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CHARACTERIZATION OF RABBIT CYTOCHROME P450IIC4 cDNA AND INDUCTION BY PHENOBARBITAL OF RELATED HEPATIC mRNA LEVELS

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SUMMARY: We have cloned cDNA containing a partial sequence of a rabbit cytochrome P-450 (designated cytochrome P450IIC4) cDNA that is a member of the cytochrome P450IIC subfamily. The cDNA contains 770 bp of which the first 429 code for the C-terminal 143 amino acids of cytochrome P450IIC4. The protein coding region of the cDNA is 98% homologous with that of cytochrome P450IIC5 and the 3' untranslated region is about 90% homologous. In contrast to the constitutive isozyme, cytochrome P450IIC5, mRNA in the liver that hybridized to the 3' untranslated region of cytochrome P450IIC4 cDNA was increased about 8-fold 24 hours after a single injection of phenobarbital.

Cytochromes P-450 (P450)[2] are mixed function oxidases that are responsible for the metabolism of a large number of lipophilic compounds (1). The P-450's form a multigene superfamily that can be divided into at least seven families (2). We have characterized three rabbit liver cDNA's that define subfamily C within the P450II[3] family (3). The rate of transcription (Govind, S., Bell, P. A. and Kemper, B., unpublished) and the level of mRNA (4) of P450IIC1 and P450IIC2 are increased by phenobarbital while that of P450IIC3, which is 70% homologous to the other two, is not. The cDNA of a fourth member of this subfamily, P450IIC5, has also been sequenced and is about 80% homologous to the other members (5). P450IIC5 has been isolated

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[2] Abbreviations used are: P450, cytochrome P-450; MOPS, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

[3] P450 nomenclature is as recommended recently (2). Previously used names for P450IIC1 through P450IIC5 are: P-450PBc1, P-450PBc2, P-450PBc3, P-450PBc4, and P-450(form 1), respectively.

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from liver microsomes of untreated rabbits and catalyzes the C-21 hydroxylation of progesterone (6,7). We now report the partial cDNA sequence of P450IIC4, a fifth member of this subfamily, and the induction of P450IIC4 or a closely related P-450 by phenobarbital.

MATERIALS AND METHODS

Isolation of RNA: Adult male New Zealand white rabbits (2 kg) were injected intraperitoneally with 100 mg/kg of 0.1% phenobarbital in 0.9% NaCl. Liver, kidney and lung tissues were removed 0, 6, 12, and 24 hr after phenobarbital treatment from animals that had been starved overnight. The tissue was rinsed in cold 0.9% NaCl, cut into small pieces, and stored in liquid nitrogen. RNA was extracted from the tissue by proteinase K digestion, phenol extraction and oligo(dT)cellulose chromatography as described previously (4).

Cloning and sequencing of cDNA: The P450IIC4 cDNA clone was selected from the same liver cDNA bank used to isolate P450IIC1, P450IIC2, and P450IIC3 cDNA's as described previously (3). The sequence of the cDNA insert was determined by the method of Maxam and Gilbert (8) except that 20 µl of acetylacetone was added after the first ethanol precipitations of the pyrimidine reactions (9). Both strands of the cDNA insert were sequenced.

RNA blot hybridization: Transfer of RNA to GeneScreen and hybridization to cDNA probes was carried out as described in the New England Nuclear GeneScreen Manual (hybridization method 2). For RNA dot blots, 1 mg/ml of tRNA or poly(A) containing RNA from the liver, kidney, or lung tissues of control or phenobarbital-treated rabbits was boiled for 1 min in 5 mM K^+-HEPES, pH 7.4, 1 mM EDTA, and 0.1 mM EGTA. Aliquots containing 0.5 or 1.0 µg of RNA were spotted on GeneScreen. For Northern blot analysis, 3 µg of poly(A) containing RNA from the liver were fractionated on a 1.5% agarose gel containing 6% formaldehyde (10). The RNA was heated to 60° for 15 min in 50% formamide, 6% formaldehyde, 20 mM Na^+-MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA before electrophoresis. RNA was transferred to GeneScreen by the capillary blot procedure as described by the manufacturer.

