# Multiplicity of Deoxyribonucleic Acid Sequences with Homology to a Cloned Complementary Deoxyribonucleic Acid Coding for Rat Phenobarbital-Inducible Cytochrome P-450†

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ABSTRACT: We previously identified cDNA clones for rat cytochrome P-450 of the phenobarbital-inducible type by sequence analysis [Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., & Muramatsu, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2793–2797]. With these cloned cDNAs as probe, the multiplicity of phenobarbital-inducible cytochrome P-450 gene in rat genome was investigated by three approaches. The first approach was the Cot analysis of the total rat liver DNA under conditions of DNA excess. With internal and external markers used as gene-number standards, the reassociation kinetics were studied, which suggested the presence of approximately six genes or gene-like sequences hybridizable to phenobarbital-inducible cytochrome P-450 cDNA per rat haploid genome. The second was the isolation of the cytochrome P-450 genes from a rat genomic library. From a screening of about $1 \times 10^6$ plaques, nine clones with an approximately 15-kb insert were isolated. Restriction maps and Southern blot analysis of the cloned DNAs showed that six out of nine isolated clones contained DNA inserts independent of one another. The third was Southern blot analysis of rat genomic DNA with restriction enzyme *Eco*RI. Approximately 12 positive bands were demonstrated with the cDNA probe, seven to eight of which showed the same mobilities as the fragments in the isolated six genomic clones, suggesting that some other genes or gene-like DNA sequences remained to be cloned.

Cytochrome P-450 in hepatic microsomes is a group of hemoproteins that catalyze the oxidation of a variety of substrates, including fatty acids, steroids, and other endogenous substrates, as well as exogenous compounds such as drugs, hydrocarbons, and chemical carcinogens (Sato & Omura, 1978). One of the important features of cytochrome P-450 is that, in addition to constitutive forms, there are various forms of the cytochrome that are induced by administration of various xenobiotics. For example, administration of phenobarbital (pb) or 3-methylcholanthrene to rats results in increased synthesis of a specific type of cytochrome P-450 in the liver (Sato & Omura, 1978).

Although many investigators have been concerned with the studies on molecular multiplicity of cytochrome P-450 by isolating each form of cytochrome P-450 with routine purification methods of protein, it seems difficult to purify it in a truly homgeneous state, because of minor differences or microheterogeneity in various forms of cytochrome P-450. Therefore, we studied this subject with the recent developed technology of gene cloning, which would provide more precise and detailed insight into the molecular multiplicity as well as their induction mechanisms.

cDNA clones for cytochrome P-450 were previously constructed from reverse transcription of the poly(A) RNA from pb-treated rat liver (Fujii-Kuriyama et al., 1981). The amino acid sequences deduced from the nucleotide sequences of these cDNAs coincide with those reported for the N-terminal 21 amino acids by Botelho et al. (1979) for cytochrome P-450 of the pb-inducible type. Previous data on the hybridization kinetics of these cDNA with total RNAs from normal or pb-treated rat liver further confirmed that inserts of these cDNA clones really contained information for the "pb-inducible" form of cytochrome P-450 (Fujii-Kuruyama et al., 1982b).

We describe in this paper studies with the Cot and Southern blot analyses of rat liver DNA to quantitate the gene dosage of cytochrome P-450 with the cloned cDNAs as probe and with isolation of recombinant phages containing the cytochrome P-450 gene sequences from a rat genomic library.

## Materials and Methods

*cDNA Clones.* Two bacterial clones containing cDNA sequences of cytochrome P-450 of the pb-inducible form were previously isolated and characterized by sequence analysis (Fujii-Kuriyama et al., 1981, 1982a). A bacterial clone (pcP-450pb-2) contained a 902-nucleotide cDNA insert representing 61% of the coding sequence of cytochrome P-450 mRNA, and the other clone (pcP-450pb-1) contained more than a 1200-nucleotide cDNA consisting of 926 nucleotides of the coding and approximately 300 nucleotides of the 3′-untranslated sequences, respectively. The combination of both cloned cDNAs covered about 90% of the cytochrome P-450 mRNA sequence. The outline of restriction maps of these cDNAs is illustrated in Figure 5. The cloned cDNAs were prepared as described previously (Fujii-Kuriyama et al., 1981).

*Preparation of Rat Liver DNA.* Rat liver DNA was prepared by the procedure of Gross-Bellard et al. (1973). DNA was sheared to an average length of 300 bp by sonication.

*Nick Translation of DNAs.* DNAs were labeled by nick translation with [$\alpha$-$^{32}$P]dCTP (260 Ci/mmol, Radiochemical Centre) as described by Maniatis et al. (1975). The specific activity of the [$^{32}$P]cDNA used was $(0.5–2) \times 10^8$ cpm/$\mu$g.

*Isolation of Single-Copy DNA.* Sheared and denatured rat liver DNA was incubated to a Cot of $5 \times 10^3$, and the single-stranded DNA was isolated by hydroxylapatite fractionation as described (Sakai et al., 1978). The same incubation and fractionation was repeated once again for the single-stranded DNA. This DNA was then renatured extensively to a Cot of $6 \times 10^4$ to obtain double-stranded single-copy DNA, and the renatured DNA was labeled with [$^3$H]dCTP

