PHARMACOGENETICS AND GENOMICS

Identification and phenotype characterization of two *CYP3A* haplotypes causing different enzymatic capacity in fetal livers

Background: **The fetal liver cytochrome P450 (CYP) 3A enzymes metabolize potentially toxic and teratogenic substrates and drugs in addition to endogenous hormones and differentiation factors. CYP3A7 is the most abundant CYP in the human liver during fetal stages and the first months of postnatal age and shows a large interindividual variability of unknown molecular basis.**

Methods: **A new variant gene (*CYP3A7*2*), which carries a mutation in exon 11 of *CYP3A7* causing a T409R substitution, was identified by direct sequencing. Genotype analysis was performed by use of polymerase chain reaction followed by restriction enzyme analysis. CYP3A7.2 activity was assessed in heterologous expression systems and human fetal liver microsomes.**

Results: **The frequency of *CYP3A7*2* was 8%, 17%, 28%, and 62% in white, Saudi Arabian, Chinese, and Tanzanian individuals, respectively. By use of human HEK293 cells, no significant differences in expression between CYP3A7.1 and CYP3A7.2 were found and fetal livers homozygous for *CYP3A7*2* had similar or higher CYP3A7 protein contents than *CYP3A7*1* livers. Kinetic studies showed that CYP3A7.2 was a functional enzyme with a significantly higher catalytic constant (k_{cat}) as compared with CYP3A7.1 ($P < .05$). Interestingly, fetal livers that expressed CYP3A7.2 also expressed CYP3A5 protein, and we found a linkage disequilibrium between the *CYP3A7*2* and *CYP3A5*1* alleles that was subject to interethnic differences. Determination of the alprazolam 1-hydroxylation rate revealed that CYP3A5 plays a significant role in the metabolism of CYP3A substrates in the fetal liver.**

Conclusion: **We have identified 2 different CYP3A phenotypes in the fetal liver—one that is the result of a *CYP3A7*1/CYP3A5*3* haplotype causing CYP3A7.1 but no CYP3A5 expression and another with higher detoxification capacity, inherent in the *CYP3A7*2/CYP3A5*1* haplotype, where CYP3A5 and a more active form of CYP3A7 are expressed. (Clin Pharmacol Ther 2005;77:259-70.)**

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Cytochrome P450 (CYP) comprises a large superfamily of enzymes that catalyze the biotransformation of endogenous substrates and xenobiotics. Genes in families 1, 2, and 3 encode the most relevant enzymes catalyzing the biotransformation of xenobiotics, including a wide variety of drugs and toxicants, and have a

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large impact on disease treatment.[1] Polymorphic CYP variants are of importance for individual susceptibility to adverse drug reactions or lack of therapeutic effect of drugs at ordinary dosages. The CYP3As are the most abundant P450s in the human liver and small intestine, and their activity is made up by the expression of CYP3A4, CYP3A5, and CYP3A7, whereas the low expression levels of CYP3A43 and improper folding in mammalian systems rule out any significant contribution of this enzyme to CYP3A activity.[2,3] The expression of CYP3A enzymes is regulated in a developmental manner.[4-6] CYP3A4, which is involved in the metabolism of 50% of currently used therapeutic drugs, has an insignificant or very low expression in the fetus but constitutes 30% to 50% of total hepatic and intestinal CYP in adults.[7,8] CYP3A5 expression starts at the fetal stage and continues through adulthood,[6,9] but a frequent single nucleotide polymorphism (SNP) within intron 3 of CYP3A5 (6986G>A, *CYP3A5*3* allele) results in the absence of the CYP3A5 protein.[10] Individuals with at least 1 *CYP3A5*1* allele mainly have correctly spliced *CYP3A5* messenger ribonucleic acid (mRNA) and functional protein. CYP3A7 is the major CYP expressed in the fetal liver, in which it accounts for 50% of the total CYP content.[11] Its expression starts after 50 to 60 days of gestation and continues up to 6 months of postnatal age.[5,6] In adults, CYP3A7 expression is much lower, although some individuals have low but significant *CYP3A7* gene expression in the liver and intestine.[12]

CYP3A7 shares substrate specificity with CYP3A4 and CYP3A5 but has unique activity toward many xenobiotics and endogenous substrates. Thus it plays an important role in the metabolism of key steroids in the adrenals and gonads, such as dehydroepiandrosterone and testosterone, and also in that of potentially toxic and teratogenic endogenous substrates such as retinoic acid.[13-16] In addition, CYP3A7 is involved in the metabolic clearance of many xenobiotics that reach the fetus when taken during pregnancy, such as therapeutic drugs for women with long-term medical conditions (for example, heart disease, seizure disorders, coagulopathies, or acquired immunodeficiency syndrome) and substances of abuse such as cocaine, cannabinoids, and methadone.[17-20]

Because of the important roles of CYP3A7, the substantial interindividual expression differences found in fetal livers[9] are expected to have a significant impact in interindividual differences in embryotoxicity and teratogenicity. Polymorphism in *CYP3A7* could explain the expression variability of CYP3A7; however, no enzyme variants have been described so far. Herein, we identify a novel SNP in the *CYP3A7* coding region that results in the replacement of Thr409 with Arg, which alters the enzyme kinetics. The corresponding allele, *CYP3A7*2* (approved by the Human CYP Allele Nomenclature Committee),[21] is in linkage disequilibrium with *CYP3A5*1* and, therefore, with functional CYP3A5 enzyme in the liver, allowing the identification of high and low CYP3A activity haplotypes in the fetus.

METHODS

Materials. δ-Aminolevulinic acid, isopropyl β-D-thiogalactoside (IPTG), glucose, galactose, and nicotinamide dinucleotide phosphate, reduced (NADPH), were purchased from Sigma-Aldrich (St Louis, Mo). Protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany). Dehydroepiandrosterone (DHEA) [1,2,6,7-^{3}H(N)] (specific activity, 74 Ci/mmol) was purchased from Perkin-Elmer (Foster City, Calif). P450-Glo CYP3A7 assay was purchased from Promega (Madison, Wis). Lipofectamine-2000, Minimum Essential Medium, fetal bovine serum (FBS), streptomycin, penicillin, nonessential amino acids, and sodium pyruvate were from Invitrogen (Stockholm, Sweden). The expression vectors pCWori+, pCW′/NPR, and pCW′/3A4#17 were kindly provided by Dr Elisabeth Gillam (University of Queensland, Brisbane, Australia). Alprazolam, 4−hydroxy (OH)–alprazolam, and 1-OH-alprazolam were a gift from Leif Bertilsson (Karolinska Institutet, Stockholm, Sweden).