The hybridization probe was a 390 bp Eco R1-Pst I fragment of the cDNA that corresponded to the 3' untranslated region of P450IIC4 mRNA. The DNA was labeled with [^{32}P]dCTP by nick translation (11) to a specific activity of 1-4 x 10^8 cpm/µg.

RESULTS

A rabbit liver cDNA bank (3) was screened with a cloned cDNA fragment that hybridized selectively to liver RNA from phenobarbital-treated rabbits. Three near full length cDNA's for P450IIC1, P450IIC2 and P450IIC3 from this bank have been described previously (3). A fourth clone with a partial cDNA sequence distinct from the other three by restriction mapping was also isolated. The sequence of this cDNA, designated P450IIC4, is shown in figure 1A. The insert contained 770 bp with an open reading frame of 426 bp from the

A

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ACGGATGCTG TGATACATGA GGTCCAGAGA TTCATTGACC TCCTTCCCAC TAACCTGCCC CATGCAGTGA CCCGAGATGT TAGATTCAGA    90
                       A              C

AACTACTTTA TCCCTAAGGG TACAGACATC ATAACATCAC TGACATCCGT GCTACATGAT GAAAAAGCAT TTCCTAACCC AAAGGTATTT   180
                          G

GACCCTGGGC ACTTTCTGGA TGAGAGTGGC AACTTTAAGA AGAGTGACTA CTTCATGCCT TTCTCAGCAG GAAAACGGAT GTGTGTGGGA   270
       A

GAGGGCCTGG CCCGCATGGA GCTGTTTTTG TTCCTGACCT CCATTTTGCA GAACTTTAAA TCGCAATCTC TGGTTGAGCC AAAGGACCTA   360
                                                          CT                           G

GACATCACTG CAGTTGTCAA TGGATTTGTT TCTGTGCCAC CTTCGTTCCA GCTCTGCTTC ATTCCTATTT GAAAGAGAAC AAACTCTCTG   450
                                               C A

CCTCTGTGCT GTTGTCTGCA ACTCCTTCTC TTCAGAGGCA TTGTCTATCC CTTCCTCTAT CAGAGACACC CTCCATGACT TCTCTCACAT   540

CTTACTATTC CATCAGATCC ACCAGATCCA GTGAACACAC AACCACAATT AAAGGAGTTT AGTGGGATCT TATCGCAGGG ATA--TCTGC   630
                        --------G TG      T      T C     T                          A         TC

TGTTTCCTAC ATTCTGCAAC ACCTATGCTG AATTCCACAT TTGATAATTC ATATCAATGC TGCTTTACCG AGTCTTATCA CTATAAAAC-   720
                           CA     T           CC    -                        A                    C

-----AGAAA TAAATATTAA AATATGACAA TTTAGACCCA TACTTCAGTC    770
AAGAA T          A T G                          C
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B

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TDAVIHEVQR FIDLLPTNLP HAVTRDVRFR NYFIPKGTDI ITSLTSVLHD EKAFPNPKVF DPGHFLDESG NFKKSDYFMP FSAGKRMCVG    90
       I                                                                                      *

EGLARMELFL FLTSILQNFK SQSLVEPKDL DITAVVNGFV SVPPSFQLCF IPI   143
                      L                        Y
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Table I

Percent similarities of the cDNA sequences of members of the P450IIC subfamily

	C1	C2	C3	C4	C5
C1	-	72	51	62	64
C2	88	-	52	56	61
C3	74	73	-	44	53
C4	78	76	76	-	91
C5	81	79	74	98	-

Near complete sequences were used for the C1/C2/C3 (3) and C5 (5) comparisons and the 3' 770 bp were used for C4 comparisons. The lower left matrix compares the protein coding regions and the upper right matrix compares the 3' untranslated regions. Similarities, determined by the Wilbur and Lipman method (18), were calculated by dividing the number of matches by the number of bases not in gaps plus the number of gaps.

beginning of the sequence followed by a TGA (overlined) termination codon. Two potential polyadenylation signals (underlined) were present near the 3' end of the sequence but no polyadenylate region was present in the cDNA clone so it is not known whether these sequences are functional signals.

