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Sequence Analysis, *In Vitro* Translation, and Expression of the cDNA for Rat Liver Minoxidil Sulfotransferase

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SUMMARY

A cDNA encoding minoxidil sulfotransferase (Mx-ST), a rat liver cytosolic sulfotransferase that catalyzes the 3′-phosphoadenosine 5′-phosphosulfate-dependent sulfate conjugation of minoxidil and *p*-nitrophenol, has been isolated from a λgtII cDNA library constructed from poly(A)$^+$ RNA isolated from female Sprague-Dawley rat liver. The largest cDNA, designated Mx-STb, consists of 1245 base pairs and contains an open reading frame of 291 amino acids. The predicted size of the protein translated by Mx-STb is 33,909 Da; however, the molecular mass of the pure protein [*Biochem. J.* **270**:721–728 (1990)] is estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 35,000 Da. The size of the protein obtained by *in vitro* translation of Mx-STb is identical to that of the pure protein. Results of initial studies of the expression of Mx-STb in COS-1 cells indicate that the expressed protein displays characteristic Mx-ST and *p*-nitrophenol sulfotransferase activity, is recognized by rabbit polyclonal antibodies raised against pure rat liver Mx-ST, and migrates at approximately 35,000 Da during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This paper presents the cloning and expression of a rat phenol sulfotransferase for which the physical, immunological, and kinetic properties are known. Isolation of the cDNA for Mx-ST will aid in the investigation of the heterogeneity, the tissue localization, and the characterization of the kinetic properties of this important drug-metabolizing enzyme, with respect to other similar phenol sulfotransferases present in rat liver cytosol.

Sulfation is a major conjugation reaction involved in the biotransformation of a wide variety of drugs, carcinogens, and endogenous compounds possessing a hydroxyl or nitrogen functional group. This reaction is catalyzed by the family of enzymes termed the STs. The mechanism of sulfation involves the transfer of the sulfonate moiety from PAPS to an acceptor compound, to form a sulfate ester. A major role of the STs in drug metabolism is the conversion of a substance into a more readily excretable form, via formation of its sulfate or sulfamate conjugate. Less frequently, conjugation with a sulfonate group can also serve to convert a substrate to its biologically active form, as seen with the bioactivation of Mx (1, 2).

Mx is used as a therapeutic vasodilating agent in the treatment of hypertension and, upon recognition of its hair growth-stimulatory properties, has been used for the treatment of conditions such as alopecia areata and alopecia androgenetica (3). Recent reports have indicated that Mx-S, rather than Mx, is the therapeutically active form of the drug and is responsible for both the hair growth-stimulatory and smooth muscle-vasodilatory effects observed with Mx treatment (1, 2). Mx is sulfated by PAPS-dependent PSTs in both rat and human liver cytosol (4, 5). The mechanism by which Mx-S elicits its effects is still unclear, but recently Meisheri *et al.* (6), as well as Groppi *et al.* (7), have postulated that protein sulfation may be involved.

Recently, Hirshey and Falany (4) reported the purification to apparent homogeneity of a unique rat liver cytosolic ST, Mx-ST, that is capable of sulfating the *N*-oxide moiety of Mx, as well as small phenols, such as PNP. Because of its ability to conjugate small phenols and its immunological similarity to the human liver P-PST, Mx-ST is considered a member of the family of PSTs present in rat liver cytosol; however, Mx-ST is

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ABBREVIATIONS: ST, sulfotransferase; Mx, minoxidil; PST, phenol sulfotransferase; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PNP, *p*-nitrophenol; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; P-PST, phenol-sulfating phenol sulfotransferase; Mx-S, minoxidil *N,O*-sulfate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; kb, kilobases.

physically, immunologically, and kinetically distinct from the other forms of PST identified in rat liver (8–11). Mx-ST has also been specifically immunolocalized to the outer root sheath of rat hair follicles, where it may be involved in the sulfation and hair growth-stimulatory activity of Mx (12).

At least eight different forms of PST activity have been identified and isolated from rat liver cytosol (8–11). Very little is known about the molecular structure or heterogeneity of rat PST cDNAs, because to date only a single preliminary report of a rat PST cDNA sequence has appeared (13). Unfortunately, the biochemical, kinetic, or physical properties of the enzyme encoded by this PST sequence were not reported. However, based on preliminary amino acid sequence information on Mx-ST, we suspected a high degree of similarity between Mx-ST and PST-1.

The present paper describes the cloning, sequence analysis, molecular characterization, *in vitro* translation, and expression in mammalian cells of the cDNA coding for rat liver Mx-ST. This represents one of the first reports of the cloning and characterization of a rat PST cDNA in which the physical and biochemical properties of the protein encoded by that cDNA are known. The isolation and characterization of the cDNA for Mx-ST will also provide a valuable probe for the investigation of the functions of this enzyme in drug metabolism, as well as in hair growth and development.

Experimental Procedures

Materials

Restriction enzymes and DNA-modifying enzymes were purchased from United States Biochemicals (Cleveland, OH) or New England Biolabs (Beverly, MA). [α-^{35}S]dATP (3000 Ci/mmol), [α-^{32}P]dCTP (800 Ci/mmol), and [^{35}S]methionine (1232.7 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Nick-translation kits were purchased from Amersham, Inc. (Arlington Heights, IL). The MAX-Iscript *in vitro* transcription kit and the ReticLysate IVT translation kit were purchased from Ambion Inc. (Austin, TX). Magnagraph nylon transfer membrane was purchased from Micron Separation Inc. (Westborough, MA). Nitrocellulose paper was purchased from Schleicher & Schuell (Keene, NH). Oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX). Packagene was obtained from Promega-Biotec (Madison, WI). Livers were obtained from male or female Sprague-Dawley rats (175–200 g), from Charles Rivers Breeders (Portage, MI) and Zivic Miller Laboratories (Zelionople, PA). COS-1 cells were purchased from the American Type Culture Collection (Rockville, MD). FBS, DMEM, and lipofectin reagent were obtained from GIBCO-BRL (Grand Island, NY). All other reagents were of molecular biology grade.

