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Sequence Analysis and Expression of the cDNA for the Phenol-Sulfating Form of Human Liver Phenol Sulfotransferase

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SUMMARY

A cDNA encoding the human liver phenol-sulfating form of phenol sulfotransferase (P-PST) has been isolated and characterized from a λUni-Zap XR human liver cDNA library. P-PST is the major form of phenol sulfotransferase involved in drug and xenobiotic metabolism in human liver. P-PST is also responsible for the sulfation and activation of minoxidil to its therapeutically active sulfate ester. The full length cDNA, P-PST-1, is 1206 base pairs in length and encodes a 295-amino acid protein with a molecular mass of 34,097 Da. The translation sequence of P-PST-1 is 96% similar to the amino acid sequences of five peptides derived from the purified protein. *In vitro* transcription and translation of P-PST-1 generated a protein that comigrates with immunoreactive P-PST from human liver. Significant increases in sulfotransferase activity toward two P-PST-specific substrates, minoxidil and 4-nitrophenol, were detected in cytosol prepared from COS-7 cells transfected with P-PST-1 in the expression vector p-SV-SPORT-1. Northern blot analysis of human liver RNA detected a transcript of approximately 1300 nucleotides in length. Characterization of P-PST at the molecular level provides insight into the structure and heterogeneity of this major class of drug-metabolizing enzymes.

Sulfation is one of the major conjugation reactions involved in the biotransformation of a wide variety of drugs, xenobiotics, and endogenous compounds in human tissues. The STs catalyze the transfer of the sulfonate group from PAPS to an acceptor compound to form either a sulfate ester or a sulfamate (1). Three major forms of cytosolic ST have been identified in human liver, *i.e.*, a steroid/bile acid ST, dehydroepiandrosterone ST, and two forms of PST (2).

A wide range of endogenous and exogenous phenolic compounds are sulfated by the human cytosolic PSTs, and enzyme activity has been identified in many human tissues including liver, adrenal, lung, brain, platelets, jejunum, and placenta (3–9). Sulfation usually results in decreased biological activity and an increased rate of excretion of the acceptor compound; however, in several cases sulfation greatly enhances the activity of compounds such as cholecystokinin (10) and the antihypertensive agent Mx (11–13). Sulfation has also been implicated in rodents in the bioactivation of several compounds, such as *N*-hydroxy-2-acetylaminofluorene and 7-hydroxymethyl-12-methylbenz[*a*]anthracene, to reactive electrophiles (2). Most of the characterization of PST activity and of the heterogeneity of the PSTs has involved investigation of the forms of PST present in rat liver. At least six different forms of PST have been isolated and characterized from rat liver cytosol (14).

In contrast to the multiplicity of forms of PST in rats, only two distinct forms of PST activity have been identified in human tissues, M-PST and P-PST (2–9). These enzymes have been shown to be functionally distinct, based on differences in substrate (15) and inhibitor specificity (7) and thermal stability (8). Although there is some degree of substrate overlap, at micromolar substrate concentrations M-PST selectively sulfates catechol and phenolic amines, such as dopamine and norepinephrine (3). At similar substrate concentrations P-PST selectively conjugates neutral phenols, such as phenol and PNP, as well as a variety of other substrates, including triiodothyronine and Mx (3, 15–18).

M-PST and P-PST have been purified to homogeneity from

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ABBREVIATIONS: ST, sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PST, phenol sulfotransferase; P-PST, phenol-sulfating form of phenol sulfotransferase; M-PST, monoamine-sulfating form of phenol sulfotransferase; Mx, minoxidil; rMx-ST, rat liver minoxidil sulfotransferase; PNP, *p*-nitrophenol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; SSC, standard saline citrate; MET, methionine; bp, base pair(s); PCR, polymerase chain reaction; AST, arylsulfotransferase.

human blood platelets (19) and liver (9), respectively. Both M-PST and P-PST appear to exist as homodimers in their active forms, with approximate subunit molecular masses of 34 kDa and 32 kDa, respectively. Although M-PST and P-PST are distinct proteins, antibodies raised in rabbits to platelet M-PST cross-react strongly with P-PST (20). The liver is a relatively rich source of P-PST activity, and P-PST activity in different individuals is 3.5–30-fold higher than M-PST activity (9). If only two forms of PST are present in human liver, understanding the biochemical and molecular properties of these enzymes will greatly aid in the investigation and appreciation of the role of the PSTs in human drug metabolism and homeostasis.

This report describes the molecular cloning, characterization, sequence analysis, and expression in mammalian cells of a cDNA encoding human liver P-PST. The expressed enzyme displays ST activity toward PNP and Mx, specific substrates for P-PST (2). The data in the present study provide important insights into the structure, heterogeneity, and activity of this major class of drug-metabolizing enzymes.

