

Conservation of the Hydroxysteroid Sulfotransferase *SULT2B1* Gene Structure in the Mouse: Pre- and Postnatal Expression, Kinetic Analysis of Isoforms, and Comparison with Prototypical *SULT2A1*

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A novel mouse hydroxysteroid sulfotransferase cDNA has been cloned, and organization of its gene structure has been determined. The new mouse sulfotransferase, *SULT2B1a*, and its closely related isoform, *SULT2B1b*, are derived from a single *SULT2B1* gene as a result of an alternative exon I and differential splicing. Thus, the only structural distinction between the two *SULT2B1* isoforms is at their amino-terminal ends. Importantly, in contrast to the prototypical mouse hydroxysteroid sulfotransferase *SULT2A1*, the *SULT2B1* isoforms have a predilection for cholesterol. Real-time RT-PCR reveals that the *SULT2B1a* isoform is most abundantly expressed in the brain and spinal cord, whereas *SULT2B1b* and *SULT2A1* are weakly, if at all, expressed in the central nervous system. On the other hand, the *SULT2B1b* isoform is the most

prominent hydroxysteroid sulfotransferase expressed in skin, whereas *SULT2A1* is strikingly expressed in the liver. The substrate specificities and differential expression patterns of the three *SULT2* isozymes strongly suggest that they have distinct biologic roles to play. Of further interest, the mouse *SULT2B1* and *SULT2A1* genes are differentially expressed during embryonic development, with the former being expressed at all stages from E8.5–E19, whereas the latter is not expressed until E19. It is speculated that, during embryonic development, *SULT2B1b* is required for production of cholesterol sulfate essential for normal skin development, whereas *SULT2B1a* produces pregnenolone sulfate, an essential neurosteroid during development of the central nervous system. (*Endocrinology* 144: 1186–1193, 2003)

A SIGNIFICANT DEVELOPMENT in the field of cytosolic sulfotransferases has been the discovery of a hydroxysteroid sulfotransferase isozyme in humans and mice that is structurally unique and distinct from all previously cloned mammalian hydroxysteroid sulfotransferases, as well as other cognate cytosolic sulfotransferases (1, 2). Whereas *SULT2A1* is the prototypical mammalian hydroxysteroid sulfotransferase, the new *SULT2* subfamily has been designated *SULT2B1* (3). The original cloning of the mouse ortholog of *SULT2B1* revealed a single cDNA (2). We have now cloned a second isoform of mouse *SULT2B1*, determined the *SULT2B1* gene structure, and resolved that the two mouse *SULT2B1* isoforms arise from a single gene as a result of an alternative exon I. Thus, the mouse *SULT2B1* isoforms structurally differ only at their amino termini.

To gain insight into functionality of the mouse *SULT2* subfamilies and perhaps a clue as to their physiologic relevance, we have overexpressed and purified the three mouse *SULT2* isozymes for use in examining substrate preferences and performing kinetic analyses. Furthermore, we have determined tissue expression patterns by real-time PCR as well as expression during embryonic development by traditional RT-PCR.

Abbreviations: CNS, Central nervous system; EST, expressed sequence tags; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione-S-transferase; k_{cat} , catalytic constant; K_m , Michaelis-Menten constant; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; pI, isoelectric point; RACE, rapid amplification of cDNA ends; RT, reverse transcription; TLC, thin-layer chromatography; UTR, untranslated region.

Materials and Methods

Materials

Cholesterol, steroids, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), 2-hydroxypropyl- β -cyclodextrin, and iodine crystals were obtained from Sigma (St. Louis, MO). [3 H]cholesterol (60 Ci/mmol), [3 H]pregnenolone (14–17.5 Ci/mmol), and [3 H]DHEA (60 Ci/mmol) were purchased from Perkin-Elmer Life Science Products (Boston, MA). Silica gel thin-layer chromatography (TLC) plates were procured from Analtech (Newark, DE). Organic solvents were obtained from Mallinckrodt-Baker (Phillipsburg, NJ).

RNA extraction

Female mice at 5 months of age (C57BL/6; Charles River Laboratories, Inc., Wilmington, MA) were used for isolation of total RNA from the hypothalamus and adrenal glands. Total RNA was extracted using the Absolutely RNA RT-PCR Miniprep Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The use of animals was carried out under an approved protocol and in accordance with NIH Guidelines for the Care and Use of Animals.

Cloning of mouse *SULT2B1a* cDNA

A mouse expressed sequence tags (EST) database was searched using Basic Local Alignment Search Tool (BLAST) and a bait sequence that corresponded to a region of the *SULT2B1b* cDNA (GenBank accession no. AF026072), presumably located in the second exon, based on the genomic organization of the human *SULT2B1* gene previously reported (1). A single EST clone (GenBank accession no. BE863542), with a partially different sequence from the mouse *SULT2B1b* cDNA, was obtained. To isolate the full-length cDNA of the EST clone, 1 μ g hypothalamic total RNA was reverse transcribed with random hexamer primers, using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). Subsequently, high-fidelity PCR with *Pfu* Turbo Hotstart DNA polymerase (Stratagene) was performed using the EST clone-specific

sense primer (5'-GTCGACG_(nt122)TATGACATCACGGGACTGCTG-TGGTG-GAG_(nt151)-3') and mouse SULT2B1b-specific antisense primer (5'-GCGGCCGCT_(nt1086)TTATT-GTGAGGATCCTGGGTTGGGGTCAT-C_(nt1057)-3'). *Underlines* indicate *Sall* and *NotI* restriction sites, respectively. PCR conditions were: denaturing at 95 C for 3 min, followed by 45 cycles of denaturing at 95 C for 30 sec, annealing at 62 C for 30 sec, and extension at 72 C for 2 min. PCR products were purified, subcloned into pCR2.1-TOPO vector (Invitrogen), designated pCR-mSULT2B1a, and sequenced on both strands. The analysis to determine genomic organization was carried out by aligning the mouse SULT2B1a and SULT2B1b cDNAs with the mouse genome draft sequence (GenBank accession no. AC073774) derived from mouse chromosome 7. Briefly, information on mouse SULT2B1 was searched for at the UniGene web site (<http://www.ncbi.nlm.nih.gov/unigene/>). Using the MacVector 7.0 Clustal W program, homologous regions between the mouse genome and the mouse SULT2B1 cDNAs were determined.

