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Human Hydroxysteroid Sulfotransferase SULT2B1 Pharmacogenomics: Gene Sequence Variation and Functional Genomics[S]

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ABSTRACT

The human hydroxysteroid sulfotransferase (SULT) 2B1 gene is a member of the cytosolic SULT gene superfamily. The two SULT2B1 isoforms, SULT2B1a and SULT2B1b, are encoded by a single gene as a result of alternative transcription initiation and alternative splicing. SULT2B1b catalyzes the sulfonation of 3β-hydroxysteroid hormones and cholesterol, whereas SULT2B1a preferentially catalyzes pregnenolone sulfonation. We used a genotype-to-phenotype approach to identify and characterize common sequence variation in *SULT2B1*. Specifically, we resequenced all exons, splice junctions, and ~2.5 kb of the 5′-flanking regions (FRs) for each isoform using 60 DNA samples each from African-American and Caucasian-American subjects. We observed 100 polymorphisms, including four nonsynonymous coding single nucleotide polymorphisms and one 6-base pair deletion—all within the "shared" region of the open reading frame. Functional genomic studies of the wild type (WT) and five variant allozymes for each isoform performed with a mammalian expression system showed that variant allozyme activities ranged from 64 to 88% of WT for SULT2B1a and from 76 to 98% for SULT2B1b. Relative levels of immunoreactive protein were similar to those for enzyme activity. Luciferase reporter gene constructs for 2.5 kb of the *SULT2B1b* 5′-FR displayed a cell line-dependent pattern of variation in activity. Finally, deletion of the proline-rich SULT2B1 carboxyl terminus resulted in intracellular protein aggregate formation and accelerated degradation of the truncated protein. These studies resulted in the identification of common *SULT2B1* gene sequence variation, as well as insight into the effects of that variation on the function of this important steroid-metabolizing enzyme.

The human hydroxysteroid sulfotransferase (SULT) 2B1 isoforms are members of the human cytosolic SULT gene superfamily (Her et al., 1998; Freimuth et al., 2004). The two SULT2B1 isoforms, SULT2B1a and SULT2B1b, are encoded by a single gene but differ at their N termini as a result of alternative transcription initiation and alternative splicing (Her et al., 1998). SULT gene family members catalyze the sulfonation of both exogenous and endogenous compounds—including drugs, hormones, and neurotransmitters (Falany, 1997a; Freimuth et al., 2004; Nimmagadda et al., 2006). Although pharmacogenomic studies have been performed for many members of this gene family (Raftogianis et al., 1997, 1999; Thomae et al., 2002, 2003; Adjei et al., 2003; Hildebrandt et al., 2004, 2007), no systematic studies of *SULT2B1* pharmacogenomics have been performed. SULT2B1b is the most widely expressed of the two SULT2B1 isoforms and preferentially catalyzes the sulfonation of cholesterol and 3β-hydroxysteroid hormones (Geese and Raftogianis, 2001; Lee et al., 2003). It is expressed in the prostate and placenta, as well as tissues such as skin and platelets (Her et al., 1998; Geese and Raftogianis, 2001; Javitt et al., 2001; Higashi et al., 2004; Yanai et al., 2004). In contrast, SULT2B1a cata-

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ABBREVIATIONS: SULT, sulfotransferase; AA, African-American; CA, Caucasian-American; FR, flanking region; ORF, open reading frame; SNP, single nucleotide polymorphism; WT, wild type; DHEA, dehydroepiandrosterone; UTR, untranslated region; RRL, rabbit reticulocyte lysate; MAF, minor allele frequency; VNTR, variable number of tandem repeats; TV, truncated variant; kb, kilobase; bp, base pair; GFP, green fluorescent protein; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

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lyzes pregnenolone sulfonation and is expressed in fewer tissues than is SULT2B1b (Her et al., 1998; Geese and Raftogianis, 2001; Fuda et al., 2002). Both SULT2B1 isoforms can catalyze dehydroepiandrosterone (DHEA) sulfonation (Her et al., 1998; Geese and Raftogianis, 2001). In skin, cholesterol sulfate plays a role in barrier formation and in keratinocyte differentiation (Epstein et al., 1984). Abnormally high levels of cholesterol sulfate are present in the skin of patients with X-linked ichthyosis (Hazan et al., 2005). Although SULT2B1a is expressed in fewer tissues than SULT2B1b, its sulfonated product, pregnenolone sulfate, is a neurosteroid that is capable of modulating the function of other neurotransmitters (Lee et al., 2003).

The human *SULT2B1* gene was cloned in 1998 and maps to chromosome 19q13.3, telomeric to the other known human hydroxysteroid SULT, *SULT2A1* (Her et al., 1998). The proteins encoded by *SULT2B1* share 48% amino acid sequence identity with that encoded by *SULT2A1* (Her et al., 1998). SULT2B1b includes a unique 23-amino acid segment at its N terminus that is critical for cholesterol sulfonation (Fuda et al., 2002); however, deletion of the unique 8-amino acid N terminus for SULT2B1a has no effect on pregnenolone sulfonation (Fuda et al., 2002). Finally, unlike all other members of the cytosolic SULT gene family, both of the SULT2B1 isoforms have a 53-amino acid proline-rich C terminus (Her et al., 1998). Loss of this proline-rich "tail" does not influence the catalytic activity of either isoform (Fuda et al., 2002), suggesting that this structural feature might have other functional roles.

In the present studies, we used a genotype-to-phenotype research strategy (Weinshilboum and Wang, 2004) to identify common sequence variation in the *SULT2B1* gene, followed by characterization of the functional consequences of that variation. Specifically, we resequenced all exons, exon-intron splice junctions, ~2.5 kb of the 5′-flanking regions (5′-FRs) for each isoform, and a portion of the 3′-untranslated region (UTR) using 60 DNA samples (120 alleles), each from African-American (AA) and Caucasian-American (CA) subjects. One hundred polymorphisms were observed, five of which altered the encoded amino acid sequence. Functional genomic studies were then performed with recombinant allozymes for both isoforms and for common 5′-FR haplotypes for *SULT2B1b*, the most widely expressed of the two isoforms (Her et al., 1998; Geese and Raftogianis, 2001; Javitt et al., 2001; Fuda et al., 2002). Subcellular localization studies of SULT2B1b protein and preliminary studies of the functional consequences of loss of the proline-rich carboxyl terminus were also performed. These observations provide a foundation for future studies of the possible relationship of common variation in *SULT2B1* sequence with individual variation in disease risk or drug response.

Materials and Methods

DNA Samples. DNA from 60 AA and 60 CA subjects was obtained from the Coriell Cell Repository (Camden, NJ). These samples (sample sets HD100AA and HD100CAU) were deposited by the NIGMS, National Institutes of Health. The samples had been anonymized before deposit, and all subjects had provided written informed consent for the use of their DNA for research purposes. These studies were reviewed and approved by the Mayo Clinic Institutional Review Board.

SULT2B1 **Gene Resequencing.** The human *SULT2B1* consensus sequence used in our studies was that of *Homo sapiens* chromosome 19 genomic contig NT_011109.15. Because the *SULT2B1* gene can be transcribed to form two mRNAs, designations for nucleotide locations were based on the distance to the nearest ATG translation initiation codon. Specifically, numbering for nucleotides within the *SULT2B1b* 5′-FR and exon 1B began at the "A" in the ATG for the SULT2B1b cDNA, with nucleotides 5′ to that position assigned negative numbers and those located 3′ to that position assigned positive numbers. Numbering for nucleotides within the *SULT2B1a* 5′-FR and all other exons, including exon 1A, began at the A in the ATG for the SULT2B1a cDNA. Nucleotides located within introns were numbered based on their distance from the closest splice site, using positive and negative numbers for distances from 5′- and 3′-splice sites, respectively. Because the amino acid sequences of the two SULT2B1 isoforms differ at their N termini, the numbering scheme for amino acid sequences within the shared region of the isoforms was based on the sequence of SULT2B1a, whereas sequences within the isoform-specific regions were numbered based on their distance from the methionine encoded by the transcription initiation codon within that isoform. Nearly 7 kb of *SULT2B1* sequence, including all seven exons, intron-exon splice junctions, and ~2.5 kb of 5′-FR upstream of both exons 1A and 1B, were resequenced for each of the 120 DNA samples studied. Primers used in gene resequencing are listed in the supplemental table. All amplicons were sequenced on both strands in the Mayo Clinic Molecular Biology Core Facility using ABI 3730xl DNA sequencers (Applied Biosystems, Foster City, CA) and Dye Terminator sequencing chemistry. To exclude polymerase chain reaction-induced artifacts, independent amplifications were performed for SNPs that were observed only once or for any amplicon that had an ambiguous chromatogram.

