Published in final edited form as:

Drug Metab Dispos. 2008 September ; 36(9): 1930–1937. doi:10.1124/dmd.108.022020.

mRNA Distribution and Heterologous Expression of Orphan Cytochrome P450 20A1

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Abstract

Cytochrome P450 (P450) 20A1 is one of the so-called “orphan” P450s without assigned biological function. mRNA expression was detected in human liver and extrahepatic expression was noted in several human brain regions, including substantia nigra, hippocampus, and amygdala, using conventional polymerase chain reaction and RNA dot blot analysis. Adult human liver contained 3-fold higher overall mRNA levels than whole brain, although specific regions (i.e., hippocampus and substantia nigra) exhibited higher mRNA expression levels than liver. Orthologous full-length and truncated transcripts of P450 20A1 were transcribed and sequenced from rat liver, heart, and brain. In rat, the concentrations of full-length transcripts were 3–4 fold higher in brain and heart than liver. *In situ* hybridization of rat whole brain sections showed a similar mRNA expression pattern as observed for human P450 20A1, indicating expression in substantia nigra, hippocampus, and amygdala. A number of N-terminal modifications of the codon-optimized human P450 20A1 sequence were prepared and expressed in *Escherichia coli*, and two of the truncated derivatives showed characteristic P450 spectra (200–280 nmol P450/l). Although the recombinant enzyme system oxidized NADPH, no catalytic activity was observed with the heterologously expressed protein when a number of potential steroids and biogenic amines were surveyed as potential substrates. The function of P450 20A1 remains unknown; however, the sites of mRNA expression in human brain and the conservation among species may suggest possible neurophysiological function.

P450 monooxygenases catalyze the introduction of oxygen into a vast range of molecules and are known to have diverse functions in endogenous and exogenous metabolism (Ortiz de Montellano, 2005). P450s in Families 1–3 are primarily involved in the metabolism of exogenous compounds; i.e., drugs and environmental pollutants, while Families 4–51 consist of enzymes primarily involved in the bioconversion of endogenous compounds; i.e., steroids, fatty acids, vitamins, and eicosanoids (Guengerich, 2005). To date 57 human P450

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^SThis paper contains Supplemental Data.

(CYP or P450, P450 indicating the nucleotide or protein, while CYP indicating the gene in question) genes are known (<http://drnelson.utmem.edu/CytochromeP450.html>). The P450s can be divided into six major groups based on their main substrates: steroids, vitamins, fatty acids, eicosanoids, xenobiotics, and unknown (Nelson et al., 1996; Guengerich et al., 2005). It is noteworthy, however, that a number of known P450s have assigned substrates in more than one of these groups and some substrates cannot be assumed to be true physiological substrates based only on their conversion rates (e.g. testosterone 6 β -hydroxylation for hepatic P450 3A4), in the absence of other evidence of function. Although extensive research efforts have been directed towards elucidating the endogenous and exogenous functions of individual P450s, one-fourth of the human P450s still have not been assigned a catalytic activity and remain “orphans” (Guengerich et al., 2005; Guengerich, 2008; Stark and Guengerich, 2007) (based on the terminology previously used for nuclear receptor proteins without known ligands (e.g. (Mangelsdorf and Evans, 1995)). Our present working list of P450 orphans includes ~12 human P450s: 2A7, 2S1, 2U1, 2W1, 3A43, 4A22, 4F22, 4V2, 4 $\times$ 1, 4Z1, 20A1, and 27C1 (Guengerich et al., 2005; Stark and Guengerich, 2007).

Human P450 20A1 has, to our knowledge, only been mentioned in one publication (Marek et al., 2007), in which a 230-bp mRNA transcript was reported in stellate cells prepared from chronically damaged liver. No information is available about either expression sites or function. *In silico*-generated mRNA expression data from the NCBI suggests P450 20A1 expression in kidney, liver, and brain (<http://www.ncbi.nlm.nih.gov/>). Only one P450 20A1 cDNA transcript (NM_177538.2, UniGene ID Hs.4460659) has been reported to the NCBI, coding for a putative 462 amino acid protein (NP_803882.2). The gene is located on chromosome 2q33.2 and, on the basis of phylogenetic information, P450 20A1 appears to be the only human P450 Family 20 member. P450 20A1 EST sequences have recently been reported from kidney, brain, placenta, and urogenital tissues, according to the NCBI UniGene database (<http://www.ncbi.nlm.nih.gov/UniGene/>). Orthologous nucleotide sequences have been found in a number of mammals, including rat (84% identity), mouse (85%), chimpanzee (99.6%), and chicken (72%) (<http://drnelson.utmem.edu/CytochromeP450.html>). None of these (putative) proteins has been studied to date.

In this work, P450 20A1 mRNA was found in human adult liver and brain, with a highly selective expression in brain tissues, i.e., substantia nigra, hippocampus, basal ganglia, and amygdala. mRNA expression analysis of the rat ortholog was also studied. Heterologous expression in *Escherichia coli* confirmed that this is a P450 enzyme. Some potential activities in the metabolism of steroids, model P450 substrates, and brain neurotransmitters by an N-terminal-optimized version of P450 20A1 were investigated.