5'-Untranslated region (UTR) of mouse SULT2B1a cDNA

To confirm the 5'-UTR, 5'-rapid amplification of cDNA ends (RACE) was employed using the SMART RACE cDNA Amplification Kit according to the manufacturer's instructions (BD Biosciences, San Diego, CA). Briefly, a mixture of 0.3 μ g hypothalamic total RNA and SMART II A oligonucleotide was used to carry out a reverse transcription (RT) reaction using a 5'-RACE CDS primer provided with the kit. PCR was performed using universal primer mix A and SULT2B1a-specific primer (5'-T_(nt224)CATTCACACGCCAGTGAGGAGTGTC_(nt199)-3'). PCR conditions were: heat denature at 94 C for 5 sec and annealing/extension at 72 C for 2 min, 5 cycles; heat denature at 94 C for 5 sec, annealing at 70 C for 10 sec, and extension at 72 C for 1 min, 5 cycles; heat denature at 94 C for 5 sec, annealing at 68 C for 10 sec, and extension at 72 C for 1 min, 35 cycles. PCR products were analyzed by electrophoresis using 2% agarose gels. After gel purification, PCR products were subcloned into pCR2.1-TOPO vector and sequenced.

mRNA expression analysis of adult mouse SULT2A1, SULT2B1a, and SULT2B1b isozymes by real-time RT-PCR

Total RNA, isolated from mouse skin and small intestine, was obtained from OriGene Technologies, Inc. (Rockville, MD), whereas total RNA isolated from all other mouse tissues was obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA). RT was performed using the ThermoScript RT-PCR system according to the manufacturer's instructions (Invitrogen). Briefly, using 3 μ g total RNA as a template, first-strand cDNA was made using 25 pmol oligo(deoxythymidine) 20 and 25 ng random hexamer primer (Invitrogen) in a 20- μ l reaction vol. After heat denaturing at 65 C for 5 min, RT was carried out at 25 C for 10 min and then 60 C for 50 min.

Real-time PCR was performed using a fluorescence temperature cycler (LightCycler) and SYBR Green I as a double-stranded DNA-specific binding dye, according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). This technique continuously monitors the cycle-by-cycle accumulation of fluorescently labeled PCR product. Amplifications were carried out using 1 U Platinum *Taq* DNA Polymerase (Invitrogen), 0.5 μ M of each primer, 3 mM MgCl₂, 10 \times Platinum *Taq* DNA polymerase buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 0.2 mM deoxynucleotide triphosphate, 1 mg/ml BSA, 1 μ l 1:2000 diluted SYBR Green I nucleic acid gel stain (BioWhittaker, Inc. Molecular Applications, Rockland, ME), and 2 μ l 1:5 diluted cDNA in a total vol of 20 μ l. The real-time PCR conditions were: preheat denature at 95 C for 5 min, annealing at 59 C for 10 sec, and extension at 72 C for 7 sec; cycle number, 45. SYBR Green I fluorescence was detected at 72 C at the end of each cycle to monitor the amount of PCR product formed during that cycle. A melting curve analysis of the amplification products was performed at the end of the PCR run by rapidly increasing the temperature to 95 C; followed by immediate cooling to 65 C for 15 sec; after which, the temperature was gradually increased to 95 C at a rate of 0.1 C/sec with continuous measurement of fluorescence to confirm amplification of specific transcripts. The melting temperature profile for all samples of SULT2A1, SULT2B1a, and SULT2B1b, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), demonstrated single peaks at 88, 89, 88, and 89 C, respectively. The interassay and intraassay

coefficients of variation were calculated to be 7.4% and 5.7%, respectively, using SULT2B1b primer and skin cDNA.

Primer sequences used were: 5'-A_(nt28)TGATGTCAGACTATAAT-TGGTTTGAAGGC_(nt57)-3' (sense) and 5'-A_(nt319)GGTTATGAGTCGTG-TCTTCTTATTG_(nt291)-3' (antisense) for SULT2A1, 5'-A_(nt200)-CACTCCTCACTGGCGTGTGAATG_(nt223)-3' (sense) and 5'-T_(nt551)TG-AAGGCGCTTATGATGGTCTCGC_(nt527)-3' (antisense) for SULT2B1a, and 5'-T_(nt95)GTGGAGCTCGTCTGAGAAAAATGTTTCCG_(nt124)-3' (sense) and the same primer as SULT2B1a (antisense) for SULT2B1b. Primers were designed to recognize a different exon in each gene; furthermore, the appropriate size of PCR products was verified by agarose gel electrophoresis. External cDNA standards for SULT2A1, SULT2B1a, and SULT2B1b were produced by inserting PCR products, which were generated using the same primers as noted above, and liver, brain, and skin cDNAs as templates, into the pCR2.1 vector using the TOPO TA Cloning kit (Invitrogen). Vector constructs were used to transform XL1-blue (Stratagene), and plasmid DNA was prepared by QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). Inserts of control vectors for the SULT2s were verified by sequencing. The concentration of each standard was determined by measuring the OD₂₆₀ and the copy number calculated.

Mouse GAPDH was quantified to normalize SULT2 mRNA levels, and the final results are expressed as the ratio of the copy number of a specific SULT2 to the copy number of GAPDH. Primer sequences for GAPDH were: 5'-A_(nt140)ACGACCCCTTCATTGAC_(nt157)-3' (sense) and 5'-T_(nt330)CCACGACATACTCAGCAC_(nt312)-3' (antisense). The appropriate size of PCR product was verified by agarose gel electrophoresis. Quantitative standards were generated similarly to those for the SULT2s by cloning the product obtained by PCR amplification of skin cDNA.

mRNA expression analysis of embryonic mouse SULT2A1, SULT2B1a, and SULT2B1b isozymes by RT-PCR