SULT2B1 Transient Expression. The SULT2B1a and SULT2B1b WT cDNA sequences were cloned into the eukaryotic expression vector pCR3.1 (Invitrogen, Carlsbad, CA). Variant constructs for nonsynonymous coding SNPs identified during *SULT2B1* resequencing were created by site-directed mutagenesis using circular polymerase chain reaction (see supplemental table). The sequences of all constructs were confirmed by sequencing the inserts in both directions. These constructs were then transiently expressed in COS-1 cells, with pSV-β-galactosidase (Promega, Madison, WI) cotransfection to make it possible to correct for variation in transfection efficiency. Cells were harvested 48 h after transfection, and cytosol preparations containing recombinant SULT2B1 proteins were stored at −80°C for use in functional genomic experiments.

SULT2B1 Allozyme Activity Assays. Recombinant SULT2B1 allozyme activity was assayed using a modification of a radiochemical assay described elsewhere (Thomae et al., 2002). Specifically, the 160 μl of the final reaction mixture contained 0.3 mM $MgCl_2$, 50 μM DHEA, and 100 μl of COS-1 cell cytosol diluted in 5 mM potassium phosphate buffer, pH 6.5, that contained 1.5 mg/ml BSA and 1.54 mg/ml dithiothreitol, plus 50 μl of a "cocktail" that contained 25 μl of 50 mM potassium phosphate buffer, pH 5.5, 25 μl of 7.4 mg/ml dithiothreitol and 0.4 μM [^{35}S]adenosine 3′-phosphate-5′-phosphosulfate. After incubation at 37°C for 20 min, the reaction was stopped by adding 100 μl of a 1:1 mixture of 0.1 M barium acetate and 0.1 M $Ba(OH)_2$, and the reaction tubes were vortexed. Fifty microliters of 0.1 M $Ba(OH)_2$ and 50 μl of 0.1 M $ZnSO_4$ were added, followed by 400 μl of distilled water. The samples were vortexed once again and centrifuged for 10 min. The supernatant (0.5 ml) was aspirated and added to 5 ml of Bio-safe II liquid scintillation counting fluid (Research Products, Mt. Prospect, IL) before measurement of radioactivity in a liquid scintillation counter.

SULT2B1 Allozyme Western Blot Analyses. A rabbit polyclonal antibody directed against residues 335 to 350 of SULT2B1a (residues 350–365 of 2B1b) that was described previously (Her et al., 1998) was used to perform most of the Western blot analyses. All amino acid substitutions present in variant allozymes occurred outside of this region, with the exception of a two-amino acid deletion

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variant. To avoid possible artifacts, Western blot analysis for variant allozymes that contained the two-amino acid deletion was repeated using two antibodies directed against the N terminus of both SULT2B1a (residues 1–8) and SULT2B1b (residues 1–18). COS-1 cytosol preparations were subjected to SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels, followed by Western blot analysis performed as described previously (Ji et al., 2005). The gels were loaded based on the cotransfected β-galactosidase activity to correct for possible variation in transfection efficiency. Variant allozyme protein levels were expressed as a percentage of the density of the WT protein band on the same gel.

***SULT2B1b* 5′-FR Haplotype Reporter Gene Studies.** Previous studies have demonstrated that SULT2B1b is the most widely expressed of the two SULT2B1 isoforms (Her et al., 1998; Geese and Raftogianis, 2001). Therefore, SULT2B1b was selected for functional studies of the effects of 5′-FR haplotypes on transcription. Luciferase reporter gene constructs were created for all common *SULT2B1b* 5′-FR haplotypes (frequencies ≥5% in either population or haplotypes that were present in both of the ethnic groups studied). 5′-FR sequences containing the desired haplotypes were amplified from the same genomic DNA samples that had been used to perform the gene-resequencing studies. Forward and reverse primers had *ACC65I* and XhoI restriction sites at their 5′ ends (see supplemental table for similar sequences), respectively, to facilitate cloning of the amplicons into pGL3-Basic (Promega) upstream of the firefly luciferase gene ORF. Each of the inserts was sequenced in both directions to ensure that the correct sequence was present in the construct.

To test the possible influence of upstream sequence variation on transcription, two sets of reporter gene constructs were created and designated pGL3-L (long), constructs that contained 2.5 kb of *SULT2B1b* 5′-FR sequence, and pGL3-S (short), constructs that contained 0.7 kb of 5′-FR sequence. These two sets of constructs were then transiently transfected into cells that were also cotransfected with a pRL-thymidine kinase construct that encoded *Renilla* luciferase. The cells were harvested after 48 h, followed by dual-luciferase assay (Promega). Results were reported as the ratio of firefly luciferase light units to *Renilla* luciferase light units, and values were also expressed as a percentage of the activity of the appropriate pGL3-L WT construct. All assays were performed in triplicate; i.e., three independent transfections were performed, and each experiment was repeated twice for a total of six independent determinations.

SULT2B1b Subcellular Localization Studies. MCF-7 cells, cells that express endogenous SULT2B1b protein, were cultured on coverslips. The cells were fixed with 3% paraformaldehyde and were permeabilized with 0.5% Triton X-100. The coverslips were then incubated with rabbit polyclonal anti-human SULT2B1b antibody, followed by incubation with fluorescein isothiocyanate-conjugated goat anti- rabbit IgG antibody (Southern Biotechnology Associates, Inc. Birmingham, AL). To study the subcellular localization of recombinant SULT2B1b protein, a green fluorescent protein (GFP)-SULT2B1b fusion construct (GFP-WT) was created by cloning the WT SULT2B1b cDNA into the pEGFPC2 vector (Clontech, Palo Alto, CA), downstream of the GFP ORF. A truncated GFP-labeled variant lacking the 53-amino acid proline-rich “tail” at the C terminus was also created (GFP-TV). COS-1 cells were transfected with GFP-tagged expression constructs, and in some cases, the cells were also treated with 20 μM MG132 for 20 h before fixation with 3% formaldehyde. 4′,6-Diamidine-2-phenylindole was used to stain the nuclei before the slides were viewed with a Zeiss LSM510 Confocal Laser-Scanning Microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) using 364- and 488-nm filters for the excitation of blue and green fluorochromes, respectively.

SULT2B1 in Vitro Translation and Degradation. Expression constructs for truncated SULT2B1a and SULT2B1b variants that lacked the SULT2B1 proline-rich tail were created by using site-directed mutagenesis to introduce a translation termination codon immediately before the final 53 amino acids at the C terminus of the protein. Both WT and “truncated” proteins were then synthesized in vitro using the TNT-coupled rabbit reticulocyte lysate (RRL) system (Promega), followed by protein degradation studies as described elsewhere (Wang et al., 2003; Ji et al., 2005).

Data Analysis. DNA sequence data from the gene-resequencing studies were analyzed using Mutation Surveyor (SoftGenetics, LLC, State College, PA). Average levels of recombinant allozyme activity, immunoreactive protein, and reporter gene data were compared by analysis of variance using Prism (GraphPad, San Diego, CA), followed by a post hoc test (Tukey’s multiple comparison test) if a significant *F*-score was observed. Linkage disequilibrium among *SULT2B1* polymorphisms was determined by calculating D' values, a method that is independent of allele frequency (Hartl and Clark, 2000; Hendrick, 2000). Haplotypes were inferred computationally as described by Schaid et al. (2002). Values for π, θ, and Tajima’s D were determined as described by Tajima (1989a,b). Graphical presentations of population-specific D' values across the *SULT2B1* gene were generated using Haploview and Locusview 2.0.

Results

Human *SULT2B1* Gene Resequencing. Human *SULT2B1* was resequenced using DNA samples from 60 AA and 60 CA subjects. Approximately 7 kb of DNA was sequenced for each sample. A total of 100 sequence variants were observed, including four nonsynonymous coding single nucleotide polymorphisms and one 6-bp deletion—all located within the “shared” ORF region of the two isoforms (Fig. 1; Table 1). These polymorphisms resulted in the following alterations in encoded amino acids, with locations of sequence alterations based on the SULT2B1a sequence: L36S, D176N,

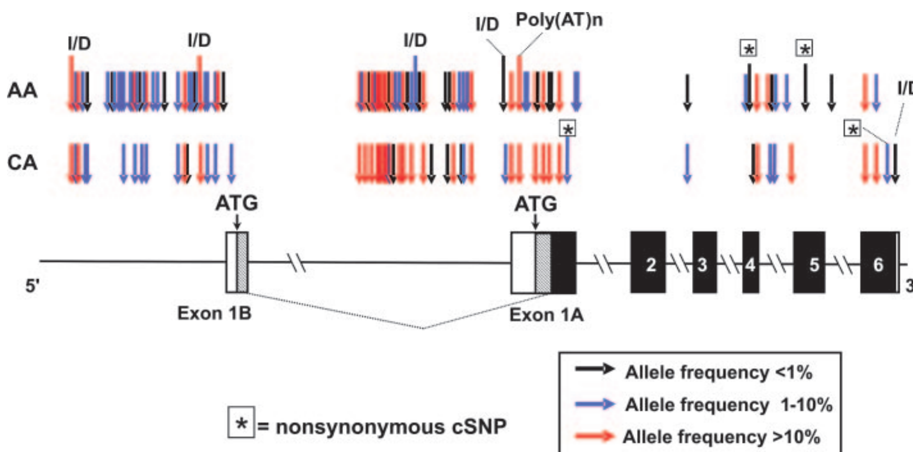

Fig. 1. Human *SULT2B1* genetic polymorphisms. Arrows indicate the locations of polymorphisms, with different colors indicating minor allele frequencies. Black and cross-hatched rectangles represent portions of exons encoding the open-reading frame. Open rectangles represent portions of exons encoding 5′- and 3′-UTRs. I/D is insertion/deletion. *, nonsynonymous coding single nucleotide polymorphisms.