Methods

Construction of rat liver λgt11 cDNA library. Total RNA was isolated from livers of female Sprague-Dawley rats by the method of Chomczynski and Sacchi (14). The cDNA library was synthesized in our laboratory with poly(A)$^+$ RNA isolated from total RNA by oligo(dT)-cellulose chromatography, as described by Davis *et al.* (15). The first strand of the cDNA was synthesized with avian myeloblastosis virus reverse transcriptase and an oligo(dT) primer. The second strand was synthesized using an RNase H and DNA polymerase I procedure (15). cDNAs larger than 300 base pairs were size-selected after agarose gel electrophoresis, blunted with T4 polymerase, and ligated to *Eco*RI linkers (Promega). The cDNAs were then digested with *Eco*RI, purified by agarose gel electrophoresis, and ligated into the *Eco*RI site of λgt11. The recombinant phage were packaged using Packagene (Promega) and grown in *Escherichia coli* Y1090. The library contained approximately 1.2×10^6 independent recombinant phage.

Isolation of Mx-ST cDNA. The female Sprague-Dawley λgtII library was grown in *E. coli* Y1090 on 150-mm-diameter Petri dishes at a phage concentration of 30,000–40,000 plaques/plate (approximately 80% of phage were recombinants). The probe utilized for the detection and isolation of the Mx-ST cDNA was an antisense oligonucleotide (27 nucleotides in length) synthesized to an internal sequence of a putative rat liver PST cDNA (PST-1, bases 690–716) described by Ozawa *et al.* (13). PST-1 was isolated from a λgt11 male Sprague-Dawley rat liver cDNA library by using antiserum raised against a purified PNP-ST. However, the purification and the characterization of the kinetic, immunological, and physical properties of PST-1 or the anti-PST antibody have not been reported, thereby providing inadequate support for the identification of this clone.

Approximately 250,000 independent clones from the λgt11 female rat liver cDNA library were screened with the ^{32}P-kinased oligonucleotide, and purification of positively hybridizing plaques was carried out as described by Sambrook *et al.* (16). Hybridization of the cDNA library with the radiolabeled 27-mer oligonucleotide probe was performed at 42°, using approximately 1.5×10^6 dpm/ml of hybridization buffer (10× Denhardt's solution, [1 × Denhardt's solution is 0.02% each of Ficoll *Type 400), polyvinalpyrolidone, and bovine serum albumin], 50% formamide, 800 mM NaCl, 10 mM Tris·HCl, pH 7.2, 1 mM EDTA, 0.5% SDS, 100 μg/ml poly(A)$^+$, 100 μg/ml yeast tRNA). The filters were washed at 50° with 3× SSC (1× SSC = 150 mM NaCl, 50 mM sodium citrate, pH 7.0) containing 0.5% SDS and then with 2× SSC containing 0.5% SDS and were exposed to X-ray film at −70°, with an intensifying screen.

Positive plaques were purified by cycles of dilution and rescreening until a single positive plaque-forming unit could be isolated. Phage DNA was purified from liter cultures of phage grown in *E. coli* Y1090 cells, using a cesium chloride gradient procedure (16). The Mx-ST cDNA inserts were isolated from the λgt11 phage by digestion with *Eco*RI and were purified by using an agarose gel electrophoresis and DE-81 paper procedure (16). The Mx-ST cDNAs were then subcloned into the *Eco*RI site of pGem 7zf (Promega), for preparation of large amounts of the Mx-ST cDNAs for characterization, sequence analysis, and transfection studies.

DNA sequencing. The Mx-ST cDNAs in pGem 7zf were sequenced by using the Sequenase 2.0 dideoxynucleotide procedure (United States Biochemicals), with [^{35}S]dATP as a radioactive label. pGem 7zf possesses T7 and SP6 sequencing primer sites on opposite sides of a multiple cloning site, allowing for sequence analysis of both strands of the cDNA. For sequencing of the complete cDNA, restriction fragments of the cDNAs were resolved by agarose gel electrophoresis, recovered from the gels by elution onto DE-81 paper, and subcloned into appropriately digested pGem 7zf for double-stranded sequencing. DNA fragments synthesized with Sequenase 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 programs of the University of Wisconsin Genetics Computer Group (17).

Amino acid composition analysis of purified rat liver Mx-ST. In preparation for amino acid analysis, affinity-purified male rat liver Mx-ST was further purified by HPLC, as described previously (4). After HPLC, Mx-ST was frozen at −70°, and the amino acid composition analyses were performed by the Protein Structure Facility of the University of Iowa, using a Beckman 6300 amino acid analyzer. For Mx-ST, 24-, 48-, and 72-hr HCl hydrolyses were performed and the amino acid composition was calculated for a subunit molecular mass of 35,000 Da. Performic acid oxidation was carried out for the determination of cysteine and methionine residues.