Expression profile of the SULT2 isozymes during mouse embryonic development (Inbred of FVB) was analyzed using RAPID-SCAN gene expression panels (OriGene Technologies, Inc.). Lyophilized cDNAs were suspended in 25 μ l H₂O, and 4- μ l (for detection of SULT2 mRNAs) and 2- μ l (for detection of β -actin mRNA) aliquots were used as templates. Primers used were: 5'-GTCGACGTATGATGTCAGACTATA-ATTGGTTTGAAGGC-3' (sense) and 5'-AGGTTATG-AGTCGTGGTC-TTCTTATTG-3' (antisense) for mouse SULT2A1, 5'-ACACTCCTC-ACTG-GCGTGTGAATG-3' (sense) and 5'-TTGAAGGCGCTTATGA-TGGTCTCGC-3' (antisense) for mouse SULT2B1a, and 5'-TGTGG-AGCTCGTCTGAGAAAAATGTTTCCG-3' (sense) and 5'-TTGAA-GGCGCTTATGATGGTCTCGC-3' (antisense) for mouse SULT2B1b. Expected sizes of SULT2A1, SULT2B1a, and SULT2B1b PCR products were, respectively, 299 bp, 341 bp, and 300 bp. PCR conditions were: denaturing at 95 C for 5 min, followed by 35 cycles of denaturing at 94 C for 15 sec, annealing at 55 C for 15 sec, and extension at 72 C for 30 sec. Each PCR product was subcloned and sequenced. To check quality and quantity of cDNA used, PCR was also performed using β -actin-specific primers provided with the kit. PCR products were analyzed by electrophoresis using 3% agarose gels.

Construction of mouse SULT2A1, SULT2B1a, and SULT2B1b expression vectors

To isolate SULT2A1 cDNA, RT-PCR was employed. One microgram of adrenal total RNA was reverse transcribed using ThermoScript RT-PCR System (Invitrogen), and the cDNA was amplified with mouse SULT2A1-specific primers: 5'-GTCGACGTATGATGTCAGACTATA-ATTGGTTTGAAGGC-3' (sense), 5'-GCGGCCGCTTATTTCCATGG-GAACATCCTGGGGGAATC-3' (antisense). *Underlines* indicate *Sall* and *NotI* sites, respectively. PCR conditions with high-fidelity DNA polymerase were: denaturing at 95 C for 3 min, followed by 45 cycles of denaturing at 95 C for 30 sec, annealing at 62 C for 30 sec, and extension at 72 C for 2 min. The PCR product was subcloned into pCR 2.1-TOPO vector (Invitrogen) and sequenced. After digestion with *Sall* and *NotI*, insert DNA was ligated to *Sall/NotI*-digested pGEX-6P-1 (Amersham Biosciences, Piscataway, NJ). SULT2B1b cDNA (GenBank accession no. AF026072), kindly provided by Dr. Ming-Cheh Liu (University of Texas Health Center, Tyler, TX), was digested with *EcoRI* and *NotI* and ligated into *EcoRI/NotI*-digested pGEX-6P-1 vector. To generate SULT2B1a ex-

pression vector, pCR-mSULT2B1a was digested with *Sall* and *NotI*, and the insert DNA fragment was ligated to *Sall/NotI*-digested pGEX-6P-1 vector. Proper clones were verified by sequencing.

Generation of recombinant mouse SULT2A1, SULT2B1a, and SULT2B1b

Glutathione-S-transferase (GST) gene fusion system (Amersham Biosciences) was employed for purification of bacterially overexpressed recombinant proteins. Briefly, plasmids were used for transformation of BL21-Gold (DE3) pLysS (Stratagene). Vectors were mixed with competent cells, incubated on ice for 30 min, heat-shocked at 42 C for 45 sec, and incubated on ice for 2 min. Bacterial cultures were added to 100 ml Luria-Bertani broth and grown overnight at 26 C in ampicillin medium to minimize expressed proteins in inclusions bodies. Additional Luria-Bertani broth containing ampicillin was then added to increase culture volumes to 1 liter, and incubations continued until the OD_{595 nm} reached 0.6, at which time, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 50 μM. After overnight incubation, cells were collected by centrifugation, and the bacterial pellets were frozen at -80 C. Pellets were extracted by sonication in iced PBS, to which had been added a protease inhibitor cocktail tablet (Roche Molecular Biochemicals) and 1 mg/ml lysozyme (Sigma). The sonication step was carried out for 1 min and repeated 4 times on ice. Extracts were centrifuged at 204,000 × *g* for 1 h at 4 C. Supernatants were collected, mixed with 1 ml glutathione Sepharose 4B resin (Amersham Biosciences), and incubated for 1–3 h at 4 C. The resin mixture of GST-fusion protein was transferred to a plastic column and washed with a 25-column volume of cleavage buffer [50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol]. Forty microliters (80 U) of PreScission Protease (Amersham Biosciences) in 1 ml cleavage buffer were applied per 1 ml column resin and incubated overnight at 4 C. After elution, protein concentrations were determined by BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL) using BSA as standard. To check the quality of recombinant proteins, SDS-PAGE was performed using a NuPAGE 10% Bis-Tris gel (Invitrogen) and a standard procedure. Degree of purity, determined by densitometry, was based on expected protein size. For imaging analysis, Quantity One Software (Bio-Rad Laboratories, Inc., Hercules, CA) was used.

Kinetic analyses of mouse SULT2A1, SULT2B1a, and SULT2B1b

Sulfotransferase activity was determined using radiolabeled cholesterol, pregnenolone, and DHEA. A 20-μl reaction vol contained a specific substrate, 0.1 mM PAPS, and a purified enzyme preparation as described above: SULT2A1 (2 μg), SULT2B1a (0.2 μg), SULT2B1b (0.1 μg) in the cholesterol assay; SULT2A1 (1 μg), SULT2B1a (2 μg), SULT2B1b (1 μg) in the DHEA assay; SULT2A1 (1 μg), SULT2B1a (0.4 μg), SULT2B1b (0.1 μg) in the pregnenolone assay, in 0.1 mM Tris-HCl buffer (pH 7.5) containing 5 mM MgCl₂, 0.2 mM 2-hydroxypropyl-β-cyclodextrin, and 4% ethanol (vol/vol). Reactions were carried out at 37 C for 5 min and stopped at 100 C for 5 min. After adding 10 μl of 5 mg/ml of a respective sulfoconjugated standard as a carrier, 5 μl of aliquots were applied to silica gel-TLC plates, and chromatography was carried out using the solvent system of chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1). After development, TLC plates were dried and exposed to iodine vapor to visualize location of the sulfoconjugated products. The iodine-adsorbed spots were excised, the silica was placed into counting vials containing 5 ml scintillation cocktail, and the radioactivity was determined by liquid scintillation spectrometry.