The protein sequence derived from the cDNA is shown in figure 1B. The protein coding region of P450IIC4 cDNA was about 75% similar to those of the P450IIC1, P450IIC2 and P450IIC3 cDNA's (Table 1) clearly establishing P450IIC4 as a member of the P450IIC subfamily. The 3' untranslated region as expected was less similar. P450IIC4 contains a cysteine (residue 88 in figure 1B) which corresponds to the cysteine at about residue 450 in other P450's and serves as a ligand for the heme moiety (12). The beginning of the derived

Figure 1. Comparison of P450IIC4 and P450IIC5 cDNA and protein sequences. The cDNA sequence and derived protein sequence of P450IIC4 are shown in A and B, respectively. Only differences in the P450IIC5 sequences from those of P450IIC4 are indicated below the sequences. Dashed lines indicate gaps introduced into the sequences to maximize similarity by the Wilbur and Lipman method (18). In panel A, the TGA termination codon is overlined and two potential polyadenylation signals are underlined. In panel B, ten of the amino acids that are part of the "analogous peptide" (13) are overlined. An asterisk is above the cysteine residue thought to be a ligand for the heme moiety (12).

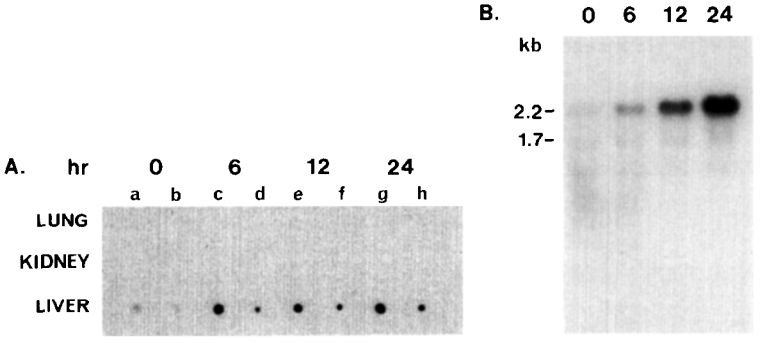

Figure 2. Phenobarbital induction of mRNA hybridizing to P450IIC4 cDNA. Rabbits were treated with phenobarbital for 0, 6, 12, or 24 hr. The hybridization probe was a DNA fragment from the 3' untranslated region of the cDNA. In panel A, RNA was analyzed by dot blot hybridization. Either 1.0 µg (a,c,e,g) or 0.5 µg (b,d,f,h) of poly(A) containing RNA from rabbit lung, kidney or liver tissue was spotted on GeneScreen. In panel B, 3 µg of liver poly(A) containing mRNA was fractionated and analyzed by Northern blotting as described in methods. RNA standards, derived from SP6 RNA polymerase transcripts, were run in parallel to estimate the sizes of the two RNA bands indicated in kb on the right side of the autoradiogram.

sequence corresponds to part of the "analogous peptide" region (13) which is conserved in most P450's. Nine of the ten amino acids in this region (overlined) are identical with those in rat P450IIB1 (P-450b) (14).

P450IIC4 was very closely related to another member of this subfamily, P450IIC5 (5). In the coding region 98% similarity was present in the two sequences, and high homology of 91% was retained in the 3' untranslated region (Table 1). Differences between the sequences of P450IIC4 and P450IIC5 are shown in figure 1. In the protein coding region only 8 differences in nucleotide sequence were observed which led to 3 changes in the 143 derived amino acids. P450IIC5 is a constitutive enzyme that catalyzes the 21-hydroxylation of progesterone in the liver (6).