Experimental Procedures

Materials. Restriction enzymes and DNA-modifying enzymes were purchased from United States Biochemical or New England Biolabs. Nick-translation kits, [α-^{35}S]dATP (3000 Ci/mmol), [α-^{32}P]dCTP (800 Ci/mmol), and [^{35}S]MET (1232.7 Ci/mmol) were purchased from New England Nuclear. The MAXIscript *in vitro* transcription kit and the ReticLysate IVT translation kit were from Ambion Inc. The λUni-Zap XR human liver cDNA library was purchased from Stratagene. pGEM-7zf, pGEMEX 2, and pSP-72 were purchased from Promega. COS-7 cells were purchased from the American Type Culture Collection. pSV-SPORT-1, fetal bovine serum, Dulbecco's modified Eagle medium, and Lipofectin were obtained from GIBCO-BRL. Human liver specimens were obtained at the time of removal of other organs for donation, through the Organ Procurement Program at the University of Rochester (Rochester, NY). All other reagents were of molecular biology grade.

ST assays. P-PST activity was determined using PNP and Mx as substrates. PNP ST and dopamine ST activities were assayed using 4 μM PNP and 10 μM dopamine, respectively, as previously described by Hirshey and Falany (21). PNP sulfate and dopamine sulfate were separated from PAPS using the barium precipitation procedure of Foldes and Meek (22). Mx ST was assayed using a Mx concentration of 6.7 mM, as described by Falany and Kerl (18). Mx sulfate was separated from PAPS using the alkaline organic extraction procedure of Johnson and Baker (23).

Immunoblot procedure. The preparation and characterization of rabbit anti-human M-PST antibodies have been described previously (20). Proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose paper using a Bio-Rad Transblot unit and the buffer system described by Towbin *et al.* (24). The nitrocellulose membrane was blocked for 1 hr with 3% gelatin in 100 mM Tris·HCl, pH 7.5, containing 500 mM NaCl (Tris-buffered saline). Incubation with a 1/5000 dilution of rabbit anti-human PST antibody in Tris-buffered saline containing 0.1% Tween 20 and 1% gelatin was carried out overnight at room temperature with gentle shaking. Immunoconjugates were detected using goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) at a 1/5000 dilution. Protein concentrations were determined by the procedure of Bradford (25), using bovine γ-globulin as a standard.

Sequence analysis of peptides derived from purified P-PST. P-PST was purified from human liver cytosol as described previously (9). Initial attempts to sequence the amino-terminal end of pure P-PST were unsuccessful, apparently as the result of a blocked amino terminus on the protein. Therefore, amino acid sequence was obtained from peptides generated by tryptic and cyanogen bromide cleavage of P-PST. P-PST was resolved by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane, and peptide fragments were generated *in situ* by cyanogen bromide treatment as described by Yuen *et al.* (26). For digestion with trypsin, 50 μg of purified P-PST were suspended in 50 μl of 0.4 M ammonium bicarbonate, pH 8.0, and the pH of the mixture was adjusted to 7.5–8.0 with NaOH. P-PST was reduced in the presence of 4 mM dithiothreitol at 50° for 15 min and then acetylated in the presence of 8 mM iodoacetamide in the dark for 15 min. The volume was adjusted to 200 μl with water, and 5 μl of trypsin (1.0 mg/ml in 0.1% TFA) were added. After a 24-hr incubation at 37°, the reaction was stopped by the addition of 5.0 μl of 10% TFA. After lyophilization, the trypsin- and cyanogen bromide-generated peptides were suspended in water containing 0.1% TFA and were separated by reverse phase high performance liquid chromatography on a Vydac C-18 column (46 mm × 250 mm), using a linear gradient of 0–80% (v/v) acetonitrile in the presence of 0.1% TFA. Elution of the peptides from the column was monitored at 215 nm and 280 nm with a Perkin Elmer model 235 diode array detector. Peaks absorbing at both wavelengths were collected and stored at −70° before sequence analysis. The amino acid sequences of the isolated peptides were determined at The Upjohn Company using a 471A gas phase protein sequenator (Applied Biosystems, Inc.) connected on-line to an ABI 120 (high performance liquid chromatography) phenylthiohydantoin analyzer. Data were collected and yields were calculated on a Nelson Analytical 3000 Series chromatography system.

Isolation of P-PST cDNAs. Human P-PST cDNAs were isolated from a λUni-Zap XR human liver cDNA library by using the cDNA for rMx-ST as a probe (27). Approximately 350,000 phage were grown in *Escherichia coli* XL1-Blue, plated on 150-mm Petri dishes at approximately 40,000 phage/dish, and blotted onto nitrocellulose filters (28). The filters were denatured, baked *in vacuo*, and prehybridized for 2 hr at 55° in a solution containing 6× 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0, 0.5% SDS, 10× Denhardt's solution (0.1% Ficoll (w/v)/0.1% polyvinylpyrolidone (w/v)/0.1% bovine serum albumin (w/v)), and 200 μg/ml denatured salmon sperm DNA. The filters were then hybridized overnight at 55° in the same solution with approximately 1.5 × 10^6 dpm/ml ^{32}P-nick-translated Mx ST cDNA (5 × 10^7 dpm/μg). Subsequently, the filters were washed three times in 3× SSC/0.5% SDS at 55°, dried, and exposed to autoradiographic film for 18 hr at −70° in the presence of an intensifying screen.

