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Sequence Analysis and Homology Modeling Suggest That Primary Congenital Glaucoma on 2p21 Results from Mutations Disrupting Either the Hinge Region or the Conserved Core Structures of Cytochrome P4501B1

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Summary

We recently reported three truncating mutations of the cytochrome P4501B1 gene (*CYP1B1*) in five families with primary congenital glaucoma (PCG) linked to the GLC3A locus on chromosome 2p21. This could be the first direct evidence supporting the hypothesis that members of the cytochrome P450 superfamily may control the processes of growth and differentiation. We present a comprehensive sequence analysis of the translated regions of the *CYP1B1* gene in 22 PCG families and 100 randomly selected normal individuals. Sixteen mutations and six polymorphisms were identified, illustrating an extensive allelic heterogeneity. The positions affected by these changes were evaluated by building a three-dimensional homology model of the conserved C-terminal half of *CYP1B1*. These mutations may interfere with heme incorporation, by affecting the hinge region and/or the conserved core structures (CCS) that determine the proper folding and heme-binding ability of P450 molecules. In contrast, all polymorphic sites were poorly conserved and located outside the CCS. Northern hybridization analysis showed strong expression of *CYP1B1* in the anterior uveal tract, which is involved in secretion of the aqueous humor and in regulation of outflow facility, processes that could contribute to the elevated intraocular pressure characteristic of PCG.

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Introduction

Severe primary congenital glaucoma (PCG) manifests itself during the neonatal or infantile period. The disease is characterized by high intraocular pressure (IOP), which, if uncontrolled, leads to optic-nerve damage and permanent loss of vision (De Luise and Anderson 1983). PCG is likely to be due to maldevelopment of the anterior chamber angle of the eye, thus interfering with the aqueous-humor outflow (Allen et al. 1955; Barkan 1955; Maumenee 1958; Kupfer and Kaiser-Kupfer 1979; Anderson 1981). The resulting increase in IOP produces ocular enlargement (buphthalmos), corneal edema, and progressive optic-nerve cupping. PCG has a higher incidence in Gypsies (1:1,250) and in the Middle East (1:2,500) as compared with that in Western societies (1:5,000–1:22,000 births) (Francois 1980; Jaffar 1988; Gencik 1989). An autosomal recessive pattern of inheritance is well documented (Francois 1980; Gencik 1989; Turacli et al. 1992). Families presenting with affected individuals in successive generations have raised the possibility that a pseudodominant form of the disease may also exist. This may be due, however, to the marriage of homozygous and heterozygous individuals (Stoilov et al. 1997).

Recently, we reported the identification of three truncating mutations in the human cytochrome P4501B1 gene (*CYP1B1* [MIM 601771]) in five families with PCG linked to locus GLC3A (MIM 231300) on chromosome 2p21 (Sarfarazi et al. 1995; Akarsu et al. 1996; Stoilov et al. 1997). It has long been speculated that members of the cytochrome P450 superfamily may be directly involved in regulation of the processes of growth and differentiation (Nebert 1990, 1991). Therefore, our finding could be the first direct confirmation of this hy-

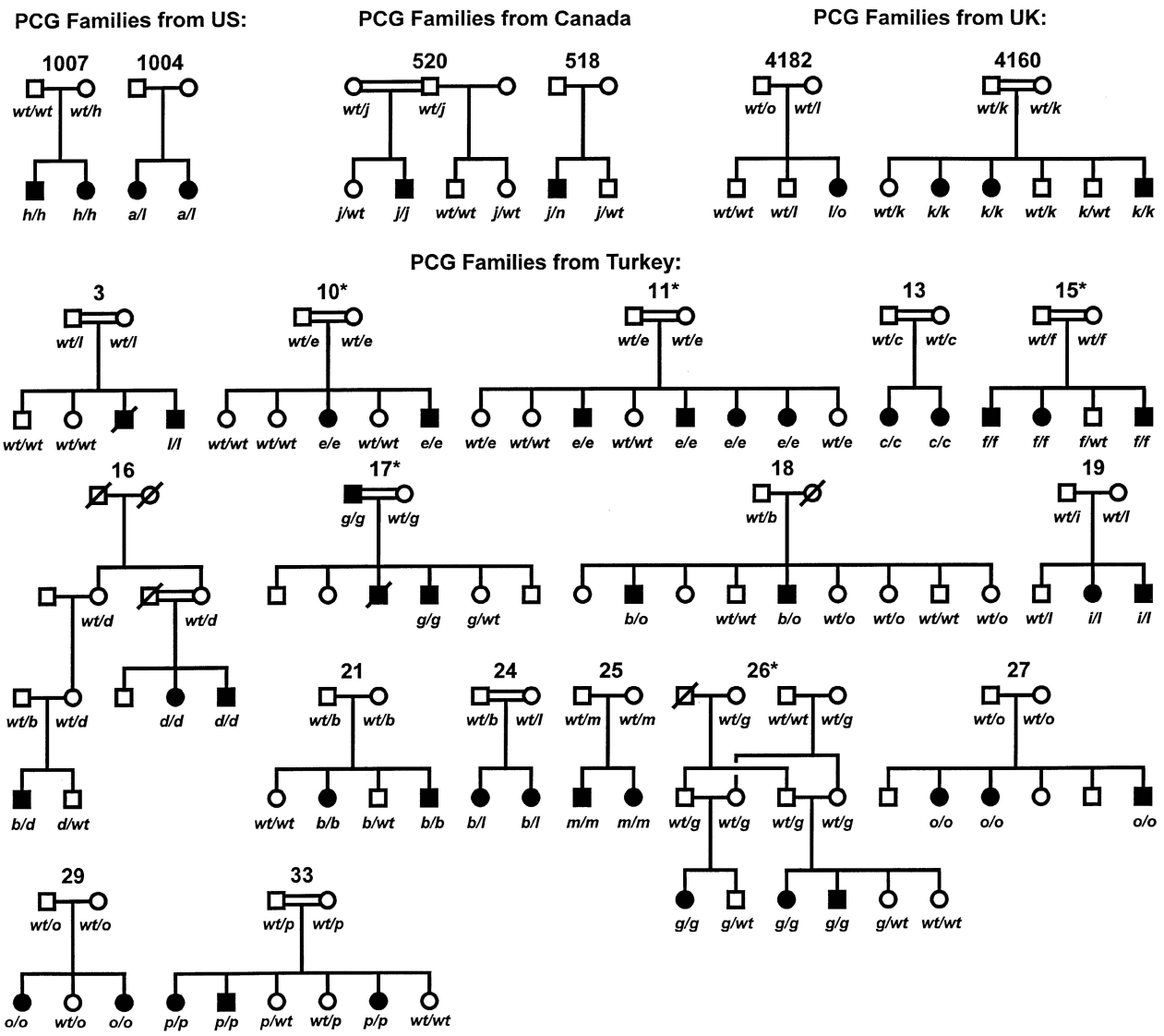

Figure 1 Pedigrees of the 22 PCG families in the present study. *CYP1B1* mutant alleles segregating in each family are defined in terms of the one-letter code used in table 1. wt = Wild-type allele. Absence of genotype data indicates that a DNA sample from that individual was not available for analysis. Pedigrees reported elsewhere are denoted by an asterisk (*).