Amino acid sequence analysis of purified rat liver Mx-ST. Mx-ST was purified from male Sprague-Dawley rat liver cytosol, for amino acid sequencing studies, as described by Hirshey and Falany (4). Because the amino-terminal end of Mx-ST was not available for sequencing, internal amino acid sequences of Mx-ST were obtained from HPLC-purified cyanogen bromide-generated fragments of puri-

fied Mx-ST, prepared following the method of Yuen *et al.* (18). Briefly, approximately 100 μg of purified Mx-ST were resolved by SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Inc., Bedford, MA), and visualized by Ponceau S staining. The Mx-ST band was cut from the filter, placed in a microcentrifuge tube, and cleaved *in situ* with 100 μl of 10 mg/ml fresh cyanogen bromide (Kodak, Rochester, NY), in 70% formic acid, overnight in the dark at room temperature. The next morning, the formic acid solution was removed, the filter was washed with 70% isopropanol/5.0% TFA, and the two solutions were combined and lyophilized. The cyanogen bromide-generated peptide fragments were then resuspended in water containing 0.1% TFA and were separated by reverse phase HPLC on a Vydac C-18 column (46 mm × 250 mm), using a linear gradient of 0–80% acetonitrile in the presence of 0.1% (v/v) TFA. Elution of peptides from the Vydac C-18 column was monitored, with a Perkin Elmer model 235 diode array detector, at wavelengths of 215 nm and 280 nm. After HPLC, peaks absorbing at both wavelengths were frozen at −70° before sequence analysis. The amino acid sequence of the isolated peptides was determined by using a 470A gas phase protein sequenator (Applied Biosystems, Inc.) connected on-line to an ABI 120A (HPLC) phenylthiohydantoin analyzer. Data were collected and yields were calculated on a Nelson Analytical 3000 Series chromatography system.

Northern blot analysis of rat liver RNA. Total RNA was prepared from liver samples of 10-week-old male and female Sprague-Dawley rats by using the acid guanidinium thiocyanate method of Chomczynski and Sacchi (14). Total RNA (10 μg) from each sample was resolved by electrophoresis in a 1.5% agarose-formaldehyde denaturing gel, transferred to a nitrocellulose filter, and baked *in vacuo* for 2 hr at 80°. The filter was then washed for 2 hr in 10× Denhardt's solution, 50% formamide, 800 mM NaCl, 10 mM Tris·HCl, pH 7.2, 1 mM EDTA, 0.5% SDS, 75 μg/ml poly(A)$^+$, 50 μg/ml yeast tRNA. Next, the filter was hybridized at 42° overnight in fresh solution containing 1 × 10^6 dpm/ml of the nick-translated [^{32}P]Mx-ST cDNA (5 × 10^7 dpm/μg). The filter was washed at 65° with 3× SSC, 2× SSC, and then 1× SSC and was exposed to autoradiography film for 3 days, with an intensifying screen, before development.

Southern blot analysis of rat genomic DNA. Genomic DNA was prepared from the livers of male Sprague-Dawley rats by the method of Strauss (19). Aliquots (15 μg) of genomic DNA were exhaustively digested with restriction enzymes with six-base recognition sites (*Eco*RI, *Bam*HI, *Hind*III, and *Kpn*I), and the digested genomic DNA was resolved by electrophoresis in an 0.8% agarose gel. DNA fragments were transferred to nylon membranes, according to the manufacturer's instructions, and were hybridized overnight, at 42° (high stringency) or 35° (low stringency), to nick-translated [^{32}P]Mx-STb (approximately 1.5 × 10^6 dpm/ml), in 50% formamide, 5× SSC, 10 mM Tris, pH 7.5, 4× Denhardt's, 200 μg/ml calf thymus DNA, 200 μg/ml poly(A)$^+$, 0.1% SDS. Filters were washed (at 42° or 35°) with 3× SSC containing 0.5% SDS, followed by 2× SSC containing 0.5% SDS, and were exposed to autoradiography film at −70° for 3 days.

***In vitro* transcription of Mx-STb.** Mx-STb cDNA was isolated from pGem 7zf by *Eco*RI digestion and was subcloned into the *Eco*RI site of pSV-SPORT-1. pSV-SPORT-1 (BRL) is a mammalian expression vector containing the SV40 early promoter, as well as SP6 and T7 RNA polymerase promoter sites. Mx-STb was subcloned into this vector for expression studies of the cDNA in cultured COS-1 cells. Insertion of the Mx-STb cDNA in the correct orientation for transcription by SP6 RNA polymerase expression was checked by sequence analysis. pSV-SPORT-1-Mx-STb was linearized with *Hind*III, and RNA transcripts were synthesized by using the MAXIscript SP6 transcription kit. The integrity and size of the generated transcripts were monitored by denaturing agarose-formaldehyde gel electrophoresis.

***In vitro* translation of Mx-ST cDNAs.** Transcripts of Mx-STb generated by *in vitro* transcription were translated with a ReticLysate IVT kit, using [^{35}S]methionine for labeling of the synthesized proteins. Samples of the *in vitro* translation reactions were suspended in SDS-PAGE loading buffer (containing 5% 2-mercaptoethanol and 2.5% SDS), heated to 95°, and resolved by SDS-PAGE in 12.5% polyacrylamide gels, as described previously (4). The gels were vacuum-dried at 65° and exposed to autoradiography film overnight, to detect the newly translated proteins. Detectable levels of newly translated protein were obtained with 0.1-μg aliquots of Mx-STb RNA transcripts.

Transient transfection of cells. COS-1 Green monkey kidney cells were maintained in DMEM with high glucose, supplemented with 10% heat-inactivated FBS. Transfections were carried out by a lipofectin procedure, according to the manufacturer's instructions (GIBCO-BRL). Briefly, cells were plated in 60-mm dishes at least 18 hr before transfection. When cells were approximately 80% confluent, they were washed two times with serum-free medium, followed by addition of 2.5 ml of serum-free medium to each dish. Next, 10 μg of supercoiled pSV-SPORT-1 or pSV-SPORT-1-Mx-STb cDNA (premixed with 30 μg of lipofectin) were applied dropwise to each dish. Cells were incubated at 37° in 5% CO_2 for 8 hr, followed by addition of 2.5 ml of DMEM containing 20% FBS to each dish. The cells were then incubated for 3 days in medium containing 10% FBS. The medium was changed after the first 24 hr.