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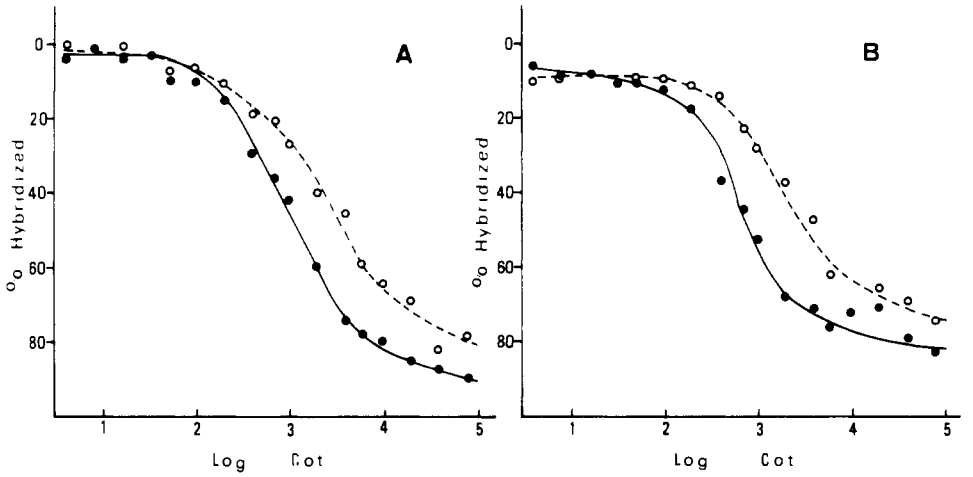


FIGURE 1: Reassociation kinetics of cytochrome P-450 cDNA with total liver DNA. Cytochrome P-450 cDNA was isolated from plasmid clone pcP-450pb-1 (see Figure 5) and highly labeled with [$^{32}$P]dCTP by nick translation. Rat unique-copy DNA was prepared as described under Materials and Methods and nicktranslated with [$^{3}$H]dCTP. $^{3}$H-labeled unique-copy DNA was used as an internal standard of reassociation kinetics of single gene copy. 1000 cpm of $^{32}$P-labeled cytochrome P-450 cDNA (0.02 ng) and 1500 cpm of [$^{3}$P-labeled unique-copy DNA (1 ng) were mixed togehter with 180 $\mu$g of sheared rat liver DNA in 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.5 M NaCl. After being sealed in capillaries, they were denatured at 95 °C for 5 min and incubated at 65 °C up to the indicated Cot values. After incubation, the reaction mixture was divided into two parts. One of them was assayed for single-stranded and double-stranded DNA by hydroxylapatite fractionation with 0.12 and 0.4 M sodium phosphate (pH 6.8), respectively. After the $Cl_3CCOOH$-insoluble radioactivity of each fraction was counted, the ratios of double-stranded DNA to total DNA were calculated. The other was assayed for hybrid formation by $S_1$ nuclease digestion as described previously (Sakai et al., 1978). The amount of duplex formed at each incubation time was normalized by taking the radioactivity of each probe as 100%. (A) Hydroxylapatite assay; (B) $S_1$ nuclease assay; (●) $^{32}$P-labeled cytochrome P-450 cDNA; (○) $^{3}$H-labeled unique-copy DNA.

(23 Ci/mmol, Radiochemical Centre) by nick translation (Sakai et al., 1978).

*Cot Analyses.* For quantitation of gene dosage of the cytochrome P-450 in rat DNA, we analyzed the reassociation kinetics of [$^{32}$P]cDNA with the total rat liver DNA used as a driver. Two kinds of calibration standards were used. One is $^{3}$H-labeled "single-copy" DNA prepared as described above and used as an internal standard. The other is an artificial standard mixture of known gene-copy numbers prepared as follows. The cloned cDNA of cytochrome P-450 (pcP-450pb-1), which had been digested with BglII to give three fragments of 550, 400, and 250 bp (Fujii-Kuriyama et al., 1982a), was mixed with sheared *Escherichia coli* DNA in appropriate ratios to give the following standards: zero gene (only *E. coli* DNA); one gene (*E. coli* DNA:cDNA = 1 mg:0.3 ng); three genes (*E. coli* DNA:cDNA = 1 mg:0.9 ng); six genes (*E. coli* DNA:cDNA = 1 mg:1.8 ng). These proportions were calculated on the basis of a value of 4.1 pg of DNA/rat haploid genome (Albert et al., 1953) and the length of the cDNA (1250 bp) (Fujii-Kuriyama et al., 1982a).

The experimental procedures were as reported previously (Sakai et al., 1978). In brief, [$^{32}$P]cDNA (pcP-450pb-1 insert) was mixed either with sheared liver DNA or with the standard DNA mixture in 20 mM Tris-HCl[1] (pH 7.5), 0.1 mM EDTA, and 0.5 M NaCl. After denaturation of the reaction mixture, the hybridization reactions were allowed to proceed at 65 °C to the desired Cot in sealed siliconized capillaries. The hybrid formation was assayed either by S1 nuclease treatment or by hydroxylapatite fractionation as described previously (Sakai et al., 1978).

*Screening of Rat Gene Library for Cytochrome P-450 Clones.* Approximately 1 × 10$^{6}$ plaques from a rat gene library [a gift from T. D. Sargent, R. B. Wallace, and J. Bonner (*Eco*RI gene library)] were screened as described by Benton & David (1977). Nitrocellulose filters were peeled off from each petri dish, and the DNA in the filters was denatured, neutralized, and baked as described (Benton & Davis, 1977). Plaque hybridization was done at 65 °C for 18 h with [$^{32}$P]cDNAs prepared from a mixture of plasmid clones pcP-450pb-1 and -2 as a probe as described by Grunstein & Hogness (1975). Positive hybridization plaques were picked and replated several times until all the plaques in the plates gave positive signals of hybridization.

*Growth of Recombinant Phages and Preparation of Phage DNA.* Phages were grown and purified by CsCl density gradient centrifugation as described by Blattner et al. (1977). DNA was prepared from the phages by proteinase K treatment and then by phenol extractions (Blattner et al., 1977).