Methods

P450 20A1 mRNA Analysis

A human Multiple Tissue Expression Array 3TM (BD Biosciences-Clontech, Palo Alto, CA), with approximately 0.01–1 μ g mRNA (normalized to β -actin mRNA) from 75 normal non-diseased tissue-specific polyA⁺ RNAs, was hybridized with a 48-bp P450 20A1 probe (nucleotides 789–839 of the natural P450 20A1 sequence, Fig. 1 and Supplemental Data Fig. 1). The probe design was analyzed (compared to all known 57 human sequences) using the

BLAST algorithm and found to selectively detect P450 20A1 (NM_177538.2). The blot was hybridized using a ^{32}P -labeled oligonucleotide probe for P450 20A1, according to the manufacturer's instructions (BD Biosciences-Clontech). The array was blocked using ExpressHyb solution containing salmon sperm DNA (1.5 mg, 20 min, 60°C). The multiple tissue expression array was hybridized at 60°C overnight, washed four times (20 min each in $2\times$ SSC containing 0.05% SDS (w/v) at room temperature), and finally washed 1 h in $0.1\times$ SSC containing 0.1% SDS (w/v) at 60°C before development (Molecular Imager FX Phosphorimager, Bio-Rad, Hercules, CA). Three independent experiments (on two different membranes purchased from the supplier) using the same probe (*vide supra*) yielded very similar results.

Isolation of Human P450 20A1 cDNA

Sense and antisense primers were designed to copy the full-length *CYP20A1* gene (see Supplemental Data Fig. S1–Fig. S3). PCR was performed using 1 μl (100 ng) cDNA template, 20 pmol of each primer, and 2.5 U Taq *Pfu* Ultra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA) in a total volume of 50 μl of buffer (10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl, 2.5 mM MgCl_2 , and 0.2 mM dNTP). PCR was run as follows: 95°C for 5 min, followed by 33 cycles (denaturation at 95°C for 30 s; annealing at 60°C, for 30 s; extension at 72°C for 90 s in each cycle), and a final extension step (72°C, 10 min). PCR yielded a fragment with the expected size of a P450 20A1 amplicon from cDNA synthesized from liver. The amplified PCR product was inserted into a pCR II-Topo TA cloning vector (Invitrogen, Carlsbad, CA) and the insert was sequenced using BigDye Terminator chemistry on a ABI 3730xl DNA Analyzer in the Vanderbilt facility.

RT PCR Analysis of P450 20A1 Expression

Total polyA⁺ RNA samples from human adult organs, including brain and a number of human brain regions (cerebellum, basal ganglia, amygdala, hippocampus, and substantia nigra), were obtained from Ambion Inc. (Austin, TX) and Stratagene (La Jolla, CA). Aliquots of total RNA (1 μg) were reverse transcribed using a two-step Enhanced Avian RT reaction (SigmaAldrich, St. Louis, MO) containing a deoxyribonucleotide mixture (10 mM each dNTP), random nonamers (50 μM in H_2O), enhanced AMV RT (20 U/ml in 200 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol, 0.2% Triton X-100 (w/v), and 50% glycerol (v/v)), $10\times$ buffer for AMV RT (500 mM Tris-HCl buffer (pH 8.3) containing 400 mM KCl, 80 mM MgCl_2 , and 10 mM dithiothreitol), and RNase inhibitor (20U/ml) in a total volume of 20 μl and used for first strand synthesis (25°C, 25 min; 42°C, 50 min) according to the manufacturer's protocol (SigmaAldrich), with 1 μl cDNA used as template for each PCR. The PCR amplification was performed at 94°C for 5 min, followed by 33–40 cycles of annealing (94°C, 30s; 60°C, 30 s; 72°C, 90 s) and extension (72°C, 10 min). PCR products were analyzed on 2% (w/v) agarose gels and stained with ethidium bromide; the results were recorded with a gel camera (Kodak Edas 290, DyNALight Dual Intensity Illuminator). PCR products were ligated into the pCR2.1 TOPOTM vector according to the manufacturer's protocol and sequenced using the manufacturer's primers (Invitrogen).

Real-time RT PCR of Human P450 20A1

Real-time RT-PCR reactions included 25 µl PCR Master Mix RT² SYBR Green/Fluorescein (SuperArray Bioscience, Frederick, MD) and 1 µl first strand cDNA template (corresponding to 30–50 ng cDNA). “Housekeeping gene” GAPDH and 18S RNA RT² qPCR primer assay sets were purchased from SuperArray Bioscience, and β-actin primers were designed using freely available primer design programs. The program was set at 95°C (3 min), followed by 40 cycles of 95°C (10 s)/55°C (45 s). A melting curve analysis was preformed after each run to establish that only one insert was formed. RT²-qPCR primer assay sets for human P450 20A1 (Reference sequence ID NM_177538.2) were purchased from SuperArray Bioscience. Specific primers for both the human P450 20A1 transcripts and rat splice variants were designed using freely available primer software (see Supplemental Data Fig. S2A). The iScript One-Step RT-PCR KitTM with SYBR Green (Bio-Rad, Hercules, CA), coupling RT, and real-time PCR cycling was used for some reactions. Each sample was analyzed in triplicate using a MyIQ Single-Color Real-Time PCR Detection SystemTM (Bio-Rad, Hercules, CA) in MicroAmpTMOptical 96-well reaction plates (Bio-Rad). The iCyclerTM software calculates the threshold cycle at which fluorescence reaches a selected level. After amplification a melting curve was performed to ensure specific product formation. The real-time PCR results were analyzed using the iCyclerTM software, and the P450 20A1 expression levels were calculated using the comparative C_t method and normalized to the levels of two housekeeping genes, GAPDH and 18S RNA. The expression of the housekeeping genes was analyzed as described above. To detect the human P450 20A1 sequence, forward (h20A1_F, 5'-TCTGAGATTGGAAAAGGCTTTCTA) and reverse primers (h20A1_R, 5'-AGGTTCCCTTGTTACTAAGGAGTCAATG) were used to produce a 197 bp product (Supplemental Data Fig. S2A).

