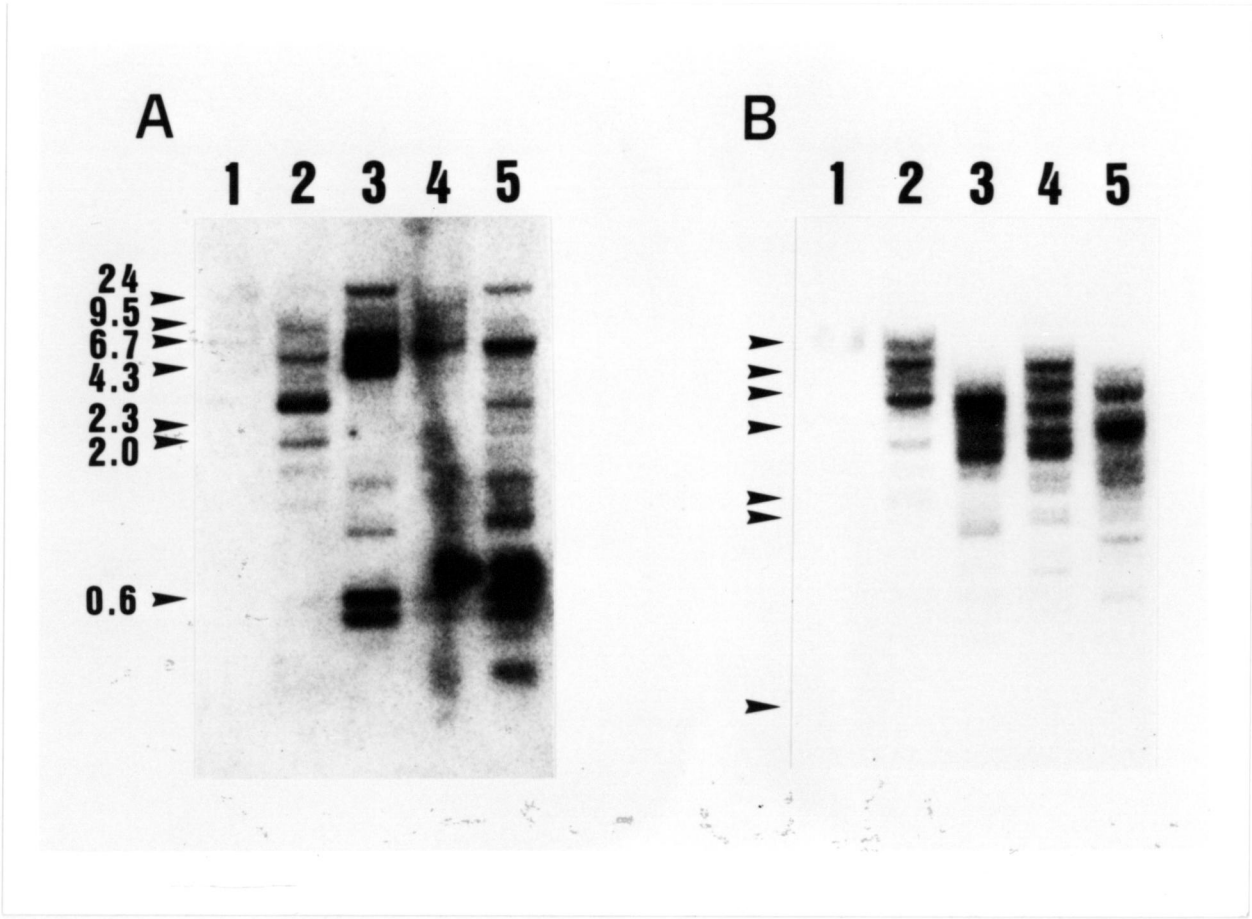

Fig. 22. Southern blot analysis with various cDNA probes. Total rabbit liver genomic DNA was digested with various restriction enzymes, electrophoresed on a 1 % agarose gel, and transferred to a nitrocellulose filter. Hybridization was carried out under high stringent conditions as described in "Materials and Methods". λDNA-HindIII fragments were used as a size marker (lane 1). (A) The XmaI-fragment of pPH-1 was used as a probe. Digestion was performed with EcoRI (lane 2), BamHI (lane 3), HindIII (lane 4), and PstI (lane 5). (B) The HindIII-BamHI fragment of pPH-2 was used as a probe. Digestion was performed with BamHI (lane 2), EcoRI (lane 3), HindIII (lane 4), and PstI (lane 5).

