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Human Cytochrome P-450 PB-1: A Multigene Family Involved in Mephenytoin and Steroid Oxidations That Maps to Chromosome 10

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Summary

The cytochrome P-450 monooxygenase system possesses catalytic activity toward many exogenous compounds (e.g., drugs, insecticides, and polycyclic aromatic hydrocarbons) and endogenous compounds (e.g., steroids, fatty acids, and prostaglandins). Multiple forms of cytochrome P-450 with different substrate specificities have been isolated. In the present paper we report the isolation and sequence of a cDNA clone for the human hepatic cytochrome P-450 responsible for mephenytoin (an anticonvulsant) oxidation. The mephenytoin cytochrome P-450 is analogous to the rat cytochrome P-450 form termed PB-1 (family *P450C2C*). We also report that human PB-1 is encoded by one of a small family of related genes all of which map to human chromosome 10q24.1-10q24.3. The endogenous role of this enzyme appears to be in steroid oxidations. This cytochrome P-450 family does not correspond to any of the hepatic cytochrome P-450 gene families previously mapped in humans.

Introduction

We live in a chemically challenging environment. A major component of an organism's defense against chemical adversity is the "super family" of heme-containing proteins termed cytochrome P-450s. These enzymes are monooxygenases possessing catalytic activity toward a wide variety of foreign compounds, converting them to products that are more readily excreted from the body (Lu and West 1980; Nebert and Negishi 1982). In addition to this function, these proteins also have an endogenous role in the biosynthesis and/or degradation of steroid hormones (Jefcoate 1986; Waterman et al. 1986) and will also metabolize prostaglandins and leukotrienes, fatty acids, and vitamin D. Biochemical analysis and the application of recombinant-DNA methodology have identified multiple forms of cytochrome P-450 encoded by distinct multigene families (Adesnik and Atchison 1986; Wolf 1986). To date at least six different families involved in foreign-compound metabolism have been identified, and it is likely that several more will be found (Nebert et al. 1987).

In man there are at least two distinct pharmacogenetic responses involving the P-450 system in Caucasian populations (Mahgoub et al. 1977; Küpfer and Preisig 1984; Wolf 1986). These responses have been associated with two distinct P-450 families and are characterized by the metabolism of the marker drugs debrisoquine (an antihypertensive agent) and mephenytoin (an anticonvulsant). In Europeans a polymorphism associated with a low activity in the 4-hydroxylation of debrisoquine occurs at a frequency of 9% (Evans et al. 1980). Similarly, poor metabolizers of mephenytoin exist at a frequency of 5% (Küpfer and Preisig 1984). Both these traits are inherited as autosomal recessives (Eichelbaum 1984; Jurima et al. 1985). These metabolic

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differences assume wider clinical importance if, in addition to being associated with adverse drug side effects, they also serve as markers for altered susceptibility to environmental toxins and carcinogens. For example, the debrisoquine polymorphism has been associated with susceptibility to drug-induced Parkinson disease (Barbeau et al. 1985) and also with susceptibility to lung and liver cancer (Idle and Ritchie 1983; Ayesh et al. 1985). The use of recombinant-DNA techniques will be of central importance in categorizing individuals into different polymorphic groups with respect to oxidative drug metabolism. With this in mind, we report the isolation of a human hepatic cDNA clone that corresponds to the rat P-450 designated PB-1 (Waxman and Walsh 1983; Wolf et al. 1984, 1986). Proteins within the PB-1 family in man appear to be responsible for mephenytoin oxidation (Shimada et al. 1986) and, by inference, may be responsible for the polymorphism associated with this activity. We also report the chromosomal location of the human PB-1 gene family and its genomic complexity. (In our original paper the protein defined as PB-1 [Wolf et al. 1984] was equated with the form described as form b by Ryan et al. [1982]. However, N-terminal sequencing shows it to be equivalent to the form described by Waxman and Walsh [1983] as PB_1. Recently a new nomenclature has been proposed for the P-450 gene superfamily, and this is listed by Nebert et al. [1987]. PB-1 is a member of the gene family *P450C2C*.)

Material and Methods

Plasmids

The rat PB-1 P-450 clone pTF-1 has been described elsewhere (Freidberg et al. 1986). Inserts derived from pTF-1 were used to screen a human liver cDNA library (Woods et al. 1982) by means of the in situ colony filter-hybridization technique (Grunstein and Hogness 1975). Positive colonies were subsequently grown in bulk for isolation of plasmids (Maniatis et al. 1982).

Isolation of DNA

Mouse liver DNA and human liver DNA were isolated as described elsewhere (Hill et al. 1985). Human DNA from the lymphoblastoid cell line AKLO was a gift from Dr. D. J. Porteous. Human/rodent somatic cell–hybrid DNA and human placental DNA was isolated as described elsewhere (Lund et al. 1983; Porteous 1985).

Insert Isolation and Preparation of Radioactive Probes

The *Pst*I inserts from pTF-1 and pHL5,5 were isolated by means of agarose-gel fractionation as described elsewhere (Hill et al. 1985). The inserts from pHL5,5 were subcloned into the *Pst*I site of the pEMBL vector (Dente et al. 1983) and reisolated to prevent contamination of insert probes. For radiolabeling of inserts, the nick-translation (Rigby et al. 1977) or random-priming (Feinberg and Vogelstein 1983, 1984) methods were used.

DNA Analysis

Restriction-enzyme digestions were performed according to the manufacturers specifications, using up to a fourfold excess of enzyme. Digested DNA was fractionated electrophoretically on 0.8%–1.0% agarose gels and transferred to nitrocellulose or Hybond® (Amersham) by means of the method of Southern (1975). The probes were hybridized to filters at 68 C in a solution containing 0.7 M NaCl and 0.75 M sodium citrate (= 5 × SSC), 2 × Denhardt's (1 × Denhardt's = 0.02% BSA/0.02% Ficoll/0.02% polyvinylpyrrolidone), 10% dextran sulphate, 0.1% SDS (0.5% for Hybond®), 0.1% sodium pyrophosphate, and 100–150 μg denatured salmon-sperm DNA/ml. After hybridization overnight, the filters were washed at 68 C in a solution containing 0.01 M NaCl/0.0015 M sodium citrate, 0.1% SDS, and 0.1% sodium pyrophosphate. After drying, filters were exposed under an intensifying screen at −70 C with Kodak XAR-5 film.

