ISOLATION OF A NEW CANINE CYTOCHROME P450 cDNA FROM THE CYTOCHROME
P450 2C SUBFAMILY (CYP2C41) AND EVIDENCE FOR POLYMORPHIC DIFFERENCES
IN ITS EXPRESSION

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ABSTRACT:

Two members of the canine cytochrome P4502C subfamily [CYP2C21 and CYP2C41 (sequence has been submitted to Genbank with accession number AF016248)] were cloned from three beagle liver cDNA libraries. The two canine CYP2C cDNAs exhibited 70% nucleotide and amino acid identity as well as 74–83% nucleotide and 67–76% amino acid identity with the human CYP2Cs. Canine CYP2C41 is more homologous to the human CYP2Cs than CYP2C21. The two canine CYP2C cDNAs exhibited a slightly lower nucleotide and amino acid identity (66–77%) with the rat P450Cyps, 2C11 and 2C12. Reverse transcription-polymerase chain reaction-based restriction enzyme tests for CYP2C21 and 2C41 mRNAs as well as polymerase chain reaction-based tests for genomic DNA were developed. CYP2C21 cDNA was present in the livers of all dogs tested (N = 9), but CYP2C41 was present in only 1 of the 9 (11%). Genomic tests found that the gene coding for CYP2C21 was also present in all dogs tested (N = 25), of which 15 were beagles and 10 mixed breeds. In contrast, the gene coding for CYP2C41 was present in only 16% (4 out of 25) of the dogs. An even distribution of the CYP2C41 gene was found between the sexes and between beagles and mixed breeds. This unique polymorphism in the canine CYP2C subfamily may be a source of variability in the metabolic clearance in dogs of xenobiotics that are metabolized by the cytochrome P450 2C subfamily of enzymes.

The important role cytochrome P450 (P4501) plays in the oxidative metabolism of xenobiotics as well as many endogenous substances such as steroids and fatty acids has long been recognized (Guengerich, 1990). Multiple P450 isozymes have been purified from various species. Of the most commonly used laboratory animals, rat, mouse, and rabbit P450s have been the most intensively studied. The development of molecular biology techniques has led to the identification of rare P450s that would be difficult to isolate using standard protein purification techniques and has allowed greater insights into genetic heterogeneity and polymorphisms of P450 expression.

Despite the use of the dog in safety evaluation and efficacy studies of new drugs, knowledge concerning the canine P450 system is limited. At present, only a small number of canine liver P450 isozymes have been identified and characterized. The most intensively studied of these has been CYP2B11. This P450 has been isolated from dog liver microsomes (Duignan et al., 1987), identified by cloning techniques (Graves et al., 1990), and expressed in cDNA expression systems (Kedzie et al., 1991, 1993; John et al., 1994). Other canine P450 cDNAs identified by cloning techniques include two members of the CYP1A subfamily (Fukuta et al., 1992; Uchida et al., 1990), a member of the CYP2D subfamily (CYP2D15) (Sakamoto et al., 1995), and a member of the CYP3A subfamily (Ciaccio et al., 1991).

Komori et al. (1989) purified a microsomal protein from male beagle dog liver (P-450-D1) and proposed that it belonged to the CYP2 subfamily based on similarities in N-terminal amino acid sequence and catalytic activities to rat CYP2C11. Subsequently, Uchida et al. (1990) isolated an 1875-base pair (bp) cDNA clone (DM1–1) from a cDNA library from the liver of a male beagle dog, belonging to the CYP2 subfamily (CYP2C21). However, this clone was not full length. Therefore, it was not possible to determine whether it was identical to P-450-D1 isolated by Komori et al. (1989).

At present, four genes have been identified in the human CYP2C subfamily (Goldstein and de Morais, 1994) and five genes in the rat CYP2C subfamily (Soucek and Gut, 1992). Most of these genes are constitutively expressed, and some members are polymorphic in each of these species. In the rat, several members of the CYP2C subfamily are gender specific in their expression. The specific aim of this study was to determine whether additional members of the CYP2C subfamily exist in the dog using molecular cloning techniques.

Materials and Methods

Tissue and Blood. Frozen liver tissue samples from untreated male and female beagle dogs were generously provided by M. Faletto and C. J. Serabjit-Singh at Glaxo-Wellcome (Research Triangle Park, NC) and by D. D. Christ at the DuPont-Merck Pharmaceutical Company (Wilmington, DE). Male and female canine whole blood samples from mixed and beagle breeds were obtained from the College of Veterinary Medicine, North Carolina State University (Raleigh, NC).

