A Novel Polymorphic Cytochrome P450 Formed by Splicing of CYP3A7 and the Pseudogene CYP3AP1*

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The cytochrome P450 3A7 (CYP3A7) is the most abundant CYP in human liver during fetal development and first months of postnatal age, playing an important role in the metabolism of endogenous hormones, drugs, differentiation factors, and potentially toxic and teratogenic substrates. Here we describe and characterize a novel enzyme, CYP3A7.1L, encompassing the CYP3A7.1 protein with the last four carboxyl-terminal amino acids replaced by a unique sequence of 36 amino acids, generated by splicing of CYP3A7 with CYP3AP1 RNA. The corresponding CYP3A7-3AP1 mRNA had a significant expression in liver, kidney, and gastrointestinal tract, and its presence was found to be tissue-specific and dependent on the developmental stage. Heterologous expression in yeast revealed that CYP3A7.1L was a functional enzyme with a specific activity similar to that of CYP3A7.1 and, in some conditions, a different hydroxylation specificity than CYP3A7.1 using dehydroepiandrosterone as a substrate. CYP3A7.1L was found to be polymorphic due to a mutation at position 6 of the first splicing site of CYP3AP1 (CYP3A7_39256T→A), which abrogates the pseudogene splicing. This polymorphism had pronounced interethnic differences and was in linkage disequilibrium with other functional polymorphisms described in the CYP3A locus: CYP3A7*2 and CYP3A5*1. Therefore, the resulting CYP3A haplotypes express different sets of enzymes within the population. In conclusion, a novel mechanism, consisting of the splicing of the pseudogene CYP3AP1 to CYP3A7, causes the formation of the novel CYP3A7.1L having a different tissue distribution and functional properties than the parent CYP3A7 enzyme, with possible developmental, physiological, and toxicological consequences.

The cytochrome P450s (CYPs)1 of families 1–3 are the most prominent enzymes catalyzing the biotransformation of exogenous compounds in the liver and have a major role in drug metabolism. The CYP3A subfamily has a special relevance because CYP3A7 and CYP3A4 metabolize a large number of therapeutic drugs and are the most abundant CYPs in fetal and adult liver, respectively. The human CYP3A locus is located on chromosome 7q21-q22.1 and contains four genes, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, and three pseudogenes, CYP3AP1, CYP3AP2, and CYP3AP3 (1, 2). The CYP3A genes were acquired by gene duplications and divergence of the proteins during evolution, generating enzymes with different functions and expression patterns. The pseudogenes probably represent recombination events followed by deletions resulting in a non-functional combination of exons: exons 1, 2, and 13 (CYP3AP1), exons 1 and 2 (CYP3AP2), and exon 1 (CYP3AP3) (1).

Developmental- and tissue-specific mechanisms regulate the expression of the CYP3A enzymes. Among adults, CYP3A4 is the dominant CYP in liver and intestines, being involved in the metabolism of more than 50% of currently used therapeutic drugs (3). CYP3A5 can be present at relevant levels in both fetal and adult liver, although in a polymorphic manner (4). The low expression of CYP3A43 and improper folding in mammalian systems rule out any significant contribution of this enzyme to CYP3A activity (5, 6). In fetal liver, where the number of expressed xenobiotic metabolizing enzymes is small, CYP3A7 is present at high levels (7–9). CYP3A7 plays an important role in the metabolism of key steroids in the adrenals and gonads, of potentially toxic and teratogenic endogenous substrates such as retinoic acid (10–13), and of many xenobiotics that reach the fetus, such as therapeutic drugs for women and substances of abuse (14–17).

The important role of CYP3A7 in the metabolism of endogenous and exogenous compounds, added to the fact that most drug-metabolizing P450 genes are not expressed during fetal stages, results in a well conserved gene with few allelic variants (18). In contrast, the adult liver contains multiple CYPs with different substrate specificities to facilitate the metabolism and elimination of a wide range of compounds. Here we describe a mechanism that generates a novel CYP3A7 enzyme (CYP3A7.1L) by splicing between the pseudogene CYP3AP1 and CYP3A7. The novel mRNA, containing 13 exons of CYP3A7 and 2 additional exons from CYP3AP1 at the 3′ end (CYP3A7-3AP1, GenBank™ accession number AF315325) (1) (see Fig. 1), is found at relevant amounts in several tissues and encodes a functional enzyme.