RNA Analysis

RNA isolation and gel electrophoresis were as described elsewhere (Meehan et al. 1984). Hybridization conditions were the same as those used for DNA blots. RNA blots were washed in a solution of 0.3 M NaCl/0.03 M sodium citrate, 0.1% SDS, and 0.1% pyrophosphate.

In Situ Hybridization

Chromosomes were prepared from peripheral blood lymphocytes stimulated with PHA and cultured in RPMI 1640 with 15% fetal calf serum in the usual way. After 72 h the cells were synchronized by the addition of 200 μg BrdU/ml, cultured overnight, and released the following morning by replacing the BrdU with 2×10^{-5} M thymidine. Culture was continued for 5 h 15 min, colcemid was added for 40 min, and harvesting and fixation were carried out in

Table 1

Segregation of PB-1 P-450 with Human Chromosomes in Cell Hybrids

Hybrid Cell	Human Chromosomes																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
1[a]	−	+	−	(+)	(+)	−	−	+	−	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−
2[a]	−	+	+	−	+	−	−	+	−	+	−	+	−	+	−	−	−	+	−	−	+	−	−	−
3[a]	+	+	+	−	−	+	+	+	−	+	−	+	−	+	−	+	+	+	−	−	+	+	+	−
4[a]	−	−	+	−	+	−	−	−	−	+	+	+	+	+	+	−	+	+	−	+	+	+	+	−
5[a]	−	−	−	−	−	−	+	−	−	+	+	−	−	+	+	−	+	−	−	−	+	−	+	−
6[a]	−	−	+	−	−	−	−	−	−	+	+	−	+	−	+	−	+	+	−	+	+	+	−	−
7[a]	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−	−	−	−	−	−	+	+	−
8[a]	−	+	−	−	−	+	+	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	+	−
9	−	+	+	−	+	−	−	+	+	−	−	+	−	+	−	−	−	+	−	−	+	−	−	−
10	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	+	+	+	−	+	−	−
11[b]	+	−	+	−	+	+	−	−	+	−	+	+	−	+	−	+	−	−	−	−	+	−	−	−
12[b]	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
13	−	−	+	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	+	−
14	−	−	−	−	−	+	−	+	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+	−
15	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+	+	+	−	−	+	−	−	−	−
16[b]	+	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	−	+	−	−
17[b]	−	+	−	+	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	+	−
18[b]	+	−	+	−	−	+	−	−	−	−	+	−	+	−	−	−	−	−	+	−	+	+	−	+
19	+	+	+	−	+	+	+	+	−	−	−	+	−	+	−	+	+	+	−	−	−	−	+	−
20	−	+	+	−	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−
21	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+
22	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
23	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
24	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
26	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
27	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
28	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
% concordant	53	71	64	64	64	57	61	67	89	100	71	71	69	69	69	46	75	71	61	71	71	69	64	64

NOTE.—A plus sign denotes presence in >20% of the metaphases analyzed; a plus sign in parentheses denotes presence in 10% of the metaphases analyzed; and a minus sign denotes absence in the metaphases analyzed.

[a] Chromosome 10–containing hybrids.

[b] Contain unidentified human marker chromosomes and some identified translocations (see Lund et al. [1983]). However, these abnormal human chromosomes did not give rise to any aberrant segregation patterns for the PB-1 P-450 cDNA clone.

the usual way. Chromosome preparations were air-dried on acid alcohol–cleaned slides.

pHL5,5 (0.5 μg) was labeled with ^{3}H-dATP, dCTP, and dTTP (Amersham) by means of random oligonucleotide–primed synthesis (Feinberg and Vogelstein 1983, 1984) to a specific activity of ~10^7 dpm/μg. To eliminate hybridization of the repeat sequence, the labeled probe was denatured and annealed with 200 μg of total human DNA in 5 × SSC in a volume of 80 μl for 30 min at 70 C. The remainder of the hybridization mix (tRNA, formamide, dextran sulphate) was added as described elsewhere (Gosden et al. 1986) to make final concentrations of 50% formamide, 10% dextran sulfate 2 × SSC, 400 μg tRNA/ml. The chromosomes were treated with RNAse A, and the chromosomal DNA was denatured in 70% formamide, 0.6 × SSC at 70 C, and the probe was denatured at 70 C for 5 min and applied to the slides as described elsewhere (Gosden et al. 1986). The slides were incubated at 37 C overnight and washed in 50% formamide, 1 × SSC at 40 C (4 × 5 min) and in 1 × SSC at 40 C (4 × 4 min) before being dehydrated through an alcohol series and dipped in Ilford L4 liquid nuclear emulsion. Slides were exposed at 4 C for 7 days, developed and fixed as described above, and thoroughly dried. They were then stained in Hoescht 33258 (50 μg/ml in 2 × SSC) for 20 min and were flooded with 2 × SSC in a petri dish and exposed to ultraviolet light for 17 min, before being rinsed thoroughly in 2 × SSC and

stained in Wright's stain for 8 min. This procedure produced clear G-bands, enabling simultaneous analysis of karyotype and grain distribution.

DNA Subcloning and Sequencing

The three *Pst*I fragments from pHL5,5 were subcloned into M13mp18 (BRL) (Messing 1983) and propagated in *Escherichia coli* JM101. DNA sequencing was performed with a BRL ^{35}S sequencing kit according to the manufacturer's protocol. After a sequence of 250–300 nucleotides had been determined, sequencing proceeded by using a synthetic 18mer primer made with an Applied Biosystems DNA synthesizer. Overlapping and complementary sequences were read for verification and tested by means of restriction mapping of pHL5,5. Each sequence was determined a minimum of five times.

