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Cloning And Molecular Characterization Of The Phenol Sulfating Form Of Human Liver Phenol Sulfotransferase.

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Cloning and molecular characterization of the phenol sulfating form of human liver phenol sulfotransferase

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CLONING AND MOLECULAR CHARACTERIZATION OF THE PHENOL SULFATING FORM OF HUMAN LIVER PHENOL SULFOTRANSFERASE

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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Sulfation of drugs and xenobiotics usually results in their decreased biological activity and increased excretion; however, certain compounds, such as the antihypertensive drug minoxidil, are converted by sulfation to biologically active forms. The predominant enzyme involved in the sulfation of many drugs, including minoxidil, is the hepatic phenol sulfotransferase, P-PST. To date, none of the human phenol sulfotransferases have been characterized at the molecular level. In order to gain insight into the structure, heterogeneity, and activity of P-PST, a cDNA encoding this enzyme has been isolated from a human liver library using a rat liver P-PST cDNA encoding minoxidil sulfotransferase. Upon sequencing, the largest clone isolated, P-PST2-2, was found to be truncated at the 5' end. The polymerase chain reaction, PCR, was employed to obtain the missing 5'sequence. Ligation of the PCR-amplified 5' cDNA end with P-PST2-2 resulted in a 1220 bp full length cDNA, P-PST-1, encoding a polypeptide of 295 amino acids with a molecular mass of 34,097

Daltons. In vitro transcription and translation verified the ability of P-PST-1 to encode a protein that co-migrates with immunoreactive P-PST from human liver on SDS-PAGE analysis. Cytosol from COS-7 cells transfected with P-PST-1 in the vector pSV-SPORT displayed 118- and 13- fold more sulfotransferase activity toward P-PST specific substrates minoxidil and p-nitrophenol, than did control cells ($p<0.0005$). Proteins encoded by the eight known mammalian sulfotransferase cDNA were used to generate a consensus sequence. Several potential phosphorylation motifs in the P-PST-1 encoded protein sequence were identified by computer analysis. On Northern blot analysis, P-PST-1 hybridized with one message of approximately 1300 bp in human liver RNA under conditions of moderate stringency. Following digestion of genomic DNA with several restriction endonucleases, Southern blot analysis revealed that P-PST-1 annealed with 1 to 3 DNA fragments under conditions of high and moderate stringency. These data suggest that unlike the large family of rat PSTs and the extensive families of other human drug metabolizing enzymes, the human PST family is small.

Abstract Approved by: **Committee Chairman** D S Barnes

Program Director

Date 9/22/92 **Dean of Graduate School** W. A. Sibley

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
APS	adenosine 5'-phosphosulfate
BAST I	bile acid sulfotransferase I
CCK	cholecystokinin
cDNA	copy DNA
CK2	casein kinase 2
COMT	catechol-o-methyl transferase
CNS	central nervous system
DCNP	2,6-dichloro-4-nitrophenol
DEAE	diethylaminoethyl
DEPC	diethyl pyrocarbonate
DHEA	dehydroepiandrosterone
DGGE	denaturing gradient gel electrophoresis
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
kD	kilodaltons
K_m	Michaelis-Menten constant

LIST OF ABBREVIATIONS (Continued)

M	molar
MAO	monoamine oxidase
min	minute
ml	milliliter
mM	millimolar
M-PST	monoamine-sulfating form of PST
NMEM	N-ethylmaleimide
N-OH-AAF	N-hydroxy-2-acetylaminofluorene
PAGE	polyacrylamide gel electrophoresis
PAP	3',5'-diphosphoadenosine
PAPS	3'phosphoadenosine-5'phosphosulfate
PCP	pentachlorophenol
PCR	polymerase chain reaction
PKC	protein kinase C
PNP	p-nitrophenol
pI	isoelectric point
PP_i	pyrophosphate
PST	phenol sulfotransferase
P-PST	phenol-sulfating form of phenol sulfotransferase
P-PST-1	cDNA of phenol sulfating form of human phenol sulfotransferase
PVDF	polyvinylidene difluoride
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate

LIST OF ABBREVIATIONS (Continued)

ST	sulfotransferase
SMP-2	senescence marker protein-2
TBS	tris-buffered saline
TL	thermolabile
TTFA	trifluoroacetic acid
T_m	melting temperature
TPST	tyrosylprotein sulfotransferase
TS	thermostable
µg	microgram
x g	times gravity

INTRODUCTION

The liver is situated in a strategic site in the body, interposed between the digestive tract and the general circulation. Drugs, dietary compounds and other potentially toxic xenobiotics that are absorbed from the gastrointestinal tract are transported through the liver before they can be carried by the circulation to other tissues in the body. One of the major roles the liver plays is to selectively transform some of these exogenous substances into biologically inactive, charged species that are more readily eliminated from the body than the parent compounds. The same or related enzymes are also responsible for processing a number of endogenous compounds such as steroids, catecholamines, and bioactive peptides. Exposure of both endogenous and exogenous compounds to the hepatic enzymes has profound effects on their biological activity.

The reactions of enzymatic biotransformation are classified as Phase-I and Phase-II reactions (Benet and Sheiner 1985). The lipid soluble characteristics of most drugs, although important for bioavailability, result in tissue retention and poor urinary excretion. Phase-I reactions convert drugs to more polar metabolites via oxidation, reduction or hydrolysis, and often introduce or

unmask functional groups for further metabolism. Phase-II or conjugation reactions involve the addition of bulky charged groups to drugs or their metabolites, generally resulting in biologic inactivation and increased excretion. After conjugation with a hydrophilic moiety, the water solubility of many drugs is increased, resulting in greater urinary excretion. The endogenous substrates for conjugation include sulfate, glucuronic acid, acetate, glutathione, and amino acids.

Drugs are usually converted concurrently or consecutively to multiple metabolites, and there is often competition between several of the conjugating enzymes for the same substrate. The outcome of this competition is highly variable, and is influenced by the existence of multiple allelic forms among many of the drug metabolizing enzymes in the population, and by differences in the relative affinities of these enzymes for the common substrate.

Pharmacogenetics involves the study of idiosyncratic responses to drugs that have a hereditary basis (Meyer 1990). The existence of the allelic forms often remains undetected until an idiosyncratic response is precipitated by administration of a particular drug. However, once the allelic forms are identifiable, the information can have far reaching pharmacologic implications. For example, the use of isoniazid in the treatment of tuberculosis led to the identification of genetically distinct individuals classified as either fast or slow acetylators (Weinstein 1975). A number

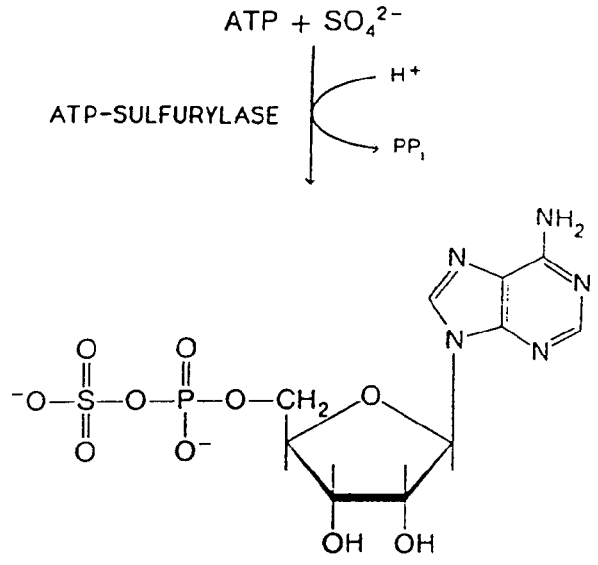
of drugs, including hydralazine, procainamide, and sulfonamides, undergo biotransformation by N-acetylation. Slow acetylators are more often prone to drug-induced toxicities than rapid acetylators, such as isoniazid-induced peripheral nerve damage, and to procainamide- and hydralazine-induced lupus erythematosus (Meyer 1990). Allelic variants often display different affinities for a given substrate, which can lead to various pharmacologic consequences within the population. The relative affinities of different conjugating systems for a given substrate, have a large role in determining the metabolic fate of that substance. One enzyme system that generally has high substrate affinity catalyzes sulfate conjugation.

The Sulfation Reaction

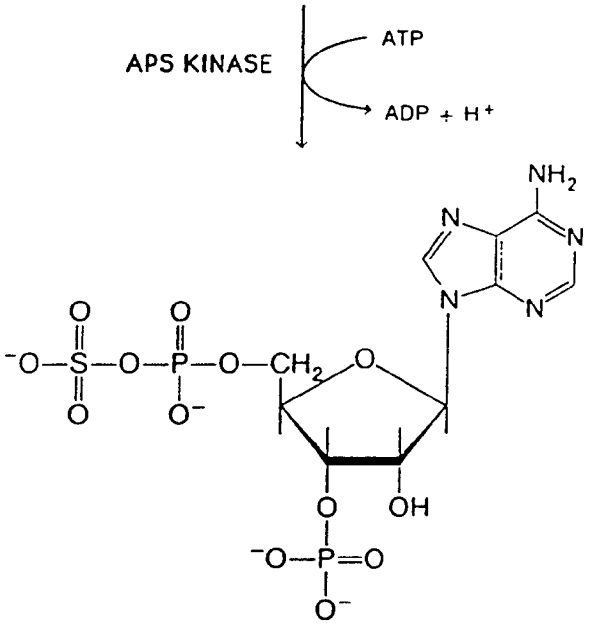
The sulfation of xenobiotics was first reported in 1875 by Baumann who isolated phenyl sulfate from the urine of a patient who ingested phenol (as reviewed by Mulder and Jakoby 1990). Sulfation received relatively little attention for many years largely because it was considered to be a means of disposal of the products of oxidative drug metabolism. The biosynthetic mechanism of sulfation remained largely unknown until 1956 when the structure of the activated form of sulfate, 3'phosphoadenosine-5'phosphosulfate (PAPS) (Figure 1), was first elucidated by Hilz and Lipmann (1955). PAPS is formed in the cell cytosol from ATP and inorganic sulfate in a two-step reaction. ATP-sulfurylase catalyzes the first step in which adenosine 5'-phosphosulfate (APS) is formed. The

Figure 1. Synthesis and Structure of PAPS.

PAPS, the activated form of sulfate, is a cosubstrate for all mammalian sulfotransferases. A two-step reaction leads to its formation. The first step, the formation of adenosine 5'-phosphosulfate (APS), is catalyzed by ATP-sulfurylase, and the second step to form 3'phosphoadenosine-5'phosphosulfate (PAPS) is catalyzed by APS kinase.



Adenosine-5'-phosphosulfate (APS)



3-Phosphoadenosine-5'-phosphosulfate (PAPS)

forward reaction is unfavorable, but the overall production of PAPS *in vivo* is promoted by the hydrolysis of pyrophosphate and the favorable APS kinase reaction (Schmidt et al. 1982). APS kinase catalyzes a second reaction in which APS is phosphorylated to PAPS. All sulfate esterifications require PAPS as the sulfate donor. The hydroxyl group in phenols, alcohols, or (N- substituted) hydroxylamines is the most common acceptor group for sulfation; however, thiols and amines may also serve as sulfate acceptors (Mulder and Jakoby 1990).

Sulfation and glucuronidation are competing pathways in the liver for a number of substances including phenolic substrates (Mulder and Jakoby 1990). Sulfation is generally the dominant pathway at low concentrations due to the high affinity it displays towards its substrates. As the substrate concentration increases, however, sulfation becomes saturated and glucuronidation begins to predominate. The primary cause for nonlinear pharmacokinetics of sulfate conjugation which occurs at elevated substrate concentrations, is thought to be the depletion of inorganic sulfate (Levy 1986). Although PAPS is present in low concentrations *in vivo*, it appears to be synthesized rapidly. The inorganic sulfate required for PAPS synthesis is derived mainly from the catabolism of sulfur containing amino acids, methionine and cysteine. Carrier-mediated transport is the likely mechanism by which sulfate is rapidly taken up in the cells (Schwarz 1982). In man, the serum sulfate concentration is approximately 0.3 mM, but this

can be decreased by a diet low in sulfur-containing amino acids or by administration of a large dose of a substrate for sulfation, such as acetaminophen (Levy 1986). Decreased levels of sulfate in the CNS and plasma resulting from drug administration may modify the disposition of endogenous substrates such as neurotransmitters which are also subject to sulfate conjugation.

The outcome of competition between sulfation and other conjugating systems for the same substrate is influenced by the heterogenous distribution of drug metabolizing enzymes in the liver. The basic unit of the liver, the acinus, is a group of hepatocytes perfused by a terminal portal venule and hepatic arteriole (Gumucio and Traber 1991). Blood moves from these vessels into hepatic sinusoids, perfusing the hepatic cells in a sequential manner. Blood modified by the hepatocytes exits the acinus by way of the hepatic venule to the systemic circulation. Approximately 20 to 25 hepatocyctes span the distance between the terminal portal venule and the hepatic venules. The sequential perfusion of the hepatocytes results in a progressive modification of the sinusoidal blood composition. The hepatocytes of the acinus have been arbitrarily divided into three zones: zone 1 or periportal, zone 2 or mid-zonal, and zone 3 or pericentral (Gumucio and Traber 1991). Although little is known regarding the distribution of enzymes in man, in rats increased concentrations of cytochrome P-450s, epoxide hydrolases, glutathione S-transferases and UDP-glucuronyltransferases are

known to exist in zone 3, whereas sulfotransferases (STs) have an increased presence in zone 1 (Pang 1990). It is likely, therefore, that a drug would first encounter a ST as it traverses the liver. The same STs are also involved in conjugation of a variety of endogenous substances such as steroids, bile acids and catecholamines.

The Biological Significance of Sulfate Esters

Peptides, Proteins, and Other Macromolecules

Several classes of endogenous compounds are sulfated in the Golgi apparatus by membrane-bound STs that are distinct from the cytosolic forms that participate in drug and xenobiotic metabolism. These compounds include many secreted proteins that are sulfated at tyrosine residues, sulfolipids, glycosaminoglycans, and glycoproteins. This aspect of sulfation is described in brief below.

Tyrosine Sulfates

Sulfation of proteins frequently occurs on tyrosine residues of secreted proteins. As much as 1% of the tyrosine residues of total protein in an organism can be sulfated, making it one of the most abundant post-translational modifications known for this amino acid (Huttner 1987). A tyrosylprotein ST (TPST) located in the trans Golgi has been characterized in various species and tissues, including the human liver (Lee and Huttner 1983; Rens-Domiano and Roth 1989; Lin and Roth 1990). Similarities in biochemical properties of TPST from several species suggest that protein and peptide

sulfation is a well-conserved process and is an essential post-translational modification.

Tyrosine sulfation has been shown to have several important consequences. One effect results in alteration of the biological activity of a compound. For example, the ability of cholecystokinin (CCK), secreted from the small intestine, to stimulate pancreatic secretions is greatly increased by tyrosine sulfation (Jensen et al. 1981). The circulating form of CCK always exists in the sulfated state, whereas gastrin, a highly related peptide of gastric origin, is sulfated on approximately 50% of the circulating molecules. The ability of gastrin to stimulate gastric acid secretion is not affected by sulfation, but it does result in the ability of the peptide to stimulate pancreatic secretions in a manner similar to CCK (Brand, Andersen and Rehfeld 1984).

The sensitivity of specific sites within proteins to proteolytic cleavage can be altered by tyrosine sulfation (McDermott et al. 1985). Aminopeptidases have been implicated in the inactivation of several neuropeptides including leucine enkephalin. Sulfation of leucine enkephalin results in loss of biological activity, but the modification makes the peptide more resistant to aminopeptidase hydrolysis.

Tyrosine sulfation appears to play a role in the intracellular transport of secretory proteins. Most tyrosine sulfated proteins studied to date are secretory (Huttner 1987). Vitellogenin 2 of *Drosophila melanogaster* is sulfated on Tyr 172. After site-directed mutagenesis of this residue

prevented the protein from undergoing sulfation, the appearance of the unsulfated vitellogenin 2 at the cell surface was significantly slower than that of the sulfated wild-type protein. Inhibition of sulfation with chlorate likewise retarded the appearance of wild type vitellogenin 2 at the cell surface.

A recent investigation identified a membrane-associated protein that contains tyrosine sulfate although its function has not been determined (Liu and Baenziger 1986). A ST involved in the tyrosine sulfation of this membrane protein, P61, isolated from A431, a human fibroblast cell line, appeared to be distinct from the Golgi-associated enzyme that mediates sulfation of secreted proteins. The similarity of sulfate and phosphate, suggests that tyrosine sulfation may be analogous to tyrosine phosphorylation, a process involved in growth regulation. However, there is no evidence to suggest tyrosine sulfation is reversible.

Sulfolipids

Sulfolipids are concentrated in the central and peripheral nervous sytems and the reproductive organs where they play vital roles in membrane function. For example, maintenance of the correct membrane potential is dependent on sulfolipids which form an annulus around the electrogenic membrane sodium/potassium ATPase (Hansson, Karlsson and Samuelsson 1978). The potassium ion first binds to a sulfate ester before being transferred to the channel. Sulfolipids are also important components in the microenvironment of the

beta-endorphin and opiate receptor. Due to the reversible manner in which sulfolipids cluster in a lipid bilayer, they are thought to be involved in regulating receptor protein conformation and activation of enzymes bound near the sulfolipid cluster (Cestaro et al. 1983).