Materials. Restriction endonucleases were purchased from New England Biolabs. ZAP-cDNA synthesis and ZAP-cDNA Gigapack Gold cloning kits were purchased from Stratagene, QIAamp blood and tissue kits and plasmid

1 Abbreviations used are: P450, cytochrome P450; bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction.

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kits from Qiagen, and cDNA synthesis kits from Gibco-BRL. Transfer membranes and (\(^{32}\)P)dATP (6000 Ci/mmol) were from Du Pont Chemical Co., and kanamycin, ampicillin, and tetracycline were obtained from Sigma.

**RNA Isolation.** RNA was extracted from livers from nine beagle dogs (five females and four males) by the method of Chomczynski and Sacchi (1987). Five \(\mu\)g of RNA was reversed-transcribed into cDNA, using a cDNA synthesis kit (Gibco-BRL) and random hexamers or oligo(dT) (for library synthesis) as primers.

**cDNA Library Construction and Screening.** Three Uni-ZAP XR cDNA libraries were constructed using the ZAP cDNA synthesis kit from Stratagene. Three libraries were constructed from hepatic RNA from two male beagle dogs and one female beagle. Poly(A\(^\ast\)) RNA was prepared using oligo(dT)-cellulose columns (Gibco-BRL) and reverse-transcribed using oligo(dT) as a primer. The cDNA was sized by fractionation on a Sephacryl S-500 spin column, ligated to Uni-ZAP XR arms, and packaged into *Escherichia coli* XL1-Blue MRF\(^\ast\) cells using a Gigapack Gold III packaging kit as described by the manufacturer (Stratagene).

Approximately \(5 \times 10^9\) recombinant phage plaques from each library were screened on nylon filters by plaque hybridization. The probe used to screen the cDNA libraries was a 275-bp hepatic canine cDNA PCR product based on the sequence data of canine CYP2C21 (DM1–1) cDNA (Uchida et al., 1990). The forward and reverse primers were 5'-CTGTTGCTCCTGCAATGTG-3' and 5'-AGTCCCGAGGGTTACTAAG-3', respectively. The PCR reaction involved an initial denaturing step of 94°C for 2 min, denaturation at 94°C for 20 sec, annealing at 55°C for 10 sec, and extension at 72°C for 20 sec for 38 cycles. The amplified 275-bp fragment was purified on a 2% agarose gel and extracted using a MicroPure 0.22/Microcon 30 kit (Amicon, Beverly, MA). The probe was labeled with \(^{32}\)P using random primer labeling (Gibco-BRL). Recombinant phage were isolated by three rounds of screening and excised using ExAssist helper phage and the SOLR strain of *E. coli* (Stratagene). Phagemid cDNA was isolated using a plasmid midi kit (Qiagen) and sequenced using the cycle sequencing reaction with fluorescence-tagged dye terminators from a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). Modified T3 and T7 primers were used to obtain sequence for subsequent sequencing. Sequence comparisons were made using the University of Wisconsin GCG software package.

**Genomic DNA from Liver and Blood.** Purified genomic DNA from either liver or blood was extracted using the QIAamp tissue and blood kits, according to the manufacturer’s recommendations. Hepatic DNA was obtained from 9 beagles, and blood DNA was obtained from another 19 dogs of both sexes, 9 beagles and 10 of mixed breeds.

**Genetic Test to Discriminate between CYP2C21 and CYP2C41 cDNAs.**

The strategy is outlined in fig. A, B, and C. Two specific primers were used to amplify exons 4 through 8 of both CYP2C21 and CYP2C41. The exon nomenclature is based on the human 2C genes (de Morais et al., 1993). The forward and reverse primers were 5'-CTGTTGCTCCTGCAATGTG-3' and 5'-CCATGAGTACACTTCTC-3', respectively. The underlined C in the forward primer represents a mismatch with the CYP2C41 sequence. The PCR reaction involved an initial denaturing step of 94°C for 1 min, followed by 38 cycles of denaturing at 94°C for 20 sec, annealing at 55°C for 10 sec, and extension at 72°C for 30 sec with a final extension of 5 min at 72°C. The 767-bp PCR product of this amplification was digested overnight at 37°C in separate reactions with \(Nde\)I, which digests the CYP2C21 fragment into two smaller fragments of 359 and 408 bp but does not digest CYP2C41, and \(Bbs\)I, which digests the fragment from CYP2C21 into two smaller fragments of 654 and 113 bp but does not digest CYP2C21. The reactions were electrophoresed on a 3% agarose gel.