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TABLE 1

Human *SULT2B1* genetic polymorphisms

Polymorphism locations, alterations in nucleotide and amino acid sequences, and MAFs with 95% confidence intervals (CIs) for the two ethnic groups studied are listed. Polymorphisms within exons are boxed. Numbering for nucleotides within the *SULT2B1b* 5′-FR and exon1B begins at the A in ATG for the SULT2B1b cDNA, with negative nucleotides located 5′ and positive nucleotides located 3′ to that position. Numbering for nucleotides within the *SULT2B1a* 5′-FR and all the other exons, including exon 1A, begin at the A in the ATG for the SULT2B1a cDNA. Nucleotides located within introns (IVS) are numbered based on their distance to the nearest 5′- or 3′-splice site, using positive and negative numbers, respectively. Novel polymorphisms are labeled with an * in the first column. Polymorphisms with *rs* numbers were discovered in the course of the present study.

Human *SULT2B1* Polymorphisms

	#	Gene location	Nucleotide location	Nucleotide change	Amino Acid change	MAF				dbSNP designation
						AA	95%CI	CA	95%CI	
*	1	1b 5'-FR	(-2307)	Δ CCCTT		0.375	(0.288,0.468)	0.100	(0.053,0.168)	
*	2	1b 5'-FR	(-2262)	G > A		0.042	(0.014,0.095)	0.067	(0.029,0.127)	
*	3	1b 5'-FR	(-2204)	A > C		0.483	(0.391,0.576)	0.167	(0.105,0.246)	
*	4	1b 5'-FR	(-2144)	G > T		0.033	(0.009,0.083)	---	---	
*	5	1b 5'-FR	(-2088)	C > T		---	---	0.025	(0.005,0.071)	
	6	1b 5'-FR	(-2010)	C > T		0.008	(0,0.046)	0.067	(0.029,0.127)	rs279450
*	7	1b 5'-FR	(-1732)	C > T		0.025	(0.005,0.071)	---	---	
*	8	1b 5'-FR	(-1669)	T > C		0.008	(0,0.046)	---	---	
*	9	1b 5'-FR	(-1620)	A > C		0.083	(0.041,0.148)	---	---	
*	10	1b 5'-FR	(-1566)	A > G		0.017	(0.002,0.059)	---	---	
*	11	1b 5'-FR	(-1530)	C > T		0.083	(0.041,0.148)	---	---	
*	12	1b 5'-FR	(-1529)	A > G		0.033	(0.009,0.083)	0.058	(0.024,0.116)	
	13	1b 5'-FR	(-1431)	G > A		0.058	(0.024,0.116)	---	---	rs11878685
*	14	1b 5'-FR	(-1422)	G > T		0.017	(0.002,0.059)	---	---	
*	15	1b 5'-FR	(-1417)	G > A		0.133	(0.078,0.207)	0.025	(0.005,0.071)	
*	16	1b 5'-FR	(-1399)	A > G		0.083	(0.041,0.148)	---	---	
*	17	1b 5'-FR	(-1389)	G > A		0.008	(0,0.046)	---	---	
	18	1b 5'-FR	(-1359)	T > A		---	---	0.017	(0.002,0.059)	rs4802488
*	19	1b 5'-FR	(-1339)	T > C		0.083	(0.041,0.148)	---	---	
*	20	1b 5'-FR	(-1309)	T > C		0.192	(0.126,0.274)	0.075	(0.035,0.138)	
*	21	1b 5'-FR	(-1125)	G > A		0.058	(0.024,0.116)	---	---	
*	22	1b 5'-FR	(-1030)	A > C		0.025	0.005,0.071)	---	---	
*	23	1b 5'-FR	(-976)	G > A		0.008	(0,0.046)	---	---	
*	24	1b 5'-FR	(-710)	G > A		0.067	(0.029,0.127)	0.025	(0.005,0.071)	
*	25	1b 5'-FR	(-653)	A > G		0.250	(0.175,0.337)	0.150	(0.091,0.227)	
*	26	1b 5'-FR	(-636)	C > G		---	---	0.008	(0,0.046)	
*	27	1b 5'-FR	(-629)	C > T		0.017	(0.002,0.059)	---	---	
*	28	1b 5'-FR	(-618)	G > A		0.067	(0.029,0.127)	---	---	
*	29	1b 5'-FR	(-598)	G > A		0.008	(0,0.046)	---	---	
*	30	1b 5'-FR	(-550)	C > T		0.017	(0.002,0.059)	---	---	
*	31	1b 5'-FR	(-423)	Δ TGAA		0.333	(0.250,0.425)	0.125	(0.072,0.198)	
*	32	1b 5'-FR	(-385)	C > T		0.067	(0.029,0.127)	---	---	
*	33	1b 5'-FR	(-375)	G > A		0.083	(0.041,0.148)	0.025	(0.005,0.071)	
*	34	1b 5'-FR	(-231)	A > C		0.033	(0.009,0.083)	---	---	
	35	1b 5'-FR	(-194)	C > G		0.167	(0.105,0.246)	0.092	(0.047,0.158)	rs12609189
*	36	1b 5'-FR	(-97)	G > A		0.008	(0,0.046)	---	---	
*	37	1b 5'-UTR	(-21)	C > T		---	---	0.017	(0.002,0.059)	*rs16982137*
*	38	Ex 1b	151	C > T		---	---	0.008	(0,0.046)	*rs16982141*
*	39	In 1b	(+100)	G > A		0.067	(0.029,0.127)	---	---	
	40	1a 5'-FR	(-2570)	G > A		0.308	(0.227,0.399)	0.575	(0.481,0.665)	rs3760800
*	41	1a 5'-FR	(-2541)	G > A		0.042	(0.014,0.095)	---	---	
	42	1a 5'-FR	(-2485)	G > A		0.358	(0.273,0.451)	0.575	(0.481,0.665)	rs3760802
*	43	1a 5'-FR	(-2417)	A > G		0.008	(0,0.046)	---	---	
	44	1a 5'-FR	(-2387)	C > T		0.308	(0.227,0.399)	0.567	(0.473,0.657)	rs3760803
	45	1a 5'-FR	(-2350)	G > A		0.317	(0.235,0.408)	---	---	rs8109679
	46	1a 5'-FR	(-2342)	T > C		0.025	(0.005,0.071)	---	---	rs8109498
	47	1a 5'-FR	(-2249)	C > G		0.308	(0.227,0.339)	0.575	(0.481,0.665)	rs3826827
	48	1a 5'-FR	(-2243)	T > G		0.308	(0.227,0.339)	0.575	(0.481,0.665)	rs4802490
	49	1a 5'-FR	(-2146)	G > C		0.308	(0.227,0.339)	0.575	(0.481,0.665)	rs4802491
	50	1a 5'-FR	(-2115)	A > G		0.533	(0.440,0.625)	0.167	(0.105,0.246)	rs3760804
	51	1a 5'-FR	(-2108)	T > C		0.308	(0.227,0.399)	0.583	(0.490,0.673)	rs7257121
*	52	1a 5'-FR	(-2067)	C > T		0.008	(0,0.046)	0.017	(0.002,0.059)	
	53	1a 5'-FR	(-2050)	C > G		0.467	(0.375,0.560)	0.842	(0.764,0.902)	rs3760805