Glycosaminoglycans and Glycoproteins

A group of membrane-bound STs are involved in sulfation at glycosyl residues of many glycosaminoglycans or glycoproteins (Huxtable 1986). Glycosaminoglycans generally consist of disaccharide repeating units and are found in high concentrations in skin, cartilage, bone, and connective tissue. The ability of these highly negatively charged molecules to bind metal ions allows them to perform a number of vital functions. Sulfated glycosaminoglycans appear to promote matrix stabilization and to be indirectly involved in the maintenance of differentiated cell morphology and growth control (Hook 1984). In growing cells there is a shift toward less sulfated glycosaminoglycans, and the sulfate content of glycosaminoglycans from the surface of nonadhering myeloma cells was also found to be much lower than the sulfate content of adhering cells.

The presence of sulfate groups on glycosaminoglycans also creates anionic binding sites, as in the case of heparin sulfate. The anticoagulant effect of heparin is critically dependent on the synthesis of the 3-O sulfate esters of the N-acetylglucosamine residues (Lindahl, Feingold and Roden 1986). This sulfate group is involved in binding heparin to

antithrombin III, the serine protease inhibitor that mediates the action of heparin by neutralizing several activated clotting factors.

One function that has been suggested for carbohydrate sulfation of glycoproteins present inside secretory granules is a role in the packaging of secretory products. For example, sulfation is a shared feature of several proteins secreted from the pituitary including lutenizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone and pro-opiomelanocortine (Mulder and Jakoby 1990).

Bile Acids, Steroid and Thyroid Hormones, and Biogenic Amines

Sulfation of bile acids, steroids and the biogenic amines occurs through the action of a group of cytosolic STs. Many of the same enzymes also participate in the metabolism of drugs and other xenobiotics.

Bile Acids

Bile acids, the principal metabolites of cholesterol, are excreted into bile mostly as N-acyl amidates with glycine and taurine (Barnes 1991). Chenodeoxycholic acid and cholic acid are formed in the liver and are termed "primary" bile acids. In general, bile acids undergo a highly efficient enterohepatic circulation; however, they are subject to modification by intestinal bacteria. The resulting "secondary" bile acids are lithocholic acid and deoxycholic acid. Whereas deoxycholic acid has a high aqueous solubility, lithocholic acid is relatively insoluble (Small 1971).

Deoxycholic acid and its conjugates accumulate in the enterohepatic circulation to form up to 25% of the total biliary bile acids in man. In contrast, the hepatotoxic lithocholate fails to accumulate due to the formation of the 3-alpha-sulfate which is a poor substrate for the ileal active transport system for bile acids (Low-Beer, Tyor and Lack 1969). In addition to lithocholic acid, other poorly soluble monohydroxy bile acids such as 3-beta-hydroxy-5-cholenoic acid occur in the neonatal period and in patients with liver diseases. However, sulfation facilitates their intestinal and urinary excretion. In animals that fail to sulfate monohydroxy bile acids efficiently, administration of these bile acids or their precursors frequently leads to hepatic cirrhosis and biliary tract damage. Rabbits, which have no measurable hepatic bile acid ST activity, are highly susceptible to hepatotoxicity when fed the lithocholic acid precursor chenodeoxycholic acid (Fisher et al. 1974). Rhesus monkeys sulfate bile acids inefficiently and have been shown to develop cirrhosis when fed large doses of chenodeoxycholic acid (Federowski et al. 1978). It is also likely that humans with inborn errors involving a deficiency in bile acid sulfation would be susceptible to liver disease (Barnes 1991).

Several inborn errors in bile acid metabolism have been described in humans that result in the accumulation of bile acids retaining the planar configuration of cholesterol (Clayton et al. 1987; Setchell et al. 1988). Bile acids with this configuration are the preferred substrates for the bile

acid STs (Barnes et al. 1986, 1989). As a result, patients with these inborn errors have a high urinary output of the bile acid sulfate conjugates.

Steroid and Thyroid Hormones

Sulfation is a major conjugation reaction for steroids in humans, occurring at the aliphatic hydroxyl groups of the hydroxysteroids as well as the phenolic hydroxyl groups present in the estrogens. STs having activity toward the aliphatic hydroxyl groups often have an overlapping spectrum of steroid substrates; however, they are distinct from those displaying activity with the aromatic hydroxyl group of steroids (Mulder and Jakoby 1990).

Generally, sulfation abolishes the biological effects of steroids. Some steroid sulfates, however, have important functions as intermediates of metabolism and as storage forms of the parent compound. Estrone sulfate is the predominant form of circulating estrogen in humans (Huxtable 1986). This conjugate has a long half-life in the blood, thereby making it available to many tissues. Estrone and estrone sulfate are both taken up by the liver, but their metabolic fates differ. Estrone is glucuronidated and excreted into the bile, whereas estrone sulfate is either reduced to estradiol sulfate or hydrolyzed to free estrone and returned to the circulation (Huxtable 1986). Estrone sulfate acting as an inactive, transportable form of the hormone is taken up at extrahepatic sites, such as the uterus and pituitary. Membrane bound sulfatases with activity toward estrone sulfate have been

identified in several tissues where they appear to regulate the conversion of the hormone to the active form (Rose 1982).

Target cell sensitivity of hormonal steroids is generally dictated by the specific steroid receptors that are involved in the regulation of the hormone-responsiveness genes. However, the presence of specific hormone-inactivating enzymes within the target cell can effectively control the availability of the active hormone, providing another mechanism of control. In the uterine endometrium of humans and other mammalian species, progesterone up-regulates estrogen ST activity, resulting in inactivation of 17-beta-estradiol and estrone. Estrogen ST activity is highest during the secretory phase of the cycle, when estrogen action and cell proliferation need to be blunted in favor of the secretory processes. The rapidly proliferating cells of endometrial carcinomas appear to contain low levels of estrogen ST activity. The use of progesterone in the treatment of endometrial carcinomas results in marked elevation of estrogen ST activity, thereby decreasing the biologically active estrogen concentration (Buirchell and Hahnel 1975).

In humans, dehydroepiandrosterone (DHEA) is primarily synthesized in the adrenal cortex. The high levels of DHEA sulfate synthesized and secreted from the human adrenals provide the precursor for androgens and estrogens synthesized in the gonads and other tissues (Hobkirk 1985). The presence of the sulfate moiety increases the plasma half-life of this

hormone and allows it to accumulate at micromolar concentrations. DHEA sulfate is also an important estrogen precursor during fetal development. It is secreted from the neo-cortex of the fetal adrenals and transported in the fetal circulation to the placenta where it is desulfated and converted into estrogens. Approximately 50% of the 17-beta-estradiol and greater than 90% of the estriol in the maternal circulation originates from fetal DHEA sulfate, although the physiological significance of these elevated concentrations is unclear.

In the catabolism of the two major biologically active forms of thyroid hormone, T_3 and T_4, sulfation may preceed deiodination. The sulfate conjugates have been shown to undergo more rapid deiodination than their nonsulfated counterparts (Otten, Mol, and Visser 1983). A three-fold variation was seen in the level of human liver enzyme activity responsible for this modification; however, further studies will be necessary to determine the significance of this finding (Young, Gorman, and Weinshilboum 1988).

Catecholamines

Sulfation of the catecholamine neurotransmitters, such as dopamine, norepinephrine and epinephrine, terminates their biological activity (Roth 1986b). Catecholamine metabolism *in vivo* involves the combined activities of ST, monoamine oxidase (MAO), and catechol-O-methyl transferase (COMT). Sulfation predominantly occurs on the 3-position of the aromatic ring and therefore the ST competes with COMT for inactivation. In

the peripheral nervous system, sulfation appears to be a major pathway for processing catecholamines. Greater than 99% of the total dopamine and approximately 75% of total norepinephrine and epinephrine in human plasma exist as the sulfate conjugate. The contribution of sulfation in terminating catecholamine action in the CNS has been more difficult to assess (Roth 1986b).

In crude preparations of human frontal cortex, sulfation has been estimated to account for 5 to 15% of dopamine and norepinephrine metabolism (Roth, Rivett and Resnkers 1982); however, this may not reflect events occurring in the intact human brain. The contribution of each enzymatic pathway involved in catecholamine metabolism in part depends on its location, concentration, and accessibility of the substrates for the enzymes in each of the cell types. Using antibodies raised against the form of human ST involved in the metabolism of catecholamines, immunohistochemical studies were performed to determine the cellular localization of this enzyme in several areas of human brain (Zou, Pentney, and Roth 1990). The ST was found to be compartmentalized in specific neurons in the CNS, suggesting greater participation of sulfation in neurotransmitter inactivation in these regions than was indicated in studies involving crude brain preparations.

Several studies have suggested that altered or impaired ST activity may have important physiological consequences in relation to neurological and behavioral disorders. Tyramine is the product of tyrosine decarboxylation (Weiner and Taylor

1985). It is similar in structure to the catecholamines and is acted upon by some of the same enzymes involved in catecholamine metabolism. When patients who manifest diet-associated migraine headaches were challenged with oral doses of tyramine, there was a significant decrease in the urinary sulfate metabolite of this amine when compared to that of the normal population (Youdim, Bonham-Carter, and Sandler 1971; Glover et al. 1983). Tyramine sulfation, after oral administration, is also decreased greater than two-fold in depressed patients than in the normal controls (Sandler, Bonham-Carter, and Walker 1983; Harrison et al. 1983). This data suggests a defect in the sulfation mechanism in patients with a history of tyramine-sensitive migraine headaches and some forms of depression.

Sulfate conjugation of bioactive agents has the potential to have profound physiological consequences in humans. In addition, many drugs and biologically active compounds ingested from the environment undergo sulfate conjugation, often resulting in significant pharmacological effects.

Drugs and Other Xenobiotics

Many drugs, including acetaminophen, corticosteroid derivatives, estrogen derivatives, L-dopa, isoproterenol, metoclopramide, triamterene, and minoxidil, undergo sulfation (Buu, Duhaime, and Kuchel 1982; Roth, Rivett, and Renskers 1982; Brunton 1990: Falany 1990; Mulder and Jakoby 1990). In the past, sulfation of drugs has not received much attention because, in spite of its comparatively high substrate

affinity, it is a low capacity system (Mulder and Jakoby 1990). Although the sulfate conjugates of many drugs are found in urine, the percentage of a dose eliminated in this form is generally low.

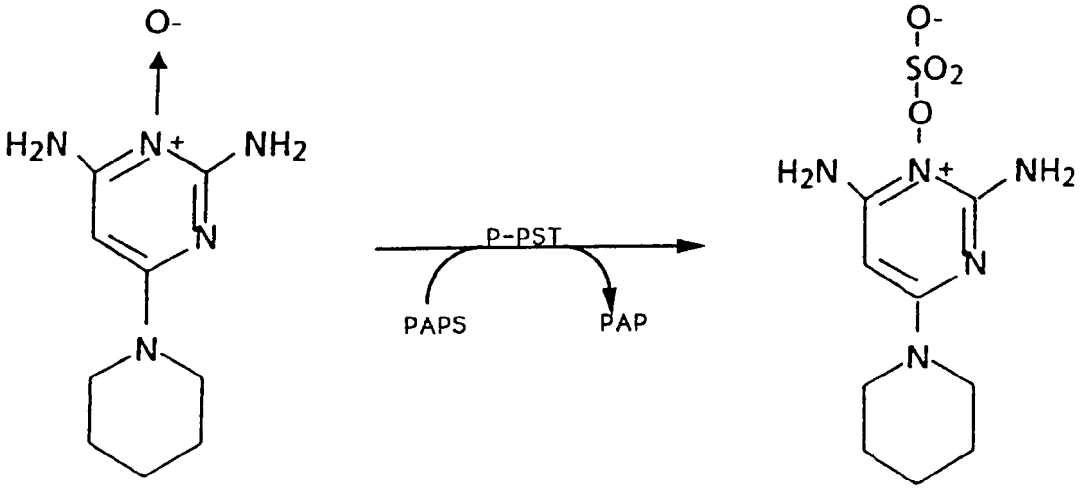
The ease with which the STs are saturated is a critical factor in the disposition of many drugs. For example, the phenolic group of acetaminophen is a substrate for both ST and UDP-glucuronyltransferase activities (Flower, Moncada, and Vane 1985). At low plasma concentrations acetaminophen is predominantly excreted as the sulfate ester; however, as the plasma concentration increases, the ST becomes saturated and the glucuronide metabolite begins to predominate. At still higher plasma levels, both conjugating systems become saturated and acetaminophen becomes a substrate for a cytochrome P-450 enzyme that results in its oxidation to a quinoline. This species is a reactive electrophile that combines with glutathione and tissue macromolecules, resulting in cell death and tissue necrosis (Flower, Moncada, and Vane 1985).

Although sulfation is generally thought of as a means by which biological action is terminated, the reactive nature of the sulfate conjugates of several drugs and environmental carcinogens actually results in the formation of the biologically active species. In these cases the low capacity of the system does not reflect its biological importance.

Minoxidil, a pyrimidine N-oxide derivative, is a potent vasodilator that induces hypertrichosis of facial and body hair (DuCharme et al. 1973; Clissold and Heel 1987). Initial investigations into the mechanism by which minoxidil induces direct vasodilation indicated that the sulfate ester formed at the pyrimidine N-oxide was the active species (McCall et al. 1983) (Figure 2). The sulfate metabolite results in the formation of an inner salt, with increased lipid solubility in comparison with the parent compound. Minoxidil sulfate is thought to induce vasodilation by opening potassium channels in smooth muscle cells (Meisheri, Cipkus, and Taylor 1988). A recent investigation suggests the molecular mechanism by which minoxidil sulfate produces its vascular effects is due to its intrinsic ability to donate the sulfate group to smooth muscle membrane proteins (Meisheri, Oleynek, and Puddington 1991). After exposure of rabbit mesenteric arteries to ^{35}S-minoxidil sulfate, more than 30% of the radiolabel was retained two hours after removal of the drug. When the piperidine ring of minoxidil sulfate was labeled with tritium, retention of the radiolabel was not detected. SDS-PAGE analysis of ^{35}S-labeled proteins isolated from the rabbit arteries after drug exposure showed preferential labeling of a 116 kD protein. The relationship between this protein and the potassium channel is not known, but covalent modification of a protein that is involved with potassium channel function is consistent with the observation that the pharmacologic

Figure 2. Sulfation of Minoxidil.

The prodrug minoxidil is converted in humans to its active species by sulfation at the N-oxide catalyzed by P-PST. The intrinsic ability of minoxidil sulfate to nonenzymatically donate the sulfate group to endogenous proteins is thought to be the mechanism by which the drug produces its effects (Meisheri, Oleynek and Puddington 1991; Groppi et al. 1990). Minoxidil sulfate is more lipophilic than the parent compound due to the formation of an inner salt between the sulfate group and one of the amine groups (McCall et al. 1983).



Minoxidil

Minoxidil Sulfate

half-life of minoxidil exceeds its serum half-life (Meisheri, Oleynek, and Puddington 1991).

Minoxidil sulfate is considered to be very active in terms of its ability to donate the sulfate group nonenzymatically to nucleophiles, including proteins. In an in vitro study, over 50 peptides were screened in order to characterize sulfation by minoxidil sulfate. It revealed that sulfation of peptides preferentially occurred at N-termini and on histidine residues, whereas serine, threonine, and tyrosine residues were not sulfated. The sulfated peptides were stable in exponentially growing cells with a half-life of approximately 12 hours (Groppi et al. 1990).

In addition to its use in the treatment of hypertension, minoxidil is frequently used topically in the treatment of alopecia. The sulfate conjugate has also been defined as the active species involved in stimulating hair growth (Buhl et al. 1990). The presence of minoxidil sulfate in cultured hair follicles induced a rapid expression of an undefined hair protein, suggesting the drug action on the hair follicle was not simply the result of increased blood flow to the region. Using antibodies to the rat ST involved in minoxidil sulfation, the enzyme has been localized within the cytoplasm of epithelial cells in the lower outer root sheath of anagen hair follicles in rats (Dooley et al. 1991). This region of the hair follicle surrounds the epithelial cells of the inner root sheath and cortex, which undergo differentiation and

keratinization to form the hair shaft. The enzyme was not detected in dermal blood vessels.

Sulfation also appears to be involved in the activation of several environmental carcinogens. Metabolism of chemical carcinogens to reactive electrophiles capable of reacting with the nucleophilic groups of nucleic acids and proteins is thought to be an important step in the carcinogenesis by these compounds (Miller 1978). The aromatic amines are common environmental contaminants. They can be metabolized by N-acetylation and N-hydroxylation to hydroxamic acids that can be further metabolized by conjugation with sulfate or glucuronide. The sulfate conjugate is highly unstable and rapidly rearranges to reactive nitrenium ions (Mulder and Jakoby 1990). The potent liver carcinogen N-hydroxy 2-acetyl aminofluorene (N-OH-2AAF) is metabolized to several species capable of binding to macromolecules, but it appears that sulfation is the major mechanism of toxicity (Miller et al. 1985). Administration of the sulfation inhibitor pentachlorophenol (PCP) resulted in a significant decrease in covalent binding of the fluorene nucleus to macromolecules, whereas administration of sulfate in sulfate-depleted animals increased both the toxicity of the compound and the levels of covalently bound metabolites (Miller et al. 1985).

