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Isolation of cDNAs Coding for Three Different Forms of Liver Microsomal Cytochrome P-450 from Polychlorinated Biphenyl-Treated Beagle Dogs

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SUMMARY

Three different cDNA clones, namely DM1-1, Dah1, and Dah2, encoding hepatic cytochrome P-450, were isolated from a cDNA library in λgt11 constructed from liver RNA of polychlorinated biphenyl-treated beagle dogs. DM1-1 was 1857 base pairs (bp) long and encoded a polypeptide of 457 residues. Dah1 was 2394 bp long and contained an entire coding region for 524 amino acid residues. In addition, Dah2 was 1623 bp long and had an open reading frame consisting of 503 amino acid residues, although it lacked the translational initiation codon. Judging from the similarity of the nucleotide and amino acid sequences, forms of cytochrome P-450 encoded by DM1-1, Dah1, and Dah2 were judged to belong to the P450IIC, P450IA1, and P450IA2 subfamilies, respectively. Northern blot analysis of RNA from various tissues, using the specific 3′ noncoding regions of Dah1 and Dah2 as probes, indicated that mRNAs for P-450(Dah1) and P-450(Dah2) were not detectable in tissues from untreated dogs, except for P-450(Dah2) in livers. Polychlorinated biphenyl induced both mRNAs in liver, kidney, and lung, especially in the kidney.

Cytochrome P-450 in hepatic microsomes catalyzes the oxidation of a variety of endogenous and exogenous substrates, including steroids, fatty acids, drugs, and toxicants. It has been widely accepted that there are many forms of cytochrome P-450. Based on the similarity of nucleotide sequences of the forms, the forms of cytochrome P-450 are classified into subfamilies (1). Although the substrate specificity of each form of cytochrome P-450 is not narrow, the amount and the catalytic properties of each form of cytochrome P-450 affect the capacity to metabolize compounds in liver microsomes, presumably leading to changes in pharmacological and toxicological consequences *in vivo*. Thus, studies on the interspecies homology of cytochrome P-450 may provide useful information for prediction of the extent of actions of drugs and toxicants in animal species.

Among a number of experimental animals, beagle dogs are commonly used in the pharmaceutical industry in pharmacokinetic as well as toxicokinetic examinations for new drug development. Despite this rather routine research, only limited information is now available on the molecular properties of cytochrome P-450 in beagle dogs.

From the toxicological point of view, dogs are known to be unique experimental animals that show bladder carcinoma upon treatment with 4-aminobiphenyl (2). 4-Aminobiphenyl is *N*-hydroxylated by cytochrome P-450 before exerting its toxicity. We previously reported that rat P-450d catalyzes efficiently the *N*-hydroxylation of various promutagens including 4-aminobiphenyl (3). Thus, the presence in beagle dogs of a form of cytochrome P-450 corresponding to rat P-450d had been expected. However, no reports supporting this idea had appeared until our recent studies. Although investigations have included the purification of cytochrome P-450 from dog livers (4–8), no direct comparison of dog cytochrome P-450 with forms of cytochrome P-450 from other animal species has been performed on a molecular basis, including comparison of their primary structures. Thus, the aim of this study was to further clarify the molecular sequences of cytochrome P-450 from beagle dogs.

We report herein the nucleotide and the amino acid sequences of three forms of cytochrome P-450 that showed great similarities to those of rat P-450c (9), P-450d (10), and P-450(M-1) (11), respectively. The expression of dog P450IA genes in liver and extrahepatic tissues was also examined by Northern blot analysis.

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ABBREVIATIONS: PCB, polychlorinated biphenyl; MOPS, 3-(*N*-morphorino)propanesulfonic acid; 1× SSC, 0.15 M NaCl, 15 mM Na-citrate (pH 7.0); 1× Denhardt's solution, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin; Kb, kilobases; bp, base pair.

Materials and Methods

Animals. Adult male beagle dogs weighing about 10 kg were given a single intraperitoneal injection of PCB (Kaneclor KC-500) in corn oil, at a dose of 400 mg/kg, and were killed 5 days later. Livers were immediately excised and used for RNA preparation.

Materials. Restriction endonucleases and other DNA-modifying enzymes were purchased from Nippon Gene (Toyama), Takara Shuzo (Kyoto), and NEB (Beverly, MA). cDNA synthesis, λgt11 cloning, M13 cloning and sequencing kits, [α-^{32}P]dCTP (3000 Ci/mmol), and [α-^{35}S] dATP (1000 Ci/mmol) were purchased from Amersham International (London). Nitrocellulose filters were obtained from Toyo Roshi (Tokyo) or Schleicher & Schuell (FRG). pHPah1 (12) was kindly supplied by Dr. R. Sato of the Institute for Protein Research, Osaka University, and pcP-450(M-1)-4 (11) by Dr. Y. Fujii-Kuriyama of Tohoku University. Other materials are described in the following sections.

