

cDNA Cloning and Initial Characterization of CYP3A43, a Novel Human Cytochrome P450¹

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ABSTRACT

The RACE amplification technology was used on a novel CYP3A-like exon 1 sequence detected during the reverse transcriptase/polymerase chain reaction analysis of human CYP3A gene expression. This resulted in the identification of cDNAs encompassing the complete coding sequence of a new member of the CYP3A gene subfamily, CYP3A43. Interestingly, the majority of the cDNAs identified were characterized by alternative splicing events such as exon skipping and complete or partial intron inclusion. CYP3A43 expression was detected in liver, kidney, pancreas, and prostate. The amino acid sequence is 75% identical to that of CYP3A4 and CYP3A5 and 71% identical to CYP3A7. CYP3A43 differs from CYP3A4 at six amino acid residues, found within the putative substrate recognition sites of CYP3A4, that are known to be determinants of substrate selectivity. The N terminus of CYP3A43 was modified

for efficient expression of the protein in *Escherichia coli*, and a 6X histidine tag was added at the C terminus to facilitate purification. CYP3A43 gave a reduced carbon monoxide difference spectra with an absorbance maximum at 450 nm. The level of heterologous expression was significantly lower than that observed for CYP3A4 and CYP3A5. Immunoblot analyses revealed that CYP3A43 comigrates with CYP3A4 in polyacrylamide gel electrophoresis but does separate from CYP3A5. Monooxygenase assays were performed under a variety of conditions, several of which yielded reproducible, albeit low, testosterone hydroxylase activity. The findings from this study demonstrate that there is a novel CYP3A member expressed in human tissues, although its relative contribution to drug metabolism has yet to be ascertained.

Human cytochromes P450 3A are considered the major drug-metabolizing subfamily and are localized to the organs most associated with drug disposition, including the liver, gastrointestinal tract, and kidney. Of the CYP3A isoforms, CYP3A4 is the most abundant and metabolizes approximately 50% of the drugs currently in use (Thummel and Wilkinson, 1998). CYP3A5 is also found in the liver but at levels 10 to 30% of 3A4. Instead, CYP3A5 is the predominant form in the kidney (Wrighton and Vandenbranden, 1989). The third functional CYP3A is CYP3A7, which was originally isolated from fetal liver (Kitada et al., 1987). CYP3A7 expression in adult liver is questionable and certainly much lower than CYP3A4 and CYP3A5. However, there has never been definitive evidence suggesting that there are only three human members of the CYP3A subfamily. Recently, a genomic contig containing a putative gene for a fourth human CYP3A,

CYP3A43, has been sequenced (GenBank accession number AC011904). With the release of the first draft of the human genome, there seems little chance that additional members of the CYP3A subfamily will be discovered, making the identification of CYP3A43 of particular significance. Equipped with the knowledge that there are only four CYP3A members in humans, the role of each can be more precisely established, and the substrate specificities unraveled.

CYP3A5 and CYP3A7 exhibit approximately 85% amino acid sequence identity to CYP3A4 but only partially overlapping substrate specificities. Other minor CYP3A proteins could contribute to the activities that have thus far been attributed to CYP3A4 or CYP3A5, and the relatively high activity and broad substrate specificity of CYP3A4 may complicate the discovery and analysis of other cytochromes P450 in human tissue (Houston and Kenworthy, 2000). For example, it was recently found that a small amount of the predominant rat CYP2B1 in purified preparations of the minor form 2B2 resulted in an incorrect assessment of the functional properties of CYP2B2 (Strobel and Halpert, 1997). Because certain variants of these proteins comigrate in polyacryl-

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ABBREVIATIONS: P450, cytochrome P450; PCR, polymerase chain reaction; RT, reverse transcriptase; IgG, immunoglobulin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; DLPC, dilauroyl-L-3-phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; MOPS, 3-(N-morpholino)propanesulfonic acid; 7-BFC, 7-benzoyl-4-(trifluoromethyl)coumarin; bp, base pair(s); RACE, rapid amplification of cDNA ends.

amide electrophoresis, the contribution of the contaminating CYP2B1 was not discovered until CYP2B2 was isolated from a heterologous expression system. In addition, *in vivo* analyses of the metabolism of multiple CYP3A substrates have shown only weak correlations, and the reasons for this have not been well defined (Kinirons et al., 1994; Kenworthy et al., 1999). It is within the realm of possibility that other cytochromes P450 that are similar to CYP3A4 and are recognized by the same antibodies may have partially overlapping substrate specificities. If CYP3A43 contributes to the metabolism of some of the substrates tested, some metabolism rates reported to be catalyzed by CYP3A4 would seem artificially high, resulting in a poor correlation.