Safrole is a carcinogenic alkylbenzene that occurs naturally in a variety of plants with a mechanism of toxicity that is analogous to that of N-OH-2AAF (Miller et al. 1985). Sulfation of the 1'-hydroxy metabolite of safrole results in

the formation of a highly reactive C,O-sulfonate. The resulting electrophile is stabilized by resonance between the vinyl and benzyl groups. Administration of PCP reduced DNA adduct formation to 15% and the average number of hepatomas per animal to less than 10% of that observed in animals not treated with the ST inhibitor.

The use of brachymorphic mice provided additional evidence of the involvement of sulfation in carcinogen activation (Miller et al. 1985). Brachymorphic mice are characterized by a deficiency in the biosynthesis of PAPS. When 1'-hydroxysafrole was administered to brachymorphic mice, the level of DNA adduct formation in the liver was only 15% of the level seen in the liver of phenotypically normal mice given the same treatment. The multiplicity of hepatomas thatdeveloped in the PAPS deficient mice were no more than 10% of that observed in normal littermates.

The mechanism by which sulfation activates carcinogens has been described for a number of these compounds in rats and mice; however, very little is known concerning the sulfation of these compounds in humans. Preliminary data indicates that there are important species differences. For example, N-OH-AAF does not appear to undergo sulfation in human liver cytosol (Falany 1991), whereas N-hydroxy-2-aminofluorene and N-hydroxy-4-aminobiphenyl were substrates for human cytosolic STs and resulted in DNA adduct formation (Chou, Lang and Kadlubar 1991.)

The Cytosolic Sulfotransferases

There are two broad categories of cytosolic STs: the phenol STs (PST) and the steroid STs. A large number of enzymes representing members of both categories of cytosolic STs have been isolated from rat livers, and recent reports of the cDNA sequences encoding several of these proteins have allowed their molecular characterization (Nash et al. 1988; Ogura et al. 1989, 1990a; Ozawa et al. 1990; Demyan et al. 1992; Hirshey et al. 1992; Otterness et al. 1992).

Steroids share the cyclopenta-alpha-phenanthrene skeleton with bile acids, and there appears to be some substrate overlap among the enzymes catalyzing the sulfation of members of these two classes. The rat hydroxysteroid ST, STa, is known to be involved in the activation of carcinogenic hydroxymethyl arenes, such as 7-hydroxymethylbenz-alpha-anthracene (Ogura et al. 1990b). Ogura et al. also determined that STa has high activity toward DHEA. The cDNA encoding this enzyme was isolated and sequenced (Ogura et al. 1990a), and the encoded protein was found to have high homology to the N-terminal sequence of purified bile acid ST I (BAST I) (Barnes et al. 1989), with identity in 31 of 36 residues. BAST I also has high substrate affinity for DHEA. Both BAST I and STa are expressed in much higher levels in females than in males. STa also has 73.7% homology with the deduced amino acid sequence of the senescence marker protein (SMP-2) (Chatterjee et al. 1987), with a homology of 92% in four specific regions corresponding to approximately 60% of the

total encoded sequences. SMP-2 was isolated in an investigation of age-related changes in hepatic protein expression associated with androgen sensitivity, although the function of the protein is not known. Like BAST I and STa, SMP-2 exists in higher concentrations in female than in male rats of all ages. The mechanism of sex-related expression of SMP-2 is predominantly androgenic repression. It is thought that STa is an isoenzyme of SMP-2 (Ogura et al. 1990).

The deduced sequence of the protein encoded by the rat cDNA for a PST, PST-1 (Ozawa et al. 1990) appears to have low sequence homology with the known steroid STs. The amino acid sequence homology of STa and PST-1 is 41%, indicating that these proteins belong to distinct classes of enzymes, although the existence of several regions of high homology suggest they may have evolved from a common ancestral gene. These proteins are further distinguished by differences in sex-related expression; the PSTs are more highly expressed in male rats than in female rats (Wu and Straub 1976; Sekura and Jakoby 1981; Borchardt and Schasteen 1982; Hirshey and Falany 1990).

Only three distinct cytosolic STs have been isolated from human tissues; two isoenzymes of PST and a single form of steroid/bile acid ST (DHEA ST). The steroid/bile acid ST purified from human liver has been named DHEA ST because DHEA is the most rapidly conjugated endogenous substrate tested; however, all of the steroids tested, including estradiol, pregnenolone, and testosterone, were conjugated to some degree (Falany, Vazquez, and Kalb 1989). The enzyme also conjugates

bile acids (Radominska et al. 1990). There was no evidence for the formation of bile acid sulfates by an enzyme other than the DHEA ST.

As discussed above, the sulfation of bile acids and endogenous steroids has several important consequences which also apply to drugs of a steroidal nature. The majority of drug and xenobiotic sulfation, however, occurs as a result of PST activity.

The Phenol Sulfotransferases

Sulfation of phenolic compounds is a widely occurring reaction that is involved in Phase-II reactions of the drug and xenobiotic metabolizing systems. A variety of biologically active endogenous substances also undergo extensive sulfoconjugation at phenolic hydroxyls *in vivo*. The family of cytosolic enzymes responsible for sulfation at phenolic hydroxyls has been designated as the aryl STs or PSTs. Table 1 lists the PSTs that have been isolated from animal tissues and highlights some of their physical characteristics. A large amount of information regarding the PSTs has come from enzymes present in male rat livers, a source of a heterogenous group of PSTs displaying distinct, but often overlapping, substrate specificities.

Rat Phenol Sulfotransferases

At least six different cytosolic forms of PST have been isolated from rat livers. Four of these activities have been isolated and designated PST I-IV in order of their elution from a DEAE column (Sekura, Duffel, and Jakoby 1981). These

Table 1

Comparison of purified mammalian phenol sulfotransferases

PST	pH Optimum	Mr (kD)	Subunit Mr (kD)*	pI	Selected Kms	
Rat PST I	6.5	64	35	8.1	PAPS Phenol PNP	6.5 µM 1.8 mM 1.6 mM
Rat PST II	6.5	64	35	6.9	PAPS Phenol PNP	12 µM 2.6 mM 2.5 mM
Rat PST III	5.5	61	33.5	6.4	-	
Rat PST IV	5.5	61	33.5	5.8	PAPS Dopamine	58 µM 160 µM
Rat Minoxidil ST	6.5-6.8	68	35	4.7	PAPS Minoxidil PNP	5 µM 625 µM 0.5 µM
Rat N-OH-AAF ST	6.3	68	38.2	5.7	-	
Borchardt's Rat PST	5.5-6.4	69	70	-	PAPS PNP	2.5 µM 3.6 µM
Paracetamol ST (Rat)	-	-	35	-	-	
Human M-PST	7.0	69	34	4.7	PAPS Dopamine	0.35 µM 2.9 µM
Human P-PST	8.5	68	32	4.7	PAPS Phenol	0.5 µM 2.8 µM

*As determined by SDS-PAGE

enzymes have been placed in two families based on substrate specificities and similar physical characteristics. Substrates for the first family, containing PST I and II, are simple phenolic compounds such as 2-naphthol. They display pH optima of 6.5 with 2-naphthol as substrate and have a subunit molecular weight of 35 kD. PST III and PST IV comprise the second family, with a pH optima toward 2-naphthol of 5.5 and a subunit molecular weight of 33.5 kD. PST III activity has been found to deteriorate at -80° at all stages of purification and is unstable to isoelectric focusing; therefore, it has not been well characterized (Sekura, Duffel and Jakoby 1981). In addition to simple phenolic compounds, PST IV is capable of sulfating catecholamines, the N-terminal tyrosyl residue of several small peptides, including the CCK heptapeptide and several enkephalins, and hydroxylamines (Sekura and Jakoby 1981).

PST IV activates the hydroxylamine N-OH-AAF to the ultimate toxic form of this carcinogen. Several similarities exist between PST IV and the N-OH-AAF ST (Wu and Straub 1976), including activity towards N-OH-AAF, male specific expression, and similar isoelectric points (pI). Male rats, expressing more of these ST activities, are more susceptible to the carcinogenic effect of N-OH-AAF (Wu and Straub 1976). A separate PST isolated from male rat livers (Borschardt and Schasteen 1982) displays activity toward catecholamines, a characteristic shared with PST IV. However, these two enzymes

differ in subunit molecular weight, salt sensitivity, and K_m for PAPS.

The minoxidil ST is a unique PST that is responsible for the majority of sulfating activity at the N-oxide of minoxidil (Hirshey and Falany 1990). It also represents approximately 11% of the cytosolic p-nitrophenol (PNP) ST activity in the male rat liver. Expression of minoxidil ST activity appears to be tissue specific. When PNP and minoxidil ST activities were determined in cytosol prepared from male rat livers, kidneys, intestines and brains, detectable levels of PNP ST activities were present in all tissues, whereas measurable levels of minoxidil ST activity were detectable only in the liver. There is also a two-fold higher level of expression of this enzyme in males than in females; therefore, the enzyme was isolated from the liver of male rats (Hirshey and Falany 1990).

Minoxidil ST was separated from other forms of rat PST that are capable of sulfating small phenols, such as PNP, by a combination of anion exchange, hydroxyapatite, and ATP-agarose affinity chromatography (Hirshey and Falany 1990). During the purification procedure, the minoxidil ST/PNP ST specific activity ratio was monitored and found to increase approximately 8-fold. Purification of the enzyme to homogeneity was determined by SDS-PAGE and reversed-phase HPLC analysis. PST IV and N-OH-AAF ST have activity with N-hydroxy compounds, but their ability to sulfate at N-oxides is not known. The minoxidil ST differs from PST IV and N-OH-AAF STs

in its subunit molecular mass, pI, kinetic properties, amino acid composition, and immunological properties. For example, the pI of minoxidil ST (4.7) (Falany unpublished observation) is lower than that of PST IV (5.8) (Sekura, Duffel, and Jakoby 1981) and N-OH-AAF ST (5.7) (Wu and Straub 1976). There is no evidence of cross-reactivity of rabbit polyclonal antibodies prepared against PST IV (Duffel et al. 1991) and minoxidil ST (Hirshey and Falany 1990). The minoxidil ST K_m for PNP (0.5 μM) is significantly lower than that reported for most rat PSTs including PST IV (170 μM) (Sekura, Duffel, and Jakoby 1981; Sekura and Jakoby 1981; Hirshey and Falany 1990). The only other rat PST with a similar K_m is the enzyme isolated by Borchardt and Schasteen (K_m = 3.6 μM) (1982). This enzyme, however, does not appear to function as a dimer, in contrast to the other rat PSTs. A further distinguishing characteristic of the minoxidil ST is that it displays substrate inhibition at PNP concentrations greater than 1.2 μM, a concentration frequently exceeded in assays of the other rat PSTs (Hirshey and Falany 1990).

The purification of another rat PST, the paracetamol (acetaminophen) ST, has recently been reported (Coughtrie and Sharp 1990). In addition to displaying activity toward phenol and alpha-naphthol, this enzyme is also capable of forming a N-sulfate with metoclopramide. The paracetamol ST has the same subunit molecular mass as the minoxidil ST and it is possible that they are the same protein. The low specific activity toward PNP reported for the paracetamol ST (0.008

nmol/min/mg) (Coughtrie and Sharp 1990) may reflect substrate inhibition at the concentration of PNP (250 μM) employed in the assay.

Human Phenol Sulfotransferases

In contrast to the multiplicity of forms of rat PST, humans appear to have only two functionally distinct forms of PST that are present in all human tissues studied, including the platelets, liver, intestine, brain, lung, adrenal, and placenta (Rein, Glover, and Sandler 1981a, 1982, 1984; Reiter et al. 1983; Sundaram and Weinshiboum 1985; Baranczykk-Kuzma, 1986; Falany et al. 1990). One form is the phenol sulfating form of PST (P-PST), which is capable of conjugating simple phenols such as PNP and 2-naphthol at micromolar concentrations; the other is the monoamine form of PST (M-PST) that conjugates monamines such as dopamine (Rein, Glover, and Sandler 1981b). At higher substrate concentrations, however, phenol and PNP can serve as substrates for M-PST (Reiter and Weinshilboum 1982), while at millimolar concentrations dopamine and other monoamines can be conjugated by P-PST (Campbell 1987). Dopamine is the substrate most often used to measure M-PST activity, while PNP is the preferred substrate to measure P-PST activity.

Although the two PSTs have distinct differences in substrate specificity, a number of factors suggest that M-PST and P-PST are related. As noted earlier, the proteins are cross-immunoreactive (Heroux, Falany, and Roth 1989), whereas there is no cross-immunoreactivity with the other form of

human cytosolic ST, the DHEA-ST (Falany et al. 1990). P-PST and M-PST are similar in size, pI, and K_m for PAPS, and both appear to function as homodimers (Heroux and Roth 1988; Falany et al. 1990). At millimolar concentrations there is also some substrate overlap. P-PST and M-PST activities assayed in cytosol prepared from several different normal human livers demonstrated that the activities varied by approximately 10-fold and 3-fold, respectively (Falany et al. 1990). The same investigators concluded that the ratio of P-PST to M-PST activity in the individual livers varied from 30-fold to 3.5-fold. This is consistent with the previous observation that M-PST and P-PST activities in platelets are independently regulated (Weinshilboum 1986).

Human M-PST and P-PST are distinguishable by a number of physical properties. For example, M-PST is considerably more heat-labile than P-PST, resulting in their designation by some investigators as the thermolabile (TL) and thermostable (TS) forms of PST, respectively (Reiter et al. 1983). P-PST is further distinguished from M-PST by displaying increased sensitivity to inhibition by 2,6-dichloro-4-nitrophenol (DCNP) (Rein, Glover and Sandler 1982). Although there is some degree of overlap, at micromolar concentrations substrate specificities are quite distinct. Both enzymes appear to be equally expressed in males and females (Falany 1991), in sharp contrast to the rat STs.

Human M-PST, like rat PST IV, readily esterifies the catecholamine neurotransmitters, such as dopamine, epinephrine

and norepinephrine, as well as a number of their metabolites. Several biochemical features, however, distinguish these two forms of PST. The M-PST Km for dopamine is 2.9 μM (Roth, Rivett, and Resnkers 1982), whereas the rat PST IV Km for this substrate is 160 μM (Sekura and Jakoby 1981). The only animal species that has a Km for dopamine comparable to human M-PST is the African green monkey (Roth, Rivett, and Resnkers 1982). Primates may have a unique form of PST that is specific for the catecholamines. The reaction mechanisms of the rat and human enzymes also differ. Human M-PST proceeds by an ordered reaction mechanism with PAPS as the leading substrate (Whittemore, Pearce, and Roth 1985), whereas the rat PST IV proceeds by a reversible rapid equilibrium random reaction mechanism (Duffel and Jakoby 1981). Rat PST IV is capable of sulfating N-terminal tyrosine residues in small peptides, an activity that is lacking in M-PST. These enzymes also differ in their salt sensitivity and pH optima (see Table 1).

An isoenzyme of M-PST has been purified from human platelets by a combination of anion exchange, gel filtration and PAP-agarose affinity chromatography (Heroux and Roth 1988). DEAE anion exchange chromatography revealed the presence of two peaks of dopamine sulfating activity. The second peak to elute from the column, M_{II}-PST, was subjected to further purification. The native molecular weight of this enzyme is 69,000 and the subunit molecular weight is approximately 34,000, suggesting that M_{II}-PST exists as a homodimer *in vivo*. Immunoblot analysis using polyclonal

antibodies raised to human platelet M_{II}-PST reacted with proteins of 32 and 34 kD from human platelet supernate (Heroux, Falany, and Roth 1989). The 34 kD protein coeluted with M-PST activity during DEAE-cellulose chromatography, whereas the 32 kD protein coeluted with P-PST activity, indicating that these proteins are structurally related. Immunoreactive M-PST was also detected in placenta.

Human P-PST has activity toward a number of neutral phenolic compounds, including many drugs such as minoxidil (Falany and Kerl 1990) and acetaminophen (Reiter and Weinshilboum 1982). In addition, P-PST is capable of sulfating the tyrosyl residues of peptides regardless of the position of the tyrosine within the peptide chain (Whittemore, Jeffery, and Roth 1984), in contrast to rat PST IV that sulfates only at N-terminal tyrosine residues. Whittemore et al. also identified leucine enkephalin, growth hormone releasing factor, angiotensin II, and oxytocin as substrates for P-PST. Triiodothyronine is a substrate for both P-PST and M-PST with similar K_m values for both enzymes. Quantitatively, however, the most important sulfating activity toward this substrate in the liver is P-PST (Young et al. 1988).