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TABLE 1—*continued*

*	54	1a5'-FR	(-2001)	T > G		—	—	0.008	(0,0.046)	
*	55	1a5'-FR	(-1994)	G > A		0.042	(0.014,0.095)	—	—	
*	56	1a5'-FR	(-1941)	C > T		0.067	(0.029,0.127)	0.150	(0.091,0.227)	
*	57	1a5'-FR	(-1896)	G > C		0.233	(0.161,0.319)	0.267	(0.190,0.355)	
*	58	1a5'-FR	(-1776)	G > A		0.008	(0,0.046)		—	
	59	1a5'-FR	(-1720)	G > A		0.083	(0.041,0.148)	0.158	(0.098,0.236)	rs2665579
*	60	1a5'-FR	(-1650)	Δ TTCC		0.050	(0.019,0.106)	—	—	
*	61	1a5'-FR	(-1647)	A > C		0.033	(0.009,0.083)	—	—	
*	62	1a5'-FR	(-1608)	C > G		0.008	(0,0.046)	—	—	
	63	1a5'-FR	(-1505)	C > T		0.233	(0.161,0.319)	0.267	(0.190,0.355)	rs3760808
*	64	1a5'-FR	(-1314)	C > T		—	—	0.008	(0,0.046)	
*	65	1a5'-FR	(-1162)	T > C		0.008	(0,0.046)	—	—	
*	66	1a5'-FR	(-1086)	C > T		—	—	0.008	(0,0.046)	
*	67	1a5'-FR	(-1036)	A > T		0.008	(0,0.046)	—	—	
	68	1a5'-FR	(-1031)	G > A		0.325	(0.242,0.417)	0.267	(0.190,0.355)	rs2665580
*	69	1a5'-FR	(-965)	T > C		0.067	(0.029,0.127)	—	—	
*	70	1a5'-FR	(-946)	C > T		—	—	0.008	(0,0.046)	
*	71	1a5'-FR	(-923)	CTC > (-)		—	—	0.017	(0.002,0.059)	
*	72	1a5'-FR	(-861)	A > G		0.075	(0.035,0.138)	—	—	
	73	1a5'-FR	(-745)	G > A		0.325	(0.242,0.417)	0.425	(0.335,0.519)	rs4801766
*	74	1a5'-FR	(-451)	(AT)n(n=12>14,13)		0.317(14)	(0.235,0.408)	0.025(14)	(0.005,0.071)	
						0.058(13)	(0.024,0.116)	0.083(13)	(0.041,0.148)	
*	75	1a5'-FR	(-402)	T > C		0.075	(0.035,0.138)	—	—	
*	76	1a5'-FR	(-399)	C > T		0.075	(0.035,0.138)	—	—	
	77	1a5'-FR	(-333)	G > A		0.267	(0.190,0.355)	0.283	(0.205,0.373)	rs7249118
*	78	1a5'-FR	(-318)	C > T		0.008	(0,0.046)	0.000	(0,0.030)	
*	79	1a5'-FR	(-275)	C > T		0.192	(0.126,0.274)	0.125	(0.072,0.198)	
*	80	1a5'-FR	(-183)	C > T		0.008	(0,0.046)	0.100	(0.053,0.168)	
*	81	1a5'-UTR	(-10)	A > C		0.008	(0,0.046)	—	—	
	82	Ex 1A	75	C > T	Pro25	0.158	(0.098,0.236)	0.100	(0.053,0.168)	rs2544794
*	83	Ex 1A	107	T > C	Leu36Ser	—	—	0.017	(0.002,0.059)	*rs16982149*
*	84	In 1a	(+23)	C > T		0.017	(0.002,0.059)	—	—	*rs16982150*
*	85	In 1a	(+24)	G > A		0.017	(0.002,0.059)	—	—	*rs16982151*
	86	In 2	(-10)	C > T		0.008	(0,0.046)	0.042	(0.014,0.095)	rs3745726
*	87	Ex 4	525	C > T	Phe175	0.025	(0.005,0.071)	—	—	*rs16982154*
*	88	Ex 4	526	G > A	Asp176Asn	0.008	(0,0.046)	—	—	*rs16982158*
*	89	Ex 4	555	G > A	Lys185	—	—	0.008	(0,0.046)	*rs16982159*
	90	Ex 4	592	C > T	Leu198	0.258	(0.183,0.346)	0.350	(0.265,0.442)	rs2302948
*	91	In 4	(+88)	C > A		0.100	(0.053,0.168)	0.083	(0.041,0.148)	*rs16982163*
	92	In 4	(+94)	G > A		0.008	(0,0.046)	0.042	(0.014,0.095)	rs4802495
*	93	In 4	(+172)	A > G		0.017	(0.002,0.059)	—	—	
	94	In 4	(-41)	C > T		0.050	(0.019,0.106)	0.200	(0.133,0.283)	rs3815691
*	95	Ex 5	644	G > A	Arg215His	0.008	(0,0.046)	—	—	*rs16982169*
*	96	In 5	(+3)	G > A		0.008	(0,0.046)	—	—	*rs16982170*
	97	Ex 6	789	C > T	Cys263	0.225	(0.154,0.310)	0.417	(0.327,0.510)	rs1132054
	98	Ex 6	903	C > T	Asp301	0.058	(0.024,0.116)	0.192	(0.126,0.274)	rs1052131
*	99	Ex 6	989	C > T	Pro330Leu	—	—	0.025	(0.005,0.071)	*rs17842463*
*	100	Ex 6	1009	Δ AGCCCC	Ser337Pro338/--	—	—	0.008	(0,0.046)	*rs16989366*

R215H, P330L, and deletion of Ser337/Pro338. All five of these alterations in nucleotide sequence had relatively low minor allele frequencies (MAFs) (0.8–2.5%). It should be emphasized that, since we resequenced only 120 alleles for each ethnic group, we were not able to reliably differentiate polymorphisms with low minor allele frequencies from rare variants. Therefore, the data in Table 1 include 95% confidence interval estimates for the MAF values. A 5-bp CCCTT insertion and a 4-bp TGAA deletion, both of which were quite common in the two populations studied (MAF >10%), were present within the *SULT2B1b* 5′-FR. The TGAA sequence was part of a variable number of tandem repeats (VNTR), with six TGAA repeats as the major allele and five as the minor allele. Within the *SULT2B1a* 5′-FR, two relatively infrequent deletion polymorphisms, as well as a common VNTR composed of AT repeats, were observed. Three alleles were observed for this AT VNTR, with $n = 12$ as the major allele and $n = 13$ and 14 as minor alleles (Table 1). Eighty-six of the 100 polymorphisms observed were present in DNA from AA subjects, and 55 were present in DNA from CA subjects (Fig. 1). Sixty of these polymorphisms were novel, with 40 present in the public dbSNP database (www.ncbi.nlm.nih.gov/SNP) (Table 1).

We also calculated measures of nucleotide diversity (Tajima, 1989b). π and θ are two common measures of nucleotide diversity. π is the average heterozygosity per site, and θ is a population mutation measure that is theoretically equal to the neutral mutation parameter. Values for π and θ in CA samples were slightly lower than those for samples obtained from AA subjects (Table 2). Tajima's D, a test of the neutral hypothesis, was also estimated for both populations (Tajima, 1989b). Neither of the values for Tajima's D differed signifi-

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TABLE 2
Estimates for π, θ, and Tajima's D values for *SULT2B1* in two ethnic groups
Values are parameter estimate means ± S.E. P values refer to Tajima's D.

Population	Values for π, θ, and Tajima's D			
	π	θ	Tajima's D	P Values
CA	11.7 ± 5.9	10.1 ± 2.7	0.49	0.64
AA	14.9 ± 7.4	16.9 ± 4.0	−0.19	0.86

cantly from those predicted by the neutral hypothesis (Table 2).

***SULT2B1* Linkage Disequilibrium and Haplotype Analysis.** Linkage disequilibrium and haplotype analyses were also performed. Haplotypes are proving increasingly useful for association studies (Furihata et al., 2006). Linkage disequilibrium was estimated by calculating D' values for all possible pairwise combinations of polymorphisms. D' values are equal to 1.0 when two polymorphisms are maximally associated, and they are zero when the polymorphisms are randomly associated (Hendrick, 2000). Graphical representation of population-specific D' values across the *SULT2B1* gene for AA and CA DNA is shown in Fig. 2. The figure shows that there may be at least two "haplotype blocks" for AA DNA within the 54 kb of DNA included in the area resequenced, but only one haplotype block was clearly defined for the CA population. Obviously, because we only resequenced a portion of *SULT2B1*, we are not able to clearly define the total number of haplotype blocks that might be present in these two ethnic groups. However, the current version of the HapMap (January, 2007), although containing many fewer SNPs than our resequencing effort, shows a similar haplotype block structure for this gene. Haplotype analysis resulted in the identification of three unequivocal and 24 computationally inferred *SULT2B1* haplotypes that were present at frequencies of 1% or greater (Table 3) (Excoffier and Slatkin, 1995; Long et al., 1995; Schaid et al., 2002). Because our in depth resequencing resulted in the identification of 100 sequence variants, 60 of which were novel, a very large number of haplotypes with frequencies below our 1% threshold were inferred—72 in samples from AA subjects and 55 in samples from CA subjects. It was for that reason that nearly 60% of the haplotypes for AA samples and 40% for CA samples fell below the 1% threshold for inclusion in Table 3. Although 1% was the threshold used to select the haplotypes listed in Table 3, the table also lists any haplotype that included polymorphisms that altered the encoded amino acid sequence. Haplotype designations were based on encoded amino acid sequences, with **1* being the WT or the most common encoded sequence. The designations (**2*, **3*, etc.) were then assigned based on the locations of variant amino acids, starting at the amino terminus of the protein. Letter designations (**1A*, **1B*, **1C*, etc.) for alleles that encoded identical amino acid sequences were assigned based on descending allele frequencies, starting with the most frequent allele in the AA population sample.