Positive clones were purified by repeated cycles of dilution and rescreening (28). The λUni-Zap phage contains the cDNAs within a pBluescript vector. The cDNAs were isolated from the λ vector in pBluescript by coinfection with R408 helper bacteriophage in *E. coli* XL1-Blue. Clones containing the five largest cDNA inserts, ranging in size from 550 to 1121 bp, were subjected to preliminary nucleotide sequence analysis. Partial sequences of the 3′ ends of these cDNAs were identical and analysis of the 5′ ends indicated that none of the clones contained an initiation codon. The largest cDNA, P-PST2-2, appeared to be close to full length.

A PCR procedure was used to isolate the missing 5′ end of the open reading frame and part of the 5′ nontranslated region of P-PST. Phage DNA isolated from the amplified λUni-Zap XR human liver cDNA library was used as the template in PCR reactions. The 17-mer T3 primer, which recognizes the Bluescript arm adjacent to the 5′ end of the multiple cloning site, was used as the sense primer. A specific primer corresponding to bases 375–393 of the P-PST-1 cDNA (Fig. 1) was used as the antisense primer. Template DNA (0.1 μg) and primers (1.0 μg each) were subjected to 30 cycles of denaturation for 1 min at 94°, annealing for 2 min at 50°, and extension at 72° for 3 min, with a final extension of 7 min at 72°. PCR reactions were extracted with chloroform, precipitated with ethanol, and digested with 40 units each of *Eco*RI and *Sac*I. The restricted PCR products were then resolved in a 1.5% agarose gel. DNA fragments ranging in size from 250 to 600 bp were isolated using DE-81 paper (28) and were subcloned into the

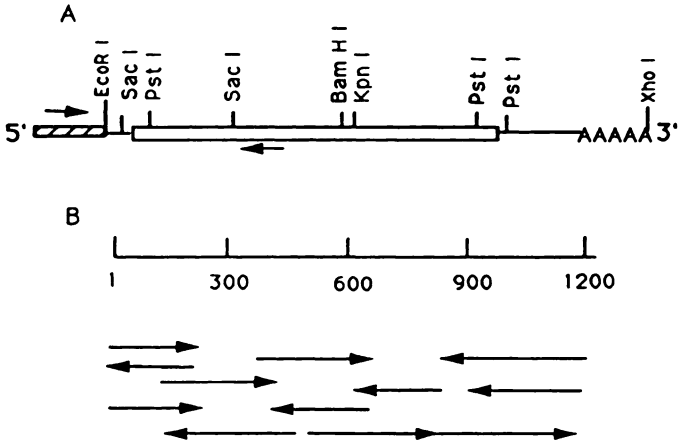

Fig. 1. Partial restriction map and strategy for obtaining P-PST-1 cDNA. A, *Open box*, coding region of the cDNA. *Single lines*, 5′ and 3′ noncoding regions. *Hatched box*, pBluescript sequence at the 5′ end of the cDNA. The cloning sites in pBluescript were *Eco*RI and *Xho*I. *Arrows*, positions and directions of the primers used to generate the PCR-amplified DNA containing the 5′ end of P-PST-1. B, Partial strategy for sequencing P-PST-1 cDNA. *Arrows*, direction of selected sequence determinations. These represent both directional and shotgun clones. The position of each coding nucleotide was sequenced at least four times and at least once in each strand.

*Eco*RI/*Sac*I sites of pGem-7zf (Promega) for sequencing. Two DNA fragments, 280 and 304 bp in length, possessing an ATG initiation codon in-frame with the translation sequence for P-PST2–2, were identified by sequence analysis. The 304-bp DNA fragment was isolated by complete digestion with *Eco*RI and partial digestion with *Sac*I (Fig. 1) and was used in the construction of a full length cDNA. The 1121-bp P-PST fragment was subcloned into pSV72 and the 205-bp *Eco*RI/*Sac*I fragment was removed. The 304-bp PCR-generated *Eco*RI/*Sac*I fragment containing the 5′ end of the open reading frame was ligated into the *Eco*RI/*Sac*I sites of the pSP-72 cDNA construct, producing the final 1206-bp full length cDNA, P-PST-1.

DNA sequence analysis. The cDNAs were subjected to double-stranded sequencing using the dideoxynucleotide chain termination procedure with Sequenase 2.0 (United States Biochemical) and [α-^{35}S] dATP to label the synthesized DNA fragments. Internal sequence of P-PST2–2 in pBluescript was obtained by subcloning directional and shotgun restriction fragments into pGEM-7zf for sequencing with T7 and SP6 primers. *Sau*3A and *Alu*I were used to generate the fragments for shotgun cloning. The ^{35}S-labeled products of the sequencing reactions were resolved in 6% polyacrylamide-urea gels using a buffer gradient of 0.5–2.5× TBE (1× TBE = 89 mM Tris-borate, 2 mM EDTA). Sequence gels were read manually and analyzed using the University of Wisconsin Genetics Computer Group programs (29).