Construction of cDNA library of PCB-treated dog liver. Total RNA was prepared from the liver, kidney, small intestine, and lung of PCB-treated and untreated beagle dogs by the guanidium thiocyanate method (13). Poly(A)$^+$RNA was enriched from the total liver RNA of PCB-treated dogs by oligo(dT)-cellulose (Pharmacia Co., Uppsala) column chromatography (14) and then subjected to sucrose density gradient centrifugation (5 to 25% sucrose). Fractions containing mRNA hybridizable with a rabbit cDNA fragment of pHPah1, which encoded a 3-methylcholanthrene-inducible rabbit cytochrome P-450 (12), were pooled. The rabbit cDNA fragment used as a probe was labeled by nick translation with [α-^{32}P]dCTP. A 5-μg portion of mRNA (18 to 28 S) was used for cDNA synthesis. The construction of a cDNA library in λgt11 was performed according to the protocols for the cDNA synthesis and cloning kits (Amersham).

Screening of cDNA library. Approximately 4.0 × 10^4 recombinant phage plaques of λgt11 were screened by *in situ* plaque hybridization (15), using the *Pst*I-*Nco*I fragment (425 bp) of pcP-450(M-1)-4 (11) as a probe. In a similar manner, approximately 6.0 × 10^4 plaques were screened using the *Nco*I-*Xmn*I fragment (443 bp) of pHPah1 (12) as a probe. Positive plaques were identified by repeated screening. Phage DNAs of positive plaques were purified by means of standard protocols (16). Insert cDNA fragments of these positive phage DNAs were subcloned into pUC18 and mapped by various restriction enzymes. The cDNA fragments were subcloned into M13 mp18 and mp19 and sequenced by the dideoxy method (17) using [α-^{35}S]dATP. The sequencing strategy for each cDNA is shown in Fig. 1.

Northern blot analysis. Total RNA (3 μg) from liver, kidney, small intestine, and lung was electrophoresed in a 1% agarose gel containing 20 mM formaldehyde, 40 mM MOPS (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA. RNA was transferred to a nitrocellulose filter and hybridized with a ^{32}P-labeled probe, as described previously (18). Hybridization was carried out at 42° in 50 mM sodium phosphate buffer (pH 6.5) containing 50% formamide, 5× SSC, 5× Denhardt's, 100 μg/ml heat-denatured salmon sperm DNA, and cDNA fragments that were ^{32}P-labeled by nick translation. The filters were washed twice with 2× SSC containing 0.1% sodium dodecyl sulfate, at 55° and then autoradiographed at −80° (Fuji film, Tokyo).

Results

Isolation of cDNAs for beagle dog cytochrome P-450. By screening approximately 4.0 × 10^4 plaques using the 5′-terminal region of a rat cDNA fragment, the *Pst*I-*Nco*I fragment of pcP-450(M-1)-4, as a probe, a dog cDNA clone, namely DM1-1 (about 1.9-kb long), was obtained. *Eco*RI digests of the DM1-1 insert gave two fragments (0.4 and 1.5 kb). The restriction map of DM1-1 is shown in Fig. 1.

From the same cDNA library (approximately 6.0 × 10^4 plaques), five positive clones were isolated using the *Nco*I-*Xmn*I fragment of the rabbit P450IA1 cDNA clone pHPah1 as a probe. One of them (termed Dah2) displayed a different restriction enzyme map from that of the other four clones. Among these four cDNA clones, the longest one (2.4 kb) was termed Dah1. Fig. 1 also shows the restriction enzyme maps of these two different cDNA clones.

Sequence analysis of DM1-1. Fig. 2 shows the nucleotide and the deduced amino acid sequences of DM1-1. This clone was 1,875 bp long, coding for a polypeptide of 457 amino acid residues. Poly(A) and poly(A) additional signal (AATAAA) were also found in the 3′ noncoding region. When the nucleotide and the deduced amino acid sequences of DM1-1 were compared with those of other forms of mammalian hepatic cytochrome P-450, it was suggested that P-450(DM1-1)[2] belonged to the P450IIC gene family. As shown in Table 1, DM1-1 showed the greatest similarity with MP-8 (19, 20), which seems to encode a cytochrome P-450 catalyzing tolbutamide methyl hydroxylation in human livers. Great similarity was also seen between DM1-1 and Hp1-1 (21), which is a member

[2] We indicate the name of cytochrome P-450 encoded by a cDNA as P-450( ); e.g., P-450(DM1-1) indicates a form of cytochrome P-450 encoded by DM1-1.