Recently, we have analyzed the expression pattern of human 3A isoforms in various tissues. This approach revealed the presence of a 3A-like exon 1 that differed from the known 3A genes. Using PCR primers based on this 3A-like sequence, the complete coding sequence of a novel human cytochrome P450, CYP3A43, has been determined. CYP3A43 expressed in *Escherichia coli* produced a reduced carbon monoxide difference peak at 450 nm and was recognized by anti-3A12 IgG. In addition, RT/PCR results provide the first evidence that a fourth member of the CYP3A subfamily is expressed in several human tissues.

Experimental Procedures

Materials. Oligonucleotide primers were obtained from Cybergene AB (Huddinge, Sweden) and the University of Texas Medical Branch Recombinant DNA Laboratory, National Institute of Environmental Health Sciences Center (Galveston, TX). Restriction enzymes, Luria-Bertani Broth, and Terrific Broth were purchased from Life Technologies (Grand Island, NY). The pGEM5 vector was purchased from Promega (Madison, WI). The Expand High Fidelity PCR kit and Rapid Ligation kit were purchased from Roche (Indianapolis, IN). The GeneClean II kit was obtained from Bio101 (Carlsbad, CA). CHAPS, DLPC, DOPC, phosphatidylserine, tetracycline, ampicillin, δ -aminolevulinic acid, isopropyl β -D-thiogalactoside, and MOPS were purchased from Sigma (St. Louis, MO). 7-BFC was purchased from Gentest (Woburn, MA). Acrylamide was obtained from National Diagnostics (Atlanta, GA). Nitrocellulose, ammonium persulfate, and TEMED were purchased from Bio-Rad (Hercules, CA). Talon metal affinity resin and Marathon ready cDNA from human liver were purchased from CLONTECH (Palo Alto, CA), and Qiagen Ni-NTA from Qiagen (Valencia, CA). [14 C]Progesterone was purchased from New England Nuclear (Boston, MA), and [14 C]testosterone was obtained from Amersham Pharmacia (Piscataway, NJ). CYP3A5 cDNA was kindly provided by Dr. Frank Gonzalez (National Cancer Institute, Bethesda, MD). The cDNA was cloned into the expression plasmid pKK233-2 (obtained from Amersham Pharmacia) with an N-terminal modification described previously (Gillam et al., 1995) and a C-terminal 4X histidine tag to produce pKK3A5H. CYP3A5 was expressed in *E. coli* and purified as described previously for CYP3A4 (Domanski et al., 1998). CYP3A4 was purified previously (Domanski et al., 1998). Silica thin-layer chromatography plates were purchased from J.T. Baker (Phillipsburg, NJ), and other reagents and supplies were obtained from standard sources.

cDNA Cloning. Human liver cDNA was prepared as described before (Finta and Zaphiropoulos, 2000a). RACE amplification (Frohman et al., 1988) on Marathon ready cDNA from human liver was performed in a two-step PCR approach using an initial and subsequently various nested primers, essentially following the recommendations of the kit, with Expand polymerase. A *SalI* site overhang was present in each of the nested primers, allowing cloning of the resulting products into a *SalI*-*NotI* digested pGEM5 vector. The primers

used for PCR amplification were as follows: 3A4 exon 1 forward (position 30–48 in GenBank accession number M18907); 3A43 exon 1 forward, 5' AAC TCA GAA GAC AGA GCT GAA A; 3A43 exon 1 forward, nested, 5' GCG TCG ACT TTG CCA TGG AAA CAT GGG TTC; 3A43 exon 3 forward, nested, 5' GCG TCG ACT TTG GAA TTT TGA CAG AGA ATG TAA TG; 3A43 exon 10 forward, nested, 5' GCG TCG ACT GAT CTG GAG CTT GTG GCC CAG.

Full Length cDNA Construct. The following forward and reverse primers were used with *NotI* and *SalI* overhangs, respectively: exon 1 forward, 5' GCG CGG CCG CGA TGG ATC TCA TTC CAA ACT TT; exon 13 reverse, 5' GCG TCG ACA AAG TCA GGG TCC ACT TGT AA. Human liver cDNA was amplified for thirty cycles using Expand polymerase for 1 min at 94°C, 1 min at 58°C and 1 min at 72°C with sequential 4 sec increases at each of the extension steps.

Analysis of CYP3A43 Tissue Distribution. The human I, II, and fetal human cDNA panels were subjected to 25, 30, 35, and 40 cycles of PCR amplification for 5 s at 92°C, 20 sec at 52°C and 30 sec at 72°C using the following primers: exon 1 forward, 5' ACA GAG CTG AAA AAG AAA AC; Exon 2 reverse, 5' TAG AAC AAA ATA GTT CCC AG. Products started to be detected at cycle 35 and were more clearly visible after 40 cycles. The PCR products were cloned and sequenced to ensure that they represent 3A43 and not misprimed sequences.