Northern blot analysis of human liver RNA. The livers were obtained from the Organ Procurement Program at the University of Rochester, at the time of removal of other organs for transplantation. The livers were immediately placed on ice and were frozen within 30 min of removal from the donor. Samples of the livers were ground in liquid nitrogen, and total RNA was prepared from normal human liver samples using the acid guanidinium thiocyanate method of Chomczynski and Sacchi (30). Poly(A)$^+$ RNA was isolated from total RNA by oligo(dT)-cellulose chromatography (31). Samples of total, non-poly(A)$^+$, and poly(A)$^+$ RNA were resolved by electrophoresis in 1.5% agarose-formaldehyde gels (31), transferred to nylon membranes, and baked *in vacuo* for 2 hr at 65°. The filters were hybridized overnight at 42° with 1.5 × 10^6 dpm/ml ^{32}P-nick-translated P-PST2–2, in 50% formamide, 5× SSC, 10 mM Tris·HCl, pH 7.5, 4× Denhardt's, 200 μg/ml calf thymus DNA, and 0.1% SDS. The filters were washed at 10-min intervals twice in 3× SSC/0.5% SDS at room temperature, twice in 3× SSC/0.5% SDS at 65°, and once in 1× SSC/0.5% SDS at 65° and were then exposed to autoradiographic film for 48 hr at −70°, with an intensifying screen.

Southern blot analysis of human liver DNA. Genomic DNA was obtained from normal human liver specimens as described by Strauss (32). Aliquots (10 μg) of DNA were digested overnight with 50 units of *Pst*I, *Sac*I, or *Hind*III and were resolved by electrophoresis in a 0.8% agarose gel. The gel was pretreated and transferred by capillary action to a nylon membrane according to the manufacturer's instructions (Micron Separations Inc.). The membrane was baked *in vacuo* at 65° for 2 hr and hybridized overnight with 1.5 × 10^6 dpm/ml ^{32}P-nick-translated P-PST2–2, as described above. The filters were washed twice for 10 min in 3× SSC/0.5% SDS at room temperature and once each in 1× SSC/0.5% SDS at 37°, 1× SSC/0.5% SDS at 65°, and 0.2× SSC/0.5% SDS at 65°, followed by exposure to autoradiographic film with an intensifying screen at −70° for 24 hr.

***In vitro* transcription and translation of P-PST-1.** In order to facilitate directional cloning of P-PST-1 into the mammalian expression vector pSV-SPORT-1, P-PST-1 was isolated as an *Eco*RI/*Xho*I restriction fragment from pSP-72 and subcloned into the *Eco*RI/*Xho*I sites in pGEMEX 2. The complete P-PST-1 cDNA was then removed from pGEMEX 2 as an *Eco*RI/*Hind*III fragment and subcloned into the *Eco*RI/*Hind*III sites of pSV-SPORT-1. pSV-SPORT-1 contains the SP6 RNA polymerase promoter site in the same orientation as the SV40 early promotor. pSV-SPORT-P-PST-1 was linearized with *Hind*III and RNA transcripts were synthesized using the MAXIscript SP6 transcription system. Transcripts were translated with a Retic-Lysate IVT system in the presence of [^{35}S]MET to label the translated proteins. Translation reactions were resolved by SDS-PAGE, gels were dried *in vacuo*, and the size of the translated polypeptides was determined by autoradiography for 16 hr. To verify that the translated protein comigrated with P-PST from human liver, human liver cytosol was resolved on the same polyacrylamide gel, transferred to nitrocellulose, and detected with rabbit anti-human PST antibodies (20).

Transient expression of P-PST-1 in COS-7 cells. The ability of P-PST-1 to express P-PST activity was investigated in transfected COS-7 cells. COS-7 Green monkey kidney cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Transfection of cells was accomplished using a liposome-mediated procedure (Lipofectin; GIBCO-BRL), according to the manufacturer's instructions. Each 60-mm plate of COS-7 cells was transfected with 10 μg of supercoiled pSV-SPORT-1 or pSV-SPORT-P-PST-1 and incubated at 37° for a total of 72 hr.

In order to detect expression of P-PST, cells were washed three times with phosphate-buffered saline and harvested in 10 mM triethanolamine, pH 7.4, containing 1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride. The cells were then homogenized in a Teflon-glass homogenizer, and cytosol was recovered after centrifugation at 100,000 × *g* for 1 hr. Aliquots of the cytosol were assayed for PNP, dopamine, and Mx sulfation activities as described previously, and immunoreactive P-PST was detected by immunoblotting using the anti-human PST antibodies.