*Gel Electrophoresis, Blotting, and Filter Hybridization.* The DNA preparations obtained were digested with various restriction endonucleases as recommended by the suppliers. Restricted DNAs were electrophoresed on 0.8–1.2% agarose gels (Fujii-Kuriyama et al., 1981). *Hin*dIII-digested λ phage DNA fragments or *Hin*dIII-digested SV 40 DNA fragments were used as molecular weight markers. Specific DNA fragments were isolated, if necessary, from agarose gels as described by Tabak & Flavell (1978). DNA fragments were transferred to nitrocellulose filters by a slight modification of the method of Southern (1975). DNA fragments on filter strips were hybridized with $^{32}$P-labeled cytochrome P-450 cDNA at 65 °C for 18 h as described by Maniatis et al. (1975).

## Results

*Hybridization Kinetics of Cytochrome P-450 cDNA with Rat Liver DNA.* Figure 1 presents the hybridization kinetics of the cytochrome P-450 gene sequence in rat liver DNA with [$^{32}$P]cDNA used as a probe. In this experiment, we used $^{3}$H-labeled "unique-copy gene" DNA as an internal standard, and furthermore, we utilized cDNA (pcP-450pb-1) with a very high specific activity (1 × 10$^{8}$ cpm/$\mu$g) in order to attain a true vast excess of driving total DNA, which was necessary for an accurate determination of $Cot_{1/2}$ values for the

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

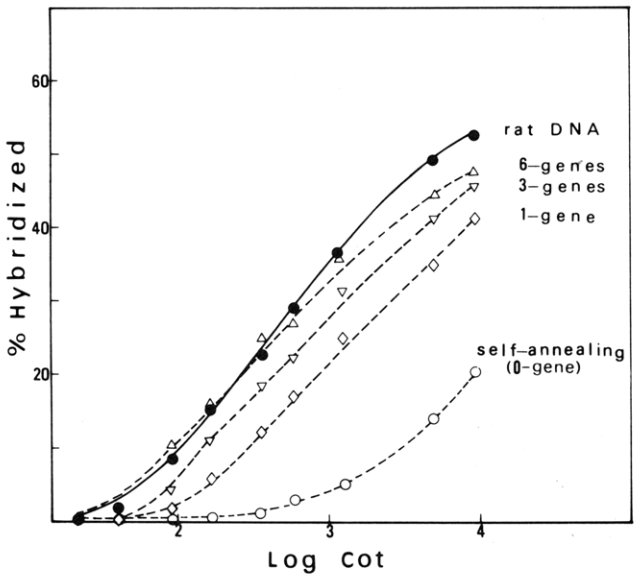


FIGURE 2: Comparative analysis of hybridization kinetics of cytochrome P-450 cDNA between rat liver DNA and gene-number standards. The gene frequency of cytochrome P-450 in rat liver DNA was determined by hybridization kinetics of $^{32}$P-labeled cytochrome P-450 cDNA with total rat liver DNA, in comparison with zero-gene, one-gene, three-gene, and six-gene standards. Gene-number standards were prepared by the addition of appropriate amounts of cytochrome P-450 cDNA (pcP-450pb-1) to *E. coli* DNA. The assumptions used in preparing these standards are described under Materials and Methods. Hybridization was performed in the same manner as described in Figure 1. The extent of hybridization was assayed by hydroxylapatite fractionation. (●) Total liver DNA; (○) zero-gene, (◇) one-gene, (▽) three-gene, and (△) six-gene standard.

unique-copy DNA. The hybridization curves obtained with the nuclease SI assay and the hydroxylapatite column assay showed essentially the same pattern. $Cot_{1/2}$ values for $^{3}$H-labeled unique DNA and $^{32}$P-labeled P-450 cDNA (pcP-450pb-1) were (2.5–2.8) × $10^3$ and (0.5–1.0) × $10^3$, respectively. The $Cot_{1/2}$ value for $^{32}$P-labeled P-450 cDNA was 3–5-fold lower than that for the $^{3}$H-labeled unique-copy DNA, indicating that the gene number for cytochrome P-450 is not unique, but has several values in rat haploid DNA. The several genes for the cytochrome was confirmed and quantitated more accurately in reference to the reassociation kinetics of standard mixtures of known gene numbers.

As shown in Figure 2, the hybridization reaction with rat DNA proceeded at a rate similar to that with the six-gene standard. Under our experimental conditions, hybrid formation was also observed in the zero-gene standard but at much higher Cot values than 1 × $10^3$, which was probably due to the self-annealing of [$^{32}$P]cDNA probe. This effect of self-annealing could be neglected in estimation of gene dosage of cytochrome P-450 because of its much higher $Cot_{1/2}$ value.

From these results, we conclude that there exist approximately six genes or gene-like sequences that have homology with the cloned cDNA corresponding to the pb-inducible cytochrome P-450 in rat haploid genome.

*Isolation and Identification of Recombinant Phages Containing Cytochrome P-450 Sequence.* To verify the multiplicity of the cytochrome P-450 gene in the rat, we isolated genomic clones for the cytochrome by screening a rat gene library in λ Charon 4A with the cloned cDNA as a probe. After about 1 × $10^6$ plaques were screened, nine recombinant phages hybridizing with the cDNA were isolated and designated here pgP-450pb-1 to -9. Since three of them (pgP-450pb-2, -3, and -4) showed the same patterns of restriction cleavage and Southern blot analysis (data not shown), we regarded these clones as identical and used the pgP-450pb-2 clone for further studies. The DNA of each clone was digested with *Eco*RI and subjected to agarose gel electrophoresis (Figure 3A). Three large fragments (23, 19.8, and 10.9 kb) in all the clones corresponded to Charon 4A vector DNAs, and all the other bands showed the inserted fragments derived from rat DNA. The DNA was transferred to a nitrocellulose filter and then hybridized with [$^{32}$P]cDNA. The $^{32}$P-labeled probe used here was a mixture of cloned cDNAs of pcP-450pb-1 and -2, representing approximately 90% of the cytochrome P-450 mRNA sequence. The autoradiograph of the Southern blot analysis is shown in Figure 3B. All the genomic clones contained inserted sequences hybridizing with the cDNA probe.