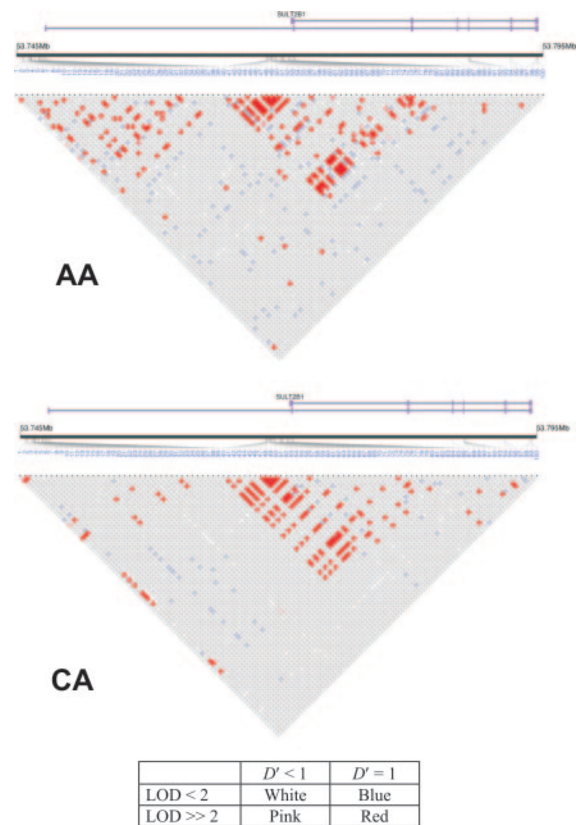

Fig. 2. Human *SULT2B1* linkage disequilibrium in DNA from AA and CA subjects. D' values were calculated for each polymorphism pair. All values shown in color were statistically significant ($P < 0.05$). Numbers identifying individual polymorphisms are those listed in Table 1.

SULT2B1 Allozyme Functional Genomics. The functional consequences of polymorphisms that altered the SULT2B1 amino acid sequence were studied using recombinant proteins. A total of 12 expression constructs were created, six for each isoform. These constructs were then transiently expressed in COS-1 cells, together with cotransfected pSV-β-galactosidase as a control for transfection efficiency. COS-1 cells were used to perform these studies to ensure the presence of mammalian mechanisms for post-translational modification and protein degradation. The recombinant allozyme proteins were then used to assay SULT2B1 activity and to perform quantitative Western blot analyses. Levels of SULT2B1 allozyme enzyme activity and immunoreactive protein, expressed as a percentage of WT after correction for transfection efficiency, are shown in Fig. 3. Although some of the recombinant allozymes differed significantly from the WT, none had less than 64% WT activity. For example, with DHEA as substrate, SULT2B1a allozymes had 64 to 88% of the activity of the WT allozyme, whereas SULT2B1b allozymes had 76 to 98% of the activity of WT. The range of SULT2B1a-immunoreactive allozyme protein levels was 82 to 109%, and that for SULT2B1b allozymes varied from 79 to 112% WT. These values were determined by quantitative Western blot analysis. Only the two-amino acid deletion variant was located in an area included in the epitope used to generate the antibody to perform these studies. Therefore, that variant was also studied with two separate N-terminal antibodies—one for SULT2B1a and another for SULT2B1b. Levels of allozyme activity were significantly correlated with levels of immunoreactive protein for SULT2B1b allozymes, but not for SULT2B1a allozymes (Fig. 3, C and D). However, these relatively small differences among allozymes in levels of activity and immunoreactive protein may not be of major functional significance in vivo.

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TABLE 3

Human *SULT2B1* haplotypes with frequencies of 1% or greater

If a haplotype encoded a variant amino acid sequence, it was included in the table, even if its frequency was less than 1%. Nucleotide locations are designated as described in the legend for Table 1. Variant nucleotides compared with the "reference sequence" (i.e., the most common allele in AA subjects) are highlighted by shading. "Unequivocal" haplotypes are indicated by black against grey in the column listing allele designations. All other haplotypes were inferred. I, insertion; D, deletion.

Allele	Frequency		Nucleotide Location																					
			1b 5'-FR																					Intron 1b
Designations	AA	CA	(-2307)	(-2262)	(-2204)	(-2144)	(-1732)	(-1621)	(-1530)	(-1529)	(-1422)	(-1417)	(-1399)	(-1389)	(-1339)	(-1309)	(-1030)	(-653)	(-618)	(-598)	(-423)	(-375)	(-194)	i1b(100)
*1A	0.117	---	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1B	0.058	0.064	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1C	0.050	---	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	D	G	G	G
*1D	0.050	---	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1E	0.042	---	I	G	C	G	C	C	T	A	G	G	G	G	C	C	A	G	A	G	D	G	C	A
*1F	0.025	---	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1G	0.025	---	I	G	C	G	C	A	C	A	G	G	A	G	T	C	A	G	G	G	I	G	C	G
*1H	0.017	---	I	G	C	G	C	A	C	A	G	A	A	G	T	T	A	A	G	G	I	G	C	G
*1I	0.017	---	I	G	C	G	C	A	C	A	G	G	A	G	T	T	C	G	G	G	I	G	C	G
*1J	---	0.284	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1K	---	0.061	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1L	---	0.043	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1M	---	0.025	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1N	---	0.017	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	D	G	G	G
*1O	---	0.017	I	A	C	G	C	A	C	A	G	G	A	G	T	T	A	G	G	G	I	G	C	G
*1P	---	0.015	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1Q	---	0.011	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1R	---	0.011	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*1S	---	0.008	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*2A	---	0.008	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*2B	---	0.008	D	G	A	G	C	A	C	G	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*3	0.008	---	I	G	C	T	T	A	C	A	G	G	A	A	T	T	A	G	G	A	D	A	C	G
*4	0.008	---	I	A	C	G	C	A	C	G	T	G	A	G	T	T	A	G	G	G	I	G	C	G
*5A	---	0.008	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*5B	---	0.008	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*5C	---	0.008	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G
*6	---	0.008	D	G	A	G	C	A	C	A	G	G	A	G	T	T	A	A	G	G	I	G	C	G

Allele	Frequency		Nucleotide Location																															
			1a 5'-FR																					Exon 1a		Intron 2	Exon 4		Intron 4			Exon 5		
Designations	AA	CA	(-2570)	(-2485)	(-2387)	(-2350)	(-2249)	(-2243)	(-2146)	(-2115)	(-2108)	(-2050)	(-1941)	(-1896)	(-1720)	(-1505)	(-1031)	(-923)	(-745)	(-451)	(-333)	(-275)	(-183)	75	107	i2(-10)	526	592	i4(88)	i4(94)	i4(-41)	644	789
*1A	0.117	---	G	G	C	G	C	T	G	G	T	C	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	C	G	C
*1B	0.058	0.064	A	A	T	G	G	G	C	A	C	G	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	T	C	G	C	G	C
*1C	0.050	---	G	G	C	A	C	T	G	G	T	C	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	C	G	C
*1D	0.050	---	G	G	C	A	C	T	G	G	T	C	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	C	G	C
*1E	0.042	---	G	G	C	G	C	T	G	G	T	C	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	C	G	C
*1F	0.025	---	G	G	C	G	C	T	G	A	T	G	C	C	G	T	A	I	A	D	G	C	C	C	T	C	G	C	C	G	C	G	C
*1G	0.025	---	G	A	C	G	C	T	G	G	T	C	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	C	G	C
*1H	0.017	---	G	G	C	A	C	T	G	G	T	C	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	C	G	C
*1I	0.017	---	A	G	T	G	G	G	C	A	C	G	C	G	G	C	G	I	G	I	G	T	C	C	T	C	G	C	C	G	C	G	C
*1J	---	0.284	A	A	T	G	G	G	C	A	C	G	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	C	G	C
*1K	---	0.061	G	G	C	G	C	T	G	G	T	C	C	G	A	C	G	I	A	I	A	C	C	C	T	C	G	C	C	G	C	G	T
*1L	---	0.043	A	A	T	G	G	G	C	A	C	G	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	C	G	T
*1M	---	0.025	G	G	C	G	C	T	G	A	T	G	T	C	G	T	A	I	A	D	G	T	C	C	T	C	G	C	C	G	C	G	T
*1N	---	0.017	G	G	C	G	C	T	G	A	T	G	T	C	G	T	A	I	A	I	G	C	C	C	T	C	G	C	C	G	C	G	T
*1O	---	0.017	G	G	C	G	C	T	G	A	T	G	C	C	G	T	A	I	A	I	A	C	C	C	T	C	G	C	C	G	C	G	C
*1P	---	0.015	A	A	T	G	G	G	C	A	C	G	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	T	C	G	C	G	T
*1Q	---	0.011	G	G	C	G	C	T	G	A	T	G	T	C	G	T	A	I	A	D	G	T	C	C	T	C	G	C	C	G	C	G	C
*1R	---	0.011	A	A	T	G	G	G	C	A	C	G	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	C	G	T	G	C
*1S	---	0.008	A	A	T	G	G	G	C	A	C	G	C	C	G	C	G	I	G	D	G	C	C	C	T	C	G	T	C	G	C	G	C
*2A	---	0.008	A	A	T	G	G	G	C	A	C	G	C	G	G	C	G	I	G	D	G	C	T	C	C	C	G	T	C	G	T	G	T
*2B	---	0.008	A	A	T	G	G	G	C	A	C	G	C	G	G	C	G	I	G	D	G	C	T	C	C	C	G	T	C	G	T	G	T
*3	0.008	---	A	A	T	G	G	G	C	A	C	G	C	G	A	C	G	I	A	I	G	C	C	C	T	C	A	T	C	G	C	G	C
*4	0.008	---	G	G	C	G	C	T	G	G	T	C	C	G	A	C	G	I	A	I	A	C	C	C	T	C	G	C	C	G	C	A	T
*5A	---	0.008	A	A	C	G	G	G	C	A	C	G	C	G	G	C	G	I	G	D	G	C	C	C	T	C	G	C	A	G	C	G	C
*5B	---	0.008	G	G	C	G	C	T	G	A	T	G	C	G	G	T	A	I	A	I	A	C	C	C	T	C	G	C	C	G	C	G	T
*5C	---	0.008	G	G	C	G	C	T	G	A	T	G	T	C	G	T	A	D	A	D	G	T	C	C	T	T	G	C	C	A	C	G	T
*6	---	0.008	G	G	C	G	C	T	G	A	T	G	C	C	G	T	A	I	A	I	A	C	C	T	T	C	G	T	C	G	C	G	T