Results and Discussion

Molecular characterization of P-PST cDNAs. The human liver P-PST cDNAs were isolated from a λUni-Zap XR cDNA library using a cDNA encoding a rat liver PST, rMx-ST, as a probe. rMx-ST has been purified and cloned in our laboratory (27), and several lines of evidence support the close relationship of this enzyme to P-PST. Rabbit anti-rMx-ST antibodies cross-react with human P-PST (21) and, conversely, rabbit anti-human PST antibodies cross-react with rMx-ST (20). Purified human P-PST and rMx-ST display similar substrate reactivities (9, 18, 21), and cyanogen bromide-generated peptides derived from both purified proteins show a high degree of similarity; in one peptide, eight of 10 amino acids were

Fig. 2. Nucleotide and deduced amino acid sequences of P-PST-1. The amino acid sequence is numbered at the *right*. The P-PST-1 cDNA contains 70 nucleotides of 5′ nontranslated sequence. ***, stop codon. *Dashed line*, amino acid sequences of peptides derived from tryptic and cyanogen bromide cleavage of purified P-PST. The differences between the translation sequence of P-PST-1 and the peptide sequences are noted.

identical and the two different amino acids were conservative substitutions. Duffel *et al.* (33) have reported that antibodies raised to rat liver AST IV, a major form of PST in rat liver, do not react with rMx-ST, indicating that rMx-ST is immunologically more closely related to P-PST than to AST IV.

A number of positive clones were isolated from the human liver cDNA library using the rMx-ST cDNA as a probe, and five clones were characterized. The longest of these clones, P-PST2–2, contained an open reading frame encoding 290 amino acids and 251 bp of the 3′ nontranslated region and a poly(A)$^+$ tract; however, P-PST2–2 lacked an ATG initiation codon, indicating that the clone was not full length.

Isolation of the 5′ end of the P-PST cDNA used a PCR procedure, and two DNA fragments (280 and 304 bp in length) were isolated. Each cDNA contained 207 bp of sequence identical to the 5′ end of P-PST2–2 and possessed an in-frame ATG initiation codon, adding five amino acids to the amino-terminal end of the P-PST2–2 translation product (Fig. 1). The

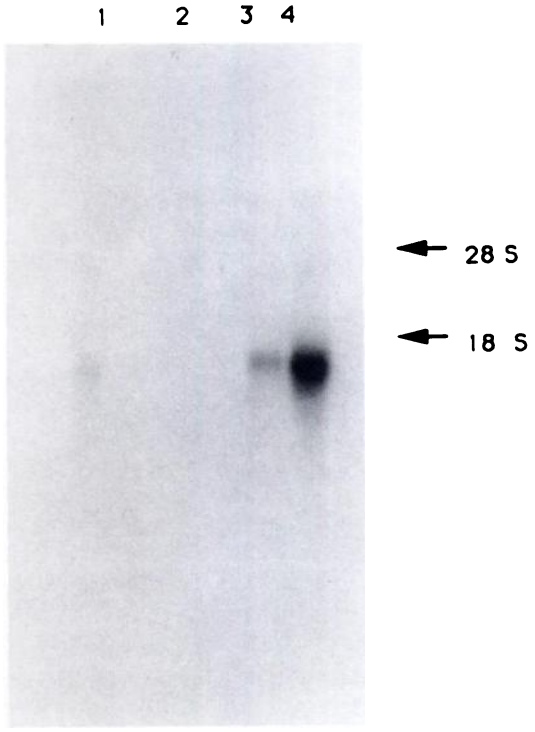

Fig. 3. Northern blot analysis of the P-PST mRNA. Total and poly(A)$^+$ RNA were obtained from a normal human liver (white woman of age 66) as described in Experimental Procedures. RNA samples were resolved in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled P-PST2–2. The RNA fractions were as follows: *lane 1*, total RNA (100 μg); *lane 2*, tRNA and rRNA (100 μg); *lane 3*, poly(A)$^+$ RNA (5 μg); *lane 4*, poly(A)$^+$ RNA (20 μg). *Arrows in the right margin*, migration of human liver rRNA.

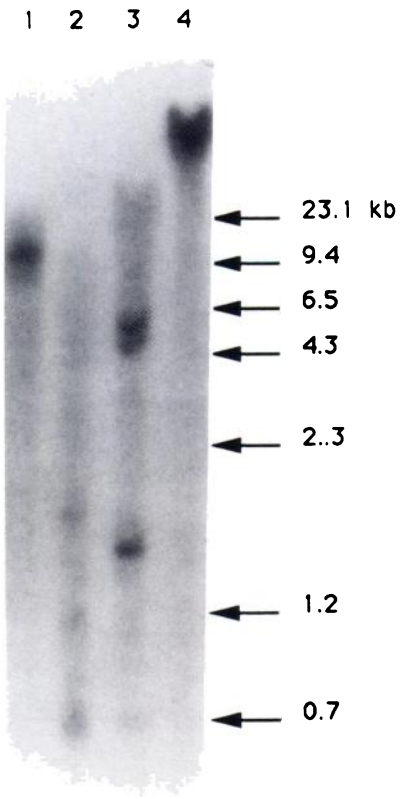

Fig. 4. Southern blot analysis of the PST genes. High molecular weight genomic DNA obtained from human liver (white woman of age 66) was digested with an excess of *Hind*III (*lane 1*), *Pst*I (*lane 2*), or *Sac*I (*lane 3*) and was resolved adjacent to undigested genomic DNA (*lane 4*) in a 0.8% agarose gel, as described in Experimental Procedures. The DNA was transferred to a nylon membrane and hybridized with 1.5 × 10^6 dpm/ml ^{32}P-nick-translated P-PST2–2. λDNA digested with *Hind*III or *Bst*EII was used for size standards.

larger fragment also contained 70 bp of the 5′ nontranslated region. The 1206-bp full length cDNA, P-PST-1, encodes a protein of 295 amino acids with a predicted molecular mass of 34,097 Da (Fig. 2). The cDNA terminates in a poly(A)$^+$ tract, and three putative polyadenylation signal sequences, AATAAA (34), are located 11, 16 and 21 bases upstream from the beginning of the poly(A)$^+$ tract.