Clone pgP-450pb-1 (lane 1) showed seven *Eco*RI fragments of the inserted DNA (the smallest band of 0.1 kb was barely detectable in this figure), and three of them (2.1, 2.5, and 3.1 kb) hybridized with the cDNA probe. Although a faint hybridization band was seen at the 1.7-kb position in the autoradiography with a slight smear, it was not observed repro-

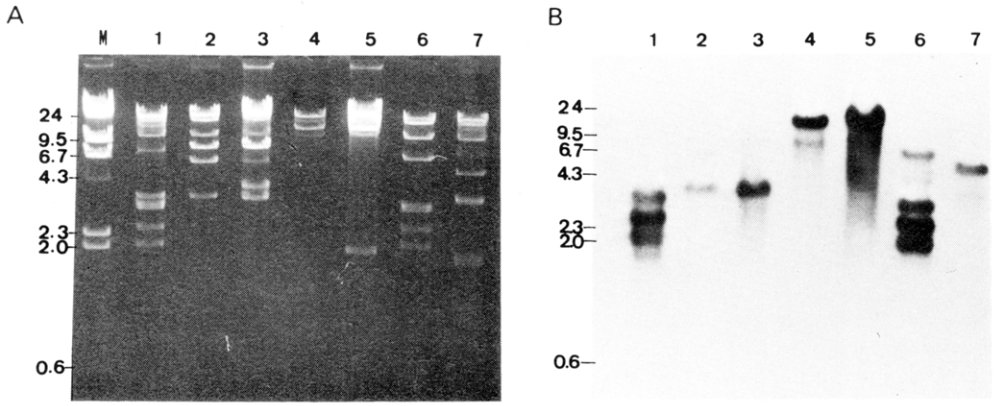


FIGURE 3: Blot hybridization analysis of recombinant phage DNAs. Approximately 1 × $10^6$ plaques from the rat gene library of charon 4A that contained *Eco*RI partially digested fragments of rat chromesomal DNA were screened with $^{32}$P-labeled cytochrome P-450 cDNA (a mixture of equal amounts of the inserts from pcP-450pb-1 and -2) as a probe. Nine positive clones were obtained, seven of which revealed different restriction and hybridization patterns from one another and were designated as indicated. DNAs were extracted from those clones, and 1-μg portions of them were digested with *Eco*RI (5 units) for 3 h and then subjected to agarose gel electrophoresis. (A) 0.2 (lane 4) or 1 (the other lanes) μg of DNAs of each digestion were electrophoresed on a 0.8% agarose gel. (B) Restriction fragments of (A) were transferred from the gel to a nitrocellulose filter by the method of Southern (1975) and hybridized at 65 °C for 16 h with $^{32}$P-labeled cytochrome P-450 cDNA probe prepared from the plasmid clones pcP-450pb-1 and -2. The filter was washed twice with 0.1 × SSC at 65 °C for 1 h and autoradiographed for 16 h at −80 °C. Lanes 1, 2, 3, 4, 5, 6, and 7 correspond to clones pgP-450pb-1, -2, -5, -6, -7, -8, and -9, respectively. (M) *Hin*dHIII-digested λ phage DNA as size markers (shown in kilobase).

dicibly. Clone pb-8 (lane 6) contained inserted DNA that was digested into five fragments by *Eco*RI, and four of them (2.1, 2.5, 3.1, and 6.5 kb) hybridized with the cDNA probe. Three fragments (2.1, 2.5, and 3.1 kb) were commonly seen in both clones pb-1 and -8. As will be described later, these two clones most probably contain overlapping sequences with each other. The genomic DNA from pgP-450pb-2 (lane 2) showed two hybridizing fragments with a size of 3.4 and 5.9 kb. Although the hybridization band of 5.9 kb appeared only weakly, this band was reproducibly observed, and the equivalent band was found in DNA staining gels. Clone pb-5 (lane 3) showed 8.0-, 3.9-, 3.3-, and 0.6-kb fragments of the inserted DNA. An additional faint band of approximately 6.0 kb also was occasionally observed in DNA staining. This may occur due to partial digestion of the enzyme or to some unknown reasons. Only the 3.3-kb fragment of this clone hybridized with the cDNA probe (Figure 3B, lane 3). In the DNA from pgP-450pb-6 (lane 4), a fragment with the size of 14 kb was seen between two vector fragments, and there was no detectable fragment smaller than the 10.9-kb vector arm on the gel (Figure 3A, lane 4). This 14-kb fragment hybridized with the cDNA probe (Figure 3B, lane 4). Although a weak but clear hybridization band was also seen at the 8.8-kb region in Figure 3A, we could not detect the DNA fragment corresponding to this band in an ethidium bromide staining gel. In addition, in the restriction map of this clone (shown later), we could not find any *Eco*RI site in the sequence of the inserted DNA. The reason for occurrence of this weak hybridization band of 8.8 kb is not clear. This is not considered to be due to simply a partial digestion by the enzyme, because we observed complete digestion of λ phage DNA by the enzyme as a control under the same conditions. The inserted DNA from pb-7 (lane 5) was digested into 13- and 1.5-kb DNA fragments by *Eco*RI, and only the 13-kb fragment hybridized with the cDNA probe. A dark smear was observed in lane 5 of the hybridization experiment; this might occur from some artificial degradation of the 13-kb fragment during *Eco*RI digestion because this smear diminished in other experments. Clone pb-9 (lane 7) showed seven *Eco*RI fragments of the inserted DNA, and three of them (5.0, 3.4, and 0.9 kb) hybridized with the cDNA probe. The hybridization signals at 3.4 and 0.9 kb were weak but highly reproducible. The intensities of the hybridization signal varied among the genomic clones and could be due to either length of homologous parts or degree of homology in hybridizable fragments of cloned genomic DNA sequences to the cDNA probe.

