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Molecular Cloning and Characterization of Rat ST1B1 and Human ST1B2 cDNAs, Encoding Thyroid Hormone Sulfotransferases[1]

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Human and rat cDNAs encoding thyroid hormone sulfotransferases have been isolated from their liver cDNA libraries. The isolated sulfotransferases, termed rat ST1B1 and human ST1B2, share 77 and 74% homologies at nucleotide and deduced amino acid levels. These forms showed less than 36 and 56% homologies to hydroxysteroid and aryl sulfotransferases, indicating that they constitute a new gene subfamily of aryl sulfotransferase. Expression of ST1B1 and ST1B2 in COS-1 cells resulted in the appearance of 33.0 and 32.5 kDa proteins, respectively, whose mobilities were identical with proteins detected in rat and human livers in Western blots using antibodies raised against ST1B1 and ST1B2 produced in *Escherichia coli*. The recombinant forms catalyzed sulfation of *p*-nitrophenol, 3,3′,5-triiodothyronine (T_3) and dopamine, but not of β-estradiol and dehydroepiandrosterone. ST1B1 and ST1B2 showed higher affinities for formation of T_3 sulfate (apparent K_m 40.2 and 63.5 μM, respectively) than did thermostable phenol sulfotransferase ST1A3 (apparent K_m 413 μM) or thermolabile phenol sulfotransferase ST1A5 (apparent K_m 180 μM). These data indicate that the newly characterized sulfotransferases constitute a distinct ST1 subfamily of enzymes catalyzing the sulfation of T_3 as a typical endogenous substrate in rats and humans.

Key words: aryl sulfotransferase, molecular cloning, new gene subfamily, recombinant form, thyroid hormone.

Sulfate conjugation plays a key role in biotransformation of drugs and xenobiotics, and of endogenous compounds such as steroids, neurotransmitters and bile acids (*1, 2*). The conjugation is regarded as a detoxification in most cases, but it may also be involved in the bioactivation of chemicals (*3*).

Sulfation[3] of 3,3′,5-triiodothyronine (T_3), a major biologically active form of thyroid hormone, has long been known (*4*). Thyroxine secreted by the thyroid gland is converted enzymatically in peripheral tissues by outer ring deiodination to an active form, T_3, or by inner ring deiodination to an inactive form, 3,3′,5′-triiodothyronine. Both T_3 and 3,3′,5′-triiodothyronine are further deiodinated to form diiodothyronines, including 3,3′-diiodothyronine. Sulfation and glucuronidation of iodothyronines constitute a major pathway for their metabolism in mammals. Phenolic ring sulfation is shown to facilitate the subsequent deiodination of iodothyronines (*5*). These data suggest a primary role of sulfation in the irreversible inactivation of thyroid hormone (*4*).

On sulfotransferases involved in the metabolism of iodothyronines, Sekura *et al.* (*6*) found that aryl sulfotransferases I and IV purified from rat livers catalyzed the sulfation of a variety of iodothyronine derivatives. Gong *et al.* (*7*) showed a marked sex-related difference in the hepatic sulfating activity of T_3 in mice and rats. Young *et al.* (*8*) reported that the hormone was a substrate for at least three phenol sulfotransferases in humans. These results suggest that multiple forms of aryl (phenol) sulfotransferases participate in the sulfation of iodothyronine derivatives in rat and human tissues. The exact contribution of individual forms in both rat and human, however, remains to be established. In addition, these data do not exclude, but rather suggest, the possible contribution of an uncharacterized form for thyroid hormone sulfation in these species. Sulfotransferases have been characterized using purified or partially purified enzymes. Sulfotransferases show mutually overlapping substrate specificities and similar molecular weights (30-36 kDa) (*9*). In addition, the existence of heterodimeric forms of sulfotransferases was also reported (*10*). These results suggest the possibility

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Abbreviations: T_3, 3,3′,5-triiodothyronine; *p*-NP, *p*-nitrophenol; β-E_2, β-estradiol; DHEA, dehydroepiandrosterone; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; RACE, rapid amplification of cDNA end; RT-PCR, reverse transcriptase-PCR; ORF, open reading frame; UTR, untranslated region.

[3] Sulfotransferase mediates the transfer of SO_3^- from PAPS to phenols, alcohols, and amines. In this study, "sulfation" is used to indicate these reactions collectively.

that other forms are included in the purified enzymes previously used.

Molecular cloning studies from our laboratory and others showed that rat and human livers contain multiple forms of sulfotransferases involved in the metabolism of phenols (*9*). A form (ST1B1) encoded by RST-7[4] was isolated from a rat liver cDNA library (*11*). In preliminary experiments, we showed the deduced amino acid sequence and the high sulfating activity of T_3 of recombinant ST1B1 (*9*). Sakakibara *et al.* (*12*) reported the purification of Dopa/Tyrosine sulfotransferase from rat livers. Amino acid sequences of the three peptide fragments were identical with parts of the amino acid sequence of ST1B1.

To better understand the properties of sulfotransferase, a human cDNA library was screened using rat RST-7 as a probe to isolate the human counterpart in the present study. The results indicate that both the rat (ST1B1)[5] and human forms (ST1B2)[5] constitute a distinct group of aryl sulfotransferase (ST1B) catalyzing the sulfation of T_3 as a typical endogenous substrate.

MATERIALS AND METHODS

Materials—Restriction endonucleases, DNA modifying enzymes, reverse transcriptase (RAV-2), and TaKaRa Ex Taq were purchased from Takara Shuzo (Kyoto). DNA labeling kit was purchased from Nippon Gene (Toyama). 7-Deaza Sequenase Ver. 2.0 kit was purchased from U.S. Biochemical. A λgt11 cDNA library of a human liver was obtained from CLONTECH (Palo Alto, CA). Mammalian expression vector (pCMV4) was kindly provided by Dr. David W. Russall (University of Texas South Western Medical Center, Dallas, TX). Human liver samples (HL100, HL102, HL112, and HL116) were kindly provided by SRI International Toxicology Laboratory (Menlo Park, CA). 3′-Phosphoadenosine 5′-phosphosulfate (PAPS), DTT, T_3, *p*-nitrophenol (*p*-NP), β-estradiol (β-E_2), dopamine, dehydroepiandrosterone (DHEA), nitro blue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate, and alkaline phosphatase conjugated goat anti-rabbit IgG were purchased from Sigma Chemical (St. Louis, MO). [α-^{32}P]dCTP (3,000 mCi/mmol) and [α-^{35}S]dCTP (3,000 mCi/mmol) were obtained from Amersham Japan (Tokyo). [α-^{35}S]PAPS (2,000 mCi/mmol) was obtained from New England Nuclear (Boston, MA). The QIAexpress and Ni-NTA spin kits were purchased from Qiagen (Chatsworth, CA), Bio-Rad Protein Assay kit and SDS-PAGE Molecular Weight Standards (Low Range) were from Bio-Rad (Richmond, CA) and peroxidase-conjugated goat anti-rabbit IgG was from Cappel Laboratories (Boston, MA). Dulbecco's modified Eagle's medium and fetal calf serum were obtained from GIBCO (Gaithersburg, MD) and Mitsubishi Kasei (Tokyo), respectively. All other chemicals used were of the highest grade available.