Sequencing of CYP3A7 and genotyping. The *CYP3A7*2* allele was detected after polymerase chain reaction (PCR) amplification of CYP3A7 complementary deoxyribonucleic acid (cDNA) from fetal liver (see reference 21 for allele nomenclature) (*CYP3A7*1* GenBank accession No. NM_000765.2). The deoxyribonucleic acid (DNA) fragment containing *CYP3A7*2* was amplified by use of 5′-ATCTCATCCCAAACTT GGCCG-3′ forward and 5′-TCAGGCTCCACTT ACGGTCT-3′ reverse primers, purified by use of the Wizard PCR Kit (Stratagene, La Jolla, Calif), sequenced by use of the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif), and analyzed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). The SNP detected was confirmed at the genomic level by direct sequencing.

*CYP3A7*2* genotyping was performed by restriction fragment length polymorphism (RFLP). A 385−base pair (bp) region covering parts of exon 11 and intron 11 of *CYP3A7* was amplified by PCR by use of 5′-CACCTATGATACTGTGCTACAGT-3′ forward and

5′-TGTGGTTTGTAAAGTGTGACAATG-3′ reverse primers. Then 10 μL of this PCR fragment was incubated with *Gsu*I overnight and separated by electrophoresis in an agarose gel. The *CYP3A7*1* allele resulted in the 385-bp undigested PCR fragment, whereas the *CYP3A7*2* allele resulted in 2 DNA fragments of 177 and 208 bp. *CYP3A5*1* and *CYP3A5*3* genotypes were assessed by allele-specific PCR as previously described.[22] *CYP3A7*1C* was analyzed as previously described.[12]

To determine whether *CYP3A7*2* could be in linkage disequilibrium with other polymorphisms in the *CYP3A7* coding region, the *CYP3A7* full coding region was amplified with 5′-CAGGAAAGCTCCACACA CAC-3′ forward and 5′-TGGGGCACAGCTTTCTT AAA-3′ reverse primers, by use of the cDNA from 8 fetal and 19 adult livers from white subjects. The PCR fragments were purified and sequenced as described.

Subjects. Allelic frequencies were determined by use of genomic DNA from unrelated individuals from different populations. The study included 101 volunteers from the Zaragoza area in northern Spain,[23] 47 Swedish volunteers,[22,24] 96 Chinese volunteers,[25] 70 Saudi Arabian volunteers,[26] and 90 Tanzanian volunteers. The genomic DNA from Swedish liver samples was extracted by use of the QIAamp Tissue Kit (Qiagen, Valencia, Calif). This study was approved by the Ethical Committee at Karolinska Institutet, Stockholm, Sweden.

Heterologous expression of CYP3A7*1 and CYP3A7*2. Full-length *CYP3A7*1* and *CYP3A7*2* cDNAs were PCR-amplified by use of Elongase (Invitrogen) and reverse-transcribed human liver ribonucleic acid (RNA) from individuals homozygous for the different alleles. The cDNAs were cloned in appropriate expression vectors, and the inserted sequences were analyzed by the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and ABI PRISM 377 DNA Sequencer (Applied Biosystems), to rule out PCR artifacts in the cloned sequences. Quantification of expressed CYP holoenzyme was performed by carbon monoxide–binding spectral analyses.[27]

For heterologous expression in *Escherichia coli*, the PCR was carried out with a forward primer that introduced an *Nde*I site at the 5′ end (5′-AAGGCATATG GATCTCATCCCAAACTTGGCCG-3′) and a reverse primer containing an *Xba*I site at the 5′ end (5′-CTTATCTAGATCAGGCTCCACTTACGGTCT-3′). After *Nde*I/*Xba*I digestion of the CYP3A7 cDNAs, DNA fragments were cloned into pCWori+ expression vector digested with the same restriction enzymes. *Bam*HI/*Xba*I fragments from these plasmids were subcloned into pCW′/hNPR, which contained human NADPH CYP reductase,[28] to generate a bicistronic vector. To achieve high levels of CYP expression, the DNA sequence corresponding to the N-terminal part of the protein was modified by digestion of the CYP3A7 bicistronic vectors with *Nde*I/*Stu*I, DNA purification to eliminate the small DNA fragments liberated, and ligation of the *Nde*I/*Stu*I fragment from pCW′/3A4#17.[29] The final bicistronic expression vectors contained the pCW′/3A4#17 sequence until the *Stu*I site followed by the sequence of *CYP3A7*1* or *CYP3A7*2* from amino acids 83 to 503. CYP expression was achieved as previously described,[30] with IPTG induction for 24 hours at 30°C and with the use of a bacteria strain, Rosetta (DE3) (Novagen, Madison, Wis), which contains a chloramphenicol-resistant plasmid that provides transfer RNAs that are rare in *E coli*. Bacteria fractioning was performed as previously described.[28]

For heterologous expression in *Saccharomyces cerevisiae*, CYP3A7 cDNAs were amplified by PCR with a forward primer that introduced 3 A residues in front of the translation start to increase the expression efficiency,[31] a *Bam*HI site at the 5′ end (5′-AAGGGGA TCCAAAATGGATCTCATCCCAAACTTGGCCG-3′), and a reverse primer containing a *Kpn*I site at the 5′ end (5′-CTTAGGTACCTCAGGCTCCACTTACGG TCT-3′). After double digestion of the amplified DNA with *Bam*HI/*Kpn*I, fragments were cloned into the pYeDP60 expression vector,[32] generating the plasmids V60-*3A7*1* and V60-*3A7*2*, respectively. The *S cerevisiae* strain W(R) engineered to overexpress the yeast CYP reductase[33,34] was used to express *CYP3A7*1* and *CYP3A7*2*, essentially as described elsewhere.[33,35] In brief, the yeast was transformed with the pYeDP60-derived expression vectors and grown to high density with glucose as the main energy source; thereafter galactose was added to induce expression. After expression, the cells were harvested and mechanically disrupted,[36] and microsomes containing the recombinant enzyme were isolated by differential centrifugation (20,000*g* for 10 minutes plus 100,000*g* for 60 minutes).