pothesis. Herein, we present a comprehensive list of mutations and polymorphic sites identified by direct sequencing of the translated regions of *CYP1B1* in members of 22 PCG families and 100 randomly selected normal individuals (50 British and 50 Turkish). The positions affected by these changes were evaluated with the assistance of a three-dimensional model of the conserved C-terminal half of *CYP1B1* (Graham-Lorence and Peterson 1996), which was build by homology modeling.

Subjects and Methods

Subjects

Twenty-two PCG families (fig. 1) from Turkey, the United States, Canada, and the United Kingdom were ascertained (Barsoum-Homsy and Chevrette 1986; Turacli et al. 1992; Sarfarazi et al. 1995; Akarsu et al. 1996). The affected individuals included in this study had an aggressive form of glaucoma with an early onset (at age 0–3 years), manifested by symptoms of increased IOP and corneal edema, including excessive tearing, photophobia, buphthalmos, and cloudy cornea. Optic-nerve cupping and ruptures of Descmet's membrane were determined, where possible. Individuals presenting with other associated ocular or systemic anomalies were excluded. Therefore, the type of glaucoma discussed in the present article represents isolated congenital glaucoma, according to the Shaffer and Weiss (1970) classification, and isolated trabeculodysgenesis, according to (Hoskins

Table 1

PCG Mutations in *CYP1B1*

Code	Exon	DNA Change	Predicted Effect	Location	Amino Acid Deleted	Restriction Site	PCG Families	No. (%) of PCG Chromosomes (n = 28)	Ethnic Origin	Reference
a	2	G517→C	Trp57→Cys	Hinge region	...	...	1004	1 (3.57)	Hispanic	Present study
b	2	G528→A	Gly61→Glu	Hinge region	...	*Taq*I$^+$	16, 18, 21, 24	5 (17.85%)	Turkish	Present study
c	2	847insT	Frameshift	...	377	*Xho*I$^-$	13	1 (3.57)	Turkish	Present study
d	2	G1187→T	Glu281→stop	...	262	...	16	1 (3.57)	Turkish	Present study
e	2	1209insC	Frameshift	...	254	...	10, 11	1 (3.57)	Turkish	Stoilov et al. (1997)
f	3	Large deletion	Splicing error	...	...	...	15	1 (3.57)	Turkish	Stoilov et al. (1997)
g	3	1410del13	Frameshift	...	189	*Xho*I$^-$	17, 26	2 (7.14%)	Turkish	Stoilov et al. (1997)
h	3	G1439→T	Gly365→Trp	Helix J	...	...	1007	1 (3.57)	U.S.	Present study
i	3	C1482→T	Pro379→Leu	Helix K	...	...	19	1 (3.57)	Turkish	Present study
j	3	G1505→A	Glu387→Lys	Helix K	...	...	518, 520	2 (7.14%)	Hispanic, French Canadian	Present study
k	3	G1515→A	Arg390→His	Helix K	...	*Cfo*I$^-$	4160	1 (3.57)	Pakistani	Present study
l	3	1546dup10	Frameshift	...	140	*Nla*III$^+$	3, 19, 24, 1004, 4182	5 (17.86%)	U.S., British, Turkish	Present study
m	3	C1656→T	Pro437→Leu	Meander	...	...	25	1 (3.57)	Turkish	Present study
n	3	G1691del	Frameshift	...	95	...	518	1 (3.57)	Hispanic	Present study
o	3	C1751→T	Arg469→Trp	Heme binding	...	*Aci*I$^-$	18, 27, 29, 4182	4 (14.29%)	British, Turkish	Present study
p	3	1749dup27	Frameshift	...	76	*Bfa*I$^+$	33	1 (3.57)	Turkish	Present study

Note.—Restriction assays for some of these mutations are also presented in figure 7.

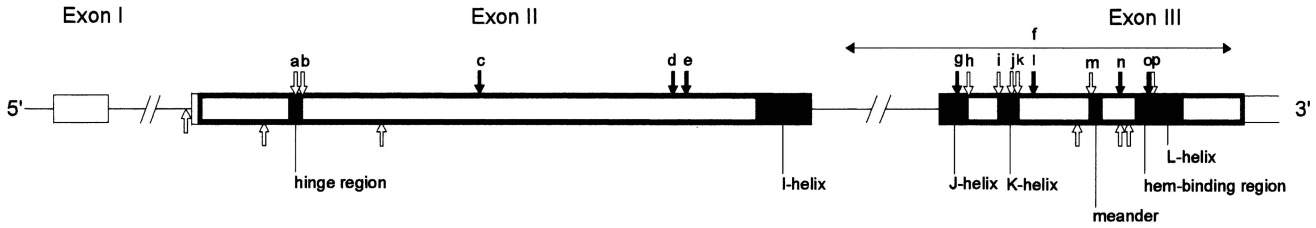

Figure 2 Observed sequence changes in *CYP1B1*. The translated regions are boxed by the thicker lines; and the conserved regions are depicted as blackened. Mutations that are predicted to truncate the open reading frame are indicated by the blackened downward-pointing arrows, whereas the missense mutations are indicated by unblackened downward-pointing arrows. All mutations are defined in terms of the one-letter code used in table 1. Polymorphic positions are indicated by unblackened upward-pointing arrows. The horizontal line below mutation "f" denotes a splicing event.

et al.'s (1984) anatomic classification of developmental glaucomas. Members of these families have identified themselves as German American (family PCG-1007), Hispanic (families PCG-518 and PCG-1004), French Canadian (family PCG-520), British (family PCG-4182), Pakistani (family PCG-4160), and Turkish (families PCG-3–PCG-31). All subjects provided informed consent prior being included in this study.