Isolation of Rat P450 20A1 and Sequence Analysis

cDNA was synthesized as described above from rat liver, heart, and brain total RNA (Ambion Inc., Austin, TX) using random hexamers or oligo dT primers. Forward (5'-ATGCTGGACTTCGCCATCTTCGCCGTG) and reverse (5'-CTTTCGGAGACAGTGATCCAAGT) primers were used to amplify the rat P450 20A1 cDNA (NM_199401.1) (Supplemental Data Fig. S3). The PCR products were purified using a QIAGEN Gel Extraction KitTM (Qiagen, Valencia, CA) and ligated into a PCR2.1 TOPO vector (TOPO TA Cloning KitTM for sequencing, Invitrogen). Chemically-competent One-shotTM *E. coli* cells (Invitrogen) were transformed, and single clones were used to prepare plasmid DNA. Plasmid DNA was isolated from overnight Luria-Bertani medium cultures of selected clones using a FastPlasmid KitTM (Eppendorf, Westbury, NY). Plasmids were digested using *EcoRI* (New England Biolabs, Ipswich, MA), and the digestion fragments were visualized after separation using 1 and 2% agarose (w/v) gels. Sequencing of the products was performed in the Vanderbilt facility using the M13 (–20) forward (5'-TGTAACGACGGCCACT) and reverse (5'-CAGGAAACAGCTATGAC) primers according to the manufacturer's instructions. All DNA sequences were analyzed using the OK BLAST similarity search tool (Altschul et al., 1997).

A full-length transcript was ligated into the pCW monocistronic vector. Screening for full-length transcripts of rat P450 20A1 was performed using exon-specific primers, and putative full-length transcripts were submitted for sequencing (see Supplemental Data Fig. S2A, Fig. S3, and Fig. S4).

Real-time and RT PCR of Rat P450 20A1

Real-time RT-PCR reactions (25 μ l) were performed essentially as for human P450 20A1 using primers directed against exon-specific regions of rat P450 20A1 (reference sequence NM_199401.1) (Supplemental Data Fig. S2A, Fig. S4). A standard curve was constructed using a 10-fold dilution series of full-length rat P450 20A1 transcripts from rat heart.

In situ Hybridization Fluorescence of Rat P450 20A1 mRNA

Male Fischer 344 rats (~200 g), from Charles River Laboratories (Charles River, Wilmington, DE), were anesthetized with pentobarbital. The brains were removed, rapidly frozen on dry ice, and kept at -80°C until use. Sections (12–18 μm) were cut from frozen rat brains in a cryostat. *In situ* hybridization fluorescence sections were thawed to room temperature before a hybridization solution (IsHyb *In Situ* Hybridization (ISH) Kit, BioChain Institute, Hayward, CA) was added, and sections were pre-hybridized in a humidified chamber (2 h, 42°C). The sections were then covered with the DIG-labeled P450 20A1 sense and antisense probes (Supplemental Data Fig. S5), respectively, and diluted in hybridization solution (50% deionized formamide (v/v), 20% (v/v) $20\times$ SSC, $5\times$ Denhardt's reagent, and 100 μg denatured sheared herring sperm DNA/ml in 0.20 M potassium phosphate buffer, pH 7.2) overnight at 42°C . After the hybridization step, sections were rinsed five times with $1\times$ SSC at 55°C , followed by a cooling step at room temperature before washing three times with $1\times$ TBS buffer and then blocking using 3% bovine serum albumin (w/v) in TBS (23°C , 60 min, gyratory shaking at 60 rpm). Sections were incubated with an anti-DIG alkaline phosphatase antibody (Roche, Basel; 1:5000 dilution) overnight at room temperature. Immunoreactivity was visualized by incubation of the sections with a 5-bromo-4-chloro-3-indolyl phosphate/nitrobluetetrazolium alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA) and mounted using Histomount (Zymed, San Francisco, CA). Identification of regions was done in collaboration with M. Andersson (Dept. Biochemistry, Vanderbilt Univ.).

Optimization of P450 20A1 and Vector Preparation

Automatic codon optimization and oligonucleotide design for PCR-based gene synthesis were performed using DNAWorks 3.1 from Information Technology, National Institutes of Health (<http://helixweb.nih.gov/dnaworks>) (Wu et al., 2006a). The amino acid sequence and the native cDNA sequence information for human P450 20A1 (NM_177538.2) were obtained from NCBI GenBank sequences (Fig. 1, Supplemental Data Fig. S1). The codons were optimized to fit the codon biases of *E. coli* (Supplemental Data Fig. S1, Table S1). A number of N-terminal sequences were added to the constructs in order to increase the level of protein expression (Fig. 2, Supplemental Data Fig. S6). In brief, a number of overlapping synthons were designed to span the cDNA sequence and used for primary PCA, followed by a one-step PCR reaction (94°C , 5 min; 94°C , 30 s; 58°C , 30 s; 72°C , 2 min (30 cycles); 72

