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Co-Regulation of *CYP3A4* and *CYP3A5* and Contribution to Hepatic and Intestinal Midazolam Metabolism

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ABSTRACT

We recently demonstrated that a variant allele of *CYP3A5* (*CYP3A5*3*) confers low CYP3A5 expression as a result of improper mRNA splicing. In this study, we further evaluated the regulation of CYP3A5 in liver and jejunal mucosa from white donors. For all tissues, high levels of CYP3A5 protein were strongly concordant with the presence of a wild-type allele of the *CYP3A5* gene (*CYP3A5*1*). CYP3A5 represented greater than 50% of total CYP3A content in nearly all of the livers and jejuna that carried the *CYP3A5*1* wild-type allele. Overall, CYP3A5 protein content accounted for 31% of the variability in hepatic midazolam hydroxylation activity. Improperly spliced mRNA (*SV1-CYP3A5*) was found only in tissues containing a *CYP3A5*3* allele. Properly spliced *CYP3A5* mRNA (*wt-CYP3A5*) was detected in all tissues, but the median *wt-CYP3A5* mRNA was 4-fold higher in *CYP3A5*1/*3* livers compared with *CYP3A5*3/*3* livers. Differences in *wt-CYP3A5* and *CYP3A4* mRNA content explained 53 and 51% of the interliver variability in CYP3A5 and CYP3A4 content, respectively. Hepatic CYP3A4 and CYP3A5 contents were not correlated when all livers were compared. However, for *CYP3A5*1/*3* livers, levels of the two proteins were strongly correlated ($r = 0.93$) as were *wt-CYP3A5* and *CYP3A4* mRNA ($r = 0.76$). These findings suggest that *CYP3A4* and *CYP3A5* genes share a common regulatory pathway for constitutive expression, possibly involving conserved elements in the 5′-flanking region.

CYP3A contributes to the metabolism of numerous therapeutic agents and endogenous molecules. Substrates of CYP3A include benzodiazepines, hydroxymethyl glutaryl-CoA reductase inhibitors, dihydropyridine calcium channel blockers, human immunodeficiency virus protease inhibitors, antiepileptics, chemotherapeutics, and immunosuppressants (Guengerich, 1999). Interindividual differences in the oral bioavailability and systemic clearance of CYP3A substrates can be attributed in large part to variable expression of CYP3A in the liver (Thummel et al., 1994) and mucosal epithelium of the small intestine (DeWaziers et al., 1990; Paine et al., 1996, 1997). CYP3A4 is the dominant CYP3A isoform in the liver and small intestine of most white adults, whereas CYP3A7 is primarily a fetal enzyme (Kitada and Kamataki, 1994). More recently, human CYP3A43 has been identified and cloned (Domanski et al., 2001), although its contribution to hepatic or extrahepatic CYP3A-dependent drug clearance is thought to be negligible (Westlind et al., 2001). CYP3A5 is also found in the liver and intestinal mucosa (Wrighton et al., 1990; Paine et al., 1997) and other extrahepatic tissues, including the kidney (Haehner et al., 1996), lung (Kivistö et al., 1996), and prostate gland (Yamakoshi et al., 1999). Its expression is polymorphic, with readily detectable levels in 25 to 30% and very low or undetectable levels in 70 to 75% of livers and small intestines examined (Wrighton et al., 1990; Paine et al., 1997; Tateishi et al., 1999).

The genetic basis for polymorphic CYP3A5 expression was first examined by Jounäidi et al. (1996). These investigators demonstrated an interesting but inconsistent association between low levels of protein and a single nucleotide polymorphism (SNP) in exon 11 resulting in a T398N substitution. This structural change was thought to reduce CYP3A5 stability and decrease steady-state tissue levels of the enzyme. More recently, polymorphic CYP3A5 expression in adult liver and small intestine was strongly correlated with a single nucleotide polymorphism, A>G, within intron 3 of the *CYP3A5* gene (nt 22,893 in AC005020) and designated as

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ABBREVIATIONS: SNP, single nucleotide polymorphism; nt, nucleotide; wt, wild-type; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BTE, basic transcription element; PXR, pregnane X receptor.

TABLE 1
Demographics of white tissue donors

	Livers	Small Intestines
Number of subjects	60	31
Age (years): median (range)	46 (7–70)	47 (12–64)
Sex: Male/Female	29/31	14/17

*CYP3A5*3* (Kuehl et al., 2001). This mutation creates a cryptic consensus splice site and results in the production of improperly spliced mRNA containing exon 3B (*SV1-CYP3A5* mRNA) and a small amount of properly spliced mRNA (*wt-CYP3A5* mRNA). In contrast to correctly spliced *wt-CYP3A5* mRNA, the aberrant *SV1-CYP3A5* mRNA contains an exon derived from the intron 3 sequence of *CYP3A5* and encodes a protein that is truncated at amino acid 102.

To further understand the mechanism and functional significance of the *CYP3A5*3* polymorphism, we characterized the *CYP3A5* genotype and phenotype for a large panel of livers and small intestines from white donors. Specifically, we measured *wt-CYP3A5*, *SV1-CYP3A5*, and *CYP3A4* mRNA levels, CYP3A5 and CYP3A4 protein levels, and CYP3A-dependent midazolam hydroxylation activities and correlated these parameters with *CYP3A5* genotype.

Materials and Methods

General Reagents

NADPH and alkaline phosphatase-conjugated secondary antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Midazolam, 1′-hydroxymidazolam, 4-hydroxymidazolam, and $^{15}N_3$-midazolam were kindly provided by Roche Laboratories (Nutley, NJ). $^{15}N_3$-labeled metabolites of midazolam were generated enzymatically from $^{15}N_3$-midazolam as described previously (Schmiedlin-Ren et al., 1997). *N*-methyl-*N*-(*t*-butyl-dimethylsilyl) trifluoroacetamide was obtained from Pierce Chemical (Rockford, IL). CYP3A5-specific antibody (A235) was purchased from BD Gentest (Woburn, MA). SDS-polyacrylamide gel electrophoresis reagents (37.5:1 bis-acrylamide, ammonium persulfate, and *N*,*N*,*N*′,*N*′-tetra-methyl-ethylene-diamine) were purchased from Bio-Rad Laboratories (Hercules, CA). Nitrocellulose was purchased from Schleicher & Schuell (Keene, NH). 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium reagent was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Tissue Samples

Samples of human liver (n = 60) and jejunal mucosa (n = 31) from white donors were obtained from the University of Washington School of Pharmacy Human Tissue Bank (Seattle, WA). Paired human liver and intestinal samples were obtained from 10 persons. Demographic information for the donors is summarized in Table 1. Human liver microsomes and homogenate from jejunal mucosa were prepared according to previously published protocols (Paine et al., 1997). Protein concentrations were determined by the method of Lowry et al. (1951).

Isolation of Tissue DNA and RNA

DNA was isolated from liver and jejunal mucosal samples using commercially available kits (QIAGEN GmbH, Hilden, Germany). Total RNA was isolated from tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA). Samples for RNA analysis (24 livers and 17 small intestines) were selected based on the observed *CYP3A5* genotype. For livers, 1 *CYP3A5*1/*1*, 12 *CYP3A5*1/*3*, and 11 *CYP3A5*3/*3* tissues were studied. For jejuna, 1 *CYP3A5*1/*1*, 1 *CYP3A5*1/*3*, and 15 *CYP3A5*3/*3* tissues were studied. There was no evidence of significant mRNA degradation in the selected samples.