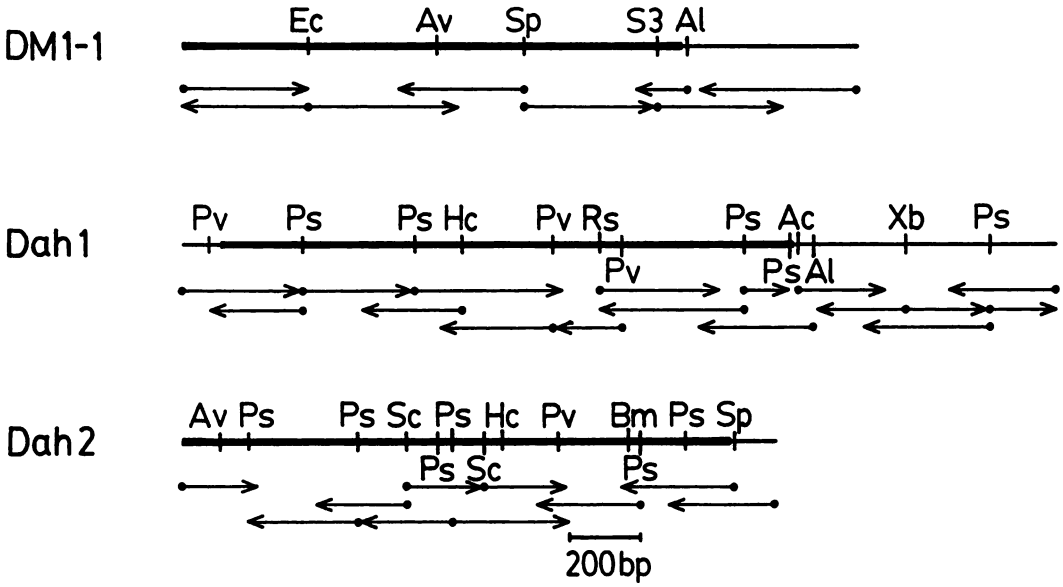

Fig. 1. Restriction enzyme maps of three different cDNA clones for hepatic cytochrome P-450 of dog. *Thick* and *thin lines*, coding and noncoding region, respectively. *Arrows*, extent and direction of sequencing. Each sequence was repeated at least two times. *Ac*, *Acc*I; *Al*, *Alu*I; *Av*, *Ava*I; *Bm*, *Bam*HI; *Ec*, *Eco*RI; *Hc*, *Hin*cII; *Ps*, *Pst*I; *Pv*, *Pvu*II; *Rs*, *Rsa*I; *S3*, *Sau*3A; *Sc*, *Sac*I; *Sp*, *Sph*I; *Xb*, *Xba*I.

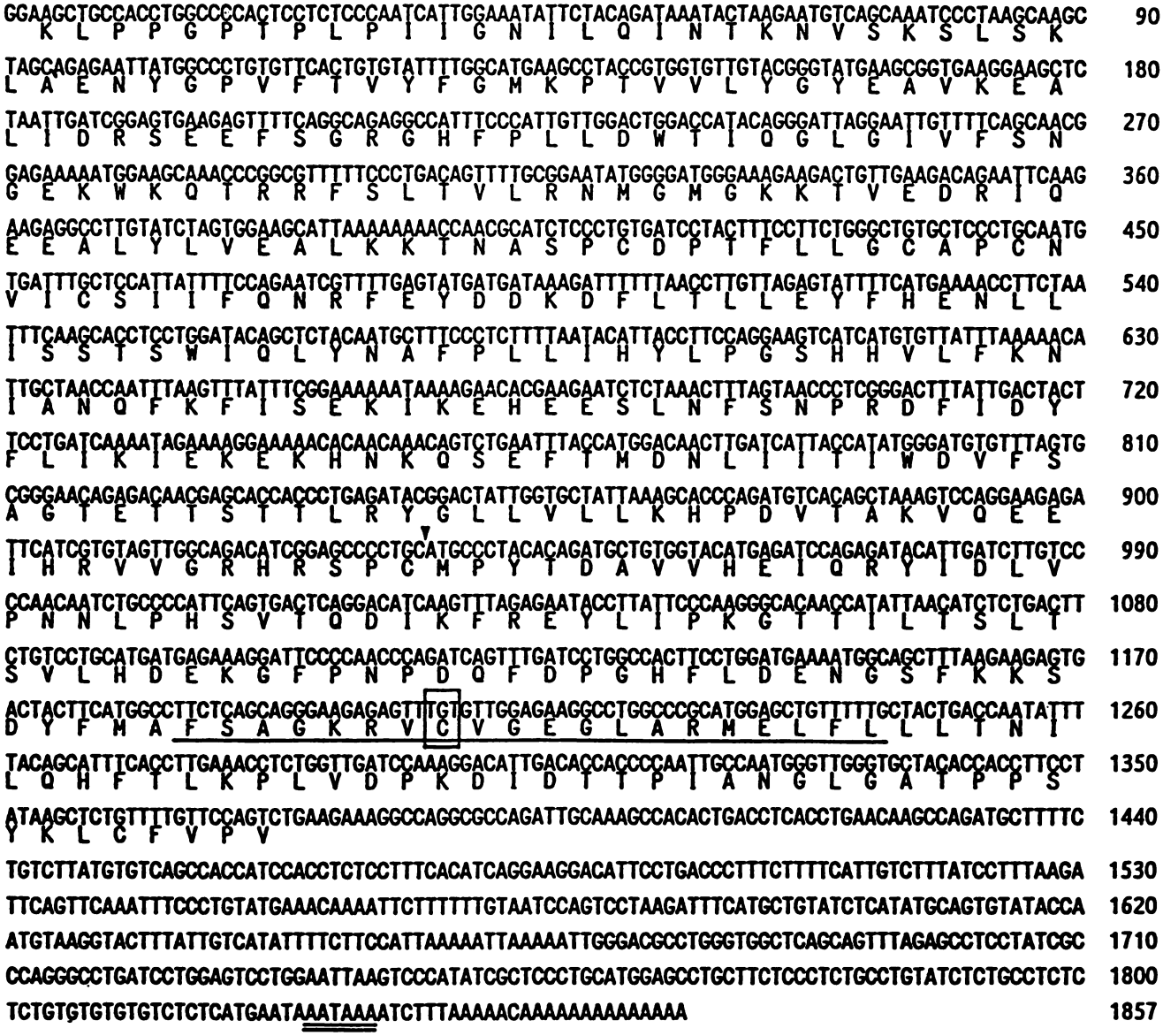

Fig. 2. Nucleotide and deduced amino acid sequences of DM1-1. The cysteinyl residue that acts as the fifth ligand to heme iron is *boxed* and the heme-binding region including the cysteinyl residue is *underlined*. *Double underline*, poly(A) additional signal. *Arrowhead*, deletion site of six amino acid residues.

