Isolation and Characterization of the Human Cytochrome P450 CYP1B1 Gene*

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Previously, we identified a novel human cytochrome P450 cDNA that is inducible by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and represents the first member of a new subfamily designated cytochrome P4501B1 (CYP1B1; Sutter, T. R., Tang, Y. M., Hayes, C. L., Wo, Y. P., Jabs, E. W., Li, X., Yin, H., Cody, C. W., and Greenlee, W. F. (1994) J. Biol. Chem. 269, 13089–13099). Here, we report on the isolation and initial characterization of the CYP1B1 gene. The CYP1B1 gene maps to human chromosome 2 at 2p21–22 and contains three exons and two introns. The putative open reading frame starts in the second exon and is 1629 base pairs in length. Southern analysis using DNA probes directed to each of the three exons confirms that CYP1B1 is a single copy gene. Human CYP1B1 differs from its two most closely related members of the cytochrome P450 superfamily, CYP1A1 and CYP1A2, in the number of exons (3 versus 7) and chromosome location (2 versus 15). A single transcription initiation site was identified by primer extension analysis and S1 nuclease mapping. Based on nucleotide sequence analysis, the CYP1B1 gene lacks a consensus TATA box in the promoter region and contains nine TCDD-responsive enhancer core binding motifs (5'-GGCTG-3') located within a 2.5-kilobase pair genomic fragment 5'-ward of the transcription initiation start site. Deletion analysis of chloramphenicol acetyltransferase reporter gene constructs containing 5' CYP1B1 genomic fragments indicates that a region from −1022 to −835 containing three of the nine core binding motifs contributes to the TCDD-inducible expression of CYP1B1.

The expression of genes encoding proteins involved in the metabolism of xenobiotics, acute inflammatory responses, management of oxidative stress, and growth regulation is al-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U56438.

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1 The abbreviations used are: TCDD/dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, Ah receptor; ARNT, AhR nuclear translocator; bp, base pair(s); CAT, chloramphenicol acetyltransferase; DRE, dioxin-responsive enhancer; kb, kilobase pair(s); PCR, polymerase chain reaction; TIS, transcription initiation site.

2 In accordance with the rules recommended by the P450 Nomenclature Committee, for all cytochrome P450s referred to in this report, the italicized CYP will be used for the gene and cDNA, and the nonitalicized CYP will be used for the mRNA and protein. P450 families are designated by an Arabic number, and subfamilies are designated by a letter. Multiple subfamily members are indicated by an Arabic number that follows the subfamily letter designation (e.g. CYP1A1 is the first A subfamily member of the CYP1 family).
genomic DNA fragment that contains the entire CYP1B1 gene and includes both 5′- and 3′-flanking sequences.

**EXPERIMENTAL PROCEDURES**

**Materials**—A human genomic library prepared from the human lung fibroblast WI-38 cell line and a SequeMan 2.0 sequencing kit were purchased from Promega (Madison, WI). An in situ hybridization kit was purchased from Oncor Inc. (Gaithersburg, MD).

**Isolation of Genomic Clone**—The human genomic library was screened using standard methods (17). The probe used in library screening was a 489-bp PCR-amplified cDNA fragment located at the 3′-end of a cDNA clone, p128 (7). The probe was labeled with 32P-dCTP at a specific activity of 10^8 cpm/μg by nick translation (18). Prehybridization and hybridization were conducted at 40°C for 4 h and 16 h, respectively, in a solution containing 5 mM NaCl, 0.1% SDS, 100 mg/ml denatured salmon sperm DNA, and 30% formamide. The blots were washed twice for 30 min in 6× SSC (1× SSC, 0.15 M sodium citrate, pH 7.0), 0.1% SDS at room temperature, then washed for 30 min in 0.5× SSC, 0.1% SDS at 42°C, and subjected to autoradiography.

