Comparison of Genomic and cDNA Sequences of Guinea Pig CYP2B18 and Rat CYP2B2: Absence of a Phenobarbital-Responsive Enhancer Module in the Upstream Region of the CYP2B18 Gene

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ABSTRACT: Potential mechanisms were investigated whereby CYP2B18, a cytochrome P450 gene exhibiting high constitutive expression but only low levels of phenobarbital-inducibility in the guinea pig liver, may be differentially regulated versus the highly inducible rat CYP2B2 gene. To comparatively assess potential regulatory sequences associated with CYP2B18, a guinea pig genomic library was screened enabling isolation of the CYP2B18 gene. The genomic screening process resulted in the identification of at least four closely-related CYP2B18 genes, designated here as CYP2B18A-D. Of these isolates, CYP2B18A exhibited sequence identical to that of the CYP2B18 cDNA. Further, the deduced amino acid sequence of the CYP2B18 cDNA was identical to that of N-terminal and internally-derived peptide sequences obtained in this investigation from CYP2B18 protein isolated from guinea pig liver. Genomic structural sequences were derived for CYP2B18A, together with the respective 5′-upstream and intronic regions of the gene. Comparison of the CYP2B18A and CYP2B2 gene sequences revealed the lack of repetitive LINE gene sequences in CYP2B18A, putative silencing elements that effect neighboring genes, although these sequences were present in both 5′-upstream and 3′-downstream regions of CYP2B2. We determined that the phenobarbital-responsive enhancer module was absent from the 5′-upstream region as well as the intronic regions of CYP2B18A gene. We hypothesize that the compromised phenobarbital inducibility of CYP2B18A stems from its lack of a functional phenobarbital responsive enhancer module.

KEYWORDS: CYP2B18; CYP2B2; CYP2B6; Gene Structure; Intronic Sequence; Phenobarbital-Responsive Enhancer Module

INTRODUCTION

Cytochromes P450 (P450) belonging to the CYP2B subfamily catalyze the hydroxylation of a large number of structurally diverse xenobiotics [1]. In the rat, constitutive expression of the CYP2B1 and CYP2B2 genes is extremely low, however the transcription of each gene is markedly activated following phenobarbital (PB) treatment [2]. The PB-responsive enhancer module, or unit (PBREM/PBRU), has been identified as a conserved region located upstream of PB-inducible genes such as the CYP2B subfamily [3–8] and functions to drive the induction process. A nuclear receptor heterodimer consisting of the constitutive androstane receptor (CAR) and retinoid X receptor alpha (RXRα) has been identified as playing a critical role in trans-activating gene transcription via the PBREM [9]. In support of this concept, CAR-deficient mice lack PB-inducibility of Cyp2b10 [10]. A recent study from our laboratory examined the Qdj:SD rat, a mutant strain that lacks responsiveness to PB-mediated CYP2B2 in-
duction. We demonstrated that no mutations or deletions exist within the PBREM sequence in the CYP2B2 gene of these animals [11]. This observation suggested a role of other region(s), distinct from the PBREM, that may also participate in the PB induction process. In this respect, several non-PBREM regions, such as RBP-Jκ/NFκB, have been suggested to be involved in the regulation of CYP2B2 gene expression [12,13]. Further studies are needed to better elucidate the basis of these alternative regulatory schemes.

It is of interest that the constitutive and inducible nature of expression of the CYP2B subfamily varies greatly in different animal species. For example, in untreated guinea pigs, CYP2B18 is one of the major forms of P450 expressed in the liver, in clear distinction to rat CYP2B1/2 [14,15]. In further contrast, PB treatment of guinea pigs results in only a minor increase, less than two fold, of the specific content of liver microsomal CYP2B18, compared to the high levels of induction observed for the orthologous rat CYP2B members [14,15]. To help elucidate the mechanisms that underlie these differences in species expression and inducer responsiveness, we hypothesized that the respective regulatory region sequences may be divergent and that alternative regulatory schemes.

Materials and Methods

Materials

A guinea pig liver cDNA library (cloning vector, λgt11; Clontec Laboratories, Inc., Palo Alto, CA, USA) and genomic library (Stratagene, La Jolla, CA, USA) were obtained from the sources indicated. A cosmid and genomic library (Stratagene, La Jolla, CA, USA) were obtained from standard commercial suppliers. All other materials were of the reagent grade commercial kits (Amersham Biosciences, Piscataway, NJ, USA). Hepatic CYP2B18 protein was a gift from Dr. Frank J. Gonzalez (National Institute of Health, Bethesda, MD, USA). Hepatic CYP2B18 protein was labeled, either fluorescently for cDNA cloning, or with alkaline phosphatase for genomic DNA cloning, using commercial kits (Amersham Biosciences, Piscataway, NJ, USA). All other materials were of the reagent grade and obtained from standard commercial suppliers.