*Southern Blot Analysis of Total Rat DNA.* It is necessary to know whether DNA fragments in the genomic clones that hybridized with the cDNA probe are actually present in the total genomic DNA. Therefore, we performed a blot hybridization experiment with total liver DNA. *Eco*RI-digested rat liver total DNA was electrophoresed on an agarose gel in parallel with seven cloned DNAs, transferred to the filter, and hybridized with the cDNA probe. As shown in Figure 4 (the autoradiography of cloned DNAs is the same as that in Figure 3 and omitted in this figure), three different conditions of *Eco*RI digestion resulted in the same restriction (Figure 4B) and hybridization pattern (Figure 4A) with each other, indicating the complete digestion by the enzyme. All of them showed approximately 12 visible bands. The bands equivalent to the seven to eight *Eco*RI fragments containing cytochrome P-450 sequence in the cloned DNAs are also found in the total liver DNA. The remaining five bands, indicated by asterisks, may show additional cytochrome P-450 sequences not represented in the isolated clones. It should be noted that the

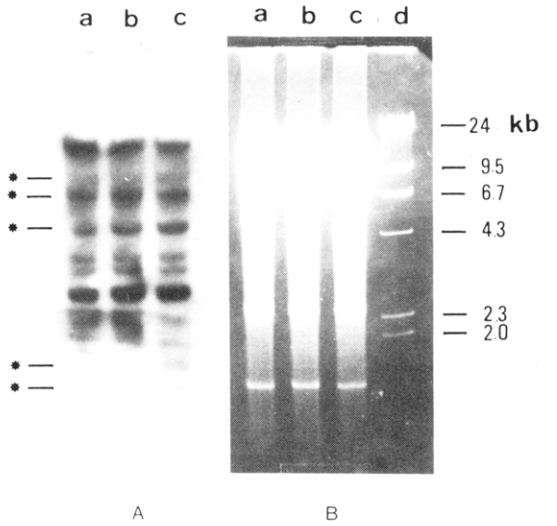


FIGURE 4: Southern blotting of total rat liver DNA. Total DNA was prepared from the liver of normal rat as described under Materials and Methods. The DNA (20 μg) was digested with 5 (lane a), 10 (lane b), and 20 units (lane c) of *Eco*RI for 3 h, and 15-μg portions of each were electrophosed on a 0.8% agarose gel in parallel with seven cloned DNAs. They were blotted and hybridized with [$^{32}$P]cDNA prepared from pcP-450pb-1 and -2 (B) as described in Figure 3 (B). The restriction patterns and the autoradiographs of the seven cloned DNAs are omitted in this figure. The bands marked by asterisks on the left are those that were not represented in the cloned DNAs. The size markers of *Hin*dHIII-digested γ DNA fragments are shown in kilobase.

hybridization signal of the 2.7-kb band was much stronger than the others, suggesting that the nucleotide sequence of this segment may be common to most cytochrome P-450 related gene sequences.

*Restriction Maps of Genomic Clones.* To orient these cloned genomic DNAs the Southern blot analysis was performed with 5′- and 3′-end fragments of the cloned cDNA used as a probe. DNAs from seven genomic clones were digested by *Eco*RI and then transfered onto the nitrocellulose paper as described. *Pst*I/*Bam*HI (0.35 kb) and *Hin*dIII/*Pst*I (0.45 kb) fragments of the cDNA clones pcP-450pb-2 and -1, respectively, were used as 5′ and 3′ probes (restriction maps of the cDNA clones are summarized in Figure 5A). The results of these blot hybridization experiments are shown in Figure 5.

Restriction cleavage maps for these seven genomic clones were determined by a combination of complete and partial digestions of restriction enzymes as described by Smith & Birnstiel (1976). The location of the cytochrome P-450 gene sequence within the recombinant DNA was determined by the blot hybridization of the *Eco*RI, *Bam*HI, *Hin*dIII, *Eco*RI/*Bam*HI, or *Eco*RI/*Hin*dIII fragments with [$^{32}$P]cDNA as a probe. These results are summarized in Figure 6.

The genomic DNAs cloned in pgP-450pb-1 and -8 showed hybridizable bands at the 2.1-kb position with the 5′ probe, while with the 3′ probe a hybridization band of 6.5 kb was observed in pb-8 and only a faint but reproducible hybridization band of 2.5 kb in pb-2 and -8 (panels B and C of Figure 5, lanes 1 and 6).

Sequence overlap between clones pgP-450pb-1 and -8 was comfirmed by digesting the inserted DNAs with such additional enzymes as *Kpn*I and *Xba*I. As shown in Figure 6, clone pgP-450pb-1 contained *Eco*RI fragments of 2.1, 2.5, and 3.1 kb plus additional fragments of 0.1, 3.6, and 3.3 kb extended to the 5′ end, while clone pgP-450pb-8 had 2.1, 2.5, and 3.1 kb plus additional fragments of 6.5 and 3.3 kb to the 3′ direction. The *Kpn*I and *Xba*I sites in the 2.1-, 2.5-, and 3.1-kb