EXPERIMENTAL PROCEDURES

Materials—Protease inhibitor mixture tablets were from Roche Applied Science. Dehydroepiandrosterone (DHEA), 16a-hydroxy-DHEA, glucose, galactose, and NADPH were purchased from Sigma. 1,2,6,7...
Heterologous Expression of CYP3A7.1 and CYP3A7.1L in Yeast—Full-length CYP3A7 and CYP3A7.1L coding regions were PCR-amplified from human liver cDNA using Elongase (Invitrogen). The forward primer was the same for CYP3A7 and CYP3A7-3AP1, 5'-AAGGGAGATC-CAAAAAATGGATCTCATCCCAAAATTTGCCC-3' and introduced three A residues in front of the translation start to increase the expression efficiency in yeast (24) and a BamHI site at the 5' end. The reverse primers contained a KpnI site at the 5' end: CYP3A7, 5'-CTTAGTGAT-CTCAGGTCCTCACTAAGGTCT-3' and CYP3A7-3AP1, 5'-AAGGGAGATC-CAAAAAATGGATCTCATCCCAAAATTTGCCC-3'. The underlined nucleotides correspond to the restriction enzyme sites. Amplification of the amplified DNA with BamHI/KpnI, the fragments were cloned into the yEpD60 expression vector (25). Plasmids from positive transformed bacterial colonies were isolated, and the sequence of the insert was examined using the ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems) and analyzed on an ABI Prism 377 DNA sequencer (Applied Biosystems). The Saccharomyces cerevisiae strain WR(26) was constructed by sub-stitution of the natural promoter of the cytochrome P450 reductase (CPR1) gene by the galactose-inducible and glucose-repressed promoter GAL10-CYC1 in the strain W303-1B: MATa ade2-1 his3-11-15 leu2-3, 112-trpl-1 ura3-1 (26, 27), and the strain INVSc1-HR: MAT a his3-1 leu2 trp1-289 ura3-52 (pFL3-35 human reductase) expressing human NADPH-generating system. An ORF encoding the galactose inducible promoter in front of the cDNA encoding CYP3A7.1 or CYP3A7.1L. To achieve high expression levels, the yeast cells were 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 as described previously (28). Microsomes containing the recombinant enzyme were thereafter prepared by differential centrifugation (20,000 × g for 10 min plus 100,000 × g for 60 min), and protein concentration was determined according to Bradford (29). Production of active CYP3A7.1L in WR(26) cells proved to be difficult, and different growth and induction conditions were tested. A successful CYP3A7.1L overexpression was achieved at 28 °C and 7 h of galactose induction. The W(?) data in Table II was generated using this preparation of CYP3A7.1L and the parallel preparations for CYP3A7.1 and control yeast transformed with empty vector.

Immunoblot Analysis—Microsomal proteins were electrophoresed in SDS-polyacrylamide gels using the Mini-PROTEAN II electrophoresis cell (Bio-Rad), transferred to Hybond-C extra nitrocellulose membranes (Amersham Biosciences), and incubated with a CYP3A antibody (Genentech, catalog numbers A254 and 458223) following the manufacturer’s instructions. Both antibodies, A254 and 458223, recognize CYP3A4, CYP3A5, and CYP3A7 (this latter antibody was a gift from the program of the University of Dundee/Biotechnology and Biology Research Council/Department of Trade and Industry/Pharmaceutical Industry). Both strains WR(26) and INVSc1-HR were used to express CYP3A7.1 and CYP3A7.1L, essentially as described elsewhere (26, 28). In brief, the yeast cells were transformed with the yEpD60 expression vectors containing a galactose-inducible promoter in front of the cDNA encoding CYP3A7.1 or CYP3A7.1L. Plasmids were transformed into yeast transformed with empty vector.

Microsomes were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with a CYP3A antibody (Genentech, catalog numbers A254 and 458223) followed by the manufacturer’s instructions. Both antibodies, A254 and 458223, recognize CYP3A4, CYP3A5, and CYP3A7. Both antibodies were raised in rabbits against synthetic peptides (using heterologous expression systems). A254 is a monoclonal antibody, and 458223 is polyclonal antibody produced by using rat CYP3A2 as immunogen. After washing, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody, and the proteins were visualized using the SuperSignal West Pico chemiluminescence method (Pierce) and scanned using LAS-1000 (Fujiﬁlm, Düsseldorf, Germany). The relative intensity of each band was determined by Image Gauge V3.6. The CYP3A7.1 and CYP3A7.1L content was determined from standard curves derived from a microsomal sample quantified with respect to the apo-CYP3A7 content determined by peptide-speciﬁc antibody and peptide-conjugated lysozyme, kindly provided by Robert J. Edwards, Imperial College London, UK, and Sarah C. Sim, Karolinska Institutet.