Screening of cDNA Library—Human ST1B2-5′ clone was isolated from a λgt11 human liver cDNA library using rat RST-7 (*11*) as a probe (Fig. 1, line 1). The phages (about 9.6×10^5) were plated on 150-mm Petri dishes at 8.0×10^4 phages/dish with *Escherichia coli* strain Y1088 and blotted onto nitrocellulose membranes. The membranes were denatured, exposed to UV light (UV cross-linker, SPECTRONICS), then incubated in prehybridization solution [6×SSC (90 mM sodium citrate, pH 7.0 and 0.9 M NaCl), 20×Denhardt's solution, 0.5% SDS and 1 μg/ml single strand salmon sperm DNA] at 52°C for 1 h. After hybridization with ^{32}P-labeled RST-7 probe (10^7 cpm/ml), the membranes were washed three times at 55°C in 2×SSC containing 0.1% SDS for 15 min, then dried and subjected to autoradiography overnight at −70°C. Positive clones were rescreened by repeated cycles of dilution, and the phage DNAs were purified as described previously (*13*). The insert DNAs were subcloned into pUC19.

Amplification of cDNA by PCR—A 3′-portion of sequence (ST1B2-3′; Fig. 1, line 2) containing a termination codon and the 3′-untranslated region (UTR) of the ST1B2 cDNA was obtained by a rapid amplification of cDNA end (3′-RACE) procedure (*14*) with HST1B-5 and poly-T primers (Table I). Using human liver cDNAs isolated from the library, a cDNA including the complete open reading frame (ORF) of ST1B2, designated ST1B2-ORF (Fig. 1, line 3), was obtained by PCR with HST1B-ORF-5′ and HST1B-ORF-3′ primers (Table I). Using similar protocol, ST1A5 cDNA was obtained by PCR with ST1A5-5′ and ST1A5-3′ primers (Table I) from the human liver cDNAs. The PCR was performed with a thermal cycler (Gene Amp PCR System 2400, PERKIN ELMER) in a reaction mixture (30 μl) containing 5 ng of template cDNA, 20 pmol each of 5′- and 3′-primers, 0.2 mM each of four deoxynucleoside triphosphates, 1.5 units of TaKaRa Ex Taq, and 3 μl of Ex Taq buffer. After an initial denaturation at 94°C for 2 min, the amplification was started for 30 cycles, for 1 min at 94°C for denaturation, 2 min at 55°C for annealing, 2 min at 72°C for extension, and a final extension period of 7 min at 72°C. The amplification products were purified, digested with *Xba*I and *Xma*I (ST1B2-3′), *Bam*HI and *Hin*dIII (ST1B2-ORF), or *Eco*RI (ST1A5 cDNA), then subcloned into *Xba*I and *Xma*I, *Bam*HI and *Hin*dIII, or *Eco*RI sites of pUC19, respectively.

cDNAs, designated 6xH ST1B2[6] (Fig. 1, line 4) and 6xH ST1B1 cDNAs, which contained nucleotides corresponding to 17 extra amino acid residues at their N-terminal, were constructed by PCR with primers, as shown in Table I (ST1B2-Lys and HST1B-ORF-3′ for ST1B2, and ST1B1-Lys and ST1B1-3′ for ST1B1). In similar way, 6xH ST1A3 and 6xH ST1A5 cDNAs were obtained by PCR with their primers, as shown in Table I (ST1A-Lys and ST1A-3′) from ST1A3 and ST1A5 cDNAs, as templates, respectively.

DNA Sequencing and Sequence Analysis—Insert cDNAs obtained by the plaque-hybridization, 3′-RACE or PCR were subcloned into suitable sites of M13mp18 and M13mp19 for their single-strand sequencing. Sequencing was done by M13 site-direction and shotgun restriction fragment procedures combining with a dideoxynucleotide chain termination method (*15*). Electrophoresis was performed using 6% polyacrylamide-urea buffer gradient gels.

[4] RST-7 is a cDNA isolated from a rat liver cDNA library by use of antibody against purified *N*-hydroxy-2-acetylaminofluorene sulfotransferase I.

[5] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession number D89479 for ST1B2 and D89375 for ST1B1.

[6] 6xH ST1B2 is a fusion protein having 17 additional amino acids at the N-terminal. 6xH ST1B1, 6xH ST1A3, and 6xH ST1A5 also contain these amino acid residues at their N-terminals.

TABLE I. **Oligonucleotide primers used for PCR.** Recognition sites of restriction endonuclease in the oligonucleotides are underlined. Initiation and termination codons for translation are boxed. Bold lines indicate the sequences translated to ArgArgArgArgLys amino acid residues, which are a proteolytic recognition site of enterokinase, cleaving at the C-terminal side of lysine. Numbers of nucleotides (nt) of ST1A5-5′,ST1A5-3′, HST1A-Lys, and HST1A-3′ indicate the nucleotide sequences derived from Ref. *21*.

3′-RACE primers

HST1B-5 (nt, 301-320, sense) 5′- GGTATAGAACAATTGGAGAA -3′

Poly-T (3′-RACE adaptor antisense) 5′- GCCCCGGG $(dT)_{18}$ -3′
Sma I

PCR primers

HST1B-ORF-5′ (nt, 14-35, sense) 5′- GCGGATCCCTGGTATTAAAATGCTTTCCCC -3′
*Bam*HI

HST1B-ORF-3′ (nt, 902-925, antisense) 5′- GCAAGCTTTTTAGACACTTTAAATCTCTGTGC -3′
*Hin*dIII

ST1A5-5′ (nt, (-6)-16, sense) 5′- GCGAATTCAGGAACATGGAGCTGATCCAGG -3′
*Eco*RI

ST1A5-3′ (nt, 1032-1052, antisense) 5′- GCGAATTCTCTCAAATTCGTGGCCTATTG -3′
*Eco*RI

PCR primers used for prokaryotic expression

ST1B2-Lys (nt, 25-50, sense) 5′- GCGGATCCGATGACGATGACAAAATGCTTTCCCCAAAAGATATTCTGCG -3′
*Bam*HI

ST1B1-Lys (nt, 39-58, sense) 5′- GCGGATCCGATGACGATGACAAAATGGGTACTGCAGAAGATGT -3′
*Bam*HI

ST1B1-3′ (nt, 921-940, antisense) 5′- GCAAGCTTGTTTAGGCACTCTGAATATC -3′
*Hin*dIII

HST1A-Lys (nt, 1-18, sense) 5′- GCGGATCCGATGACGATGACAAAATGGAGCTGATCCAGGAC -3′
*Bam*HI

HST1A-3′ (nt, 873-894, antisense) 5′- GCAAGCTTCCCCTCTCACAGCTCAGAGCGG -3′
*Hin*dIII

Sequence data read manually were analyzed by use of Gene Works software (IntelliGenetics, CA).