P-PST has been purified from human liver using anion exchange, gel filtration, and PAP-agarose affinity chromatography (Falany et al. 1990). The native molecular weight of P-PST is 68 kD with a subunit molecular weight of 32 kD, as determined by SDS-PAGE, suggesting that this protein

functions in vivo as a homodimer. As mentioned earlier, antibodies to purified M-PST cross react with P-PST (Heroux, Falany and Roth 1989). The same investigators determined that immunoreactive P-PST is present in human liver, adrenal, platelet and placental tissue.

In comparison to the rat liver PSTs, P-PST appears to be most similar to the minoxidil ST (Hirshey and Falany 1990). They have similar native molecular weights, pIs, and substrate specificities. The structural similarity of these proteins is also suggested by cross-immunoreactivity: antibodies raised to the rat enzyme also reacted with human P-PST, and human anti-PST antibodies react to rat minoxidil ST (Heroux, Falany, and Roth 1989; Hirshey and Falany 1990). Cyanogen bromide generated peptides derived from both purified proteins showed a high degree of similarity; in one peptide, 8 out of 10 amino acids were identical and the two different residues were conservative substitutions (Falany, unpublished observation).

As with M-PST, two types of P-PST have been identified by their elution during DEAE-cellulose anion exchange chromatography; however, each liver contained only one type of activity (Whittemore, Pearce, and Roth 1986; Campbell, VanLoon, and Weinshilboum 1987; Heroux and Roth 1988; Falany et al. 1990). These two activities, P_1-PST and P_2-PST, have been reported to be differentially sensitive to thermal inactivation (Campbell, VanLoon and Weinshiboum 1987). This difference is noted when the cytosol is prepared in phosphate buffer, but both activities are equally sensitive to thermal

inactivation when prepared in triethanolamine buffer (Falany et al. 1990). Both forms of P-PST were inhibited to similar extents by incubation with the ST inhibitors phenylglyoxal or N-ethylmaleimide (NMEM), and both possess the same subunit molecular weight and immunoreactivity. They are also equally sensitive to inhibition by the P-PST specific inhibitor DCNP (Whittemore, Pearce, and Roth 1986), further suggesting that P_1-PST and P_2-PST are very similar and probably represent different allelic forms of the enzyme. The only difference that has been noted for these proteins is sensitivity of enzyme activity to sodium chloride (Whittemore, Pearce, and Roth 1986). P_1-PST was inhibited 50% in the presence of 100 mM sodium chloride, with complete inhibition occurring at a concentration of 300 mM, whereas P_2-PST was relatively insensitive to sodium chloride, with less than 20% loss of activity at a concentration of 300 mM. A 20% increase in P_2-PST activity was seen at sodium chloride concentrations from 50 to 100 mM. M-PST was intermediate in its sodium chloride sensitivity, with 50% inhibition occurring in 300 mM sodium chloride.

Due to the high degree of variation of both M-PST and P-PST activity in the population, studies have been undertaken to determine the heritability of these enzymes (Weinshilboum 1990). Inheritance was the major factor responsible for individual differences in the levels of activity of platelet M-PST and P-PST. To determine if the effects of inheritance were polygenic, that is, due to the effects of many genetic

loci or mendelian, due to the effects of polymorphism at a single genetic locus, segregation analysis was performed. The findings indicated that genetic polymorphism was responsible for the genetic control of PST activity. The data for P-PST further suggested a single genetic locus with alleles for high and low levels of activity that had gene frequencies of 0.2 and 0.8, respectively (Weinshilboum 1990).

The existence of individuals with high and low levels of sulfating ability may have significant effects on their predisposition to drug responsiveness or toxicity. It may also indicate increased susceptibility to certain environmental carcinogens, as it has been determined that some of the genetic polymorphisms of drug metabolism are associated with an increased risk of developing a certain disease. For example, there is a statistically significant association between the rapid acetylator phenotype and the incidence of colorectal cancer (Lang et al. 1986); the slow acetylator phenotype, however, is associated with an increased risk of bladder cancer (Hassen et al. 1985).

Consequences of the existence of several sulfator phenotypes are not known. The PSTs have a wide range of substrates, including drugs and procarcinogens. Molecular characterization of P-PST would assist in the design of a method of phenotyping members of the population, thus enabling further studies to be conducted.

To date, none of the cDNA sequences for the human PSTs has been reported. Molecular characterization of human P-PST

will assist in investigating the relationship between P-PST and other mammalian STs in an attempt to define functionally active sites and provide information as to the mechanism of sulfate transfer. The protein encoded by the P-PST cDNA sequence can be analyzed for the presence of regulatory and other known sequence motifs, and can be used to search the protein data banks for identification of homologous proteins.

The apparent lack of heterogeneity among the human PSTs, their widespread tissue distribution, and their activity with a wide range of substrates suggest their importance has not been fully comprehended. If only two forms of PST are present in human liver, molecular characterization of these enzymes will greatly aid in the investigation and appreciation of the role of the PSTs in human drug and xenobiotic metabolism as well as their role in the metabolism of endogenous substrates.

Specific Aims

The specific aims of this dissertation are to clone and characterize the cDNA encoding the human P-PST by:

1. obtaining the full-length sequence for the cDNA,
2. verifying in an in-vitro system the ability of the cDNA to be transcribed and translated in the presence of ^{35}S-methionine into a protein of the correct size,
3. transiently expressing the cDNA in COS cells to characterize the ST activity encoded by the cDNA,
4. determining the size and number of poly (A)+ RNA fragments present in the liver that hybridize to the cDNA, and

5. analyzing the restriction patterns of genomic DNA probed with the cDNA as an indicator of the number and size of related genes in the human genome.

Preliminary Work Done in this Laboratory

Prior to these dissertation studies on P-PST, several important experiments were carried out by research assistants Tom Dowling, Mary Vazquez, and Rena Lu, and by predoctoral fellow Kathleen Comer. This work has been included in the manuscript "Sequence analysis and expression of the cDNA for the phenol-sulfating form of human liver phenol ST" which has been submitted for publication and is described below.

Sequence Analysis of Peptides Derived from Purified P-PST

P-PST was purified from human liver as previously described (Falany et al. 1990). Initial attempts to sequence the amino-terminal end of P-PST were unsuccessful, apparently the result of a blocked amino-terminus on the protein. Therefore, amino acid sequence information was obtained from peptides generated by tryptic and cyanogen bromide cleavage of P-PST. In the generation of peptides by cyanogen bromide digestion, P-PST was resolved by SDS-PAGE, transferred electrophoretically to a PVDF membrane, and peptide fragments were generated *in situ* as previously described (Yuen et al. 1989). Digestion with trypsin was accomplished by suspending 50 µg of purified P-PST in 50 µl of 0.4 M ammonium bicarbonate, pH 8.0, adjusting the pH of the mixture to 7.5-8.0 with sodium hydroxide and reducing P-PST in the presence

of 4 mM dithiothreitol (DTT) at 50°C for 15 min. P-PST was subsequently alkylated in the presence of 8 mM iodoacetamide in the dark for 15 min. The volume of the reaction was adjusted to 200 µl with water, and 5 µl of trypsin 1.0 mg/ml in 0.1% trifluoroacetic acid (TFA), was added. After a 24 hour incubation at 37°C, the reaction was stopped by the addition of 5.0 µl of 10% TFA. Following lyophilization of the P-PST tryptic and cyanogen bromide digests, the resultant peptides were suspended in water containing 0.1% TFA and separated by reversed-phase HPLC on a Vydac C-18 column (46 mm x 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 °C prior to 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 online to an ABI 120 (HPLC) PTH analyzer. Data were collected and yields calculated on a Nelson Analytical 3000 Series Chromatography System.

Isolation of P-PST cDNAs

Human P-PST cDNAs were isolated from a lambda Uni-Zap XR human liver cDNA library (Stratagene) using the cDNA for rat liver minoxidil ST as a probe (Hirshey et al. 1991). The rat minoxidil ST cDNA was cloned in our laboratory and several lines of evidence support the close relationship of this

enzyme to human P-PST. Rabbit polyclonal antibodies developed to the two proteins demonstrated cross-immunoreactivity (Heroux, Falany and Roth 1989; Hirshey and Falany 1990). Purified human P-PST and minoxidil ST displayed similar substrate reactivities (Falany et al. 1990; Falany and Kerl 1990; Hirshey and Falany 1990) and cyanogen bromide generated peptides derived from both purified proteins showed a high degree of similarity. In one peptide, 8 out of 10 amino acids were identical and the two different amino acids were conservative substitutions (Falany unpublished observation).

Approximately 350,000 phage were grown in E. coli XL1-Blue, plated on 150 mm Petri dishes at approximately 40,000 phage/dish, and blotted onto nitrocellulose filters. The filters were denatured, baked in vacuo, and prehybridized for 2 hours at 55°C in a solution containing 6x SSC, 0.5% sodium dodecyl sulfate (SDS), 10x Denhardt's solution (50x Denhardt's = 1% each of type 400 ficoll, polyvinylpyrrolidone, and bovine serum albumin), and 200 µg/ml denatured salmon sperm DNA (Sambrook, Fritsch, and Maniatis 1989). The filters were then hybridized overnight at 55°C in the same solution with approximately 1.5 x 10^6 dpm/ml of the [^{32}P]nick-translated minoxidil ST cDNA (5 x 10^7 dpm/µg). Subsequently, the filters were washed three times in 3x SSC/0.5% SDS at 55°, dried, and exposed to autoradiograph film for 18 hours at -70°C in the presence of an intensifying screen. Positive clones were purified by repeated cycles of dilution and rescreening. The lambda Zap II phage vector contained the cDNA insert within a

pBluescript vector. The cDNAs were isolated from the lambda vector in pBluescript by coinfection with R408 helper bacteriophage in E. coli XL1-Blue as described by the manufacturer.

EXPERIMENTAL PROCEDURES

Materials

Sequenase Version 2.0 DNA sequencing kits were purchased from United States Biochemical (Cleveland, Ohio). Restriction enzymes were purchased from New England Biolabs (Beverly, Massachusetts), Promega (Madison, Wisconsin), or Gibco-BRL (Grand Island, New York). Nick-translation kits, [alpha-^{35}S]dATP (3000 Ci/mmol), [alpha-^{32}P]dCTP (800 Ci/mmol), and [^{35}S]methionine (1232.7 Ci/mmol] were purchased from New England Nuclear (Boston, Massachusetts). Oligonucleotides were obtained from Research Genetics (Huntsville, Alabama). The GeneAmp PCR kit was purchased from Perkin Elmer (Norwalk, CT). Magnagraph nylon membranes were purchased from Micron Separation Inc. (Westbourgh, Massachusetts). Nitrocellulose paper was obtained from Schleicher and Schuell (Keene, Hew Hampshire). The MAXIscript™ *in vitro* transcription kit and the ReticLysate IVT™ translation kit were from Ambion Inc. (Austin, Texas). The lambda Uni-Zap XR human liver cDNA library and T4 DNA ligase were purchased from Stratagene (La Jolla, California). pGEM-7zf, pGEMEX 2, and pSP72 were purchased from Promega (Madison, Wisconsin). COS-7 cells were purchased from American Type Culture Collection (Rockville, Maryland). Fetal bovine serum was purchased from Hyclone

Laboratories (Logan, Utah). PAPS was obtained from Pharmacia (Piscataway, New Jersey). Minoxidil was obtained from the Upjohn Company (Kalamazoo, New Jersey) and p-nitrophenol was obtained from the Sigma Chemical Company (St. Louis, Missouri) pSV-SPORT-1, fetal bovine serum, Dulbecco's modified Eagle medium (DMEM), and Lipofectin™ were obtained from Gibco-BRL (Grand Island, New York). Sequencing grade trypsin was obtained from Boehringer Mannheim (Indianapolis, Indiana). 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, New York). All other reagents were of molecular biology grade.

Sulfotransferase Assays

P-PST activity was determined using PNP and minoxidil as substrates. Human PST assays using PNP (10 μM) were performed as previously described (Falany, Vazquez, and Kalb 1989) in a volume of 0.25 ml containing 10 μM [^{35}S]PAPS (0.05 μCi/nmol). Reactions were incubated for 15 min at 37°. Sulfated products were separated from reactants using a barium precipitation procedure (Foldes and Meek 1973).

Minoxidil ST activity was assayed as previously described using 7 mM minoxidil and 10 μM [^{35}S]PAPS (Falany and Kerl 1990) in a volume of 0.17 ml. Reactions were incubated for 15 min at 37°. Minoxidil N,O-sulfate was separated from PAPS using an alkaline extraction procedure (Johnson and Baker 1987). This procedure takes advantage of the fact that minoxidil

sulfate is more hydrophobic than minoxidil and is quantitatively extracted into the organic phase of the solvent system. [^{35}S]PAPS remains in the aqueous phase.

Immunoblot Procedure

The preparation and characterization of rabbit anti-human M-PST antibodies have been described previously (Heroux, Falany and Roth 1989). Proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose paper using a Bio-Rad Transblot unit and the buffer system of Towbin (Towbin, Staehelin, and Gordon 1979). The nitrocellulose membrane was blocked with 3% gelatin in 100 mM Tris-Cl, pH 7.5, containing 500 mM NaCl (TBS), for 1 hour. Incubation with a 1:10,000 dilution of the rabbit anti-human PST antiserum was carried out overnight at room temperature with gentle shaking in TBS containing 0.1% Tween 20 and 1% gelatin. Immunoconjugates were detected using goat anti-rabbit IgG alkaline phosphatase conjugate (Bio Rad) at a 1:5,000 dilution. Protein concentrations were determined by the procedure of Bradford (1976), using bovine gamma globulin as a standard.

Molecular Characterization of P-PST cDNAs

The cDNAs cloned into the lambda Zap II vector can be automatically excised by coinfection with R408 helper bacteriophage and recircularized to generate subclones in pBluescript. Clones containing the five largest cDNAs, with inserts ranging in size from 550 to 1121 base pairs, were subjected to preliminary sequence analysis. Partial sequences

of the 3'-ends of the cDNAs were identical, although none contained a full-length open reading frame. The largest cDNA, P-PST2-2, appeared to be close to full-length, but did not contain an ATG initiation codon.

A polymerase chain reaction (PCR) procedure was used to isolate the missing 5'-end of P-PST (Figure 3). Host bacteria, XL1 Blue, were infected at low multiplicity (approximately 5 x 10^7 plaque forming units per 10^{10} bacterial cells) with phage from the amplified lambda Uni-Zap XR human liver cDNA library; the phage DNA was isolated (Sambrook, Fritsch, and Maniatis 1989) to serve as the template in PCR reactions. Isolation of the phage DNA involved growing the phage-infected bacteria at 37°C for 12 hours, at which time mass bacterial cell lysis occurred. Phage DNA was purified by a procedure involving a glycerol step gradient (Sambrook, Fritsch, and Maniatis 1989).

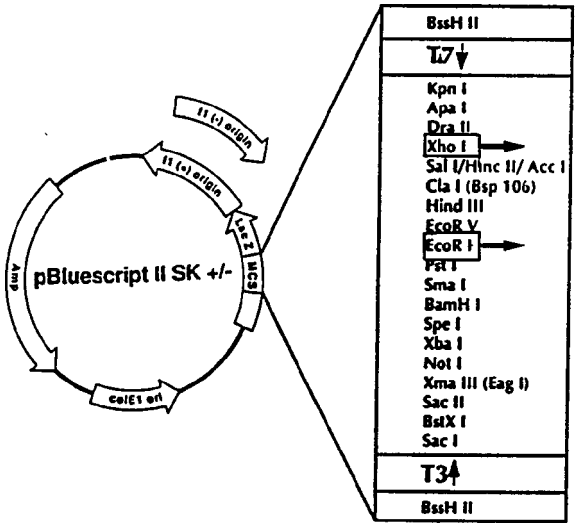
The pBluescript vector located within the lambda Zap II vector contained the T3 primer binding site located upstream from the 5'-end of the multiple cloning site. The 17-mer T3 primer was used as the nonspecific sense primer. A specific primer corresponding to bases 375 to 393 of the P-PST-1 cDNA was used as the antisense primer. This primer was selected from a region 3' to a Sac I restriction endonuclease site in P-PST2-2 in order to facilitate subsequent ligation of the PCR amplified product to the 3' end of P-PST2-2. In addition, the primer was selected on the basis of its 53% G + C composition, and lack of 3' complementarity with the T3 primer (Innis and

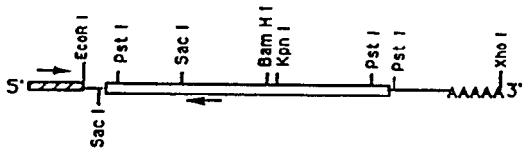
Figure 3. Strategy for Obtaining the 5' End of the P-PST cDNA.

Analysis of 5 clones isolated from the lambda UniZap XR cDNA library indicated that all lacked the initiation codon. In order to obtain this sequence information, a procedure involving use of the polymerase chain reaction (PCR) was employed.

Lambda DNA isolated from the library served as the template in the PCR reactions. The cDNAs in the library were inserted within the pBluescript at the EcoR I and Xho I sites. The T3 binding site flanked the 5' end of the multiple cloning site of pBluescript; therefore, the T3 primer was selected as the nonspecific sense primer.