Dehydroepiandrosterone Hydroxylation Assay and Identiﬁcation of Metabolites—Measurements of DHEA activity were performed in a final volume of 0.5 ml with 100 mM potassium phosphate buffer, pH 7.4, containing 15–70 mM [3H]DHEA and between 250 and 500 µg of yeast microsomal protein. The reaction mixtures were preincubated at 37 °C for 5 min before the addition of NADPH to a final concentration of 2 mM. The reaction was terminated by addition of 5 ml of dichloromethane, and the extraction of the metabolites was carried out by vortexing for 20 min, 10 min of centrifuga-tion at 5000 × g, and separation and drying of the organic phase.

S. C. Sim, R. J. Edwards, A. R. Boobis, and M. Ingelman-Sundberg (2005) Pharmaco- genetics, in press.
under a stream of \( N_2 \) gas. Dried extracts were dissolved in 100 \( \mu l \) of mobile phase and analyzed by high-performance liquid chromatography (HPLC). A 5 \( \mu m \) \( 4 \times 250 \) mm LiChrospher-100 RP-18 column was used, with the mobile phase being a linear gradient of methanol (MeOH) and water, starting with 35% methanol and increasing to 100% methanol over 20 min. The selection of 15 min as the elution time was based on the ability to separate the hydroxylated products. Radioactive peaks were detected using an in-line Berthold radioactivity monitor LB506C-1, and a Berthold LB5035 pump was used for the scintillation liquid at a 1.5 ml/min flow rate (QuickSafe Flow 2, Zinsser Analytic). Values greater than 0.1 pmol of metabolite were accurately detected with this method. Linearity with protein and time was observed for the production of DHEA metabolites. It should be noted that the \( ^3\)H label in position C-7 of DHEA will result in a somewhat diminished signal of the 7-hydroxylated metabolites.

For identification of the DHEA metabolites by gas chromatography mass spectrometry (GC/MS), corresponding incubation mixtures contained 50–150 \( \mu l \) unlabeled DHEA, and the reactions were stopped by the addition of FOLCH solution (CHCl\(_3\):MeOH, 2:1). After vortexing, the aqueous phase was then passed through a Sep-Pak C\(_{18}\) cartridge again, prior to a wash with 5 ml of methanol. Steroids were then eluted with 10 ml of 75\% aqueous methanol. This solution was passed through a column (40 \( \times \) 4 mm) of the strong lipophilic anion exchanger, triethylaminoxypropyl Sephadex LH-20, in bicarbonate form (30). After elution with an additional 5 ml of methanol, the total effluent from the column was taken to dryness in vacuo. The residue was then transferred with methanol to a stoppered tube and dried under nitrogen. Steroids were then redissolved in 50 \( \mu l \) of hexane. GC/MS was performed using a Finnigan SSQ 710 instrument housing a fused silica column (25 m \( \times \) 0.32 mm) coated with a 0.17-\( \mu m \) layer of cross-linked methyl silicone (Ultra 1, Hewlett-Packard, Palo Alto, CA) ending in the ion source. An on-column injection device was used. The oven temperature was 50 \( ^\circ\)C during the injection and, after 3 min, it was rapidly increased to 185 \( ^\circ\)C and then programmed to 280 \( ^\circ\)C at a rate of 5 \( ^\circ\)C/min. The electron energy was 50 eV, and repetitive scanning (30 scans/min) over the \( m/z \) range of 50–800 was started after a suitable delay. The identification of a steroid was based on the retention time and complete mass spectrum, which were compared with those of the authentic steroid. The retention indices (Kovats) for DHEA, 7\( \beta\)-hydroxy-DHEA, and 16a-hydroxy-DHEA were 2515, 2590, and 2725, respectively, and mass spectra of the two 7-hydroxylated metabolites showed one intense ion (\( m/z \) 2670, and 2725, respectively, and mass spectra of the two 7-hydroxylated metabolites).