Cells

The following human/rodent somatic cell–hybrid cell lines have been reported by our colleagues (Lund et al. 1983), the numbers in parentheses referring to their position in table 1: SK81.Wg3H.L2.15.3.2 (1), Cl21 (22), CTP41.7 (14), CTP41.3 (15), H22.6.9 (16), Horp25.14 (17), ADP3.10.7 (18), PGME25.8 (20), THYB1.33 (24), ThyB1.33.6 (25), SK81-RagG5 (12), and ALR.1B5.5AG.58A (11). The following somatic-cell hybrids are from our laboratory: SK81.Wg3H.L12.15.3 (2), SK81Wg3H.L2.38.2 (9), H22.6.13 (10), Horp25.4 (26), ADP3.10.8 (21), PGME25.9 (27), and X63.BTS4.F9.2 (3). The somatic-cell hybrids IWILA4.9 (28) (Nabholz et al. 1969); DUR4.3 (4) and CTP41.A2 (20) (Heisterkamp et al. 1982); 3W4C15 (5), DT1.2R (6), and CTP34B4 (19) (Hobart et al. 1981); and Horp9.5 (7) (van Heyningen et al. 1975) have been described elsewhere. SIF4A31 (13) and SIF-15 (8) were gifts from Dr. S. Povey.

The characterization of the hybrids was accomplished by means of established methods (Lund et al. 1983).

Results

Identification of Human PB-1 P-450 cDNA

cDNA inserts from the rodent PB-1 clone pTF-1 (Freidberg et al. 1986) were used to screen a human liver cDNA library constructed in pKT218 (Woods et al. 1982). Approximately 10,000–20,000 colonies were screened, and two colonies were identified that hybridized to the pTF-1 insert at high stringency in

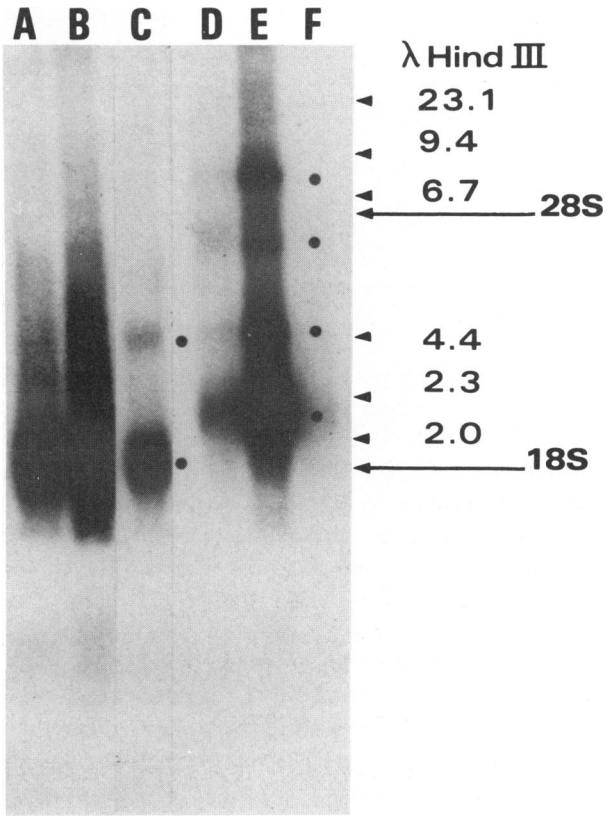

Figure 1 Northern blot analysis of human and rodent liver RNA samples with the PB-1 cDNA clone pHL5,5. Lane A, rat liver control poly-A$^+$ (1 μg); lane B, PB-induced rat liver poly-A$^+$ (1 μg); lane C, C57 BL/6 mouse liver control total RNA (10 μg); lane D, normal human male liver total RNA (10 μg); lane E, normal human male liver poly-A$^+$ (2.5 μg); lane F, a black dot (•) indicates multiple transcripts in human tracks. Size markers were denatured λ*Hin*dIII and 28S and 18S ribosomal RNA markers. All these samples were run on the same gel, but superfluous tracks were removed.

colony filter-hybridization assays. On the basis of their mobility on agarose gel, these clones were subsequently found to contain inserts of ~2,000 bp (pHL5,5) and 700 bp (pHL1,5). The larger plasmid (pHL5,5) was used for further analysis. *Pst*I digestion of pHL5,5 released three fragments of ~900, ~700, and ~400 bp, indicating the presence of two internal *Pst*I sites in the pHL5,5 insert. To characterize pHL5,5 further, Northern blots of rodent and human liver mRNAs were performed (fig. 1). It has been demonstrated elsewhere (Wolf et al. 1984, 1987; Freidberg et al. 1986) that PB-1 P-450 protein and mRNA have a high constitutive level in rodent liver and can be marginally induced by administration of phenobarbital. The pattern of expression in figure 1

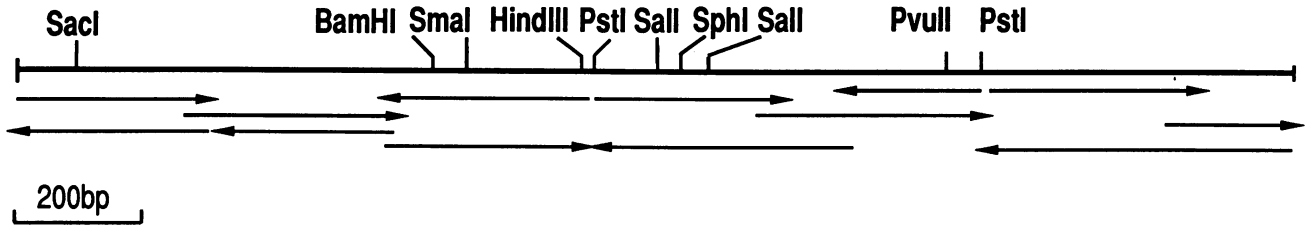

Figure 2 Restriction map and sequence strategy of the cDNA insert from pHL5,5 corresponding to human PB-1 P-450. The *PstI* fragments were subcloned into M13 mp18 and sequenced by means of the dideoxy method. The arrows indicate the segments and direction of sequences read from synthetic probes (see Material and Methods).