Amino acid sequence analysis

Multiple alignment analysis was carried out using the MacVector 7.0 system, which is based on the Clustal W algorithm (4).

Results

Cloning of mouse SULT2B1a

The novel mouse hydroxysteroid sulfotransferase was cloned by searching the mouse EST databank and designated

SULT2B1a to distinguish it from the originally reported mouse SULT2B1 isozyme (2), which we have designated SULT2B1b. The terminology we are using is based on the identical genomic organization of human *SULT2B1*, as noted below, and is thus consistent with the nomenclature used for the human ortholog (1). The 1242-bp mouse SULT2B1a cDNA has been submitted to GenBank and assigned the accession no. AF478566. The 5'-RACE procedure indicated that the 5'UTR is 123 bp in length and contains an in-frame stop codon located 18 bp upstream of the translation initiation codon (data not shown). Based on a search of the mouse genome draft sequence, within 100 bp upstream of the 5'-end of the cDNA, there is neither a TATAAA nor a CAAT box, nor is it a GC-rich region. Interestingly, however, the sequence TCCACTTT, which completely matches the *initiator* consensus motif (PyPyPyA₊₁NA/TPyPy; Refs. 5 and 6) is found 13 bp downstream of the 5'-end of the longest cDNA; furthermore, 4 of six clones obtained by 5'-RACE were initiated from the A₊₁ in the *initiator* sequence. In the coding region that is common to both SULT2B1 cDNAs, there are 4 nucleotide differences (249C/T, 474C/T, 609T/C, and 1088C/T) (SULT2B1a/SULT2B1b; numbering begins with A of the initiation ATG codon). The first three nucleotide differences are silent, whereas the fourth nucleotide variant located near the carboxy termini results in different amino acids, *i.e.* a serine in SULT2B1a and a phenylalanine in SULT2B1b (see Fig. 2). Forty-four EST clones were checked regarding the 1088C/T bp difference, with 32 clones demonstrating a C and 12 indicating a T (data not shown), suggesting that this particular nucleotide variant probably represents a true polymorphism.

Mouse SULT2B1 gene structure

SULT2B1a and SULT2B1b are derived from a common mouse *SULT2B1* gene that is composed of exons IA, IB, and II–VI (Fig. 1A). The SULT2B1a cDNA is encoded by exon IA and exons II–VI, whereas the SULT2B1b cDNA is encoded by exon IB, the 3'-terminal portion of exon IA, and exons II–VI (Fig. 1A). All mouse *SULT2B1* exon-intron splice junction patterns conform to the gt-ag rule (7), as indicated in Fig. 1B.

Amino acid analysis of the mouse SULT2B1 isoforms and comparison with mouse SULT2A1

Mouse SULT2B1a consists of 372 amino acids, with a calculated molecular weight of 42,201 and an estimated isoelectric point (pI) of 5.10. Mouse SULT2B1b contains 338 amino acids, with a calculated molecular weight of 38,404 and an estimated pI of 4.85. For comparison, mouse SULT2A1 contains 285 amino acids, with a calculated molecular weight of 33,326 and an estimated pI of 7.25.

Based on the amino acid sequence alignment (Fig. 2), it is apparent that the SULT2B1 isoforms are identical except for their amino-terminal ends, where the greater length of the amino terminus unique to SULT2B1a accounts for its greater size. The mouse SULT2B1 isoforms are considerably longer than the prototypical mouse hydroxysteroid sulfotransferase, SULT2A1, a feature attributable primarily to the extended amino- and carboxy-terminal ends of the former proteins. Overall, the three proteins are 37% identical; however, if the amino- and carboxy-terminal ends of the SULT2B1 isoforms are excluded from the analysis, amino acid identi-