The location of restriction endonuclease sites present in P-PST2-2 are indicated above the stick figure. A specific anti-sense primer was selected to a region of P-PST 2-2 due to its location 3' to a Sac I restriction endonuclease site. PCR amplification of the 5' end of the cDNA indicated the presence of a Sac I site, indicated below the stick figure, in the 5' noncoding region. The open boxed area represents the coding region of the cDNA. Arrows indicate the positions and directions of the primers. Single lines represent the 5' and 3' noncoding regions. The hatched box area represents pBluescript sequence at the 5'-end of the cDNA.





SENSE PRIMER: (T3) 5' ATTAACCCTCACTAAAG 3'

ANTISENSE PRIMER: 5' TGTGTCTTCAGGAGTCGTG 3'

Gelfand 1990). The calculated melting temperatures (T_ms) of the T3 and P-PST specific primers were approximately 46°C and 58°C, respectively. PCR reactions contained 200 µM of each deoxynucleoside triphosphate, 50 mM potassium chloride, 10 mM Tris-HCl, pH 8.3, gelatin (100 µg/ml), and 2.0 units of AmpliTaq DNA polymerase in a volume of 100 µl. The magnesium chloride concentration was varied from 1.5 to 3.0 mM. Template DNA (0.1 µg) and primers (1.0 µg each) were subjected to 30 cycles of denaturation for one min at 94°C, annealing for two min at 50°C, and extension at 72°C for 3 min with a final extension of 7 min at 72°C.

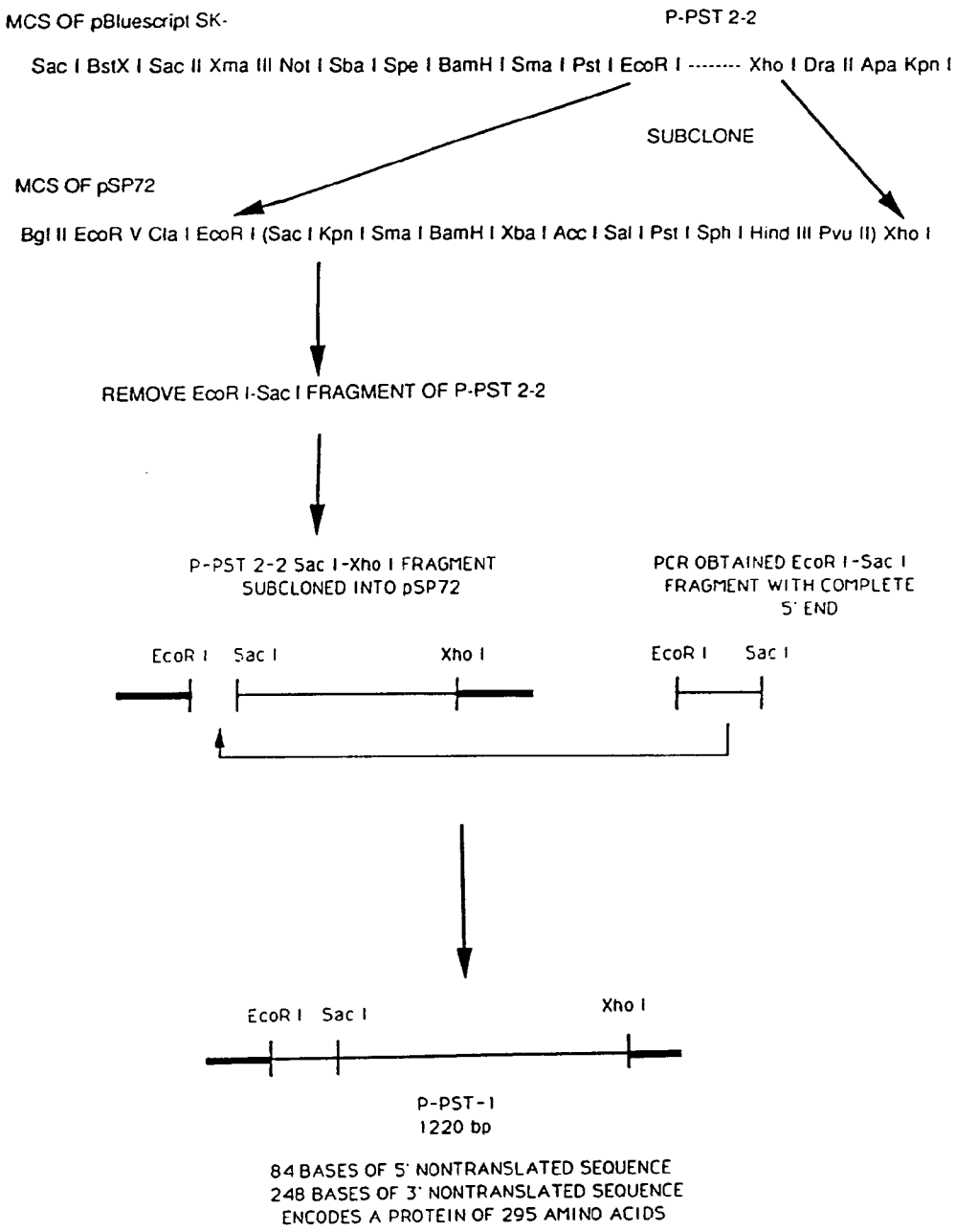
PCR reactions were extracted with chloroform, ethanol precipitated, and digested with 40 units each of EcoR I and Sac I for 2 hours. The restricted PCR products were then resolved in a 1.5% agarose gel. DNA fragments ranging in size from 250 to 600 bp were isolated using DE-81 paper (Sambrook, Fritsch, and Maniatis 1989) and subcloned into the EcoR I-Sac I sites of pGem-7zf. pGEM-7zf encodes the alpha fragment of beta-galactosidase which complements the omega fragment of beta-galactosidase encoded by the host. Together, these two fragments produce functional beta-galactosidase and in the presence of the inducer IPTG and the chromagen, Xgal, produce colonies with blue color. However, DNA inserted into the plasmid destroys the ability to produce the alpha fragment of beta-galactosidase and therefore the colonies remain white, allowing color selection of insert-containing plasmids. The pGem-7zf constructs were transfected into E. coli DH5-alpha.

Plasmid preparations were digested with EcoR I and Sac I and subjected to Southern blot analysis using a probe designed from the 5' end of P-PST2-2. Two DNA fragments possessing an ATG initiation codon in-frame with the translation of P-PST 2-2 were identified by sequence analysis. The larger DNA fragment was isolated by complete digestion with EcoR I for 2 hours, followed by partial digestion with Sac I for 15 min, due to the presence of an internal Sac I site. This fragment was then used in the construction of a full-length cDNA (Figure 4). The EcoR I-Xho I fragment of P-PST2-2 was subcloned into pSP-72 and the EcoR I-Sac I fragment removed. The PCR generated EcoR I-Sac I fragment was ligated into the EcoR I-Sac I sites of the pSP-72 cDNA construct to generate the full-length cDNA, P-PST-1.

DNA Sequence Analysis

The cDNAs were subjected to sequencing using the dideoxynucleotide chain termination procedure with Sequenase 2.0 (USB) and [alpha-^{35}S]dATP. The dideoxy chain termination method of sequencing (Sanger, Nicklen, and Coulson 1977) involved annealing an oligonucleotide primer to the template DNA, extending the primer using limiting concentrations of the deoxynucleoside triphosphates in order to incorporate the labeled dATP, and terminating chain elongation by the incorporation of dideoxynucleotides. Plasmid DNA was prepared by a small-scale alkaline lysis procedure and was subjected to double-stranded sequencing (Kraft et al. 1988). Double stranded sequencing involved preliminary denaturation with 0.2

Figure 4. Obtaining a Full-Length P-PST cDNA.
In order to obtain a full-length P-PST cDNA, the insert from the truncated cDNA, P-PST2-2, was isolated as an EcoR I-Xho I fragment and subcloned into pSP72. The EcoR I-Sac I fragment was removed from this clone and the PCR amplified DNA digested with EcoR I and Sac I was inserted into the pSP72 construct. This produced the 1220 bp full length cDNA, P-PST-1. Abbreviation: MCS, multiple cloning site.


MCS OF pBluescript SK-
P-PST 2-2
Sac I BstX I Sac II Xma III Not I Sba I Spe I BamH I Sma I Pst I EcoR I -------- Xho I Dra II Apa Kpn I
SUBCLONE
MCS OF pSP72
Bgl II EcoR V Cla I EcoR I (Sac I Kpn I Sma I BamH I Xba I Acc I Sal I Pst I Sph I Hind III Pvu II) Xho I
REMOVE EcoR I-Sac I FRAGMENT OF P-PST 2-2
P-PST 2-2 Sac I-Xho I FRAGMENT SUBCLONED INTO pSP72
PCR OBTAINED EcoR I-Sac I FRAGMENT WITH COMPLETE 5' END
EcoR I
Sac I
Xho I
EcoR I
Sac I
EcoR I
Sac I
Xho I
P-PST-1
1220 bp
84 BASES OF 5' NONTRANSLATED SEQUENCE
248 BASES OF 3' NONTRANSLATED SEQUENCE
ENCODES A PROTEIN OF 295 AMINO ACIDS

normal sodium hydroxide, neutralization, and ethanol precipitation before proceeding with the standard sequencing protocol.

Sequence information from the 5' and 3' ends of the cDNA clones in pBluescript was obtained by using the T3 and T7 primers that recognize sequences flanking the multiple cloning site of this vector. P-PST2-2 was subjected to internal sequence analysis by subcloning directional and shotgun restriction fragments into pGEM-7zf for sequencing with T7 and SP6 primers. Directional subcloning involved digesting the vector and the P-PST2-2 insert with the following pairs of restriction endonucleases: EcoR I-Sac I; BamH I-Sac I; Pst I-Sac I; BamH I-Pst I; and EcoR I-BamH I. These enzymes were chosen as the result of information provided by preliminary endonuclease mapping of P-PST2-2 that showed the presence of BamH I, Sac I, and Pst I restriction endonuclease sites. Shotgun cloning was accomplished by digesting the P-PST2-2 insert with Alu I or Hae III and subcloning into the vector pGem-7zf digested with Sma I. Alu I and Hae III were frequent cutting restriction endonucleases that cleaved their respective recognition sequences at the axis of symmetry, yielding blunt ends. This allowed ligation of DNA restricted with these enzymes into vectors that had been digested with any blunt cutting restriction endonuclease, such as Sma I. PCR amplified DNA was digested with EcoR I and Sac I and subcloned into pGEM-7zf restricted with the same enzymes. The products of the sequencing reactions were resolved in 6%

polyacrylamide-urea gels using a buffer gradient of 0.5-2.5x TBE (1x TBE = 89 mM Tris-borate, 2 mM EDTA). Sequencing gels were read manually and analyzed using the University of Wisconsin Genetics Computer Group's (GCG) programs (Devereus, Haeberli, and Smithies 1984).

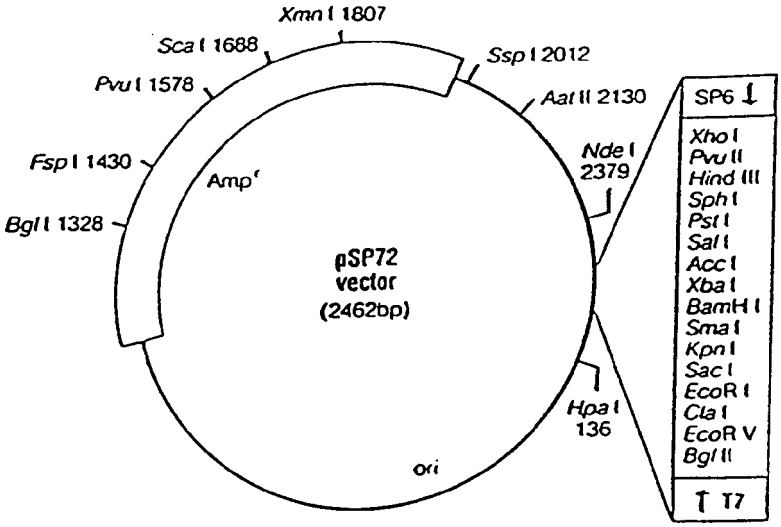
The P-PST cDNA sequence was compared with other protein sequences in the GCG SwissProt data base for identification of homologous sequences and the generation of a consensus sequence. The sequence was also analyzed by the GCG program Motifs for the presence of known sequence motifs.

In Vitro Transcription and Translation of P-PST-1

In order to facilitate directional subcloning into the mammalian expression vector, pSV-SPORT-1, P-PST-1, constructed in the vector pSP-72 as described above, was isolated as an EcoR I-Xho I fragment from pSP-72 and subcloned into the EcoR I-Xho I sites in pGEMEX 2 (Figure 5). The complete P-PST-1 cDNA was then removed from pGEMEX 2 as an EcoR I-Hind III fragment and subcloned into the EcoR I-Hind III sites of pSV-SPORT-1 (Figure 5). pSV-SPORT-1 contains the SP6 RNA polymerase promoter site; therefore, the SP6 RNA polymerase was used in the generation of RNA transcripts. pSV-SPORT-PST-1 was linearized with Hind III and RNA transcripts were synthesized using the MAXIscript™ SP6 transcription system. Placental ribonuclease inhibitor (60 units) was added to transcription reactions to assist in the preservation of full-length RNA molecules. Transcription reactions were incubated with 25 units of SP6 RNA polymerase at 37°C for 1 hour, at

Figure 5. Subcloning P-PST-1 into pSV-SPORT-1 Expression Vector.

P-PST-1 was isolated from pSP72 by digestion with EcoR I and Xho I and subcloned into the corresponding sites of pGEMEX-2. The cDNA was then isolated by digestion of this construct with EcoR I and Hind III, and subcloned into pSV-SPORT-1. This vector contains the SV40 origin of replication, early promoter, small t intron, and poly $(A)^+$ addition site.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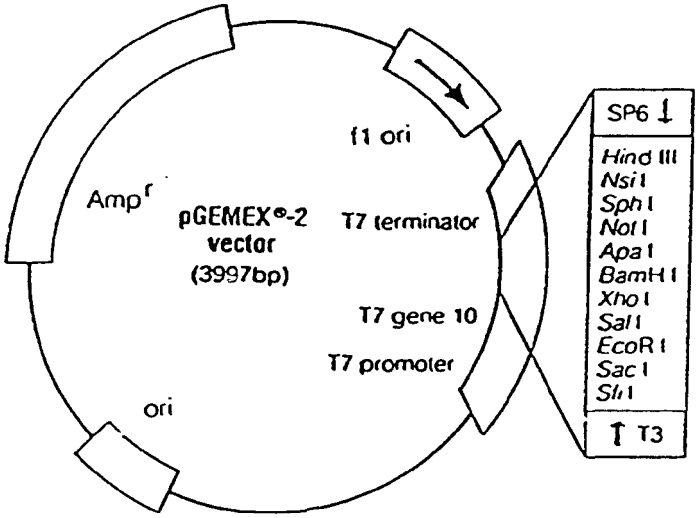
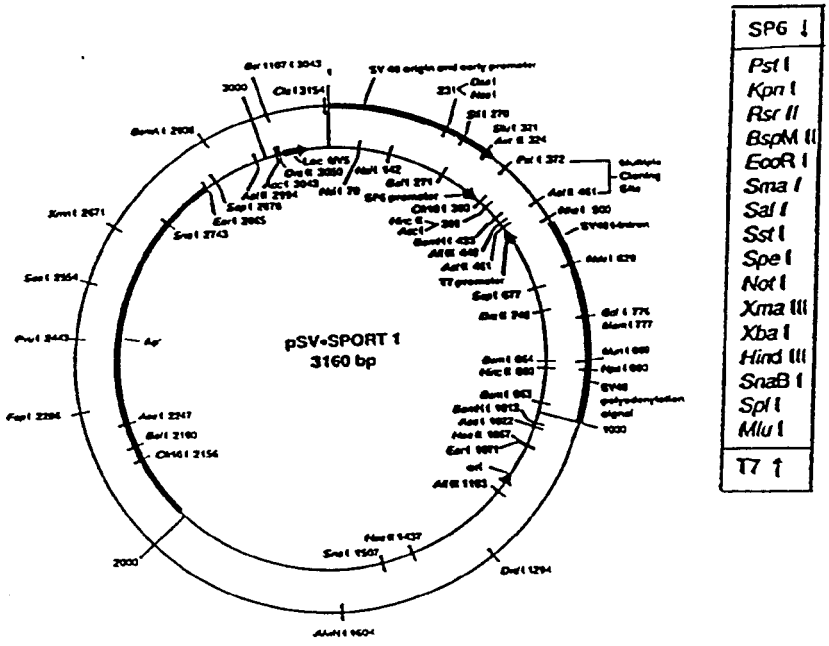
Sca I 1688
Ssp I 2012
Pvu I 1578
Aat II 2130
Nde I 2379
Fsp I 1430
Amp^r
Bgl I 1328
pSP72 vector (2462bp)
Hpa I 136
ori
SP6
Xho I
Pvu II
Hind III
Sph I
Pst I
Sal I
Acc I
Xba I
BamH I
Sma I
Kpn I
Sac I
EcoR I
Cla I
EcoR V
Bgl II
T7


f1 ori
Amp^r
pGEMEX®-2 vector (3997bp)
T7 terminator
T7 gene 10
T7 promoter
ori
SP6
Hind III
Nsi I
Sph I
Not I
Apa I
BamH I
Xho I
Sal I
EcoR I
Sac I
Sli I
T3


pSV-SPORT 1 3160 bp
SP6
Pst I
Kpn I
Rsr II
BspM II
EcoR I
Sma I
Sal I
Sst I
Spe I
Not I
Xma III
Xba I
Hind III
SnaB I
Spl I
Mlu I
T7

which time an additional 25 units of SP6 RNA polymerase were added and incubation continued for another hour.