Cloning CYP3A43 cDNA into the pSE380 Expression Vector. For efficient expression in *E. coli*, PCR was used to modify the N terminus of CYP3A43. Amino acid residues 2 to 12 were deleted and residues 13 to 18 were modified to coincide with the corresponding sequence described by Barnes et al. for P450 17 α (Barnes et al., 1991; Gillam et al., 1993). In addition, a 6X histidine tag was added at the C terminus and an *NcoI* site that interfered with cloning was removed (C to A at bp 540 of cDNA). A two-step PCR methodology was used. Plasmid pGEM5 containing the entire coding region of CYP3A43 was used as the template. In the first step, primer 5' *NcoI*, incorporating an *NcoI* site and the 17 α sequence (5' GCC GGC CCA TGG CTC TGT TAT TAG CAG TTT TTC TGG TAC TCC TCT ATA TTT ATG GG), and primer Δ *NcoI*, to remove an additional *NcoI* site (5' GTG ATT ACA TCC ATT GTG TAG GCC CC), were used to produce a fragment containing 532 bp. This product, A, and primer 6X his (5' CCG GGC ACT AGT TCA ATG GTG ATG GTG ATG GTG GGG TCC ACT TGT AAT CCC), were used in the second PCR, again using the 3A43-containing pGEM5 clone. The 1520-bp product of the second PCR was digested with *NcoI* and *SpeI*. The desired fragment was purified with the GeneClean II kit, ligated to pSE380 treated similarly, and transformed into DH5 α cells. Resulting colonies were checked for the presence of the appropriately sized pS3A43H. DNA was purified with the Qiagen Midi kit and verified by sequencing (Protein Chemistry Laboratory, University of Texas Medical Branch, Galveston, TX).

Heterologous Expression and Purification of CYP3A43. CYP3A43 was expressed in *E. coli* as described previously (Domanski et al., 1998) with the following modifications. Plasmid pS3A43H was transformed into TOPP3 cells. Single colonies were inoculated into 2 ml of Luria-Bertani broth containing ampicillin (50 μ g/ml) and tetracycline (15 μ g/ml). The cultures were grown overnight at 37°C. A 1/1000 dilution of culture was inoculated into 20 ml of Luria-Bertani media containing ampicillin (50 μ g/ml) and tetracycline (15 μ g/ml) and was grown as described above. Fifteen microliters of this culture was added to 250 ml of TB media containing ampicillin (50 μ g/ml). The cultures were grown 2 to 2.5 h at 37°C at 250 rpm, after which δ -aminolevulinic acid (80 mg/L) and isopropyl β -D-thiogalactoside (1 mM) were added. The flasks were incubated at 30°C for 72 h at 190 rpm.

Solubilized membranes were prepared as described previously (John et al., 1994; Richardson et al., 1995; Harlow and Halpert, 1997). For preparations that were used for purification, the procedure was performed with the modifications outlined previously (Domanski et al., 1998). His-tagged CYP3A43 was purified by column chromatography using Qiagen Ni-NTA Agarose. Resin (1 ml)

was equilibrated with 5 ml of buffer A (100 mM MOPS, pH 8.0, 10% glycerol) plus 0.5% CHAPS and 0.5 M KCl. Solubilized membrane preparations of CYP3A43 from 12 250-ml cultures were loaded onto the column at 9 ml/h. The column was then washed with 10 bed volumes of buffer A plus 0.5% CHAPS and 0.5 M KCl followed by 10 bed volumes of buffer A alone. CYP3A43 was eluted with buffer A containing 0.5 M imidazole. The P450-containing fraction was dialyzed overnight in two 1-l changes of MOPS buffer (100 mM MOPS, pH 7.3, 10% glycerol, 0.2 mM dithiothreitol, 1 mM EDTA). The P450 content was determined by reduced carbon monoxide difference spectra.

Western Blot Analysis. Purified samples of CYP3A4, CYP3A5, and CYP3A43 (1 pmol per lane of each) were run separately, in pairs, or in a mix of all three on an 8.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with anti-3A12 IgG as described previously (Ciaccio and Halpert, 1989; Kedzie et al., 1991). Human anti-3A4 IgG, raised against a C-terminal peptide, was obtained from Gentest and immunoblotting was performed according to the manufacturer's instructions.