Determination of CYP3A5 Genotype

The primers used to amplify *CYP3A5* exons and introns from genomic DNA were described previously (Kuehl et al., 2001). Forward and reverse primers were tailed with universal sequencing primers (−40 M13 and −28 M13, respectively). Primer pairs were used for 35 cycles to amplify genomic DNA; the following conditions were used in each cycle: 95°C for 15 s, 61°C for 30 s, and 72°C for 1 min. Unincorporated nucleotides and primers were removed by incubating the PCR product with shrimp alkaline phosphatase and exonuclease for 30 min at 37°C followed by 15 min at 80°C. Amplified *CYP3A5* fragments were sequenced using BigDye Ter-

TABLE 2
RNA primers and probes

wt-CYP3A5 mRNA	
Forward primer	5′ gggTCTCTggAAATTTgACACAgAg 3′
Reverse primer	5′ CTgTTCTgATCACgTCgggATCT 3′
Probe	5′ 6FAM-ATgTggggAACgTATgAAggTCAACTCCCT-TAMRA 3′
SV1-CYP3A5 mRNA	
Forward primer	5′ TCTCTggAAATTTgACACAgAgTgC 3′
Reverse primer	5′ CAgCAAgAgTCTCACACAggAgC 3′
Probe	5′ 6FAM-AATgTggggTATCTCTTCCCTgTTTggACCA-TAMRA 3′
CYP3A4 mRNA	
Forward primer	5′ CACAgATCCCCCTgAAATTAAgCTTA 3′
Reverse primer	5′ AAAATTCAggCTCCACTTACggTg 3′
Probe	5′ 6FAM-AggACTTCTTCAACCAgAAAAACCCgTTgTTCT-TAMRA 3′
CYP3A5 mRNA	
Forward primer	5′ ACAgATCCCCTTgAAATTAgACACg 3′
Reverse primer	5′ CTTAgggTTCCATCTCTTgAATCCA 3′
Probe	5′ 6FAM-AAggACTTCTTCAACCAgAAAAACCCATTgTTCTA-TAMRA 3′
GAPDH mRNA	
Forward primer	5′ TCCTgCACCACCAACTgCTT 3′
Reverse primer	5′ gAggggCCATCCACAgTCTT 3′
Probe	5′ 6FAM-CACTCATgACCACAgTCCATgCCATCAC-TAMRA 3′

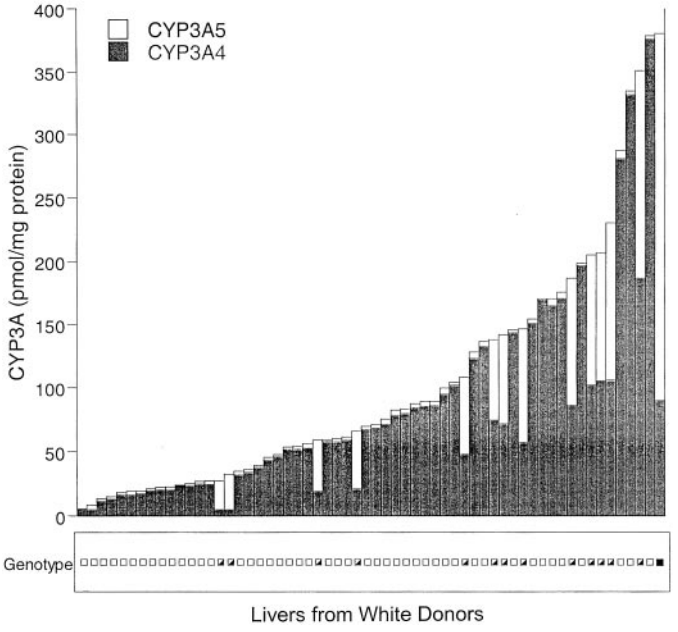

Fig. 1. *CYP3A5* genotype and CYP3A4 and CYP3A5 protein contents as a fraction of total CYP3A in livers. Genotype and microsomal CYP3A content were determined as described under *Materials and Methods*. ■, ◪, and □ correspond to livers with a *CYP3A5*1/*1*, *CYP3A5*1/*3*, and *CYP3A5*3/*3* genotype, respectively.

minator (Applied Biosystems, Foster City, CA), and products were resolved by polyacrylamide gel electrophoresis or capillary gel electrophoresis. The resultant trace files were basecalled by phred and assembled by phrap (http://www.genome.washington.edu). Polyphred (Nickerson et al., 1997) was used to detect potential heterozygosity. To be a true variant, the variant-containing sequence generated by the forward primer had to be consistent with that generated by the reverse primer.

Quantitation of CYP3A mRNA

Primers. Nucleotide sequences for primers and probes specific for *CYP3A5*, *CYP3A4*, and GAPDH mRNA are listed in Table 2. PCR primers and the dual-labeled probe for the allele-specific human *CYP3A5* mRNA assays were designed using the primer design software Primer Express (Applied Biosystems). Probes were synthesized by IDT, Inc. (Coralville, IA). The quantitation probe for wild-type (*wt-CYP3A5*) mRNA was specific for the exon 3/exon 4 boundary. The quantitation probe for aberrant *SV1-CYP3A5* mRNA arising from the intron 3 mutation of the *CYP3A5*3* allele was designed to match the exon 3/exon 3B boundary. The quantitation probe for total *CYP3A5* mRNA was specific for exon 13. The 18S ribosomal RNA gene was quantitated using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems).

Reverse Transcription. For each liver or jejunal RNA sample, 2 μg of total RNA was treated with 0.25 units of DNase I (Roche Diagnostics, Mannheim, Germany) at 37°C for 30 min. DNase was inactivated by heating at 70°C for 5 min. Reverse transcription was performed on liver RNA samples using random hexamers, according to the manufacturer's instructions for the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), with the exception that the resulting cDNA was not treated with RNase H. Similarly, reverse transcription was performed on intestinal RNA samples using 200 ng of oligo(dT)$_{12-18}$ primers (Invitrogen). Resulting cDNA from liver and intestinal RNA samples were diluted to a final volume of 100 μl.

Real-Time Quantitative PCR. The liver or jejunal PCR mixture (final volume, 24 μl) consisted of 4 μl of cDNA, the appropriate forward and reverse primers (0.4 μM each), 100 nM TaqMan probe, and 1× TaqMan Universal PCR Master Mix (Applied Biosystems). Amplification and detection were performed with the ABI 7700 system with the following PCR reaction profile: 95°C for 10 min and 40 cycles of 95°C for 20s and 62°C for 1 min. Reported liver mRNA data were normalized to 18S ribosomal RNA, although normalization to GAPDH mRNA resulted in qualitatively similar data. Jejunal *CYP3A5* mRNA data were normalized to GAPDH mRNA. The PCR amplicons used to quantify the gene expression levels for *CYP3A5* and *CYP3A4* were examined using BigDye Terminator cycle sequencing, and the subsequent products were analyzed on a ABI377 Automated DNA Sequencer (Applied Biosystems). The resultant DNA sequences were aligned using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI) and were specific for the respective genes.

Western Blot Analysis

CYP3A4 (purified from human liver) and CYP3A5 (purified from a heterologous baculovirus/insect cell expression system) were used as reference standards (specific contents were 12.2 and 11.7 nmol of spectral cytochrome P450/mg of protein). Total protein concentration for the CYP3A standards was determined by the method of Lowry et al. (1951), using bovine serum albumin as a reference protein. Total cytochrome P450 content was determined from the CO-binding spectra (Omura and Sato, 1964).

Immunoquantitation of CYP3A4 and CYP3A5 was performed as described by Paine et al. (1997), with minor modifications. Briefly, liver microsomal protein (3 μg for livers expressing at least one *CYP3A5*1* allele and 20 μg for *CYP3A5*3/*3* livers) and jejunal homogenates (50 μg) were resolved by electrophoresis on 9% acrylamide gels. To control for matrix effects, liver microsomes or jejunal homogenates with nearly undetectable CYP3A4 or CYP3A5 levels were added to CYP3A4 or CYP3A5 standard curves. The amount of protein added to CYP3A4 or CYP3A5 standard curves was

TABLE 3
CYP3A phenotype as a function of *CYP3A5* genotype
Data are reported as median (range).