TABLE 1

Comparison of the nucleotide and the deduced amino acid sequences of DM1-1 with those of forms or mammalian cytochrome P-450

The nucleotide and the deduced amino acid sequences of DM1-1 were compared with those of P-450 (M-1) (11), p1-8 (31), Hp1-1 (21), MP-8 (19), phP450j (22), NF25 (23), and hP_1-450 (24).

Species	Trivial name	Gene locus	Identity	
			Nucleotide	Amino acid
			%	
Rat	M-1	CYP2C11	72	66
Rabbit	p1-8	CYP2C5	72	67
Human	Hp1-1	CYP2C8	74	66
	MP-8	CYP2C10	76	68
	phP450j	CYP2E1	63	58
	NF25	CYP3A4	49	26
	hP_1-450	CYP1A1	47	30

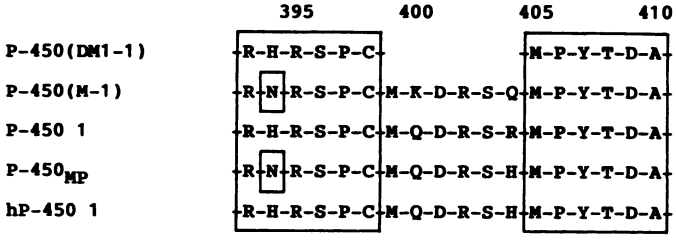

Fig. 3. Deletion of six amino acid residues in the deduced sequence of DM1-1, as compared with other forms of cytochrome P-450 in the P450IIC subfamily. The deduced amino acid sequence of DM1-1 is aligned with those of other forms of cytochrome P-450, rat P-450(M-1) (11), rabbit P-450 1 (31), human $P\text{-}450_{MP}$ (19), and P-450 1 (21). Residues identical to those of DM1-1 are *boxed*. *Numbers*, the amino acid sequence of P-450(M-1).