Steroid Hydroxylase Assays. CYP3A43 was assayed under a number of conditions. All assays were carried out with 10 pmol of P450 in 100 μ l with a final methanol concentration of 1% for substrate delivery. Except for the cumene hydroperoxide-dependent assays, the reactions were started by the addition of 1 mM NADPH. All incubations were carried out for 5 to 10 min, the reactions were stopped with 50 μ l of tetrahydrofuran, and the metabolites were resolved by thin-layer chromatography as described previously (Domanski et al., 1998). Steroid hydroxylase assays contained 50 to 150 μ M [14 C]progesterone or 50 to 200 μ M [14 C]testosterone. First, some reactions were carried out in 50 mM HEPES, pH 7.6, containing 15 mM MgCl₂, 0.1 mM EDTA, and 0.04% CHAPS (Domanski et al., 1998). These reconstitutions contained a range of molar ratios of P450 to rat NADPH-P450 reductase (1:2 to 1:8), varying molar ratios of P450 to cytochrome b₅ (1:0 to 1:2), and 0.1 mg/ml DOPC. A second set of reactions was run in 50 mM HEPES, pH 7.6, plus 30 mM MgCl₂, 0.1 mM EDTA, and 0.02% sodium cholate (Ueng et al., 1997). These reconstitutions included P450 to rat NADPH-P450 reductase molar ratios of 1:2 and 1:4, molar ratios of P450 to cytochrome b₅ of 1:0 and 1:2, and 0.1 mg/ml of a 1/1/1 mix of DOPC, DLPC, and phosphatidylserine. Third, other reactions were conducted in 0.1 M KPO₄ buffer, pH 7.4, containing 0.04% CHAPS (Imaoka et al., 1992). These reconstitutions contained a range of molar ratios of P450 to rat NADPH-P450 reductase (1:4 to 1:8), varying molar ratios of P450 to cytochrome b₅ (1:0 to 1:2), and 0.1 mg/ml of a 1/1/1 phospholipid mix. Fourth, cumene hydroperoxide-dependent assays were conducted. The reactions contained either 50 mM HEPES, pH 7.6, 30 mM MgCl₂, and 0.1 mM EDTA (Ueng et al., 1997) or 0.1 M KPO₄, pH 7.4, and 0.1 mg/ml 1/1/1 phospholipid mix (Imaoka et al., 1992). The reactions were started by the addition of 150 μ M cumene hydroperoxide in acetone (1% final concentration).

Results

cDNA Cloning. Using a primer from the 5' untranslated region of the 3A4 cDNA (see *Experimental Procedures*) in RT/PCR amplification from human liver RNA, a PCR product was obtained that encompassed the complete coding sequence of an exon 1 and differed from the known sequences of 3A4, 3A5, and 3A7. Because this exonic sequence had a methionine codon at the equivalent position as the known CYP3As and an open reading frame, it was likely to represent part of a novel 3A P450. To obtain the remaining sequence, RACE amplification was performed using primers designed from this exon 1. Twenty cloned products were analyzed from human liver but none extended beyond exon 3 of this new 3A P450. To obtain additional sequence informa-

tion, RACE was performed using a nested primer from the newly identified exon 3. The longest clones obtained extended up to exon 10, and this sequence information was used to design an additional nested primer that allowed the cloning of the remaining sequences, including the last exon, exon 13. The coding sequence of this P450 and its comparison with 3A4, 3A5, and 3A7 are shown in Fig. 1. This sequence is identical with a putative cytochrome P450 genomic sequence recently identified within the High Throughput Genomic Se-