°C, 10 min). In order to decrease the error frequency the sequence was manually divided into two synthons (Synthon 1: 850 bp, Synthon 2: 601 bp) and later ligated together using an *MscI* site (Supplemental Data Table S1, Fig. S7). An *NdeI* restriction site spanning the start codon was introduced at the 5'-end sequence, and the 3'-flanking sequence contained an *XbaI* restriction site. The resulting insert of the correct size was ligated into the pCW vector for both monocistronic and "bicistronic" vectors (the latter containing a human NADPH-P450 reductase cDNA downstream of the cDNA insert, between the *NdeI* and the *XbaI* sites) (Parikh et al., 1997). Positive selected clones were sequenced using an Applied Biosystems Big Dye system in the Vanderbilt facility. To facilitate Ni²⁺-nitrilotriacetate affinity purification and other future applications, a (His)₅ tag was added to the C-terminal end of the native protein. N-Terminal mutations were introduced into the native pCW 20A1 construct by PCR-based mutagenesis. The cDNA of P450 20A1 was amplified between the *NdeI* and the *XbaI* sites using 5'-PCR primers containing the desired mutations and the 3' outermost oligonucleotide of Synthon 2 (see Supplemental Data Fig. S6). *Pfu* Ultra High-Fidelity™ DNA polymerase (Stratagene, La Jolla, CA) was used for the PCR amplification at an annealing temperature of 58–60°C. All PCR-products were purified using agarose gel electrophoresis and double digested using *NdeI* and *XbaI* before ligation into the vector. All modifications were confirmed by nucleotide sequence analysis in the Vanderbilt facility.

Heterologous Expression of P450 20A1

Expression of P450 20A1 constructs was performed in both *E. coli* DH5α cells and pGroEL/ES12 DH5α cells, the latter containing a chaperone system. Both the plasmids pGro12 ES/EL and each of the twelve constructs of pCW 20A1 were transformed into *E. coli* DH5α competent cells and selected on Luria-Bertani medium plates containing 50 µg ampicillin/ml or 50 µg ampicillin plus 20 µg kanamycin/ml, respectively. Single colonies were grown overnight in Luria-Bertani liquid media (100 µg ampicillin/ml alone or with 50 µg kanamycin/ml) at 37°C with 225 rpm gyrorotary shaking. Each overnight culture was then inoculated (1:100 dilution) into 50 ml of Terrific Broth containing 100 µg ampicillin/ml, 50 µg kanamycin/ml (when used), and a 0.025% (v/v) mixture of a trace element mixture (Sandhu et al., 1993) in a 250-ml flask. The cultures were incubated at 37°C with gyrorotary shaking at 200 rpm until the OD₆₀₀ reached 0.5; P450 20A1 expression was induced with 1.0 mM D-isopropyl-β-galactoside and 0.5 mM 5-aminolevulinic acid. The addition of arabinose (1 mg/ml) was used to initiate pGroEL/ES12 transcription in the case of chaperones. The incubation continued at 27°C with gyrorotary shaking at 200 rpm for another 40 h. Expression levels were monitored from 24 to 48 h. Large scale expression for constructs 11 and 12 (Fig. 2) was performed in 2.8-l Fernbach flasks containing 1 l media, essentially in the same manner as for small scale trials, except that for construct 12 (with chaperones) the cultures were incubated at 28°C after induction with gyrorotary shaking at 200 rpm for 40 h in a New Brunswick Innova 4300 shaker (New Brunswick Scientific, Edison, NJ).

P450 concentrations were estimated with a CO-difference spectra assay (Omura and Sato, 1964) using an OLIS/Aminco DW2a spectrophotometer (On-Line Instrument Systems, Bogart, GA).

Assay of Cholesterol Oxidation

All bicistronic membrane samples (prepared using a vector also containing NADPH-P450 reductase) were tested using an NADPH oxidation assay (A_{340} , $\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) before use in the experiments with potential substrates. Assays of cholesterol oxidation were performed using a general procedure previously described (Wu et al., 2006a). Bicistronic membranes containing human P450 20A1 (~100 pmol) or human P450 7A1 (100 pmol) (positive control, a generous gift of Dr. I. Pikuleva, University of Texas, Galveston, TX) (plus 200 pmol NADPH-P450 reductase and 30 μg L- α -dilauroyl-*sn*-glycero-3-phosphocholine in the case of purified P450 7A1) were incubated in 1.0 ml of 50 mM potassium phosphate buffer (pH 7.4) and 100 μM cholesterol together with 3 μl [$4\text{-}^{14}\text{C}$]-cholesterol (in $\text{C}_2\text{H}_5\text{OH}$, 2.66×10^5 dpm total). Reactions (at 37°C, 20 min) were initiated by the addition of an NADPH-generating system (Guengerich and Bartleson, 2006). Reactions were quenched by the addition of 1.0 ml ethyl acetate, mixed using a vortex device, and analyzed essentially as previously described (Wu et al., 2006a). HPLC separation was performed on a Zorbax octylsilane (C8) column (3 μm , 6.2 mm $\times$ 80 mm, Agilent Technologies, Palo Alto, CA) with a $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (95:5, v/v) mobile phase at a flow rate of 1.0 ml/min, connected in-line with an IN/US radio-flow scintillation counter (IN/US, Tampa, FL).

UPLC-MS Analysis of Products of Incubations with Bicistronic P450 20A1 Membranes

Incubations with potential substrates were run for 30 min (0.1 nmol of P450 20A1 and NADPH-P450 reductase in membranes prepared from bacteria expressing bicistronic vectors) at 37°C, in a similar manner as described for cholesterol oxidation. All bicistronic membrane samples were tested using the NADPH oxidation assay (A_{340} , $\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) before use in the experiments with potential substrates. Extractions were performed using ethyl acetate, and the concentrated extracts were analyzed by LC-MS.