**DNA Sequence Analysis**—Phage DNA clone was isolated (19) and digested separately with XhoI, XbaI, or SalI. The digested fragments were subcloned into either pBlueScript II or pUC18 vectors for double-stranded sequencing. Plasmid DNA was prepared (20, 21), and the DNA sequence of both strands of each subclone was determined by the dideoxynucleotide chain termination method (22). In order to obtain the sequence information in the highly GC-rich region at the 5′-end of the genomic clone, a series of nested deletion subclones were constructed (23).

**Southern Hybridizations**—Southern hybridizations were performed according to a standard protocol (17), using 10 μg of genomic DNA. Three DNA fragments corresponding to each exon were labeled by nick translation to a specific activity of 1× 10^9 cpm/μg DNA with [α-32P]dCTP and used as hybridization probes. The hybridizations were performed at 42°C for 16 h in a solution containing 6× SSC, 0.5% SDS, 100 μg/ml denatured fragmented salmon sperm DNA, 5 × Denhardt’s solution, 50% formamide, and 5.5× 10^6 cpm/ml probe. The blots were washed at 55–68°C for 30 min in 0.1 × SSC, 0.1% SDS and subjected to autoradiography.

**High Resolution Chromosome Mapping**—Slides with chromosome spreads were prepared from normal male lymphocytes cultured with bromodeoxyuridine (24). A DNA fragment in a genomic subclone (XbaI 4) containing an 8-kb fragment at the 5′-end of human CYP1B1 gene was nick translation-labeled with biotin-14-dATP (Life Technologies, Inc.). The efficiency of incorporation was about 38–47%, as determined by tritium tracer incorporation. Fluorescence in situ hybridization was performed as described (25), with modification. Probe mix (2 × SSCP, 50% formamide, 10% dextran sulfate, 12.5 ng/μl biotinylated probe, 0.5 μg/ml human Cot-1 DNA to suppress repeated sequences, and 0.5 μg/ml salmon sperm DNA) was denatured at 70°C for 5 min, preannealed at 37°C for 30 min, placed onto the slides, and hybridized at 37°C overnight. Slides were washed in 65% formamide, 2× SSC at 43°C for 20 min and two changes of 2× SSC at 37°C for 5 min each. Biotinylated probe was detected with fluorescein isothiocyanate-avidin and amplified with biotinylated anti-avidin, using reagents from the in situ hybridization kit in accordance with the manufacturer’s instructions.

**Cell Treatment and RNA Preparation**—Human SCC12(c12c2) cells were cultured as described previously (2). At 90% confluence, the cells were grown for 24 h in Dulbecco’s modified Eagle’s medium containing 50 μg/ml gentamicin and 0.1 μg/ml hCG. The (CAT) reporter gene. All constructs were named according to the position of their insert fragments relative to the TIS; for example, pCAT-basic(−2300 to +25). To generate the plasmids pCAT-basic(−1356 to +25), (−1022 to +25), (−642 to +25), and (−474 to +25), a DNA fragment in the construct pCAT-basic(−2300 to +25) was removed by a HindIII in (the vector) and a SacI (at −154) restriction digest. This fragment was replaced by HindIII/SacI fragments with various lengths (−1356 to −154, −1022 to −154, −642 to −154, and −474 to −154) from a series of plasmids that were obtained by an exonuclease III nested deletion of a pUC18 subclone containing a SacI/SacI insert of a CYP1B1 sequence spanning nucleotides 154 to 1420. Plasmid pCAT-basic(−154 to +25) was created by treating the HindIII/SacI-digested pCAT-basic(−2300 to +25) with mung bean nuclease and religated with T4 DNA ligase.