A Reverse Transcriptase-PCR (RT-PCR)—RT reaction was performed with RAV-2 (6.0 units) and four poly(A)$^+$ RNA (50 ng) samples isolated from four individual human livers as templates at 42°C for 60 min. PCR was performed under the conditions described above with primer HST1B-ORF-5′ and HST1B-ORF-3′ (Table I) as specific probes for ST1B2 cDNA. A control experiment was performed with primer ST1A5-5′ and ST1A5-3′ (Table I) as specific probes for ST1A5 cDNA. PCR products were analyzed by electrophoresis.

Expression of cDNA in COS-1 Cells—ST1B1 cDNA and ST1B2-ORF were each ligated into a *Bgl*II/*Hin*dIII site of a mammalian expression vector, pCMV4. The constructed plasmid DNAs (pCMV4-ST1B1 and pCMV4-ST1B2) were prepared using the alkaline lysis procedure followed by two cycles of CsCl/ethidium bromide equilibrium density gradient centrifugation (*13*). Introduction of plasmid DNA into COS-1 cells was performed by electroporation as described previously (*11*) with minor modifications. COS-1 cells harvested with 0.25% trypsin containing 0.1 mM EDTA were resuspended in HEPES-buffered saline (42 mM HEPES, pH 7.5, and 274 mM NaCl) at a cell density of 8×10^6 cells/ml. The suspended cells were mixed with plasmid DNA at the concentration of 50 μg of DNA/ml and kept on ice for 10 min. Electroporation was carried out at 350 V, 400 μF, and 24 ohm using a ELECTRO CELL MANIPULATOR 600 apparatus. After electroporation, cells were immediately transferred to a 90-mm diameter Petri dish containing 10 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum.

Transformed COS-1 cells were cultured for 2 days under a humidified atmosphere of 95% air-5% CO_2 at 37°C. COS-1 cells were harvested, washed with phosphate-buffered saline, and sonicated in a ice-cold solution containing 10 mM Tris-HCl (pH 7.4), 1 mM DTT, and 250 mM sucrose. The soluble fraction was prepared by centrifugation at 105,000$\times g$ for 60 min at 4°C. The resultant cytosol was stored at −80°C until use.

Expression of cDNA in Escherichia coli—6xH ST1B1, 6xH ST1B2, 6xH ST1A3, and 6xH ST1A5 cDNAs obtained by PCR were ligated into a *Bam*HI and *Hin*dIII site of a prokaryotic expression vector, pQE30, to facilitate high levels of expression and rapid purification. An overnight culture (20 ml) of *E. coli* strain M15[pREP4] harboring pQE30-6xH ST was used to inoculate 1,000 ml of Luria-Bertani medium containing 150 μg/ml ampicillin and 25 μg/ml kanamycin. The cells were grown at 37°C until A_{600} reached 0.7-0.9. Expression of the recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM, and cultivation was continued for an additional 2 h. Cells were then collected by centrifugation, resuspended in 20 ml of 50 mM sodium phosphate (pH 8.0) containing 300 mM NaCl and lysed by sonication. The soluble fraction was obtained as described above for the preparation of cytosol of COS-1 cells. The soluble fraction was applied to 1 ml of nickel-nitriloacetic acid-bound agarose that had been preequilibrated in the same buffer, then agitated on ice for 60 min.

The agarose gel was then packed in a 1 cm diameter column, washed three times with a solution of 50 mM sodium phosphate (pH 6.0) containing 300 mM NaCl and 20 mM imidazole, and sulfotransferases were eluted with the same buffer containing 250 mM imidazole instead of 20 mM. The eluted fraction was dialyzed against a solution of 10 mM potassium phosphate (pH 7.4) containing 0.1 mM DTT, 10% glycerol and 0.025% Tween-20. Concentration of proteins was determined by the method of Bradford (*16*), using BSA as the standard.

Antibody Preparation and Immunoblot Analysis—New Zealand white rabbits (2.5 kg, female) were immunized intradermally with 100 μg of the purified 6xH ST1B1 or 6xH ST1B2 in complete Freund's adjuvant, and immunity was boosted intravenously with 100 μg of the protein three weeks later. One week later, anti-sera were obtained and kept at −80°C until use. For immunoblots, cytosolic proteins were applied on 10.5% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane as described previously (*11*). ST1B1 and ST1B2 on the membrane were reacted with peroxidase-conjugated goat anti-rabbit IgG or alkaline phosphatase-conjugated goat anti-rabbit IgG, respectively.

Sulfotransferase Assays—Sulfating activities were determined by the radioactivity of metabolites formed in the presence of [^{35}S]PAPS after TLC as described previously (*11*). The reaction mixtures consisted of a substrate (100 μM *p*-NP, 60 μM T_3, 100 and 1,000 μM dopamine, 100 μM β-E_2, or 5 μM DHEA), 100 mM potassium phosphate (pH 6.6 for *p*-NP and β-E_2, pH 6.0 for DHEA) or 100 mM Tris-HCl at pH 8.0 (for T_3 and dopamine), 5 μM [^{35}S]-PAPS, 1 mM DTT, 0.3 mM $MgCl_2$ (for β-E_2 and DHEA), 1 mg/ml BSA (for *p*-NP, T_3, and dopamine), 1 mM pargyline (for dopamine), and 0.05 mg/ml of sulfotransferase-expressed cytosols in *E. coli* in a final volume of 10 μl. For measurement of sulfating activities of each substrate catalyzed by sulfotransferase-expressed cytosols, assay conditions were optimized with regard to reaction pH, substrate and enzyme concentrations, and supplements. The reaction was initiated by the addition of [^{35}S]PAPS and terminated by the addition of 10 μl of acetonitrile after incubation for the designated period at 37°C. The reaction mixture without a substrate was always included as a control. A portion (10 μl) of the reaction mixture was applied to a cellulose thin layer plate (Chromagram sheet 13255, KODAK). Metabolites on the chromatogram were developed with *n*-propanol : ammonia : water (6 : 3 : 1). The radioactive spots, which were positive only in the presence of the substrate, were scraped from the plate and quantified by use of a liquid scintillation counter (LS6500, BECKMAN). The rates of sulfation were calculated after subtraction of the respective control radioactivities and expressed as nmol of sulfate formed per mg protein per min. The apparent K_m values for T_3, *p*-NP, and dopamine were estimated by linear regression analysis of the Lineweaver-Burk plots. A range of concentrations (six points) was used to obtain optimal conditions for sulfotransferase-expressed cytosols in *E. coli*.

RESULTS

Isolation and Characterization of ST1B2 cDNA—A human liver cDNA library was screened with rat RST-7 encoding ST1B1 sulfotransferase, and four positive clones were selected as candidates for the human counterpart. These four cDNA inserts had an identical length (775 base pairs) and sequence (Fig. 1, line 1), and showed similarities to mammalian sulfotransferase cDNAs in their nucleotide sequences. The cDNA contained a possible initiation codon at bases 25-27, but lacked the 3′-side of the ORF and the 3′-UTR. Information on the complete ORF and the 3′-UTR was obtained using a 3′-RACE procedure, the full-length sequence of ST1B2 cDNA was thereby determined (Fig. 1). ST1B2 cDNA consisted of 1031 base pairs, which included 24 and 119 base pairs of the 5′- and 3′-UTRs, respectively (Fig. 2). The enzyme, ST1B2, was encoded by 888 base pairs and consisted of 296 amino acids with a molecular weight of 34,787 (Fig. 2).