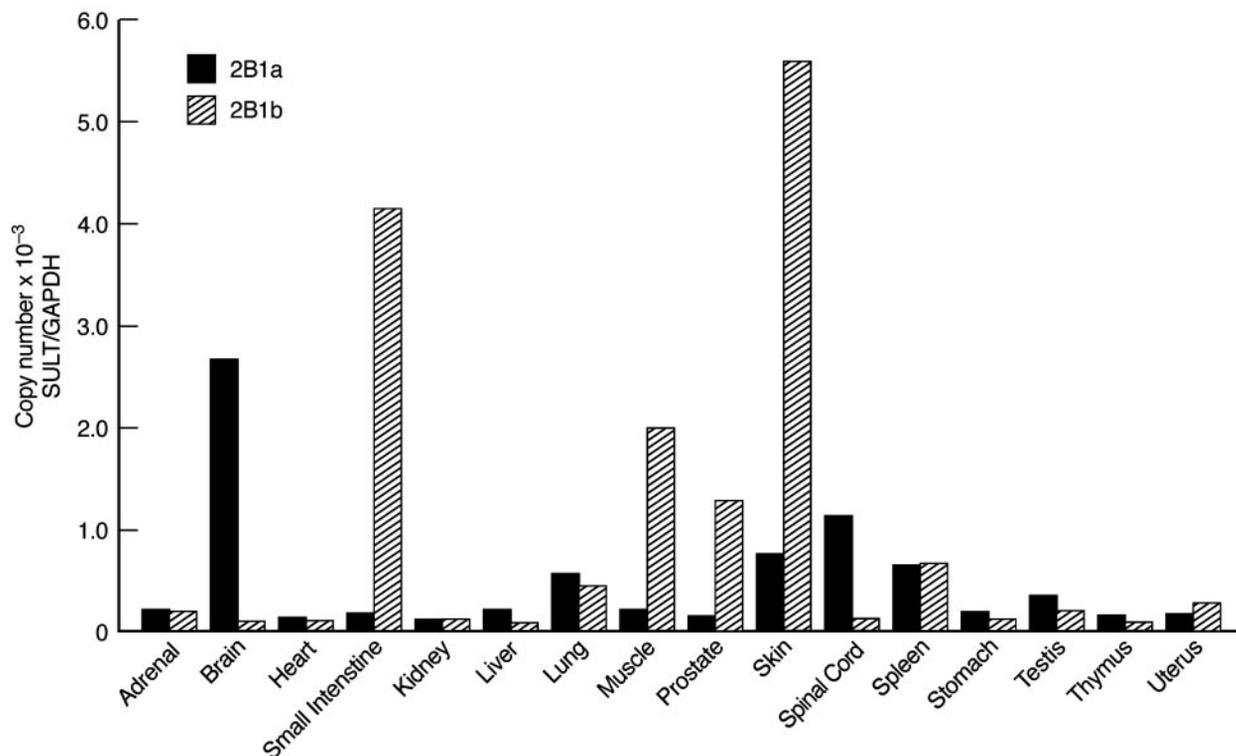


FIG. 3. Expression of mRNA for mouse SULT2B1a (solid columns) and SULT2B1b (hatched columns) in various tissues, as determined by real-time RT-PCR. Column heights represent the ratio of the mRNA copy number of each respective SULT to the mRNA copy number of GAPDH.

and spinal cord, with modest expression in the lung, skin, and spleen. SULT2B1b is most prominently expressed in skin and small intestine, with modest expression in muscle and prostate. The same tissues were also examined for expression of SULT2A1; remarkably, however, expression of this SULT2 isozyme by the liver is exceedingly high, being several orders of magnitude greater than any other tissue (data not presented). Interestingly, real-time PCR reveals that SULT2B1a is most abundantly expressed in the brain, in contrast to SULT2B1b and SULT2A1, which are weakly, if at all, expressed in this tissue (Fig. 4). On the other hand, SULT2B1b is the most prominent SULT2 isozyme expressed in skin (Fig. 4).

Mouse SULT2B1a, SULT2B1b, and SULT2A1 mRNA expression during embryonic development

Temporal expression of the SULT2 genes, using cDNA from whole embryos, reveals that mRNAs for the SULT2B1a and SULT2B1b isoforms are expressed at E8.5, the earliest time examined, whereas mRNA for SULT2A1 is clearly not expressed until E19 (Fig. 5). Furthermore, SULT2B1a mRNA has been identified in mouse brain at E15 (data not presented).

Kinetic analyses of mouse SULT2B1a, SULT2B1b, and SULT2A1

An SDS-PAGE analysis of the purified recombinant SULT2 isozymes revealed that the degree of purity was more than 90% for the three proteins (data not presented). A steady-state analysis of the SULT2 isozymes, using chole-

sterol, pregnenolone, and DHEA as substrates, is depicted in Fig. 6. Although the SULT2B1a isoform sulfonates pregnenolone more vigorously than cholesterol, it does so with an efficiency that is less than half that for cholesterol; furthermore, the Michaelis-Menten constant (K_m) for cholesterol is $0.88 \text{ M} \times 10^{-6}$, whereas the K_m for pregnenolone is $17.7 \text{ M} \times 10^{-6}$. DHEA is a relatively poor substrate for SULT2B1a, with a catalytic constant (k_{cat})/ K_m ratio that is 10-fold lower than that for pregnenolone and 20-fold lower than that for cholesterol. The SULT2B1b isoform preferentially sulfonates cholesterol, with a k_{cat}/K_m ratio of $19.4 \text{ M}^{-1}\text{sec}^{-1} \times 10^3$ vs. $2.4 \text{ M}^{-1}\text{sec}^{-1} \times 10^3$ for pregnenolone and $1.7 \text{ M}^{-1}\text{sec}^{-1} \times 10^3$ for DHEA. Additionally, SULT2B1b K_m for cholesterol is $0.87 \text{ M} \times 10^{-6}$, a value similar to the SULT2B1a K_m for cholesterol; in contrast, the K_m s for pregnenolone and DHEA are 16.4 and $19.7 \text{ M} \times 10^{-6}$, respectively. The SULT2A1 isozyme is most active with pregnenolone as a substrate (k_{cat}/K_m ratio of $2.2 \text{ M}^{-1}\text{sec}^{-1} \times 10^3$) and, to a somewhat lesser extent, with DHEA (k_{cat}/K_m ratio of $1.2 \text{ M}^{-1}\text{sec}^{-1} \times 10^3$), whereas cholesterol (k_{cat}/K_m ratio of $0.2 \text{ M}^{-1}\text{sec}^{-1} \times 10^3$) serves as a poor substrate.

Discussion

The new mouse SULT2B1 hydroxysteroid sulfotransferase (designated SULT2B1a) is akin to a previously cloned mouse SULT2B1 form (designated SULT2B1b) and is, in fact, derived from the same gene as a result of an alternative exon 1 and differential splicing. Thus, the two isoforms differ only at their amino termini. The mouse SULT2B1 gene structure is identical with that of the human SULT2B1 gene, clearly

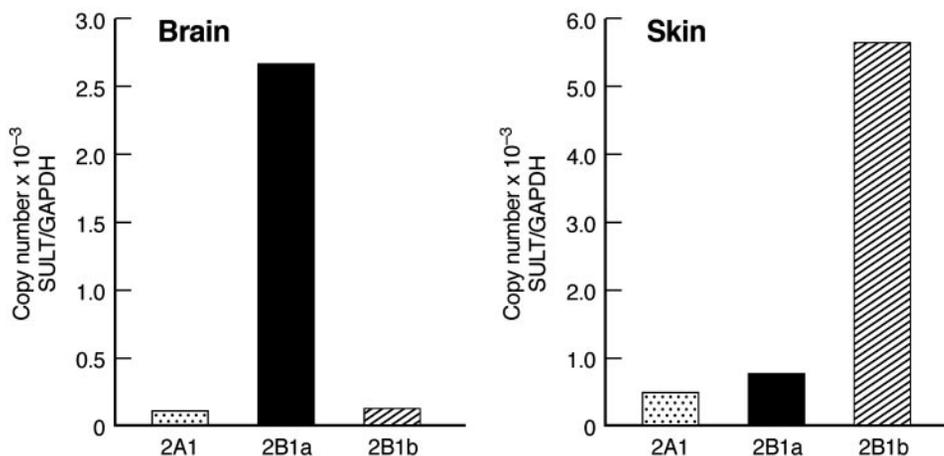
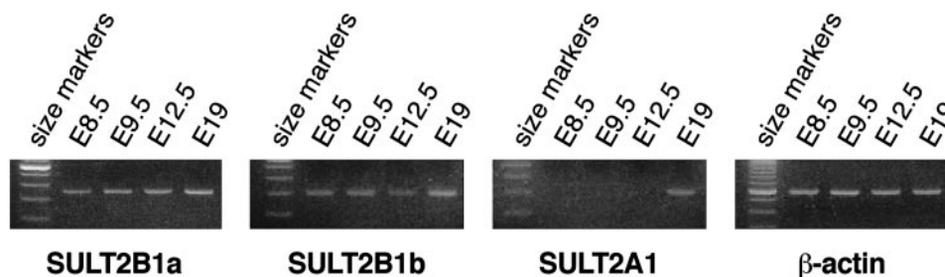