is observed when pHL5,5 is used to probe treated and untreated rodent liver RNAs, in which a major transcript is observed at approximately 18S in untreated animals (figs. 1*A*, 1C), a transcript that is increased twofold in the phenobarbital-treated animals (fig. 1*B*). The human liver sample also has a high constitutive level of expression, but the major transcript observed is larger than the rodent transcript and in fact appears to almost correspond in size to the cDNA insert seen in pHL5,5 (figs. 1*D*, 1*E*). Interestingly, minor transcripts that are larger than the major form can be seen in the rodent and human RNA samples. These transcripts may correspond to processing intermediates or to alternative transcripts (see Discussion below). The complete cDNA sequence of pHL5,5 is presented in figure 2. On the basis of this the *PstI* fragment sizes can be more accurately estimated as being 894, 640, and 430 bp, respectively (fig. 3). An open reading frame of 1,470 nucleotides was found to code for a polypeptide of 490 amino acids. The first 29 amino acids are identical to those reported for the NH_2-terminal sequence of the cytochrome P-450 that metabolizes mephenytoin. The sequence including the first ATG codon is included in the consensus sequences (A/G)NNATG(A/G) characteristic of an active start codon (Kozak 1984). There is a 3′-untranslated region of ~400 nucleotides. It is possible that there is a longer 5′ leader sequence that would account for the larger size of mRNA transcript for human PB-1 P-450; alternatively, the size difference could be due to polyA tail length. The primary coding sequence of