Mutation Screening

Three primer sets were used to amplify the translated regions of *CYP1B1* from genomic DNA: set 1 (1F, 5′-tctccagagagtcagctccg-3′; and 1R, 5′-gggtcgtcgtggctgtag-3′ [786 bp]); set 2 (2F, 5′-atggctttcggccactact-3′; and 2R, 5′-gatcttggttttgaggggtg-3′ [787 bp]); and set 3 (3F, 5′-tcccagaaatattaatttagtcactg-3′; and 3R, 5′-tatggagcacacctcacctg-3′ [885 bp]). PCR amplifications were performed in a 40-μl volume containing 1.0 μl of 20 μM stock solution for each primer oligo, 100 ng of genomic DNA, 1 U of AmpliTaq polymerase (Perkin-Elmer), 0.1 mM of each dNTP, 1.5–2.0 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCL (pH 9.3), by means of 35 cycles of amplification, each consisting of 30 s denaturation at 94°C, 30 s annealing at 56°C, and 1 min extension at 72°C. Sets 1 and 2 were amplified in the presence of 1.5 mM $MgCl_2$ and 10% dimethyl sulfoxide (Sigma); and set 3 was amplified in the presence of 2.0 mM $MgCl_2$. Dye terminator-cycle sequencing with AmpliTaq DNA polymerase FS (Perkin-Elmer) was performed according to the protocol supplied by the manufacturer. Analysis of the sequencing reactions was performed on an ABI-377 automated DNA Sequencer (Perkin-Elmer). Restriction endonucleases were purchased from Gibco BRL (in the case of *Cfo*I, *Taq*I, and *Xho*I) or New England Bio-

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                R48G  W57C  G61E   A119S      G365W P379L E387K  R390H  V432L P437L      N453S          R469W
                  ↓     ↓     ↓       ↓          ↓     ↓     ↓  ↓      ↓     ↓          ↓              ↓*
CYP1A1 (trout)  LKRLPGPKPLPIIGNML...RSDLYS...LKEKVG...NLPLLEAFILEIFRHS...HDPELWKEPSSFN...ELNKLEGEKVLVFGMGKRRCIGEAIGR  467
CYP1A1 (mouse)  LKTPPGPWGLPFIGHML...RPDLYS...LDTVIG...QLPYLEAFILETFRHS...HDRELWGDPNEFR...TLDKRLSEKVTLFGLGKRKCIGETIGR  465
CYP1A1 (g.pig)  LKSPPGPWGWPLIGHML...RPDLYS...LDTVIG...KLPYMEAFISEVFRYS...HDQKLWGDPSVFR...TVDKALSEKVTIFGLGKRRCLGEVIGR  457
CYP1A1 (human)  LKNPPGPWGWPLIGHML...RPDLYT...LDTVIG...HLPYMEAFILETFRHS...HDQKLWVNPSEFL...AIDKVLSEKVIIFGMGKRKCIGETVAR  461
CYP1A2 (trout)  LKRLPGPKPLPIIGNVL...RPDLYS...LKEKVG...NLPLLEAFILEIFRHS...HDPELWKEPSSFN...ELNKLEGEKVLVFGMGKRRCIGEAIGR  467
CYP1A2 (human)  LKSPPEPWGWPLLGHVL...RPDLYT...LDTVIG...QLPYLEAFILETFRHS...HDPELWEDPSEFR...AINKPLSEKMMLFGMGKRRCIGEVLAK  462
CYP1B1 (mouse)  WSSPPGPFPWPLIGNAA...RPPFAS...LDQVVG...NLPYVMAFLYESMRFS...HDPAKWPNPEDFD...FINKALASSVMIFSVGKRRCIGEELSK  474
CYP1B1 (rat)    WSSPPGPFPWPLIGNAA...RPPFAS...LDQVVG...NLPYVMAFLYESMRFT...HDPAKWSNPEDFD...FINKALASSVMIFSVGKRRCIGEELSK  474
CYP1B1 (human)  RSAPPGPFAWPLIGNAA...RPAFAS...LDQVVG...NLPYVLAFLYEAMRFS...HDPVKWPNPENFD...LINKDLTSRVMIFSVGKRRCIGEELSK  474
CYP2A7 (human)  GKLPPGPTPLPFIGNYL...RGEQAT...IDRVIG...KMPYMEAVIHEIQRFG...RDPSFFSNPQDFN...--QFKKSDAFVPFSIGKRYCFGEGLAR  446
CYP2B6 (human)  DRLPPGPRPLPLLGNLL...RGKIAM...IEQVIG...KMPYTEAVIYEIQRFS...HDPHYFEKPDAFN...---LKKTEAFIPFSLGKRICLGEGIAR  443
CYP2C8 (human)  RKLPPGPTPLPIIGNML...RGNSPI...IDHVIG...HMPYTDAVVHEIQRYS...HDDKEFPNPNIFD...--NFKKSDYFMPFSAGKRICAGEGLAR  439
CYP2D6 (human)  ARYPPGPLPLPGLGNLL...RPPVPI...IDDVIG...HMPYTTAVIHEVQRFG...KDEAVWEKPFRFH...--HFVKPEAFLPFSAGRRACLGEPLAR  448
CYP2E1 (human)  WNLPPGPFPLPIIGNLF...RGDLPA...IDRVIG...EMPYMDAVVHEIQRFI...YDNQEFPDPEKFK...-GKFKYSDYFKPFSTGKRVCAGEGLAR  444
CYP2F1 (human)  GKLPPGPRPLSILGNLL...RGDYPA...IDLVVG...AMPYTDAVIHEVQRFA...YDPSQFLTPQEFN...-QSFKKSPAFMPFSAGRRLCLGELLAR  443
CYP3A5 (human)  RLGIPGPTPLPLLGNVL...NRRSLG...IDAVLP...QMEYLDMVVNETLRLF...HDPKYWTEPEEFR...-KDSIDPYIYTPFGTGPRNCIGMRFAL  445
CYP4B1 (human)  MDKFPGPPTHWLFGHAL...DVYDFF...VREILG...KMTYLTMCIKESFRLY...RNSAVWPDPEVFD...NASKRHPFAFMPFSAGPRNCIGQQFAM  458
CYP5   (human)  KLGLRHPKPSPFIGNLT...RMASGL...VDVF--...GLPYLDMVIAETLRMY...HDPEHWPSPETFN...ARQQHRPFTYLPFGAGPRSCLGVRLGL  483
CYP7   (human)  TGEPPLENGLIPYLGCA...KFHFAT...NAGQ--...DLPVLDSIIKESLRLS...LDPEIYPDPLTFK...CNGLKLKYYYMPFGSGATICPGRLFAI  449
CYP11A1(human)  EIPSPGDNGWLNLYHFW...RFLIPP...VLAA--...LVPLLKASIKETLRLH...REPTFFFDPENFD...DKNITYFRNL-GFGWGVRQCLGRRIAE  466
CYP17  (human)  AKYPKSLLSLPLVGSLP...RPQMAT...IDQNVG...RLLLLEATIREVLRLR...HNEKEWHQPDQFM...TQLISPSVSYLPFGAGPRSCIGEILAR  446
CYP21A2(human)  WKLRSLHLPPLAPGFLH...RPEPLT...LDHELG...RLPLLNATIAEVLRLR...LDETVWERPHEFW...---GKNSRAL-AFGCGARVCLGEPLAR  432
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Figure 3 Multiple sequence alignment for 22 different members of the cytochrome P450 superfamily. Conserved residues affected by mutations are indicated by boldface letters. Single-letter code is used to denote the mutations (boldface, underlined, and indicated by the larger arrows) and the polymorphic sites. The invariant cysteine residue in the heme-binding region is denoted by an asterisk (*).