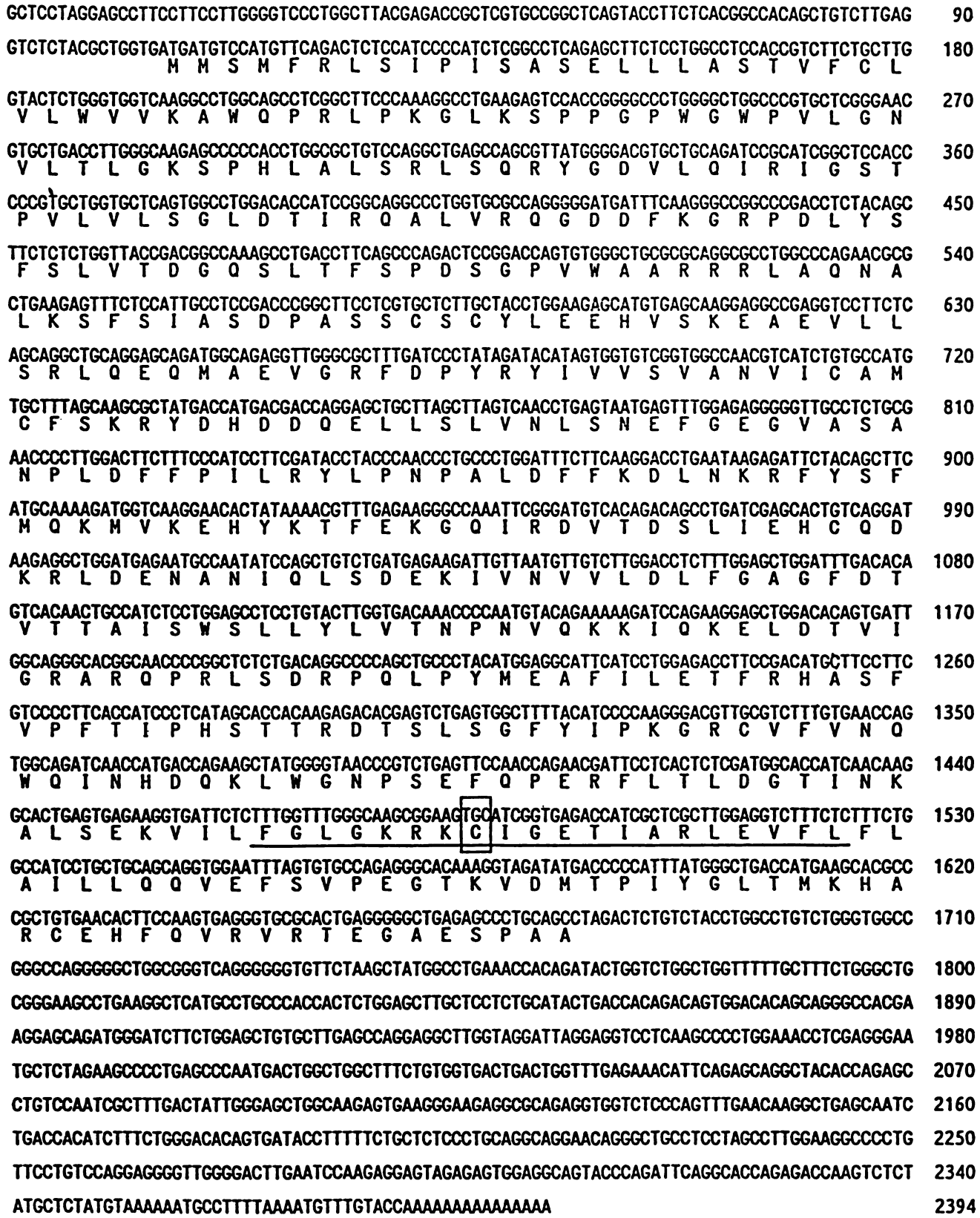

Fig. 4. Nucleotide and deduced amino acid sequences of Dah1. See Fig. 2 for other details.

of the human P450IIC family, as is MP-8. Much less similarity was seen between DM1-1 and phP450j (22), NF25 (23), and hP_1-450 (24), which are members of the human P450IIE, III, and IA gene family, respectively. It was interesting to note that P-450(DM1-1) lacked six amino acids between the 311th cysteine and 312th methionine residues (indicated by an *arrowhead* in Fig. 2), compared with those of other forms of cytochrome P-450 in the same P450IIC subfamily (Fig. 3). The

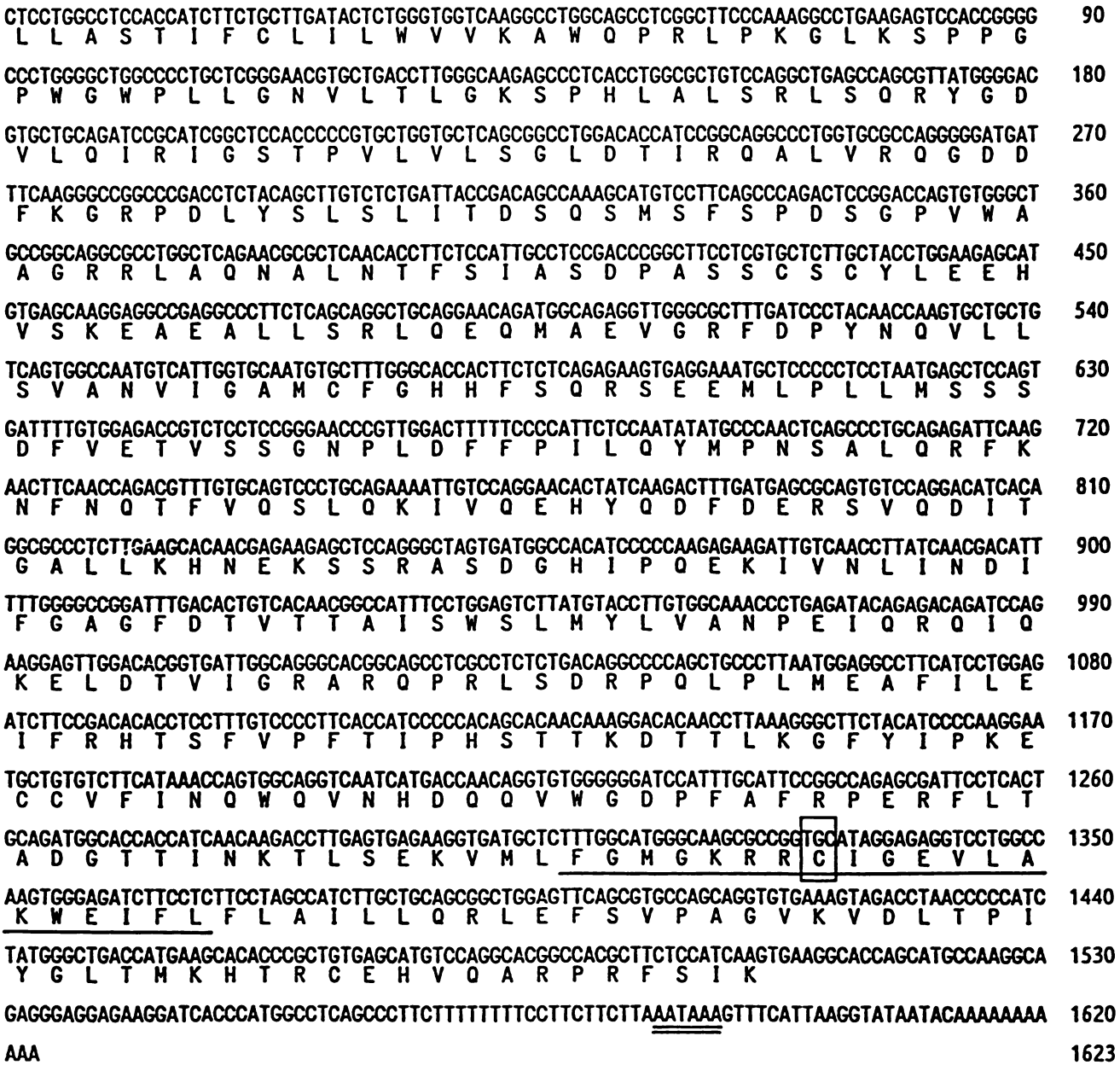

Fig. 5. Nucleotide and deduced amino acid sequences of Dah2. See Fig. 2 for other details.