The amino acid sequence of P-PST obtained by translation of P-PST-1 is 96% identical to the amino acid sequences of five peptides derived by cyanogen bromide and tryptic cleavage of purified P-PST (Fig. 2). The two differences between the sequences are that the two cysteines at amino acid residues 70 and 287 of the translated sequence were reported as glutamates in the peptide sequences. This high degree of homology supports the identification of P-PST-1 as an isoenzyme of P-PST.

Previous studies have indicated that at least two allelic forms of P-PST activity are present in human liver cytosol, based on differences in thermal stability and elution during anion exchange chromatography (9, 35, 36). Both allelic forms of P-PST were shown to possess the same subunit molecular mass and immunoreactivity, and both were inhibited to similar extents by *N*-ethylmaleimide and phenylglyoxal, indicating that they are highly related (9). The amino acid sequence differences between the translation of P-PST-1 and the peptides are possibly allelic differences; however, the allelic nature of expressed P-PST-1 has not yet been determined. Apparent allelic differences were also observed in the sequence of peptides derived from P-PST. One cyanogen bromide-derived fragment of purified P-PST (amino acids 60–74), which was isolated and sequenced twice from the same liver, shows a conservative exchange of a glutamine residue for an asparagine at amino acid residue 63 (Fig. 2).

Two distinct forms of PST, M-PST and P-PST, have been identified in human liver cytosol; however, the levels of P-PST activity and immunoreactivity in liver are significantly higher than those for M-PST (9). No clones or PCR fragments were detected during the cloning of P-PST-1, which may encode M-PST, apparently reflecting the low levels of M-PST in liver. All of the clones identified in this study were related by sequence to P-PST-1.

Northern blot analysis of total and poly(A)$^+$ RNA was used to determine both the size and number of transcripts hybridizing to P-PST2–2. Fig. 3 shows that P-PST2–2 apparently detects one transcript, approximately 1300 bp in length, in human liver RNA. No bands were detected in the rRNA/tRNA fraction obtained during oligo(dT) chromatography. Although M-PST and P-PST are thought to be related, based on immunological cross-reactivity, a different size transcript possibly corresponding to M-PST was not observed. This may reflect the low levels of expression of M-PST in human liver, but it is possible that the messages encoding M-PST and P-PST are similar in size, because their molecular masses do not differ greatly (9, 19).

Southern blot analysis of genomic DNA was performed to investigate the complexity and size of restriction fragments of

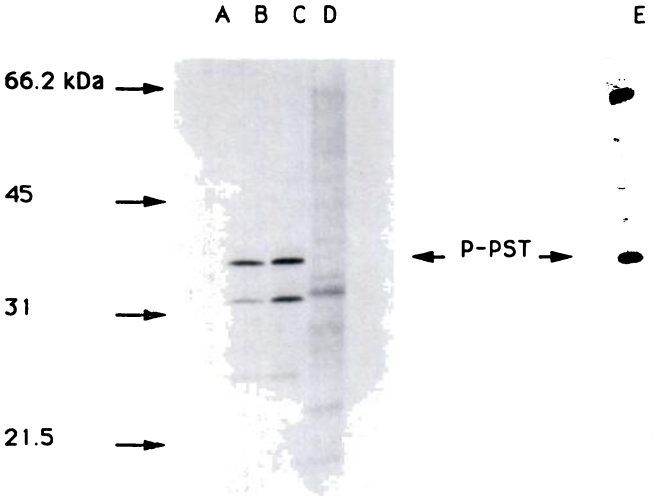

Fig. 5. *In vitro* translation of P-PST-1. RNA from *in vitro* transcription of pSV-SPORT-P-PST-1 was translated using a rabbit reticulocyte lysate system (ReticLysate IVT). Translated protein was resolved by SDS-PAGE, transferred to nitrocellulose, and exposed to autoradiographic film. The lanes represent reactions that contained no RNA (*lane A*), transcripts from pSV-SPORT-PST-1 (*lanes B* and *C*), or tobacco mosaic virus-positive control RNA (*lane D*). *Lane E*, sample of human liver cytosol (15 μg) simultaneously resolved in the same gel with *in vitro* translation reactions and then immunoblotted with rabbit anti-human PST antibodies as described in Experimental Procedures. *Arrows in the left margin*, migration of molecular mass standards (Sigma).