Comparison of ST1B2 with ST1B1—ST1B1 cDNA (1234 base pairs) encodes a sulfotransferase which is identical with the purified Dopa/Tyrosine sulfotransferase (*12*) except for Gly^{68} instead of Glu^{68}. This cDNA clone is shorter in the 5′-UTR (51 nucleotides) and longer in the 3′-UTR (56 nucleotides) than the Dopa/Tyrosine sulfotransferase cDNA clone. Substitutions of 12 nucleotides were observed between the two cDNAs (3, 8, and 1 nucleotides of the 5′- and 3′-UTRs and ORF, respectively). Sulfotransferase encoded by the Dopa/Tyrosine sulfotransferase cDNA is probably variant form of ST1B1. Nucleotide and deduced amino acid sequences of human ST1B2 were compared with those of rat ST1B1 (Fig. 2). ST1B1 and ST1B2 have high homologies with each other at the nucleotide (77%) and deduced amino acid (74%) levels,

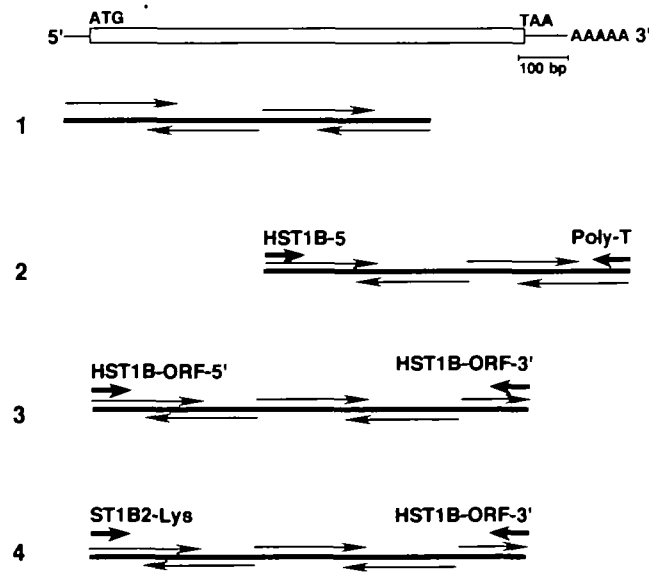

Fig. 1. **Strategy of isolation and determination of full-length cDNA.** The top diagram shows ST1B2 mRNA. The open box represents the ORF, and the solid lines represent the 5′- and 3′-UTR. Line 1 (ST1B2-5′) represents a cDNA clone obtained by screening with RST-7 as a probe. Line 2 (ST1B2-3′) represents a 3′-RACE product obtained by PCR using HST1B-5 and poly-T primers. Lines 3 (ST1B2-ORF) and 4 (6xH ST1B2 cDNA) represent PCR products containing the entire sequence of the ORF obtained using HST1B-ORF-5′ or ST1B2-Lys, and HST1B-ORF-3′ primers, respectively. The 6xH ST1B2 cDNA contained a sequence of an enterokinase cleavage site in the 5′-region of ST1B2-ORF. Boldface arrows denote primer positions and orientation. Lightface arrows denote the direction and length of sequences.

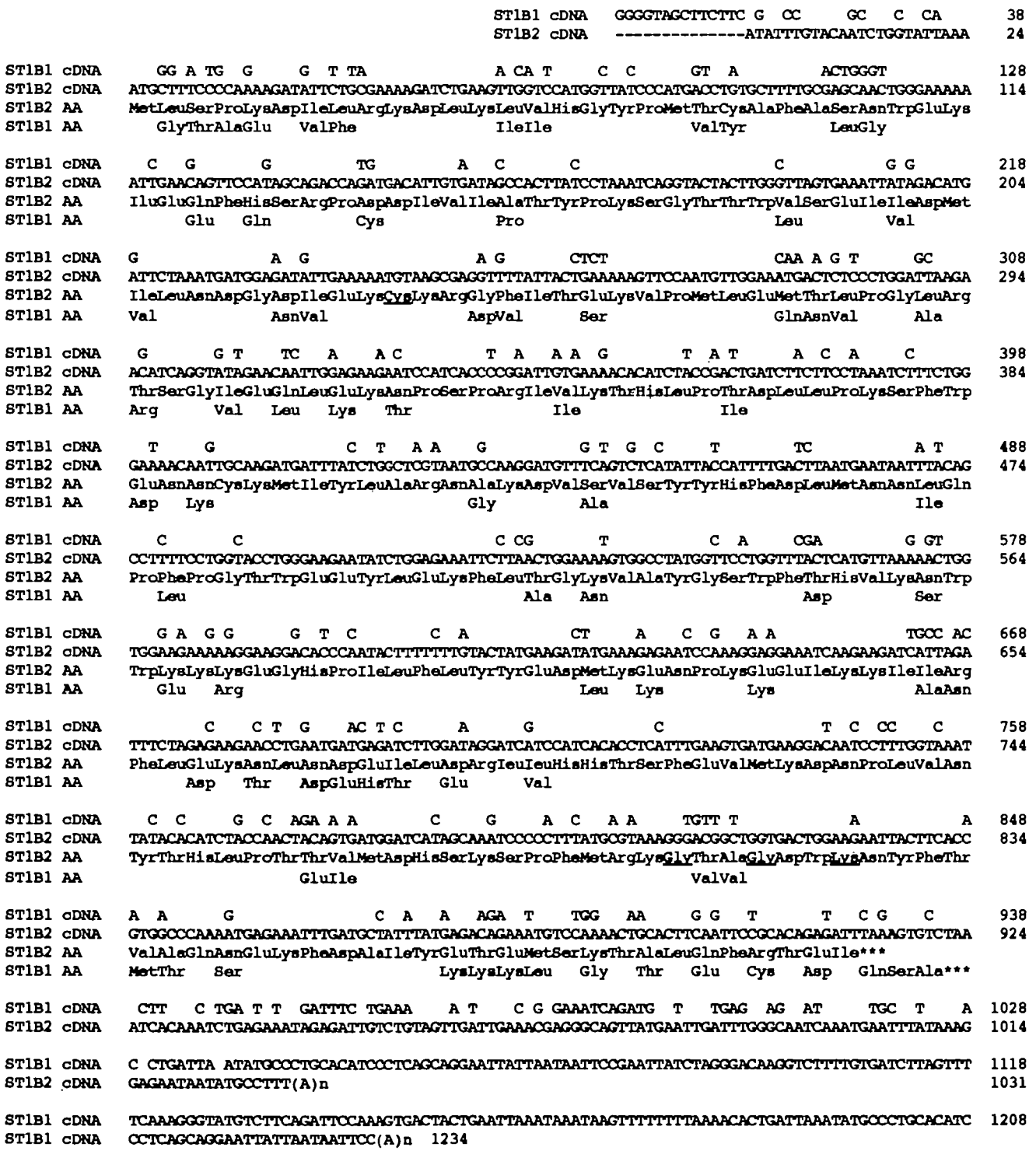

Fig. 2. **Comparison of nucleotide and deduced amino acid (AA) sequences of ST1B2 and ST1B1.** Sequences of ST1B1 that are identical to ST1B2 are not shown. Dashed bar in the 5′-UTR of ST1B2 cDNA indicates the absence of corresponding sequences. The termination codons are indicated by asterisks. Cys^{70}, Gly^{260}, Gly^{263}, and Lys^{266} residues in ST1B2 are underlined.