For expression in the adenovirus 5–transformed human embryonic kidney cell line 293 (HEK293 [CRL-1573, American Type Culture Collection, Manassas, Va]), the V60-*CYP3A7*1* and V60-*CYP3A7*2* plasmids described were digested with *Bam*HI/*Kpn*I and the insert was subcloned into pCMV4 expression vector[36] digested with *Bgl*II/*Kpn*I. The resulting expression vectors, pCMV4-*CYP3A7*1* and pCMV4-*CYP3A7*2*, were transfected by use of Lipofectamine-2000 into

Table I. *CYP3A7* and *CYP3A5* genotypes of fetal livers investigated

Fetal liver	*Duration of gestation (wk)*	*CYP3A7 genotype*	*CYP3A5 genotype*
FLU	Unknown	**1/*1*	**3/*3*
FL24	15	**1/*1*	**3/*3*
FL26	15	**1/*1*	**3/*3*
FL37	9.5	**1/*1*	**3/*3*
FL55	12	**1/*1*	**3/*3*
FL61	14	**2/*2*	**1/*1*
FL65	21	**2/*2*	**1/*1*

HEK293 cells. After transfection, the cells were maintained in Minimum Essential Medium containing 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and nonessential amino acids and sodium pyruvate. Sixty hours after transfection, the cells were harvested and homogenized in a glass Dounce homogenizer (Kontes, Vineland, NJ) in 10-mmol/L Tris–hydrochloric acid (pH 7.4) containing 0.25-mol/L sucrose, 1-mmol/L ethylenediaminetetraacetic acid, and Protease Inhibitor Cocktail Tablets (Roche). This suspension was loaded on top of 10-mmol/L Tris-HCl (pH 7.4) and 0.5-mol/L sucrose and centrifuged at 900*g* for 10 minutes. The supernatant and interface were transferred to a new tube containing 10-mmol/L Tris-HCl (pH 7.4) and 0.5-mol/L sucrose and centrifuged at 9000*g* for 10 minutes. The supernatant was transferred to a new tube, and microsomes were pelleted by centrifugation for 1 hour at 100,000*g*.

Liver tissue. Fetal human liver pieces were obtained from legal induced abortions with sociomedical indications. Gestational durations of the fetal livers are listed in Table I. All of the samples were frozen in liquid nitrogen and stored at −70°C until subcellular fractionation and isolation of RNA and genomic DNA. The study was approved by the Ethical Committee at Karolinska Institutet, Stockholm, Sweden.

Subcellular fractionation of human liver. Human fetal liver samples were homogenized in a glass homogenizer in 4 volumes of ice-cold 50-mmol/L Tris-HCl (pH 7.4) containing 0.25-mol/L sucrose and protease inhibitors. The resulting homogenate was centrifuged for 20 minutes at 10,000*g* at 4°C, and the supernatant was collected and protein concentration was measured according to Bradford.[37] When the amount of human fetal material permitted, microsomes were obtained by centrifugation at 10,000*g* for 20 minutes at 4°C, followed by centrifugation at 100,000*g* for 1 hour at 4°C. The pellet was washed and resuspended in 0.1-mol/L phosphate-buffered saline solution (pH 7.4) with 10% glycerol and protease inhibitors. Protein content was measured by the method of Bradford.[37]

Immunoquantitation of CYP3A5 and CYP3A7. Proteins from fetal livers and from heterologous systems used to express CYP3A7 were separated by 8.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis by use of the Mini-PROTEAN II electrophoresis cell (Bio-Rad, Sundbyberg, Sweden) and transferred to Hybond-C nitrocellulose membranes (Amersham Biosciences). The membranes were blocked and then incubated with primary antibodies A234, A254, or A235 (BD Gentest, Woburn, Mass), following the manufacturer's instructions. A234 recognizes CYP3A4-7, A254 recognizes CYP3A4-5-7, and A235 recognizes CYP3A5. After washing, the membranes were incubated with the corresponding horseradish peroxide–conjugated secondary antibody and the proteins were visualized by use of the SuperSignal West Pico Chemiluminescence method (Pierce Chemical, Rockford, Ill) and scanned by use of LAS-1000 (Fujifilm, Dusseldorf, Germany). For the CYP protein quantification, different amounts of standards were loaded in the gel (between 0.10 and 0.73 pmol of CYP3A5 and between 0.22 and 1.32 pmol of CYP3A7). The limits of detection under the conditions used were 6 pmol/mg protein and 15 pmol/mg protein for CYP3A5 and CYP3A7, respectively. The relative intensity of each band was determined by Image Gauge V3.6 (Fujifilm, Dusseldorf, Germany), and the CYP3A5 and CYP3A7 content for each sample was determined from standard curves. A microsomal sample quantified with respect to the apoenzyme CYP3A7 content determined by peptide-specific antibody and peptide-conjugated lysozyme as standard was kindly provided by Robert J. Edwards (Imperial College London, London, United Kingdom) and Sarah C. Sim (Karolinska Institutet, Stockholm, Sweden; 2004) and used as reference for CYP3A7 immunoquantitation. Human liver microsomes in which CYP3A5 expression had been precisely quantified previously[22] were used as standards for CYP3A5 immunoquantitation. Fifteen micrograms of protein of human adult and fetal liver microsomes was used for CYP3A5 quantification with the antibody A235 (Gentest), and 1 μg of human fetal liver microsomes was used for CYP3A7 quantification with the antibody A234 (Gentest).

Assays for CYP activities. Measurements of DHEA activity were performed in a final volume of 0.5 mL with 100-mmol/L potassium phosphate buffer (pH 7.4) containing 15-nmol/L [^{3}H]DHEA and DHEA to obtain final

concentrations between 0.2 and 100 μmol/L and 200 to 300 μg or 5 to 50 μg of microsomal protein from HEK293 cells and fetal liver, respectively. The reaction mixtures were preincubated at 37°C for 5 minutes before the reactions were started by the addition of NADPH to a final concentration of 2 mmol/L. The reactions were carried out at 37°C for 15 to 30 minutes, within the linear range of formation of metabolites. The reactions were stopped by addition of 5 mL of dichloromethane, and the extraction of the metabolites was carried out by blending in a vortex blender for 20 minutes, centrifugation for 10 minutes at 5000*g*, and separation and drying of the organic phase under a stream of nitrogen gas. Dried extracts were dissolved in 100 μL of mobile phase and analyzed by HPLC. A LiChrospher-100 RP-18 column (5 μm, 4 × 250 mm; Merck, Darmstadt, Germany), by use of a 64:36 methanol/water (vol/vol) isocratic mobile phase, was used to separate the hydroxylated products. Radioactive peaks were detected by an in-line Berthold Radioactivity Monitor LB506C-1 (Berthold, Bad Wildbad, Germany); a Berthold LB5035 pump was used for the scintillation liquid at a 1.5-mL/min flow rate (Quicksafe Flow 2; Zinsser Analytic, Frankfurt, Germany).

For the kinetic studies, 2 independent HEK293 transfections and microsome preparations for each *CYP3A7* allele were used. The mean of each independent preparation was used to calculate a mean value and to generate a Michaelis-Menten plot by use of GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, Calif) with a nonlinear regression fit to a hyperbola equation.