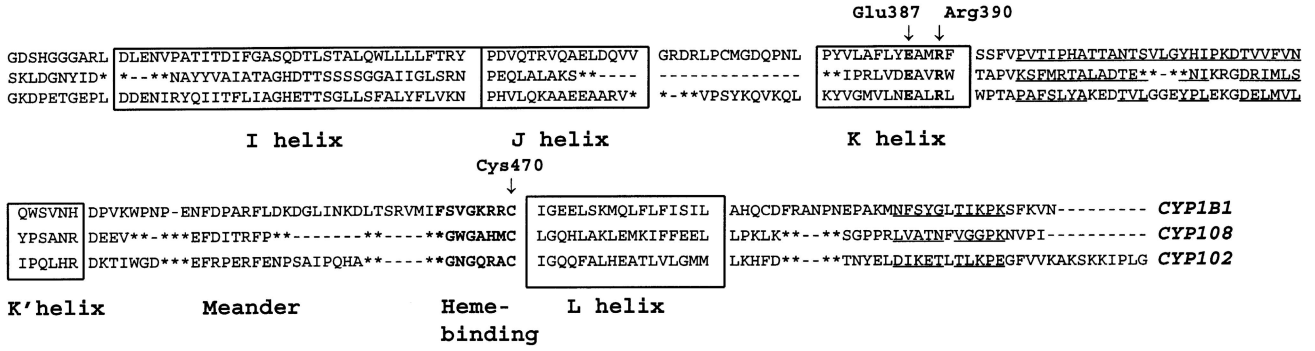

Figure 4 Sequence alignment/structure of *CYP1B1, CYP102* (BM-P) and *CYP108* (TERP), as generated by ProMod. Helical regions are boxed, and the strand regions are underlined. The invariant residues Glu387, Arg390, and Cys470 are indicated by arrows.

labs (in the case of *Aci*I), and assays were performed as recommended by the supplier.

Analysis of the CYP1B1 *Primary Structure by Comparative Sequence Alignment*

The amino acid sequences of 22 cytochrome P450 proteins belonging to CYP families 1–5, 7, 11, 17, and 21 were obtained from SwissProt and GenBank (Nelson et al. 1993). Computer-assisted sequence alignment was performed with the pattern-induced multi-sequence alignment program (PIMA) (Smith and Smith 1992; the program is available at http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html).

Construction of a Three-Dimensional Model of the Conserved C-Terminal Half of the CYP1B1 *Gene by Homology Modeling*

A three-dimensional model of the conserved C-terminal part of *CYP1B1* was constructed by homology modeling with the assistance of the protein-modeling tool ProMod (Peitsch 1996). There are two reasons to expect that this approach could produce a realistic model for the C-terminal part of the CYP1B1 protein. First, three-dimensional models based on crystallization and structural-resolution studies of several soluble bacterial P450s are available and can be used as templates (Poulos et al. 1987; Ravichandran et al. 1993; Hasemann et al. 1994). Second, all cytochrome P450 molecules share a set of conserved structural elements. Included in these structures are four helix bundles (helices D, I, and L and the antiparallel helix E), helices J and K, β-sheets 1 and 2, the heme-binding region, and the "meander" region just N-terminal of the heme-binding domain. Most of these regions are located at the C-terminal half of the molecule and are expected to be involved in the heme-binding and proper folding of the molecule (Graham-Lorence and Peterson 1996). The modeling was performed as follows: First, the complete protein sequence of CYP1B1 was screened against the ExPdb structure database (derived from the Brookhaven Protein Data Bank), in order to identify a template structure appropriate for modeling. From a series of templates, we selected the three-dimensional models for *CYP102* (P450BM-P bacterial fatty acid monooxygenase from *Bacillus magaterium*) and *CYP108* (P450TERP catalyzes the hydroxilation of a-terpineol), because, first, both of them were based on a crystallographic analysis, and, second, *CYP102* is considered to be the best model for eukaryotic P450s. The multiple sequence/structure alignment of *CYP1B1, CYP102*,, and *CYP108* generated during the first attempt and the ProMod command files were resubmitted for refinement of the primary model, by energy minimization with CHARMM (Peitsch 1996). No manual editing of this alignment was made, because the landmark residues Glu387, Arg390, and Cys470, which are absolutely invariant among all members of the cytochrome P450 family, were properly aligned. Also, the location of different helices, strands, and loops was overlapping with *CYP1B1* secondary structure, as predicted by the profile-fed neural-network system (Rost and Sander 1993), on the basis of sequence alignment of 84 different cytochrome P450s (data not shown).