	All Genotypes	*CYP3A5*1/*1*	*CYP3A5*1/*3*	*CYP3A5*3/*3*
Liver microsomal protein	(n = 60)	(n = 1)	(n = 13)	(n = 36)
CYP3A5 (pmol/mg)	3.0 (0.25–291)	291	70.6 (22.2–164)	2.5 (0.25–6.6)**
CYP3A4 (pmol/mg)	58.4 (5–376)	90.1	71.6 (5–187)	57.8 (5–376)
Total CYP3A (pmol/mg)	70.4 (5.6–381)	381	142 (27.2–351)	59.7 (5.6–379)*
Jejunal homogenate protein	(n = 31)	(n = 2)	(n = 2)	(n = 27)
CYP3A5 (pmol/mg)	0.5 (0–19.8)	14.0 (8.2,19.8)	3.6 (2.6,4.5)	0.5 (0–2.4)**
CYP3A4 (pmol/mg)	17.3 (0.5–32.8)	17.2 (14.3,20.1)	2.4 (0.9,3.8)	18.2 (0.5–32.8)
Total CYP3A (pmol/mg)	18.7 (0.5–34.1)	31.2 (28.3,34.1)	5.9 (5.4,6.4)	18.7 (0.5–33.3)

* $p < 0.01$ comparing *CYP3A5*1* carriers and *CYP3A5*3/*3* individuals.
** $p < 0.001$ comparing *CYP3A5*1* carriers and *CYP3A5*3/*3* individuals.

equivalent to that of the liver or jejunal samples being analyzed. After electrophoresis, the gels were cut using molecular weight standards as markers, and the resulting strips were placed on sheets of nitrocellulose for simultaneous electrophoretic transfer. The nitrocellulose sheets were incubated with a specific anti-CYP3A5 IgG antibody (BD Gentest) or an anti-CYP3A4 antibody (Thummel et al., 1994). Although the anti-CYP3A4 antibody cross-reacts with CYP3A5, the SDS-polyacrylamide gel electrophoresis conditions used in this study resulted in physical separation of CYP3A4 and CYP3A5 bands and thus should give a reliable quantitation of CYP3A4 content. CYP3A5 content was determined using the specific anti-CYP3A5 antibody. An integrated optical density for each 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium-developed protein band was generated using a Bio-Rad ChemiDoc and Quantity One program. CYP3A protein levels in the tissue preparations were estimated by comparison of the unknown band integrated optical density with the appropriate standard curve.

Midazolam Kinetic Protocol

All incubations (final volume, 1 ml) were performed in duplicate. Each incubation tube contained 20 to 100 μg of human liver microsomes or 50 to 100 μg of jejunal homogenate in 0.1 M potassium phosphate, pH 7.4. Appropriate controls to confirm linear product formation were performed. Midazolam (final concentration, 8 μM) was added to the diluted tissue preparations, and tubes were preincubated at 37°C for 5 min. NADPH (final concentration, 1 mM) was added to initiate the reaction. Reactions were terminated after 2 min (livers) or 4 min (jejuna) by the addition of 1 ml of ice-cold 0.1 M Na_2CO_3, pH ~11. ^{15}N-labeled metabolite internal standards were added to the samples and standards before extraction using ethyl acetate. The organic phase was transferred to a clean tube, blown down to dryness, reconstituted in acetonitrile, and derivatized using *N*-methyl-*N*-(*t*-butyl-dimethylsilyl) trifluoroacetamide. Samples and standards were analyzed for 1′-hydroxymidazolam and 4-hydroxymidazolam by negative chemical ionization gas chromatography-mass spectroscopy (Paine et al., 1997).

Statistical Analysis

Statistical analysis was performed by SPSS, version 8.0 (Chicago, IL). The phenotypic markers (protein and mRNA expression and catalytic activity) for liver and jejunal mucosa were not normally distributed. Thus, group differences based on *CYP3A5* genotype and sex were analyzed nonparametrically by the Wilcoxon two-sample test. In addition, linear regression was performed to compare CYP3A protein and mRNA levels. Stepwise multivariate regression was performed to assess the relative contribution of variability in liver microsomal CYP3A4 and CYP3A5 to interliver differences in total midazolam hydroxylation activity. *P* values less than 0.05 were considered statistically significant.

Results

Hepatic CYP3A5 Genotype and CYP3A Protein Phenotype. There was a strong concordance between the *CYP3A5* genotype and CYP3A5 protein expression among the 60 livers from white donors (Fig. 1). Overall, 77% of livers studied were *CYP3A5*3/*3*. The median (range) microsomal CYP3A5 content for *CYP3A5*3/*3* livers was 2.5 (0.25–6.6) pmol/mg of protein (Table 3). Of the livers examined, 22% displayed a *CYP3A5*1/*3* genotype. For those livers, the median CYP3A5 protein content was 70.6 (22–164) pmol/mg of protein. The single *CYP3A5*1/*1* liver had the highest CYP3A5 protein level detected, 291 pmol/mg of protein.

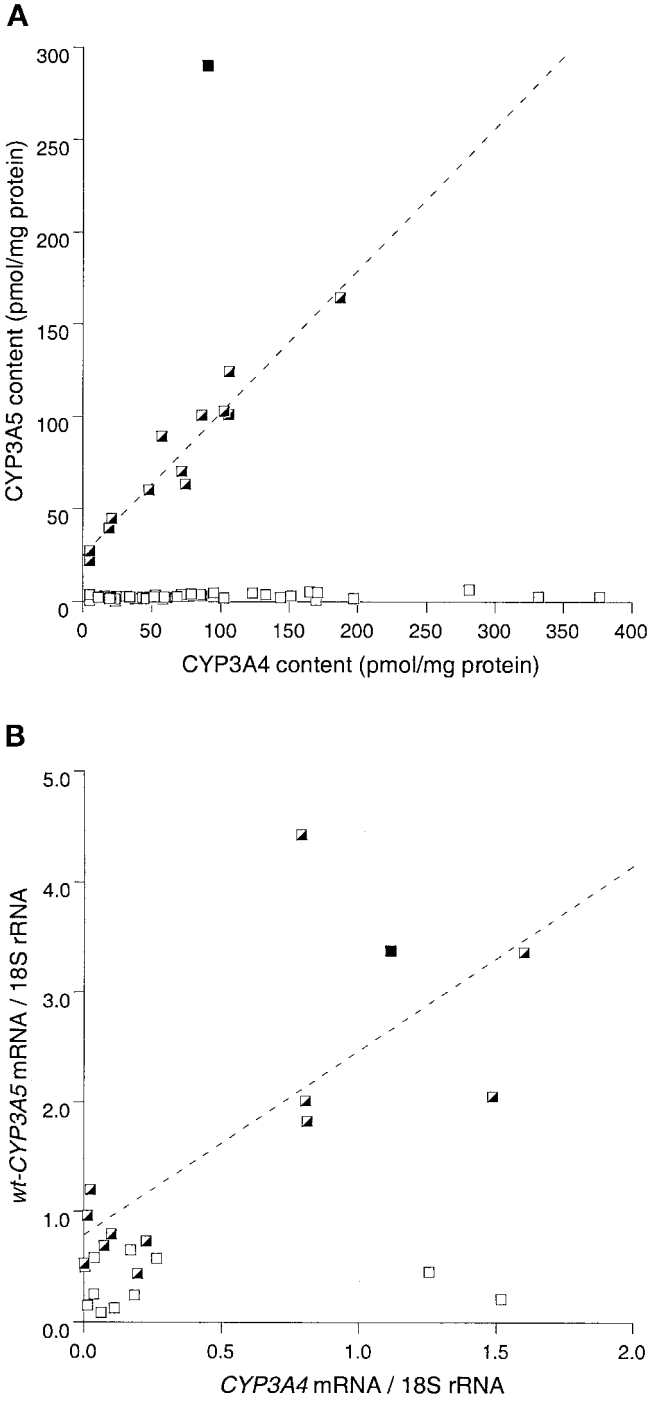

Fig. 2. Correlation between hepatic CYP3A4 and CYP3A5 phenotype. Microsomal CYP3A4 and CYP3A5 protein content for 60 livers (A) and *wt-CYP3A5* and *CYP3A4* mRNA content for a 24 liver subset (B) were measured as described under *Materials and Methods*. ■, ◪, and □ correspond to livers with a *CYP3A5*1/*1*, *CYP3A5*1/*3*, and *CYP3A5*3/*3* genotype, respectively. For each figure, the regression line is shown for the *CYP3A5*1/*3* subset of livers.