To detect the expression of Mx-ST, cytosolic fractions of the transfected COS-1 cells were prepared for enzyme assays and immunoblot analysis. Cells were washed twice with Tris buffer (10 mM Tris·HCl, pH 7.4, containing 0.25 M sucrose, 1 mM dithiothreitol, 10%, v/v, glycerol, and 1 mM phenylmethylsulfonylfluoride), harvested by scraping into cold Tris buffer, homogenized three times for 10 sec each time, using a Brinkmann Polytron, and centrifuged at 100,000 × *g*. Cytosolic samples were then assayed for the presence of Mx-ST and PNP-ST activity, as well as being separated by SDS-PAGE for immunodetection of expressed Mx-ST with the anti-rat Mx-ST antibodies (4).

Enzyme assays and SDS-PAGE. Mx-ST activity was determined by using the extraction procedure described by Johnson and Baker (20), which takes advantage of the migration of Mx-S into an ethyl acetate organic phase. PNP-ST activity was assayed by using the barium precipitation method of Foldes and Meek (21). For both substrates, [^{35}S]PAPS was used as sulfate donor, and final concentrations of Mx and PNP were 1 mM and 1.2 μM, respectively (4). Reaction conditions were identical to those previously described (4), with the exception of an increase in incubation time from 10 to 40 min. Separation of proteins was carried out in 10–15% gradient SDS-polyacrylamide gels.

Immunoblotting procedure with rabbit anti-rat Mx-ST antibodies. Immunoblot analysis of rat liver and COS-1 cell cytosolic fractions was carried out as described previously (4). Briefly, after resolution by SDS-PAGE, proteins were electrotransferred to nitrocellulose paper. An overnight incubation with a 1/5000 dilution of rabbit anti-rat Mx-ST IgG (0.5 μg of protein/μl, before dilution) or anti-Mx-ST antiserum as primary antibody was followed by detection of immunoconjugates by using a goat anti-rabbit IgG antibody-alkaline phosphatase conjugate (Bio-Rad).

Results

Molecular characterization of MX-ST cDNAs. A λgt11 cDNA library was synthesized from poly(A)$^+$ RNA isolated from female Sprague-Dawley rat liver and was used for the isolation of the Mx-ST cDNAs. A female rat liver library was used because several rat liver PSTs are reportedly much less abundant in female rat liver than in male, and this would result in a relative enrichment of Mx-ST message in female rats (4, 9, 22, 23). Using the 27-mer oligonucleotide synthesized to PST-1, two positive clones, Mx-STa and Mx-STb, were isolated after screening of approximately 250,000 phage from the female rat liver λgt11 cDNA library. The two clones, Mx-STa (995 base pairs) and Mx-STb (1245 base pairs), were identical in nucleotide sequence and possessed identical open reading frames (Fig. 1). The differences between the cDNAs are that

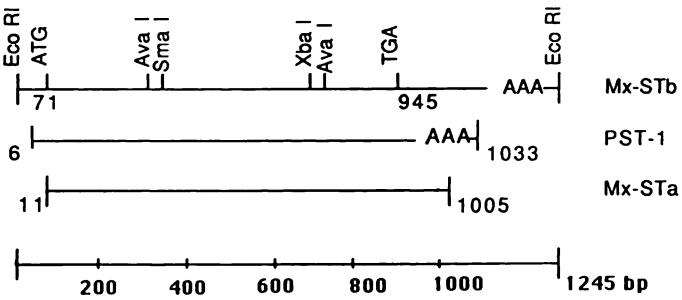

Fig. 1. Schematic representation comparing the sizes of the Mx-STb, Mx-STa, and PST-1 (13) cDNAs. An approximate scale is drawn at the *bottom*. The actual lengths of the poly(A)$^+$ tracts (AAA) are not depicted. Restriction sites and translation start (ATG) and stop (TGA) codons are indicated along Mx-STb cDNA. The 5′ and 3′ ends of the Mx-STa and PST-1 cDNAs are numbered relative to Mx-STb.

Mx-STb possesses a more extensive 3′ nontranslated region, which terminates in a poly(A)$^+$ tract, and also has a slightly longer 5′ nontranslated region than does Mx-STa. Because it is slightly larger and possesses the 3′ nontranslated region, Mx-STb was used to generate most of the data presented in this study. Also, the sequence of PST-1 (13) was identical to the sequence of Mx-STb, except that Mx-STb possessed longer 5′ and 3′ nontranslated regions (Fig. 1).

Fig. 2 shows the nucleotide sequence and translation of Mx-STb. Mx-STb is 1245 base pairs in length and contains an open reading frame, beginning at base 71, that encodes a 291-amino acid polypeptide. The predicted molecular mass, 33,909 Da, of a Mx-ST subunit calculated from the translated sequence is slightly smaller than the previously reported subunit size of 35,000 Da, estimated by SDS-PAGE analysis of the pure protein (4). The termination codon (TGA) is followed by 298 nucleotides of the 3′ nontranslated region, including a 25-base poly(A)$^+$ tract. A putative polyadenylation signal sequence, AATAAA (24), is located 21 nucleotides upstream from the beginning of the poly(A)$^+$ tract.

Northern blot analysis of total rat liver RNA was used to determine both the size and the number of messages hybridizing to Mx-STb. Fig. 3 shows that, under high stringency hybridization conditions, Mx-STb detected two different-size messages in total liver RNA isolated from both male and female rats. The larger and more abundant RNA was approximately 1350 nucleotides in length, whereas the smaller and less abundant message was about 1100 nucleotides in length. There was also a greater intensity of both bands in RNA isolated from male rats, compared with females. These results agree with the approximately 2-fold higher level of expression of Mx-ST activity and immunoreactive protein in male rat liver, compared with females, as described previously (4). In addition, Fig. 3 also shows that the ratio of intensity of the top and bottom bands in male rat liver RNA versus female RNA appeared quite similar. Identical results were obtained using poly(A)$^+$ RNA isolated from livers of male and female rats, and no bands were detected in the rRNA and tRNA fractions after oligo(dT) chromatography of total RNA (data not shown).