Expression of the Cytochrome CYP1B1 *Gene in Human Ocular and Nonocular Tissues*

Expression of the *CYP1B1* gene in human ocular and nonocular tissues was studied by northern hybridization. Total RNA extracted from ocular tissues of a pair of eyes from a 55-year-old donor (cadaver) and from a human cell line established from nonpigmented cilliary epithelium (Martin-Vasallo et al. 1989) was size fractionated by electrophoresis on denaturing 1% agarose-formaldehyde gels and was transferred to a Nytran filter. Radiolabeled probe was prepared from a 1,631-bp PCR fragment amplified from cDNA (GenBank accession

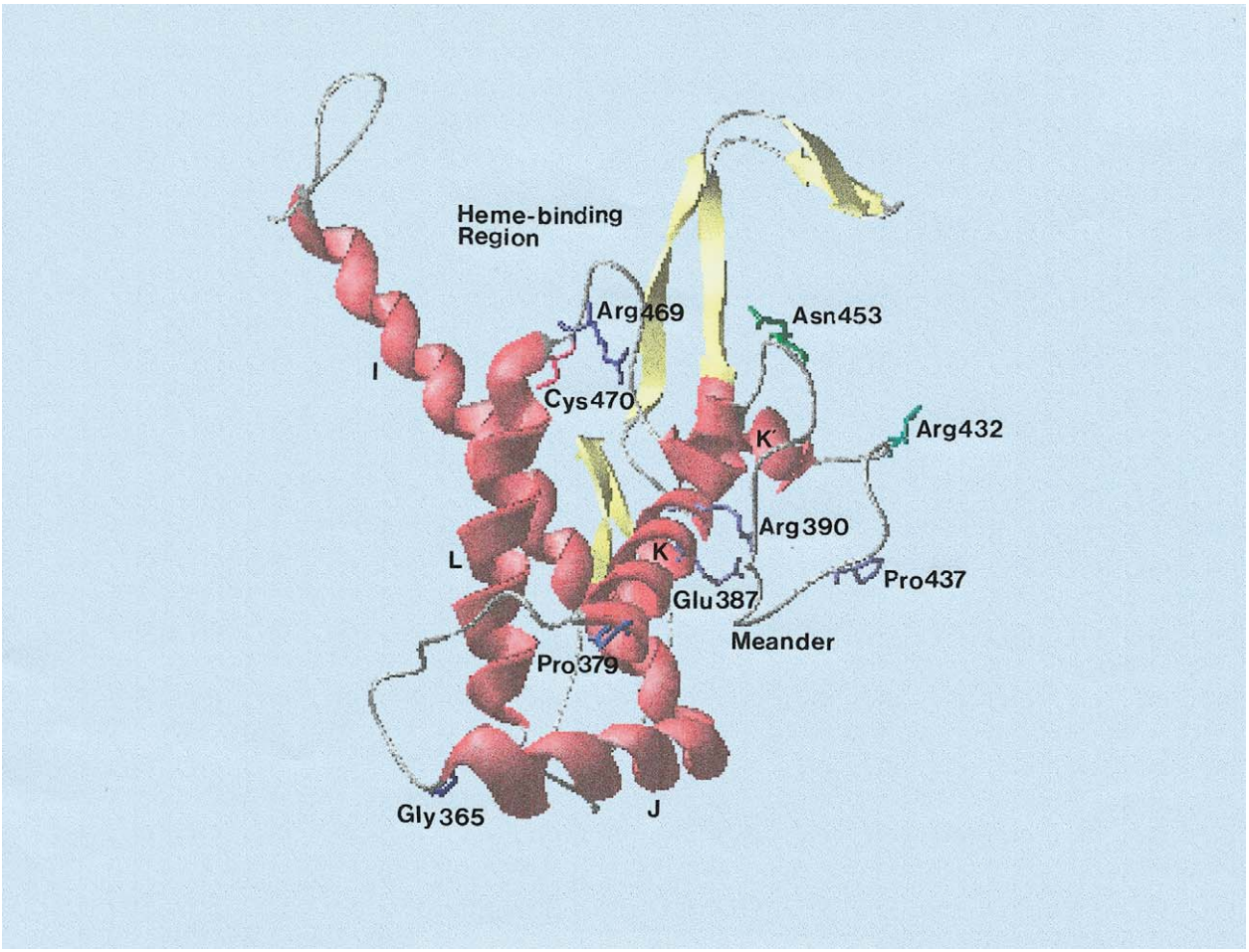

Figure 5 Three-dimensional model of the conserved C-terminal of the CYP1B1 protein. The α helices are indicated by capital letters and are red; β strands are yellow; and random coils are gray. The side chains of the amino acid residues affected by mutations are blue. The side chains of the polymorphic residues are green. The invariant Cys470 also is red.

number V03600) and containing the complete coding sequence of the *CYP1B1* gene. Expression of this gene in nonocular tissues was studied by use of the same probe and a premade blot (Clontech) containing poly(A)+ mRNA (2 μg/lane) from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

Results

CYP1B1 *Mutations*

Sequencing analysis of the *CYP1B1* translated regions in 22 PCG families resulted in identification of 16 mutations, 13 of which are novel and 3 of which have been described elsewhere (table 1 and fig. 2; Stoilov et al. 1997). Eight (50%) of these mutations were predicted to truncate the *CYP1B1* open reading frame; they include one nonsense mutation (Glu281→Stop), six frameshift mutations (847insT, 1209insC, 1410del13, 1546dup10, 1691delG, and 1749dup27), and one large deletion. We also detected eight missense mutations: Trp57→Cys, Gly61→Glu, Gly365→Trp, Pro379→Leu, Glu387→ Lys, Arg390→His, Pro437→Leu, and Arg469→Trp. All of the mutations described above segregated with the disease phenotype in one or more PCG families (fig. 1) and were not present in 200 chromosomes from randomly selected normal individuals. The segregation of the mutant *CYP1B1* alleles was consistent with autosomal recessive inheritance of the disease in all families investigated. An interesting instance was observed in family 1007, in which the affected children were homozygous for Gly365→Trp, whereas their mother was heterozygous for the same mutation. The father, however, after several rounds of sequencing, was found to be homozygous for the wild-type allele. In order to assess the question of nonpaternity in this pedigree, we also genotyped a total of 28 highly polymorphic

Table 2

Polymorphisms in *CYP1B1*

Location	Codon	Allele		Predicted Effect	Frequency in 100 Normal Individuals (%)		
		Wild Type[a]	New		Wild Type/Wild Type	Wild Type/New	New/New
Intron 1	−13[b]	cctctctctg	cctctttctg	Not applicable	52	39	9
Exon 2	48	CGG	GGG	Arg48→Gly	51	40	9
Exon 2	119	GCC	TCC	Ala119→Ser	51	40	9
Exon 3	432	GTG	CTG	Val432→Leu	11	35	54
Exon 3	449	GAT	GAC	silent	10	39	51
Exon 3	453	AAC	AGC	Asn453→Ser	59	34	7

[a] Alleles identical to the published cDNA sequence of *CYP1B1* (Sutter et al. 1994) are indicated as wild type. These data are based on analysis of 100 normal individuals, of which 50 were British and 50 were Turkish; the data from this two groups were combined because they showed remarkably identical allelic frequencies.