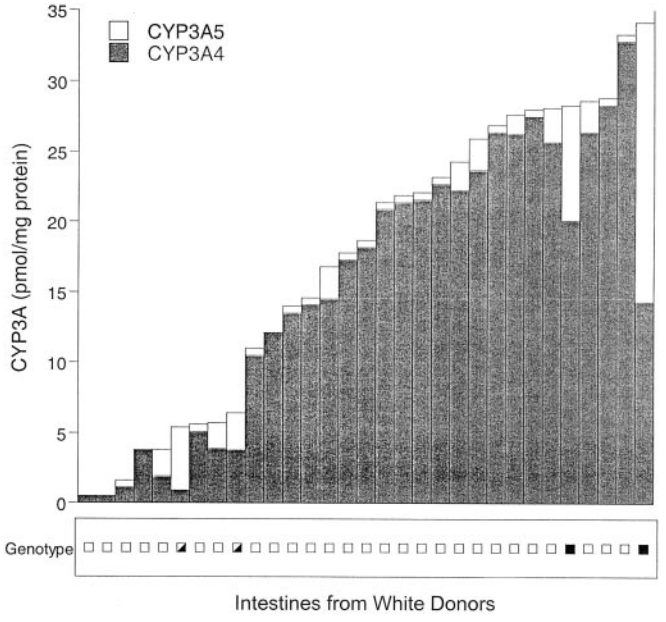

Fig. 3. *CYP3A5* genotype and CYP3A4 and CYP3A5 protein contents as a fraction of total CYP3A in jejuna. Genotype and homogenate CYP3A contents were determined as described under *Materials and Methods*. ■, ◪, and □ correspond to intestines with a *CYP3A5*1/*1*, *CYP3A5*1/*3*, and *CYP3A5*3/*3* genotype, respectively.

The total microsomal CYP3A content for our bank of livers varied considerably, from 6 to 381 pmol/mg of microsomal protein (Table 3). Wide variability in CYP3A4 expression was also noted; levels ranged from 5 (assigned value for a faint band) to 376 pmol/mg of microsomal protein. The contribution of CYP3A5 to the total CYP3A protein content was significant (Fig. 1), representing 46 to 85% of total hepatic CYP3A content in livers with at least one *CYP3A5*1* allele. There was no significant difference ($p = 0.57$) in CYP3A4 content between *CYP3A5*3/*3* livers (expressing little CYP3A5 protein) and livers containing at least one *CYP3A5*1* allele (expressing high levels of CYP3A5 protein). However, the median (range) total CYP3A content was 142 (27–351) pmol/mg of microsomal protein in *CYP3A5*1/*3* livers, whereas the median total CYP3A content in *CYP3A5*3/*3* livers was 60 (6–379) pmol of CYP3A/mg of microsomal protein. The single *CYP3A5*1/*1* liver contained 381 pmol of CYP3A/mg of microsomal protein. Thus, total CYP3A content was more than 2-fold higher for livers with at least one *CYP3A5*1* allele compared with *CYP3A5*3/*3* livers ($p = 0.007$).

There was no significant correlation between hepatic CYP3A5 and CYP3A4 content when all livers were compared ($r = 0.08$). However, when only *CYP3A5*1/*3* livers were examined, CYP3A5 and CYP3A4 levels were found to be highly correlated ($r = 0.97$, $p < 0.001$) (Fig. 2A).

Small Intestinal CYP3A5 Genotype and CYP3A Protein Phenotype. Of the 31 jejunum samples from white donors, 87% had a faint or quantifiable CYP3A5 band ranging from 0.5 (detectable but nonquantifiable) to 19.8 pmol/mg of homogenate protein (Table 3). The remaining 13% of jejunal samples had no detectable CYP3A5 protein and were assigned a value of 0 pmol/mg of homogenate protein. All jejunal tissue with a nondetectable or low level of CYP3A5 protein (≤0.5 pmol/mg homogenate) were found to be *CYP3A5*3/*3*. Median CYP3A5 protein contents were 3.6 and 14 pmol/mg of homogenate protein for two *CYP3A5*1/*3* and two *CYP3A5*1/*1* jejuna, respectively (Table 3).

CYP3A5 represented 29 to 83% of total CYP3A content in jejunal samples with at least one *CYP3A5*1* allele (Fig. 3). As expected, CYP3A5 content was significantly higher for jejuna with at least one *CYP3A5*1* allele than those homozygous for the *CYP3A5*3* allele (Table 3). Wide variability in CYP3A4 expression was noted; the values ranged from 0.5 to 32.8 pmol/mg of homogenate protein (Table 3). The median jejunal CYP3A4 content was 18.2 pmol/mg of homogenate protein for *CYP3A5*3/*3* intestines and 9.1 pmol/mg of homogenate protein for the four intestines that were *CYP3A5*1/*3* or *CYP3A5*1/*1*. Median total jejunal CYP3A content was 14 and 19 pmol/mg of homogenate protein for jejuna with at least one *CYP3A5*1* allele and *CYP3A5*3/*3* tissues, respectively. There was no difference in either CYP3A4 or total CYP3A contents between the groups. Because of the limited number of intestines found to have a *CYP3A5*1* allele, it was not possible to obtain a meaningful correlation of jejunal CYP3A5 and CYP3A4 protein content, as was performed for livers.

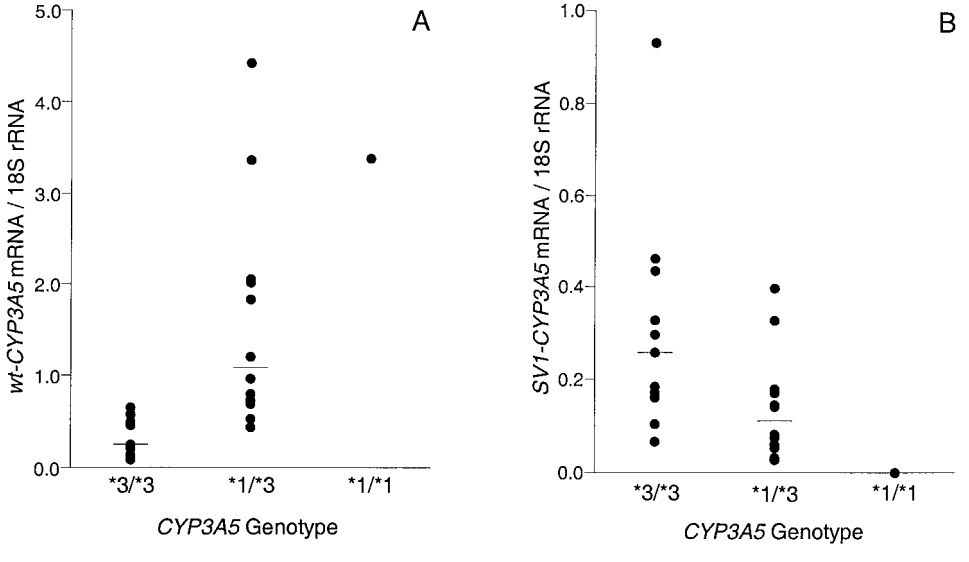

Fig. 4. Quantitation of *CYP3A5* mRNA in livers from white donors. Total mRNA was extracted from 24 livers with known *CYP3A5* genotype (1 *CYP3A5*1/*1*, 12 *CYP3A5*1/*3*, and 11 *CYP3A5*3/*3*) and subjected to quantitative RT-PCR analysis as described under *Materials and Methods*. For each genotype, *wt-CYP3A5* (A) or *SV1-CYP3A5* (B) mRNA data are presented.