	1		50
3A4	MALIPDLAME TWLLLVASLV LLYLYGTHSH GLFKKLGIPIG	PTPLPFLGNI	
3A5	MDLIPNLAVE TWLLLVASLV LLYLYGTRTH GLFKKLGIPIG	PTPLPLLGNV	
3A7	MDLIPNLAVE TWLLLVASLV LLYLYGTRTH GLFKKLGIPIG	PTPLPFLGNA	
3A43	MDLIPNFAME TWVLVATSLV LLYLYGTHSH KLFKKLGIPIG	PTPLPFLGTT	
	51		100
3A4	LSYHKGFCMF DMECHKKYGK VWGFIYDQQP	VLAITDPMI KTVLVKECYS	
3A5	LSYRQGLWKF DTECYKKYK MWGTYEGQLP	VLAITDPMI RTVLVKECYS	
3A7	LSFRKGYWTF DMECYKKYK VWGIYDQQP	MLAITDPMI KTVLVKECYS	
3A43	LFYLRGLWNF DRECKEYGE MWGLYEGQQP	MLVIMDPMI KTVLVKECYS	
	101		150
3A4	VFTNRRPFGP VGFMKSAISI AEDEEWKRLR	SLLSPTFTSG KLKEMVPIIA	
3A5	VFTNRRSLGP VGFMKSAISL AEDEEWKRIR	SLLSPTFTSG KLKEMFPIIA	
3A7	VFTNRRPFGP VGFMKNAISI AEDEEWKRIR	SLLSPTFTSG KLKEMVPIIA	
3A43	VFTNQMPFGP MGFLKSALSF AEDEEWKRIR	TLLSPAFTSV KFKEMVPIIS	
	151		200
3A4	QYGDVLVRNL RREAETGKPV TLKDVFGAYS	MDVITSTSG VNIDSLLNNPQ	
3A5	QYGDVLVRNL RREAEGKGPV TLKDIFGAYS	MDVITGTSFG VNIDSLLNNPQ	
3A7	QYGDVLVRNL RREAETGKPV TLKHVFGAYS	MDVITSTSG VSIDSLLNNPQ	
3A43	QCQDMLVRSL RQEAENKSI NLKDFFGAYT	MDVITGTLFG VNIDSLLNNPQ	
	201		250
3A4	DPFVEVTKK LKFGFLDPLF LSITVFPFLI	PILEVNLICV FPPEVNTFLR	
3A5	DPFVESTKKF LKFGFLDPLF LSIIILFFLT	PVFALNVSL FPKDITNLS	
3A7	DPFVENTKKL LRFNLDPLF LSIVKFFLT	PILEALNITV FPKVTSFTL	
3A43	DPFLKNMKKL LKLDLDFPLF LLISLFFLT	PVFALNIGL FPKDVTFLK	
	251		300
3A4	KSVKRMKESR LEDTQKHRVD FLQLMIDSQN	SKETESHKAL SDLELVAQSI	
3A5	KSVNRMKKS LNDKQKRLD FLQLMIDSQN	SKETESHKAL SDLELVAQSI	
3A7	KSVQIKESR LKETQKHRVD FLQLMIDSQN	SKDSETHKAL SDLELVAQSI	
3A43	NSIERMKESR LKDKQKHRVD FFQOMIDSQN	SKETKSHKAL SDLELVAQSI	
	301		350
3A4	IFIFAGYETT SSVLSFIMYE LATHPDVQK	LQEEIDAVLP NKAPPTYDTV	
3A5	IFIFAGYETT SSVLSFTLYE LATHPDVQK	LQKEIDAVLP NKAPPTYDAV	
3A7	IFIFAGYETT SSVLSFIIYE LATHPDVQK	VQKEIDVLP NKAPPTYDTV	
3A43	IIIFAAAYDTT STTLFFIMYE LATHPDVQK	LQEEIDAVLP NKAPVTYDAL	
	351		400
3A4	LQMEYLDVV NETLRLFPV MRERCKKD	VEINGMFIPK GVVVMIPSYA	
3A5	VQMEYLDVV NETLRLFPV IRLERTCKKD	VEINGVFIPK GSMVVIPTA	
3A7	LQLEYLDVV NETLRLFPV MRLERVCKKD	VEINGMFIPK GVVVMIPSYV	
3A43	VQMEYLDVV NETLRLFPV SVTRVCKKD	IEINGVFIPK GLAVMVIPTA	
	401		450
3A4	LHRDPKYWTE PEKFLPERFS KKNKDNIDPY	IYTPFGSGPR NCIGMRFAIM	
3A5	LHHDPKYWTE PEEFRPERFS KK.KDSIDPY	IYTPFGTGR NCIGMRFAIM	
3A7	LHHDPKYWTE PEKFLPERFS KKNKDNIDPY	IYTPFGSGPR NCIGMRFAIV	
3A43	LHHDPKYWTE PEKFCPERFS KKNKDSIDLY	RYIPFGAGPR NCIGMRFAIT	
	451		500
3A4	NMKLALIRVL QNFSFKPCKE TQIPLKLDQ	GLLQPEKPIV LKVESRDGTV	
3A5	NMKLALIRVL QNFSFKPCKE TQIPLKLDQ	GLLQPEKPIV LKVDSDGTV	
3A7	NMKLALIRVL QNFSFKPCKE TQIPLKLDQ	GLLTLEKPIV LKESRDET	
3A43	NIKLAIRAL QNFSFKPCKE TQIPLKLDQ	PILQPEKPIV LKHVLRDGT	
	501		
3A4	SGA		
3A5	SGE		
3A7	SGA		
3A43	SGP		

Fig. 1. Amino acid sequence comparison of 3A43 with 3A4 (GenBank accession number M18907), 3A5 (GenBank accession number J04813), and 3A7 (GenBank accession number D00408). The underlined sequence shows the region that was modified to ALLLAVF for bacterial expression. The underlined and shaded residues are those that have previously been studied and found to play a role in CYP3A4 substrate selectivity. The bold, italicized residues in the CYP3A43 amino acid sequence are those that differ from CYP3A4 at such sites.

quence Database (<http://drnelson.utmem.edu/Nomenclature.html>). According to the standardized P450 nomenclature, the name assigned for this P450 is 3A43.

Amino Acid Sequence Alignment of CYP3A43 with CYP3A4, 3A5, and 3A7. An amino acid alignment of CYP3A43 with the other members of the human CYP3A subfamily, illustrates that although the four P450s show significant similarity, there are notable differences. For example, CYP3A43 differs significantly from the other forms at the N terminus (underlined sequence in Fig. 1). Structure-function analyses of CYP3A4 have so far identified 17 residues that are important for substrate specificity/regioselectivity (Fig. 1, shaded, underlined text) (Harlow and Halpert, 1997; He et al., 1997; Domanski et al., 1998; Wang et al., 1998). Of these, CYP3A43 differs from CYP3A4 at six sites (Fig. 1, italic, bold text). Of these 17 sites of known importance, CYP3A43 contains unique residues at two sites conserved in the other members of the human CYP3A subfamily (V370 and V373) (Fig. 1). CYP3A also differs from CYP3A4 and/or CYP3A5 at four additional sites that are important for regioselectivity of steroid hydroxylation (L108, V369, D478, and N479) (Wang et al., 1998). In addition, a number of other putative substrate recognition site residues vary between CYP3A43 and the other CYP3A members.