Measurements of luciferin 6′ benzyl ether (luciferin BE; Promega) debenzylation were performed as recommended by the manufacturer in the P450-Glo CYP3A7 assay (Promega). The reaction volume was 50 μL, the concentration of luciferin BE was 150 μmol/L, and the reactions were incubated at 37°C for 15 minutes, when the formation of metabolites was linear. Reactions were stopped by addition of luciferin detection reagent (Promega), and the luminescence was recorded after incubation for 20 minutes at room temperature. For kinetic analysis, 1 HEK293 microsomal preparation was used in triplicate.

Alprazolam incubations were performed in 1-mL reaction mixtures (50-mmol/L Tris-HCl [pH 7.4], 500-μmol/L alprazolam, and 2-mmol/L NADPH) containing 200 μg of fetal liver microsomal protein. Methanol concentration was 0.25%. Reaction mixtures were preincubated at 37°C for 4 minutes; NADPH was then added, and the reactions were terminated after 30 minutes with the addition of 40 μL of 100-μmol/L caffeine (internal standard), 1 mL of 1-mol/L borate buffer (pH 9.0), and 4.5 mL of dichloromethane. The incubations were mixed in a vortex blender and centrifuged for 5 minutes at 5000*g*, and the lower organic phase was separated and evaporated under nitrogen atmosphere. The residues were dissolved in the HPLC mobile phase (100-mmol/L phosphate buffer [pH 6.0] containing 35% acetonitrile [vol/vol]) and immediately injected into an HPLC column (LiChrospher-100 RP-18 column [5 μm, 4 × 250 mm]) at a flow rate of 1 mL/min. The formation of metabolites was detected by monitoring absorption at 214 nm. The amount of metabolites formed was estimated from standard curves of 1-OH-alprazolam and 4-OH-alprazolam.

Statistical analysis. Data were analyzed by use of GraphPad InStat version 3.00 for Windows 95 (GraphPad Software). Statistical analysis was done by Mann-Whitney test or Student *t* test. Results are expressed as mean ± SD. Differences were considered significant when *P* values were less than .05.

RESULTS

Identification and allelic frequency of CYP3A7*2. A C-to-G change in exon 11 of *CYP3A7* (*CYP3A7*2*) that causes the amino acid substitution T409R was detected by direct sequencing of a full-length *CYP3A7* cDNA derived from human fetal liver RNA. This SNP was confirmed in genomic DNA, and an RFLP assay was set up for genotyping. Genomic DNA from unrelated individuals from different ethnic groups was used to determine *CYP3A7*2* allele frequency. *CYP3A7*2* had a higher occurrence than the wild-type allele in Tanzanians (*CYP3A7*2*, q = 0.62). The frequencies were considerably lower in Chinese subjects (q = 0.28), Saudi Arabian subjects (q = 0.17), and white subjects (q = 0.08), as shown in Table II. These results show that *CYP3A7*2* is a frequent allele with high interethnic differences in its distribution.

To determine whether *CYP3A7*2* could be in linkage disequilibrium with other polymorphisms in the *CYP3A7* coding region, we sequenced the full coding region of *CYP3A7* using the cDNA from 27 livers from white subjects. Only *CYP3A7*2* was identified, indicating that polymorphisms in the *CYP3A7* coding region are rare, suggesting an important role of CYP3A7 in the metabolism of endogenous compounds.

It has been shown that some adult individuals express CYP3A7 in the liver and gastrointestinal tract as a result of a replacement of part of the *CYP3A7* proximal promoter by the corresponding region of *CYP3A4* containing the pregnane X receptor element and yielding the *CYP3A7*1C* allele (q = 0.03 in white subjects and q = 0.06 in black subjects).[10,12] To determine whether *CYP3A7*2* is associated with *CYP3A7*1C*, 44

Table II. Allele frequencies of *CYP3A7*2* and *CYP3A5*1* in different ethnic groups and linkage disequilibrium

Ethnic group	*No. of chromosomes analyzed*	*Allele frequency†*		*Frequency*			
		*CYP3A7*2*	*CYP3A5*1*	*CYP3A7*1/ CYP3A5*3*	*CYP3A7*1/ CYP3A5*1*	*CYP3A7*2/ CYP3A5*3*	*CYP3A7*2/ CYP3A5*1*
White							
Spanish	202	0.08	0.09	0.89	0.03	0.02	0.06
Swedish	94	0.08	0.07	0.92	0	0.01	0.07
Chinese	192	0.28	0.27	0.72	0	0.01	0.27
Saudi Arabian	140	0.17	0.09	0.80	0.03	0.12	0.05
Tanzanian	180	0.62	0.79	0.20	0.17	0.01	0.62

The frequencies of the haplotypes are given for each population.
†The frequency of the allele was calculated by use of the following formula: Frequency = [2 × (No. of persons homozygous for allele) + (No. of heterozygous persons)]/[2 × (Total No. of persons)]. *CYP3A7*1* GenBank accession No. NM_000765.2.

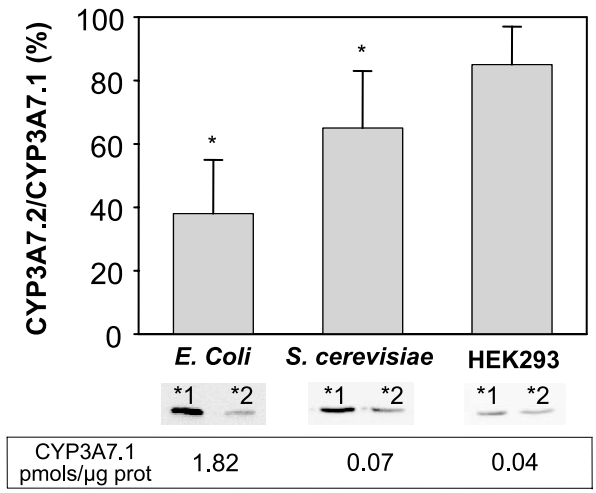

Fig 1. Stability of CYP3A7.2 protein expressed in *E coli*, *S cerevisiae*, and HEK293 cells with reference to that of CYP3A7.1. CYP3A7 was expressed in *E coli* (Rosetta), *S cerevisiae* [W(R)], and human HEK293 cells. The expression of CYP3A7.2 was immunoquantified as described in the Methods section and compared with that obtained for CYP3A7.1 in parallel preparations. The amount of CYP3A7.2 protein was expressed relative to the amount of CYP3A7.1 protein. These results were obtained from 4 independent experiments in *E coli* and HEK293 cells and 5 experiments in *S cerevisiae* cells and are expressed as mean ± SD. In the *lower panel*, the amounts of CYP3A7.1 versus the total protein in the preparations are recorded (in picomoles of CYP per microgram of protein [prot]). CYP3A7.2 expression was significantly different from CYP3A7.1 expression in *E coli* and *S cerevisiae* cells (*asterisk*, $P < .05$ [Mann-Whitney test]).