Quantitation of mRNA. Twenty-four livers with predetermined *CYP3A5* genotypes were subjected to quantitative RT-PCR for allele-specific *CYP3A5* mRNA. Primers were designed to detect either properly spliced mRNA (*wt-CYP3A5*; primer at exon 3/exon 4 boundary) or aberrantly spliced mRNA (*SV1-CYP3A5*; primer at exon 3/exon 3B boundary). *Wt-CYP3A5* mRNA was detected in all livers examined but varied considerably between and within the genotype groups (Fig. 4A). *CYP3A5*1/*3* livers had a 4-fold higher median *wt-CYP3A5* mRNA level than *CYP3A5*3/*3* livers. The single *CYP3A5*1/*1* liver exhibited a *wt-CYP3A5* mRNA level that was comparable with the highest level seen for *CYP3A5*1/*3* livers. Aberrantly spliced *SV1-CYP3A5* mRNA was not detected in the *CYP3A5*1/*1* liver but was found in all other livers containing at least one *CYP3A5*3* allele. Individual levels of the *SV1-CYP3A5* mRNA varied considerably (Fig. 4B), but the median level for livers with a *CYP3A5*3/*3* genotype was approximately 2-fold higher than that found in heterozygous livers.

For each genotype, hepatic levels of *SV1-CYP3A5* and *wt-CYP3A5* mRNA were compared. *SV1-CYP3A5* and *wt-CYP3A5* mRNA were significantly correlated: $r = 0.79$ for *CYP3A5*3/*3* livers and $r = 0.98$ for *CYP3A5*1/*3* livers (Fig. 5). A 10-fold difference in the slopes of the *SV1-CYP3A5* versus *wt-CYP3A5* mRNA regression line for *CYP3A5*3/*3* livers and *CYP3A5*1/*3* livers was noted. For *CYP3A5*1/*3* livers, *wt-CYP3A5* mRNA was correlated with *CYP3A4* mRNA ($r = 0.76$) (Fig. 2B). Among livers with at least one *CYP3A5*1* allele, *wt-CYP3A5* mRNA levels were of similar magnitude as the *CYP3A4* mRNA content (Fig. 2B). Such a finding was consistent with the comparable median level of CYP3A5 and CYP3A4 protein measured in the same livers (80 versus 64 pmol/mg of microsomal protein, respectively).

Hepatic CYP3A5 protein content was significantly correlated with *wt-CYP3A5* mRNA ($r = 0.73$; Fig. 6A). A similar correlation between hepatic *CYP3A4* mRNA and CYP3A4 protein contents was also observed ($r = 0.71$, Fig. 6B). However, there was substantial noise in both data sets, suggestive of selective mRNA or protein degradation or a partial dissociation of *CYP3A* mRNA and protein regulation.

Seventeen jejunum samples with predetermined *CYP3A5* genotypes were subjected to quantitative RT-PCR analysis to determine allele-specific *CYP3A5* mRNA content. Unfortunately, we were limited by the availability of viable mucosal samples, thus only one *CYP3A5*1/*1* and one *CYP3A5*1/*3* specimen was examined.

Median *wt-CYP3A5* mRNA was higher in jejuna with the *CYP3A5*1* allele compared with *CYP3A5*3/*3* intestines (218 and 69, respectively) (Fig. 7). Intestinal *wt-CYP3A5* mRNA and CYP3A5 protein were not correlated due to the limited number of *CYP3A5*1*-positive samples. However, *SV1-CYP3A5* and *wt-CYP3A5* mRNA were well correlated for the *CYP3A5*3/*3* group ($r = 0.73$), consistent with the strong correlation seen with liver samples.

Correlation of CYP3A Content and Midazolam Hydroxylation Activity. A comparison of midazolam hydroxylation activity and CYP3A contents in livers is shown in Fig. 8, A and B. Stepwise multiple regression analysis of the data was performed. The correlation between total midazolam hydroxylation rate and CYP3A4 content was significant although imprecise ($r = 0.75$). However, it was improved significantly by consideration of both CYP3A4 and CYP3A5

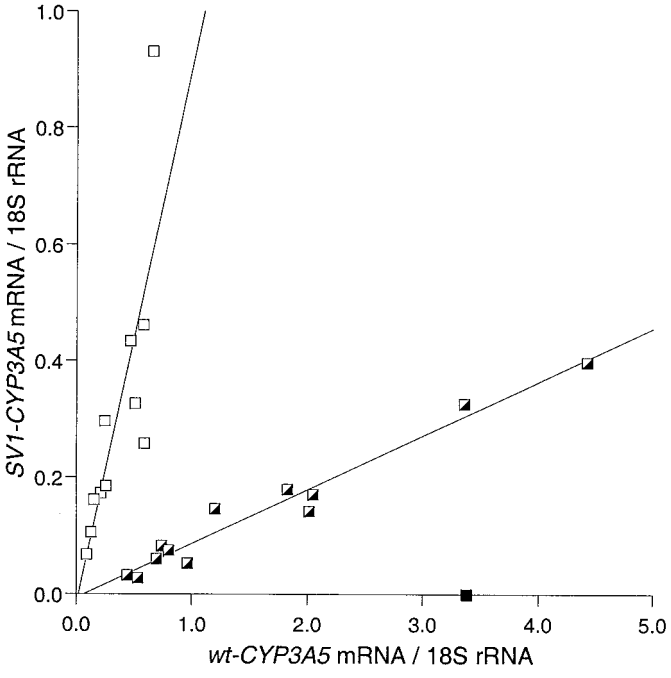

Fig. 5. Correlation between *SV1-CYP3A5* and *wt-CYP3A5* mRNA in livers from white donors. Regression lines are shown for the *CYP3A5*3/*3* ($r = 0.79$; □) and *CYP3A5*1/*3* ($r = 0.98$; ◪) genotypes.

content in the microsomes ($r = 0.94$). Overall, CYP3A4 enzyme content accounted for 57% of the variability in midazolam hydroxylation activity, whereas CYP3A5 content accounted for an additional 32%.

Corresponding to higher total CYP3A levels, livers with at least one *CYP3A5*1* allele exhibited higher rates of midazolam hydroxylation compared with those that were *CYP3A5*3/*3*. As seen in Table 4, livers that contained at least one *CYP3A5*1* allele exhibited a median rate of midazolam hydroxylation that was nearly 3-fold higher than that for *CYP3A5*3/*3* livers. Moreover, the mean 1′/-4-hydroxymidazolam product ratio was 8.2 and 5.5 for *CYP3A5*1/*3* and *CYP3A5*3/*3* livers, respectively ($p = 0.007$).

Among *CYP3A5*3/*3* livers, female donors exhibited a median hepatic hydroxylation activity that was 55% higher than that for male donors ($p = 0.008$). Moreover, in the *CYP3A5*3/*3* livers, median CYP3A4 content was 2-fold higher in women than men ($p = 0.018$), although no difference in CYP3A5 expression was seen. Gender comparisons for *CYP3A5*1* genotypes were not performed because of the small sample size.

There was no significant difference in the total midazolam hydroxylation activity between jejunal samples with at least one *CYP3A5*1* allele and *CYP3A5*3/*3* samples (Table 4), despite the fact that CYP3A5 content was significantly different (Table 3). This was attributed to the low frequency of *CYP3A5*1*-positive intestines encountered (4 of 31), highly variable CYP3A4 levels, and a generally weaker correlation between catalytic activity and CYP3A expression for jejunal homogenates compared with liver microsomes. Nonetheless, the two *CYP3A5*1/*1* intestines exhibited a higher median level of midazolam hydroxylation activity, 330 pmol/min/mg of homogenate protein, compared with 173 pmol/min/mg of homogenate protein for *CYP3A5*3/*3* intestines. Moreover,