Tissue Distribution of CYP3A43. The expression level of 3A43 in human liver is not very high, making it difficult to obtain clear signals in a dot blot assay with mRNA from human liver and from other tissues. Specifically, the Human Multiple Tissue Expression Array (Clontech) was negative when probed with sequences from 3A43. However when cDNA panels from adult and fetal human tissues were subjected to PCR amplification, 3A43 expression was detected in adult liver, kidney, pancreas, and prostate, as well as in fetal liver and skeletal muscle. The results of this tissue distribution analysis are shown in Fig. 2.

cDNA Products Generated by the Use of Exons 1 and 13 PCR Primers. The 3A43 sequence obtained from the RACE analysis was not represented in a single clone, because three sequential RACE amplifications had been performed. To obtain a single cDNA clone containing the contiguous coding sequence of 3A43, PCR amplification was performed on human liver cDNA using primers encompassing the methionine and the termination codons (see *Experimental Procedures*). Fourteen clones were analyzed, and one was identified with no nucleotide misincorporation that would alter the reading frame. The single base change present at codon 243, AAG instead of AAA, does not alter the corresponding amino acid, Lys. Interestingly, eight of the analyzed clones

did not encompass the contiguous coding sequence of 3A43. Instead, each of these revealed one or more splicing events that distinguished them from the canonical 3A43 mRNA. Two clones were found to lack exon 12 and are therefore likely to code for a nonfunctional P450, because the conserved cysteine residue would be absent and the change in the reading frame in exon 13 would result in premature termination. Exon 4 was found to be skipped in four clones, and this event would also change the reading frame. The complete intron five was found in two clones, as judged by the identity with GenBank accession number AC011904 that contains the complete 3A43 gene. The 58 bases positioned at the 5' end of intron 5 (a sequence followed by GT intronic dinucleotides in GenBank accession number AC011904) were present in three clones. A 121-base Alu-like sequence from intron 7 (position 30765–30885 in GenBank accession number AC011904, flanked by AG and GT intronic dinucleotides) was found in two clones. Collectively these observations provide evidence that at a high frequency (8 of 14), transcripts distinct from the canonical 3A43 mRNA are generated from the 3A43 locus.

Expression and Purification of CYP3A43. The N terminus of CYP3A43 was truncated (amino acids 2–12) and modified to MALLAVF to correspond to that found in CYP17 α (Barnes et al., 1991; Gillam et al., 1993). This sequence has been found to significantly increase the heterologous expression of a number of mammalian cytochromes P450 (Gillam et al., 1993; John et al., 1994; Richardson et al., 1995; Harlow and Halpert, 1997). When CYP3A43 was expressed in *E. coli* TOPP3 cells, the protein expression level was relatively low (4–28 nmol of P450/liter of culture) compared with the expression typically observed with CYP3A4 (100–300 nmol P450/liter of culture). The absorbance peak of the Fe²⁺-CO complex was found to be at 450 nm (data not shown).

For purification of CYP3A43, solubilized preparations from 12 separate 250-ml cultures were combined to produce sufficient protein (18–20 nmol) to undergo column purification. In a first attempt, Talon Metal Affinity Resin was used. In this instance, more than 80% of the protein bound to the column but would not elute, although a final concentration of up to 1 M imidazole was added. Instead, Qiagen Ni-NTA was used to purify CYP3A43 (see *Experimental Procedures*). CYP3A43 was eluted in buffer containing 500 mM imidazole and dialyzed in MOPS buffer, pH 7.3. The total yield of CYP3A43 was 18.7%. CYP3A43 was analyzed by SDS-polyacrylamide gel electrophoresis to ensure a similar level of purity compared with CYP3A4 (Fig. 3a).

Western Blot Analysis of CYP3A43. As reported by others, CYP3A4 and CYP3A5 have different electrophoretic mobilities on polyacrylamide gels and in Western blot analysis (Aoyama et al., 1989). In this study, equal amounts of CYP3A4, CYP3A5, and CYP3A43 were run together, in pairs, and singly on a SDS-PAGE. After transfer to nitrocellulose and probing with an anti-3A12 polyclonal antibody, CYP3A43 separated from CYP3A5, but CYP3A4 and CYP3A43 were found to comigrate (Fig. 3b). Both CYP3A4 and CYP3A43 were recognized by anti-3A4 IgG that was raised against a C-terminal peptide (data not shown).