**Plasmid Constructs for 5′ Mapping**—In order to generate 5′-progressive deletions of the human CYP1B1 gene, a sequence spanning −2300 to +1500 (relative to the transcription start site) was excised with PsI from a pUC18 genomic subclone, Xba I, and cloned into the PsI site of promoterless plasmid pCAT-basic (Promega). The plasmid containing the insert in the proper orientation was linearized by XbaI digestion, blunted with mung bean nuclease, digested with HindIII, and religated. The resulting construct contained the CYP1B1 sequence from −2300 to +25 upstream of the chloramphenical acetyltransferase (CAT) reporter gene. All constructs were named according to the position of their insert fragments relative to the TIS; for example, pCAT-basic(−2300 to +25). To generate the plasmids pCAT-basic(−1356 to +25), (−1022 to +25), (−642 to +25), and (−474 to +25), a DNA fragment in the construct pCAT-basic(−2300 to +25) was removed by a HindIII in (the vector) and a SacI (at −154) restriction digest. This fragment was replaced by HindIII/SacI fragments with various lengths (−1356 to −154, −1022 to −154, −642 to −154, and −474 to −154) from a series of plasmids that were obtained by an exonuclease III nested deletion of a pUC18 subclone containing a SacI/SacI insert of a CYP1B1 sequence spanning nucleotides 154 to 1420. Plasmid pCAT-basic(−154 to +25) was created by treating the HindIII/SacI-digested pCAT-basic(−2300 to +25) with mung bean nuclease and religated with T4 DNA ligase.

**Prime Extension Analysis**—The primer extension analysis was performed according to a standard protocol (17), using an oligonucleotide primer designated T49 (5′-TGGCAAAAGTCGAGGTTTCCTACAGCGTGAGG-3′). One pmol of the 32P-labeled primer (8.8× 10^4 cpm/pmol) was mixed with 150 μg of RNA and precipitated with 2 volumes of ethanol. The hybridization was carried out at 30°C for 16 h in 80% formamide. The extension reaction of primer-RNA complex was performed at 42°C for 1 h using 200 units of reverse transcriptase and then analyzed on an 8% polyacrylamide-urea gel and isolated (28). S1 nuclease mapping was performed according to the procedure of Berk and Sharp (29).

**Primary Constructs of 5′-Mapping**—In order to generate 5′-progressive deletions of the human CYP1B1 gene, a sequence spanning −2300 to −1092, and −2783 to −481 with various 3′-end deletions of the 5′-flanking region of CYP1B1 was inserted into the complete 3′-end deletion vector of pCAT-promoter (Promega). Subclone Xba I (for the fragment −3044 to −1092) and XbaI (for the fragment −3044 to −1092) and by digesting Xba I with ApIII, filling in with Klenow fragment, and cleaving with XhoI (for the fragment −3044 to −1092). The isolated fragment was then inserted into the pCAT-promoter, which had been digested with PsI, blunted-ended with mung bean nuclease, and subsequently digested with XbaI. A DNA fragment containing the sequence from −1022 to −835 was amplified by PCR using a pair of primers carrying BamHI recognition sites. After digestion with BamHI, the fragment was then inserted into the BamHI-digested pCAT-promoter to construct pCAT-promoter(−1022 to −835). The extension and nucleotide sequence of the insert were determined by DNA sequencing.

**Cell Culture and Transient Transfection**—Cultures of the human squamous cell carcinoma line SCC12(c12c2) were plated at 2.5× 10^5 to 5× 10^5 cells/60-mm dish and grown to 70–90% confluence before transfection. The transient transfection was performed by a lipofection method (30) in a serum-free medium (RBM, Clonetix) with 2.5 μg of each plasmid and 2.5 μg of pCD10, a f-galactosidase expression vector. After incubation for 24 h, the lipofection solution was replaced with Dulbecco’s modified Eagle’s medium, and the transfectants were treated with 10 μM TCDD in dimethyl sulfoxide (0.1%) or dimethyl sulfoxide alone for another 24 h. The cells were resuspended in 0.25 μl Tris-HCl (pH 7.8) and 1 μl EDTA and lysed by five frozen/thaw cycles. Extracts were clarified by centrifugation (5 min, 12,000× g),
assayed for β-galactosidase (31) and CAT (32) activities, and normalized for transfection efficiency.

RESULTS

Isolation and Mapping of the CYP1B1 Gene—Approximately 950,000 bacteriophage plaques of a human genomic DNA library were screened with a human CYP1B1 489-bp cDNA probe (Fig. 1). Two clones, designated GP48 and GP49, containing the same and largest insert (18 kb) were isolated. A region of 12 kb in clone GP48 was sequenced in both strands and found to contain DNA corresponding to the entire 5.1-kb CYP1B1 cDNA sequence and 3.0 kb of DNA upstream (5′-ward) to the cDNA sequence (Fig. 1).