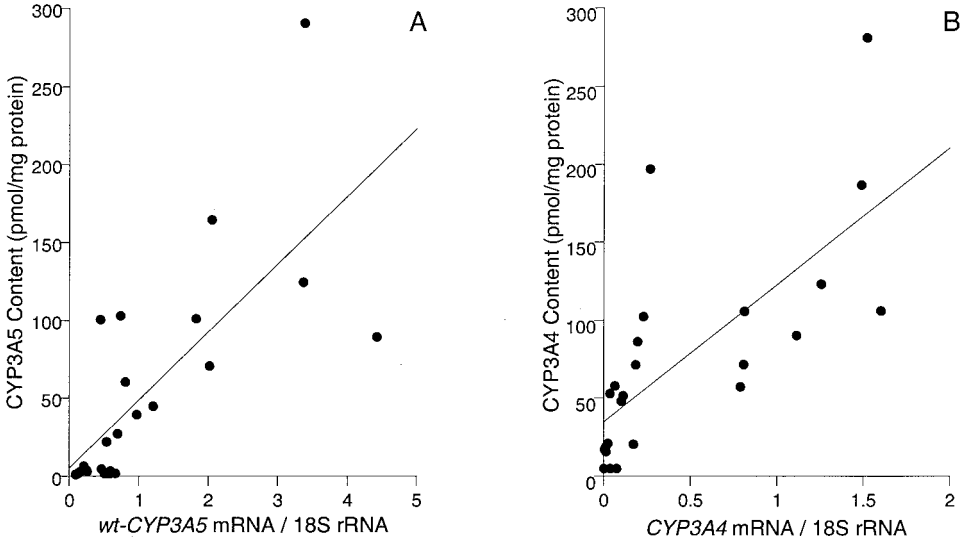

Fig. 6. Correlation between hepatic *CYP3A* mRNA and protein contents. Quantitation of *wt-CYP3A5* mRNA and CYP3A5 protein (A) and *CYP3A4* mRNA and CYP3A4 protein (B) was performed as described under *Materials and Methods*. The correlation coefficient was 0.73 and 0.71 for A and B, respectively.

the 1′/-4-hydroxymidazolam product ratio was higher for the four intestines with a *CYP3A5*1* genotype (range = 7.3–13.1) compared with the ratio for those intestines with a *CYP3A5*3/*3* genotype (range = 4.0–7.9).

Comparing the subset of 10 paired intestine-liver samples, there was no significant correlation between hepatic and intestinal CYP3A4 mRNA, CYP3A protein, and midazolam hydroxylation activity. CYP3A5 protein and mRNA contents were higher in both liver and intestinal tissue coming from the two donors that were *CYP3A5*1/*3* compared with the respective contents from the other eight paired tissues from *CYP3A5*3/*3* donors.

Discussion

Recently, we reported that polymorphic CYP3A5 expression is due primarily to a single point mutation within intron 3 that results in aberrantly spliced mRNA and truncation of the protein (Kuehl et al., 2001). We confirmed this result here with a larger set of livers from white donors. The *CYP3A5*1* allele frequency for the 81 individual organ donors studied was found to be 0.12. This value is similar to the 0.15 allele frequency that we observed in our previous study of white subjects from the United States (Kuehl et al., 2001). However, both allele frequencies are higher than the 0.05 allele frequency reported recently by Hustert et al. (2001) in their analysis of DNA from more than 350 white West Europeans (primarily German and Swiss). Because of the nature of our liver bank, it is not possible to obtain more specific ethnic information about these self-identified white persons in the U.S. Northwest (Washington, Alaska, Oregon, Idaho, and Montana). Nonetheless, the different reported *CYP3A5*1* allele frequencies simply might reflect a greater genetic diversity among the U.S. white population.

We also confirmed by quantitative RT-PCR that the intron 3 SNP provides an alternative splicing pathway and not complete nullification of the proper splicing pathway, because we detected *wt-CYP3A5* mRNA in all tissue samples examined. Median accumulation of *wt-CYP3A5* mRNA was 4-fold higher in liver samples with at least one *CYP3A5*1* allele compared with those with a *CYP3A5*3/*3* genotype (Fig. 4). Moreover, *SV1-CYP3A5* mRNA was only produced in tissues with at least one *CYP3A5*3* allele. The observation that *wt-CYP3A5* and *SV1-CYP3A5* mRNA levels were highly correlated (within each genotype) suggests that function of

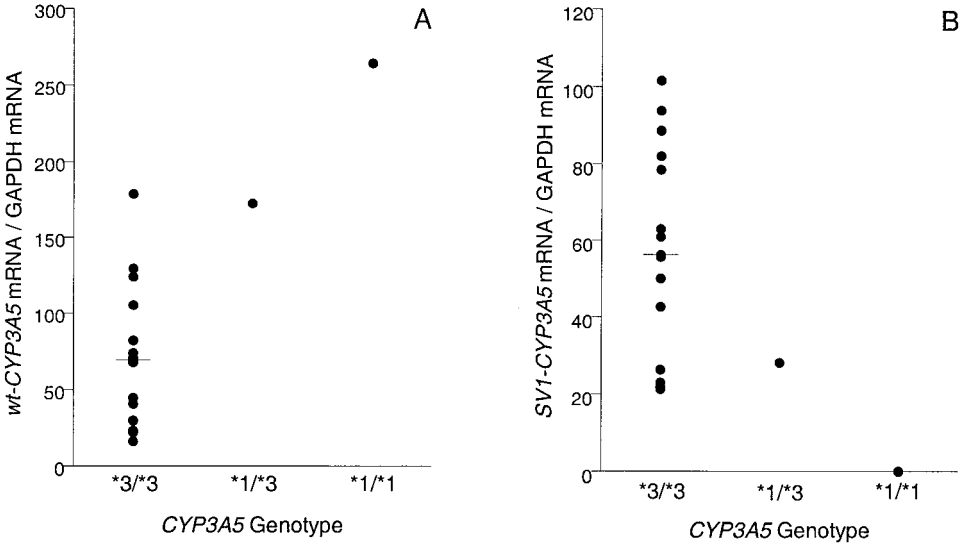

Fig. 7. Quantitation of *CYP3A5* transcripts in jejunal mucosa from white donors. Total mRNA was extracted from 17 jejunal samples with known *CYP3A5* genotype (1 *CYP3A5*1/*1*, 1 *CYP3A5*1/*3*, and 15 *CYP3A5*3/*3*) and subjected to quantitative RT-PCR analysis as described under *Materials and Methods*. For each genotype, *wt-CYP3A5* (A) or *SV1-CYP3A5* (B) mRNA data are shown.