Determination of Internal Amino Acid Sequences of CYP2B18 Protein

A purified protein specimen (30–45 μg) was partially digested in either (i) 40 mM phosphate (pH 7.25) containing 0.3 μg α-chymotrypsin, 0.1% sodium dodecyl sulfate (SDS), and 15% glycerol or (ii) 0.15 M BrCN/70% formic acid. The peptides generated were separated by SDS-polyacrylamide gel electrophoreses, and the major bands subsequently transferred to a polyvinylidene difluoride membrane. Individual bands were then excised and subjected to analysis using an automated protein sequencer (PE Biosystems ABI473A protein sequencer).

Cloning of Guinea Pig Liver CYP2B18 cDNA

The guinea pig liver cDNA library was screened by plaque hybridization using a fluorescently-labeled CYP2B2 full-length cDNA probe. After screening approximately 1 × 10^6 plaques, three positive clones (pGP4, pGP9, and pGP10) were obtained. Each clone, together with respective restriction fragments yielded by EcoRI, Pst I, and Hind III digestion, were subcloned into an M13 phage vector for DNA sequencing. The sequence of the CYP2B18A cDNA is available in the DDBJ/GenBank/EMBL database under the accession number AB115744.

Cloning of CYP2B18 Genes

A guinea pig gene library consisting of 7 × 10^6 plaques was screened by plaque hybridization with an alkaline phosphatase-labeled CYP2B18 cDNA fragment (760 bp), spanning the 2nd through the 7th exon. The fragment was generated by polymerase-chain reaction (PCR) using CYP2B18 cDNA (pGP10) as a template. Thirty-four positive clones were obtained. Because of the presence of multiple related genes (see Results section), a variable region located within exon 2 was PCR-amplified for each positive clone, and the resulting sequence information enabled identification of specific clones encoding the CYP2B18 protein. The PCR reaction mixture (50 μL) included template (1.5 × 10^6 λFIXII phage), 5 pmol each of primer and 2.5 units DNA polymerase (LA-Taq, Takara Shuzo Co., Ltd., Shiga, Japan), and was cycled according to the following conditions: 95°C for 5 min, (98°C for 10 s and 68°C for 15 min) × 30 cycles; followed by final extension (72°C for 10 min) and hold (4°C) steps. The primer sequences used were as follows: 5′-AGATTAATCCGGCAGC-GTCCGTCGTTG-3′ (sense strand; targeted to exon 2) and 5′-AAGCAACCTGCAACGTATTGAGCGC-3′ (reverse strand; targeted to exon 3). The screening analysis identified 4 of the 34 positive clones as CYP2B18 cDNAs.
Determination of Nucleotide Sequence

The DNA inserts of 2 of the 4 genomic clones confirmed to code CYP2B18 protein, pGP18A1 (insert DNA size, 18.5 kbp) and pGP18A2 (insert size 18 kbp) were PCR-amplified, and the respective DNA sequences were determined by shot-gun methods. Briefly, three regions of the CYP2B18 gene were amplified separately by PCR (PCR 1–4; Figure 1) with LA-Taq (Takara Shuzo Co., Ltd.) under the conditions suggested by the manufacturer. The PCR products were partially digested by sonication, and DNA fragments in the range of 1–3 kbp were extracted by polyacrylamide gel (3%) electrophoresis. The respective DNA fragments were subcloned into M13 vectors for sequencing. The sequence of another clone (pGP18B1; insert DNA size 21 kbp), selected randomly from 30 positive clones, was analyzed similarly. The sequence for rat CYP2B2 gene was also determined by the same methods as above. However, in this case, whole pWE’-39E cosmid DNA was prepared from transformed E.coli was fragmented without first extracting CYP2B2 gene sequences, and respective fragments in the 1–3 kbp size range were subcloned into M13. Nucleotide sequences were determined by a dye-primer method using a PE Biosystems ABI373 DNA sequencer. The derived CYP2B18 and 2B2 gene sequences were assembled by using ABI377 instrumentation assembly software. Sequence similarities between the CYP2B genes were analyzed by PipMaker (PIP: Percent Identity Plot) [17]. The sequence of the CYP2B18A gene (pGP18A1/18A2; accession number: AB115742) was registered in the DDBJ/GenBank/EMBL database together with the CYP2B18B gene (pGP18B1; AB115743).