[b] Upstream from ATG.

markers from the X and Y chromosomes and from the autosomes. For the X-linked markers, the father and daughter shared an identical haplotype, whereas, for the Y-linked markers, the father and son shared another haplotype. Also, none of the autosomal markers showed any evidence of incompatibility in this pedigree. Therefore, since we have found no evidence for nonpaternity, and since the DNA samples used for the screening were prepared from peripheral blood, we interpreted this as a case of germinal mosaicism.

Evaluation of the Mutated Positions by Multiple Sequence Alignment and Homology Modeling of CYP1B1

Comparative amino acid sequence alignment of 22 different cytochrome P450 proteins revealed that all missense mutations had occurred at highly conserved positions (fig. 3). Six mutations (Gly61→Glu, Gly365→Trp, Pro379→Leu, Glu387→Lys, Arg390→His, and Pro437→Leu) affected positions conserved among at least 19 of the 22 different cytochromes analyzed. Furthermore, the position affected by Trp57→Cys is occupied by hydrophobic residue in 19 of 22 cytochrome P450 sequences analyzed, with tryptophan presented in all members of the *CYP1B1* family. Finally, the Arg469→Trp mutation affects a position that immediately precedes the invariant Cys470. It is interesting that, in contrast to the other *CYP* families, in which this position is occupied by a neutral amino acid, in the *CYP1* family it is occupied by either arginine or lysine, both of which are basic and positively charged.

The fact that all missense mutations affected positions conserved among different members of the P450 superfamily indicates that, most probably, they are interfering with some universal properties of the cytochrome P450 molecule, such as heme binding. This was further supported when the mutated positions were matched to conserved structural elements shared by cytochrome P450

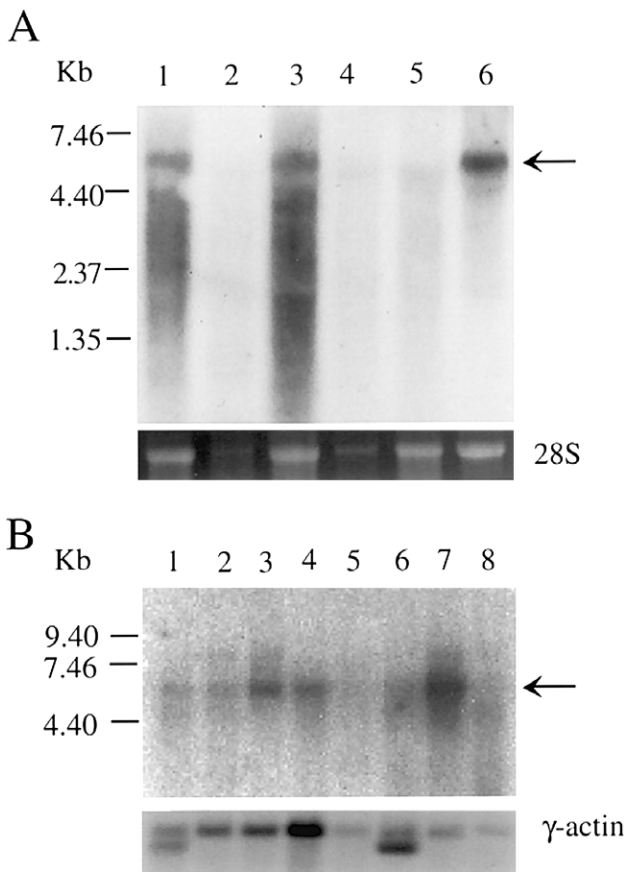

Figure 6 Northern analysis of *CYP1B1* expression. *A,* Normal ocular tissues: cilliary body (lane 1), cornea (lane 2), iris (lane 3), retinal-pigment epithelium (lane 4), retina (lane 5), and nonpigmented ciliary epithelial cell line (lane 6). Normal eyes were enucleated 2–3 h after death, and RNA was extracted ⩽24 h after death. *B,* Nonocular tissues: heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). In both *A* and *B,* the arrow on the right indicates the position of the main transcript hybridized; its size is expected to be 5.1 kb. RNA-molecular-weight markers are given on the left.

Table 3

Haplotype Analysis of PCG Chromosomes Harboring Recurrent Mutations Gly61→Glu and Arg469→Trp

	No. (Source) of Mutations[a]									
	Gly61→Glu					Arg469→Trp				
	16 (M)	18 (P)	21 (P)	21 (M)	24 (P)	18 (M)	27 (P)	27 (M)	29 (P/M)	4182 (P)
D2S1788	4	5	11	10	7	11	12	12	2	4
D2S1325	5	4	4	5	2	4	4	4	5	…
D2S177	5	2	3	7	3	5	4	5	9	5
D2S1346	5	4	4	8	4	5	4	4	5	6
D2S1348	…	3	2	6	3	3	4	4	5	…
D2S1356	3	4	6	6	1	7	2	2	3	…

[a] M = maternal; and P = paternal.

molecules. Two mutations, Gly61→Glu and Trp57→Cys were predicted to affect the proline- and glycine-rich region that follows the N-terminal transmembrane domain. This "hinge" region is characteristic of the microsomal cytochrome P450s. Its predicted function is to permit flexibility in connection between the membrane-spanning domain and the cytoplasmic portion of the molecule (Yamazaki et al. 1993). Induced mutations in the hinge region have previously been reported to interfere with the proper folding and heme-binding properties of the cytochrome P450 molecules (Yamazaki et al. 1993; Chen and Kemper 1996). The remaining six missense mutations were located in the 5′ end of exon 3 and directly affected the elements of the conserved core structures (CCS): helix J (Gly365→Trp), helix K (Pro379→Leu, Glu387→Lys, and Arg390→His), the meander region (Pro437→Leu), and the heme-binding region (Arg469→Trp) (figs. 4 and 5). The heme-binding region is also predicted to be eliminated by every one of the truncating mutations described above.

Polymorphisms in the CYP1B1 *Gene*

Sequence analysis of the translated regions in 50 Turkish and 50 British normal unrelated individuals resulted in the identification of six polymorphic sites (table 2). These positions were characterized by a high degree of variability (fig. 3) and mapped outside the CCS (fig. 5).

Analysis of CYP1B1 *Expression*

An abundant mRNA target for the *CYP1B1* probe was observed in the ciliary body and iris and in the nonpigmented ciliary epithelial cell line (fig. 6). The amounts of mRNA target in the cornea, retinal-pigment epithelium, and retina were much lower. Elsewhere we have demonstrated the presence of *CYP1B1* mRNA in the trabecular-meshwork cell line, by quantitative PCR amplification (Stoilov et al. 1997). Relatively low constitutive levels of *CYP1B1* mRNA were detected in most of the nonocular tissues studied, with the kidneys demonstrating relatively higher levels of expression. This pattern of nonocular expression is in agreement with results reported elsewhere (Sutter et al. 1994).