Southern blot analysis of total male Sprague-Dawley rat liver DNA was performed using Mx-STb as a probe, to investigate the complexity and size of genomic DNA fragments related to Mx-STb. Fig. 4 shows the pattern of fragments of rat liver DNA, digested with several restriction enzymes, that hybridize with Mx-STb under high stringency conditions. The sizes of

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                                                          GGAATTCGCAACGCCTA
CACAAAGATCCCTATCACTGAGCACCCGGAGGCAAGGCTCAGAACCCCAGGATCAGCAAC

ATGGAGTTCTCCCGTCCACCGCTAGTGCATGTGAAGGGTATCCCACTCATCAAATACTTT
MetGluPheSerArgProProLeuValHisValLysGlyIleProLeuIleLysTyrPhe    20

GCAGAGACAATTGGGCCATTGCAGAACTTCACAGCCTGGCCTGATGACTTGCTGATCAGC
AlaGluThrIleGlyProLeuGlnAsnPheThrAlaTrpProAspAspLeuLeuIleSer    40

ACATACCCAAAGTCTGGTACTACCTGGATGAGTGAGATCCTGGATATGATCTATCAGGGT
ThrTyrProLysSerGlyThrThrTrpMetSerGluIleLeuAspMetIleTyrGlnGly    60

GGCAAGCTAGAGAAGTGTGGCCGCGCCCCCATCTATGCCCGGGTACCCTTCCTTGAGTTC
GlyLysLeuGluLysCysGlyArgAlaProIleTyrAlaArgValProPheLeuGluPhe    80
                    ----------XXX---

AAATGTCCAGGGGTTCCCTCAGGTCTTGAAACTTTGGAAGAGACACCAGCCCCACGGCTC
LysCysProGlyValProSerGlyLeuGluThrLeuGluGluThrProAlaProArgLeu   100

CTTAAGACACATCTGCCCCTGTCCTTGCTCCCTCAGAGTCTGCTGGATCAGAAGGTCAAG
LeuLysThrHisLeuProLeuSerLeuLeuProGlnSerLeuLeuAspGlnLysValLys   120

GTGATCTACATTGCCCGAAATGCAAAGGATGTGGTTGTCTCCTATTATAACTTCTACAAC
ValIleTyrIleAlaArgAsnAlaLysAspValValValSerTyrTyrAsnPheTyrAsn   140

ATGGCCAAGCTGCACCCTGATCCAGGCACCTGGGACAGCTTCTTGGAGAACTTCATGGAT
MetAlaLysLeuHisProAspProGlyThrTrpAspSerPheLeuGluAsnPheMetAsp   160
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GGGGAAGTGTCCTATGGGTCGTGGTACCAGCACGTGAAGGAGTGGTGGGAGCTGAGACAC
GlyGluValSerTyrGlySerTrpTyrGlnHisValLysGluTrpTrpGluLeuArgHis   180

ACTCACCCTGTTCTCTATCTCTTCTATGAAGACATAAAGGAGAACCCCAAAAGGGAGATC
ThrHisProValLeuTyrLeuPheTyrGluAspIleLysGluAsnProLysArgGluIle   200

AAGAAGATTCTAGAGTTTTTGGGGCGCTCTCTACCCGAGGAGACTGTGGATTCCATTGTT
LysLysIleLeuGluPheLeuGlyArgSerLeuProGluGluThrValAspSerIleVal   220

CACCACACATCTTTCAAGAAAATGAAAGAGAACTGCATGACTAACTACACAACCATCCCC
HisHisThrSerPheLysLysMetLysGluAsnCysMetThrAsnTyrThrThrIlePro   240
                                    ------------------------

ACTGAGATTATGGACCACAATGTTTCTCCCTTCATGAGGAAAGGTACTACTGGGGACTGG
ThrGluIleMetAspHisAsnValSerProPheMetArgLysGlyThrThrGlyAspTrp   260
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AAAAATACCTTCACTGTAGCCCAGAATGAGCGCTTTGATGCCCACTATGCTAAGACAATG
LysAsnThrPheThrValAlaGlnAsnGluArgPheAspAlaHisTyrAlaLysThrMet   280

ACAGATTGTGACTTCAAGTTTCGTTGTGAACTATGAGTGGATTATGGCTATACTGGGAAC
ThrAspCysAspPheLysPheArgCysGluLeuEnd                           291

CAAGGCAAACTGACACAGCCCATCATGATCTCAAGTAAAATGTGATGTGTTCAATCTACT
TGTTGTATGCCTAGAGGAAATCTGAGCTAAGAGAATAGGATTGGGGATGTGGCTGAGGCA
GAGGGTTTTATCAACGCATGTCAGGAAAGCAATCAGTCCCAACACCTAAAAAGAACCTAA
AGTACAAACATGCAAAAAATAGTAAGATAAACTATATTTTACCTGAAAGAATAAATGCCA
CTGGGAAATGAAAAAAAAAAAAAAAAAAAAAAAAAAAGAATTC
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Fig. 2. Nucleotide and deduced amino acid sequences of the Mx-STb cDNA. Amino acid positions are numbered at *right*. The amino acid sequences of peptides derived from cyanogen bromide cleavage of pure Mx-ST are *underlined*, indicating sequence identity. *XXX*, undetermined amino acid residue. Note that the initial methionines for the two peptides, which are 10 amino acids in length, are assumed.

the bands reacting after specific restriction digests are as follows: EcoRI, 6.2 and 5.2 kb; *Bam*HI, 4.3 and 3.9 kb; *Hind*III, 5.5 kb; *Kpn*I, 2.4 and 1.35 kb. The size and number of DNA fragments hybridizing to Mx-STb are suggestive of the presence of one or two genes or pseudogenes, in genomic DNA, that are closely related to Mx-STb. Very similar results were also obtained in different Southern blots of restriction-digested total genomic DNA, with Mx-STb as the probe, using less stringent hybridization (50% formamide, 5× SSC, at 35°) and wash (2× SSC containing 0.5% SDS, at 35°) conditions.