**RESULTS**

Expression of CYP3A7-3AP1 mRNA in Human Liver—To search for polymorphic variants, a forward primer corresponding to CYP3A7/3’A exon 12 and a reverse primer in the 3’-untranslated region CYP3A4 were designed to amplify CYP3A4 cDNA by reverse transcription-PCR in a panel of 18 adult and 9 fetal human livers. However, a DNA fragment of a different molecular weight was unexpectedly amplified. Sequencing of this DNA revealed that it was a CYP3A7 splicing variant that consisted of two fragments of the pseudogene CYP3AP1, which are highly similar to exons 2 and 13 of CYP3A7, joined to CYP3A7 mRNA at exon 13 in an alternative splice site, similarly to the one described by Finta and Zaphopolous (1) (Fig. 1A). All exons of this novel transcript are flanked by canonical splicing sites, which strongly suggests that it is the result of an RNA splicing event. CYP3AP1, similarly to the other CYP3A genes, contains 13 exons, whereas the novel CYP3A7-3AP1 mRNA contains 15 exons, resulting in a longer CYP3A7 protein (CYP3A7.1L). The translational stop codon is located seven nucleotides downstream in the last exon from the pseudogene. In addition, the reading frame of the pseudogene regions is shifted, and the last four amino acids of CYP3A7 are replaced by a unique 36-amino-acid sequence (Fig. 1B).

As shown in Fig. 2, the expression levels of CYP3A7-3AP1 mRNA, as determined by a quantitative reverse transcription-PCR technique, was higher in fetal than in adult livers, with the exception of two fetal samples (FL61 and FL65) having extremely low CYP3A7-3AP1 mRNA levels. Using two CYP3A-specific antibodies, CYP3A7.1L was detected by Western blotting in liver samples with CYP3A7-3AP1 mRNA expression (Fig. 2A, inset; FL65, which lacks CYP3A7.1L, is used as a negative control).

To explore the molecular mechanisms responsible for the alternative splicing, CYP3A7 mRNA levels were measured and compared with the expression of CYP3A7-3AP1 mRNA. The correlation between CYP3A7-3AP1 and CYP3A7 mRNA of 22 livers, all with exception of FL61 and FL65, was high (\( r = 0.94; r = 0.97; p < 0.0001 \)). On average, the CYP3A7-3AP1 mRNA levels were 1\% of those of CYP3A7 mRNA, but substantial differences were noted between fetal and adult livers, with mean ratios of 0.5 and 1.2\%, respectively, indicating that the splicing of the pseudogene was favored in the adult livers. Interestingly, the two fetal livers with extremely low CYP3A7-3AP1 mRNA expression (FL61 and FL65) had intermediate to high expression of CYP3A7 mRNA, suggesting a genetic defect preventing the splicing of CYP3AP1.

CYP3A7_39256 T→A Prevents CYP3AP1 Splicing—Sequencing of genomic DNA from the fetal livers lacking CYP3A7-3AP1 mRNA revealed a T→A change at position 39256 of the first acceptor splicing site of CYP3AP1. The splicing acceptor site of CYP3A7 intron 1 and the similar sequence of CYP3AP1, together with the nucleotide change, are shown in Fig. 3. Interestingly, the C at position 39256 was lower in CYP3A7 mRNA of 22 livers, all with exception of FL61 and FL65, was high (\( r = 0.94; r = 0.97; p < 0.0001 \)). On average, the CYP3A7-3AP1 mRNA levels were 1\% of those of CYP3A7 mRNA, but substantial differences were noted between fetal and adult livers, with mean ratios of 0.5 and 1.2\%, respectively, indicating that the splicing of the pseudogene was favored in the adult livers. Interestingly, the two fetal livers with extremely low CYP3A7-3AP1 mRNA expression (FL61 and FL65) had intermediate to high expression of CYP3A7 mRNA, suggesting a genetic defect preventing the splicing of CYP3AP1.

Thus, this nucleotide change at the pyrimidine stretch seems to be sufficient to affect the splicing and to shift it to an alternative site in CYP3AP1. This suggested that the changes in the pyrimidine sequence of intron 1 have a significant impact on the splicing efficiency. Thus, adult livers homozygous for T at position 39256 had a higher CYP3A7-3AP1/CYP3AP1 ratio than those heterozygous in this position (\( p = 0.003 \)). The amounts of CYP3A7-3AP1 were still low when compared with CYP3A7 mRNA. Similarly, fetal livers homozygous for A did not express CYP3A7-3AP1 mRNA. Thus, the data indicated that the CYP3A7_39256 T→A change at position 39256 of the pseudogene prevents the splicing. When the CYP3A7-3AP1/CYP3AP1 ratio of adult and fetal livers homozygous for CYP3A7_39256 T/T were compared, there was also a significant difference in the expression (1.4\% versus 0.5\%, respectively, \( p = 0.001 \)), further indicating that the splicing of the pseudogene is regulated in a developmentally-specific manner.