UPLC-MS/MS analysis was performed on a Waters Acquity UPLC system (Waters, Milford, MA) connected to a ThermoFinnigan LTQ mass spectrometer (ThermoFisher, Waltham, MA). Analysis was performed in the positive or negative ion electrospray ionization mode using an Acquity UPLC BEH octadecylsilane (C18) column (1.7 μm , 1.0 mm $\times$ 100 mm). Analyses were performed using a linear gradient from Buffer A (10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ in 5% CH_3CN , v/v) to Buffer B (10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$ plus 95% CH_3CN , v/v). The temperature of the column was maintained at 55°C and the samples were injected with an autosampler system. Positive and negative ion electrospray conditions were as follow: source voltage 4 kV; source current 100 μA ; auxiliary gas flow rate setting 20; sweep gas flow rate setting 5; sheath gas flow setting 34; capillary voltage -49 V; capillary temperature 350°C; tube lens voltage -90 V. MS/MS conditions were as follow: normalized collision energy 35%; activation Q 0.250; activation time 30 ms. Data were acquired using the Xcalibur software package (ThermoElectron Corp.). Full scans were followed by data-dependent MS/MS scans of putative hydroxylated reaction products.

Results

P450 20A1 Sequence Analysis

The *CYP20A1* gene is located on chromosome 2q33. Four other P450 genes are found on the same chromosome but all at considerable distance: *CYP1B1*, *CYP26B1*, *CYP27C1*, and *CYP27A1*. The human P450 20A1 transcript (NM_177538.2, 2091 bp) is predicted to contain 13 exons and code for a putative full-length protein (NP_803882, 462 amino acids). Alignments of the human P450 20A1 cDNA sequence with mouse P450 20a1 (85% nucleotide identity) and rat P450 20A1 (84% nucleotide identity) suggested that the exon–intron boundaries are highly conserved (Supplemental Data Fig. S1). In the P450 (*CYP*) 20A1 gene, the positions of introns 1, 2, and 4 are conserved with other mammalian members of P450 Family 20 but the third intron position is different.

Four single nucleotide polymorphisms (none of which affect the coded protein) are present in the transcript, one at Phe73 (T/G) and three in exon 4: Ser97 (C/T), Lys124 (A/G), and Tyr346 (C/T). Most P450s contain the highly conserved motif FXXGXXXCXG(XXXA) in the region surrounding the heme binding Cys residue (Graham-Lorence and Peterson 1996). The putative heme-binding residue in P450 20A1 is Cys407 (Fig. 1). However, the P450 20A1 heme binding region appears to miss one residue in the first part of the conserved FXXGXXXC sequence, reading FS_GTQEC. After the Cys residue, the third residue is a Glu, while most other P450s contain a conserved Gln residue.

mRNA Expression Analysis of P450 20A1 in Human Tissues

Human P450 20A1 expression was studied using both mRNA blotting and *in situ* fluorescence hybridization. A multiple tissue mRNA blot containing mRNA from >75 different tissues was hybridized using a P450 20A1 probe directed against residues 667–718, designed to differentiate P450 20A1 from all other human P450s using a multiple BLAST alignment program. The results indicated a strong specific expression mainly in human substantia nigra, and no expression was detectable in a number of other brain tissues using this approach (Fig. 3).⁴

Real-time PCR Analysis of Human P450 20A1

In order to further analyze the tissue expression profile obtained for P450 20A1 from a multiple tissue expression mRNA blot (Fig. 3), specific primers designed to detect partial and putative full-length transcripts of P450 20A1 were used to perform independent conventional PCR of human substantia nigra, as well as whole human brain and a number of other brain regions (amygdala, hippocampus, basal ganglia, and cerebellum), along with fetal and adult liver. Bands of the expected size were detected in all tissues examined (data not presented). Real-time PCR was used to compare the levels of P450 20A1 mRNA expression in these tissues (Fig. 4). The expression level in adult liver was at least 2-fold higher than in whole brain; however, the specific brain regions hippocampus and substantia nigra contained considerably higher mRNA levels. The results were normalized to a

⁴*In situ* hybridization of human tissues was attempted, however no conclusions could be drawn from the limited number of analyses due to lack of material.

housekeeping gene; for graphic representation all of the other values are compared with adult liver (nominally set equal to 100) (Fig. 4). However, it must be noted that these mRNA samples were from single donors and the issue of inter-individual variation has not been addressed (due to the difficulty of obtaining human brain regions from multiple donors, the investigation was also limited to expression levels within single individuals).⁴

RT qPCR Analysis of Rat P450 20A1

If P450 20A1 has a specific function in parts of human brain, one might expect to see similar localization patterns in rodents, in which the issue of interindividual variation of tissues is not a factor. Real-time PCR was used to compare the mRNA levels of P450 20A1 expression in these tissues. A number of considerations have to be made when using a reference gene to normalize expression results, because the reliability of the housekeeping gene in question is important for the validity of the assay. Four common rat housekeeping genes— β -actin, GAPDH, cyclophilin A, and 18S ribosomal RNA—were investigated, GAPDH and cyclophilin A were chosen for further studies because they showed the highest and most invariant expression levels (low C_t values).

A commercially available primer set directed against rat P450 20A1 indicated higher mRNA expression in both rat liver and heart than in brain (results not shown). Judging from the relative quantitation analysis, the P450 20A1 mRNA level was 3- to 4-fold lower in brain than in heart or liver, with reference to cyclophilin A and GAPDH. The corresponding values for exon-specific primer pairs, amplifying specifically exons 2 and 3 in order to ensure full-length P450 20A1 detection (*vide infra*), was C_t 23.5 (brain) compared to C_t 24.3 (heart) and C_t 26.5 (liver). The variability may be due to contributions from alternatively spliced transcripts (*vide infra*) in the case of the commercial primer set (Fig. 5).

In situ hybridization of P450 20A1 in whole rat brain sections showed specific localization in the neurons of the substantia nigra, hippocampus, and amygdala (Fig. 6). This tissue distribution is similar to the tissue distribution pattern found in human brain by conventional and real-time PCR analysis (Figs. 4 and 6).