**Genetic Tests to Discriminate between CYP2C21 and CYP2C41 Genomic DNAs.** Figure 2, A and B, outline the strategy used for this genetic test. Two specific primers were used to amplify a 124-bp product from exon 7 of both the CYP2C21 and CYP2C41 gene. The forward and reverse primers were 5'-TGAAGAGCTACCAGGAGATT-3' and 5'-ACAAAGGTCATTGATGC-3', respectively. The underlined nucleotides do not match the CYP2C21 sequence. The PCR reaction involved an initial denaturing step of 94°C for 2 min, followed by 39 cycles of denaturing at 94°C for 20 sec, annealing at 53°C for 10 sec, and extension at 72°C for 10 sec with a final extension of 5 min. The 124-bp PCR fragment from the amplification of exon 7 is digested overnight at 37°C in separate reactions with \(Nla\)IV, which digests the fragment from CYP2C21 gene into two smaller fragments of 45 and 79 bp but does not digest CYP2C41, and \(Aci\)I, which digests the fragment from CYP2C41 into fragments of 87 and 37 bp but does not digest that from CYP2C21. The reactions were electrophoresed on a 4% agarose gel.

An allele-specific PCR test was also developed to amplify a 148- and 147-bp product from exon 4 of CYP2C21 and CYP2C41 genes, respectively. Two pairs of allele-specific forward and reverse primers were used for each of the two canine P450s. The forward and reverse primers for exon 4 of CYP2C21 were 5'-CCGTGCATCTACATTTCCTC-3' and 5'-CAGGAGGTGCTTGAATAGA-3', respectively. For CYP2C41, the forward and reverse primers for exon 4 were 5'-CATGATGATCCACTTTTGTCTT-3' and 5'-ATGGGAGCCTCAAAATCCTG-3', respectively. The PCR reaction involved an initial denaturing step of 94°C for 1 min, followed by 38 cycles of
denaturing at 94°C for 20 sec, annealing at 55°C for 10 sec, and extension at 72°C for 30 sec. A 148-bp PCR product was expected in dogs having the CYP2C21(CM1–1) clone reported by Uchida et al. (1990). Based on a comparison with human and rat CYP2C cDNAs, clone 5b seems to be only 8 bp short of the ATG start codon. The N-terminal sequence of our clone is identical with that of the canine protein (P-450-D1) isolated by Komori et al. (1989). Second, the cDNA isolated by Uchida et al. (1990) has a six-amino acid deletion compared with clone 5b. However, based on our sequence data for CYP2C21 and the strategy used by Uchida et al. (1990) the six-amino acid deletion in their CYP2C21 clone can be explained. The putative six-amino acid deletion is flanked on both sides by two SpH1 restrictive enzyme sites. Because Uchida et al. (1990) used a restrictive mapping strategy involving SpH1, they did not sequence across the flanking SpH1 restriction sites, resulting in an apparent but erroneous six-amino acid deletion.

The second group of CYP2C clones represented a new canine CYP2C, which has been designated CYP2C41. The longest insert (clone 11e) was 1.8 kilobase pairs long and had a complete open reading frame, encoding a 489-amino acid polypeptide. In contrast to CYP2C21, the CYP2C41 clones were found only in the female beagle liver cDNA library, where it accounted for 8 of 10 clones. Figure 3B shows the nucleotide and the deduced amino acid sequences of CYP2C41. Compared with rat and human CYP2Cs as well as canine CYP2C21, CYP2C41 has an amino acid deletion in exon 1 (indicated by an arrow in fig. 3B). The significance of this is not known at this time, but this three-base deletion was seen in all the CYP2C41 clones, whose insert encompassed this coding region.

Comparisons of nucleotide and amino acid identity of canine CYP2Cs with human CYP2Cs and male-specific CYP2C11 and female-specific CYP2C12 from rats are summarized in table 1. The nucleotide and amino acid identity between the two canine CYP2Cs was 70%. The two canine CYP2Cs exhibited a higher amino acid homology with the human CYP2Cs (66.6 to 75.5%) than to those of the rat (61.7 to 70.2%). Canine CYP2C41 was more homologous to the human CYP2Cs than CYP2C21.