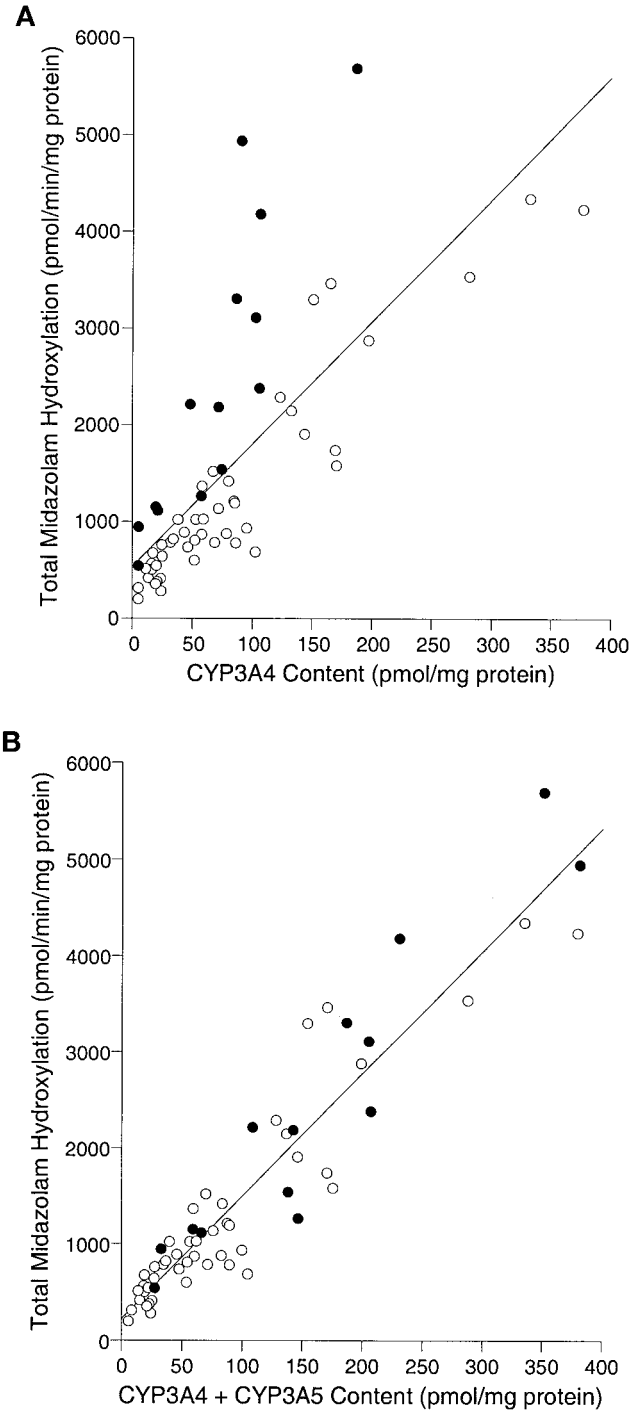

Fig. 8. Correlation between microsomal midazolam hydroxylation activity and CYP3A4 content (A) and total CYP3A content (B) for livers from white donors. ●, livers with at least one *CYP3A5*1* allele; ○, *CYP3A5*3/*3* livers. A, the correlation coefficient was 0.75 for regression of CYP3A4 content and midazolam hydroxylation activity. B, for visual purposes only, CYP3A5 contribution to the stepwise multivariate regression is illustrated by a univariate regression between the sum of CYP3A4 and CYP3A5 contents and midazolam hydroxylation activity. The resulting correlation coefficient (r = 0.93) is comparable with that obtained after the second step of the multivariate regression analysis (r = 0.94).

the spliceosome and other nuclear factors regulating mRNA splicing (Akker et al., 2001) did not differ between the livers studied. Interestingly, the ratio of *wt-CYP3A5:SV1-CYP3A5* mRNA in *CYP3A5*3/*3* livers and jejunal mucosa was approximately 1:1 (Figs. 5 and 7). This could be the result of comparable rates of production of *SV1-CYP3A5* and *wt-CYP3A5* mRNA from aberrant primary transcript or preferential splicing of *SV1-CYP3A5* mRNA that was then degraded more rapidly than *wt-CYP3A5* mRNA via nonsense-mediated mRNA decay (Gonzalez et al., 2001). The later explanation seems more likely given that the ratio of *wt-CYP3A5:SV1-CYP3A5* mRNA was approximately 10:1 in *CYP3A5*1/*3* livers.

Hepatic *wt-CYP3A5* mRNA content was highly correlated with total (nonspecific) *CYP3A5* mRNA levels (r = 0.91; data not shown). This was not too surprising given the disproportionate amount of *wt-CYP3A5* mRNA in livers carrying at least one *CYP3A5*1* allele and the comparable level of *SV1-CYP3A5* and *wt-CYP3A5* mRNA in *CYP3A5*3/*3* livers. Thus, for livers from white donors, measurement of total *CYP3A5* mRNA may be a suitable surrogate for "functional" *wt-CYP3A5* mRNA.

Alternative splicing of *CYP3A5* mRNA seems to be the single most important factor controlling the level of functional hepatic and intestinal CYP3A5 enzyme. Interestingly, there was still considerable interliver variability in CYP3A5-specific content among the *CYP3A5*1/*3* livers, and this was highly correlated with CYP3A4 protein content (Fig. 2A). Moreover, *CYP3A4* and *CYP3A5* mRNA levels were also highly correlated (Fig. 2B). In our previous report (Kuehl et al., 2001), we overlooked this association, in part because we had fewer CYP3A5-positive livers. Also, the matrix correction of the CYP3A5 standard curve for Western blot analysis permitted a more accurate quantitation of CYP3A5 content that carefully adjusted for the dynamic range of protein expression and differential matrix effect from different absolute loads of protein.

The correlation between CYP3A4 and CYP3A5 protein and mRNA contents in persons with *CYP3A5*1/*3* genotype may indicate a common regulatory pathway for control of basal hepatic *CYP3A5* and *CYP3A4* transcription and enzyme production. Inspection of the proximal 5′-flanking region of *CYP3A4* and *CYP3A5* reveals considerable sequence homology with several common response motifs (Fig. 9), including a basic transcription element (BTE), PXR response element (ER-6), and nifedipine-specific element. However, we note that there is a 57-bp insertion in the *CYP3A4* gene compared with *CYP3A5* and that this may influence the function of any one of the 5′-flanking response elements.

The proximal ER-6 element and PXR may be involved in the coordinate regulation of CYP3A4 and CYP3A5 because of their established role in mediating CYP3A4 induction by xenobiotics (Blumberg et al., 1998; Lehmann et al., 1998). However, two separate studies with PXR knockout mice have shown that basal CYP3A11 expression is not reduced by PXR gene deletion and may actually increase (Xie et al., 2000; Staudinger et al., 2001). Thus, if the ER-6 element is responsible for coordinate human CYP3A4 and CYP3A5 expression, it may involve other, as-yet-unknown transcription factors. Interestingly, there is no compelling evidence in the literature that *CYP3A5* transcription is inducible by classical PXR ligands even though it contains the proximal ER-6 response element. Presumably, *CYP3A5* gene transcription is not inducible because it lacks the distal PXR-response element cluster (Fig. 9) shown to enhance the transcription of *CYP3A4* by xenobiotics (Goodwin et al., 1999).

TABLE 4
Midazolam hydroxylation phenotype as a function of *CYP3A5* genotype
Data are reported as median (range).

	All Genotypes	*CYP3A5*1/*1*	*CYP3A5*1/*3*	*CYP3A5*3/*3*
Liver microsomal activity	(*n* = 60)	(*n* = 1)	(*n* = 13)	(*n* = 36)
1′-Hydroxylation (pmol/min/mg)	880 (174–4842)	4443	1893 (505–4842)	660 (174–3597)**
4-Hydroxylation (pmol/min/mg)	148 (26–850)	502	294 (40–850)	124 (26–699)
1′-/4-Hydroxymidazolam ratio	6.1 (4.8–13.5)	8.9	6.4 (5.7–13.5)	5.4 (4.8–6.8)
Total activity (pmol/min/mg)	1026 (200–5692)	4844	2188 (545–5692)	787 (317–4348)*
Jejunal homogenate activity	(*n* = 31)	(*n* = 2)	(*n* = 2)	(*n* = 27)
1′-Hydroxylation (pmol/min/mg)	148 (28–348)	295 (278,311)	65.5 (41,90)	148 (28–348)
4-Hydroxylation (pmol/min/mg)	26.1 (2–60.6)	35.9 (29.3,42.5)	5.8 (4.8,6.8)	26.1 (2–60.6)
1′-/4-Hydroxymidazolam ratio	6.8 (4.0–13.1)	8.4 (7.3,9.5)	10.9 (8.6,13.1)	6.3 (4.0–7.9)
Total activity (pmol/min/mg)	173 (30–409)	330 (307,353)	71.0 (46,96)	173 (30–409)

* $p < 0.01$ comparing *CYP3A5*1* carriers and *CYP3A5*3/*3* individuals.
** $p < 0.001$ comparing *CYP3A5*1* carriers and *CYP3A5*3/*3* individuals

Iwano et al. (2001) demonstrated a role for two binding domains within the *CYP3A5* promoter (CCAAT box at −78/−68 and BTE at −67/−46) that bind *trans*-acting factors NF-Y, Sp1, and Sp3 and mediate *CYP3A5* gene transcription. The ubiquitous expression of NF-Y and Sp1/3 in human tissues is consistent with the general observation that CYP3A5 is polymorphically expressed in multiple extrahepatic tissues (Kolars et al., 1994; Haehner et al., 1996; Kivistö et al., 1996; Anttila et al., 1997; Mace et al., 1998; Yamakoshi et al., 1999). The *CYP3A4* gene also contains a proximal BTE motif that binds Sp1 (Saito et al., 2001). Thus, the Sp1/BTE interaction may contribute to basal hepatic *CYP3A4* transcription, as demonstrated for *CYP3A5*, and contribute to coordinate CYP3A enzyme expression. However, CYP3A4 expression is restricted to liver and small intestinal tissues. This suggests that Sp1 binding (or some other transcription factor) may be sufficient for *CYP3A5* transcription in liver and other tissues but that basal *CYP3A4* transcription requires additional factors found only in liver and small intestine. Alternatively, there may be factors that repress CYP3A4 expression outside of the liver and small intestine.