311th cysteine and 312th methionine in the deduced amino acid sequence of DM1-1 corresponded to the 398th cysteine and 405th methionine in that of M-1.

Sequence analysis of Dah1 and Dah2. Dah1 is a cDNA clone consisting of 2394 bp and has an open reading frame of 524 amino acid residues (Fig. 4). The 5′ and 3′ noncoding regions were 105 and 717 bp, respectively. This clone lacked poly(A) additional signal (AATAAA), although it contained poly(A) at the 3′ end. There were three possible initiation codons in the nucleotide sequence of Dah1 between the 106th and the 117th nucleotides, as shown in Fig. 4. Cytochrome P-450 with N-terminal amino acid sequence identical to that of P-450(Dah1) has not been purified from liver microsomes of dogs at present. Therefore, it is not clear from which codon the translation starts.

Dah2 is a cDNA consisting of 1623 bp and codes for a polypeptide of 503 residues, although this cDNA lacked the initiation codon (Fig. 5). The 3′ noncoding region of this clone was shorter than that of Dah1. There were 80 and 75% similarities between Dah1 and Dah2, when calculated on the basis of the nucleotide and the amino acid sequences, respectively. Dot matrix analysis of the deduced amino acid sequences of Dah1 and Dah2 showed the high levels of homology at the one-third N-terminal and one-third C-terminal regions, as reported for rat cytochromes P-450 in the P450IA gene family (25) (Fig. 6).

Table 2 shows the comparison of the nucleotide and the deduced amino acid sequences of these two cDNA clones with those of other forms of cytochrome P-450 in the P450IA gene family. P-450(Dah1) displayed a greater similarity to mouse P_1-450 (26), rat P-450c (9), rabbit pHPah1 or form 6 (12, 27), and human P_1-450 (24), suggesting that P-450(Dah1) was a

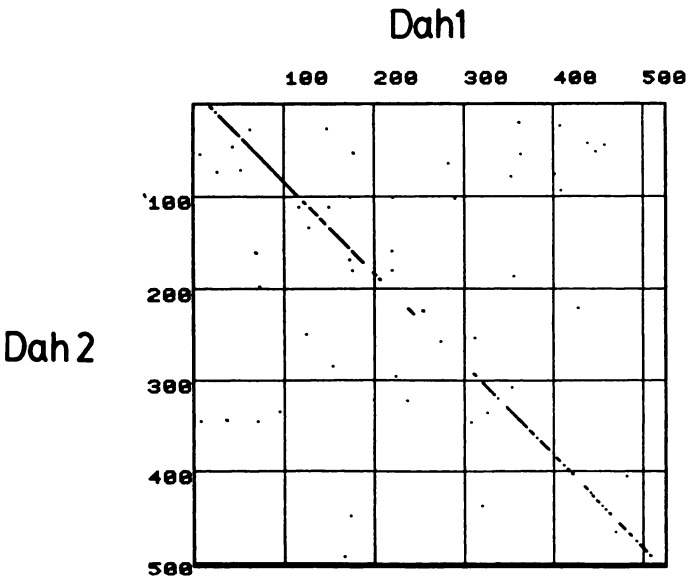

Fig. 6. Dot matrix comparison of the deduced amino acid sequences of Dah1 and Dah2. A *dot* appears at the position where three continuous amino acid residues are identical in both sequences. Dot matrix is plotted with the deduced amino acid sequence of Dah2 as *ordinate* and that of Dah1 as *abscissa*.

TABLE 2

Comparison of the nucleotide and the deduced amino acid sequences of Dah1 and Dah2 with those of other members of the P450IA subfamily

The nucleotide and the deduced amino acid sequences of Dah1 and Dah2 were compared with those of P450IA genes of mouse (26), rat (9, 10), rabbit (12, 27), and human (24, 28).

Species	Trivial name	Identity			
		Dah1		Dah2	
		Nucleotide	Amino acid	Nucleotide	Amino acid
		%			
Mouse	P_1-450	79	78	72	65
	P_3-450	73	67	77	72
Rat	P-450c	79	77	72	64
	P-450d	73	67	78	73
Rabbit	pHPah1	81	77	74	66
	pHPah2	75	69	81	76
Human	hP_1-450	84	81	76	69
	hP_3-450	77	74	84	82

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P-450(Dah2)                                          -L-L-A-S-T-I-F-
P-450-D2          A-L-S-G-M-          -A-T-G-L-L-L-A-S-T-I-F-
P-450-D3          A-L-S-Q-M-          -A-T-G-L-L-L-A-S-A-I-F-
P-450d      M-A-F-S-Q-Y-I-S-L-  -A-P-E-L-L-L-A-T-A-I-F-
hP3-450     M-A-L-S-Q-S-V-P-F-S-A-T-E-L-L-L-A-S-A-I-F-
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Fig. 7. Comparison of the deduced N-terminal amino acid sequence of Dah2 with those of purified P-450. The N-terminal amino acid sequences of P-450-D2 and P-450-D3 were determined by protein sequencing (5, 6) and those of P-450d (10) and human P_3-450 (28) are derived from the nucleotide sequences of their cDNAs. Identical residues are *boxed*. Gaps were inserted in the sequences of dog cytochrome P-450.

cytochrome P-450 in the P450IA1 gene family. On the other hand, Dah2 showed a greater similarity to mouse P_3-450 (26), rat P-450d (10), rabbit pHPah2 or form 4 (12, 27), and human P_3-450 (28), suggesting that P-450 (Dah2) was in the P450IA2 gene family. Among these mammalian species compared, dogs appeared to be slightly closer to humans in molecular evolution of these cytochrome P-450 genes.