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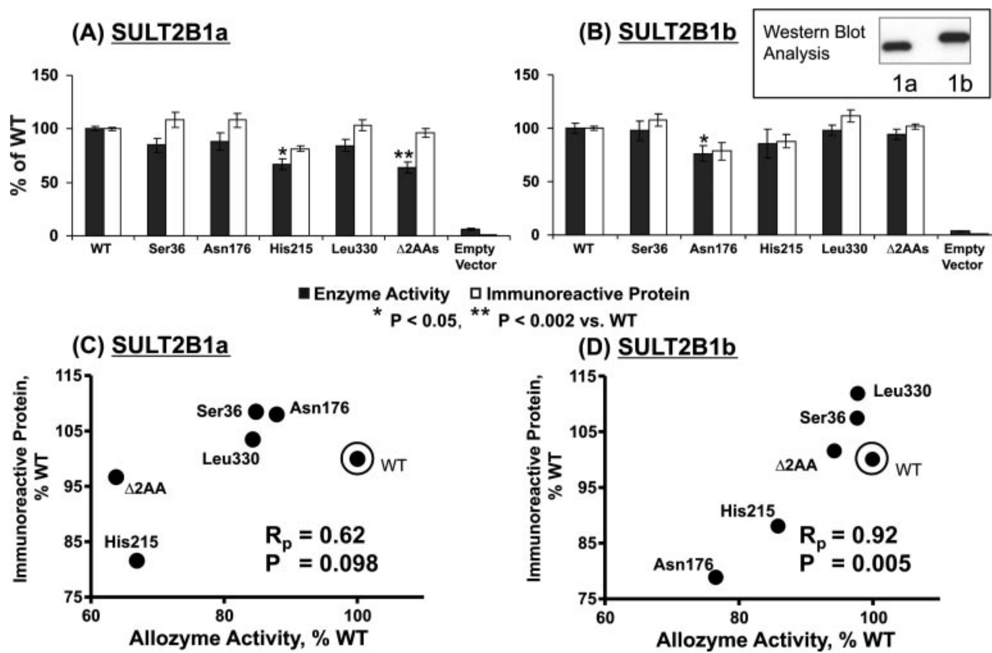

Fig. 3. SULT2B1 allozyme functional genomics. A and B, SULT2B1 allozyme activity assayed with DHEA as the sulfate acceptor substrate and immunoreactive protein levels expressed as percentages of WT are shown. Each bar represents the average of six independent transfections (mean ± S.E.M.). All values have been corrected for transfection efficiency. The insert shows Western blot analysis for WT SULT2B1a and 2B1b. C and D show correlations between average levels of immunoreactive protein and enzyme activity for SULT2B1a and SULT2B1b, respectively. The Δ2AA designation refers to data for constructs with the Ser337/Pro338 deletion.

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TABLE 4

Short (0.7 kb) *SULT2B1b* 5′-FR haplotypes

Human *SULT2B1b* 5′-FR haplotypes. The table lists haplotypes for 5′-FR sequences that were 0.7 kb in length. I, insertion; D, deletion. These haplotypes represent a subset of the data listed in Table 3.

	pGL3	Frequency		Nucleotide Location						
hap. #	Constructs	AA	CA	-653	-618	-423	-385	-375	-231	-194
1	S1	0.442	0.704	A	G	I	C	G	A	C
2	S2	0.148	0.146	G	G	I	C	G	A	C
3	S3	0.106	0.092	A	G	D	C	G	A	G
4	S4	0.065	---	G	A	D	T	G	A	C
5	S5	0.051	---	A	G	I	C	G	A	G
6	S6	0.050	0.021	A	G	D	C	A	A	C
7	---	0.033	---	A	G	D	C	G	C	C
8	S7	0.030	0.008	A	G	D	C	G	A	C

TABLE 5

Long (2.5 kb) *SULT2B1b* 2.5kb 5′-FR haplotypes

Human *SULT2B1b* 5′-FR haplotypes. The table lists haplotypes for 5′-FR sequences that were 2.5 kb in length. I, insertion; D, deletion. These haplotypes represent a subset of the data listed in Table 3.

	pGL3	Frequency		Nucleotide Location																			
hap. #	Constructs	AA	CA	-2307	-2262	-2204	-2010	-1620	-1530	-1431	-1417	-1399	-1339	-1309	-1125	-1030	-710	-653	-618	-423	-385	-375	-194
1	L1	0.367	0.646	D	G	A	C	A	C	G	G	A	T	T	G	A	G	A	G	I	C	G	C
2	L2	0.098	0.076	D	G	A	C	A	C	G	G	A	T	T	G	A	G	A	G	D	C	G	G
3	L3	0.058	---	I	G	C	C	A	C	G	A	A	T	T	G	A	G	A	G	I	C	G	C
4	L4	0.058	---	I	G	C	C	A	C	G	G	A	T	C	G	A	G	G	G	I	C	G	C
5	L5	0.050	---	I	G	C	C	C	T	G	G	G	C	C	G	A	G	G	A	D	T	G	C
6	---	0.034	---	I	G	C	C	A	C	A	G	A	T	T	A	A	G	A	G	I	C	G	G
7	---	0.025	---	I	G	C	C	A	C	G	A	A	T	T	G	A	A	A	G	D	C	A	C
8	---	0.025	---	I	G	C	C	A	C	G	G	A	T	T	G	C	G	G	G	I	C	G	C
9	L6	0.023	0.033	I	A	C	C	A	C	G	G	A	T	T	G	A	G	G	G	I	C	G	C
10	---	---	0.035	D	G	A	T	A	C	G	G	A	T	T	G	A	G	A	G	I	C	G	C
11	---	---	0.025	D	A	C	C	A	C	G	G	A	T	T	G	A	G	G	G	I	C	G	C

***SULT2B1b* 5′-FR Haplotype Reporter Gene Studies.** The functional consequences of common *SULT2B1b* 5′-FR haplotypes were studied using luciferase reporter gene constructs. *SULT2B1b* was used for these studies, because it is the most widely expressed of the two isoforms. Only 5′-FR haplotypes with frequencies of 5% or greater or haplotypes that were present in both AA and CA samples were studied. A series of "long" pGL3-L (2.5-kb 5′-FR) and "short" pGL3-S (0.7-kb 5′-FR) constructs containing common *SULT2B1b* 5′-FR haplotypes (Tables 4 and 5) were created and transfected into MCF-7 breast carcinoma, DU145 prostate cancer and JEG-3 placental choriocarcinoma cells. He et al. (2005) showed, based on Western blot analysis, that MCF-7 cells express the SULT2B1b protein. The DU145 and JEG-3 cell

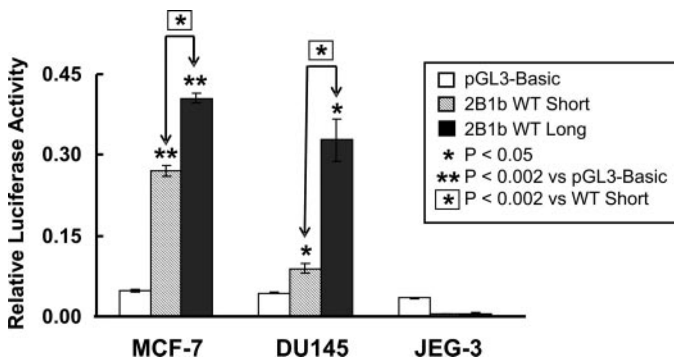

Fig. 4. Human *SULT2B1b* 5′-FR reporter gene studies for WT short and long constructs in MCF-7, DU145, and JEG-3 cell lines. Each bar represents the mean ± S.E.M. of six independent transfections.

lines were chosen for inclusion in these studies because SULT2B1b is highly expressed in both the prostate and placenta (Her et al., 1998). When WT reporter gene constructs were transfected into MCF-7 and DU145 cells, both long and short constructs could drive transcription, but no activity was observed for either long or short constructs in the JEG-3 placenta choriocarcinoma cell line (Fig. 4). Therefore, subsequent experiments performed with specific haplotypes used only MCF-7 and DU145 cells (Fig. 5). The reporter gene data for these experiments were expressed as a percentage of the level of activity for the pGL3-L WT construct in that cell line. Figure 5A also shows that the pGL3-L constructs displayed 2 to 3-fold greater activity than did the pGL3-S constructs in DU145 cells, a phenomenon that was not observed with the MCF-7 cells (Fig. 5B).