Similarly to CYP3A7 and CYP3AP1, a pseudogene containing an intron 1/exon 2 region with conserved splice sites is located downstream of CYP3A4 (CYP3AP2). However, no alternatively spliced RNA could be amplified by reverse transcription-PCR using primers at CYP3A4 exon 13 and at CYP3AP2 exon 2 with human fetal and adult liver RNA reverse-transcribed with poly(dT) or a pseudogene-exon 2-specific primer (data not shown). The cause might be inherent in the lack of an exon 13-like sequence in CYP3AP2 and, therefore, a polyadenylation site.

CYP3A7_39256 A Frequency and linkage Disequilibrium with CYP3A7*2 and CYP3A5*1—The frequency of CYP3A7_39256 A showed large interethnic differences: 8, 28, and 59\% in Caucasians, Chinese, and African Americans, re-
spectively (Table I). Previously, we described the CYP3A7*2 allele, which is in linkage disequilibrium with CYP3A5*1 and results in an association between CYP3A7 and CYP3A5 protein expression. As shown in Table I, we found that there was a complete linkage between CYP3A7_39256 A and CYP3A7*2 in Caucasians and Chinese. Of the four possible haplotypes from the combination of the CYP3A7*1 and *2 and CYP3A7_39256 A and T genes, only CYP3A7*1A (CYP3A7*1/CYP3A7_39256 T) and CYP3A7*2A (CYP3A7*2/CYP3A7_39256 A) were present in the populations studied.

Based on the amino acid present at position 409 of the protein (Thr in CYP3A7.1 and Arg in CYP3A7.2) CYP3A7.1L and CYP3A7.2L are defined. However, CYP3A7.2L could only be found with low frequency in African samples (data not shown).

In conclusion, as a result of the linkage disequilibrium of different functional polymorphisms in the CYP3A locus, the majority of Caucasian and Chinese lack CYP3A5 protein but express CYP3A7.1L and CYP3A7.1, whereas the majority of Africans express CYP3A7.2 and CYP3A5 but have no alternatively spliced CYP3A7.

Expression in Different Tissues—CYP3A7-3AP1 mRNA was detected in adult and fetal liver, and a lower expression was found in kidney, gastrointestinal adult tissues (colon, duodenum, jejunum, and ileum), and prostate (Fig. 4). Some expression was also detected using higher amounts of cDNA in pancreas, testis, and small intestine (without mucosal lining) and in the fetal tissues: lung, kidney, spleen, and thymus. In most samples, CYP3A7 and CYP3A7-3AP1 were co-expressed, in agreement with the fact that CYP3A7-3AP1 mRNA is a product of an RNA splicing event, seen at higher amounts in livers with high CYP3A7 transcription. In the majority of the samples, CYP3A7 expression was higher than that of CYP3A7-3AP1, but the relative levels were subjected to variation between different tissues (i.e. at 0.8 ng of cDNA, compare ileocecum versus kidney; at 4.0 ng of cDNA, compare prostate versus lung).

CYP3A7.1L Catalytic Activity—CYP3A7.1L contains all amino acids of CYP3A7 except for the last four, which are replaced by 36 amino acids, generated by splicing of CYP3A7 with CYP3AP1 RNA. The sequence of the last two exons of CYP3A7-3AP1 mRNA is similar to that of exon 2 and CYP3A7 mRNA, but the reading frame is shifted, resulting in a unique amino acid sequence at the carboxyl-terminal of the protein that could affect the catalytic activity or localization of the enzyme. Thus, CYP3A7.1L was heterologously expressed, and the subcellular localization and activity of the enzyme was examined. Similarly to CYP3A7.1, the heterologous expression of CYP3A7.1L proved to be difficult, and only low amounts of protein could be expressed in bacteria, yeast, and HEK293 mammalian cells. The localization of CYP3A7.1L was found to be mainly microsomal. Microsomes from cells overexpressing CYP3A7.1L were incubated with DHEA, and the highest activity was found in yeast. The different metabolites produced from DHEA were identified by GC/MS. Microsomes overexpressing CYP3A7.1 produced 16α-hydroxy DHEA as the major metabolite, and microsomes from yeast transformed with the empty vector pYeDP60 produced only low amounts of 7β-hydroxylated DHEA. When microsomes overexpressing CYP3A7.1L were incubated with DHEA, different metabolites were detected, dem-