although they differ in the number of amino acid residues. A GXXGXXK motif common to all cytosolic sulfotransferases in mammalian species (*17*) was found at Gly^{260}, Gly^{263}, and Lys^{266} in ST1B2. It has been proposed that this motif is associated with a "PAPS-binding site" (*18*) because of its homology with the consensus sequence, GXXXXGK, described as the glycine-rich phosphate-binding loop (p-loop) (*19*). In addition, a cysteine residue conserved in the ST1 gene family of sulfotransferases (*9*) was observed in ST1B2 (Cys^{70}).

Similarity of ST1B2 with Other Human Sulfotransferases—The deduced amino acid sequence of ST1B2 was

compared with those of other human sulfotransferases: two aryl sulfotransferases, ST1A2 and ST1A3 (*20*), a thermolabile phenol sulfotransferase, ST1A5 (*21*), an estrogen sulfotransferase, ST1E4 (*22*), and a DHEA sulfotransferase, ST2A3 (*23*) (Table II and Fig. 3). ST1B2 showed 53% homology to ST1A2, ST1A3, and ST1A5, similar homology to ST1E4 (56%), but lower homology to ST2A3 (36%). These results indicate that ST1B2 belongs to the ST1 family rather than the ST2 family.

A dendrogram of typical mammalian sulfotransferases based on their sequence homologies is shown in Fig. 4. The tree suggests that ST1B2 diverged from an ST1 ancestral gene together with other ST1 subfamilies of sulfotransferases.

TABLE II. **Similarity of human cytosolic sulfotransferases.** The homology (percent similarity) was calculated by use of the Gene Works software Ver. 2.3. Human sulfotransferases are arbitrarily termed from the homology of their primary structures in our laboratory.

	ST1B2	ST1A2	ST1A3	ST1A5	ST1E4
ST1A2[a]	53				
ST1A3[a]	53	96			
ST1A5[b]	53	90	93		
ST1E4[c]	56	50	50	49	
ST2A3[d]	36	34	35	35	34

[a]Aryl sulfotransferases; Ozawa *et al.* (*20*). [b]Thermolabile phenol sulfotransferase; Wood *et al.* (*21*). [c]Estrogen sulfotransferase; Aksoy *et al.* (*22*). [d]Dehydroepiandrosterone sulfotransferase; Otterness *et al.* (*23*).

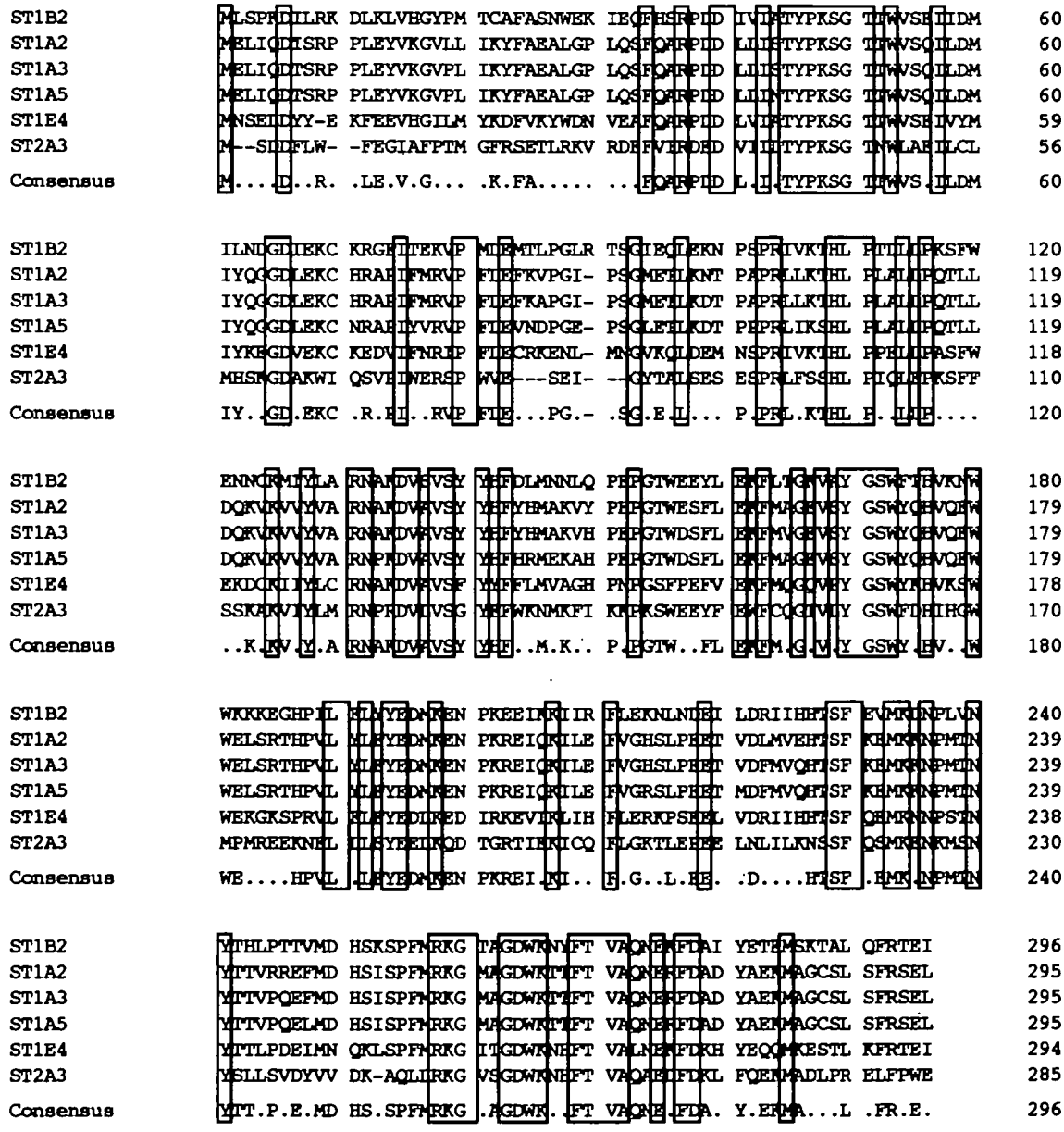

Fig. 3. **Deduced amino acid sequences of human sulfotransferases.** Amino acid sequence deduced from ST1B2 cDNA is aligned with amino acid sequences of human sulfotransferases: ST1A2 and ST1A3, phenol-sulfating forms of phenol sulfotransferase (*20*); ST1A5, a monoamine-sulfating form of phenol sulfotransferase (*21*); ST1E4, an estrogen sulfotransferase (*22*); ST2A3, a dehydroepiandrosterone sulfotransferase (*23*). Residues conserved among all forms are boxed. The consensus sequence is shown at the bottom. Dashed lines indicate unaligned amino acids between those sequences.