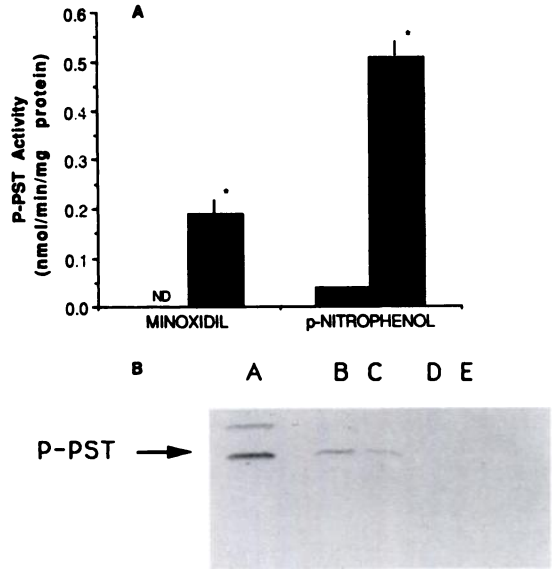

Fig. 6. Expression of P-PST in COS-7 cells. A, COS-7 cells were transfected with pSV-SPORT-1 (■) or pSV-SPORT-P-PST-1 (■) and cytosol was prepared from the cells as described in Experimental Procedures. The presence of PST activity was assayed using Mx and PNP as substrates. Specific activity is reported as the mean ± standard deviation of ST activity from four separate preparations, assayed in duplicate. *, The level of significance of difference from controls was determined to be $p < 0.0005$, using the Student unpaired *t* test. *ND*, no detectable activity. B, Protein (15 μg) from human liver cytosol (*lane A*) and cytosolic preparations, from A, of COS-7 cells transfected with pSV-SPORT-P-PST-1 (*lanes B* and *C*) or pSV-SPORT-1 (*lanes D* and *E*) were probed with rabbit anti-human PST antibodies (9) as described in Experimental Procedures.

genomic DNA that hybridize to P-PST2–2. Fig. 4 shows the pattern of fragments of human DNA digested with several restriction endonucleases that hybridized with P-PST2–2. The sizes of the bands detected are as follows: *Sac*I, 5200, 4600, and 1600 bp; *Pst*I, 1800, 1200, and 700 bp; and *Hind*III, 9800 bp. P-PST-1 contains two *Sac*I sites at bases 59 and 285, three *Pst*I sites at bases 161, 928, and 977, and no *Hind*III sites. It is possible that the entire P-PST gene is encoded within the *Hind*III 9800-bp fragment. The size and number of DNA fragments hybridizing to P-PST2–2 suggest that the number of genes closely related to P-PST-1 is small, in agreement with the possible presence of only two forms of PSTs in human tissues as indicated in the purification and kinetic studies (2). These results differ from the common pattern of drug-metabolizing enzyme families, such as UDP-glucuronyltransferases and glutathione *S*-transferases, that consist of a relatively large number of isoenzymes with overlapping substrate specificities.

***In vitro* transcription and translation of P-PST-1.** *In vitro* transcription and translation were used to verify that P-PST-1 translated a protein equivalent in size to P-PST. P-PST-1 was subcloned into an expression vector, pSV-SPORT-1, and RNA transcripts were generated using SP6 RNA polymerase. *In vitro* translation of the RNA transcripts using rabbit reticulocyte lysate and [^{35}S]MET to label the translation products revealed a major translation product from pSV-SPORT-P-PST-1 that comigrated with immunoreactive P-PST from human liver cytosol during SDS-PAGE (Fig. 5). The lower molecular weight bands probably reflect premature termination of transcription or initiation of transcription at inappropriate sites. This result indicates that P-PST-1 encodes a protein identical in size to P-PST and any post-translational modifications occurring to the enzyme do not greatly alter its mobility during SDS-PAGE.

Expression of P-PST-1 in COS-7 cells. Expression of P-PST enzyme activity in COS-7 cells was used to confirm definitively that P-PST-1 encodes P-PST. Fig. 6 shows that cytosol prepared from COS-7 cells transfected with pSV-SPORT-1 alone did not possess detectable levels of Mx ST activity and only low levels of PNP ST activity, whereas cytosol from COS-7 cells transfected with the pSV-SPORT-P-PST-1 construct displayed substantial amounts of Mx ST activity and a significant increase in PNP ST activity (Fig. 6). Mx has also been shown to be a specific substrate for P-PST and is reported not to be sulfated by M-PST (18). The ratio of PNP ST activity to Mx ST activity (ratio, 2.7:1) in the cytosol of transfected cells is similar to the ratio of these activities in human liver cytosol (18). Trace levels of dopamine sulfation activity were observed in the COS-7 cells but no significant increase in dopamine sulfation was detected in cells transfected with pSV-SPORT-P-PST-1 and 10 μM dopamine did not inhibit the activity of expressed P-PST. Dopamine at micromolar concentrations is a specific substrate for M-PST (2, 19). Also, immunoreactive P-PST was detected in cytosol obtained from the pSV-SPORT-P-PST-1-transfected COS-7 cells but not in cytosol from cells transfected with pSV-SPORT-1 alone (Fig. 6). These studies confirm that P-PST-1 encodes enzymatically active and immunologically reactive P-PST. The *in vitro* translated form of P-PST-1 is identical in size to both the expressed and liver forms of P-PST. The discrepancy between the size of P-PST predicted from the cDNA sequence and the size of the