Swedish individuals were genotyped for *CYP3A7*1C*. We found 2 subjects heterozygous for *CYP3A7*1C*; one was heterozygous for *CYP3A7*2*, and the other was homozygous for *CYP3A7*1*. To further investigate whether *CYP3A7*2* could be in linkage disequilibrium with *CYP3A7*1C*, 73 Chinese and 50 Saudi Arabian individuals were genotyped. However, none of them carried the *CYP3A7*1C* allele.

Characterization of CYP3A7*2 expression in heterologous systems. Thr409 of CYP3A7.1, which is replaced by Arg in CYP3A7.2, is located within the ERR triad, in which the Glu (E362) and first Arg (R365) derive from a highly conserved motif in the K-helix and the distal Arg (R418) derives from the meander region. The requirement of the ERR triad for the retention of the heme in a functional orientation has been demonstrated in bacterial CYPs of known structures.[38,39] SNPs in *CYP3A4* that cause amino acid changes in this region, T363M and P416L and, to a lesser extent, L373F, result in a decreased bacterial expression of the CYP3A4 enzyme.[40] To determine whether CYP3A7.2 was expressed at lower levels compared with CYP3A7.1, suitable *CYP3A7*1* and *CYP3A7*2* expression vectors were introduced into *E coli* (Rosetta), *S cerevisiae* [W(R)], and HEK293 cells. The resulting CYP3A7 expression was determined by Western blotting. Two different antibodies were used, one recognizing CYP3A4-5-7 (Gentest A254, monoclonal) and another recognizing CYP3A4-7 (Gentest A234, polyclonal), and both yielded similar results. As shown in Fig 1, the CYP3A7.2 protein content was significantly lower than wild-type CYP3A7.1 in *E coli* (Rosetta) and *S cerevisiae* W(R) (40% and 65%, respectively; $P < .05$), suggesting a lower stability of CYP3A7.2. However, in microsomes from human HEK293 cells, the CYP3A7.2 content was not significantly different from CYP3A7.1 content (85%), suggesting that the amino acid substitution T409R would not cause important differences in expression and stability in hepatocytes. The capacity of the different

heterologous systems to produce CYP3A7 protein is shown in Fig 1.

To investigate whether the T409R substitution of CYP3A7.2 could alter the folding capacity, membranes and microsomes isolated from the heterologous expressions in bacteria and yeast were investigated for the amount of holoenzyme CYP3A7 by measuring the carbon monoxide–reduced difference spectra. As shown in Fig 2, CYP3A7.2 was successfully expressed as a native, correctly folded enzyme. It has previously been shown that the expression levels of CYP3A7.1 achieved in heterologous systems are low, usually resulting in preparations with large quantities of inactive apoenzyme together with some active holoenzyme.[29] We found approximately 20-fold higher amounts of CYP3A7.1 apoenzyme than holoenzyme in bacteria and 3-fold higher amounts of the apoenzyme in yeast. No difference in these ratios was found between CYP3A7.1 and CYP3A7.2 in the bacteria and yeast (data not shown).

Expression of CYP3A7.2 and CYP3A5 in human fetal liver. To further investigate CYP3A7.2 expression and stability, we used fetal human liver samples that were identified as homozygous for *CYP3A7*1* or *CYP3A7*2* (Table I). When the small amount available of some fetal liver samples did not allow isolation of microsomes, 10,000*g* centrifugation supernatants were used for Western blotting. The CYP3A7 content in fetal liver was determined by use of a CYP3A4-7 antibody (Gentest A234). This antibody does not recognize CYP3A5, and CYP3A4 protein content in fetal livers at 94 to 168 days of gestation is negligible when compared with CYP3A7.[9] We used real-time PCR to confirm this and found that, in all of the fetal samples used in this study, CYP3A4 mRNA content was insignificant when compared with CYP3A7 mRNA content (1000-fold lower on average; data not shown). This indicates that in these Western blots the signal of the A234 antibody corresponds to CYP3A7. Fig 3, *A*, shows that the CYP3A7.2 content was higher than or similar to that of CYP3A7.1 in fetal liver, indicating that the CYP3A7.2 variant does not have a decreased stability in fetal hepatocytes. Interestingly, when a CYP3A5-specific antibody was used for detection (Gentest A235), only the 2 fetal livers homozygous for the *CYP3A7*2* allele showed CYP3A5 expression (Fig 3, *B*). To quantify the amounts of CYP3A7 and CYP3A5 protein, we used standard samples in which the amount of CYP3A5 and CYP3A7 had been precisely estimated (CYP3A5 as described in reference 22 and CYP3A7 as described in the Methods section). The CYP3A7 protein content measured in microsomes from 4 fetal livers varied from 218 to 518 pmol/mg of microsomal protein. CYP3A7.1 and CYP3A7.2 protein contents were not statistically different. With respect to CYP3A5, the 2 fetal livers with detectable CYP3A5 expression had 44 and 45 pmol of CYP3A5 per milligram of microsomal protein.

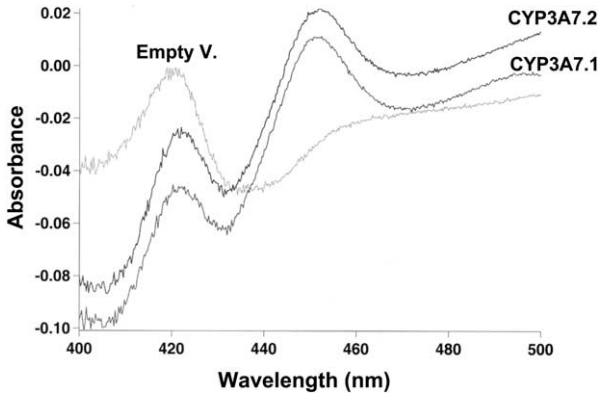

Fig 2. Carbon monoxide difference spectra of heterologously expressed CYP3A7.1 and CYP3A7.2. The spectra shown were obtained in *E coli*–BL21(DE3) strain, basically as described in the Methods section. Bacteria were transformed with expression vectors for CYP3A7.1 and CYP3A7.2 or empty control vector (V) and grown at 30°C. After induction of CYP expression with isopropyl β-D-thiogalactoside for 24 hours, membranes were isolated and used to determine the reduced carbon monoxide difference spectra at 400 to 500 nm. Between 1 and 1.5 mg of membrane protein was used for the spectra.