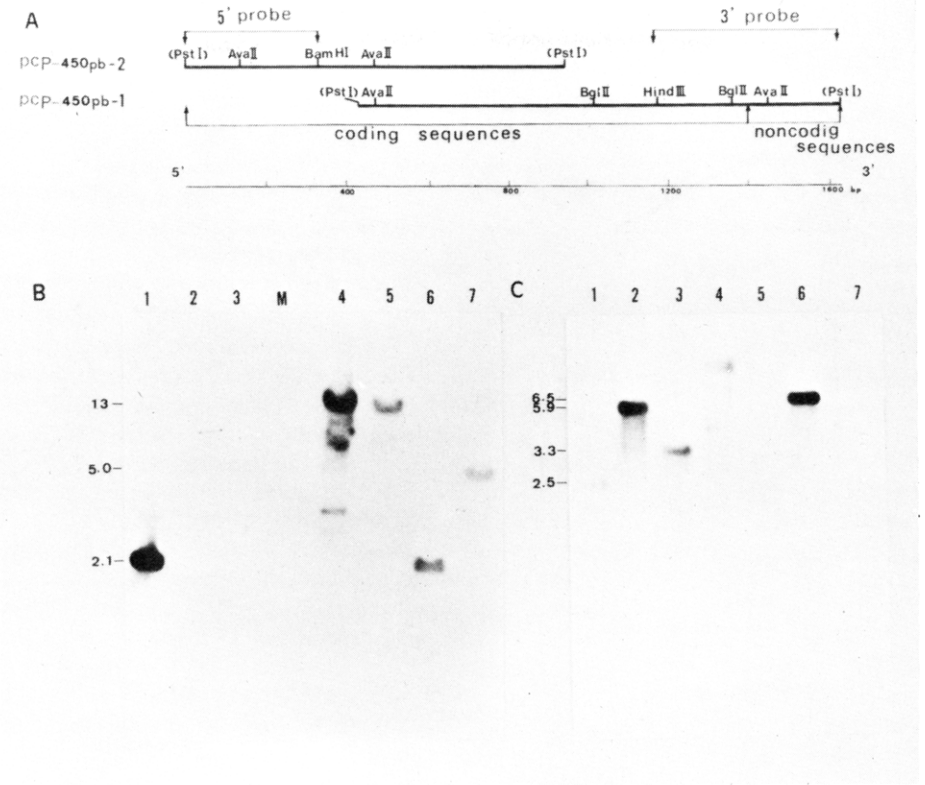


FIGURE 5: Hybridization of recombinant phage DNAs with 5′ and 3′ end fragments of cytochrome P-450 cDNAs. (A) Outline of the physical maps of cytochrome P-450 cDNAs. The restriction maps of both cDNAs were described previously (Fujii-Kuriyama et al., 1982a,b). The *Pst*I sites in parentheses were reconstructed at the 5′ and 3′ end of the inserts by the GC-tailing method. The nucleotide length of cDNA was 0.9 and 1.25 kb in clone pcP-450-b-2 and -1, respectively. The cDNA insert in clone pcP-450pb-2 contained a part of the coding sequences of the mRNA, while the one in clone pcP-450pb-1 contained a part of the coding and 3′ untranslated sequences of the mRNA. The restriction site of *Bam*HI was not detected in the cDNA of pcP-450pb-1, while *Bgl*II and *Hin*dIII sites were not contained in the cDNA of pb-2. No restriction site for *Eco*RI was found in both cDNA. (B) Autoradigraphy showing hybridization with the 5′ end fragment of the cDNA. One microgram of DNA of each genomic clone was digested with *Eco*RI (5 units) for 3 h, electrophoresed on a 0.8% agarose gel, and blotted onto a nitrocellulose filter in the same manner as described in Figure 3. The filter was then hybridized at 65 °C for 18 h with the nick-translated *Pst*I/*Bam*HI (0.35-kb) fragment of cDNA clone pbP-450pb-2 [indicated as "5′ probe" in (A)]. The filter was washed in 0.1 × SSC at 65 °C for 1 h and autoradiographed. (M) *Hin*dIII-digested λ DNA fragments used as size markers. (C) Autoradiography showing hybridization with 3′ end fragment of the cDNA. A gel similar to that in (B) was blotted onto a nitrocellulose filter. The filter was hybridized with the nick-translated *Hin*dIII/*Pst*I (0.45-kb) fragment of cDNA clone pcP-450pb-1 indicated as "3′ probe" in (A). Lanes 1, 2, 3, 4, 5, 6, and 7 correspond to genomic clones pgP-450pb-1, -2, -5, -6, -7, -8, and -9, respectively. The sizes of the representative fragments in the clones are indicated in kilobase on the left of each gel.

fragments were quite identical between both cloned DNAs. In addition, each of these fragments was cross-hybridized with the corresponding one of the other clone (data not shown). A 3.3-kb fragment was also found in both clones, but its relative location is different in the two clones; one in pb-1 was located to the left of the common three fragments while the other in pb-8 is to the right of them. Of course, these two fragments were not cross-hydridized with each other (data not shown). Therefore, as summarized in Figure 6, approximately a 23-kb DNA sequence of chromosomal DNA that contained DNA sequence homologous to the cytochrome P-450 cDNA in the middle was represented in these two clones.

Clones pgP-450pb-2 and -5 contained nucleotide sequences hybridizing with the cDNA probe that are located within 8.3-kb *Eco*RI/*Bam*HI and 2.0-kb *Eco*RI/*Hin*dII fragments of their inserted DNAs, respectively (Figure 6). These clones showed hybridization bands only with the 3′ probe but not with the 5′ probe (Figure 5B,C, lanes 2 and 3). Even if filters of the blot–hybridization were washed under less stringent conditions (1 × SSC), no fragment hybridizable with the 5′ probe was detected in the two cloned DNAs (data not shown). These clones were, therefore, supposed to lack in a nucleotide sequence homologous with the 5′ probe of the cDNA. But since considerably long DNA sequences are located to the 5′ side of the DNA sequences hybridizable with the cDNA probe in these two clones, a possibility could also be considered that sequences of the equivalent portions in these cloned DNA were so diverged that the sequence homology with the cDNA could not be detected under these conditions.