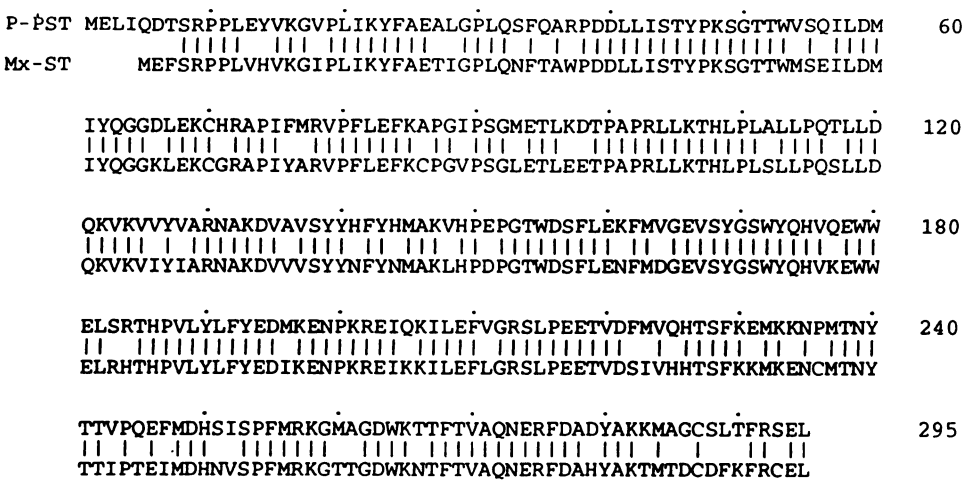

Fig. 7. Amino acid sequence comparison of human liver P-PST with rMx-ST. Deduced amino acid sequence comparison of P-PST and rMx-ST using the GAP program (30) to optimize sequence alignment. *Horizontal lines*, sequence identity.

protein as determined by SDS-PAGE is probably an artifact of the estimation of protein sizes by SDS-PAGE.

Active P-PST eluted during size exclusion chromatography with an apparent molecular mass of 68 kDa (9). The observation that expressed P-PST is enzymatically active and has a subunit molecular mass of 34,097 Da supports our previous hypothesis that P-PST exists as a homodimer in its active form (9). Two of the 34,097-Da subunits expressed in the COS-7 cells apparently associate to form the active enzyme.

Comparison of P-PST-1 with rMx-ST. As discussed previously, rMx-ST is apparently closely related to human liver P-PST, based on similarities in amino acid sequence and kinetic and immunological properties. The number of cytosolic STs detected in human liver is also small in comparison with the multiplicity of forms of ST identified in rat liver (1, 21, 37, 38). Of the multiple forms of PST in rat liver, rMx-ST has been reported to be the major rat liver PST responsible for Mx sulfation (21). Likewise, of the two forms of PST identified in human liver, only P-PST has activity toward Mx (18).

The recent isolation and characterization of a cDNA encoding rMx-ST (27) have confirmed the prediction that rMx-ST and P-PST are closely related (Fig. 7). The predicted amino acid sequences of these enzymes are 80% identical at the nucleotide and amino acid sequence levels and 89% similar at the amino acid sequence level. Based on immunological cross-reactivity (21), substrate reactivity (18, 21), and sequence similarity (Fig. 7), rMx-ST is apparently the rat homologue of human liver P-PST. Because the *N*-oxide sulfate of Mx is responsible for both of the therapeutic effects associated with Mx therapy, vasodilation and stimulation of hair growth (11–13), the identification of rMx-ST as the rat homologue of P-PST provides a rationale for using rMx-ST as an *in vivo* model for investigating the role of human P-PST in Mx-stimulated hair growth and vasodilation. However, one difference between human P-PST and rMx-ST is that P-PST has been found in most human tissues (2), whereas rMx-ST has been detected only in rat liver and the outer-root sheath of the hair follicle (20, 39).

The lack of reported nucleotide sequences for rat and human PSTs limits the comparisons that can be made between the rat and human PSTs. Investigation of the properties of the cloned enzymes will greatly aid in the investigation of the functions of and differences in sulfation in humans and rats. For example, *N*-hydroxy-2-acetylaminofluorene is sulfated and activated to a reactive electrophile in rats, apparently by AST IV; however, *N*-hydroxy-2-acetylaminofluorene is apparently not sulfated in human liver cytosol (2, 40). Also, the differences in levels of PST and steroid ST activities between male and female rat liver have not been observed in human liver.

The results of this investigation are consistent with the presence of only two forms of PST in human liver. Characterization of P-PST at the molecular level provides important insight into the structure and heterogeneity of this major class of human drug-metabolizing enzymes. Future studies involving the expression of P-PST in cultured cells will allow a more in-depth understanding of the role of P-PST in drug and xenobiotic metabolism, as well as in the modification of a number of endogenous substances.

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