RNA transcripts present in aliquots of the transcription reaction were directly translated with ReticLysate IVT™, a rabbit reticulocyte lysate system, in the presence of [^{35}S]methionine to label the translated proteins. The presence of a 5'-7-methyl guanosine cap structure on mRNAs is generally required in order to be translated efficiently in reticulocyte lysates; however, uncapped mRNAs can be translated in this system by using a buffer, provided by the manufacturer, that contains a decreased salt content. The translation reactions, including reactions containing no RNA and control RNA, were resolved by SDS-PAGE, dried in vacuo and the size of the translated polypeptides detected by autoradiography for 16 hours. 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 (Heroux, Falany, and Roth 1989).

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 DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS). COS-7 cells contain the functional large T-antigen of SV40 and produce the factors required for replication of transfected DNA that contains the SV40 origin of replication (Sambrook, Fritsch,

and Maniatis 1989). pSV-SPORT-1 is a mammalian expression vector, containing the SV40 origin of replication, early promoter, small t intron, and polyadenylation site. Expression of proteins in pSV-SPORT-1 is transient in that cell death results from the high levels of extrachromosomally replicating DNA approximately 70 to 90 hours post-transfection.

Transfection of cells was accomplished using a liposome mediated procedure (Lipofectin™, Gibco-BRL). This reagent contains cationic and neutral lipids that interact spontaneously with DNA to form a lipid-DNA complex. The fusion of this liposome complex with the tissue culture cells results in the efficient uptake of the DNA. COS-7 cells were plated in 60 mm plates 16 hours prior to transfection to achieve a confluency of approximately 50%. The plates were washed twice with serum free medium, followed by the addition of 3.0 ml of Opti-MEM™ reduced serum medium. Lipofectin™ (30 µg) was incubated at room temperature with supercoiled pSV-SPORT-1 or pSV-SPORT-P-PST-1 (10 µg) in a total of 100 µl for 15 min, after which the mixture was applied dropwise to each plate with constant swirling. The cells were incubated at 37°C under 5% CO_2/95% O_2 for 24 hours. The media was then removed and replaced with 3 ml of DMEM containing 10% FBS and the incubation continued for an additional 48 hours.

In order to detect expression of P-PST, cells were washed three times with phosphate-buffered saline and harvested in 10 mM triethanolamine-HCl buffer, pH 7.4, containing 1 mM DTT, 10% glycerol, and 1 mM phenylmethyl sulfonyl fluoride. The

cells were homogenized in a teflon-glass homogenizer and cytosol recovered after centrifugation at 100,000 x g for 1 hour at 4°C. Aliquots of the cytosol were assayed for ST activity with PNP and minoxidil, as described previously. In order to confirm that the reaction rates were being measured when they were linear with respect to time, a time course analysis was performed with reaction times of 5, 10, 20, and 30 mins.

Aliquots of transfected COS-7 cell cytosol were resolved by SDS-PAGE and electroblotted to nitrocellulose filters. As described above, immunoreactive P-PST was detected by immunoblotting using the anti-human PST antibodies. Proteins from human liver cytosol were simultaneously resolved on the gel to compare the size of the translated P-PST with the human liver P-PST.

In order to verify that the radiolabeled product detected during the PNP ST assay was PNP-sulfate, product identification was performed by HPLC analysis. A sample of PNP-sulfate was dissolved in water and its elution was monitored by resolving the sample with reversed-phase HPLC on a Brownlee Aquapore C_8 column using a linear gradient of 0-46.7% acetonitrile in 0.1% TFA at a rate of 1.5 ml/min for 20 mins. Elution of the product from the column was monitored at 260 nm using a Perkin Elmer model LC-85B spectrophotometric detector. Subsequently, 100 µl of an incubate of a PNP ST assay of transfected COS-7 cell cytosol was resolved on the column; 0.75 ml fractions of the column eluate were collected

every 30 seconds. Elution of the radioactive product was measured by liquid scintillation spectrometry.

Northern Blot Analysis of Human Liver RNA

All glassware used in RNA isolation was baked at 180°C for at least 8 hours prior before use to remove residual RNase activity. In order to chemically inactivate ribonucleases present in the solutions to be used in the preparation of RNA, the solutions were treated with 0.1% diethyl pyrocarbonate (DEPC), placed in a 37°C water bath for 2 hours, and autoclaved for 1 hour. Total RNA was prepared from normal human liver samples using the acid guanidinium thiocyanate method of Chomczynski and Sacchi (1987). An intermediate step in which the RNA was vortexed in 4 M lithium chloride was included to remove polysaccharides present in the samples (Puissant and Houdebine 1990).

Poly $(A)^+$ RNA was isolated from total RNA by oligo-dT cellulose chromatography (Davis, Dibner, and Battey, 1986). Samples of total (100 µg), transfer and ribosomal (100 µg), and poly $(A)^+$ (5 and 20 µg) RNA were resolved by electrophoresis in 1.5% agarose-formaldehyde gels, transferred to nylon membranes, and baked in vacuo for 2 hours at 65°C. The filters were hybridized overnight at 42°C with 1.5 x 10^6 dpm/ml of the [^{32}P]nick-translated PPST2-2 in 50% formamide, 5x SSC, 10 mM Tris-Cl, pH 7.5, 4x Denhardt's reagent, 200 µg/ml calf thymus DNA and 0.1% SDS.

Nick-translated P-PST2-2 was prepared according to the protocol provided by New England Nuclear with a reaction

incubation at 12°C for 1 hour. The labeled cDNA was separated from unincorporated label by size exclusion chromatography using spin column packed with Bio-Gel P-30 according to instructions from the manufacturer (Bio-Rad). The integrity of the probe was verified by resolving an aliquot of the reaction mixture in a 1% agarose gel and exposing the gel to autoradiograph film for five min at room temperature. Following hybridization, the filters were washed at 10 min intervals twice in 3x SSC/0.5% SDS at room temperature, twice in 3x SSC/0.5% SDS at 65°C, once in 1x SSC/0.5% SDS at 65°C, and then exposed to autoradiograph film for 48 hours with an intensifying screen at -70°C.

Southern Blot Analysis of Human Liver DNA

Genomic DNA was obtained from normal human liver specimens as previously described (Strauss 1989). Aliquots (10 µg) of DNA were digested overnight with 50 units of Bam H I, Kpn I, Pst I, Sac I, or Hind III and resolved by electrophoresis in a 0.8% agarose gel. The gels were pretreated and transferred by capillary action to nylon membranes according to the manufacturer's instructions (MSI). The membranes were baked *in vacuo* at 65°C for 2 hours and hybridized overnight with 1.5 x 10^6 dpm/ml of [^{32}P]nick-translated P-PST2-2, as described above. Following hybridization, the filters were washed twice for 10 min in 3x SSC/0.5% SDS at room temperature, once each in 1x SSC/0.5% SDS at 37°C, 1x SSC/0.5% SDS at 65°C, followed by exposure to autoradiograph film with an intensifying screen at -70°C for

24 hours. The filters were subsequently washed twice for 15 min in 0.2x SSC/0.5% SDS at 65°C and reexposed to autoradiograph film for 48 hours.

RESULTS

Molecular Characterization of P-PST cDNAs

The human liver P-PST cDNAs were isolated from a Uni-Zap XR cDNA library using a cDNA encoding a rat liver PST, minoxidil ST, as a probe. A number of positive clones were isolated from the cDNA library and 5 of the larger clones were characterized. The cDNAs ranged in length from 550 to 1121 bp and were identical based on restriction mapping and sequence analysis of their 3'-nontranslated regions. 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; however, this clone lacked an ATG initiation codon. Each base of the sequence was read at least four times and at least once in each direction.

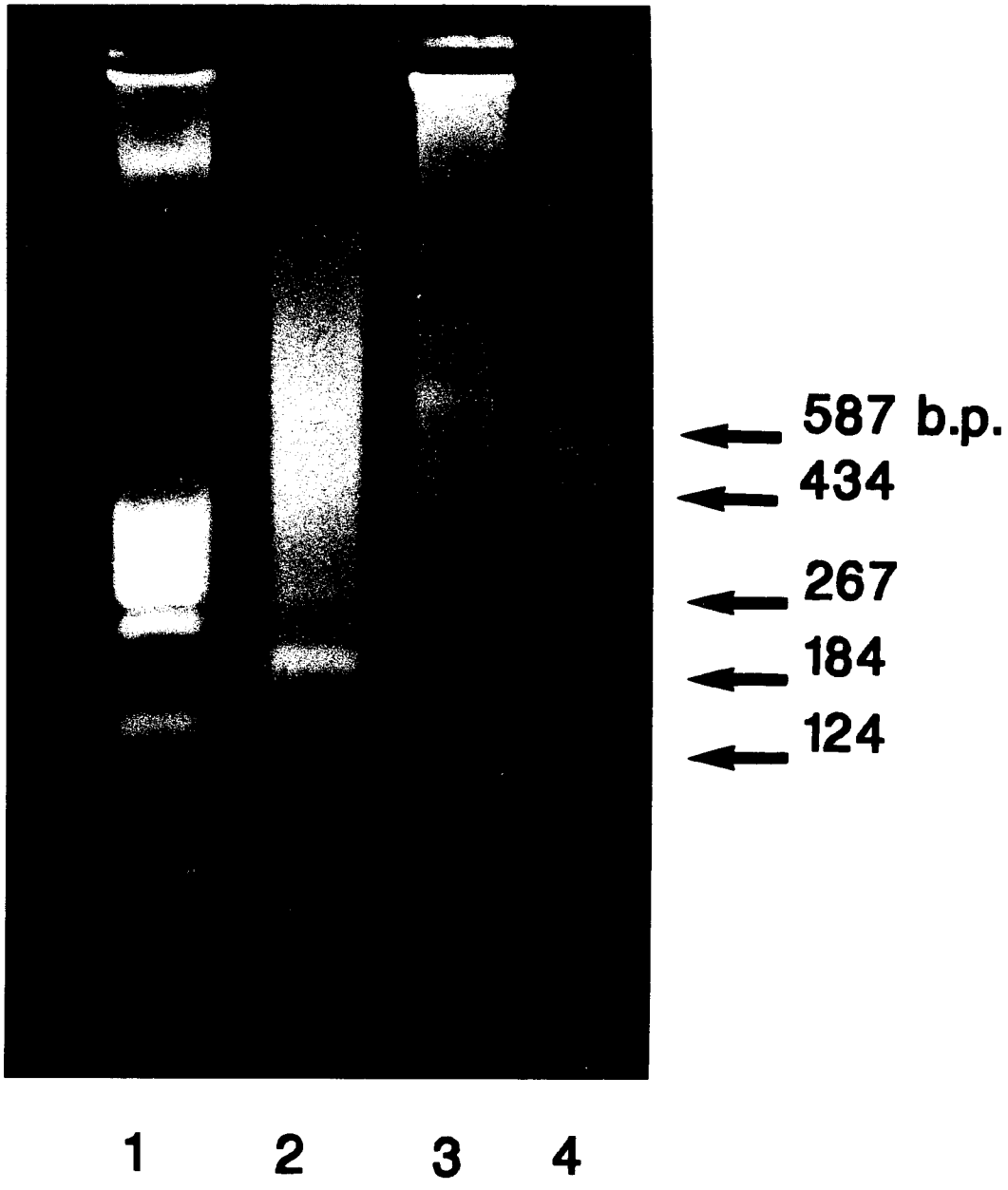
Upon characterization, all of the clones isolated from the library were truncated at the 5' end. To obtain the 5' end sequence information, a procedure involving PCR was employed (see Methods). When the PCR-amplified DNA was resolved on a 1.5% agarose gel, a diffuse band of DNA was visualized (data not shown). Slightly less nonspecific amplification was seen when the magnesium chloride concentration was lowered in PCR reaction mixture from 3.0 to 1.5 mM. The amplified DNA was digested with EcoR I, the 5' end

insertion site of the cDNAs, and Sac I, a site known to exist at nucleotide number 202 of P-PST2-2. Upon digestion of the PCR product with these enzymes, the diffuse pattern of DNA staining continued; however, two distinct products of approximately 80 and 200 bp were visualized (Figure 6). In the truncated clone P-PST2-2, the 5' EcoR I-Sac I fragment was 210 bp in length; therefore, it did not appear that either of these DNA fragments contained the missing 5'-end of the P-PST cDNA.

In order to obtain the 5' end of the cDNA, fragments ranging from 250 to 600 base pairs in length were isolated using DE81 paper, subcloned into the EcoR I and Sac I sites of pGEM-7zf and transfected into E. coli, DH5-alpha. Twelve plasmids were color selected and prepared for screening by Southern blot analysis with a probe from the 5' end of P-PST2-2. Two positive plasmids which contained EcoR I-SacI fragments were isolated. These cDNAs were subjected to sequence analysis and found to be 280 and 304 bp in length. Each of the PCR derived cDNA fragments contained 207 bp of sequence identical to the 5'-end of PPST2-2 and possessed an in-frame ATG initiation codon, adding five amino acids to the amino terminal end of the PPST2-2 translation product. The larger fragment also contained 84 bp of 5'-nontranslated region. The cDNA P-PST2-2 insert was removed from pBluescript due to the presence of a Sac I site in the polylinker that would interfere with removal of the EcoR I-Sac I fragment from the truncated clone and the subsequent insertion of the PCR

Figure 6. Restriction Digest of PCR Amplified 5' Terminal of P-PST-1.

To obtain P-PST 5' sequence information, the product of PCR amplification of DNA from a human liver cDNA library with a T3 and a P-PST specific primer was subjected to digestion with restriction endonucleases EcoR I and Sac I and resolved in a 1.5% agarose gel. Lane 1 (PCR template = P-PST2-2 truncated cDNA) served as the control. DNA fragments ranging in size from 250 to 600 bp were isolated from lanes 2-3 (PCR template = DNA from cDNA library). Lane 4 contains low molecular weight DNA standards.


587 b.p.
434
267
184
124
1
2
3
4

obtained EcoR I-Sac I fragment. In subcloning the EcoRI-XhoI insert of P-PST2-2 in pSP72, the Sac I site in the polylinker was removed and would not interfere with subsequent manipulations. The 1220 bp full-length P-PST cDNA, P-PST-1, was obtained by ligating the 304 bp EcoR I-Sac I PCR derived fragment into the EcoR I-Sac I sites of the pSP-72 construct containing the Sac I-Xho I fragment of P-PST2-2 (Figure 4).

P-PST-1 encoded a protein consisting of 295 amino acids with a predicted molecular mass of 34,097 Da (Figure 7). The cDNA contained 84 bp of 5' nontranslated region and 248 bp of 3' nontranslated region with a poly $(A)^+$ tract and three putative polyadenylation signal sequences, AATAAA, (Watson et al. 1987), located 11, 16 and 21 bases upstream from the beginning of the poly $(A)^+$ tract.

In order to aid in the identification of putative P-PST cDNAs, internal amino acid sequence of P-PST had been previously obtained from peptides generated by cyanogen bromide and tryptic cleavage of affinity purified P-PST. The amino acid sequences of five P-PST peptides, totaling 55 amino acids, (Falany unpublished observation) were 96% identical to the amino acid sequence of P-PST obtained by translation of P-PST-1 (Figure 7). The two differences in the sequence concerned the cysteines at residues 70 and 287, which were reported as glutamates in the peptide sequencing data.

In vitro Transcription and Translation of P-PST-1

The molecular mass of P-PST as estimated by SDS-PAGE (32,000 Da) is smaller than that predicted from the

Figure 7. Nucleotide and Deduced Amino Acid Sequence of P-PST-1.

The nucleotide and translation of the P-PST-1 cDNA is shown. The ATG initiation codon is preceded by 84 bp of 5' nontranslated region. The termination codon, TGA, is followed by 248 bp of 3' nontranslated region including a 19 base poly $(A)^+$ tail. Three putative polyadenylation signals (Watson et al. 1987), AATAAA, are located 11, 16, and 21 bases upstream of the poly $(A)^+$ tail.

The amino acid sequence is numbered at the right. The first 14 nucleotides at the 5' end are present in all clones sequenced from the cDNA library, therefore the 15th nucleotide (cytosine) is defined as P-PST-1 nucleotide 1. The *** indicates the stop codon. The position of each coding nucleotide was sequenced at least four times and at least once in each strand.