Splice Variants of Rat P450 20A1

RT-PCR primers designed toward rat 20A1 exons 1 and 14, respectively, were designed and used to amplify putative full-length cDNA transcripts of P450 20A1 from rat liver, heart, and brain. Transcripts of the expected size were cloned into a pCR 2.1 TOPO vector and sequenced. Sequencing of the rat P450 20A1 cDNA revealed three alternative transcripts, which differ mainly in their first exons. In addition to the full-length transcript, previously reported to the NCBI (NM_199401), alternative splicing involved exon 2 (skipped in one transcript found in rat heart and rat brain, P450 20A1s1) and exons 2 and 3 (skipped in one variant found in rat brain, P450 20A1s2) (Fig. 5, Supplemental Data Fig. S2A, Fig. S4). The existence of different splicing variants was confirmed by RT PCR of transcripts in the pCR 2.1 TOPO vector. Two alternative transcripts of P450 20A1 cDNA with exon 2 and with exons 2 and 3 deleted, respectively, were detected in rat. Deletion of exon 2 (s1) caused a frameshift and generated a stop at codon at 81 bp relative to the (ATG) start codon, leading to premature termination of translation. For the second variant (s2, missing exons 2 and 3)

the termination would be a stop codon at 94 bp relative to the starting ATG (Fig. 3A). Both of these splice variants would result in putative truncated proteins of <20 amino acids length when transcribed and translated (see Supplemental Data Fig. S4).

P450 20A1 mRNA was demonstrated to be constitutively expressed in rat brain, liver, and heart. mRNAs of both splice variants were detected in rat brain along with full-length mRNA, but liver and heart contained both full-length and transcripts missing exon 2s1 transcripts. This result implies that alternatively-spliced gene products of P450 enzymes may be generated in species- and tissue-selective manners. Full-length transcripts have been detected in all three tissues using exon-specific primers for conventional RT-PCR. Heart and liver transcripts were successfully sequenced (Fig. 5, Supplemental Data Fig. S4).

Synthesis of Codon-optimized P450 20A1 cDNA

At the beginning of this project no P450 20A1 cDNA was available, either commercially or described in the literature. cDNA was prepared for heterologous expression using PCA of a large number of overlapping oligos. The sequence was codon-optimized in order to facilitate expression in an *E. coli* expression system, following protocols for other P450s that have been successfully expressed in this laboratory (Wu et al., 2006a; Wu et al., 2006b). An alignment of the native-codon P450 20A1 sequence and two other mammalian P450 20A1 sequences is shown in Supplemental Data Fig. S6. The P450 20A1 insert was later also ligated into a bicistronic vector containing both the P450 and human NADPH P450-reductase cDNAs (Parikh et al., 1997).

Expression of N-Terminal Variants

Truncations and modifications of the N-terminus of most mammalian P450s expressed in *E. coli* have been shown to increase the expression levels (Barnes et al., 1991; Richardson et al., 1995; Barnes, 1996; Guengerich et al., 1996). The predictions of favorable truncations for a P450 are often based on alignments with close P450 family members; however in the case of human P450 20A1 this enzyme appears to be the sole member of P450 Family 20. With the assumption that the first part of the enzyme contains a membrane-binding structure that can be modified or removed, a number of different N-terminal modifications were introduced and expressed, based on previous work with P450s (Fig. 2 and Supplemental Data Fig. S6). All of these expression trials yielded expression levels < 50 nmol/l crude culture; i.e., weak ferrous-CO difference spectra. P450 20A1 constructs 11 and 12 showed reproducible expression in the range of 150–250 nmol P450/l. Modification 11 is based on the N-terminal sequence MASRQAS... in front of the proline-rich region, and 12 is based on the MALLLAFV... sequence originally used with bovine P450 17A1 (Barnes et al., 1991; Barnes, 1996) in front of the third codon. The second codon (Ala) was changed to the *E. coli*-preferred variant GCT (Barnes et al., 1991).

Expression trials were carried out using these constructs without and with co-expression of the molecular chaperones groES/EL12 in *E. coli* DH5 α under different conditions of temperature and time (Fig. 7). Membranes were prepared with a P450 recovery of 70% in the pellet fraction ($10^5 \times g$, 1 h centrifugation, Gillam et al., 1993). The peak centered near 450 nm was not seen with other constructs in which the P450 was not expressed. All

subsequent enzyme reaction assays were carried out using bicistronic membranes prepared with construct 11. Although cytochrome *b*₅ was not co-expressed in this system, previous work with P450s such as P450 3A4 have shown that the inclusion of cytochrome *b*₅ is not absolutely critical to enzymatic function in these systems (Parikh et al., 1997). Bicistronic cultures of various modifications did not produce any blue color and the enzyme is not believed to be involved in 3-indole hydroxylation (Gillam et al., 1999; Gillam et al., 2000).

Catalytic Assays

Several catalytic assays were attempted (using UPLC-MS systems) in order to investigate substrates known to be oxidized by other P450s and also compounds known to be important in the specific brain regions where P450 20A1 is localized. In order to verify that the NADPH-P450 reductase was capable of delivering electrons to this modified P450, the rate of NADPH reduction was measured, in the absence of substrate, and found to be 77 nmol NADPH oxidized/min/nmol P450 (at 23°C).

No oxidation was detected for the neurotransmitters dopamine, tyramine, serotonin, or melatonin. Further analysis with some known substrates of other P450s, e.g. arachidonic acid, docosahexaenic acid (Stark et al., 2005), and a number of steroids known to be oxidized by various P450s (including cholesterol, testosterone, progesterone, 17 β -estradiol, and androstenedione) were also investigated using methods adapted from studies with other P450s (Wang et al., 2000; Krauser and Guengerich, 2005)), all yielding negative results. The limits of detection for these assays are listed in Supplemental Data Table S2.