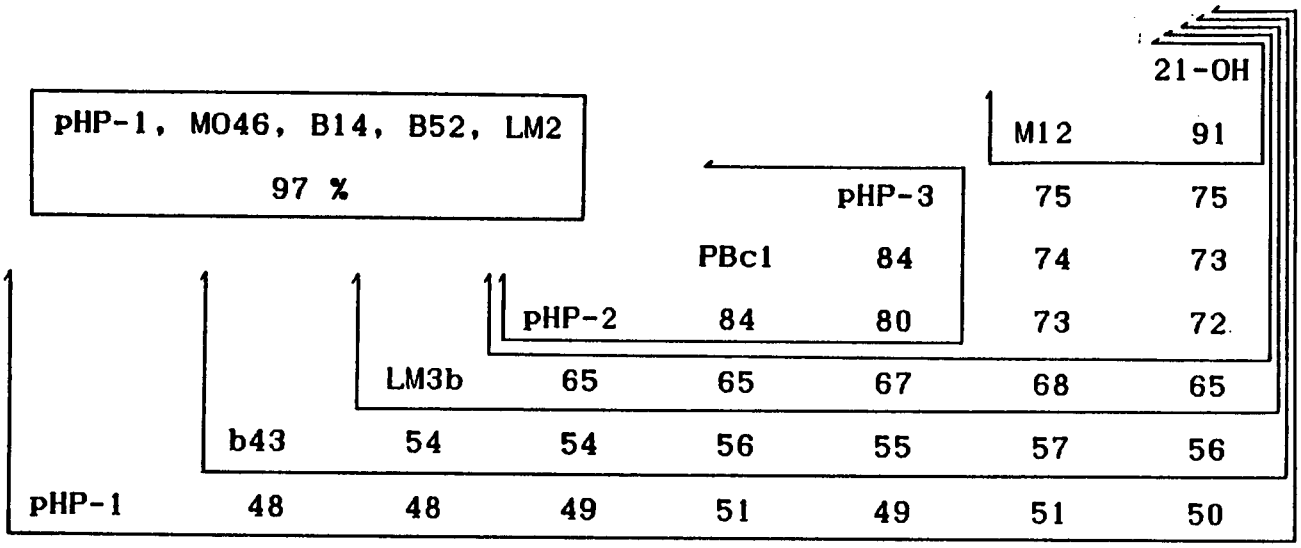

Fig. 23. Homology of amino acid sequences of various cytochrome P-450s. The numbers show percentages of matched residues.

M-----L---	----------	-*-------L	PPGP-P-P--	GN-LQ---K-	50
---S------	--G--FT-Y-	G----VV--G	-----E-L--	------G---	100
----------	-G--F-----	W---RRFSL-	--R--GMGK-	--E-R-Q-EA	150
--L---LRK-	-------T--	------N-I-	---F--RFDY	-D-----L--	200
----------	----Q----F	-------PG-	H-----N---	----------	250
-H---LD---	PRDF-D--L-	-M---***--	----------	-----F-AGT	300
-TTS-TL---	-L---K-P--	------EI--	VIG--R-P--	-DR--MPY-D	350
A--HEIQR--	-L-P---P--	------F--Y	-IPK-T----	-L-S-L----	400
-F--P--F-P	-HFL---G--	-----F-PFS	-GKR-C-GE-	--R-ELFL--	450
--ILQ-F---	--V-P---D-	-----G----	---Y------	-	491

pHP-1, pHP-2(PBc2), pHP-3, MO46, B14, B52, P-450$_1$(LM2), PBc1, Form3b(PBc3), b43-b43FP1, M12(21-OH)

Fig. 24 Conserved amino acid residues in rabbit liver cytochrome P-450s. The common amino acid residues in various rabbit liver cytochrome P-450s (listed up below the sequence) are shown in one letter. Dashes and Asterisks indicate the positions of amino acid substitutions and the gaps inserted to obtain maximal homology, respectively.