FIG. 4. Expression of mRNA for mouse SULT2A1 (stippled column), SULT2B1a (solid column), and SULT2B1b (hatched column) in brain and skin, as determined by real-time RT-PCR. Column heights represent the ratio of the mRNA copy number of each SULT to the mRNA copy number of GAPDH.

FIG. 5. Expression of mRNA for mouse SULT2B1a, SULT2B1b, SULT2A1, and β -actin in whole-mouse embryos, as determined by RT-PCR.



indicating that this gene and its products are highly conserved. There is, however, a variation on a theme at work here, in that the mouse SULT2B1a isoform is structurally at variance with its human counterpart. That is, the unique amino terminus of mouse SULT2B1a is 54 amino acids in length, whereas the comparable region in human SULT2B1a consists of only 8 amino acids (10). On the other hand, the lengths of the unique amino terminus for the mouse and human SULT2B1b isoforms are more similar, at 20 and 23 amino acids, respectively. The longer amino terminus of mouse SULT2B1a accounts for its being approximately 5% larger than SULT2B1b, which is the opposite of the case with the human counterparts, where SULT2B1b is larger than SULT2B1a.

In addition to the structural distinction between human and mouse SULT2B1a, they also differ functionally. Human SULT2B1a sulfonates cholesterol very weakly (10), whereas mouse SULT2B1a sulfonates cholesterol with a high degree of efficiency. Mouse and human SULT2B1b, on the other hand, are similar in that both avidly sulfonate cholesterol with the highest k_{cat}/K_m ratios. Thus, contrary to the human SULT2B1 isoforms, both mouse SULT2B1 isoforms sulfonate cholesterol efficiently. Mouse SULT2A1, on the other hand, weakly sulfonates cholesterol, a finding similar to that of human SULT2A1 (11). Notably, the three mouse SULT2 isozymes, particularly SULT2B1a, strongly sulfonate pregnenolone, a finding consistent with the behavior of the human SULT2 isozymes (10).

The pattern of mRNA expression of the mouse SULT2 isozymes is informative. For instance, the SULT2B1a isoform

seems to be the exclusive SULT2 isozyme expressed in the brain and spinal cord, whereas SULT2B1b and SULT2A1 are weakly, if at all, expressed in the central nervous system (CNS). On the other hand, the SULT2B1b isoform is clearly the most prominent SULT2 isozyme expressed in skin, whereas SULT2B1a and SULT2A1 are weakly expressed in this tissue. SULT2A1 is expressed in the liver at a level that overwhelms the level of expression in any other tissue; in contrast, the SULT2B1 isoforms are weakly, if at all, expressed in this organ system. It should be noted that, although the SULT2 isoform real-time PCR data were standardized using GAPDH, essentially the same results were obtained when 18S-rRNA was used for normalization (data not presented).

Cloning of the hydroxysteroid sulfotransferase SULT2B1 subfamilies in the mouse and human, which are structurally distinct from all previously cloned cytosolic sulfotransferases, represents a significant advancement in the field of biotransformation by sulfonation. Of particular importance is the fact that these new enzymes demonstrate unique substrate preferences and patterns of expression when compared with the prototypical mouse and human SULT2A1 hydroxysteroid sulfotransferases. Based on the current findings with mouse SULT2B1 and our previous report on the human SULT2B1 ortholog (10, 11), it now seems likely that this subfamily of hydroxysteroid sulfotransferases is normally involved in cholesterol metabolism via sulfonation. It should be noted that, in previous reports regarding the SULT2B1b isoform involving both the mouse (2) and human (12) orthologs, no catalytic activity with cholesterol as sub-

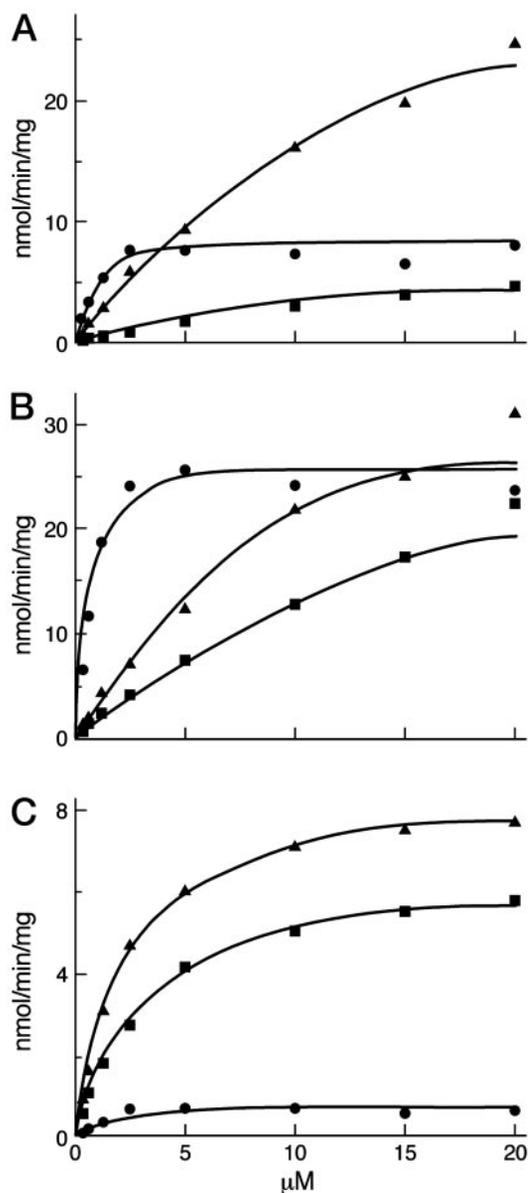


FIG. 6. Saturation analyses of mouse SULT2B1a (A), SULT2B1b (B), and SULT2A1 (C), using cholesterol (closed circles), pregnenolone (closed triangles), and DHEA (closed boxes) as substrates.

strate was found, presumably because of a difference in methodology regarding cholesterol solubility (13).