Comparison of genomic and cDNA sequences for CYP1B1 identified three exons (371, 1044, and 3707 bp in length) and two introns (390 and 3032 bp) (Fig. 1). This contrasts with the seven exons reported for the human CYP1A1 (33) and CYP1A2 (34) genes. The coding region of the CYP1B1 gene starts at the 5′-end of the second exon and ends within the last exon. The observation that the open reading frame begins within the second exon distinguishes the CYP1 family from all other cytochrome P450 superfamily members (35) and supports the previous designation (based on amino acid sequence homology) of CYP1B1 as a new CYP1 subfamily (7).

Both introns begin with the sequence GT... and end with the sequence ... AG, consistent with the GT...AG rule of intron/exon border sequences (36). The regions upstream of the 3′-end of the introns are pyrimidine-rich, and a putative lariat formation consensus sequence (Py-X-Py-T-Pu-A-Py) was identified in 6 out of 7 bases located 20–40 nucleotides upstream from the 3′-end of each intron. The presence and location of these lariat sequences are typical features of most introns (37).

Initial evidence using a single cDNA probe suggested that the human CYP1B1 subfamily did not contain multiple genes (7). Here, we have extended this analysis to include cDNA probes representing nucleotide sequences located within each of the three exons (Fig. 2). Human genomic DNA was digested with different restriction enzymes, and four samples were hybridized separately with each cDNA probe. A single band was observed for each sample (Fig. 2). These results exclude the existence of pseudogenes and indicate that the CYP1B1 gene is likely the only member of this subfamily.

High Resolution Chromosome Mapping—PCR mapping of human/rodent somatic cell hybrid panels using specific primers to the 3′-untranslated region of the CYP1B1 cDNA indicated that this gene was on human chromosome 2 (7). In this report, we have confirmed and extended these results by high resolution chromosome mapping. Twenty-eight well banded metaphases were photographed. After hybridization under conditions previously optimized on unbanded samples, 11 metaphase cells (39%) were found to have at least one pair of signals (involving both chromatids of a single chromosome). A total of 19 paired signals were seen; 15 (80%) were located midway on the p arm of chromosome 2, and 4 were located on other chromosomes. To determine the specific band location of these signals, the hybridized metaphases were rephotographed, and the color slides were aligned with the banded
photographs. Signals were located on bands 2p21–22, with most on 22 and the 21–22 band interface (Fig. 3). No other site had more than one signal.

Identification of the TIS Site—Primer extension analysis and S1 nuclease mapping were used to identify the TIS site of CYP1B1. Both methods identified a single band of the same size (Fig. 4). Comparison with a DNA sequence ladder indicated that the TIS was at an adenine residue located 27 nucleotides downstream from a TATA-like box (Fig. 5). Additional primer extension studies with two other primers and S1 nuclease mapping with two other probes confirmed that this adenine represented the only TIS for CYP1B1 (data not shown).

Initial Mapping of the CYP1B1 Regulatory Region—A 3-kb 5'-flanking region of the human CYP1B1 gene contains nine DRE recognition motifs (5'-GCGTG-3') (Fig. 5), previously shown to be required for the specific binding of the AhR/ARNT heterodimer, which is formed in response to the activation of AhR by TCDD (3, 38). Six of these motifs are located on the antisense strand (Fig. 5); however, none are contained within an enhancer sequence that completely matches the proposed consensus sequence (5' (T/G)NGCGTG(A/C)(G/C)A 3') for a functional DRE (38). In order to determine if any of the regions containing these DRE recognition motifs are involved in the TCDD-induced expression of CYP1B1, we made a series of CAT reporter gene constructs with progressive 5'-to-3' or 3'-to-5' deletions. In transient transfection assays in SCC12(c12c2) cells, the construct containing the longest 5'-end sequence (~2300 to +25) with eight of the nine DRE recognition motifs was induced 2.6-fold by TCDD (Fig. 6). The magnitude of this response was consistent with that observed for the TCDD-dependent direct transcriptional activation of the CYP1B1 gene in the same cell line, as judged by nuclear run-on analysis (2). The successive deletion of 5' fragments to nucleotide −1022 did not significantly decrease the response to TCDD. Extending the deletions to nucleotide −642, however, abolished the response. For the 3' deletion constructs examined, TCDD-inducible CAT activity was observed with a fragment from −2783 to −481, whereas deletions that resulted in the loss of DRE recognition motifs distal to position −835 and proximal to position −1022 were not responsive to TCDD. The −1022 to −835 fragment was active in either orientation (Fig. 6). Taken together, these results identify the region from −1022 to −835 as containing TCDD-responsive enhancers for CYP1B1.