The amino acid sequences of peptides derived from tryptic and cyanogen bromide cleavage of purified P-PST are indicated by the dashed line. The differences between the translation of P-PST-1 and the peptide sequences are noted.

```
P-PST-1                                                    GAATTCGGCACGAGCACACAGCAC
CCACAATCAGCCACTGCGGGCGAGGAGGGCACGAGGCCAGGTTCCCAAGAGCTCAGGAAC

ATGGAGCTGATCCAGGACACCTCCGCCCGCCACTGGAGTACGTGAAGGGGGTCCCGCTC
MetGluLeuIleGlnAspThrSerArgProProLeuGluTyrValLysGlyValProLeu    20

ATCAAGTACTTTGCAGAGGCACTGGGCCCCCTGCAGAGCTTCCAGGCCCGGCCTGATGAC
IleLysTyrPheAlaGluAlaLeuGlyProLeuGlnSerPheGlnAlaArgProAspAsp    40

CTGCTCATCAGCACCTACCCCAAGTCCGGCACTACCTGGGTAAGCCAGATTCTGGACATG
LeuLeuIleSerThrTyrProLysSerGlyThrThrTrpValSerGlnIleLeuAspMet    60
                                                              ---

ATCTACCAGGGTGGTGACCTGGAGAAGTGTCACCGAGCTCCCATCTTCATGCGGGTGCCC
IleTyrGlnGlyGlyAspLeuGluLysCysHisArgAlaProIlePheMetArgValPro    80
--------------------------Glu------------          ------------

TTCCTTGAGTTCAAAGCCCCAGGGATTCCCTCAGGGATGGAGACTCTGAAAGACACACCG
PheLeuGluPheLysAlaProGlyIleProSerGlyMetGluThrLeuLysAspThrPro   100
----------------------------

GCCCCACGACTCCTGAAGACACACCTGCCCCTGGCTCTGCTCCCCCAGACTCTGTTGGAT
AlaProArgLeuLeuLysThrHisLeuProLeuAlaLeuLeuProGlnThrLeuLeuAsp   120

CAGAAGGTCAAGGTGGTCTATGTTGCCCGCAACGCAAGGATGTGGCAGTTTCCTACTAC
GlnLysValLysValValTyrValAlaArgAsnAlaLysAspValAlaValSerTyrTyr   140

CACTTCTACCACATGGCCAAGGTGCACCCTGAGCCTGGGACCTGGGACAGCTTCCTGGAG
HisPheTyrHisMetAlaLysValHisProGluProGlyThrTrpAspSerPheLeuGlu   160
            ------------------------------

AAGTTCATGGTCGGAGAAGTGTCCTACGGATCCTGGTACCAGCACGTGCAGGAGTGGTGG
LysPheMetValGlyGluValSerTyrGlySerTrpTyrGlnHisValGlnGluTrpTrp   180

GAGCTGAGCCGCACCCACCCTGTTCTCTACCTCTTCTATGAAGACATGAAGGAGAACCCG
GluLeuSerArgThrHisProValLeuTyrLeuPheTyrGluAspMetLysGluAsnPro   200

AAAAGGGAGATTCAAAAGATCCTGGAGTTTGTGGGGCGCTCCCTGCCAGAGGAGACCGTG
LysArgGluIleGlnLysIleLeuGluPheValGlyArgSerLeuProGluGluThrVal   220
                                                  ----------

GACTTCATGGTTCAGCACACGTCGTTCAAGGAGATGAAGAAGAACCCTATGACCAACTAC
AspPheMetValGlnHisThrSerPheLysGluMetLysLysAsnProMetThrAsnTyr   240
---------

ACCACCGTCCCCCAGGAGTTCATGGACCACAGCATCTCCCCCTTCATGAGGAAAGGCATG
ThrThrValProGlnGluPheMetAspHisSerIleSerProPheMetArgLysGlyMet   260

GCTGGGGACTGGAAGACCACCTTCACCGTGGCGCAGAATGAGCGCTTCGATGCGGACTAT
AlaGlyAspTrpLysThrThrPheThrValAlaGlnAsnGluArgPheAspAlaAspTyr   280

GCGAAGAAGATGGCAGGCTGCAGCCTCACGTTCCGCTCTGAGCTGTGAGAGGGGCTCCTG
AlaLysLysMetAlaGlyCysSerLeuThrPheArgSerGluLeu***               295
          ---------Glu--------------------------

GAGTCACTGCAGAGGGAGTGTGCGAATCAAACCTGACCAAGCGGCTCAAGAATAAAATAT
CAATTGAGGGCCCGGACGGTAGGTCATGTCTGTAATCCCAGCAATTTGGAGGCTGAGGTG
GGAGGATCATTTGAGCCCAGGAGTTCGAGACCAACCTGGGCAACATAGTGAGATTCTGTT
AAAAAAATAAAATAAAATAAAACCAATTTTTAAAAAAAAAAAAAAAAAAAAGAGCTC
```

translation of P-PST-1 (34,097 Da). In vitro transcription and translation were used to verify that P-PST-1 translated a protein equivalent in size to P-PST prior to proceeding with the expression of P-PST in mammalian cells. P-PST-1 was subcloned into 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]methionine 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 (Figure 8). This result indicated that the protein encoded by P-PST-1 was identical in size to human liver P-PST.

Expression of P-PST-1 in Cos-7 Cells

Expression of P-PST enzyme activity in COS-7 cells was used to definitively confirm that P-PST-1 encoded P-PST. Minoxidil was chosen as a substrate because it is a specific substrate for P-PST and is reported not to be sulfated byM-PST (Falany and Kerl 1990). PNP is also a specific substrate for P-PST at the concentration used in the assay.

Cytosol prepared from COS-7 cells transfected with pSV-SPORT-1 did not possess detectable levels of minoxidil ST activity and only low levels of PNP sulfation activity (39 pmol/min/mg cytosol protein) (Figure 9). In contrast, cytosol from COS-7 cells transfected with pSV-SPORT-P-PST-1 displayed substantial amounts of minoxidil ST activity (189 pmol/min/mg cytosol protein) and a significant increase in PNP ST activity

Figure 8. 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 autoradiograph film as previously described. The lanes represent reactions which contained (A) no RNA; (B,C) transcripts from pSV-SPORT-PST-1; and (D) control RNA. Lane E is a sample of human liver cytosol (15 µg) simultaneously resolved in the same gel with *in vitro* translation reactions and subsequently immunoblotted with rabbit anti-human PST antibodies as described in Methods. The left margin shows the migration of molecular mass standards (Sigma).

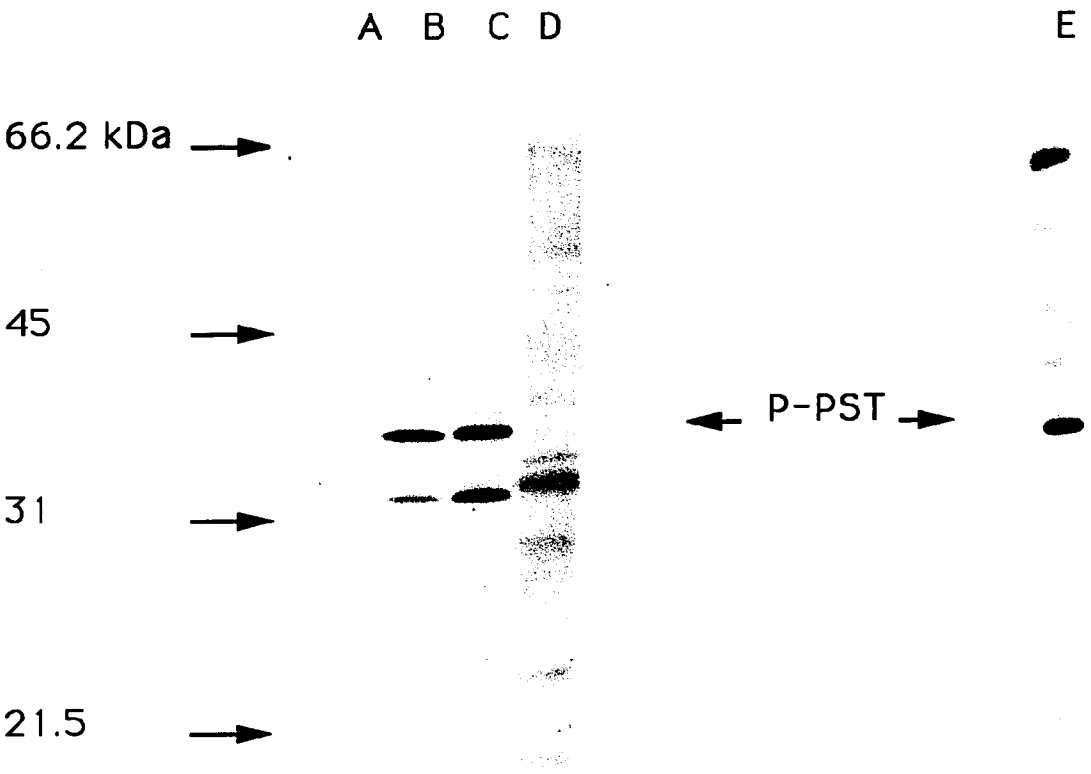
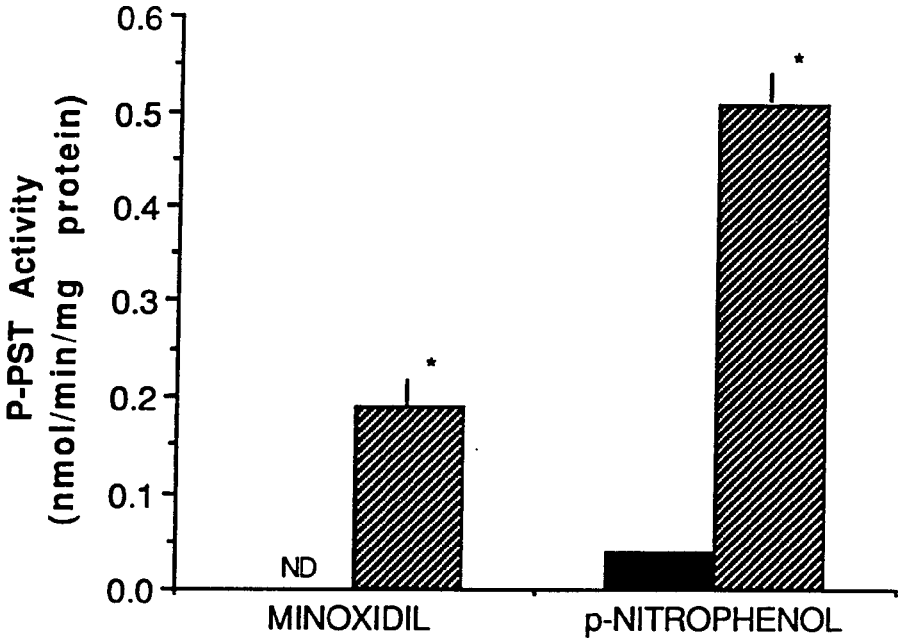

A B C D
E
66.2 kDa
45
31
21.5
P-PST

Figure 9. Expression of P-PST Activity in COS-7 Cells.
COS-7 cells were transfected with pSV-SPORT-1 (solid boxes) or pSV-SPORT-P-PST-1 (hatched boxes) and cytosol was prepared from the cells as described in Methods. The presence of PST activity was assayed using minoxidil and PNP as substrates. Specific activity is reported as the mean ST activity +/-standard deviation from four separate preparations assayed in duplicate. The level of significance of difference from controls was determined to be $p < 0.0005$ using the Students unpaired t-test. ND represents no detectable activity.


P-PST Activity
(nmol/min/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
ND
MINOXIDIL
p-NITROPHENOL

(508 pmol/min/mg cytosol protein). The ratio of PNP ST activity to minoxidil ST activity (2.7:1) in the cytosol of transfected cells is similar to the ratio of these activities in human liver cytosol (Falany and Kerl 1990). A time course analysis of product formation was performed to assure that the enzyme was not saturated during the 15 min incubation periods used in the assays. The plot revealed that reaction was linear with respect to time over the range of 15 min (Figure 10).

HPLC analysis was used to verify that the product detected in the supernatant fraction of the PNP ST assay mixture was actually PNP-sulfate. Liquid scintillation spectrometry of radioactivity in the column eluate revealed that the radioactive product eluted between 7 and 8 min (Figure 11). This was the same time interval in which the PNP-sulfate standard eluted, as indicated by its absorbance at 260 nm.

Western blot analysis of proteins in cytosol prepared from COS-7 cells transfected with pSV-SPORT-P-PST-1 and pSV-SPORT-1 was performed to determine the presence of immunoreactive P-PST. Immunoreactive protein present in the cytosol from pSV-SPORT-P-PST-1 transfected cells comigrated with immunoreactive P-PST in human liver cytosol resolved in the same gel. The presence of immunreactive protein was not detected in cytosol from cells transfected with pSV-SPORT-1 alone (Figure 12).

Figure 10. Time Course Analysis of Product Formation.
The amounts (in nmoles) of PNP and minoxidil sulfate formed in cytosol of COS-7 cells transfected with pSV-SPORT-P-PST-1 as a function of time were measured at reaction times of 5, 10, 20, and 30 min. This was performed in order to verify that saturation of the enzyme present in the cytosol had not occurred at the reaction times employed in the assays.

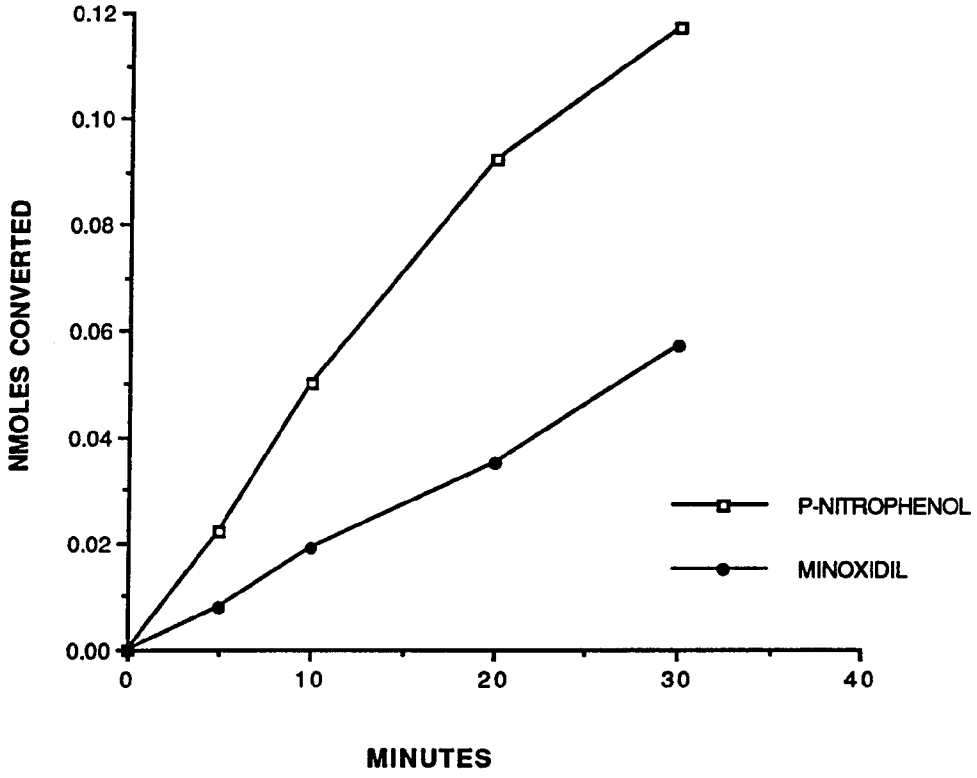

0.12
0.10
0.08
0.06
0.04
0.02
0.00
NMOLES CONVERTED
0
10
20
30
40
MINUTES
P-NITROPHENOL
MINOXIDIL

Figure 11. COS-7 Cytosol ST Assay: Product Identification.
Identification of the product formed by incubation of PNP with cytosol (25 µl) from COS-7 cells transfected with pSV-SPORT-P-PST-1 was determined by HPLC analysis. The radioactive product, as indicated by liquid scintillation spectrometry of radioactivity present in the column eluate, coeluted with the PNP sulfate standard, as monitored by absorbance at 260 nm.