Genetic Tests. CYP2C41 was found only in the cDNA library from the one female beagle liver, not in the libraries from the two male beagle livers, suggesting that CYP2C41 could be either sex-specific or polymorphic in its expression. An RT-PCR-based restriction enzyme test was developed to discriminate between CYP2C21 and CYP2C41 mRNAs, and a second PCR test was developed to discriminate between the two canine CYP2Cs at the genomic level. Exons 4 to 8 of the two CYP2C cDNAs are amplified using common primers. The PCR products are digested first with NdeI, which digests CYP2C21 to fragments of 359 and 408 but does not digest CYP2C41. The PCR products are also digested separately with BbsI, which digests CYP2C41 but not CYP2C21 (fig. 1, A and B). Figure 1C shows the results of this cDNA genetic test from the livers of the three beagles used to construct the cDNA libraries. CYP2C41 was found only in dog no. 11, the female dog liver used to construct the cDNA library, although all three dogs contained CYP2C21. cDNA prepared from livers of six additional beagles (four females and two males) were tested and found to contain CYP2C21 but not CYP2C41 (data not shown), suggesting that the difference in the expression of CYP2C41 was not sex-dependent but represents a polymorphism.

To determine whether both genes are present in all dogs or whether CYP2C41 is present in certain dogs, a PCR-restrictive enzyme test was designed to discriminate the presence or absence of the two genes in the genomic DNA (fig. 2, A and B). Figure 2C shows the results from this genomic test from blood DNA of 19 dogs of both sexes. Ten of these dogs were of mixed breeds (A–J) and 9 were beagles (K–S). Also shown in fig. 2C are the results of this genomic test using liver

**Results and Discussion**

**Canine cDNA Libraries.** Using the 275-bp canine CYP2C21 cDNA PCR product as a probe, 24 clones were isolated from three cDNA libraries. These could be divided into two members of the CYP2C subfamily. The first was similar to CYP2C21 (DM1–1) reported by Uchida et al. (1990). This CYP2C was present in all three cDNA libraries. The longest insert (clone 5b) was 1.9 kilobase pairs long and encoded a polypeptide of 487 amino acid residues.

Comparison of the sequence data of this CYP2C21 clone (fig. 3A) to the CYP2C21(DM1–1) clone reported by Uchida et al. (1990) necessitates two modifications of the original sequence. First, our clone 5b is 72 bp longer at the 5' end than the cDNA isolated by Uchida et al. (1990). Based on a comparison with human and rat CYP2C cDNAs, clone 5b seems to be only 8 bp short of the ATG start codon. The N-terminal sequence of our clone is identical with that of the canine protein (P-450-D1) isolated by Komori et al. (1989). Second, the cDNA isolated by Uchida et al. (1990) has a six-amino acid deletion compared with clone 5b. However, based on our sequence data for CYP2C21 and the strategy used by Uchida et al. (1990) the six-amino acid deletion in their CYP2C21 clone can be explained. The putative six-amino acid deletion is flanked on both sides by two SpH1 restrictive enzyme sites. Because Uchida et al. (1990) used a restrictive mapping strategy involving SpH1, they did not sequence across the flanking SpH1 restriction sites, resulting in an apparent but erroneous six-amino acid deletion.

**Fig. 2.** (A) Strategy used to genotype canine CYP2C21 and 2C41 genomic DNA utilizing PCR amplification of exon 7 followed by NlaIV digestion for CYP2C21 and by AciI for CYP2C41. (B) The predicted sizes of the digested DNA fragments for the various genotypes are shown. (C) Ethidium bromide-stained agarose gel showing PCR-restriction enzyme fragmentation patterns from the DNA from dogs A–S and by AciI for CYP2C41. (**C**) Strategy used to genotype canine CYP2C21 and 2C41 genomic DNA (**C**) Utilizing PCR amplification of exon 7 followed by NlaIV digestion for CYP2C21 and by AciI for CYP2C41. (B) The predicted sizes of the digested DNA fragments for the various genotypes are shown. (C) Ethidium bromide-stained agarose gel showing PCR-restriction enzyme fragmentation patterns from the DNA from dogs A–S
DNA of six additional beagles of both sexes. It should be noted that dog no. 11, which represents the female beagle from which the CYP2C41 cDNA was isolated, was also positive for CYP2C41 in this genomic test, supporting the validity of the test. In addition to this female beagle, three other dogs exhibited the presence of the CYP2C41 gene, dogs G, I, and K. Dogs G and I were male dogs of mixed breeds, whereas dog K was a female beagle. As seen in the results of the cDNA genetic test, CYP2C21 was present in all 25 dogs (fig. 2C).