It has been suggested that interindividual differences in constitutive hepatic CYP3A4-dependent drug clearance is predominantly genetic in origin (Ozdemir et al., 2000). However, there is no compelling evidence that SNPs in the flanking, coding, or intronic regions of the *CYP3A4* gene contribute significantly to variable in vivo drug clearance among white persons (Eiselt et al., 2001). If transcriptional factors control constitutive *CYP3A4* and *CYP3A5* transcription and contribute to interindividual differences in enzyme activity, it is possible that polymorphisms in the relevant regulatory genes may be the common source of variable basal *CYP3A4* and *CYP3A5* transcription and protein expression. Furthermore, a single DNA-based test for the relevant regulatory allele might simultaneously predict not only CYP3A4 activity but also variation in CYP3A5 activity among those carrying the *CYP3A5*1* allele.

The data we present here suggest that translational or post-translational factors might also contribute to the variability in constitutive CYP3A expression. Properly spliced *wt-CYP3A5* mRNA and CYP3A5 protein content within each *CYP3A5* genotype group varied considerably, but the parameters were only moderately correlated with each other (Fig. 6A). Similar results were seen with hepatic *CYP3A4* mRNA and protein content (Fig. 6B). Thus, it is possible that there might be interindividual variability in the efficiency of *CYP3A* mRNA translation or protein degradation rate, as suggested previously for CYP3A5 (Jounäidi et al., 1996).

The observed gender difference in liver microsomal mida-

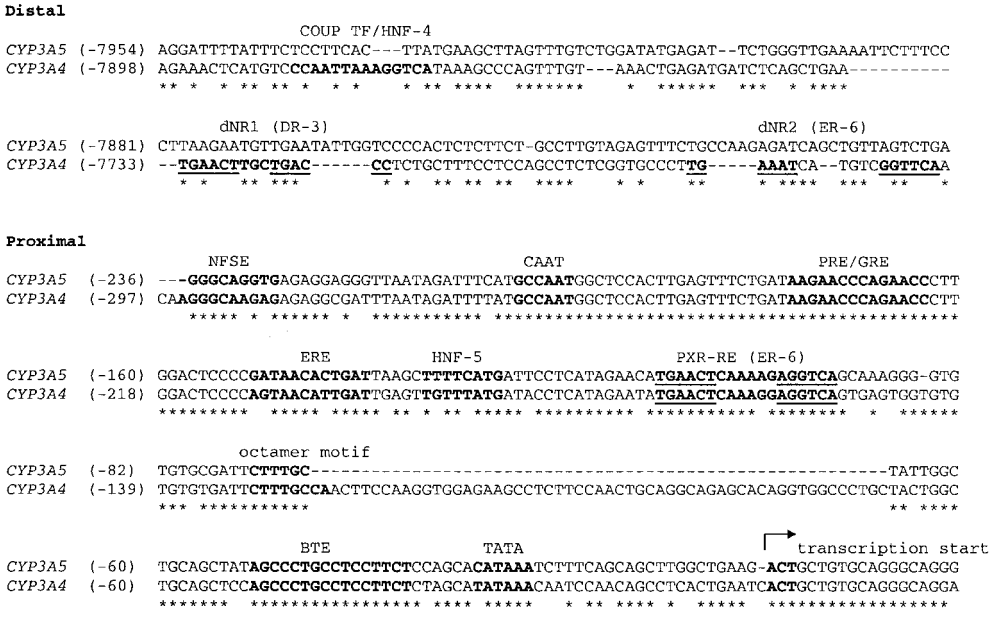

Fig. 9. Nucleotide sequence of enhancer/promoter regions for *CYP3A5* and *CYP3A4*. Sequences of distal and proximal regions of *CYP3A5* (AC005020) and *CYP3A4* (AF185589) were aligned using ClustalW (University of Washington, Seattle, WA). For *CYP3A5* and *CYP3A4*, the transcription start site occurs at nt 15,811 and nt 10,469, respectively. Transcription factor binding motifs are shown in bold, and relevant half-sites are underlined. Homology between *CYP3A4* and *CYP3A5* sequences is depicted by ⋆. NFSE, nifedipine specific element; PRE/GRE: pregnane or glucocorticoid receptor element; HNF-5, human nuclear factor-5.

zolam hydroxylation activity for *CYP3A5*3/*3* livers, although small, is consistent with reported differences in the i.v. and oral clearance of midazolam in healthy men and women (Gorski et al., 1998). We have recently confirmed this finding in a study of healthy male and female Chinese volunteers where women exhibited a higher level of CYP3A activity (unpublished data). Because there is no basis for a gender difference in *CYP3A5* or *CYP3A4* allele frequencies, the findings suggest a difference in CYP3A gene regulation. Possible candidate factors for differential regulation of *CYP3A* include sexually dimorphic circulating hormones, such as sex hormones and growth hormone, or nuclear hormone receptors, such as PXR and VDR, although a gender difference in the expression of these proteins has not been shown.

The range of hepatic CYP3A5 contents measured in this study (0–291 pmol/mg of microsomal protein) is greater than what has been reported previously (0–68 pmol/mg of protein) (Wrighton et al., 1990; Paine et al., 1997; Tateishi et al., 1999). This may simply reflect the larger study population in the present study and inclusion of a sample with a *CYP3A5*1/*1* genotype, or it may be related to the improved detection/quantitation afforded by the BD Gentest antibody.

Quantitative analysis of CYP3A5 and CYP3A4 protein demonstrated that CYP3A5 is a major contributor to the total hepatic and intestinal CYP3A pool (Figs. 1 and 3) and to total hepatic midazolam hydroxylation activity (Fig. 8B) in individual livers with at least one *CYP3A5*1* allele. For those with a *CYP3A5*1/*3* genotype, the level of CYP3A4 and CYP3A5 protein and the corresponding mRNA were comparable. Because CYP3A4 expression did not differ between different *CYP3A5* genotypes, we predict that persons who have at least one *CYP3A5*1* allele will metabolize midazolam more rapidly after oral and i.v. administration than will persons with *CYP3A5*3/*3* alleles.

Although the determination of the *CYP3A5* genotype will not equate to a specific midazolam clearance, it may indicate relative likelihood of a person having high or low CYP3A activity. As such, this information might guide initial dose selection for midazolam and other important CYP3A substrates, such as etoposide (Relling et al., 1994), lidocaine (Bargetzi et al., 1989), cyclosporine (Aoyama et al., 1989), fentanyl (Guitton et al., 1997), and nifedipine (Aoyama et al., 1989; Wrighton et al., 1990). Clearly, the most valuable application will be for narrow therapeutic index drugs. Unfortunately, only limited data are available in the literature regarding the activity of CYP3A5 toward the large number of known CYP3A4 substrates. Moreover, the published data are sometimes conflicting, as in the case of erythromycin. Wrighton et al. (1990) reported undetectable erythromycin demethylation for CYP3A5, in contrast to high activity for CYP3A4. This finding is at odds with the data of Gillam et al. (1995), who demonstrated comparable rates of erythromycin demethylation by CYP3A4 and CYP3A5. Differences in incubation conditions may explain this discrepancy, particularly the use of reconstituted purified CYP3A5 protein with optimized amounts of coenzymes and matrix constituents (Gillam et al., 1995). Unfortunately, it is not clear which system most accurately reflects the function of CYP3A5 in vivo. Further work involving pharmacogenetic studies with the drugs of interest may clarify the issue.

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