SULT2B1b Subcellular Localization. Human SULTs are cytosolic in subcellular localization (Falany, 1997a,b), but it has been reported that SULT2B1b can also localize to the nucleus of MCF-7 cells (He et al., 2004; Falany et al., 2006). Therefore, untransfected MCF-7 cells (cells that express SULT2B1b) and COS-1 cells (cells that do not express SULT2B1 but had been transfected with GFP-labeled SULT2B1b WT expression constructs) were cultured on cover slides. The MCF-7 cells were then stained to study the immunofluorescent localization of SULT2B1b with confocal microscopy. Figure 6A shows that endogenous SULT2B1b in MCF-7 cells was localized to the cytosol, as was recombinant GFP-labeled SULT2B1b expressed in COS-1 cells (Fig. 6B). Therefore, under the conditions used to perform these experiments, SULT2B1b appeared to behave as a cytosolic protein.

SULT2B1 Proline-Rich Carboxyl Terminus. The proline-rich carboxyl terminus is a unique structural feature of the two SULT2B1 isoforms among cytosolic SULTs (Her et al., 1998), and we observed a common nonsynonymous coding SNP (P330L) as well as a common synonymous SNP at nucleotide 903 within this region of the gene. Therefore, we studied the proline-rich tail, both to further define the possible functional implications of this structural feature and as a step toward future studies of common polymorphisms in this region of the *SULT2B1* gene. Specifically, a truncated variant (TV) was created that lacked the final 53 amino acids of SULT2B1. Immunofluorescence studies were then performed with this construct after the transfection of COS-1 cells. When the proline-rich tail was removed, protein aggre-

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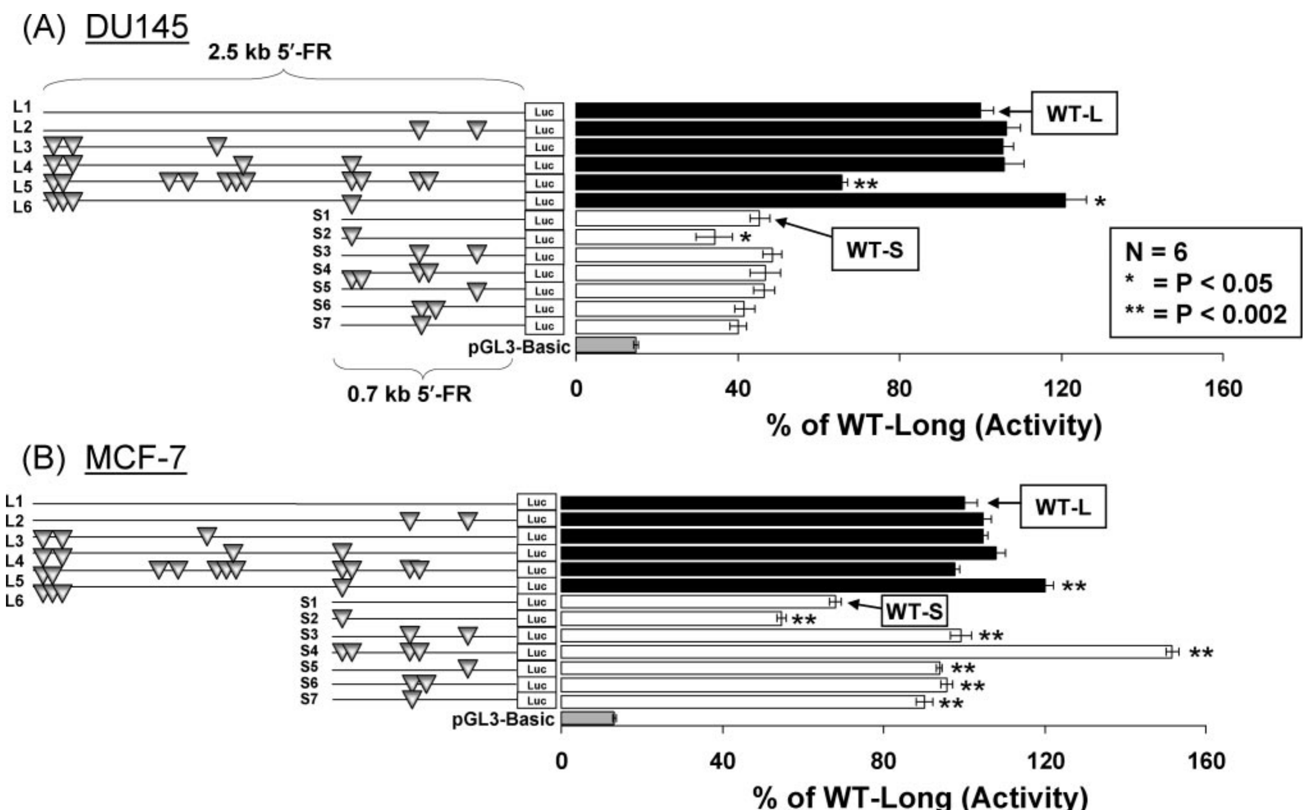

Fig. 5. Human *SULT2B1b* 5′-FR reporter gene studies. Activities of luciferase reporter gene constructs containing different *SULT2B1b* 5′-FR haplotypes are shown as a percentage of the level activity for the pGL3-L WT construct. DU145 (A) and MCF-7 (B) cells are shown. Each bar represents the mean ± S.E.M. of six independent transfections. Triangles (▽) show the locations of SNPs.

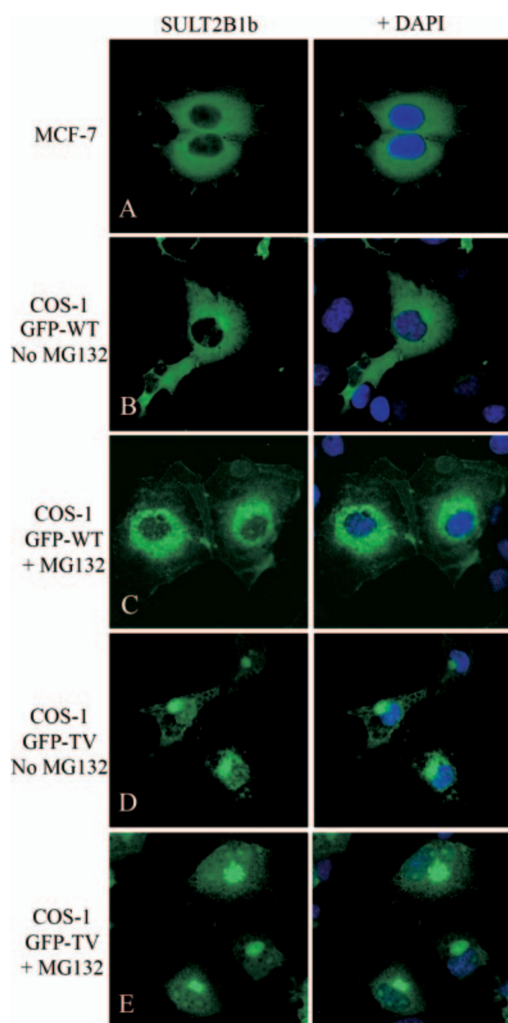

Fig. 6. SULT2B1b subcellular localization and truncated variant confocal microscopy. A, confocal fluorescence microscopy showing endogenous SULT2B1b in MCF-7 cells. B, C, D, and E, confocal fluorescence microscopy showing GFP-labeled SULT2B1b WT and TV constructs transfected into COS-1 cells. C, WT SULT2B1b after MG132 treatment. D, truncated SULT2B1b protein (TV) lacking the 53-amino acid proline-rich carboxyl terminus. E, GFP-TV-transfected cells after MG132 treatment.

gates, aggresome-like structures (Wang et al., 2005), formed in the cystosol of 22 ± 5.1% of cells (mean ± S.E.M., $n = 3$, $P < 0.05$ compared with WT) (Fig. 7). Because intracellular protein aggregation of this type is often associated with accelerated protein degradation (Wang et al., 2003), we next treated the cells with the proteasome inhibitor MG132 to determine whether the proportion of cells containing protein aggregates might increase. When the cells were treated with 20 μM MG132, a larger number of the cells showed large protein aggregates (62 ± 5.2%, $P < 0.005$) compared with cells transfected with WT-GFP-labeled SULT2B1b or with the truncated variant in the absence of MG132 (Figs. 6, D and E, and 7).