CYP3A7*2 is in linkage disequilibrium with CYP3A5*1. As mentioned, the fetal livers with *CYP3A7*2* alleles (FL61 and FL65) expressed CYP3A5 protein. Functional CYP3A5 protein expression primarily requires at least 1 *CYP3A5*1* allele,[10] and both FL61 and FL65 were identified as homozygous for *CYP3A5*1* (Table I), suggesting a linkage disequilibrium between *CYP3A7*2* and *CYP3A5*1*. This was further investigated by genotyping for the *CYP3A5*1* and *CYP3A5*3* alleles in the ethnic groups used to determine the frequency of the *CYP3A7*2* allele. Table II shows that the frequency of the *CYP3A5*1* allele is similar to that of *CYP3A7*2* in Spanish, Swedish, and Chinese populations. In contrast, Saudi Arabians had a higher frequency of *CYP3A7*2* ($q = 0.17$) than *CYP3A5*1* ($q = 0.09$), and Tanzanians had a higher frequency of *CYP3A5*1* ($q = 0.79$) than of *CYP3A7*2* ($q = 0.62$). These results

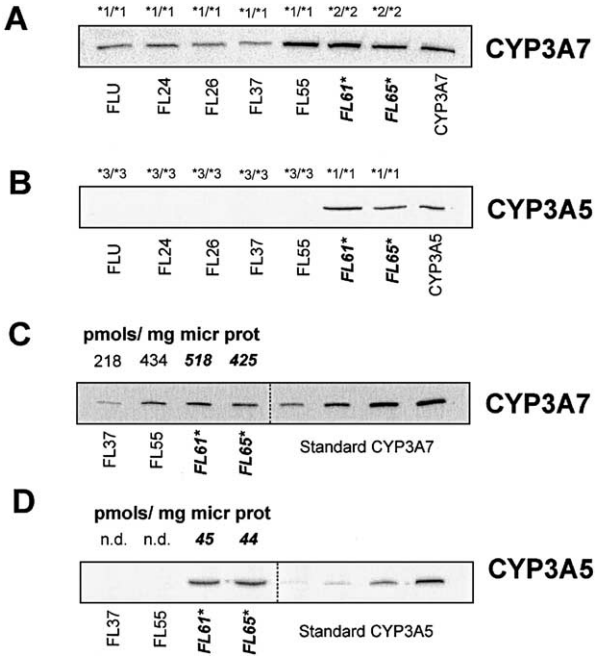

Fig 3. CYP3A7 and CYP3A5 protein levels in human fetal livers. Proteins were separated by electrophoresis and transferred to a nitrocellulose membrane. The CYP3A7 and CYP3A5 enzymes were detected with antibodies recognizing CYP3A4-7 (Gentest A234) and CYP3A5 (Gentest A235). Forty micrograms of 10,000*g* supernatant protein from 7 different human fetal livers, together with yeast-expressed CYP3A7 and CYP3A5, was used for electrophoresis, blotting, and detection of CYP3A7 (**A**) and CYP3A5 (**B**) proteins. *CYP3A7* and *CYP3A5* genotypes of the livers are indicated. The amounts of CYP3A7 (**C**) and CYP3A5 (**D**) were quantified in microsomal preparations from 4 fetal livers by use of suitable standards, as described in the Methods section. The values shown in the figure are the mean of at least 2 independent experiments. The mean values (±SD) were 218 ± 34, 434 ± 70, 518 ± 41, and 425 ± 13 pmol CYP3A7 · mg^{-1} microsomal protein (micr prot) for FL37, FL55, FL61, and FL65, respectively, in **C** and 45 ± 5 and 44 ± 2 pmol CYP3A5 · mg^{-1} microsomal protein for FL61 and FL65, respectively, in **D**. n.d., Not detectable.

indicate a linkage disequilibrium between the 2 polymorphisms studied with important interethnic differences. Thus, in Swedish and Chinese populations a strong linkage disequilibrium was observed, whereas a lower extent of linkage was found in Spanish, Tanzanian, and Saudi Arabian populations. In general, most white, Saudi Arabian, and Chinese individuals express CYP3A7.1 but have no CYP3A5 protein, whereas most Tanzanians have CYP3A7.2 and express the CYP3A5 protein.

Functional characterization of CYP3A7.1 and CYP3A7.2. DHEA is a CYP3A-specific substrate, and among the 3 main CYP3A enzymes, CYP3A7 is the dominant DHEA 16α-hydroxylase. In CYP3A7-catalyzed reactions, 16α-OH-DHEA comprises more than 95% of the total metabolites and 7β-OH-DHEA is produced only to a very small extent.[6] We used the DHEA hydroxylation assay to compare the catalytic activities of CYP3A7.1 and CYP3A7.2. HEK293 cells, transfected with *CYP3A7*1* or *CYP3A7*2* expression vectors or with the pCMV4 empty vector as a control, were homogenized, microsomes were isolated, and the CYP3A7 content was quantified. The microsomal fractions were used to measure DHEA hydroxylase activity as described in the Methods section by use of DHEA concentrations between 0.02 and 100 μmol/L. The kinetic studies showed that replacement of Thr409 with Arg produced a statistically significant 1.5-fold increase in catalytic constant (k_{cat}) for 16α-hydroxylation of DHEA (k_{cat} = 0.035 ± 0.002 s^{-1} for CYP3A7.1 and 0.052 ± 0.002 s^{-1} for CYP3A7.2; $P < .05$), indicating that CYP3A7.2 was a more efficient enzyme in this reaction (Fig 4). At DHEA concentrations near physiologic conditions (16 nmol/L), the DHEA hydroxylation catalyzed by CYP3A7.2 was 1.4-fold higher than that of CYP3A7.1. In agreement with this, use of another CYP3A7-specific substrate, luciferin BE, between 50 and 200 μmol/L, revealed a moderate increase in the rate of debenzylation (1.3-fold) catalyzed by CYP3A7.2 (data not shown).