RESULTS

Sequence of CYP2B2 and Surrounding Genes

The nucleotide sequence of a cosmid clone of rat CYP2B2, pWE’-39E, was determined by a shotgun sequencing strategy. We derived 26 kbp of CYP2B2 gene sequence, containing 7 kbp and 3 kbp of the 5′- and 3′-flanking regions, respectively, although the exact number of (G)GA repeats starting at approximately 420 bp downstream from the end of the 8th exon could not be determined. The 5′-flanking region upstream of −2.7 kbp was highly homologous with LINE (long interspersed repeated DNA element) [18] gene sequences, a characterized retrotransposon (Figure 2). High homology with LINE sequences also was identified in the 3′-flanking region of CYP2B2 gene (Figure 2). It should be noted that the draft sequence from the rat genome project currently available through database resources (Pub-Med, NCBI) does not contain the majority of the LINE gene sequences from the 5′-upstream region of CYP2B2. The reason for this database omission is not known.

Cloning of CYP2B18 cDNA

To enable sequencing the CYP2B18 gene, we first screened a guinea pig liver λgt11 cDNA library and obtained three positive clones that differed in size, but shared an identical sequence in their region of overlap (>400 bp). The combined sequence totaled 1906 bp and comprised an open reading frame except for a missing 196 bp region coding the N-terminal
region was derived from sequence of a CYP2B18A genomic clone, as discussed below. The deduced amino acid sequence consisted of 432 residues (Figure 3). The deduced internal peptide sequences as well as an N-terminal amino acid sequence derived independently (bold underline in Figure 3) were identical to those determined for the purified CYP2B18 protein. The CYP2B18 protein actually contained an additional C-terminal sequence that was absent in other member of this subfamily (Figure 3).

**Sequence of CYP2B18 Gene and the Comparison with Other Members of the CYP2B Subfamily**

Screening of a guinea pig AFXII genomic library yielded 32 positive clones. Preliminary sequencing revealed that some of the clones possessed different exonic sequences compared with that of the cDNA structures described above, indicating that multiple CYP2B18 genes existed in the guinea pig. Three (pGP18A1/pGP18A2 and pGP18B1) of 32 clones that possessed altered exonic sequences were sequenced further. The insert DNA regions of pGP18A1/pGP18A2 and pGP18B1 (21 kbp, CYP2B18B) clones were amplified by PCR, and sequenced by shotgun sequencing. The pGP18B1 clone contained approximately 2 kbp of 5'-flanking region and extended downstream through the middle of the 8th intron. The pGP18A1 + 18A2 contained approximately 4 kbp of 5'-flanking sequence and extended downstream through the middle of 6th intron. The exonic sequences of pGP18B1 were divergent from that of the CYP2B18 cDNA (homology, 91%). Differences for the deduced amino acid residues were confirmed using protein sequencing procedures (Figure 3). Although the CYP2B18B (pGP18B1) gene had correct intron–exon junctions with no stop codons present in the exonic regions, we could not detect expression of its corresponding mRNA—despite examining RNA preparations from several tissues, including liver, by reverse transcriptase-coupled PCR. On the other hand, the exonic sequences of pGP18A1/18A2 were identical with those of CYP2B18 cDNA. Consequently, we designated the gene encoding CYP2B18 protein expressed in the guinea pig tissues as CYP2B18A. Comparison of the exonic sequences of the two genes revealed several regions exhibiting hypervariable base substitutions, including that of the 2nd exon. The latter region from each of the 32 clones was amplified subsequently by PCR, and comparatively sequenced. The results, presented in Figure 4, demonstrate four different sequence patterns, suggesting the presence of at least four closely related genes (CYP2B18A–D).