Figure 3 The nucleotide sequence of PB-1 P-450 (*P450C2C*) cDNA and its predicted amino acid sequence. The amino acid sequence underlined corresponds to the NH_2-terminal sequence determined for P450MP (Shimada et al. 1986). The boxed sequence corresponds to the putative heme-binding site containing Cys 435 (Black and Coon 1986). The two internal *PstI* sites are also illustrated.

```
1
GGGGGGGGGCGGCAATGGATTCTCTTGTGGTCCTTGTGCTCTGTCTCTCATGTTTGCTT
                M  D  S  L  V  V  L  V  L  C  L  S  C  L  L  15
61
CTCCTTTCACTCTGGAGACAGAGCTCTGGGAGAGGAAAACTCCCTCCTGGCCCCACTCCT
 L  L  S  L  W  R  Q  S  S  G  R  G  K  L  P  P  G  P  T  P  35
121
CTCCCAGTGATTGGAAATATCCTACAGATAGGTATTAAGGACATCAGCAAATCCTTAACC
 L  P  V  I  G  N  I  L  Q  I  G  I  K  D  I  S  K  S  L  T  55
181
AATCTCTCAAAGGTCTATGGCCCTGTGTTCACTCTGTATTTTGGCCTGAAACCCATAGTG
 N  L  S  K  V  Y  G  P  V  F  T  L  Y  F  G  L  K  P  I  V  75
241
GTGCTGCATGGATATGAAGCAGTGAAGGAAGCCCTGATTGATCTTGGAGAGGAGTTTTCT
 V  L  H  G  Y  E  A  V  K  E  A  L  I  D  L  G  E  E  F  S  95
301
GGAAGAGGCATTTTCCCACTGGCTGAAAGAGCTAACAGAGGATTTGGAATTGTTTTCAGC
 G  R  G  I  F  P  L  A  E  R  A  N  R  G  F  G  I  V  F  S  115
361
AATGGAAAGAAATGGAAGGAGATCCGGCGTTTCTCCCTCATGACGCTGCGGAATTTTGGG
 N  G  K  K  W  K  E  I  R  R  F  S  L  M  T  L  R  N  F  G  135
421
ATGGGGAAGAGGAGCATTGAGGACTGTGTTCAAGAGGAAGCCCGCTGCCTTGTGGAGGAG
 M  G  K  R  S  I  E  D  C  V  Q  E  E  A  R  C  L  V  E  E  155
481
TTGAGAAAAACCAAGGCCTCACCCTGTGATCCCACTTTCATCCTGGGCTGTGCTCCCTAC
 L  R  K  T  K  A  S  P  C  D  P  T  F  I  L  G  C  A  P  Y  175
541
AATGTGATCTGCTCCATTATTTTCCATAAACGTTTTGATTATAAAGATCAGCAATTTCTT
 N  V  I  C  S  I  I  F  H  K  R  F  D  Y  K  D  Q  Q  F  L  195
601
AACTTAATGGAAAAGTTGAATGAAAACATCAAGATTTTGAGCAGCCCCTGGATCCAGATC
 N  L  M  E  K  L  N  E  N  I  K  I  L  S  S  P  W  I  Q  I  215
661
TGCAATAATTTTTCTCCTATCATTGATTACTTCCCGGGAACTCACAACAAATTACTTAAA
 C  N  N  F  S  P  I  I  D  Y  F  P  G  T  H  N  K  L  L  K  235
721
AACGTTGCTCTTATGAAAAGTTATATTTTGGAAAAAGTAAAAGAACACCAAGAATCAATG
 N  V  A  L  M  K  S  Y  I  L  E  K  V  K  E  H  Q  E  S  M  255
781
GACATGAACAACCCTCAGGACTTTATTGATTGCTTCCTGATGAAAATGGAGAAGGAAAAG
 D  M  N  N  P  Q  D  F  I  D  C  F  L  M  K  M  E  K  E  K  275
                                    Pst I
841
CACAACCAACCATCTGAATTTACTATTGAAAGCTTGGAAAACACTGCAGTTGACTTGTTT
 H  N  Q  P  S  E  F  T  I  E  S  L  E  N  T  A  V  D  L  F  295
901
GGAGCTGGGACAGAGACGACAAGCACAACCCTGAGATATGCTCTCCTTCTCCTGCTGAAG
 G  A  G  T  E  T  T  S  T  T  L  R  Y  A  L  L  L  L  L  K  315
961
CACCCAGAGGTCACAGCTAAAGTCCAGGAAGAGATTGAACGTGTGATTGGCAGAAACCGG
 H  P  E  V  T  A  K  V  Q  E  E  I  E  R  V  I  G  R  N  R  335
1021
AGCCCCTGCATGCAAGACAGGAGCCACATGCCCTACACAGATGCTGTGGTGCACGAGGTC
 S  P  C  M  Q  D  R  S  H  M  P  Y  T  D  A  V  V  H  E  V  355
1081
CAGAGATACATTGACCTTCTCCCCACCAGCCTGCCCCATGCAGTGACCTGTGACATTAAA
 Q  R  Y  I  D  L  L  P  T  S  L  P  H  A  V  T  C  D  I  K  375
1141
TTCAGAAACTATCTCATTCCCAAGGGCACAACCATATTAATTTCCCTGACTTCTGTGCTA
 F  R  N  Y  L  I  P  K  G  T  T  I  L  I  S  L  T  S  V  L  395
1201
CATGACAACAAAGAATTTCCCAACCCAGAGATGTTTGACCCTCATCACTTTCTGGATGAA
 H  D  N  K  E  F  P  N  P  E  M  F  D  P  H  H  F  L  D  E  415
1261
GGTGGCAATTTTAAGAAAAGTAAATACTTCATGCCTTTCTCAGCAGGAAAACGGATTTGT
 G  G  N  F  K  K  S  K  Y  F  M  P  F  S  A  G  K  R  I  C  435
1321
GTGGGAGAAGCCCTGGCCGGCATGGAGCTGTTTTTATTCCTGACCTCCATTTTACAGAAC
 V  G  E  A  L  A  G  M  E  L  F  L  F  L  T  S  I  L  Q  N  455
1381
TTTAACCTGAAATCTCTGGTTGACCCAAAGAACCTTGACACCACTCCAGTTGTCAATGGA
 F  N  L  K  S  L  V  D  P  K  N  L  D  T  T  P  V  V  N  G  475
1441
TTTGCCTCTGTGCCGCCCTTCTACCAGCTGTGCTTCATTCCTGTCTGAAGAAGAGCAGAT
 F  A  S  V  P  P  F  Y  Q  L  C  F  I  P  V  !                490
                    Pst I
1501
GGCCTGGCTGCTGCTGTCAGTCCCTGCAGCTCTCTTTCCTCTGGGGCATTATCCATCTTC
1561
ACTATCTGTAATGCCTTTTCTCACCTGTCATCTGACATTTTCCCTTCCCTGAAGATCTAG
1621
TGAACATTCGACCTCCATTACGGAGAGTTTCCTATGTTTCACTGTGCAAATATATCTGCT
1681
ATTCTCCATACTCTGTAACAGTTGCATTGACTGTCACATAATGCTCATACTTATCTAATG
1741
TTGAGTTATTAATATGTTATTATTAAATAGAGAAATATGATTTGTGTATTATAATTCAA
1801
AGGCATTTCTTTTCTGCATGTTCTAAATTAAAAAGCATTATTATTTGCTG(A)42(GA)10
(G)14(GC)10
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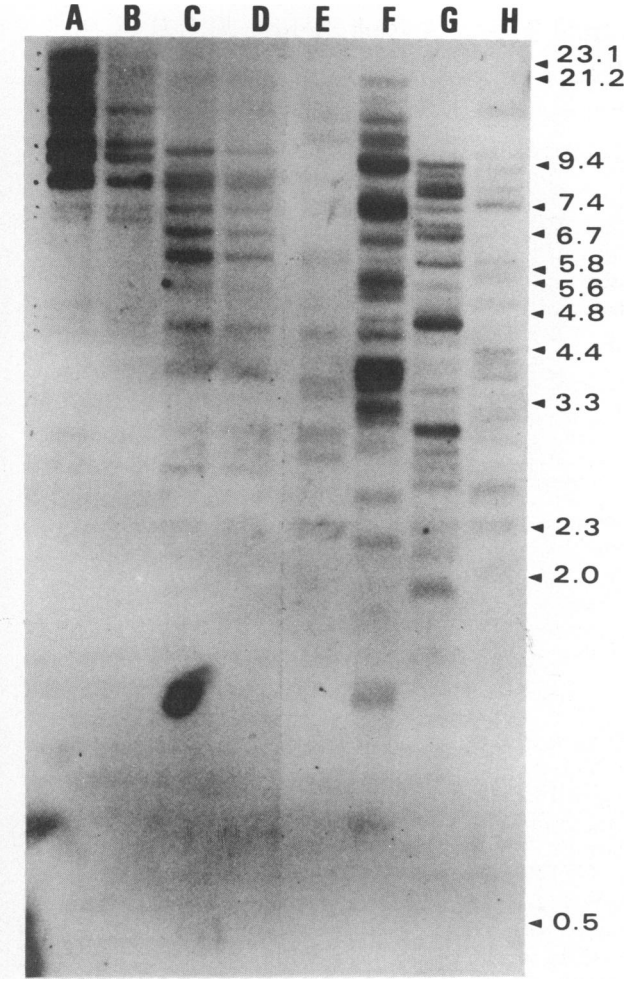

Figure 4 Southern blot analysis of primate and rodent DNAs with the PB-1 cDNA clone pHL5,5. This blot was washed at high stringency (0.01 × SSC at 68 C). The restriction enzymes used were *Bam*HI (lanes A and B) and *Eco*RI (lanes C–H). Lanes A and C, human liver DNA; lanes B and D, AKLO cell-line DNA; lane E, male chimp DNA; lane F, Chinese-hamster WG3H cell-line DNA; lane G, Wistar male rat liver DNA; lane H, C57/L male mouse spleen DNA.