Fetal liver metabolism of DHEA, luciferin BE, and alprazolam. To further investigate the catalytic capacity of CYP3A7.2, incubations of fetal liver microsomes from subjects homozygous for *CYP3A7*1* or *CYP3A7*2* were carried out with DHEA, luciferin BE, and alprazolam. Incubations at saturating concentrations (50 μmol/L) of DHEA showed a reaction rate between 2.0 and 3.5 nmol of hydroxylated metabolite per minute per milligram of microsomal protein. The contribution of CYP3A5 (only present in the CYP3A7.2 livers) to the DHEA hydroxylation in fetal liver has previously been show to be negligible.[6] As shown in Fig 5, *A*, the fetal livers FL61 and FL65, which express CYP3A7.2 and contain amounts of CYP3A7 similar to those in FL55, metabolized DHEA at a significantly higher rate compared with FL55, which expresses CYP3A7.1. The DHEA hydroxylation rates of the CYP3A7.2 fetal livers at saturating concentrations of the substrate (50 μmol/L) were 1.6- and 1.8-fold higher than the rate of the CYP3A7.1 liver (*P*

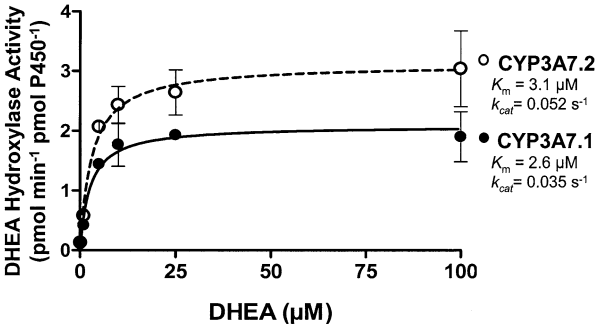

Fig 4. Kinetic analysis of dehydroepiandrosterone (DHEA) hydroxylase activity for CYP3A7.2 enzyme versus wild-type CYP3A7.1. The 2 proteins were expressed in HEK293 cells as described in the Methods section, and DHEA hydroxylase activity was measured at substrate concentrations varying from 0.2 to 100 μmol/L. *Open circles* correspond to CYP3A7.2 and *solid circles* to CYP3A7.1. The values shown are the mean of 2 independent HEK293 transfections and microsomal preparations. The Michaelis-Menten constant (K_m) and catalytic constant (k_{cat}) for the 2 enzymes were calculated by nonlinear fitting as follows: $K_m = 2.6 \pm 0.6$ μmol/L and $k_{cat} = 0.035 \pm 0.002\ s^{-1}$ for CYP3A7.1 and $K_m = 3.1 \pm 0.5$ μmol/L and $k_{cat} = 0.052 \pm 0.002\ s^{-1}$ for CYP3A7.2.

$< .05$ for FL61 and $P < .001$ for FL 65). This is in agreement with the difference in k_{cat} calculated with heterologously expressed enzymes (1.5-fold increase). Similarly, at physiologic concentrations of DHEA (16 nmol/L), 1.6- and 2.0-fold more rapid DHEA hydroxylations were found for FL61 and FL65 when compared with FL55 ($P < .01$ and $P < .001$; data not shown).

Compared with CYP3A5, CYP3A7 produces a 35-fold higher amount of debenzylated luminescent product from luciferin BE, and CYP3A4 has a 20-fold higher rate of product formation compared with CYP3A5, in all cases under the same incubation conditions (manufacturer's information). Therefore this substrate can be used to monitor CYP3A7 activity in fetal liver, without interference of CYP3A5. These results are similar to those obtained with DHEA, with liver microsomes from FL61 and FL65 having 1.9- and 2.7-fold significantly higher rates of luciferin BE debenzylation activity in comparison with those from FL55 (Fig 5, *B*).

Alprazolam metabolism to 4-OH- and 1-OH-alprazolam is catalyzed by CYP3A enzymes, with CYP3A5 being more active than CYP3A4 and CYP3A7.[41] When we used heterologously expressed

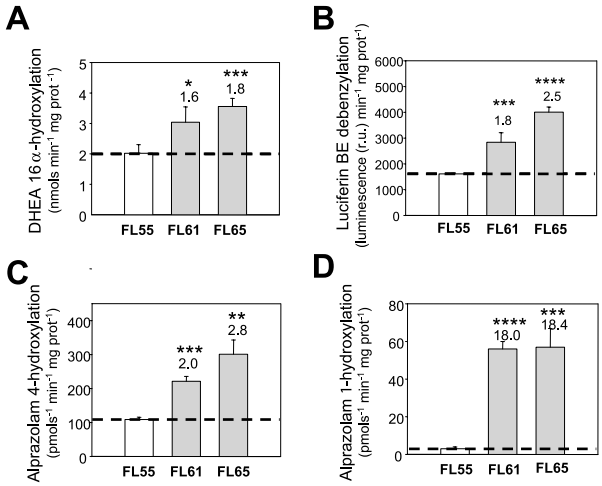

Fig 5. Rate of DHEA hydroxylation, luciferin 6′ benzyl ether (luciferin BE) debenzylation, and alprazolam 4- and 1-hydroxylation by CYP3A7.1 or CYP3A7.2 from human fetal livers. Human fetal liver microsomes were prepared from genotyped livers that belonged to haplotypes *CYP3A7*1/CYP3A5*3* (FL55) and *CYP3A7*2/CYP3A5*1* (FL61 and FL65) and incubated with 50-μmol/L DHEA (**A**), 150-μmol/L luciferin BE (**B**), or 500-μmol/L alprazolam (**C** and **D**), as described in the Methods section. Each column represents the mean ± SD of 4 (DHEA and luciferin BE) and 3 (alprazolam) independent experiments. Statistical differences in metabolization rate were analyzed by means of paired Student *t* test (*1 asterisk*, $P < .05$; *2 asterisks*, $P < .01$; *3 asterisks*, $P < .001$; *4 asterisks*, $P < .0001$; for FL55 compared with FL61 and FL65).

CYPs, we found that the rates of alprazolam 1- and 4-hydroxylation were 60- and 4-fold higher, respectively, for CYP3A5 as compared with CYP3A7.1, at a 500-μmol/L alprazolam concentration (data not shown). In addition, when compared with the rate for CYP3A7.1, moderately increased activity for CYP3A7.2 in 1- and 4-hydroxylations was found (data not shown). When alprazolam was incubated with the fetal liver microsomes, a significant increase in 4-OH-alprazolam production was detected in the *CYP3A7*2/CYPA5*1* livers as compared with the *CYP3A7*1/CYPA5*3* liver (2.0- and 2.8-fold; $P < .001$ and $P < .01$, respectively) (Fig 5, *C*); this increase is similar to that found with DHEA and luciferin BE. However, 1-hydroxylation of alprazolam was much higher for FL61 and FL65 (18.0- and 18.4-fold; $P < .0001$ and $P < .001$, respectively) when compared with liver microsomes from FL55 (Fig 5, *D*). This is in agreement with a major contribution of CYP3A5 to alprazolam 1-hydroxylation.