Sequence similarities between the CYP2B2 and CYP2B18A genes were analyzed by PipMaker [17] and the results are shown in Figure 5A. The lack of sequence similarity in the distal upstream regions of the two genes (upstream of −2.5 kb) indicates that the presence of LINE gene sequence is likely specific for CYP2B2. It was noteworthy that the PBREM was completely absent in the 5'-upstream as well as intronic regions of the guinea pig CYP2B18A gene. In the comparison between CYP2B18A and human CYP2B6 (Figure 5B), little homology was detected within their respective 5'-upstream regions. However, in contrast to the CYP2B18A vs CYP2B2 comparison, highly homologous regions were present in the 1st and 3rd introns of CYP2B18A and CYP2B6 genes, respectively.
FIGURE 3. Nucleotide and deduced amino acid sequence of CYP2B18A cDNA. The nucleotide and the deduced amino acid sequences of CYP2B18A expressed in the tissues of guinea pigs [14,15] are shown in the first and second line, respectively. The amino acid residues of CYP2B18B (see text) that differ from CYP2B18A are shown in the third line. Thin underlining indicates the region where nucleotide sequence was determined from genomic DNA (CYP2B18A gene). The sequence of CYP2B18A without a thin underline (downstream from 197th base) was determined by sequencing the pGP4 (783–2,203 bp), pGP9 (727–1,830 bp), and pGP10 (197–1,293 bp) clones which contained the regions indicated, respectively. Deduced peptide sequences, indicated by bold underlines, of CYP2B18A cDNA were matched with the sequences determined from purified protein. The region coded in the 2nd exon is boxed. The C-terminal peptide specific for CYP2B18A as compared with the other members of this subfamily from other species is shown by a bracket.

DISCUSSION

In this study, we report for the first time on the existence of multiple guinea pig CYP2B18 genes. Although the possibility that CYP2B18B–D are active genes can not be ruled out, the present results together with our previous data [14,15] strongly suggest that CYP2B18A is the predominantly and perhaps solely expressed gene in this cluster, at least in liver. While the rat CYP2B2 gene is located between LINE gene sequences, the CYP2B18A gene, which is highly expressed in the liver of untreated guinea pigs, does not contain corresponding LINE homologies. LINE gene sequences are located downstream of the rat insulin-1 gene and have...
been reported to function as a gene silencer in this context [19]. Therefore, it is possible that the low constitutive levels of rat CYP2B2 may also be negatively regulated by the presence of LINE sequence elements.

Sequence similarity between human CYP2B6 and guinea pig CYP2B18A within the 1st and 3rd introns was higher than that present between CYP2B2 and CYP2B18A, suggesting that evolutionarily, the CYP2B18A gene more closely resembles CYP2B6 than CYP2B2. This conclusion is also supported by higher similarity in cDNA sequence composition between CYP2B18A and CYP2B6 (75%), as compared to CYP2B18A and CYP2B2 (74%).

The number of putative regulatory motifs between the CYP2B18A and the CYP2B2 genes was compared. Since no PBREM was detected in the intronic sequences or the 3′-flanking region of the CYP2B2 gene, it is likely that the PBREM at ∼−2.3 kbp upstream in the CYP2B2 5′-flanking region is the sole contributor to this gene’s PB-responsiveness. However, the absence of an identifiable PBREM motif within the CYP2B18A gene is consistent with the low PB-inducible nature of this gene in guinea pig liver [14,15], although nonresponsiveness of this gene to the stimulation by PB should be confirmed using reporter constructs. The glucocorticoid responsive element (GRE) neighboring the PBREM in CYP2B2 gene has been suggested to play a role in maximizing PB responsiveness [5]. Our sequence analyses were unable to localize a corresponding GRE motif in the corresponding 5′-upstream region of CYP2B18A. We have reported that dexamethasone has no ability to increase the liver CYP2B18A as well as P450 content in guinea pig [15]. The lack of GRE in CYP2B18A gene seems to be consistent to this previous data.

Similarly, a RBP-Jκ/NF-κB site contained within the CYP2B2 promoter region has been suggested to maintain this gene in a constitutively repressed state [12]; however, the presence of this latter motif could not be identified within the 5′-upstream or intronic sequences of CYP2B18A. Since both RBP-Jκ/NF-κB and LINE gene sequences have been suggested to function as negative regulators of transcription, it appears possible that the absence of these sequences in the CYP2B18A gene is at least in part responsible for directing a high constitutive level of gene expression, in vivo. However,
absence of LINE gene sequences around CYP2B6 gene, the constitutive expression of which seems to be low, does not suggest that LINE gene is a sole regulator suppressing basal expression of CYP2B gene. It is likely that a complex interplay of regulatory regions combine to determine the constitutive and PB-inducible nature of CYP2B gene expression and that the sequence context of the CYP2B genes in different species serves to differentially modulate the respective gene expression character.

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