DISCUSSION

The results presented here on the sequence, mapping, and regulation of the human CYP1B1 gene confirm and extend our previous analysis on this cytochrome P450 (7). CYP1B1 is likely a single gene subfamily of the CYP1 family (Fig. 2; Ref. 7). Although CYP1B1, CYP1A1, and CYP1A2 most likely evolved from the same ancestral gene, CYP1B1 maps to human chromosome 2 (Fig. 3; Ref. 7), whereas CYP1A1 and CYP1A2 map to human chromosome 15 (11). This finding suggests that the CYP1B1 subfamily separated from the CYP1A family approximately 300 million years ago (39).

High resolution chromosome mapping indicates that CYP1B1 is located at 2p21–22 on human chromosome 2 (Fig. 3). It is interesting that genes known to be involved in development, signal transduction, and hormone responsiveness map to this same region. These include holoprosencephaly-2, son of
sevenless (homolog 1), calmodulin 2, and luteinizing hormone/choriogonadotropin receptor. Although the precise location of CYP1B1 relative to these genes is not known at this time, its relative proximity to them in 2p21–22 suggests a possible biological role for this P450 isozyme in the activation or inactivation of endogenous effectors of development, hormone-responsive pathways, or both.

The CYP1B1 gene has a single TIS in the absence of a consensus TATA box. For other genes, it has been shown that either a TATA box or a conserved sequence at the transcription start site called an initiator is required for accurate transcription (40, 41). For CYP1B1, an initiator sequence (TTGACTCT) flanking the TIS (−268 to −289 bp) can be found that closely matches the conserved initiator sequence (PyPyAn(T/A)PyPy) reported for terminal deoxynucleotidyltransferase (41). Of 11 Sp1 binding sequences identified in the 5′-flanking region of CYP1B1, 2 are located near the TIS at −88 and −89 (Fig. 5), which is an optimal distance for an Sp1-dependent promoter (41). Each of these functional modalities identified on the basis of nucleotide sequence has been shown to play a role in the accuracy and efficiency of transcription of the CYP1B1 promoter.3

Nucleotide sequence analysis of the 5′-flanking region of the CYP1B1 gene indicates the presence of nine DRE core recognition motifs (GCGTG) between −2260 and −2232 (Fig. 5). These DRE core motifs represent potential sites of interaction with the activated AhR/ARNT heterodimer, which mediates the transcriptional activation of certain genes by TCDD (42, 43). In addition to the DRE motifs identified in this region, matches the conserved initiator sequence (PyPyAn/T/A)PyPy) reported for terminal deoxynucleotidyltransferase (41). Of 11 Sp1 binding sequences identified in the 5′-flanking region of CYP1B1, 2 are located near the TIS at −68 and −89 (Fig. 5), which is an optimal distance for an Sp1-dependent promoter (41). Each of these functional modalities identified on the basis of nucleotide sequence has been shown to play a role in the accuracy and efficiency of transcription of the CYP1B1 promoter.3

Nucleotide sequence analysis of the 5′-flanking region of the CYP1B1 gene indicates the presence of nine DRE core recognition motifs (GCGTG) between −260 and −2320 (Fig. 5). These DRE core motifs represent potential sites of interaction with the activated AhR/ARNT heterodimer, which mediates the transcriptional activation of certain genes by TCDD (42, 43). In addition to the DRE motifs identified in this region, 3P. Wo, J. Stewart, and W. F. Greenlee, submitted for publication.