Amino acid sequence analysis of Mx-ST. The amino acid sequence of peptides derived from pure Mx-ST was compared with the translation of Mx-STb, in order to determine

Fig. 3. Northern blot analysis of male (*M*) and female (*F*) rat liver total RNA. Ten micrograms of total RNA were electrophoresed in a 1.5% agarose-formaldehyde denaturing gel. RNA was transferred to a nitrocellulose membrane and probed with ^{32}P-labeled Mx-STb, as described in Experimental Procedures. *Arrowheads at left*, migration of the 28 S and 18 S ribosomal subunits.

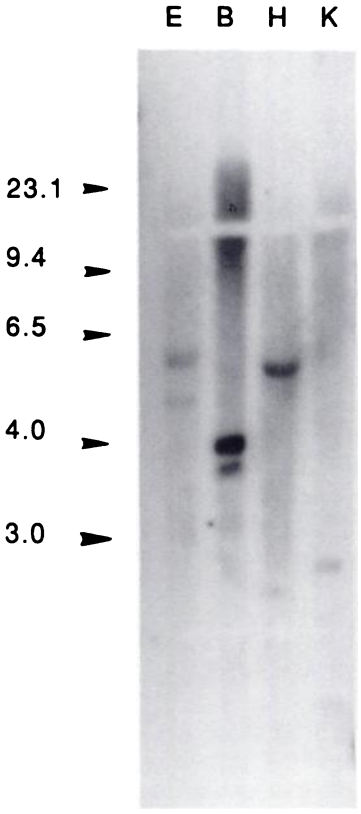

Fig. 4. Southern blot analysis of rat genomic DNA. *Eco*RI (*E*), *Bam*HI (*B*), *Hind*III (*H*), and *Kpn*I (*K*) restriction-digested rat liver genomic DNA was electrophoresed in an 0.8% agarose gel, as described in Experimental Procedures. The DNA fragments were transferred to a nylon membrane and probed with ^{32}P-labeled Mx-STb. *Arrowheads*, migration of molecular weight standards (in kb).

whether the protein encoded by Mx-STb is similar to Mx-ST. The amino-terminal end of Mx-ST was not available for sequencing; however, amino acid sequence was obtained from three cyanogen bromide fragments derived from Mx-ST. These peptides possess 100% sequence identity with the deduced amino acid sequence of Mx-STb (Fig. 2). The three peptides were 8, 10, and 10 amino acids in length, and each sequence was identified in the translation of Mx-STb. The location of the three peptides in the open reading frame of Mx-STb is not confined to one region of the Mx-STb cDNA, inasmuch as the Mx-ST peptides align to three separate regions of the translated protein. This pattern of alignments makes it less likely that the Mx-STb cDNA encodes a different PST isozyme that possesses only a small region of amino acid sequence similarity to Mx-ST. In addition, the open reading frame of Mx-STb is identical to that of PST-1.

To confirm further the identity of Mx-STb as the sequence encoding Mx-ST, the amino acid composition of Mx-ST obtained from analysis of the pure protein was compared with the composition of Mx-ST derived from the translation of Mx-STb. Table 1 shows the high degree of similarity between the mole percentage values obtained for each amino acid, based on the subunit molecular mass of the protein, as derived by these two methods.

***In vitro* transcription and translation.** To establish further that Mx-STb codes for Mx-ST, *in vitro* transcription and

TABLE 1
Amino acid composition

Amino acid	Amount[a]	
	From cDNA[b]	By analysis[c]
	mol %/mol of protein	
Cys	1.72	1.62
Asx	8.97	9.09
Glx	9.65	10.06
Ser	5.17	5.19
Gly	4.83	6.49
His	3.45	2.92
Arg	3.79	4.22
Thr	7.59	7.14
Ala	3.79	4.22
Pro	7.24	7.47
Tyr	4.83	4.55
Val	5.52	5.19
Met	3.45	4.22
Ile	5.17	4.87
Leu	8.97	9.09
Phe	5.52	5.52
Lys	7.93	8.12
Trp	2.41	ND[d]

[a] Values represent mole percentage of the amino acid residue per mole of protein.
[b] Derived from Mx-STb cDNA by using the Wisconsin Genetics Computer Group program (17).
[c] From Hirshey and Falany (4).
[d] ND, not determined.

translation of Mx-STb were used to determine the size of the protein translated by Mx-STb. The size of Mx-ST estimated by SDS-PAGE (35,000 Da) is slightly larger than the size calculated from the translation of Mx-STb (33,909 Da). Therefore, to determine whether Mx-STb encodes a protein that migrates similarly to Mx-ST during SDS-PAGE, Mx-STb was *in vitro* transcribed and translated. For these studies, Mx-STb was subcloned into the mammalian expression vector pSV-SPORT-1, and RNA transcripts were generated using SP6 RNA polymerase. The RNA transcripts were then translated *in vitro*, using a rabbit reticulocyte lysate system and [^{35}S] methionine to label the translation products. Fig. 5 shows that a single major translated product was detected, with a subunit molecular mass of approximately 35,000 Da, as determined by SDS-PAGE. Identical results were obtained when Mx-STa was *in vitro* translated (data not shown). The 35,000-Da protein comigrated with immunoreactive rat liver Mx-ST during SDS-PAGE, indicating that Mx-STb codes for a protein very similar in size to Mx-ST. Insufficient amounts of protein were translated by this system for detection of Mx-ST using the rabbit anti-Mx-ST antibodies.