Functional Characterization of CYP3A43. Neither solubilized membrane preparations nor purified samples of CYP3A43 displayed appreciable testosterone or progesterone

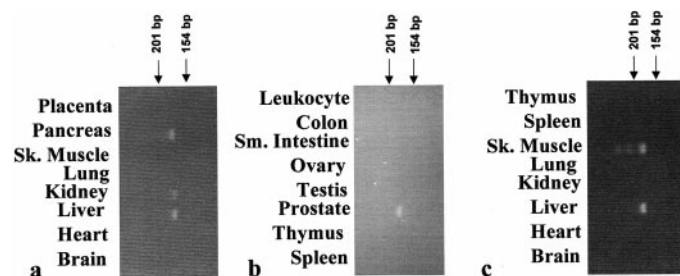


Fig. 2. Distribution of CYP3A43 mRNA in various (a and b) adult and (c) fetal tissues. The products of a 40-cycle PCR amplification with CYP3A43 primers (see *Experimental Procedures*) on adult and fetal human cDNA panels (CLONTECH) were analyzed by a 3% agarose gel electrophoresis.

hydroxylase activity when reconstituted according to our standard procedure for CYP3A4 and 3A5 (Domanski et al., 1998) (data not shown). Consequently, a number of additional assay conditions were tested. Each condition was derived from previous studies (Imaoka et al., 1992; Ueng et al., 1997) (see *Experimental Procedures*) and is similar to methods used by others for CYP3A4, CYP3A5, and CYP3A7 (Gillam et al., 1993, 1995, 1997). Conditions that resulted in a relatively low, but reproducible level of testosterone 6 β -hydroxylase activity by CYP3A43 are presented in Table 1. When similar conditions were used in progesterone hydroxylase and 7-BFC debenzylase assays, significant levels of CYP3A43 activity were not observed (data not shown).

Discussion

In this study, we report the cloning and initial characterization of a new human CYP3A, designated CYP3A43. The amino acid sequence of CYP3A43 is 75.7% identical to CYP3A4, 75.6% identical to CYP3A5, and 71.3% identical to CYP3A7. These percentages are somewhat lower than the 81 to 88% identity among CYP3A4, CYP3A5, and CYP3A7. In agreement with this finding is the observation that the 3A43 gene is not embedded within the tandemly arranged 3A4, 3A7, and 3A5 genes (Finta and Zaphiropoulos, 2000b). Moreover, comparison of the 3A43 GenBank accession number AC011904 with GenBank accession number AC069294 (version of June 27, 2000), an entry containing 25 unordered contigs spanning the 3A locus, suggests that the 3A43 gene

may be upstream of 3A4 and in the opposite orientation from all the other genes of the 3A cluster.

The apparent low expression of CYP3A43 in adult and fetal tissues has most likely prevented the isolation of this protein through conventional purification schemes that were successful for other members of the human CYP3A subfamily (Beaune et al., 1986; Kitada et al., 1987; Wrighton and Vandenberg, 1989). Instead, the advent of RT/PCR technology, and the ability to detect low levels of expression, have allowed the discovery of proteins whose function remains unknown. In addition, the apparent level of expression of CYP3A43 may not be indicative of its actual role in xenobiotic metabolism. For example, until recently, human P450 CYP2B6 had been classified as a very minor component of the human liver (Ekins et al., 1998). However, a number of recent reports have revealed a larger role for CYP2B6 than predicted (Kobayashi et al., 1999; Svensson and Ashton, 1999; Yamazaki et al., 1999). In many cases, the ability to identify CYP2B6 substrates has been improved with the development of heterologous expression systems for this enzyme.

Although heterologous expression of CYP3A43 under the conditions that have been successful for CYP3A4 and CYP3A5 produced sufficient protein for purification, the optimal conditions for this enzyme may not be similar to those for CYP3A4 and CYP3A5. This is not surprising considering the additional manipulations required to obtain reasonable levels of CYP3A5 and CYP3A7 expression (Gillam et al., 1995, 1997). For CYP3A7, at least one residue in the C terminus of the protein was found to be deleterious to heterologous expression in *E. coli*. When Thr-485 was converted to Pro, the corresponding residue in CYP3A4, CYP3A7 expression increased significantly (Inoue et al., 2000). CYP3A43 contains Pro at position 485, but other sites similarly important for efficient bacterial expression undoubtedly exist, and their identification may lead to enhancement of CYP3A43 expression in *E. coli*. For CYP3A43, the ability to use a single-column metal affinity purification scheme because of the 6X-His tag made it possible to achieve partial purification of the protein with a much smaller amount of starting material than would be necessary with traditional, multicolumn purification schemes (Gillam et al., 1993).