The allele-specific PCR test for CYP2C21 and 2C41 gave similar results. Only in the DNA from the four dogs positive for CYP2C41, exon 7 also gave a 147-bp product for exon 4 of CYP2C41, whereas

**Fig. 3.** (A) Nucleotide and deduced amino acid sequence of CYP2C21. The amino acid sequence is shown in the top line. The nucleotides underlined were not reported by Uchida et al. (1990). (B) Nucleotide and deduced amino acid sequence of CYP2C41. The heme binding region is underlined, and the cysteine residue that acts as the fifth ligand to heme iron is boxed. An arrow marks a site where CYP2C41 lacks a single amino acid compared with the human CYP2C21.
all the dogs were positive for CYP2C21 (data not shown). Thus, a 100% correlation was found between the two genomic tests. A summary of the incidence of CYP2C21 and CYP2C41 in the populations of dogs that were screened is shown in table 2. In 28 dogs, 14% (4/28) contained the CYP2C41 gene. Although this is a small population, it seems that the presence of CYP2C41 is independent of the breed (two beagles, two mixed breeds) of dog or gender (two males, two females). CYP2C21, on the other hand, was present in all 28 dogs. The expression of CYP2C41 represents the first report of a polymorphic difference in the P450 system in the dog. These results suggest that the possible mechanism for this polymorphism could be due to either a gene duplication or a gene deletion. Although this type of polymorphism is unusual, a polymorphism due to a partial gene deletion occurs in human CYP2D6 (Katoh et al., 1995; Cherevix-Trench et al., 1995). In the human glutathione S-transferase superfamily, a gene deletion seems to be the major mechanism responsible for two polymorphisms in the mu and theta families (Skoda et al., 1988; Gaedigk et al., 1991).

The substrate specificity of the two canine CYP2Cs is not known, but the polymorphism in CYP2C41 could influence the metabolism of certain drugs in this species. Studies are currently underway to heterologously express these canine CYP2Cs to address this question.

Acknowledgments. We are grateful to M. Faletto and C. J. Sera.
TABLE 1
Comparison of CYP2C21 and CYP2C41 with other CYP2Cs

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>2C21</th>
<th>2C41</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleic Acid</td>
<td>Amino Acid</td>
<td>Nucleic Acid</td>
</tr>
<tr>
<td>Canine</td>
<td>2C21&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>100.0</td>
</tr>
<tr>
<td></td>
<td>2C41</td>
<td>70.2</td>
<td>69.8</td>
</tr>
<tr>
<td>Human</td>
<td>2C8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.9</td>
<td>66.6</td>
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<td>2C9&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>69.1</td>
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<td></td>
<td>2C18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75.3</td>
<td>69.4</td>
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<tr>
<td></td>
<td>2C19&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>70.1</td>
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<tr>
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<td>2C11&lt;sup&gt;d&lt;/sup&gt;</td>
<td>71.8</td>
<td>65.6</td>
</tr>
<tr>
<td></td>
<td>2C12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67.7</td>
<td>61.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent identity in coding region.
<sup>b</sup> Uchida et al. (1990).
<sup>c</sup> Ged et al. (1987).
<sup>d</sup> Umbenhauer et al. (1987).
<sup>e</sup> Romkes et al. (1991).
<sup>f</sup> Yoshikata et al. (1997).
<sup>g</sup> Zaphiropoulos et al. (1998).

TABLE 2
Incidence of canine CYP2Cs

<table>
<thead>
<tr>
<th>Breed</th>
<th>2C21</th>
<th>2C41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed breeds</td>
<td>10/10 (100%)</td>
<td>2/10 (20%)</td>
</tr>
<tr>
<td>Beagles</td>
<td>18/18 (100%)</td>
<td>2/18 (11%)</td>
</tr>
<tr>
<td>All dogs</td>
<td>28/28 (100%)</td>
<td>4/28 (14%)</td>
</tr>
<tr>
<td>Male</td>
<td>13/13 (100%)</td>
<td>2/13 (15%)</td>
</tr>
<tr>
<td>Female</td>
<td>15/15 (100%)</td>
<td>2/15 (13%)</td>
</tr>
</tbody>
</table>

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References