Fig. 5. Nucleotide sequence of 5′-flanking region of human CYP1B1 gene. The exon sequences are shown in upper case. The TIS is in boldface and indicated by an arrow. Conserved DRE core sequences, GCGTG, are boxed. The TATA-like sequence is double-lined.
The schematics indicate the nucleotide positions for the 5′- and 3′-ends of the constructs, respectively. Circles indicate the locations of the nine core DRE recognition motifs. The ratio of the CAT activities shown on the right was obtained by comparing the normalized CAT activities measured in extracts from TCDD-treated and solvent vehicle (Me 2SO)-treated SCC12F(c12c2) cells. Values represent the mean of three experiments carried out on a minimum of triplicate samples. Replicates within a single experiment varied by less than 1%. Interexperimental variability was less than 10%.

four were found in the first intron, three were found in the coding region of the second exon, and five were found in the second intron. The significance of intronic DRE motifs and exonic motifs located in transcribed mRNA is not known at this time.

In our previous studies (2), the treatment of SCC12(c12c2) cells with TCDD resulted in a 2–3-fold increase in the rate of transcription of CYP1B1, as judged from nuclear runoff experiments. Using a transient transfection assay, we have identified a dioxin-responsive region from −1022 to −835 (relative to the TIS) containing three DREs in the human CYP1B1 gene. The mechanism accounting for the TCDD-dependent enhancement in CAT gene activity contributed from this region is under investigation. It is reasonable to propose that at least one of these three DRE motifs may interact weakly with activated AhR/ARNT complex to confer the observed enhancement of CYP1B1 expression through a synergistic effect with certain transcription factors, such as Sp1. It has been demonstrated that the presence of Sp1 binding sequences in the murine CYP1A1 gene (43) significantly augmented the response to TCDD. Although no consensus Sp1 binding motif (GGGTGG) was found in the TCDD-responsive region for the CYP1B1 gene, three copies of GGTTGG motifs were identified. The GGTTGG motif has been identified as the Sp1-binding site in the promoters of several genes (44–46). Because the affinity for Sp1 could be increased significantly with multiple repeats of the GGTTGG motif and thus result in high transcriptional activation (43), it is possible that the three GGTTGG motifs in this region of CYP1B1 might also participate in the TCDD-dependent transcriptional activation.

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FIG. 6. Deletion mapping of CAT reporter gene constructs. The constructs shown on the left were prepared as described under "Experimental Procedures." All 5′-end deletion constructs contained the first 25 bp of exon I (dark rectangle) of CYP1B1. The numbers shown next to the schematics indicate the nucleotide positions for the 5′- and 3′-ends of the constructs, respectively. Circles indicate the locations of the nine core DRE recognition motifs. The ratio of the CAT activities shown on the right was obtained by comparing the normalized CAT activities measured in extracts from TCDD-treated and solvent vehicle (Me 2SO)-treated SCC12F(c12c2) cells. Values represent the mean of three experiments carried out on a minimum of triplicate samples. Replicates within a single experiment varied by less than 1%. Interexperimental variability was less than 10%.

The constructs shown on the left were prepared as described under “Experimental Procedures.” All 5′-end deletion constructs contained the first 25 bp of exon I (dark rectangle) of CYP1B1. The numbers shown next to the schematics indicate the nucleotide positions for the 5′- and 3′-ends of the constructs, respectively. Circles indicate the locations of the nine core DRE recognition motifs. The ratio of the CAT activities shown on the right was obtained by comparing the normalized CAT activities measured in extracts from TCDD-treated and solvent vehicle (Me 2SO)-treated SCC12F(c12c2) cells. Values represent the mean of three experiments carried out on a minimum of triplicate samples. Replicates within a single experiment varied by less than 1%. Interexperimental variability was less than 10%.
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