Comparison of N-terminal amino acid sequences. When the N-terminal amino acid sequence of DM1-1 was compared with that of rat P-450(M-1) (11), it was suggested that DM1-1 lacked the N-terminal 27 residues (data not shown). The N-terminal amino acid sequence of P-450(Dah1) was not consistent with any other forms of cytochrome P-450 purified so far from dog livers. On the other hand, the N-terminal amino acid sequence of P-450(Dah2) was identical to that of P-450-D2 (5) purified from liver microsomes of PCB-treated dogs, although P-450(Dah2) lacked the N-terminal nine amino acid residues, as compared with P-450-D2 (Fig. 7).

Northern blot analysis of dog P450IA mRNA from various tissues. To determine the expression of two forms of cytochrome P-450 in the P450IA family in various tissues and the inducibility by PCB, Northern blot analysis using the cDNA fragments of each clone was carried out. Because the coding region of Dah1 and Dah2 showed 80% similarity, we used the fragments containing largely the 3′ noncoding region of Dah1 and Dah2. The similarities between the 3′ noncoding regions of these two clones were about 50%. Thus, when the *Pst*I fragment in the 3′ noncoding region of Dah1 and the *Sph*I-*Eco*RI fragment of Dah2 were used as probes, cross-hybridization between these two cDNAs was not observed under the conditions employed in this study (data not shown).

When the 3′ noncoding region of Dah1 was used as a probe, no clear band was detected in any tissue from untreated dogs, whereas a hybridizable band near 23 S was detected in liver, kidney, and lung when we analyzed RNA from PCB-treated dogs (Fig. 8a). Remarkable induction was noted in the kidney and lung. When analyzed with the 3′ noncoding region of Dah2 as a probe, it was also apparent that mRNA for P-450(Dah2) was induced by PCB pretreatment in liver, kidney, and lung (Fig. 8b). Although not very clear in the autoradiogram; RNA of the liver and kidney from PCB-treated dogs gave two bands (18 S major band and 23 S minor band), whereas RNA of the lung gave only one band near 23 S. These two species of mRNA are indicated by *arrowheads* in Fig. 8b. Furthermore, among the tissues of untreated dogs, a weak band near 18 S could be detected only in the liver when the time of exposure was extended (data not shown).

Discussion

We were capable of isolating DM1-1 from a cDNA library of livers from PCB-treated dog using rat pcP-450(M-1)-4 (11), which belonged to the P450IIC gene family, as a probe. DM1-1 encoded a polypeptide of 457 amino acid residues and was judged not to be a full length cDNA. The nucleotide and the deduced amino acid sequences of DM1-1 had the greatest similarity with MP-8, which coded for a form of cytochrome P-450 ($P\text{-}450_{TB}$) in human livers (20), supporting the conclusion that P-450(DM1-1) belonged to the P450IIC subfamily. In general, the P450IIC subfamily contains constitutive forms of cytochrome P-450, such as rat P-450(M-1) and rabbit LM3b (1). Most of them have been proven to catalyze the hydroxylation of testosterone and progesterone. In accordance with this idea, the purified preparation of P-450-D1 showed testosterone hydroxylase activities (4).

As shown in Fig. 3, we found the deletion of six amino acid residues upon comparison with corresponding forms of cyto-