The differential expression of mouse SULT2B1a, SULT2B1b, and SULT2A1, along with their distinct substrate preferences, has physiologic relevance. For instance, the almost-sole expression of SULT2B1a in the CNS and its tendency for greater activity with pregnenolone as a substrate would be in keeping with the importance of pregnenolone sulfate as a neurosteroid (14–17). Though pregnenolone sulfonation can also be carried out by SULT2A1, this sulfotransferase isozyme is not expressed in the mouse brain. The prominent expression of SULT2B1b in skin and the fact that it clearly has a predilection for cholesterol as a substrate are in keeping with the importance of cholesterol sulfate as a regulatory molecule in murine epidermal differentiation (18, 19). Likewise, cholesterol sulfate is a regulatory

molecule in human keratinocyte differentiation and development of the barrier (20, 21), and SULT2B1b is quantitatively the predominant hydroxysteroid sulfotransferase expressed in human skin (unpublished data). There is also evidence that cholesterol sulfonation in rat skin is carried out by a similar sulfotransferase subfamily (22). Finally, the robust expression of mouse SULT2A1 in liver would be in keeping with its role in general metabolism. For example, there is evidence in humans that, in addition to its involvement in the sulfonation of a variety of steroids, including androgens and estrogens, SULT2A1 is the principal bile acid sulfonating enzyme in liver (23). This is in keeping with the fact that bile acids contain a 3α -hydroxyl group and that SULT2A1 will sulfonate compounds that are 3α -hydroxylated (24, 25), whereas the SULT2B1 isoforms are specific for steroid/sterols where the 3-hydroxy group has a β -orientation (11, 12, 26).

An interesting finding is that the mouse *SULT2B1* and *SULT2A1* genes are differentially expressed during embryonic development, with the former being expressed at all stages from E8.5–E19, whereas the latter is not expressed until E19. These results suggest that expression of the *SULT2B1* gene is more critical during early development than expression of the *SULT2A1* gene, especially the expression of SULT2B1a during the early stages of brain maturation and the expression of SULT2B1b during keratinocyte differentiation and epidermal development.

Sulfoconjugated neurosteroids are widely distributed in the CNS, where they act as modulators of neurotransmitter receptors such as γ -aminobutyric acid type A, *N*-methyl-D-aspartate, and sigma 1 (14, 27). However, regardless of the physiologic importance of steroid sulfoconjugates, little is known about their synthesis in the CNS. We are aware of only a single report describing the expression of SULT2A1 mRNA in rat brain (28), although several groups have reported on hydroxysteroid sulfotransferase activity using brain cytosolic extracts (29–32). The fact that SULT2A1 mRNA has been detected in the rat brain, but not the mouse brain, suggests that there are significant species differences. It is notable that, unlike the case of sulfonated neurosteroids such as pregnenolone sulfate, there are few reports on the role of cholesterol sulfate in the CNS. One such report revealed that significant amounts of cholesterol sulfate were detectable in rat brain; furthermore, a dramatic change during early development was noted (33). However, the physiologic significance of this observation is not understood.

As noted above, the outstanding structural feature of the mouse SULT2B1 isoforms that makes them unusual and distinct from mouse SULT2A1 is their extended amino- and carboxy-terminal ends. A similar relationship exists for the human SULT2B1 and SULT2A1 orthologs (1, 11). Mouse SULT2A1 contains 285 amino acids, which is the typical size for hydroxysteroid as well as other cytosolic sulfotransferases (34), whereas the mouse SULT2B1 isoforms are 53–87 amino acids longer, a length difference of up to approximately 30%. The extended carboxy termini of the mouse SULT2B1 isoforms, which are identical, with 1 exception, are proline-rich, a finding that is similar to the human SULT2B1 orthologs (1). The extended amino termini, on the other hand, are quite distinct, both in length and amino acid composition. The mouse *SULT2B1* and *SULT2A1* genes, as well

as the genes for human *SULT2B1* and *SULT2A1*, are presumably the result of gene duplication and are thus paralogs (35). Furthermore, the mouse genes are closely linked on chromosome 7 and are syntenic to the similarly closely linked human *SULT2B1* and *SULT2A1* genes located on chromosome 19 (<http://www.ncbi.nlm.nih.gov/homology/>).

In summary, the mouse SULT2B1 isoforms have a predilection for cholesterol over that for DHEA, which is ordinarily sulfonated by the SULT2A1 subfamily of hydroxysteroid sulfotransferases (36). Of particular interest is the finding that the genes for mouse *SULT2B1* and *SULT2A1* are differentially expressed during embryonic development. Furthermore, as determined by real-time PCR, the mouse SULT2 genes demonstrate distinct expression patterns in mature animals. Notably, SULT2B1a is the exclusive SULT2 isozyme expressed in the CNS, whereas SULT2B1b is the prominent SULT2 isozyme expressed in skin. On the other hand, SULT2A1 is the manifest hydroxysteroid sulfotransferase expressed in the liver. The explicit expression patterns of the SULT2 isozymes, in association with their respective substrate predilections, have interesting physiologic implications.