To examine whether phenobarbital increases the levels of P450IIC4 mRNA, RNA was isolated from liver, kidney and lung tissues of rabbits treated with phenobarbital. The hybridization probe contained only sequence from the 3' untranslated region so that cross hybridization with P450IIC1, P450IIC2 and P450IIC3 mRNA's would not occur. RNA that hybridized to the P450IIC4 probe was not detected in lung and kidney (Fig. 2A). P450IIC4 RNA was present at low levels in the livers of untreated animals and phenobarbital increased the

level of RNA as analyzed by either dot blot or Northern blot hybridization (Fig. 2). In three RNA dot blot experiments, the fold increases over untreated animals and standard errors were 4.9 ± 0.6, 4.5 ± 0.5 and 7.5 ± 0.9 for 6, 12, and 24 hr after phenobarbital treatment, respectively. In the Northern analysis of RNA from untreated animals two bands of about equal intensity were observed that corresponded to RNA species of 1.7 and 2.2 kb. Treatment with phenobarbital increased the 2.2 kb RNA several fold, but did not greatly affect the smaller form. The 1.7 kb species may correspond to P450IIC5 mRNA which should cross-hybridize to the P450IIC4 probe and has been reported to contain 1.9 kb (5). In contrast to these results only a single RNA species was observed that hybridized with the 3' untranslated region of P450IIC5 (5) which should have detected the same RNA species that the P450IIC4 probe did, since these 2 sequences are 90% homologous. The reason for this discrepancy is not known. The single phenobarbital-responsive RNA species clearly distinguishes this mRNA from those of the other phenobarbital-inducible forms, P450IIC1 and P450IIC2, which have multiple RNA species (4).

DISCUSSION

P450IIC4 is the fifth member of the P450IIC subfamily to be described. P450IIC4 is 98% homologous in the 3' protein coding region of the cDNA to P450IIC5, which raises the possiblity that these two P450's are allelic products of the same gene. However, the lesser homology in the 3' untranslated region indicates that two separate genes code for these two proteins. P450IIC5 has been isolated from the microsomes of untreated animals and is apparently not induced by phenobarbital (6). In contrast, levels of mRNA hybridizing to the P450IIC4 cDNA were increased 7 to 8 fold. It is possible that the phenobarbital-inducible species is not P450IIC4 mRNA since a 3' untranslated region probe for P450IIC5 hybridized with up to 4 restriction fragments in a genomic DNA Southern analysis (5). This result suggests that up to 4 genes very closely related to the P450IIC5 and P450IIC4 genes may be present in the rabbit genome. In spite of this very high homology in the

coding region, at least one of these proteins is regulated quite differently by phenobarbital than P450IIC5 is.

There are several examples of differential regulation of P450 genes within a subfamily. Both constitutive and inducible genes have been reported in the P450IIB subfamily (15). In the P450IIC subfamily, P450IIC3 is constitutive while P450IIC1 and P450IIC2 are inducible (4). In the cases of the P450IIC members, homology throughout the length of the cDNA indicates that the change in response is related to changes in the regulatory region of the gene. The P450IIC4-like isozyme and P450IIC5 represent the most closely related P-450 genes yet described that respond differently to phenobarbital. The close homology suggests that they are the result of a very recent gene duplication and that either the phenobarbital responsive element was not duplicated in the P450IIC5 gene or that mutations have inactivated this region. A duplication without the regulatory region would be possible if the phenobarbital responsive element is far upstream of the promoter as the TCDD responsive element is in the P450I genes (16,17). It is also possible that the gene duplication that led to P450IIC4 and P450IIC5 occurred much earlier that the present homology indicates, and that the portions of two genes including the C-terminal and 3' untranslated regions underwent a recent gene conversion event. To definitely establish which of these possibilities is correct will require the characterization of the structure and regulation of these genes.

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