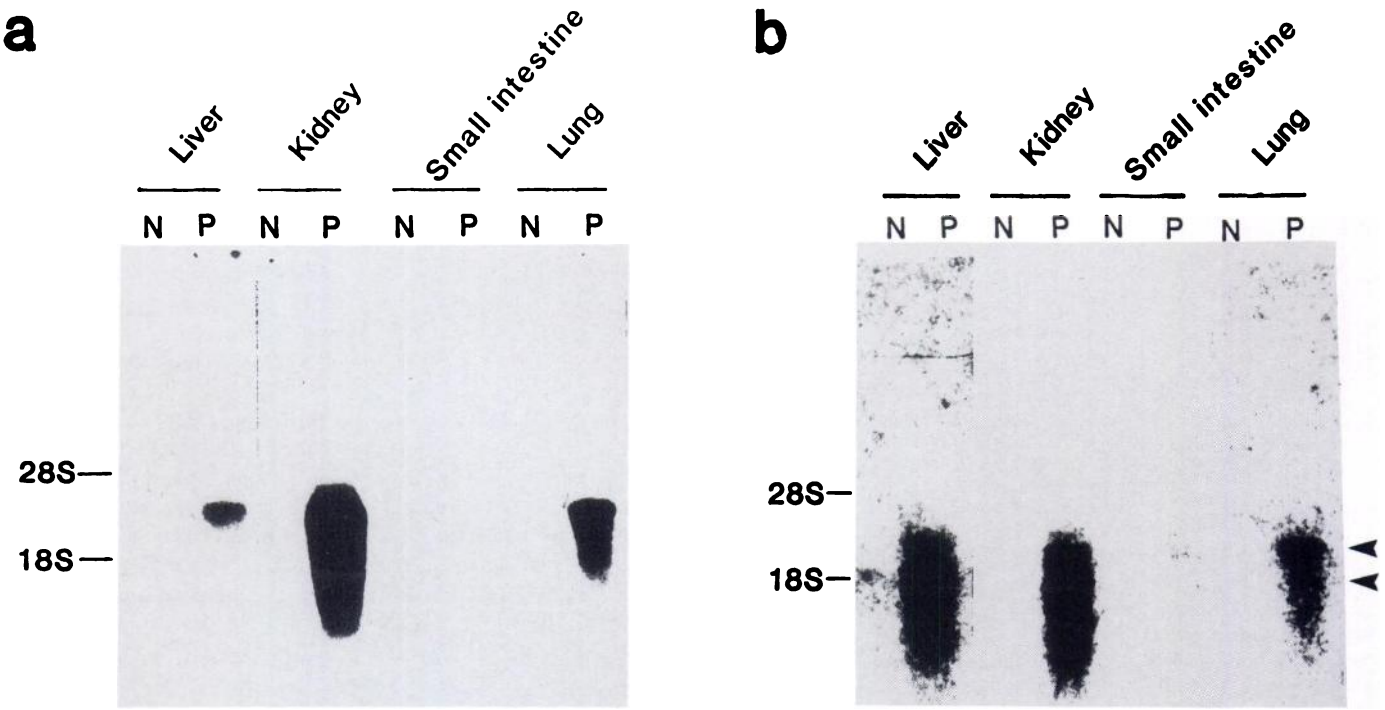

Fig. 8. Northern blot analysis of RNA for P-450(Dah1) and P-450(Dah2) in various tissues from untreated or PCB-treated dogs. *N* and *P*, total RNA (3 μg) from various tissues from untreated and PCB-treated dogs, respectively. Ribosomal RNA (18 and 28 S) was used as markers. Probes used are the *Pst*I fragment (534 bp) in the 3′ noncoding region of Dah1 (a) and the *Sph*I-*Eco*RI fragment (105 bp) of Dah2 (b). *Arrowheads*, two species of mRNA for P-450(Dah2).

chrome P-450 from other animal species. The C-terminal amino acid sequence of P-450(DM1-1) was also 10 residues shorter than that of rat P-450(M-1) (data not shown). These findings are consistent with the fact that P-450-D1 is about 3000 Da smaller than P-450-male (P-450h) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4).

Both P-450-D1 and P-450-male catalyze the hydroxylation of testosterone at the 16α-position, but P-450-D1 did not hydroxylate at the 2α-position (4). Such a great difference in a catalytic property may be caused by minor structural differences, as mentioned above. Supporting this idea, it has been observed that there were large species differences in the testosterone hydroxylation by liver microsomes (29), although it is known that the P450IIC subfamily contains multiple genes resembling each other. Regarding the testosterone 2α-hydroxylase activity, it is also possible that another form of cytochrome P-450 capable of hydroxylating testosterone at the 2α-position is present in dog livers. Although P-450(DM1-1) and P-450-D1 are assumed to be closely related forms of cytochrome P-450 in beagle dog liver, it is necessary to elucidate whether DM1-1 encodes P-450-D1 by isolating the full length cDNA for P-450(DM1-1) and comparing the deduced amino acid sequence with that of P-450-D1.

Dah1 and Dah2 were also isolated from the same cDNA library, using rabbit P450IA1 cDNA (pHPah1) as a probe. These cDNAs encoded polypeptides of 524 and 503 amino acid residues and were judged to belong to the P450IA1 and P450IA2 subfamily, respectively. Dah1 contained the entire coding region and its 3′ end had a poly(A) segment, although there was no apparent poly(A) additional signal in the 3′ noncoding region. The N-terminal sequence of P-450(Dah1) was not found in the forms of cytochrome P-450 purified from dogs so far. There were three possible initiation codons (ATG) in its sequence. Further studies are needed to decide the precise initiation codon.

In general, the P450IA gene subfamily contains only two genes, namely, P450IA1 and P450IA2. We purified two distinct forms of cytochrome P-450 (P-450-D2 and P-450-D3) from liver microsomes of PCB-treated dogs (4, 5) and obtained a cDNA clone (Dah1) in this study, all of which apparently belong to the P450IA subfamily. This line of evidence indicates that the P450IA subfamily in dogs contains at least three different genes. Further efforts may be needed to confirm this idea by isolating another cDNA coding for P-450-D3 or purifying P-450(Dah1) from livers of PCB-treated dogs. Current experiments in this laboratory have succeeded in expressing the P-450(Dah1) in yeast transformed with a recombinant plasmid constructed with an expression vector and the cDNA. The results of these experiments will be reported elsewhere.

In Northern blot analysis using the 3′ noncoding region of Dah2 as a probe, we found a minor band (23 S) and a major band (18 S) in the liver and kidney from PCB-treated dogs. The existence of these two species of mRNA may be explained by the different length of the 3′ noncoding region, as recently reported (30). The mRNAs for both P-450(Dah1) and P-450(Dah2) were expressed in greater amounts in the kidney than in the liver from PCB-treated dogs, although the reason is not clear at present.

Acknowledgments

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