Detection of ST1B2 mRNA in Human Liver by RT-PCR—To verify the expression of ST1B2 mRNA in human livers, RT-PCR was performed with poly(A)$^+$ RNAs from four individual human livers. The 923-bp PCR product was amplified in all four samples (Fig. 5) using primer HST1B-ORF-5′ and HST1B-ORF-3′ as specific probes for ST1B2 cDNA. As a control, the 1,064-bp PCR products were also amplified using primer ST1A5-5′ and ST1A5-3′ as specific probes for ST1A5 cDNA, in the same samples (Fig. 5).

Western Blot Analysis of ST1B2 in Liver Cytosols, and Expression of ST1B2 in COS-1 Cells and Escherichia coli—ST1B2 protein was detected by Western blotting of liver cytosols prepared from the same four individuals. A single 32.5 kDa band was clearly detected in each individual cytosol by use of anti-6xH ST1B2 antibody, whose migration was identical with that of COS-1 cells transfected with pCMV4-ST1B2-ORF (Fig. 6). The content of ST1B2 varied considerably among individual samples. 6xH ST1B2 expressed in *E. coli* was detected at 34.5 kDa, which was 2 kDa higher than ST1B2 expressed in COS-1 cells (Fig. 6). To determine the specificity of this antibody, its immunocross-reactivities with purified 6xH ST1A3 and 6xH ST1A5 were evaluated by immunoblot analysis. The reactivities were very low (less than 10%) compared with 6xH ST1B2. The antibody crossreacted with more than 50 ng of purified 6xH ST1A3 and 6xH ST1A5, but not with 25 ng.

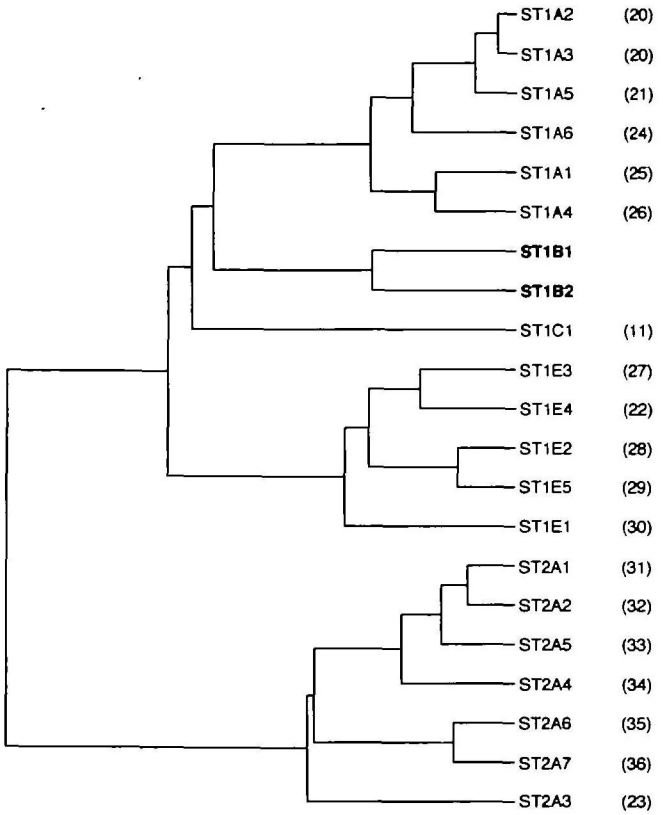

Fig. 4. **Evolutionary relationship of typical mammalian cytosolic sulfotransferases.** The evolutionary tree was made by analysis of homology of deduced amino acid sequences of 21 different cytosolic sulfotransferases in mammals by use of the Gene Works program (Intelli Genetics). References are shown on the right of each sulfotransferase. Enzymes are included in the same family (ST1 or ST2) if their amino acid sequences show more than 40% homology with each other, and they are included in the same subfamily (ST1A, ST1B, ST1C, ST1E, and ST2A) if their amino acid sequences share more than 60% homology.

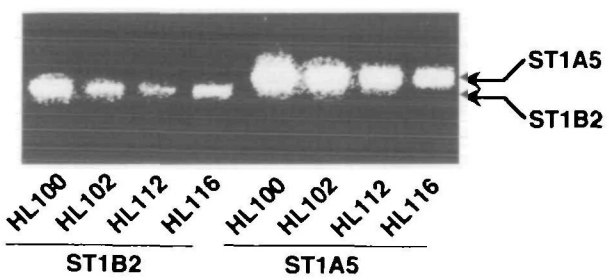

Fig. 5. **Detection of ST1B2 mRNA by the RT-PCR method.** Using 50 ng of liver poly(A)$^+$ mRNA from four human individuals as a template, RT-PCR was performed with ST1B2 primers and ST1A5 primers as described "MATERIALS AND METHODS." PCR products were visualized by staining with ethidium bromide after 1.0% agarose gel electrophoresis.

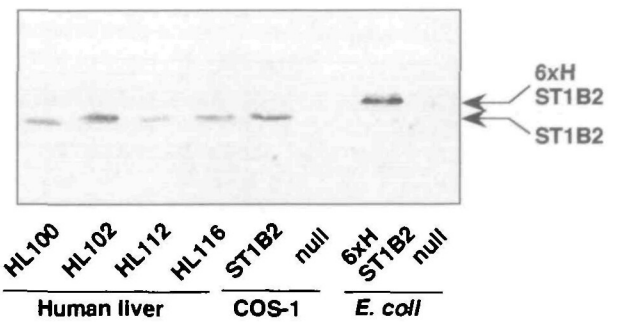

Fig. 6. **Western blot analysis of cytosolic protein of human livers, and ST1B2-transfected COS-1 cells and *Escherichia coli*.** Cytosolic proteins (human livers, 20 μg; COS-1 cells, 20 μg; and *E. coli*, 0.1 μg) were subjected to electrophoresis in 10.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was stained using rabbit anti-ST1B2 antisera, alkaline phosphatase-conjugated goat anti-rabbit IgG, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

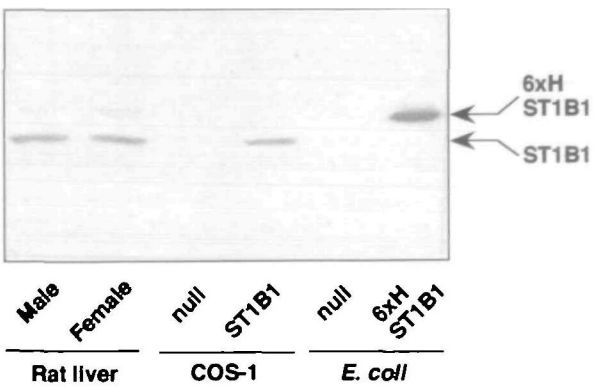

Fig. 7. **Western blot analysis of cytosolic protein of rat livers, and ST1B1-transfected COS-1 cells and *Escherichia coli*.** Cytosolic proteins (rat livers, 20 μg; COS-1 cells, 5 μg; and *E. coli*, 0.5 μg) were subjected to electrophoresis in 10.5% SDS-polyacrylamide gels. The membrane was stained using rabbit anti-ST1B1 antisera, peroxidase-conjugated goat anti-rabbit IgG, diaminobenzidine, and hydrogen peroxide.