Finally, we used a RRL system to determine whether the truncated SULT2B1 variant might be degraded more rapidly than the WT protein. WT and truncated variants for both of the SULT2B1 isoforms were synthesized using the TnT RRL system (Fig. 8A). Those recombinant proteins were then used to perform in vitro degradation assays that included an ATP-generating system and "untreated" RRL (Fig. 8B). The truncated variants for both isoforms were degraded more rapidly than were the WT proteins (Fig. 8B).

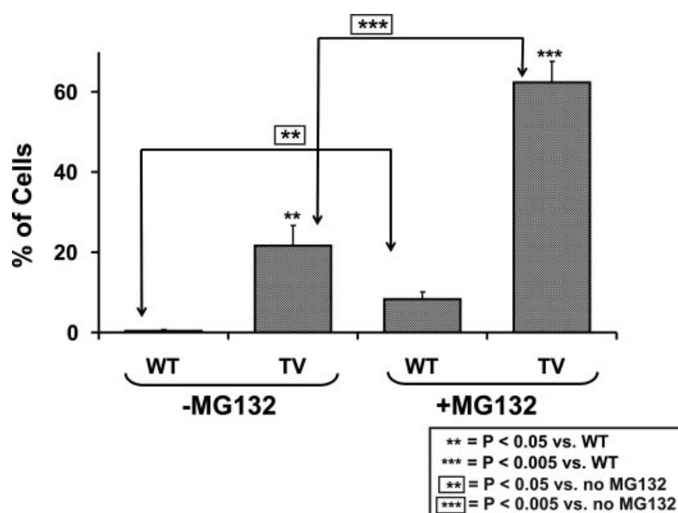

Fig. 7. Frequency of large aggregate formation in COS-1 cells transfected with either GFP-labeled WT SULT2B1b or a TV lacking the proline-rich carboxyl terminus, with or without MG132 treatment. All data represent mean ± S.E.M. for three determinations.

Discussion

Members of the human hydroxysteroid SULT family participate in the metabolism of many exogenous and endogenous compounds (Falany, 1997a; Freimuth et al., 2004; Nimmagadda et al., 2006). The two SULT2B1 isoforms preferentially catalyze the sulfonation of 3β-hydroxysteroids, including cholesterol, pregnenolone, DHEA, and many other steroids (Her et al., 1998; Javitt et al., 2001; Fuda et al., 2002; Falany et al., 2006). The sulfate conjugate of DHEA is the most abundant circulating steroid in humans (Kroboth et al., 1999), and DHEA represents the precursor for approximately 50% of androgens in men, 75% of estrogens in premenopausal women, and 100% of estrogens in postmenopausal women (Miller, 2002; Labrie, 2003). Cholesterol sulfate is the most abundant sterol sulfate in plasma (Javitt et al., 2001) and plays an important role in keratinocyte differentiation and skin barrier function (Epstein et al., 1984; Elias et al., 2002, 2004). The sulfonation of these steroid compounds is catalyzed by members of the hydroxysteroid SULT family that includes two subfamilies in humans, SULT2A1 and SULT2B1 (Freimuth et al., 2004). SULT2A1 is highly expressed in the adrenal gland, liver, and small intestine (Otterness et al., 1992, 1995), whereas SULT2B1 can catalyze steroid sulfonation in tissues where SULT2A1 is not expressed (Her et al., 1998; Geese and Raftogianis, 2001; Lee et al., 2003). As a result, genetic variation that alters SULT2B1 function could have both physiologic and pharmacologic implications.

Most studies of SULT2B1 have focused on characterization of the biochemical and physical properties of the two isoforms (Her et al., 1998; Geese and Raftogianis, 2001; Javitt et al., 2001; Fuda et al., 2002; Lee et al., 2003, 2005; He et al., 2004, 2005; Higashi et al., 2004; Yanai et al., 2004; Falany et al., 2006; Kohjitani et al., 2006). However, the possible effect on SULT2B1 function of common genetic variation has not been systematically explored. Therefore, in the present study, we

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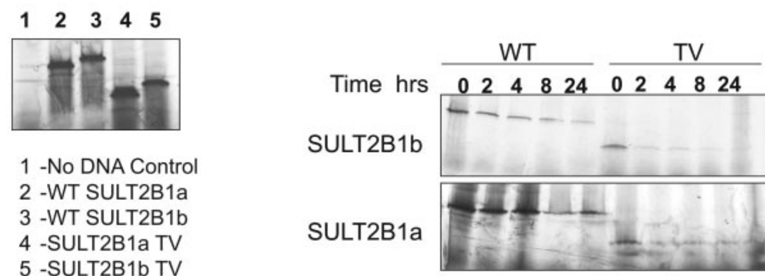

Fig. 8. SULT2B1 RRL translation and degradation. A, SULT2B1 in vitro translation in the RRL. B, SULT2B1 degradation in the RRL. The figure shows representative autoradiographs for [^{35}S]methionine radioactively labeled WT SULT2B1a and SULT2B1b as well as TV proteins lacking the 53-amino acid proline-rich carboxyl terminus at various time points during RRL degradation experiments.

used a genotype-to-phenotype strategy to identify common genetic variation and haplotypes in *SULT2B1*, followed by characterization of the functional consequences of that gene sequence variation. We began by resequencing *SULT2B1* using 120 DNA samples from two ethnic groups. That effort resulted in the identification of 100 polymorphisms. The majority of those polymorphisms were novel (Fig. 1; Table 1). Functional genomic studies were then performed with the five variant allozymes observed for each SULT2B1 isoform. Specifically, levels of immunoreactive protein and allozyme enzyme activity were determined using these recombinant proteins. The substitution of variant amino acids in the two SULT2B1 allozymes resulted in moderate but not striking alterations in function (Fig. 3). These results can now be placed within the context of those reported in a recent summary of human SULT gene resequencing and functional genomic data (Hildebrandt et al., 2007). Our resequencing studies also resulted in the identification of common polymorphisms in the 5′-FRs of both *SULT2B1a* and *SULT2B1b*, many with MAFs of greater than 10% (Fig. 1; Table 1). Therefore, a haplotype-based approach was used to create luciferase reporter gene constructs for common *SULT2B1b* 5′-FR haplotypes to study their possible effects on transcription. Because SULT2B1b is the most widely expressed isoform in human tissues, these experiments were performed only with *SULT2B1b*. A cell line-dependent effect on ability to drive *SULT2B1b* transcription was observed (Fig. 5). Because SULT2B1b is highly expressed in the placenta (Her et al., 1998; Geese and Raftogianis, 2001; He et al., 2004), JEG-3, a placental choriocarcinoma cell line, was included in the luciferase reporter gene studies. However, there was no transcriptional activity for either short or long SULT2B1b constructs in JEG-3 cells (Fig. 4). Similar results were obtained with BeWo cells, another placental choriocarcinoma cell line (data not shown). Obviously, these initial experiments will need to be followed by detailed studies of the regulation of *SULT2B1* transcription.

SULT2B1b protein has been detected in the nuclei of placental syntrophoblasts and in the cytoplasm of prostatic epithelial cells (He et al., 2004). Therefore, we also performed immunofluorescence subcellular localization studies of endogenous SULT2B1b protein in MCF-7 cells and recombinant WT protein in transfected COS-1 cells. In both of these cells, SULT2B1 was cytoplasmic in localization (Fig. 6, A and B). Finally, we made an effort to begin to study the possible function of the SULT2B1b proline-rich carboxyl terminus. Subcellular localization studies showed that a truncated SULT2B1b variant that lacked the proline-rich tail was not distributed evenly in the cytoplasm but appeared to form intracellular aggregates (Fig. 6D). When these cells were treated with the proteasome inhibitor MG132, larger aggresome-like protein aggregates were observed (Fig. 6E). Studies of other proteins have shown that intracellular protein aggregation of this type is often associated with accelerated degradation (Wang et al., 2003, 2005). Therefore, we also tested the hypothesis that truncated SULT2B1 protein might be degraded more rapidly than the WT. In vitro translation and degradation experiments demonstrated that, in the absence of the proline-rich tail, both SULT2B1 isoforms undergo accelerated degradation (Fig. 8). These observations suggest that the proline-rich domain at the C terminus of SULT2B1, a structure unique to SULT2B1 among cytosolic SULTs, appears to be necessary for protein stability. These results are compatible with recent report that SULT2B1 is more thermolabile after deletion of the proline-rich tail (He and Falany, 2006). The crystal structure of human SULT2B1b has been solved at a resolution of 2.4 Å (Lee et al., 2003), but that structure lacked the C terminus of the protein, so we were unable to use the crystal structure to help interpret our results. Ultimately, an understanding of the possible consequences of genetic variation in SULT2B1 may require increased knowledge of the function of the proline-rich carboxyl-terminal portion of the molecule.

In summary, we have identified common sequence variation in the human *SULT2B1* gene in two ethnic groups. We also characterized the functional consequences of that variation. These observations represent a foundation for future biochemical, translational, and epidemiologic studies designed to increase our understanding of the molecular genetics of *SULT2B1* expression and function, as well as the possible role of individual variation in *SULT2B1* sequence in variation in disease risk and/or drug-response phenotypes.

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