Discussion

In this study we report initial characterization of one of the orphan P450s, P450 20A1. This gene resides on human chromosome 2q33 and appears to be the only member of P450 Family 20 in both humans and rodents. Alignments between human and rodent sequences showed a high degree of sequence similarity (Supplemental Material Fig. S1). This conservation may suggest a possible physiological function. The codon-optimized P450 20A1 expressed in *E. coli* was also prepared from this human sequence.

Initial mRNA dot blot analysis suggested a highly selective expression in human substantia nigra (Fig. 3), meriting further characterization of the RNA distribution pattern of this enzyme. Further mRNA studies also established that P450 20A1 mRNA is expressed in several other brain regions including amygdala, hippocampus, basal ganglia, and cerebellum, along with human liver (Fig. 4). P450s that are constitutively expressed in liver are in some cases found to be inducible in brain (Miksys et al., 2002). P450 20A1 mRNA is expressed in both adult liver and brain. Although the overall total P450 20A1 mRNA expression level in human brain was ~2-fold lower than the corresponding value in human liver, certain brain regions (e.g. hippocampus, substantia nigra, and amygdala) exhibit high mRNA expression levels. In the case of hippocampus the increase is between 2- and 3-fold. Similar mRNA patterns were also detected in rat brain where P450 20A1 mRNA was detected in substantia nigra, hippocampus, and amygdala, as judged by *in situ* hybridization (Fig. 6).

The initial dot blot results showed expression only in the substantia nigra (Fig. 3). In general, real-time PCR is considered to be a more sensitive quantitative detection method for mRNA compared to blot analysis. With rat P450 20A1, we found evidence of the presence of two splice variants. Analysis of expression indicates that the overall P450 20A1 mRNA level is relatively low, and the presence of different transcripts does not imply the existence of several protein isoforms because both of the alternative splice variants will lead to early termination of translation. For human P450 20A1 no splice variants have been reported, although we cannot completely exclude the possibility. The difference in the patterns between the strong mRNA expression in substantia nigra detected on the dot blots (Fig. 3) and the real-time PCR analysis (Fig. 4) may be due to the presence of alternate spliced variants that do not translate into functional protein in substantia nigra, or simply to the more qualitative nature of the blot assay (Fig. 3). Recently a brain specific variant of P450 1A1 (not present in liver from the same person) was reported (Ravindranath et al., 2006), but this result has not been independently confirmed. Real-time PCR confirmed the extrahepatic expression pattern of P450 20A1 mRNA to be primarily centered in brain and showed a 2- to 3-fold increase in hippocampus and a >10-fold increase in the substantia nigra, as compared to adult liver. Because most available mRNAs are single donor mRNA samples, it is conceivable that the values may differ considerably between individuals and give rise to different expression patterns.

Although liver is the major organ involved in xenobiotic metabolism, a number of reports indicate that the P450 enzymes are present in extrahepatic tissues (e.g., lung, kidney, brain) and may contribute to the metabolism of xenobiotics in these organs (Miksys et al., 2002). Some human P450s in brain have been found to oxidize substrates involved in neurological processes and metabolism of important endogenous mediators, e.g. cholesterol and neurotransmitters (Bogdanovic et al., 2001; Yu et al., 2003). Many P450s found in brain also exhibit regional distribution (Strobel et al., 2001; Wu et al., 2005). Other P450s show primary expression in brain. P450 26B1 and the recently described P450 46B1 are involved in the metabolism of the endogenous substances *all-trans* retinoic acid and cholesterol, respectively (Bogdanovic et al., 2001; Trofimova-Griffin and Juchau, 2002; Mast et al., 2003). Drugs used in treatment of common neurological and mental diseases (e.g., clozapine, phenytoin, and antidepressants) exhibit specific function in certain brain regions (Kusumi et al., 1995; Riedl et al., 1998).

At this point only limited information is available about the regulation and function of this new orphan P450. The tissue localizations of many of these P450s may provide clues as to their putative function, although their specific roles in endogenous metabolism may be difficult to predict based solely on their localization. An important question involving the brain P450s is whether they primarily are involved in the metabolism of endogenous substrates or in drug and xenobiotic metabolism (Miksys et al., 2002). The development of better approaches to defining functions of the orphan proteins will be important for our understanding of these enzymes, and the exact function of this particular orphan remains to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M.V. Martin, M. Andersson, and M. Dostalek for technical assistance.

Supported in part by the by Henning and Johan Trone Holst stiftelse (K.S.), Svenska Läkaresällskapet och Apotekar societeten (K.S.), and USPHS grants R37 CA090426, T32 ES007028, and P30 ES000267 (F.P.G.).