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References

- Her C, Wood TC, Eichler EE, Mohrenweiser HW, Ramagli IS, Siciliano MJ, Weinshilboum RM 1998 Human hydroxysteroid sulfotransferase SULT2B1: two enzymes encoded by a single chromosome 19 gene. *Genomics* 53:284–295
- Sakakibara Y, Yanagisawa K, Takami Y, Nakayama T, Suiko M, Liu M-C 1998 Molecular cloning, expression, and functional characterization of novel mouse sulfotransferases. *Biochem Biophys Res Commun* 247:681–686
- Nagata K, Yamazoe Y 2000 Pharmacogenetics of sulfotransferase. *Annu Rev Pharmacol Toxicol* 40:159–176
- Thompson JD, Higgins DG, Gibson TJ 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Smale ST 1997 Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochim Biophys Acta* 1351:73–88
- Lo K, Smale ST 1996 Generality of a functional initiator consensus sequence. *Gene* 182:13–22
- Mount SM 1982 A catalogue of splice junction sequences. *Nucleic Acids Res* 10:459–472
- Kakuta Y, Pedersen LG, Carter CW, Negishi M, Pedersen LC 1997 Crystal structure of estrogen sulphotransferase. *Nat Struct Biol* 4:904–908
- Pedersen LC, Petrotchenko EV, Negishi M 2000 Crystal structure of SULT2A3, human hydroxysteroid sulfotransferase. *FEBS Lett* 475:61–64
- Fuda H, Lee YC, Shimizu C, Javitt NB, Strott CA 2002 Mutational analysis of human hydroxysteroid sulfotransferase SULT2B1 isoforms reveals that exon 1B of the *SULT2B1* gene produces cholesterol sulfotransferase, whereas exon 1A yields pregnenolone sulfotransferase. *J Biol Chem* 277:36161–36166
- Javitt NB, Lee YC, Shimizu C, Fuda H, Strott CA 2001 Cholesterol and hydroxycholesterol sulfotransferases: identification, distinction from dehydroepiandrosterone sulfotransferase, and differential tissue expression. *Endocrinology* 142:2978–2984
- Meloche CA, Falany CN 2001 Expression and characterization of the human 3 β -hydroxysteroid sulfotransferases (SULT2B1a and SULT2B1b). *J Steroid Biochem Mol Biol* 77:261–269
- De Caprio J, Yun J, Javitt NB 1992 Bile acid and sterol solubilization in 2-hydroxypropyl-beta-cyclodextrin. *J Lipid Res* 33:441–443
- Baulieu EE, Robel P, Schumacher M 2001 Neurosteroids: beginning of the story. *Int Rev Neurobiol* 46:1–31
- Alomary AA, Fitzgerald RL, Purdy RH 2001 Neurosteroid analysis. *Int Rev Neurobiol* 46:97–115
- Engel SR, Grant KA 2001 Neurosteroids and behavior. *Int Rev Neurobiol* 46:321–348
- Plassart-Schiess E, Baulieu EE 2001 Neurosteroids: recent findings. *Brain Res Brain Res Rev* 37:133–140
- Kagehara M, Tachi M, Harii K, Iwamori M 1994 Programmed expression of cholesterol sulfotransferase and transglutaminase during epidermal differentiation of murine skin development. *Biochim Biophys Acta* 1215:183–189
- Denning MF, Kazanietz MG, Blumberg PM, Yuspa SH 1995 Cholesterol sulfate activates multiple protein kinase C isozymes and induces granular cell differentiation in cultured murine keratinocytes. *Cell Growth Differ* 6:1619–1626
- Kawabe S, Ikuta T, Ohba M, Chida K, Ueda Y, Yamanishi K, Kuroki T 1998 Cholesterol sulfate activates transcription of transglutaminase 1 gene in normal human keratinocytes. *J Invest Dermatol* 111:1098–1102
- Hanley K, Wood L, Ng DC, He SS, Lau P, Moser A, Elias PM, Bikle DD, Williams ML, Feingold KR 2001 Cholesterol sulfate stimulates involucrin transcription in keratinocytes by increasing Fra-1, Fra-2, and Jun D. *J Lipid Res* 42:390–398
- Rearick JL, Calhoun ES 2001 Purification and characterization of cholesterol sulfotransferase from rat skin. *Biochem Cell Biol* 79:499–506
- Falany CN 1991 Molecular enzymology of human liver cytosolic sulfotransferases. *Trends Pharmacol Sci* 12:255–259
- Falany CN, Wheeler J, Oh TS, Falany JL 1994 Steroid sulfation by expressed human cytosolic sulfotransferases. *J Steroid Biochem Mol Biol* 48:369–375
- Falany CN 1997 Enzymology of human cytosolic sulfotransferases. *FASEB J* 11:206–216
- Geese WJ, Raftogianis RB 2001 Biochemical characterization and tissue distribution of human SULT2B1. *Biochem Biophys Res Commun* 288:280–289
- Mellon SH, Vaudry H 2001 Biosynthesis of neurosteroids and regulation of their synthesis. *Int Rev Neurobiol* 46:33–78
- Shimada M, Yoshinari K, Tanabe E, Shimakawa E, Kobashi M, Nagata K, Yamazoe Y 2001 Identification of ST2A1 as a rat brain neurosteroid sulfotransferase mRNA. *Brain Res* 920:222–225
- Aldred S, Waring RH 1999 Localization of dehydroepiandrosterone sulphotransferase in adult rat brain. *Brain Res Bull* 48:291–296
- Rajkowski KM, Robel P, Baulieu EE 1997 Hydroxysteroid sulfotransferase activity in the rat brain and liver as a function of aging. *Steroids* 62:427–436
- Mensah-Nyagan AG, Beaujean D, Do-Rego J-L, Mathieu M, Vallarino M, Luu-The V, Pelletier G, Vaudry H 2000 *In vivo* evidence for the production of sulfated steroids in the frog brain. *Comp Biochem Physiol [B]* 126:213–219
- Beaujean D, Mensah-Nyagan AG, Do-Rego J-L, Luu-The V, Pelletier G, Vaudry H 1999 Immunocytochemical localization and biological activity of hydroxysteroid sulfotransferase in the frog brain. *J Neurochem* 72:848–857
- Iwamori M, Moser HW, Kishimoto Y 1976 Cholesterol sulfate in rat tissues. *Biochim Biophys Acta* 441:268–279
- Luu-The V, Bernier F, Dufort I 1996 Steroid sulfotransferases. *J Endocrinol* 150:S87–S97
- Fitch W 2000 Homology a personal view on some of the problems. *Trends Genet* 16:227–231
- Kong A-NT, Tao D, Ma M, Yang L 1993 Molecular cloning of the alcohol/hydroxysteroid form (mST_{al}) of sulfotransferase from mouse liver. *Pharmacol Res* 10:627–630