On the other hand, clones pgP-450pb-7 and -9 showed hybridization bands with the 5′ probe but not with the 3′ probe (Figure 5B,C, lanes 5 and 7). However, under less stringent conditions of the filter washing (1 × SSC), the hybridization of the 3′ probe could be clearly detected with the 13-kb *Eco*RI/*Eco*RI fragment in pb-7 but not with any fragments in pb-9 (data not shown). It is, therefore, supposed that pb-7 should contained the nucleotide sequence slightly diverged from that in the 3′-end region of the cDNA. The nucleotide sequence in the pb-9 clone hybridizing with the cDNA located within the 6.0-kb sequence in the 3′ end of the inserted DNA (Figure 6) and total length of one of the cytochrome P-450 genes that we have recently sequenced (to be published elsewhere) is approximately 14 kb. Therefore, it could be considered that pb-9 clone lacks in the sequence with homology to the 3′ probe of cDNA.

The 14-kb *Eco*RI fragment of clone pgP-450pb-6 showed hybridization bands with both 5′ and 3′ probes (Figure 5B,C, lane 4), but the hybridization signal of this clone with the 3′ probe is rather weak. Sequence analysis of this clone showed that this is due to shortness of the cloned DNA sequence

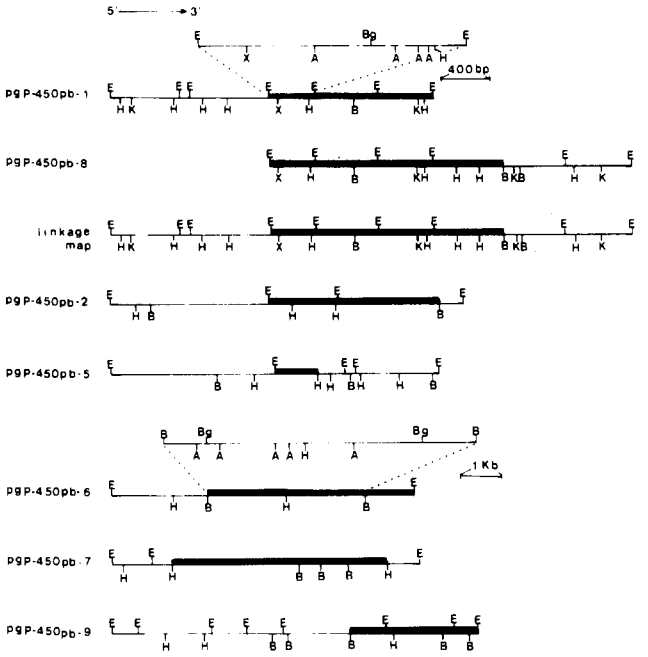


FIGURE 6: Restriction cleavage maps of genomic clones. The 5′ to 3′ direction is indicated by an arrow on the top. Restriction maps were constructed by complete and partial digestions of the DNAs from each clone according to the method of Smith & Birnstiel (1976). The *Kpn*I and *Xba*I sites were described only in the maps of pgP-450pb-1 and -8 in order to confirm the linkage of these clones. The linkage of clone pgP-450pb-1 and -8 is drawn under the restriction map of clone pgP-450pb-8. The *Ava*II and *Bgl*II sites were determined for the 2.1-kb *Eco*RI fragment of pgP-450pb-1 and the 7.0-kb *Bam*HI fragment of pgP-450pb-6, which are shown in the expanded scales. The regions hybridizing with the cDNAs were determined by a series of blot hybridizations with *Eco*RI-, *Hin*dIII-, *Bam*HI-, *Eco*RI/*Hin*dHIII-, or *Eco*RI/*Bam*HI-digested DNAs from each clone with $^{32}$P-labeled cytochrome P-450 cDNAs (a mixture of the inserts of pcP-450-b-1 and -2) as probes and are indicated by bold lines. The direction of each genomic DNA was determined by the experiment described in Figure 5. (E) *Eco*RI; (H) *Hin*dIII; (B) *Bam*HI; (K) *Kpn*I; (X) *Xba*I; (A) *Ava*II; (Bg) *Bgl*II.

homologous to the 3′ probe, and therefore, this clone lacks in the very 3′-terminal region of the cytochrome P-450 gene. Several bands smaller than the 14-kb fragment also appeared in Figure 5B, lane 4, but these bands were not reproducible. This is not considered to be due to simply a partial digestion by the enzyme because higher concentration of the enzyme did not improve the situation. This might occur from either partial enzyme digestion due to partial methylation of DNA that renders the sequence resistant to the enzyme digestion or to some unknown reasons. The nucleotide sequence of the inserted DNA of pb-6 has recently been analyzed, the results of which show that pb-6 contains nucleotide sequences completely identical with those of the cDNA probe and some intervening sequences in the insert (to be published elsewhere).

## Discussion

From a Cot analysis of total liver DNA, we estimated approximately six genes or gene-like DNA sequences for phenobarbital-inducible cytochrome P-450 in rat haploid genome. This number roughly coincides with that of the isolated genomic clones that were screened from about $1 \times 10^6$ plaques with the cDNA as a probe. However, Southern blot analysis of total liver DNA digested with *Eco*RI indicated that all the bands hybridizable with the cloned cDNA were not represented in the isolated six genomic clones. Seven or eight of the approximately 12 DNA bands in total genomic DNA that hybridized with the cDNA were found in the six genomic clones. Therefore, a few DNA sequences related to the cytochrome P-450 gene remain to be cloned. These results clearly indicate the presence of multiple genes or gene-like DNA sequences even for one type (phenobarbital-inducible) of cytochrome P-450.

Our preliminary results show that the cDNA clone of methylcholanthrene-inducible cytochrome P-450 does not appear to cross-hybridize with that of the phenobarbital-inducible one under less stringent conditions of washing (1 × SSC), therefore suggesting the presence of another gene family for the cytochrome P-450 (unpublished observations).