ABBREVIATIONS

DIG	digoxigenin
EST	expressed sequence tag
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
P450	cytochrome P450 (also termed “heme thiolate P450”. (Palmer and Reedijk, 1992); <i>CYP</i> indicating the gene in question)
PBS	phosphate-buffered saline (15 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl)
PCA	polymerase chain assembly
PCR	polymerase chain reaction
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline (0.10 M Tris-HCl (pH 7.5) containing 0.15 M NaCl)
SSC	15 mM sodium citrate buffer (pH 7.0) containing 150 mM NaCl)
UPLC	ultra performance liquid chromatography

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CCCTCATGCAACTGGAGTTTTAAAGGAACATCATAAAAGAACGAAAAGGAAGGAACCTTCAGTCAACATATTTTGACTCCTTAGTACAAGGGAACCTTAATGACCAACAG
P--S--C--N--W--S--F--K--E--H--H--K--R--T--K--R--K--E--L--Q--S--T--Y--F--D--S--L--V--Q--G--N--L--N--D--Q--Q

20A1n	native sequence	<u>ML</u> DFAIFAVTFLLALVGAVLYLYPASRQAAGIPGITPTEE
20A1 #8	P450 2C11 rat (modified)	MAKTKH <u>S</u> GIPGITPTEE
20A1 #9	P450 17A1 bovine	<u>MALLLAVFV</u> LYLYPASRQAAGIPGITPTEE
20A1 #10		<u>MAS</u> RQAAGIPGITTPTEE
20A1 #11		<u>MAS</u> RQASGIPGITTPTEE
20A1 #12		<u>MALLLAFL</u> DFAIFAVTFLLALVGAVLYLYPASRQAAGIPGITITPTEE

Fig. 2.

N-Terminal sequences used for heterologous expression of P450 20A1 membranes in *E. coli* (Wu et al., 2006b) (see also Supplemental Material Figs. S1, S6).

Fig. 3. mRNA dot blot analysis of human P45020A1 expression in individual human tissues. A ^{32}P -labeled oligonucleotide was selected for optimal recognition of P450 20A1 mRNA (and includes codons of the longer sequence, Fig. 1). A commercial blot (Clontech) with mRNA samples from individual human tissues was probed. This nitrocellulose blot was hybridized and washed according to the protocol described in Methods. The only positive region was A3, substantia nigra. The darker spots on the right side of the blot are artifacts and not positive results. The list of RNA samples follows (some zones were intentionally not loaded by the manufacturer, as controls: —): A1, whole brain; A2, cerebellum, left; A3, substantia nigra; A4, heart; A5, esophagus; A6, colon, transverse; A7, kidney; A8, lung; A9, liver; A10, leukemia, HL-60; A11, fetal brain; A12, yeast total mRNA; B1, cerebral cortex;; B2, cerebellum, right; B3, accumbens nucleus; B4, aorta; B5, stomach; B6, colon, descending; B7, skeletal muscle; B8, placenta; B9, pancreas; B10, HeLa S3; B11, fetal heart; B12, yeast tRNA; C1, frontal lobe (brain); C2, corpus callosum; C3, thalamus; C4, atrium, left; C5, duodenum; C6, rectum,; C7, spleen; C8, bladder; C9, adrenal gland; C10, leukaemia, K-562;

C11, fetal kidney; C12, *E. coli* tRNA; D1, parietal lobe; D2, amygdala; D3, pituitary gland; D4, atrium, right; D5, jejunum; D6,—; D7, thymus; D8, uterus; D9, thyroid gland; D10, leukemia, MOLT-4; D11, fetal liver; D12, *E. coli* DNA; E1, occipital lobe; E2, caudate nucleus; E3, spinal cord; E4, ventricle, left; E5, ileum; E6,—; E7, peripheral blood leukocyte; E8, prostate; E9, salivary gland; E10, Burkitt's lymphoma, Raji; E11, fetal spleen; E12, poly r(A); F1, temporal lobe; F2, hippocampus; F3,—; F4, ventricle, right; F5, ileocecum; F6,—; F7, lymph node; F8, testis; F9, mammary gland; F10, Burkitt's lymphoma, Daudi; F11, fetal thymus; F12, human C₀t-1 DNA; G1, postcentral gyrus of cerebral cortex; G2, medulla oblongata; G3,—; G4, interventricular septum; G5, appendix; G6,—; G7,—; G8, ovary; G9,—; G10, colorectal adenocarcinoma, SW480; G11, fetal lung; G12, human DNA (100 ng); H1, pons; H2, putamen; H3,—; H4, apex of the heart; H5, colon, ascending; H6,—; H7 trachea; H8,—; H9,—; H10, lung carcinoma, A549; H11,—; H12, human DNA, 500 ng.

Fig. 4.

Real time PCR of human P450 20A1. (A) Tissue distribution of 20A1 mRNA. The relative levels of P450 20A1 were determined using real-time PCR in the indicated tissues, using 18S RNA as a reference standard. P450 20A1 (longer variant) specific primers and SYBR Green PCR Mix™ were used for analysis. The relative expression was calculated using the C_t method (Livak method, from the manufacturer's instructions). For graphic presentation the graphs have been normalized and all values are compared with adult liver expression (set at 100). (B) Tissue distribution of 20A1 mRNA. The relative levels of P450 20A1 were

determined using real-time PCR for adult liver, hippocampus, and substantia nigra, using GAPDH as a reference standard.

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Fig. 5. Real-time PCR of rat P450 20A1. (A) Schematic representation of rat P450 20A1 splice variants. (B) Conventional RT-PCR analysis of rat heart, liver, and brain.

Fig. 6.
In situ hybridization for P450 20A1 in rat whole brain tissue. Representative photomicrographs are shown for (A) amygdala and (B) substantia nigra. The control slides (no probe, same conditions) are shown in parts C and D (controls for A and B, respectively). Hybridization was carried out with a DIG-labeled alkaline phosphatase cDNA antisense probe. Immunoreactivity was visualized by incubation of the sections by a BCIP/NBT Alkaline Phosphatase Substrate Kit™ (Vector Laboratories). Original magnification $\times 200$.

Fig. 7. Expression of bicistronic P450 20A1 construct in *E. coli*. An Fe^{2+} -CO vs. Fe^{2+} difference spectrum was recorded using 1/2 dilutions of whole cell extracts and reducing with $\text{Na}_2\text{S}_2\text{O}_4$.