Discussion

CYP1B1 is the only known member of the cytochrome P450 subfamily of *CYP1B*. The human gene, which, in a previous study (Tang et al. 1996), had been mapped to the 2p21-22 region, consists of three exons (one of which is noncoding) and two introns (Tang et al. 1996; Stoilov et al. 1997). Because of its map position, *CYP1B1* was considered as a candidate gene for PCG (GLC3A), which, in a previous study, had been linked to the 2p21 region (Sarfarazi et al. 1995). Recently, we reported three truncating mutations, in the *CYP1B1* gene, that segregated with the disease phenotype in five PCG families (Stoilov et al. 1997). These data strongly suggested that mutations affecting *CYP1B1* are responsible for the PCG phenotype that is associated with the GLC3A locus.

This article has described the identification and characterization of 22 allelic variants of the human *CYP1B1* gene. Sixteen of these segregated with the disease phenotype in one or more PCG families and were not present in 200 chromosomes from randomly selected normal individuals. Since these mutations were predicted either to truncate the protein or to substitute exclusively highly conserved residues with an established functional importance, they were considered to be disease-causing mutations (table 1). Six allelic variants (1) showed no relation to the disease phenotype in the affected families, (2) were observed in the general population and (3) affected exclusively poorly conserved positions. These changes were considered to be naturally occurring polymorphisms (table 2).

Members of these 22 PCG families have identified their ethnic origin as Turkish, British, French Canadian, German American, Pakistani, or Hispanic. Recently, linkage to locus GLC3A has been reported, both in families from Saudi Arabia (B. A. Bejjani and J. R. Lupski,

personal communication) and in the Gypsy population (Roms) of Slovakia (Plasilova et al. (1998)). Taken together, these data indicate that mutations in the *CYP1B1* gene may be responsible for a substantial number of PCG cases, both in the high-incidence regions of the Near East and Middle East and in the relatively low-incidence regions of Europe and North America. However, a much lower proportion of the PCG cases are also caused by mutations in at least two other unknown genes, one of which (i.e., the GLC3B locus) is known to be located on 1p36 (Akarsu et al. 1996).

The 16 mutations identified in this study are part of a diverse group of DNA rearrangements and include 8 missense mutations, 1 nonsense mutation, 6 frameshifts (deletions, insertions, or duplications), and 1 large deletion. Eleven mutations (68.75%) were observed on a single PCG-bearing chromosome. Five mutations (Gly61→Glu, 1410del13, Glu387→Lys, 1546dup10, and Arg469→Trp) either were recurrent, in which case they may have originated from a single ancestral mutation event, or arose independently more than once. In the Turkish population, association of both Gly61→Glu and Arg469→Trp with at least three different alleles for the closest flanking markers, D2S177 and D2S1346 (table 3), indicated that these mutations were most probably introduced by several independent mutational events.

Segregation of the mutant *CYP1B1* alleles was consistent with the autosomal recessive inheritance of the disease, in all the families studied. Two mutant alleles were inherited by every affected individual. Their normal siblings either had two normal alleles or were heterozygous for a single mutant allele segregating in that particular family. The penetrance of the disease was 100%, and no phenocopies were observed.

Currently, cDNAs for >300 cytochrome P450 have been cloned and sequenced (Nelson et al. 1993), and three-dimensional models based on crystallization and structural-resolution studies of several soluble bacterial P450s also have become available (Poulos et al. 1987; Ravichandran et al. 1993; Hasemann et al. 1994). Therefore, we utilized these resources and evaluated the effects of the observed mutations in the context of understanding the structure and function of the cytochrome P450 molecule.

Truncating Mutations

The mutations predicted to truncate the *CYP1B1* open reading frame are expected to eliminate 79–377 amino acids from the -COOH terminus of the *CYP1B1* polypeptide. Therefore, if a stable protein is synthesized, then every one of the mutant molecules would lack at least the heme-binding region, which is essential for the function of the cytochrome P450 molecules. We therefore expect that these mutations will result in functional null alleles. Alternatively, these mutations may interfere with RNA metabolism by the nonsense-mediated mRNA-decay mechanism (Baserga and Benz 1988).

Missense Mutations

Trp57→Cys.—The position affected by this mutation is located in the hinge region. In CYP1B1 protein this region has the sequence 51PPGPFAWP58. Substitution of the proline residues in the corresponding segments of *CYP2C11* and *CYP2C2* produced defective heme incorporation and reduced enzymatic activity (Yamazaki et al. 1993; Chen and Kemper 1996). Trp57→Cys affects the position preceding the last proline residue. There is a hydrophobic residue at this position in 19 of 22 *CYP* sequences analyzed by us, with tryptophan being present in all *CYP1B1* molecules. It is possible that the substitution of nonpolar and hydrophobic tryptophan by polar cysteine interferes with the proper folding of the *CYP1B1* molecule, in a manner similar to that suggested for the flanking hydrophobic proline residues. Identification of Trp57→Cys confirms the importance of the hinge region in the normal functioning of the cytochrome P450 molecule, as has been suggested by earlier studies. It also indicates, however, that proline residues are probably not the sole determinants of the hinge region.

Gly61→Glu.—This mutation (fig. 7*B*) is located 3 amino acids downstream from the last proline residue of the hinge region as defined by Yamazaki et al. (1993). Although both proline and glycine residues are found in this region, Yamazaki et al. concluded that folding of the P450 molecule seems to depend mainly on the proline residues, because, first, they are more conserved, and, second, their substitution results in a complete loss of heme incorporation. However, no functional studies on glycine mutants have been reported. Also, according to multiple sequence alignments provided both in the present article and in previous reports (Yamazaki et al. 1993; Chen and Kemper 1996), the glycine residue affected by Gly61→Glu is one of the most highly conserved positions in this region. It is, then, quite possible that Gly61 is a functional part of the hinge region and acts in a way similar to that described for the induced mutants in this region.

Gly365→Trp and Pro379→Leu.—These two mutations affect highly conserved positions marking the end and beginning of helix J and helix K, respectively (fig. 5). Both glycine and proline are potential turns and helix breakers frequently observed at the N- and C-termini of a helical structures. Their substitution by a residue lacking such properties would most probably affect the orientation of helices J and K, both of which belong to the CCS.