We have recently determined the primary structure of one of the phenobarbital-inducible cytochrome P-450 genes by sequence analysis of two cloned DNAs, one of which is pgP-450pb-6 and contained the first 964 coding nucleotides from the initiation codon of the mRNA [see Fujii-Kuriyama et al. (1982a)]. These results indicate that the gene cloned in pb-6 was most probably expressed in the liver of pb-treated rats. From analogy of the determined gene structure, it seems rather strange that DNAs from clones pgP-450pb-2 and -5 did not hybridized with the 5′ probe of the cDNA, in spite of the fact that these cloned DNAs contained a considerably long stretch of DNA located to the 5′ side of their DNA sequences hybridizable with the cDNA. This could be explained by one of these alternatives: (1) the nucleotide sequences in the 5′-end region of these isolated DNA sequence should be so diverged that their equivalent sequences did not have sufficient homology for hybridization to the 5′ probe; (2) there could exist unusually large intervening sequences so that the 5′ equivalent sequences were far beyond the 5′ end of these cloned DNAs; or (3) these isolated DNA sequences could be artifact products caused by rearrangement of DNA fragments during construction of the gene library. In this connection, all the cloned DNA sequence may not represent true genes for the cytochrome P-450. Some are probably pseudogenes as is often the case with other eukaryotic genes. Further work is necessary to clear these points.

As seen in Figures 3 and 5, the intensities of the hybridization bands were considerably different among genomic clones. These differences may be attributed to the differences of the nucleotide sequences among those cloned DNA. Indeed, our preliminary data showed that partial nucleotide sequences of pb-1 and 6 were slightly different from each other. Partial sequences of pb-1 and -6 are 90% and 100% homologous, respectively, to a part of the cDNA sequence (to be published elsewhere). In addition, as seen in Figure 6, the cleavage maps of several restriction enzymes were also markedly different among the clones. How and where these differences are in the sequences of the genomic DNAs hybridizable with cytochrome P-450 cDNA are most intriguing questions to be elucidated, and sequencing of these cloned DNAs is currently under study.

Our previous data with cDNA clones of cytochrome P-450 showed that their coding nucleotide sequence was 1473 bases and contained one cleavage site each for *Bam*HI and *Hin*dIII but no site for *Eco*RI. On the other hand, as shown in Figure 6, some genomic clones of the cytochrome, whose lengths range from 2 to 11 kb, contained more than two cleavage sites for *Bam*HI (clones pgP-450pb-7 and -9) and *Hin*dIII (clones pgP-450pb-1, -2, and -8) and more than one cleavage site for *Eco*RI (clones pgP-450pb-1, -8, and -9) in the regions hybridizing with the cDNA. These results suggest the presence of intervening sequence(s) in the cloned DNA sequences related to cytochrome P-450 gene. In the linkage of clones pgP-450pb-1 and -8, it was seen that the total length of the

fragments hybridizing with the cDNA probe extended to more than 11 kb with 5′ and 3′ flanking regions of considerable length, which showed no hybridization signal with the cDNA. Therefore, clones pgP-450pb-1 and -8 may cover the whole sequence of the cytochrome P-450 gene.

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**Registry No.** Cytochrome P-450, 9035-51-2; phenobarbital, 50-06-6.

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# A Stereochemical and Positional Isotope Exchange Study of the Mechanism of Activation of Isoleucine by Isoleucyl-tRNA Synthetase from *Escherichia coli*†

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ABSTRACT: Isoleucyl-tRNA synthetase from *Escherichia coli* catalyzes the activation of [$^{18}O_2$]isoleucine by adenosine 5′-[(*R*)-$\alpha$-$^{17}$O]triphosphate with inversion of configuration at phosphorus. Moreover, isoleucyl-tRNA synthetase does not catalyze positional isotope exchange in adenosine 5′-[$\beta$-$^{18}O_2$]triphosphate in the absence of isoleucine or in the presence of the competitive inhibitor isoleucinol, which effectively eliminates the possibility of either adenylyl–enzyme or adenosine metaphosphate intermediates being involved. Together, these observations require that isoleucyl-tRNA synthetase catalyzes the activation of isoleucine by associative "in line" nucleotidyl transfer. The synthesis of adenosine 5′-[(*R*)-$\alpha$-$^{17}$O]diphosphate and its conversion to adenosine 5′-[(*R*)-$\alpha$-$^{17}$O]triphosphate is described and an explanation provided for the reported differences between the treatment of adenosine 5′-[(*S*)-$\alpha$-thiodiphosphate] with cyanogen bromide and bromine in [$^{18}$O]water.

The aminoacyl-tRNA synthetases are a family of enzymes that activate amino acids with ATP and then transfer them to their cognate tRNA (Söll & Schimmel, 1974; Schimmel & Söll, 1979). The high fidelity observed in the translation of genetic information in protein biosynthesis is made possible by the ability of the aminoacyl-tRNA synthetases to selectively bind and couple their respective amino acid and tRNA and to hydrolyze ("edit") mischarged tRNA (Fersht, 1981). The activation of an amino acid by ATP to form an aminoacyl adenylate can be studied in the absence of its cognate tRNA with most aminoacyl-tRNA synthetases, and it is now accepted that all aminoacyl-tRNA synthetases adopt this two-step process (Kim et al., 1977):

$$H_3N^+\cdot CHR\cdot CO_2^- + MgATP \rightleftarrows H_3N^+\cdot CHR\cdot CO\cdot AMP + MgPP_i$$

$$H_3N^+\cdot CHR\cdot CO\cdot AMP + tRNA \rightleftarrows H_3N^+\cdot CHR\cdot CO\cdot tRNA + AMP$$

The structural diversity of the aminoacyl-tRNA synthetases is rather surprising. They occur as monomers, dimers, and tetramers and manifest considerable differences in subunit molecular weight (Kiselev & Favorova, 1974). There is however evidence to suggest that the larger subunits may have arisen by gene duplication and fusion (Kalousek & Konigsberg, 1976; Waterson & Konigsberg, 1974; Koch et al., 1974; Kula, 1973). The question we wish to consider concerns the

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