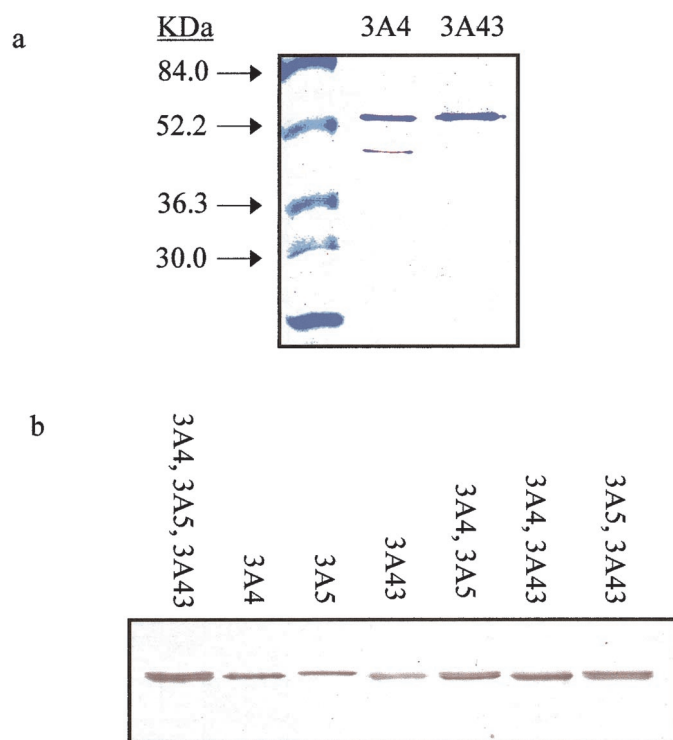


Fig. 3. Analysis of CYP3A43 protein. (a) SDS-polyacrylamide gel electrophoresis. Metal affinity-purified samples of CYP3A4 and CYP3A43 (1 pmol) were run on an 8.5% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. (b) Immunoblot analysis of CYP3A4, CYP3A5 and CYP3A43. Each P450 (1 pmol) was run on an 8.5% polyacrylamide gel separately, in pairs, or all mixed. Proteins were transferred to nitrocellulose and probed with anti-3A12 IgG.

TABLE 1

Testosterone hydroxylase assays of CYP3A4 and CYP3A43

In the assays listed, the concentration of testosterone was 200 μ M and a 1:1:1 mix of DOPC/DLPC/phosphatidylserine was included. The values reported represent the average of two assays performed in duplicate.

Reconstitution Conditions	Testosterone 6 β -OH	
	CYP3A4	CYP3A43
	nmol/min/nmol	
HEPES buffer (pH 7.6) ^a		
1:2:0 ^b plus 0.02% Na cholate, 15 mM MgCl ₂ , 0.1 mM EDTA	14.8	0.2
1:2:2 plus 0.02% Na cholate, 15 mM MgCl ₂ , 0.1 mM EDTA	44.4	0.3
KPO ₄ buffer (pH 7.4) ^c		
1:4:0 plus 0.04% CHAPS	13.2	0.2
1:4:1 plus 0.04% CHAPS	10.2	0.3
1:4:2 plus 0.04% CHAPS	10.6	0.3

^a Ueng et al. (1997).

^b 1:2:0 refers to the molar ratio of P450 to rat NADPH-P450 reductase to cytochrome b₅.

^c Imaoka et al. (1992).

The lack of monooxygenase activity observed with our standard CYP3A4 reconstitution conditions, prompted a more thorough investigation of optimal conditions for CYP3A43. Although a relatively low level of testosterone 6 β -hydroxylase activity was obtained, the substrate specificity of CYP3A43 may be different enough from other CYP3A forms that the appropriate CYP3A43 substrates were not tested in this study. For example, 7-BFC is a good substrate of CYP3A4, but CYP3A5 shows little activity (K. K. Khan, Y.-Q. He, J. R. Halpert, unpublished observations). In addition, testosterone is a very good substrate of CYP2B1 but is a poor substrate of CYP2B6 (Yang et al., 1998). The substitutions identified in CYP3A43 compared with the other CYP3As are unlikely to explain the low activity observed (Fig. 1), because substitutions made previously in CYP3A4 at substrate recognition site residues that differ between CYP3A4 and CYP3A43 have not resulted in total loss of activity (Fig. 1) (He et al., 1997; Wang et al., 1998).

In conclusion, a new (and probably the last) human member of the CYP3A subfamily has been identified, cloned, and heterologously expressed. Initial studies indicate that CYP3A43 is expressed in the liver, kidney, pancreas, and prostate. Analyses of multiple clones of CYP3A43 cDNA revealed that a high frequency of alternatively spliced forms of CYP3A43 may be produced. Although cytochrome P450 genes are generally considered to result in single gene products (Nelson et al., 1996), the presence of 3A43 variants suggests that this may not necessarily be true. In fact, there is precedent in the scientific literature that alternative splicing may generate protein isoforms with distinct characteristics (Flouriot et al., 2000; Lazaridis et al., 2000; Rekasi et al., 2000). Moreover, the 3A5 gene (GenBank accession number AC005020) also generates alternative spliced mRNA forms at a high frequency. However, these variant mRNAs were originally interpreted as representing "3A5 pseudogenes" (Schuetz and Guzelian, 1995; GenBank accession numbers L26985 and X90579). Comparison of the "3A5 pseudogene" sequences with entry AC005020 clearly establishes that these contain partially spliced intronic segments of the 3A5 gene (Finta and Zaphiropoulos, 2000b). Given the recent evidence for the high frequency of alternative splicing in the human genome (Croft et al., 2000), it may be of particular interest to examine whether certain variant CYP3A mRNAs have the capability to code for proteins with P450-like functions.

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