human PB-1 P-450 can be aligned with rat P-450 forms PB-1 and f (Freidberg et al. 1986; Gonzalez et al. 1986) without the need for any insertions or deletions. At the amino acid level these rat P-450 forms show >70% homology to human PB-1 P-450. PB-1 also shows very significant homology (70%) to rabbit P-450 form I (Tukey et al. 1985). Significant amino acid homology of 50% was seen with rat P-450 b (*P450-2B*) (Fuji-Kuriyama et al. 1982). Other features characteristic of P-450s were noted, including a hydrophobic leader sequence and a possible cysteine-containing heme-binding region centered in the region of Cys435 (Black and Coon 1986).

Genomic Complexity of Human PB-1 P-450

To determine the genomic complexity of the PB-1 P-450 gene, we probed Southern blots of human and rodent genomic DNA (fig. 4). Our experiments were initially hampered by the presence of a low-level repeat in pHL5,5, but high-stringency washing (0.01 × SSC at 68 C) removed this background and revealed a unique pattern of hybridization of some complexity (fig. 4). In humans the amount of hybridization DNA detected by pHL5,5 ranged from 70 to 150 kb, depending on the restriction enzyme used, whereas in rodent samples the pattern observed was more complex. This result implies that PB-1 either is a member of a small multigene family or corresponds to a very large gene. We favor the former interpretation because of the multigenic nature of PB-1 in rodents (Freidberg et al. 1986; Gonzalez et al. 1986; Wolf et al. 1986*b*), rabbits (Tukey et al. 1985; Leighton et al. 1984), and humans (Adams et al. 1985; Shimada et al. 1986). To investigate this hypothesis, the DNA of one individual was restricted with several different enzymes and probed with the separate *Pst*I fragments from pHL5,5 (fig. 3). The 894-bp fragment represents the 5′ end of the PB-1 transcript, the 640-bp fragment represents the middle coding region of the PB-1 transcript, and the 430-bp fragment represents the 3′ end (mostly untranslated region) of the molecule and also contains the low-level repeated sequence (fig. 5). To derive a minimum gene count for the PB-1 locus in the human genome, we probed identical Southern blots of human DNA with the isolated *Pst*I fragments. Any DNA fragments that hybridize to both the 5′ and 3′ probes must represent separate and distinct genes or pseudogenes. Therefore, the fragments in common give a value for the minimum number of genes. As can be seen in figure 5, there are a number of DNA fragments in common when the 894-bp and 640-bp *Pst*I fragments are used to probe *Bam*HI- and *Eco*RI-digested human DNA (signified by the dots). The 430-bp probe gives a smear at this stringency wash (2 × SSC at 68 C). These bands vary in intensity, but in each restriction-enzyme digest and for both probes there are at least two major fragments in common. If the experiment is repeated at higher-stringency washing (0.01 × SSC at 68 C), only two fragments are seen to be in common (data not shown). These data indicate that the PB-1 P-450 is encoded by a gene that is a member of a

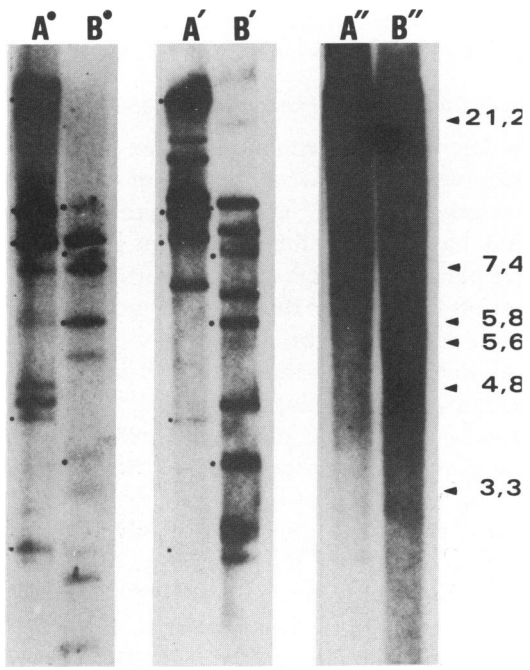

Figure 5 Complexity analysis of the PB-1 human DNA pattern. Three identical Southern transfers of *Bam*HI- (lanes A) and *Eco*RI- (lanes B) digested human DNA were probed with the three individual *Pst*I fragments from the pHL5,5 cDNA insert. The fragments used as probes were the 894-bp *Pst*I fragment (lanes A• and B•) corresponding to the 5′ half of the cDNA, the 640-bp *Pst*I fragment (lanes A′ and B′) corresponding to the 3′-coding half of the cDNA, and the 430-bp *Pst*I fragment (lanes A″ and B″) corresponding to the 3′-untranslated region of the cDNA. A black dot (•) indicates that fragments are in common.

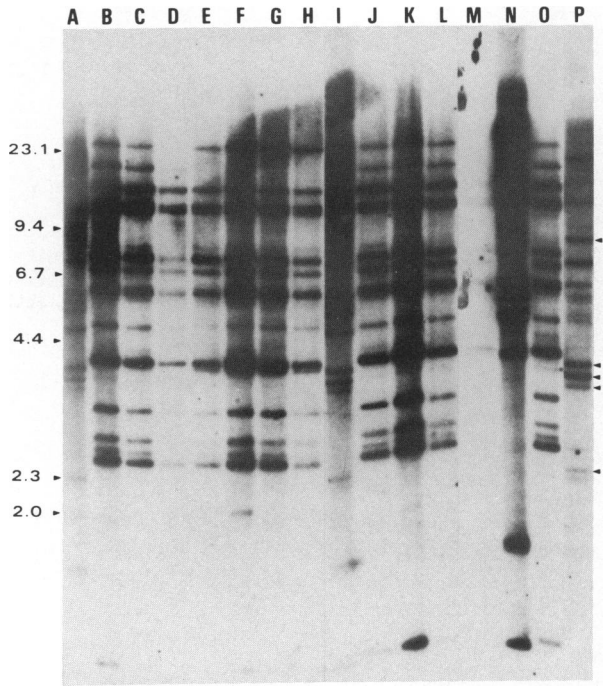

Figure 6 Southern blot analysis of a panel of human/rodent hybrid cells probed with the 894- and 638-bp *Pst*I fragments from the pHL5,5 cDNA insert. Lane A, hybrid 1; lane B, hybrid 24; lane C, hybrid 22; lane D, hybrid 15; lane E, hybrid 18; lane F, hybrid 21; lane G, hybrid 17; lane H, hybrid 10; lane I, hybrid 1; lane J, hybrid 27; lane K, hybrid 20; lane L, hybrid 14; lane M, hybrid 12; lane N, WG3H (control hamster DNA); lane O, C57/L (mouse control DNA); lane P, human female placenta DNA (only ¼ of the amount loaded compared with hybrid DNA). An arrowhead on the right hand side (◀) indicates a fragment that was mapped to chromosome 10 in this particular experiment. Only hybrid 1 (lanes A and I) was positive for human bands (see table 1 for the karyotype of hybrids, and see Material and Methods for their origin).

family of related genes containing two or more members.

Chromosomal Localization of Human PB-1 P-450