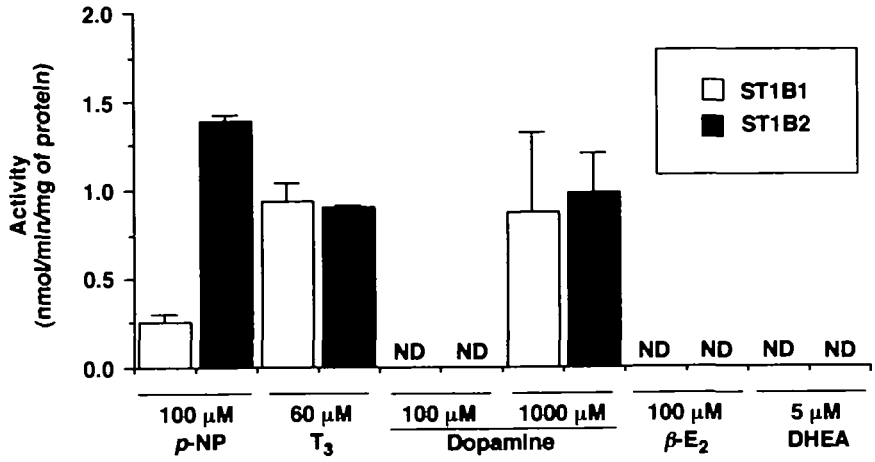

Fig. 8. **Sulfotransferase activities of recombinant ST1B1 and ST1B2 expressed in *Escherichia coli*.** Sulfotransferase activities were measured in triplicate under the conditions described in "MATERIALS AND METHODS," at the concentrations of 100 μM at pH 6.6 for *p*-NP, 60 μM at pH 8.0 for T_3, 100 and 1,000 μM at pH 8.0 for dopamine, 100 μM at pH 6.6 for β-E_2, and 5 μM at pH 6.0 for DHEA. Incubations were carried out at 37°C for 15 min (*p*-NP, dopamine, and DHEA) or 20 min (T_3 and β-E_2). ND, not detectable.

TABLE III. **Kinetic parameters for sulfations of *p*-nitrophenol and 3,3′,5-triiodothyronine by ST1B1 and ST1B2 expressed in *Escherichia coli*.** Individual concentrations used for K_m values in *p*-NP were 10, 20, 40, 50, 100, and 200 μM for ST1B1, and 5, 10, 20, 40, 50, and 100 μM for ST1B2. Individual concentrations used for K_m values in T_3 were 5, 10, 20, 40, 50, and 100 μM for ST1B1 and ST1B2. Parameters are derived from the analyses of Lineweaver-Burk plots and represent mean by four separated experiments.

Enzyme	*p*-Nitrophenol			3,3′,5-Triiodothyronine		
	K_m (μM)	V_{max} (nmol/min/mg of protein)	V_{max}/K_m ($\times 10^3$)	K_m (μM)	V_{max} (nmol/min/mg of protein)	V_{max}/K_m ($\times 10^3$)
ST1B1	115.9	0.542	4.68	40.2	1.27	31.6
ST1B2	24.1	5.1	211.6	63.5	1.59	25.0

TABLE IV. **Kinetic parameters for sulfations of 3,3′,5-triiodothyronine, *p*-nitrophenol, and dopamine by recombinant ST1B2, ST1A3, and ST1A5 expressed in *Escherichia coli*.** Concentrations of sulfate acceptor substrates for K_m values are described below. Parameters are derived from the analyses of Lineweaver-Burk plots and represent the means of four separate experiments.

	3,3′,5-Triiodothyronine	*p*-Nitrophenol	Dopamine
ST1B2	63.5 μM[a]	24.1 μM[a]	350 μM[e]
ST1A3	413 μM[b]	0.57 μM[c]	722 μM[e]
ST1A5	180 μM[b]	2980 μM[d]	3.1 μM[f]

[a]5, 10, 20, 40, 50, and 100 μM. [b]10, 20, 40, 50, 100, and 200 μM. [c]0.1, 0.2, 0.4, 0.5, 1, and 2 μM. [d]100, 200, 400, 500, 1,000, and 2,000 μM. [e]50, 100, 200, 400, 500, and 1,000 μM. [f]1, 2, 4, 5, 10, and 20 μM.

Using rabbit anti-6xH ST1B1 antibody, immunoreactive ST1B1 protein in rat livers was also investigated. A single 33.0 kDa band was detected in cytosol preparations from rat livers of both sexes and ST1B1 cDNA-expressed COS-1 cells (Fig. 7). Similar to the case of ST1B2, 6xH ST1B1 was detected at a 2 kDa higher position than ST1B1 transfected in COS-1 cells (Fig. 7).

Catalytic Properties of ST1B2 and ST1B1 Expressed in Escherichia coli—Sulfation of a typical ST1 substrate, *p*-NP, was quantitated using cytosols of ST1B2- and ST1B1-expressed *E. coli* (Fig. 8). 6xH ST1B2 and 6xH ST1B1 catalyzed the sulfation of *p*-NP at 1.39 and 0.26 nmol/mg of protein/min, respectively, in the presence of 100 μM *p*-NP.

Sulfating properties of ST1B1 and ST1B2 toward an endogenous substrate, T_3, were also studied (Fig. 8). 6xH ST1B2 and 6xH ST1B1 sulfated T_3 at 0.89 and 0.93 nmol/mg of protein/min, respectively, in the presence of 60 μM T_3.

The abilities of ST1B2 and ST1B1 to sulfate other substrates including dopamine, β-E_2 and DHEA were also examined. Dopamine was sulfated by 6xH ST1B2 and 6xH ST1B1 at the respective rates of 0.97 and 0.87 nmol/mg of protein/min in the presence of 1,000 μM dopamine, but not in the presence of 100 μM (Fig. 8). No detectable activities towards β-E_2 and DHEA were observed under the present assay conditions (Fig. 8), although activities towards β-E_2 and DHEA were observed with human liver cytosol preparations under these conditions (data not shown).