Arg390→His and Glu387→Lys.—These two residues

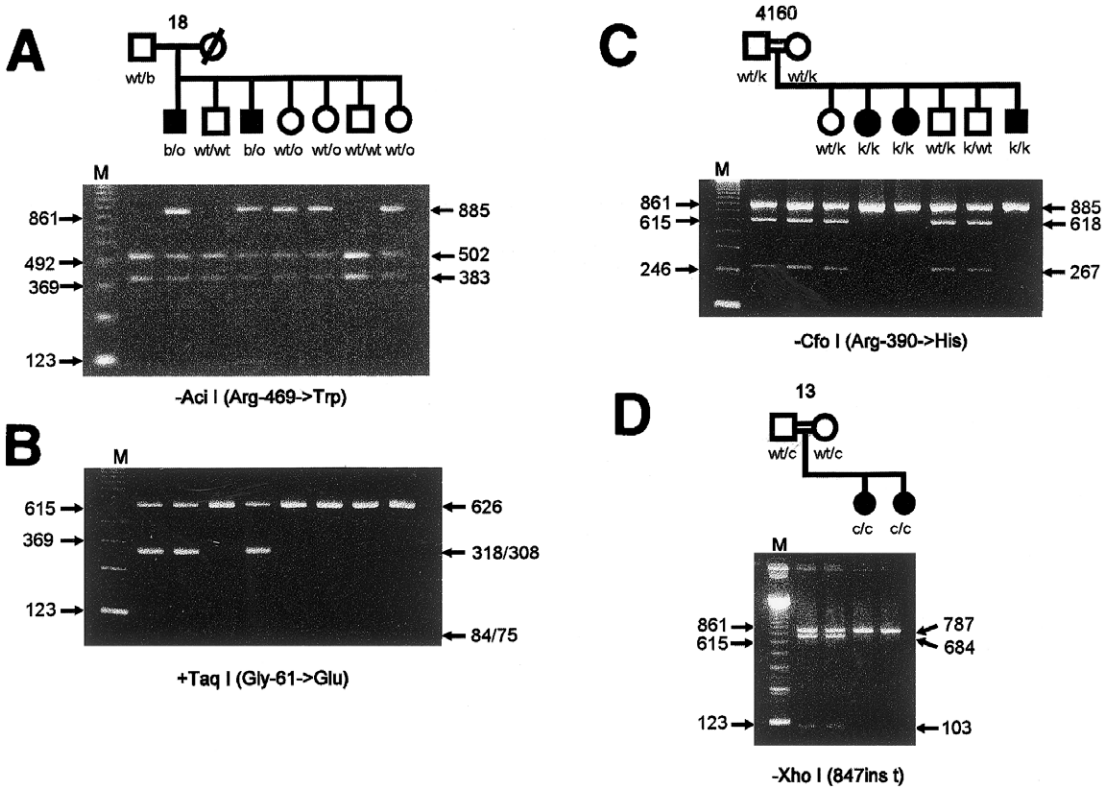

Figure 7 Restriction-endonuclease assays for Arg469→Trp (*A*), Gly61→Glu (*B*), Arg390→His (*C*), and 847insT (*D*). Sizes of the marker bands (in nucleotides) are given on the left side of each panel, whereas allele sizes are given on the right side of each panel. Sizing was performed with a 123-bp ladder (GibcoBRL). Restriction-endonuclease digestion was performed according to the manufacturer's instructions, and the samples were analyzed on 2% agarose gel. The genotypes are defined in terms of the one-letter code used in figure 1.

are located in the conserved helix K. They form the consensus sequence GluXXArg, which is absolutely conserved among all members of the cytochrome P450 superfamily. Arg390 (fig. 4) and Glu387 (fig. 4) are one helical turn apart and are predicted to form a salt bridge. The parallel orientation of their side chains is more transparent in the three-dimensional model (fig. 5). The exceptional conservation of this motif indicates that it is essential for the normal function of the P450 molecule. However, it is still unclear exactly what this function is.

Pro437→Leu.—This mutation is located in the meander region (fig. 5; Graham-Lorence and Peterson 1996), which precedes the heme-binding region. This mutation affects the first proline residue from the proposed consensus sequence xPcxFxPE+a (x = any amino acid; a = aromatic residue; c = charged residue; and + = positively charged residue).

Arg469→Trp (fig. 7*A*).—The cysteine in the heme-binding region (Cys470 in *CYP1B1*), which provides the axial heme ligand, is absolutely conserved among members of the cytochrome P450 superfamily (fig. 3). In the three-dimensional model shown in figure 5, this residue is colored red. The arginine affected by Arg469→Trp immediately precedes this invariant Cys470. We analyzed 86 different P450 sequences and found no sequence in which the invariant cysteine is preceded by tryptophan. It is possible that the large and rigid side group of tryptophan interferes with the normal conformation of the cysteine pocket, which contains a number of highly conserved positions, including Phe463 (part of the cysteine pocket in P450cam—5-exo-hydroxylase of camphor), Gly466 (which initiates a hairpin turn) and Arg468 (which interacts with the heme propionate group).

In conclusion, our data indicate that mutations identified in this study most probably interfere with the heme-binding ability of the *CYP1B1* molecule, either by deleting the heme-binding region or by affecting discrete components of the hinge region and/or the CCS, which are determinants for the proper folding and heme-binding ability of the cytochrome P450 molecules.

Expression of CYP1B1 *in Ocular and Nonocular Tissues*

Previous studies detected *CYP1B1* mRNA in most of the nonocular tissues investigated (Sutter et al. 1994).

Also, tumor-specific expression of CYP1B1 protein has been reported recently (Murray et al. 1997). It has also been demonstrated that the *CYP1B1* promoter is constitutively active (Wo et al. 1997). In the course of this study the presence of *CYP1B1* mRNA in different ocular tissues was analyzed at the mRNA level by northern hybridization (fig. 6). Strong signals were generated from tissues representing the anterior uveal tract of the eye: the cilliary body, the nonpigmented ciliary epithelium, and the iris. These structures have highly specialized functions, including accommodation, regulation of outflow facility, and formation of aqueous humor. *CYP1B1* can influence these processes by participating in the metabolism of regulatory molecules such as steroids and derivatives of archidonic acid. Such molecules could be synthesized locally or could delivered to the cilliary body via the bloodstream. It has been shown that *CYP1B1* can catalyze the 4-hydroxylation of 17β-estradiol (Hayes et al. 1996). Unfortunately, apart from their importance in the excretion of E1 and E2, the physiological role for hydroxyestrogens remains unclear. Therefore, further studies are required for an understanding both of the true nature of substrates being metabolized by the *CYP1B1* gene and of their involvement in the overall development of the eye.

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