It would be of interest to know the interrelationships of the different members of the PB-1 gene family, i.e., whether they map to a similar or to distinct chromosomal locations. A panel of rodent/human somatic-cell hybrids was used to determine the chromosomal location of the PB-1 genes in the human genome. Initial mapping experiments were hindered by the conservation of PB-1 sequences in rodents and in humans and by the much greater genomic complexity of the rodent PB-1 locus. The use of the *Bam*HI digests of somatic cell–hybrid DNA gave the clearest pattern in that it gave resolvable human fragments on a rodent background when a mixture of the 894-bp and 638-bp *Pst*I fragments were used as probes. This enzyme also has the property of not being variant for the PB-1 gene family in the human population (R. R. Meehan, J. R. Gosden, D. Rout, N. D. Hastie, T. Friedberg, M. Adesnik, R. Buckland, V. van Heyningen, J. Fletcher, N. K. Spurr, J. Sweeney, and C. R. Wolf, unpublished observations), and therefore the different sources of our hybrids will not be compromised by RFLPs.

A major triplet of human PB-1 fragments can be observed at ~4 kb, and a minor fragment at 2.3 kb (fig. 6). In our initial screen the 894-bp and 640-bp cDNA probes found only one hybrid containing hybridizable human fragments. Our analysis indicated that human chromosome 10 was concordant with this signal (table 1). To confirm this assignment we looked at a range of other chromosome 10–containing hybrids from rat, mouse, and Chinese-

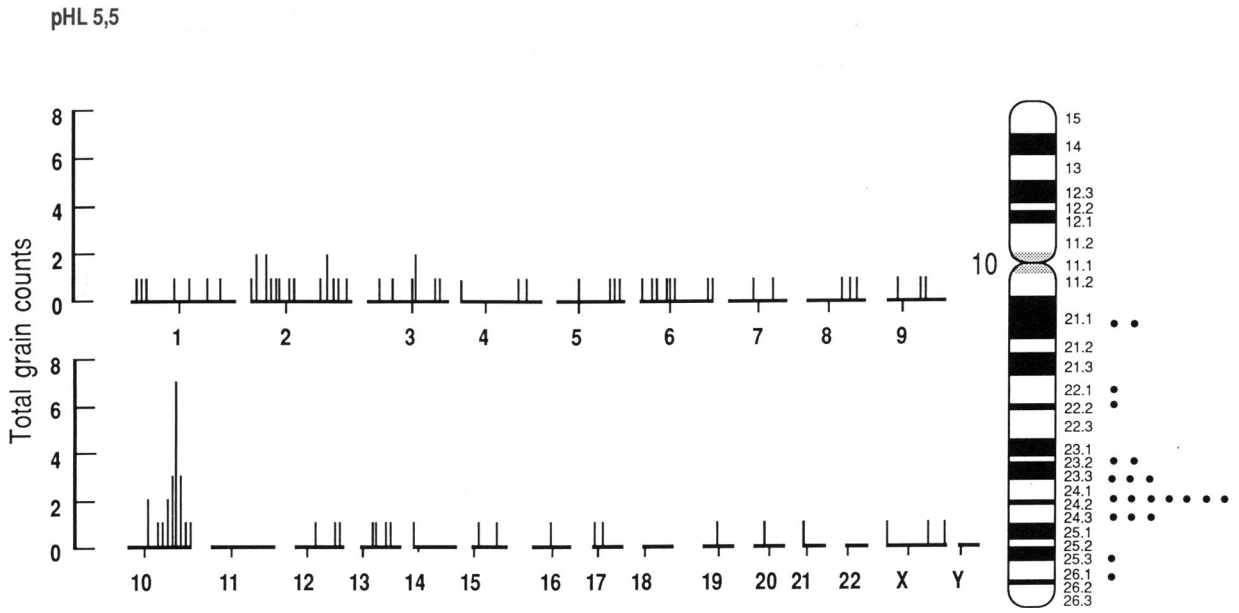

Figure 7 Autoradiographic grain distribution after in situ hybridization of pHL5,5 to human chromosomes. The horizontal axis shows the chromosomes, with the centromeres indicated by a vertical bar. The vertical axis shows the total grains found at each site in the 23 cells analyzed. Note the peak of grains in the long arm of chromosome 10; the insert shows a detailed grain distribution on that chromosome.

hamster fusions to human cells. These hybrids gave a 100% concordance between the human signal and chromosome 10 (table 1). Three hybrids deserve special mention; numbers 2 and 9 are sister subclones with an almost identical human chromosome content, except that hybrid 2 contains a chromosome 10 and hybrid 9 contains chromosome 9. In this case only hybrid 2 was positive for hybridization to human fragments. The third mouse hybrid of note is hybrid 3, which has a diploid number of human chromosomes 10, which gave an increased signal intensity for human bands when probed for human PB-1 sequences. To reduce the complexity of hybridization and to map more of the PB-1 human fragments, we probed duplicate blots of hybrid DNAs with the 894-bp and 638-bp *Pst*I fragments, respectively. On the basis of this analysis we noted that all the PB-1 DNA fragments appeared to map to chromosome 10, indicating that the PB-genes are probably linked (data not shown).

To ascertain whether PB-1 P-450 was clustered on human chromosome 10, we used pHL5,5 as the probe to metaphase spreads of human blood lymphocytes. The cDNA probe was annealed with total human DNA to eliminate hybridization of the repeat sequence (Sealey et al. 1985; Porteous et al. 1987). Twenty-three cells were analyzed with a total of 111 grains. Of these 111, 21 grains (23%) were located on chromosome 10; and of these 21, 13 (62%) were in the region 10q24.1-10q24.3, with a peak at 10q24.2 (see fig. 7). On the basis of these results we concluded that the PB-1 P-450 genes do appear to be clustered on human chromosome 10. In accordance with the new nomenclature we designated this locus *P450C2C*.

Discussion

The extremely wide diversity of cytochrome P-450 enzymes involved in foreign-compound metabolism, together with the ability to handle a vast variety of substrates, suggests that the cytochrome P-450 system is complex. However, the application of recombinant-DNA methodology to this system has both simplified and reinforced this initial conclusion. A number of distinct cytochrome P-450 gene families have been identified (Adesnik and Atchison 1986; Wolf 1986), including those of the highly inducible hepatic P-450s, e.g., 3-methylcholanthrene (*P450-1*), phenobarbital (*P450-2B*), and pregnenolone-16 α-carbonitrile (*P450-3*). In humans the *P450C1* and *P450C2A* P-450 genes have been mapped to chromosome 15 (Hildebrand et al. 1985) and chromosome 19 (Phillips et al. 1986), respectively.