Kinetic Characterization of Expressed ST1B2 and ST1B1 in Escherichia coli—The ranges of substrate concentrations for *p*-NP and T_3 were used to determine kinetic properties of ST1B2 and ST1B1. The data obtained with 6xH ST1B2 and 6xH ST1B1 are shown in Table III. Apparent K_m and V_{max} values of 6xH ST1B2 and 6xH ST1B1 for *p*-NP were calculated as 24.1 μM and 5.1 nmol/mg of protein/min, and 115.9 μM and 0.542 nmol/mg of protein/min, respectively. Under similar conditions, apparent K_m and V_{max} values of 6xH ST1B2 and 6xH ST1B1 for T_3 were calculated as 63.5 μM and 1.59 nmol/mg of protein/min, and 40.2 μM and 1.27 nmol/mg of protein/min, respectively.

Apparent K_m values of 6xH ST1B2 for *p*-NP, T_3, and dopamine were also compared with those of 6xH ST1A3 and 6xH ST1A5, which corresponded to phenol- and monoamine-sulfating forms of phenol sulfotransferases, respectively (Table IV). The apparent K_m value for T_3 was lowest with 6xH ST1B2 (63.5 μM) among the three forms. However, apparent K_m values were lowest with 6xH ST1A3 for *p*-NP and with 6xH ST1A5 for dopamine, at respective values of 0.57 and 3.1 μM. These apparent K_m values of 6xH ST1A3 for *p*-NP and 6xH ST1A5 for dopamine were similar to the values previously reported with recombinant HAST1 (corresponding to ST1A3) and HAST3 (corresponding to ST1A5) activities, respectively (*37*). 6xH ST1B2 was also enzymatically active in sulfation of *p*-NP and dopamine with intermediate affinities (24.1 and 350 μM, respectively) among the three forms.

DISCUSSION

Sulfation facilitates the subsequent deiodination of T_3 and is thus considered to be an essential step for elimination of its hormonal activities in the body (*4*). To identify the functional sulfotransferase, a human cDNA library was screened using rat ST1B1 cDNA as a probe to isolate the human counterpart, ST1B2 cDNA (Fig. 2). Primary structures of over 20 different forms of mammalian sulfotransferases have now been elucidated by cDNA cloning (Fig. 4). The deduced amino acid sequences of 5 different sulfotransferases are compared with that of ST1B2 (Fig. 3 and Table II). While human ST1B2 shares 74% homology with rat ST1B1, human ST1B2 showed much less homology with other human sulfotransferases, ST1A2/3/5 (53%), ST1E4 (56%), and ST2A3 (36%). These results indicate that ST1B2 and ST1B1 constitute a new gene subfamily (ST1B) of aryl sulfotransferase.

Using ST1B1 and ST1B2 expressed in COS-1 cells and in bacteria, their immunochemical and catalytic properties were determined. An *E. coli* system was mainly used for preparation of antibodies and characterization of substrate specificities. The *E. coli*-expressed enzymes showed the identical substrate specificities and similar kinetic parameters to the enzymes expressed in COS-1 cells, although the former are fusion proteins having 17 additional amino acids at their N-terminals (Fujita, K. *et al.*, unpublished results). The recombinant ST1B2 and ST1B1 catalyzed the sulfation of *p*-NP, T_3, and dopamine, but not of β-E_2 and DHEA (Fig. 8). These results indicate that ST1B2 and ST1B1 have substrate specificities distinct from other aryl sulfotransferase subfamilies (ST1A, ST1C, and ST1E). ST1B1 and ST1B2 catalyzed the sulfation of T_3 with high affinities and rates (Table III).

In human liver, two forms of aryl sulfotransferase have been shown to catalyze sulfation of phenols (*38*). The 32.0 kDa phenol-preferring sulfotransferase (also referred to as thermostable phenol sulfotransferase; *20, 39*) and the 34.0 kDa monoamine-preferring sulfotransferase (also referred to as thermolabile phenol sulfotransferase; *21, 39*) correspond respectively to what we have termed ST1A2/3 and ST1A5. To further clarify the immunochemical and enzymatic properties of ST1B2 as a human T_3 (aryl) sulfotransferase, ST1A3 and ST1A5 were expressed in *E. coli*. In Western blotting using anti-6xH ST1B2 antibody, ST1B2 protein was detected as 32.5 kDa protein in human livers (Fig. 6). This antibody did not crossreact with 25 ng of 6xH ST1A3 or 6xH ST1A5. In addition, the molecular weights of these forms were clearly distinct from that of ST1B2 estimated by SDS-PAGE (data not shown). Furthermore, as shown in Table IV, the K_m for T_3 of 6xH ST1B2 (63.5 μM) was one order of magnitude lower than those of 6xH ST1A3 (413 μM) and 6xH ST1A5 (180 μM), and previously purified forms of phenol sulfotransferases (K_m 81 to 217 μM) from human liver reported by Young *et al.* (*8*). *p*-NP and dopamine were the preferred substrates for 6xH ST1A3 (K_m 0.57 μM) and 6xH ST1A5 (K_m 3.1 μM), respectively, whereas 6xH ST1B2 showed intermediate affinities for these substrates (Table IV).

In our previous studies (*7*), hepatic sulfation of T_3 was shown to be 4–8 times higher in male rats than female rats. The major sulfotransferase ST1A1 and its mRNA were detected in livers of male and female rats, and showed no sex-related difference (*9*). Levels of ST1C1 mRNA and the protein were, however, higher in livers of male rats than female rats (*11*). ST1B1 protein was also detected in livers of the both sexes without clear sex-related difference (Fig. 7). In our preliminary experiments, T_3-sulfation was mediated by recombinant ST1B1 and ST1C1, but not by ST1A1 (Fujita, K. *et al.*, unpublished results). These results indicate that the higher rate of T_3 sulfation in male rats than female rats is mainly caused by the male-dominant expression of ST1C1 in livers. ST1B1 is also capable of catalyzing the sulfation of T_3 (Fig. 8) in rats of both sexes. The K_m value (40.2 μM) of 6xH ST1B1 is 3 times lower than that of rat liver cytosol (114 μM) reported previously (*40*). This form is thus considered to be a main form of T_3 sulfotransferase. In addition, our data on the recombinant ST1B1 are roughly consistent with those on purified Dopa/Tyrosine sulfotransferase (*12*) in terms of substrate specificity but not apparent K_m value for *p*-NP (115.9 μM-ST1B1 versus 30.9 mM-Dopa/Tyrosine sulfotransferase). One amino acid change may account for the difference between the two enzymes.

In human liver, no sex-related difference was observed in T_3-sulfating activity (*7*). In addition, human ST1C form (mRNA and protein), the ortholog of rat ST1C1, is not detected in the liver (*41*), and ST1B2 was capable of catalyzing sulfation of T_3 (Fig. 8). These findings indicate that ST1B1 and ST1B2 are the main forms of T_3 sulfotransferases in rat and human livers, respectively. We are studying further the roles of ST1B1 and ST1B2 as T_3-sulfotransferases *in vivo*.

In summary, the present study has shown by cDNA isolation and expression, the structural, immunochemical, and functional properties of two new sulfotransferases, rat ST1B1 and human ST1B2. These forms constitute a new ST1 subfamily (ST1B), which is characterized by sulfation of T_3.

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