Expression of Mx-ST in COS-1 cells. Table 2 provides confirmation that Mx-STb does, indeed, encode Mx-ST. Cytosolic extracts prepared from COS-1 cells transfected with pSV-SPORT-1-Mx-STb showed significantly greater ST activity towards Mx and PNP than did cytosolic extracts transfected with pSV-SPORT-1 alone. Furthermore, immunoblot analysis of cytosolic fractions obtained from COS-1 cells transfected with the Mx-STb cDNA revealed a protein that migrated at 35,000 Da and was recognized by the anti-rat Mx-ST polyclonal antibodies (Fig. 6). Fractions from control cells transfected

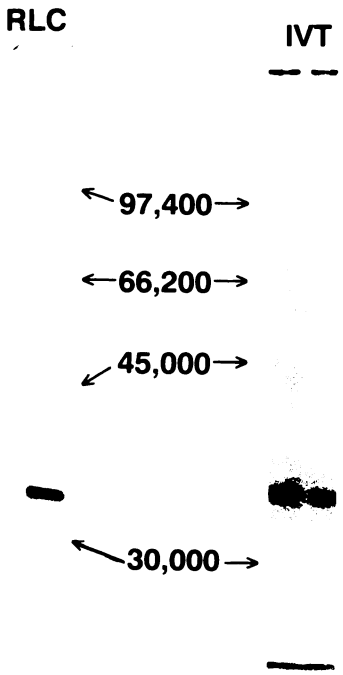

Fig. 5. *In vitro* translation of Mx-STb. *Left*, male rat liver cytosol (60 μg) was resolved by SDS-PAGE in a 12.5% gel, transferred to nitrocellulose, and probed with a 1/5000 dilution of anti-rat Mx-ST IgG fraction. *Right*, aliquots of the *in vitro* translation reactions (performed as described in Experimental Procedures) were resolved in a 12.5% polyacrylamide gel. The gel was dried and exposed to autoradiography film overnight. *Arrows*, migration of molecular weight standards.

TABLE 2

Mx-ST and PNP-ST activities in cytosol from COS-1 cells transfected with Mx-ST

Cytosol was prepared from COS-1 cells as described in Experimental Procedures. Cells were transfected with pSV-SPORT-1 or pSV-SPORT-1-Mx-STb (pSP-Mx-ST). Specific activity is reported as the mean ST activity ± standard deviation from four separate preparations, assayed in duplicate. One unit of activity is defined as 1 pmol of substrate conjugated/min. The level of significance of difference from controls was determined with Student's unpaired *t* test.

	Mx-ST activity	PNP-ST activity
	units/mg	*units/mg*
pSV-SPORT	0.90 ± 0.35	39.90 ± 2.32
pSP-MxST	5.25 ± 0.61[a]	57.45 ± 4.10[b]

[a] $p < 0.0005$.
[b] $p < 0.005$.

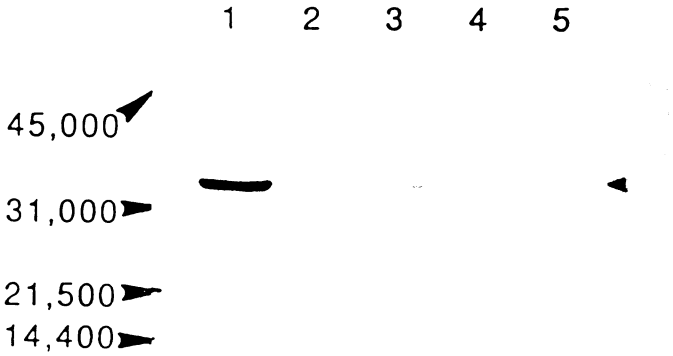

Fig. 6. Immunoblot analysis of recombinant and rat liver Mx-ST proteins. Proteins were separated in a 10–15% gradient SDS-polyacrylamide gel, electrophoretically transferred to nitrocellulose, and reacted with polyclonal rabbit antiserum (1/5000 dilution) raised against rat Mx-ST (4). *Lane 1*, rat liver cytosol [60 μg; prepared according to a previously published method (4)]; *lanes 2* and *4*, COS-1 cell cytosol (60 μg of protein) from cells transfected with pSV-SPORT-1; *lanes 3* and *5*, COS-1 cell cytosol (60 μg of protein) from cells transfected with pSV-SPORT-1-Mx-STb cDNA. *Arrowheads at left*, migration of molecular weight standards. *Arrow next to the band in the photograph*, immunoreactive Mx-ST, migrating at 35,000 Da.

with pSV-SPORT-1 alone did not contain this immunoreactive protein. Again, as seen with the *in vitro* translated product of the Mx-STb cDNA, the expressed protein migrated at a molecular mass identical to that of immunoreactive Mx-ST in rat liver cytosol. These studies confirm that Mx-STb encodes enzymatically active Mx-ST.

Discussion

The cDNA for rat liver Mx-ST has been cloned, sequenced, translated *in vitro*, and expressed *in vivo* in a mammalian cell line. Data presented in this report confirm that Mx-STb cDNA encodes Mx-ST and not a closely related PST. Also, the deduced amino acid sequence obtained from Mx-STb is identical to the sequence obtained from peptide fragments generated from the affinity-purified enzyme. In addition, *in vitro* translation studies indicate that Mx-STb encodes a protein of molecular mass identical to that of the immunoreactive protein in rat liver cytosol, as detected with rabbit anti-rat Mx-ST polyclonal antibodies (Fig. 5, *left*). Furthermore, results from expression studies in COS-1 monkey kidney cells indicate that the protein encoded by this cDNA is capable of catalyzing the sulfation of Mx and PNP, has a molecular mass of 35,000 Da, and is also recognized by polyclonal antibodies raised to rat liver Mx-ST. The minor discrepancy between the size of Mx-ST predicted from its cDNA sequence and the size of the

protein determined by SDS-PAGE analysis is most likely an artifact of the estimation of protein sizes by SDS-PAGE.