The PB-1 P-450 (*P450C2C*) also appears to be a multigene family, with a minimum of two genes located on human chromosome 10. In contrast to the highly inducible hepatic P-450s, PB-1 is expressed at a high basal level in adult rodents (Waxman and Walsh 1983; Wolf et al. 1984; Gonzalez et al. 1986), constituting as much as 0.2% of hepatic mRNA (Freidberg et al. 1986). Western blots of human liver microsomes with antibodies to rat liver PB-1 yielded two bands (Adams et al. 1985). Also, purification schemes from human liver microsomes have yielded two distinct P-450 forms structurally related to rat PB-1 that have activity in the hydroxylation of mephenytoin (Shimada et al. 1986). However, it is possible that this gene family is more extensive. In rodents, there is evidence for the existence of as many as six related hepatic forms of PB-1 P-450 (Haniu et al. 1984; Wolf et al. 1986, 1987). Our Northern blots of human liver mRNA suggest that there are multiple-sized transcripts corresponding to PB-1; this view is reinforced by the complexity of the DNA pattern detected by the PB-1 cDNA probe.

In view of the structural homology between the rat PB-1 genes and the human enzymes involved in mephenytoin metabolism (Shimada et al. 1986), it would appear that the human PB-1 cDNA is part of the *P450C2C* gene family. In the case of mephenytoin, two phenotypes can be recognized, the so-called extensive metabolizer and poor metabolizer (Eichelbaum 1984; Küpfer and Preisig 1984). Population and family studies have established that these differences are genetic in character and that the poor-metabolizer phenotype is inherited as an autosomal recessive (Jurima et al. 1985; Inaba et al. 1986). Comparative intersubjective studies had shown discordance between the mephenytoin phenotype and the debrisoquine oxidative phenotype (Jurima et al. 1985). Subsequently, the P-450s responsible for debrisoquine hydroxylation (Distlerath et al. 1985; Gut et al. 1986) and those associated with polymorphic metabolism of other drugs in man (e.g., phenacetin O-deethylation [Distlerath et al. 1985] and nifedipine oxidation [Guengerich et al. 1986]) have been purified. None of these P-450s show significant structural or biochemical homology with either the mephenytoin P-450 (Shimada et al. 1986) or each other.

It is important to note that all adverse drug side effects so far identified as being due to P-450s have been associated with constitutively expressed genes and not with the highly inducible forms *P450-1* and *P450-2B* (Wolf 1986), a result corroborating the importance of the former proteins in pharmacogenetics. However, to date no clinically important effects have been ascribed to the mephenytoin polymorphism. The potential of this defect can be underlined by the following observations: In rodent models, the *P450C2C* locus is also a multigene family that is concordant with constitutive aryl-hydrocarbon hydroxylase (AHH) activity (R. R. Meehan, J. R. Gosden, D. Rout, N. D. Hastie, T. Friedberg, M. Adesnik, R. Buckland, V. van Heyningen, J. Fletcher, N. K. Spurr, J. Sweeney, and C. R. Wolf, unpublished observations), an activity that has been associated with carcinogen activation (Nebert and Jensen 1979). Indeed, antibodies raised to PB-1 P-450 inhibit both 7-ethoxyresorufin metabolism (associated with AHH) and aflatoxin B_1 activation in uninduced rat microsomes (Ishii et al. 1986; Wolf et al. 1986). Therefore, in the absence of the induced P-450s that have high activity in the metabolism of these compounds, the constitutively expressed PB-1 P-450 would to some degree determine the metabolic fate of polycyclic aromatic hydrocarbons and various other human carcinogens. In addition to the potential role of PB-1 proteins in these reactions, sequence analysis and functional studies of the rodent enzymes indicates that members of this family also play a role in the control of the oxidation and degradation of steroid hormones, e.g., the 2-, 15α-, and 16α-hydroxylation of estradiol and the 16α-hydroxylation of testosterone (Cheng and Schenkman 1983; Waxman 1984). The results described in the present paper would also assign these functions to human chromosome 10. The use of recombinant inbred strains of mice has indicated that the PB-1 genes are clustered (R. R. Meehan, N. D. Hastie, and C. R. Wolf, unpublished observations), so it is probable that the PB-1 genes on human chromosome 10 are also closely linked to each other. The in situ hybridization would appear to confirm this hypothesis, centering the human PB-1 genes on 10q24.2, which has been designated the *P450C2C* locus. Our assignment of PB-1 P-450 to human chromosome 10 also provides much needed molecular markers for this chromosome. Human Gene Mapping 8 (Grzeschik and Kazazian 1985) shows only 28 mapped loci for chromosome 10 (including fragile sites), and of these only eight were molecular probes. We are currently identifying RFLPs for pHL5,5 for use with families that have been phenotyped for mephenytoin hydroxylation to see whether there is a correspondence between particular RFLPs and the mephenytoin phenotype.

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