FIG. 1. Structure of CYP3A7-3AP1 mRNA and CYP3A7.1L protein. A, white rectangles represent the 13 exons of CYP3A7; gray rectangles indicate the splicing junctions from CYP3AP1 that are spliced to CYP3A7. B, the novel carboxy-terminal sequence of CYP3A7.1L is shown. The arrows indicate the splicing junctions of CYP3AP1 exons. The amino acid sequence encoded is shown together with the lengths of the different CYP3A7 proteins. The stop codons are underlined.
onstrating that CYP3A7.1L is a functional enzyme. In W(R) cells, 16/9251, 16/9251-, and 16/9252-OH DHEA were produced (Fig. 5), and in INVSc1HR cells, only 16/9251-OH DHEA could be detected (Table II). The difference in the production of metabolites could be caused by different protein-protein interactions of CYP3A7.1L with the overexpressed P450 reductases. Western blotting analysis showed that the amounts of CYP3A7.1L and CYP3A7.1 in W(R) and INVSc1HR strains were different, with a 50-fold lower CYP3A7.1L/CYP3A7.1 ratio in the INVSc1HR cells (Table II). This is likely a result of different interactions of

![Fig. 2. CYP3A7-3AP1 mRNA and CYP3A7.1L expression in human liver. A, the content of CYP3A7-3AP1 mRNA was measured in 15 adult (AL) and 9 fetal (FL) human livers by quantitative real-time PCR, and the amounts were normalized by the content of β-actin. The CYP3AP1 genotype (CYP3A7_39256 A or CYP3A7_39256 T) of the different samples is shown. Inset, CYP3A7.1L is immunodetected in a fetal liver microsomal sample with CYP3A7-3AP1 mRNA expression (FL37) but not in FL65, which lacks CYP3A7-3AP1 expression. The CYP3A-specific antibodies used for Western blot analysis are described under "Experimental Procedures." Ab-1 corresponds to A254, and Ab-2 is 458223. In fetal livers, the mean ratio CYP3A7-3AP1/CYP3A7 mRNA is 0.5%, if an identical ratio is assumed at protein level (identical translation efficiency and stability of CYP3A7.1 and CYP3A7.1L proteins); 2 pmols of CYP3A7.1L/mg of microsomal protein is reasonable in fetal liver (18). R, recombinant; NS, nonspecific. B, correlation between CYP3A7-3AP1 and CYP3A7 mRNA. When all livers, except FL61 and FL65 with no quantifiable CYP3A7-3AP1 mRNA, were analyzed, the correlation was: r² = 0.94; r = 0.97; p < 0.0001. ru, relative units. C, differences in CYP3A7-3AP1/CYP3A7 mRNA ratio between adult livers and fetal livers with different CYP3AP1 genotypes. The mean value in the different groups is indicated with a horizontal line, and the number of livers in each group is shown between brackets. **, p < 0.01, significantly different expression with respect to adult CYP3A7_39256 T/T livers (AL T/T) using Mann-Whitney test.

![Fig. 3. Intron 1-exon 2 boundaries in CYP3A7 and CYP3AP1. Intronic sequences are in lowercase, and exons are shown in uppercase. The consensus splicing acceptor site is shown at the bottom. The nucleotides that are identical to the consensus splicing sequence are underlined. The polymorphism in CYP3AP1 that abolishes the pseudogene splicing (CYP3A7_39256 T→A) is marked with a star.

provenstrating that CYP3A7.1L is a functional enzyme. In W(R) cells 7α-, 16α-, and 7β-OH DHEA were produced (Fig. 5), and in INVSc1HR cells, only 16α-OH DHEA could be detected (Table II). The difference in the production of metabolites could be caused by different protein-protein interactions of CYP3A7.1L with the overexpressed P450 reductases. Western blotting analysis showed that the amounts of CYP3A7.1L and CYP3A7.1 in W(R) and INVSc1HR strains were different, with a 50-fold lower CYP3A7.1L/CYP3A7.1 ratio in the INVSc1HR cells (Table II). This is likely a result of different interactions of
CYP3A7.1L with the yeast and human reductases or could reflect differences in CYP3A7.1L carboxyl-terminal folding and stability.