This study represents one of the initial reports of the expression and unambiguous identification of a cloned ST isolated from mammalian cells. The initial purification and characterization of rat liver Mx-ST had also suggested that the active form of the enzyme was most probably a homodimer, made up of two 35-kDa subunits, because the active enzyme eluted with an apparent molecular mass of 66–68 kDa during gel filtration chromatography (4). The expression of Mx sulfation activity after the transfection of Mx-STb supports this conclusion, because the enzymatically active enzyme was obtained during the expression studies, apparently from the association of two expressed 33,909-Da subunits. In addition, the isolation of only two cDNAs, Mx-STa and Mx-STb, with identical open reading frames, also suggests that identical subunits make up Mx-ST.

Comparison of the two cDNAs isolated from the female rat liver cDNA library shows Mx-STb to be the putative full-length cDNA, whereas Mx-STa possesses an incomplete 3′ nontranslated region, lacking a poly(A)$^+$ tail (Fig. 1). In addition, Mx-STb encodes an open reading frame identical to that of rat liver PST-1 cDNA (13); however, Mx-STb possesses a 3′ nontranslated region that is 212 base pairs longer than that of than PST-1 (Fig. 1). The Mx-STb and PST-1 cDNAs both appear to be completely processed, because both are polyadenylated; however, they may respond to different polyadenylation signal sequences. Comparison of the 3′ nontranslated regions of the Mx-STb and PST-1 cDNAs reveals the consensus polyadenylation signal sequence, AATAAA (24), 20 bases upstream from the poly(A)$^+$ tail of Mx-STb, whereas PST-1 contains the sequence AAGTAAA, 20 bases upstream from its poly(A)$^+$ tail. If PST-1 was generated by alternate processing and polyadenylation, this process would result in a shorter mRNA with a translated region identical to that of Mx-STb.

Northern blot analysis of total cellular RNA from male and female rat liver suggests the presence of two messages that are highly homologous to Mx-STb (Fig. 3). The larger message, 1350 nucleotides, probably corresponds to Mx-STb, because its size is slightly larger than that of Mx-STb cDNA (1245 base pairs), which is larger than the smaller RNA (1100 nucleotides) present on the Northern blot. The smaller message detected by Mx-STb possibly represents the alternatively processed form of the same Mx-ST message, because the smaller message agrees more with the size of PST-1 (1028 base pairs). Further, the identity of the 1100-nucleotide message as another processed form of Mx-ST message is supported by the higher levels of both RNAs in male versus female rat liver. Also, the apparent relative levels of the messages are constant in male and female rats. The larger message is consistently more abundant, suggesting that the polyadenylation site in Mx-STb is more well recognized than the altered site in PST-1. The lower levels of the smaller RNA may be the result of a slightly altered and less efficient polyadenylation identifier sequence in PST-1. Therefore, there are possibly two different processed forms of the same message, which are differentially processed by polyadenylation, present *in vivo*. It is also possible that the shorter message represents an allelic form of Mx-ST or a different, highly homologous, mRNA and the short 3′ nontranslated region of PST-1 is a cloning artifact.

The number of messages observed in Northern blot analysis is consistent with the detection of only two hybridizing species by Southern blot analysis of genomic DNA. Fig. 4 shows the pattern of fragments obtained when restriction enzyme-digested total rat liver DNA was hybridized with the Mx-STb cDNA, using relatively stringent hybridization conditions. Under less stringent hybridization conditions, no increase in the number of bands hybridizing to the cDNA was observed. The relatively small number (one or two) of bands that hybridized to Mx-STb under high stringency conditions was surprising, in light of the reported heterogeneity of the rat aryl STs at the protein level. It was expected that the cDNA would hybridize to more genes but, as seen with immunoblot analysis of Mx-ST in rat liver cytosol (4), sequence differences appear to be sufficient to allow probes, both antibody and nucleotide, to distinguish between enzymatically similar PST enzymes. Because a relatively long probe was used in these blots, it is difficult to distinguish between short regions of sequence identity and longer regions of overall high sequence homology. Further analysis using shorter probes designed to specific regions of the cDNA will help to answer the question of the relationship of the two bands to each other and, additionally, to other STs present in rat liver cytosol.

Our laboratory has previously reported that human liver P-PST is responsible for Mx sulfation and that rat liver Mx-ST and human liver P-PST exhibit similar substrate specificity and immunological similarity (4, 5). Furthermore, recent data (not shown) from our laboratory indicate that the two proteins share amino acid sequence homology, as well as nucleotide sequence homology. As previously mentioned, Mx-S has the ability to sulfate proteins nonenzymatically, resulting in unique post-translational modifications of proteins in target cells. The sulfation of various smooth muscle cell proteins may be central to the mechanism by which Mx-S produces its vasodilatory effect (6, 7). Similarly, the donation of a sulfate moiety to target proteins present in hair follicle cells may very well be linked to the hair growth-stimulatory properties of the drug, inasmuch as Mx-ST has been immunolocalized to the outer root sheath of the rat hair follicle, where it is proposed to bioactivate Mx and, thereby, stimulate hair growth (12). Therefore, studies of the role of rat Mx-ST in hair growth and drug metabolism may provide a model system for the functions of human liver P-PST.

An in-depth understanding of the biochemical and kinetic properties of Mx-ST will provide information for understanding the pharmacokinetics of drugs metabolized by sulfation, as well as for designing therapeutic drugs with regard to activation or inhibition of their sulfation. Characterization of Mx-ST at the molecular level, therefore, is important as a step in the improved understanding of the role of this enzyme in drug metabolism. Isolation of the cDNA provides a means to study the enzyme without the interference of similar enzyme activities that are otherwise difficult to separate by protein purification methods. Further studies in cultured cells will allow for determination of the kinetic properties of this enzyme in an *in vivo* system where the effects of sulfation of drugs and chemicals can be monitored and, to some degree, manipulated.

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