No. of AA	pHP-1	MO46	B52	B14	LM2
20	Leu(CTC)				Phe
35	Pro(CCC)				Ser
53	His(CAC)	Arg(CGC)			Arg
57	Gln(CAG)	Arg(CGG)			Arg
104	Leu(TTG)	Met(ATG)			Val
114	Thr(ACC)	Ile(ATC)			Ile
174	Val(GTC)	Val(GTC)			Ile
209	Thr(ACC)	Ile(ATC)	*Ile(ATC)		Ile
221	Ser(TCG)	Ser(TCG)	*Pro(CCG)		Pro
248	Ser(AGC)	Ser(AGC)	*Thr(ACC)		Ser
251	Arg(AGG)	Lys(AAG)	*Lys(AAG)		Lys
254	Glu(GAA)	Ala(GCA)	*Ala(GCA)		Ala
286	Gln(CAG)	Gln(CAG)	*Arg(CGG)		Gln
294	Thr(ACG)	Ser(TCG)	*Thr(ACG)		Ser
314	Leu(CTG)	Leu(CTG)	Met(ATG)	Leu(CTG)	Met
363	Ile(ATC)	Ile(ATC)	Val(GTC)	Ile(ATC)	Ile
365	Ala(TTA)	Phe(TTC)	Phe(TTC)	Phe(TTC)	Phe
367	Val(GTG)	Val(GTG)	Ala(GCG)	Val(GTG)	Val
370	Met(ATG)	Thr(ACG)	Met(ATG)	Thr(ACG)	Thr
417	Asp(GAC)	Asn(AAC)	Asp(GAC)	Asn(AAC)	Asn
420	Leu(CTG)	Leu(CTG)	Leu(CTG)	Met(ATG)	Leu
423	Ile(ATT)	Asn(AAT)	Asn(AAT)	Asn(AAT)	Asn
425	Gly(GGC)	Cys(TGC)	Gly(GGC)	Gly(GGC)	Gly
480	Val(GTG)	Leu(CTG)	Val(GTG)	Val(GTG)	Val

Table I. The summary of the amino acid substitutions in the PB-inducible major forms of rabbit cytochromes P-450 (52). Asterisks indicate the preliminary data from clone MO3. Underlined amino acid was replaced by Gly(GGC) in clone B54 which has a shorter insert than MO46.

	pHP-1 (LM2)		pHP-2	pHP-3	LM3b	21-OH
Ala	23	(24)	15	19	13	21
Arg	37	(38)	26	24	19	24
Asn	13	(15)	30	30	16	23
Asp	25	(24)	23	24	27	25
Cys	4		13	12	10	12
Gln	17	(16)	15	16	16	12
Glu	29	(28)	27	28	34	33
Gly	35		28	28	31	27
His	15	(14)	12	10	14	10
Ile	25	(27)	26	26	33	31
Leu	68	(64)	55	54	59	57
Lys	19	(20)	29	33	34	31
Met	8		12	16	10	12
Phe	39	(41)	35	32	33	32
Pro	32		30	28	32	30
Ser	31	(32)	31	27	29	30
Thr	28	(26)	29	32	29	24
Trp	1		3	3	4	3
Tyr	12		13	13	15	12
Val	30		38	35	32	38
Total	491		490	490	490	487
MW	55,700		55,800	55,700	—	—

Table II. Comparison of amino acid compositions of rabbit liver cytochrome P-450s deduced from their nucleotide sequences shown in Fig. 12, 13, and 15 together with those of P-450 LM2, LM3b, and 21-hydroxylase.

ACKNOWLEDGEMENTS

The auther wishes to express his sincere gratitude to Professor R. Sato for his support and useful suggestions. He is also grateful to Dr. Y. Imai for his appropriate advices and suggestions. He is indebted to Dr. Y. Fujii-Kuriyama of Cancer Institute for his kindly supplying pcP-450pb4. His thanks are also due to Dr. S. Tsunazawa for his help in amino acid sequencing of P-450_1, P-450_2, and P-450^*. All members of this laboratory are also gratefully acknowledged for their encouragements and technical supports.