**DISCUSSION**

The highly polymorphic drug-metabolizing enzyme genes diversified through the coevolution of plants and animals. The differences in drug-metabolizing enzyme allele frequencies across human populations was suggested to reflect differences in xenobiotic environments mainly introduced by the adaptation to diet composition over thousands of years (31). Specifically, the wide range of structurally different substrates metabolized by CYP3A enzymes, which is illustrated by their involvement in the biotransformation of more than 50% of all therapeutic drugs currently used, suggests that they might have been exposed to multiple selective pressures and that different variants may be the targets of these pressures. However, the key role of CYP3A4 and CYP3A7 in the metabolism of endogenous substrates, such as steroid hormones or bile acids (32, 33), imposed limitations to this variation and resulted in well conserved genes with rare deleterious mutations and low variability. In agreement with this, only two CYP3A7 enzymes have been described (CYP3A7.1 and CYP3A7.2) (18), both representing active enzymes. However, the pressure on these

**TABLE I**

Allele frequencies of CYP3A7_39256 A and CYP3A7*2 in different ethnic groups and linkage disequilibrium between them

| Ethnic group | No. of chromosomes analyzed | Allele frequency | Haplotype frequency |
|--------------|----------------------------|-----------------|--------------------|
|             |                            | CYP3A7_39256 A  | CYP3A7*2           | CYP3A7*1A | CYP3A7*2A |
| Caucasians  |                            | 0.07            | 0.07               | 0.93      | 0.07      |
| Spanish     | 130                        | 0.09            | 0.09               | 0.91      | 0.09      |
| Swedish     | 90                         | 0.28            | 0.28               | 0.72      | 0.28      |
| Chinese     | 192                        | 0.59            | 0.62               |           |           |
| Africans    | 48/180                     | 0.28            | 0.28               |           |           |

*The frequency of the allele was calculated by using the formula: frequency = \( \frac{2 \times \text{number of people homozogous for the allele} + \text{number of heterozygous people}}{2 \times \text{total number of people}} \). CYP3A7*1 and CYP3A7_39256 T, GenBank TM TM accession number AF280107.

*The frequencies of the haplotypes are calculated for each population. Haplotypes and allele combinations are: CYP3A7*1A (CYP3A7*1/CYP3A7_39256 T; proteins: CYP3A7.1 and CYP3A7.1L), CYP3A7*2A (CYP3A7*2/CYP3A7_39256 A; proteins: CYP3A7.2).

* Data from NCBI, database of single nucleotide polymorphisms (dbSNP) build 124. CYP3A7_39256 T>A or rs2740565 was genotyped in 23 samples of African American descent from the Coriell Cell Repository. For European American and Chinese descent a 0.04 and 0.15 allele frequency is found using 24 samples from the Coriell Cell Repository.

* Data from Rodriguez-Antona et al. (18).

**FIG. 4.** Tissue distribution of CYP3A7-3AP1 mRNA. Adult and fetal human cDNA panels (BD Biosciences) were used to amplify by PCR the cDNAs of CYP3A7 and CYP3A7-3AP1, using 40 cycles of PCR, 0.8 or 4.0 ng of cDNA, and specific primers, as described under “Experimental Procedures.” When 32 PCR cycles were used, the higher expression of CYP3A7 when compared with CYP3AP1-3A7 could be appreciated in adult and fetal liver (data not shown). The number in brackets indicates the number of pooled tissues, in all cases corresponding to Caucasians. The small intestine sample lacks mucosal lining. asc, ascendent; tr, transversal; des, descendent; Sk, skeletal; S intest, small intestine; Leukoc, leukocytes; F, fetal; Neg cont, negative control.

**FIG. 5.** HPLC metabolite profile of DHEA after incubation with microsomes from W(R) yeast expressing CYP3A7.1L and CYP3A7.1. The HPLC chromatograms resulting from DHEA incubations with microsomes from W(R) cells (overexpressing the yeast P450 reductase) overexpressing CYP3A7.1L or CYP3A7.1 and from W(R) cells transformed with empty vector (pYeDP60) are shown. Similar incubations were analyzed by GC/MS, and the different metabolites were identified. Inset, heterologous expression of CYP3A7.1 and CYP3A7.1L in W(R) cells. Ten and 20 µg of W(R) microsomal protein were analyzed by Western blot. The 32 extra amino acids of CYP3A7.1L carboxyl-terminal result in a protein of lower mobility than CYP3A7.1.