DISCUSSION

CYP3A7 accounts for more than 50% of total CYP content in fetal liver[11] and plays an important role in the metabolism of xenobiotics to which the fetus is exposed, as well as endogenous substrates. This enzyme and CYP3A5 are apparently the major fetal CYP enzymes active in the metabolism of drugs and other xenobiotics. Therefore variant *CYP3A* haplotypes can significantly influence the pharmacokinetics, carcinogenicity, and metabolism of exogenous, as well as endogenous, compounds in the fetal liver. In addition, CYP3A7 is also expressed in the liver and intestines of some adults and in the adrenal gland, prostate, and thymus,[42] tissues in which it could play an important role. In this study we report a novel *CYP3A7* allele, present at various frequencies in different populations, that encodes a functional CYP3A7 variant enzyme (CYP3A7.2) which is associated with the protein expression of CYP3A5 in fetal livers, yielding phenotypes of high and low CYP3A metabolic activity.

The frequency of the novel *CYP3A7*2* allele differs between populations (Table II), from 8% in white subjects to the highest frequency of 62%, found in Tanzanians. In addition, we found a linkage disequilibrium between *CYP3A7*2* and *CYP3A5*1*. The association between these 2 polymorphisms, which are separated by 36 kb, is also subjected to interethnic differences. There is a high linkage in Chinese and Swedish populations and a lower linkage in Spanish, Tanzanian, and Saudi Arabian populations (Table II). The most frequent haplotype in white subjects was identified as *CYP3A7*1/CYP3A5*3*, whereas *CYP3A7*2/CYP3A5*1* was the most common haplotype in Tanzanians. These haplotypes result in different CYP3A phenotypes—individuals expressing CYP3A7.1 but with defective CYP3A5 expression and individuals who express both CYP3A7.2 and CYP3A5. In addition, we also found some evidence suggesting a linkage disequilibrium between *CYP3A7*2* and *CYP3A7*1C*, which is associated with CYP3A7 expression in adult liver and intestine.[12]

The Thr409, which is changed to Arg in CYP3A7.2, is conserved among human CYP3As, and it is located within a region (ERR triad) that is necessary for bacterial holoenzyme stability.[38,39] Human HEK293 cells showed no major differences between CYP3A7.1 and CYP3A7.2 expression levels and suggested a normal stability of the CYP3A7.2 enzyme. *E coli* and *S cerevisiae* cells, however, had a reduced expression of CYP3A7.2 enzyme, as compared with the wild-type CYP3A7.1, but this does not necessarily indicate a factual instability of the CYP3A7.2 enzyme because different heterologous expression systems have various capabilities for correct folding (Fig 1). The functional expression of CYP3A7.2 in vivo is strongly supported by experiments using microsomes from fetal livers. Thus, using a panel of 7 human fetal livers, we found that livers homozygous for *CYP3A7*2* contained similar or higher amounts of CYP3A7 protein compared with *CYP3A7*1* livers (Fig 3). The CYP3A7 content of 4 livers varied between 218 and 518 pmol $\cdot$ mg^{-1} microsomal protein. These quantities are similar to those estimated by Stevens et al[6] (311 pmol $\cdot$ mg^{-1} protein, in the same age group) and higher than the value estimated by Shimada et al[13] (100 pmol $\cdot$ mg^{-1} protein).[43] In our study the CYP3A5 protein contents of the 2 *CYP3A5*1* homozygous livers were 44 and 45 pmol of CYP3A5 per milligram of microsomal protein, values that are similar to those found in *CYP3A5*1/*3* heterozygous adult livers[22] and are in accordance with a previous study that showed that the content of fetal liver CYP3A5 was generally independent of age.[6] In the same study the maximum CYP3A5 content, by use of 115 livers with detectable CYP3A5 protein, was estimated to be 25 pmol $\cdot$ mg^{-1} microsomal protein. The difference between this value and our calculated CYP3A5 content could be caused by the different standards used. In our study the standards consisted of carrier protein–coupled peptides used by Westlind-Johnsson et al,[22] whereas Stevens et al[6] used baculovirus-expressed CYP3A5. In any case, CYP3A5 levels were considerably lower than those of CYP3A7, which are, in turn, comparable in fetal liver to those of CYP3A4 in adult liver.[22]

CYP3A7.2 formed a spectrally active holoenzyme (Fig 2), and the catalytic activity of the enzyme was slightly different by use of 2 CYP3A7-specific substrates. Kinetic analysis with DHEA showed that CYP3A7.2 had a 1.5-fold significantly higher k_{cat} than CYP3A7.1, when the 2 variants were expressed in HEK293 cells. Consistently, fetal livers expressing CYP3A7.2 had an increased metabolism of the CYP3A7-specific substrates DHEA and luciferin BE when compared with livers expressing CYP3A7.1 (Fig 5, *A* and *B*). Interestingly, we found an 18-fold increase in 1-hydroxylation of alprazolam in the fetal livers with *CYP3A7*2/CYP3A5*1* haplotype when compared with those having the *CYP3A7*1/CYP3A5*3* haplotype (Fig 5, *D*). The reason for the large differences found in alprazolam 1-hydroxylation is the major contribution of CYP3A5 to this reaction. In the adult liver, because of the fact that CYP3A5 catalyzes the same reactions as CYP3A4,[41] the contribution of CYP3A5 to total CYP3A metabolism is mainly determined by its relative expression to CYP3A4. In the fetal liver, the

amount of CYP3A5 is lower than that of CYP3A7, but there are major differences in substrate specificity between the 2 enzymes (ie, 16α-DHEA hydroxylation and alprazolam 1-hydroxylation). Thus CYP3A5 can have a significant contribution to fetal CYP3A metabolism for CYP3A5-specific substrates such as alprazolam.

In conclusion, we have identified a new polymorphism that results in a CYP3A7 variant enzyme (CYP3A7.2) which has a moderately increased catalytic activity and which is in linkage disequilibrium with *CYP3A5*1*. This allelic linkage results in haplotypes that are causing different CYP3A phenotypes during the fetal stage and the first months after birth, and they exhibit important interethnic differences in their distribution (Table II). The 2 most common phenotypes are (1) individuals in whom CYP3A7.1 is the only CYP3A enzyme present in the liver and corresponds to the frequent *CYP3A7*1/CYP3A5*3* white haplotype and (2) individuals who have a *CYP3A7*2/CYP3A5*1* haplotype, which is most frequent in Africa, and who express 2 catalytically different CYP3A enzymes, CYP3A7.2 and CYP3A5, that will provide more active and versatile biotransformation pathways for drugs and other xenobiotics, as well as endogenous compounds.

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All authors declare that there are no conflicts of interest in relation to this study.

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