CYP3A7.1L with the yeast and human reductases or could reflect differences in CYP3A7.1L carboxyl-terminal folding and stability.
genes to diversify their functions is evidenced by mechanisms such as trans-splicing between CYP3A4, CYP3A5, and CYP3A43 mRNAs, which generates new enzymes without modification of the genes (34). In addition, this work showed that a novel CYP3A7-derived enzyme (CYP3A7.1L), generated by an alternative splicing of CYP3A7 with CYP3A1P1, is a functional enzyme. The corresponding mRNA, containing almost full sequence of CYP3A7 plus two exons from regions of the CYP3AP1 pseudogene (CYP3A7-3AP1), was found mainly in liver, kidney, and gastrointestinal tract. Interestingly, we found that the splicing efficiency between CYP3A7 and CYP3A1P1 was regulated in a developmental- and tissue-specific manner (Fig. 2c), expression differences between fetal and adult liver and Fig. 4, tissue differences). This opened the possibility that CYP3A7.1L could be the major splicing form in certain tissues or at specific developmental stages.

A single nucleotide polymorphism at -6 of the first acceptor splice site of CYP3A1P1 (CYP3A7_39256 T→A) prevents the alternative splicing and results in no CYP3A7.1L expression (Fig. 3). Important interethnic differences in the frequency of CYP3A7_39256 A were found (Table I), being the predominant allele in Africans but less represented in Chinese and Caucasian populations. In addition, a linkage disequilibrium of CYP3A7_39256 A with CYP3A7*2 and CYP3A5*1 indicates that usually, specific sets of CYP3A enzymes are expressed with ethnic differences in their frequency. Two major haplotypes were identified: CYP3A7*1/CYP3A7_39256 T/CYP3A5*3, which results in the expression of CYP3A7.1 and CYP3A7.1L and is predominant in Caucasians and Chinese, and CYP3A7*2/CYP3A7_39256 A/CYP3A5*1, expressing CYP3A7.2 and CYP3A5 proteins and being the predominant in Africans (Fig. 6).

Expression of CYP3A7.1L in heterologous expression systems showed that CYP3A7.1L was a microsomal enzyme able to hydroxylate DHEA. Interestingly, the catalytic activity of the enzyme was shifted depending on the expression system used; in yeast overexpressing the yeast P450 reductase, DHEA was hydroxylated at 7α-, 16α-, and 7β-positions, whereas in yeast overexpressing human P450 reductase, DHEA was hydroxylated only at the 16α-position. Miller et al. (35) showed that CYP3A4 catalyzes DHEA 7β-, 16α-, and 7α-hydroxylations, whereas CYP3A7 hydroxylates DHEA at 16α- and 7β-but not at 7α-positions. Similarly to Stevens et al. (36), they also showed that CYP3A7 was a more efficient 16α-DHEA hydroxylase than CYP3A4 and that CYP3A7 was a less efficient 7β-hydroxylase than CYP3A4. CYP3A7 and CYP3A4 share a 88% identity, and there is a large overlap in substrate specificity; however, there are differences in activity, probably caused by changes near the active site. The different hydroxylase specificity of CYP3A7.1L in the yeast systems, which does not occur for CYP3A7.1 (Table II), could be the result of different interactions of the longer carboxyl-terminal of CYP3A7.1L with the human and yeast reductases; alternatively, it could be caused by the different stability and catalytic activity of the enzyme.

Thompson et al. (36) showed that a relatively homogeneous class of haplotypes, spanning 150 kb, including CYP3A4, CYP3A7 and CYP3A5, were driven to near fixation frequency in the non-African populations, suggesting the action of varying natural selective pressures. The authors proposed that lack of CYP3A5 protein (CYP3A5*3) conferred a selective advantage by influencing salt and water retention and risk for hypertension and could have been the target for the selection pressures in this locus. On the other hand, a different allele, in linkage disequilibrium with CYP3A5*3, could have conferred the selective advantage. The Caucasian most common haplotype consists, among other polymorphisms, of CYP3A4*1A, CYP3A7*1, CYP3A7_39256 T, and CYP3A5*3, whereas the African most common haplotype contains CYP3A4*1B, CYP3A7*2, CYP3A7_39256 A, and CYP3A5*1. An altered expression of CYP3A4*1B is controversial (19, 37), and CYP3A7.1 and CYP3A7.2 have differences in catalytic activity but are both functional enzymes (18). Therefore, it is unlikely that these alleles could have provided a significant adaptation advantage. On the other hand, CYP3A7_39256 T allele results in the
formation of a different CYP3A enzyme, which might have different catalytic activity than CYP3A7 and has different expression patterns than CYP3A4. Therefore, CYP3A7_39256 T could have contributed to the complex evolutionary history of this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region. When chimpanzee (Ensembl, scaffold 37686) and human this genomic region.

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A Novel Polymorphic Cytochrome P450 Formed by Splicing of CYP3A7 and the Pseudogene CYP3AP1
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