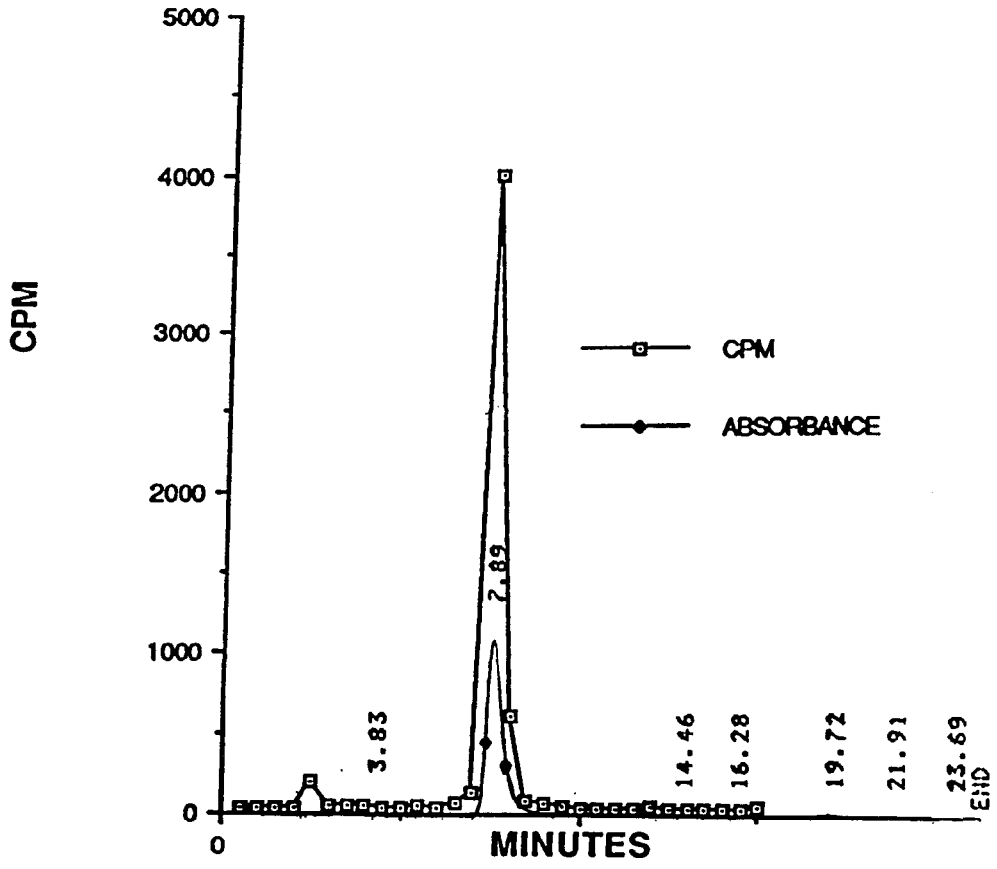
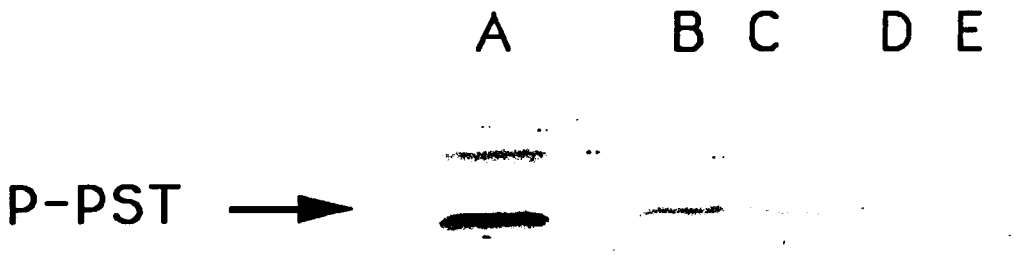

5000
4000
3000
2000
1000
0
CPM
0
MINUTES
CPM
ABSORBANCE
2.89
3.83
14.46
16.28
19.72
21.91
23.69
END

Figure 12. Expression of Immunoreactive P-PST in COS-7 Cells.

Protein (15 µg) from human liver cytosol (lane A), and cytosolic preparations of COS-7 cells transfected with pSV-SPORT-P-PST-1 (lanes B,C) and pSV-SPORT-1 (lanes D,E) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane was probed with rabbit anti-human PST antibodies as described in Methods.


A
B C
D E
P-PST

Northern Blot Analysis of Human Liver RNA

RNA was obtained from a normal human liver and Northern blot analysis of total and poly $(A)^+$ RNA was used to determine both the size and number of messages hybridizing to P-PST2-2. Figure 13 shows that P-PST2-2 detects one message, approximately 1300 bp in length, in both total and poly $(A)^+$ human liver RNA. No bands were detected in the ribosomal and transfer RNA fraction obtained during the isolation of poly $(A)^+$ RNA by oligo-dT chromatography.

Southern Blot Analysis

Southern blot analysis of genomic DNA was performed under conditions of moderate and high stringency to investigate the complexity and size of restriction fragments of genomic DNA which hybridize to PPST2-2. Figure 14 shows the pattern of fragments of human DNA digested with several restriction endonucleases which hybridized with P-PST2-2 after washing at a final stringency of 0.2x SSC/0.5% SDS at 65°. 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. The same bands were visualized after washing at a final stringency of 1x SSC/0.5% SDS at 65°.

Sequence Analysis and Comparison with Other Mammalian Sulfotransferases

The GCG program Motifs was used to analyze the P-PST cDNA sequence for the presence of sequence motifs by searching for patterns in the PROSITE Dictionary of Protein Sites and Patterns (Devereux, Haeberli, and Smithies 1984). Four common

Figure 13. Northern Blot Analysis of Human Liver RNA.

Total and poly(A)+ RNA were obtained from a normal human liver (W/F/66) as described in Methods. RNA samples were resolved in a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, hybridized with ^{32}P labeled P-PST2-2, washed and autoradiographed as described in Methods. The RNA fractions were as follows: total RNA (100 µg)(lane 1), transfer and ribosomal RNA (100 µg)(lane 2), poly (A)+ RNA (5 µg)(lane 3), and poly (A)+ RNA (20 µg)(lane 4). The arrows in the right margin show the migration of the 28S and 18S ribosomal subunits of human liver RNA.

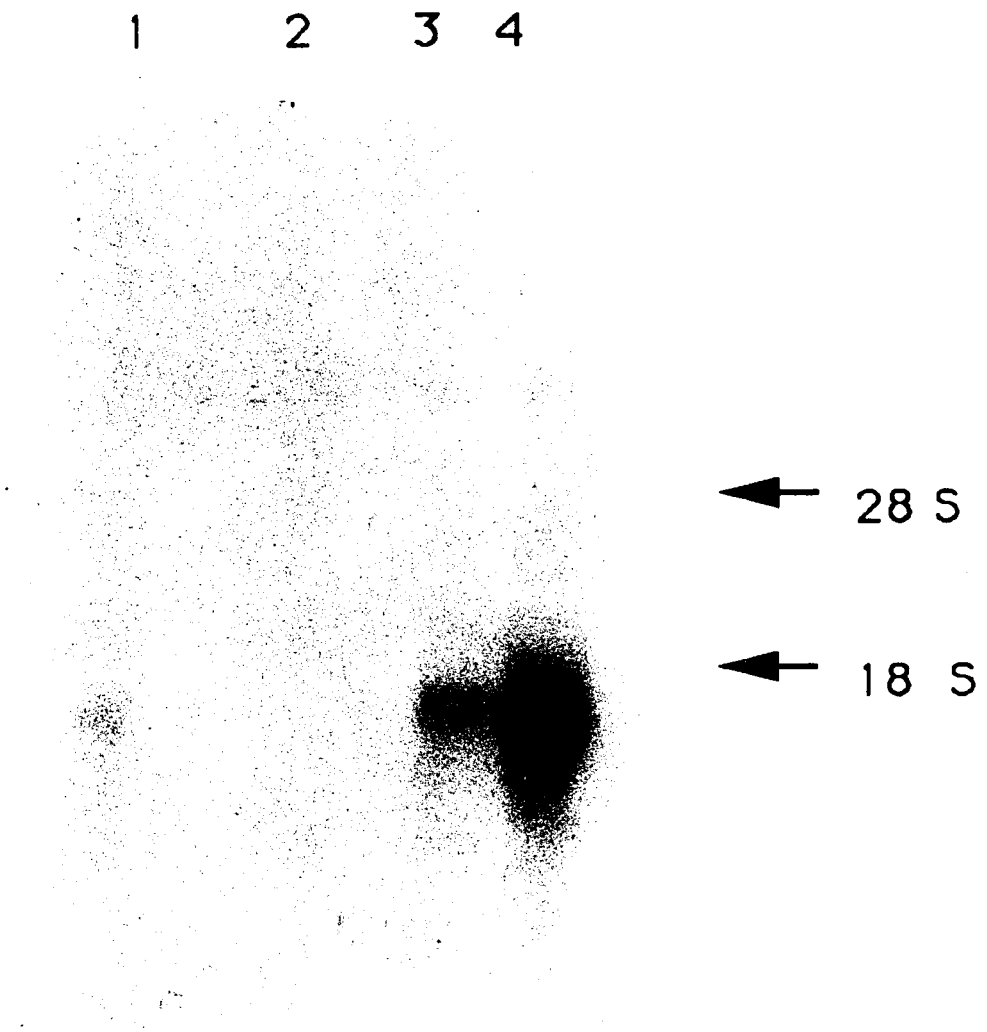
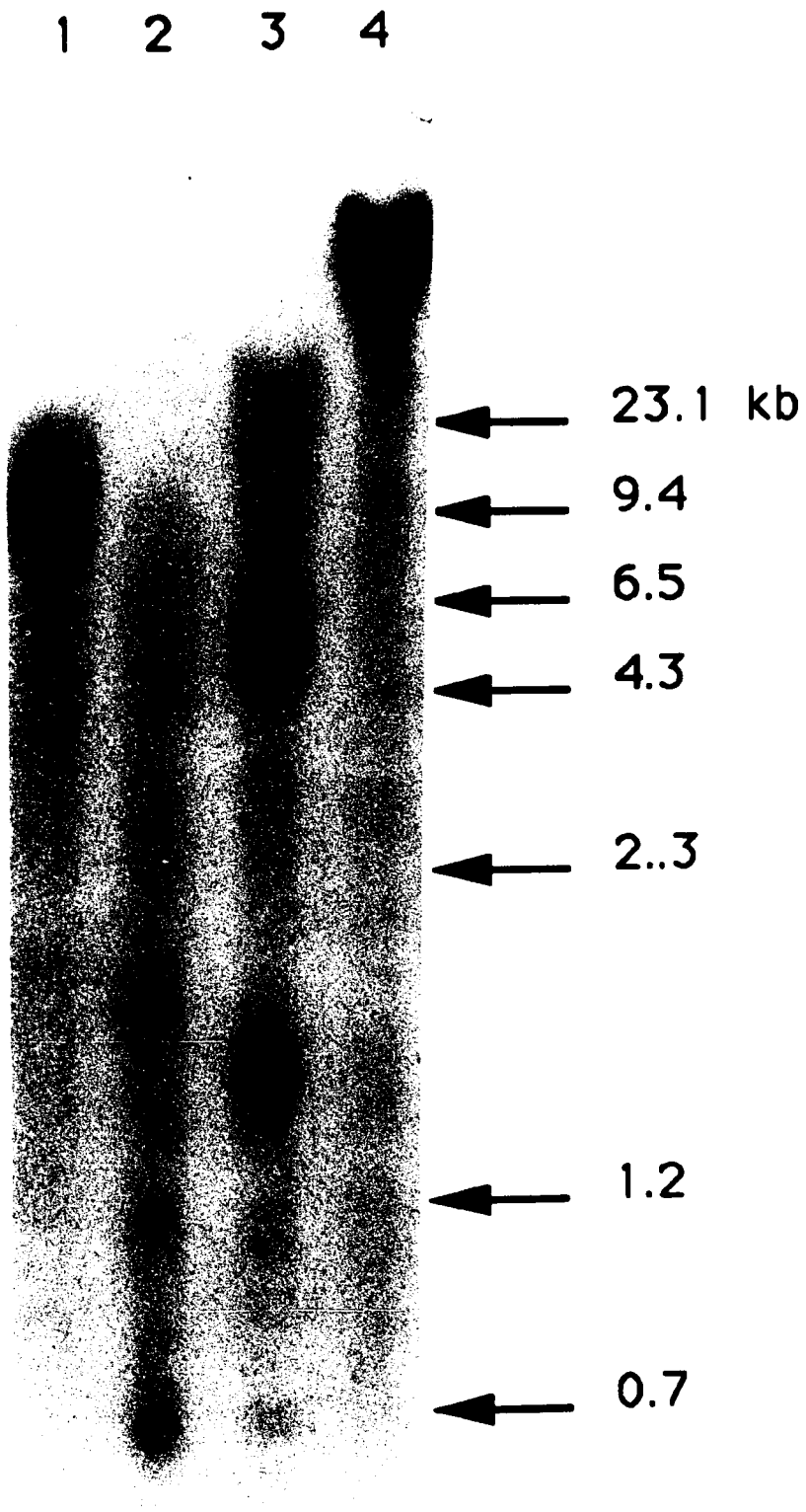

1
2
3
4
28 S
18 S

Figure 14. Southern Blot Analysis of Human Liver DNA.
High molecular weight genomic DNA obtained from a human liver (W/F/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. The DNA was transferred to a nylon membrane, hybridized with 1.5 x 10^6 dpm/ml of the [^{32}P]nick-translated P-PST 2-2, washed, and autoradiographed as described in Methods. Fragments of DNA digested with Hind III or BstE II were used as size standards.


1
2
3
4
23.1 kb
9.4
6.5
4.3
2..3
1.2
0.7

motifs were found which included an N-glycosylation site, casein kinase 2 (CK2) phosphorylation sites, N-myristoylation sites, and protein kinase C (PKC) phosphorylation sites (Table 2).

The recent isolation and characterization of a cDNA encoding the rat liver minoxidil ST in our laboratory (Hirshey et al. 1992) have allowed for the comparison of the encoded proteins at the primary amino acid level (Figure 15). The amino acid sequence of P-PST-1 (295 residues) is 80% identical and 89% similar to the rat liver minoxidil ST (291 residues). These enzymes are also 80% identical at the nucleotide sequence level.

The GCG program PileUp was used to generate a consensus sequence from the sequence of eight related cDNAs, seven of which are known to be STs. PileUp creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. The dendogram created by PileUp displays the clustering relationships used to create the alignment (Figure 16). The vertical branch lengths are proportional to the similarity between the sequences; however, this is not a phylogenetic tree. Figure 17 displays the consensus sequence. No other proteins with significant homology to the consensus sequence were identified in a search of the SwissProt data base.

Table 2

Sequence motifs present in P-PST-1

	AA#		
N-GLYCOSYLATION SITE		N-{P}-[ST]-{P}	
	239: KNPM	TNYTT	VPQEF
N-MYRISTOYLATION SITE		G-{EDRKHPFYW}-(2)-[STAGCN]{P}	
	88: EFKAP	GIPSGM	ETLKD
	153: VHPEP	GTWDSF	LEKFM
	286: AKKMA	GCSLTF	RSEL
CK2 PHOSPHORYLATION SITE		[ST]-x(2)-[DE]	
	91: APGIP	SGME	TLKDT
	95: PSGME	TLKD	TPAPR
	117: ALLPQ	TLLD	QKVKV
	157: PGTWD	SFLE	KFMVG
	214: EFVGR	SLPE	ETVDF
	228: MVQHT	SFKE	MKKNP
PKC PHOSPHORYLATION SITE		[ST]-x-[RK]	
	7: ELIQD	TSR	PPLEY
	95: PSGME	TLK	DTPAP
	228: MVQHT	SFK	EMKKN
	290: AGCSL	TFR	SEL

AA = AMINO ACID
{} = PROHIBITED AMINO ACID
[] = REQUIRED AMINO ACID
_ = MODIFIED AMINO ACID

Figure 15. Amino Acid Sequence Comparison of Human Liver P-PST with Rat Liver Minoxidil ST.

Deduced amino acid sequence comparison of P-PST-1 and minoxidil ST. The GAP program (Devereux, Haeberli and Smithies 1984) was used to optimize sequence alignment. Numbering is according to the longer sequence, P-PST-1. Horizontal lines indicate sequence identity.

```
P-PST MELIQDTSRPPLEYVKGVPLIKYFAEALGPLQSFQARPDDLLISTYPKSGTTWVSQILDM    60
Mx-ST     MEFSRPPLVHVKGIPLIKYFAETIGPLQNFTAWPDDLLISTYPKSGTTWMSEILDM

      IYQGGDLEKCHRAPIFMRVPFLEFKAPGIPSGMETLKDTPAPRLLKTHLPLALLPQTLLD   120
      IYQGGKLEKCGRAPIYARVPFLEFKCPGVPSGLETLEETPAPRLLKTHLPLSLLPQSLLD

      QKVKVVYVARNAKDVAVSYYHFYHMAKVHPEPGTWDSFLEKFMVGEVSYGSWYQHVQEWW   180
      QKVKVIYIARNAKDVVVSYYNFYNMAKLHPDPGTWDSFLENFMDGEVSYGSWYQHVKEWW

      ELSRTHPVLYLFYEDMKENPKREIQKILEFVGRSLPEETVDFMVQHTSFKEMKKNPMTNY   240
      ELRHTHPVLYLFYEDIKENPKREIKKILEFLGRSLPEETVDSIVHHTSFKKMKENCMTNY

      TTVPQEFMDHSISPFMRKGMAGDWKTTFTVAQNERFDADYAKKMAGCSLTFRSEL        295
      TTIPTEIMDHNVSPFMRKGTTGDWKNTFTVAQNERFDAHYAKTMTDCDFKFRCEL
```

Figure 16. Dendogram Generated During Sulfotransferase Sequence Alignment

The GCG program Pileup creates a multiple sequence alignment beginning with pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. The final alignment is made by a series of progressive, pairwise alignments that include increasingly dissimilar sequences until all sequences have been included in the final alignment. Although this is not a phylogenetic tree, the distance along the vertical axis is proportional to the difference between sequences.

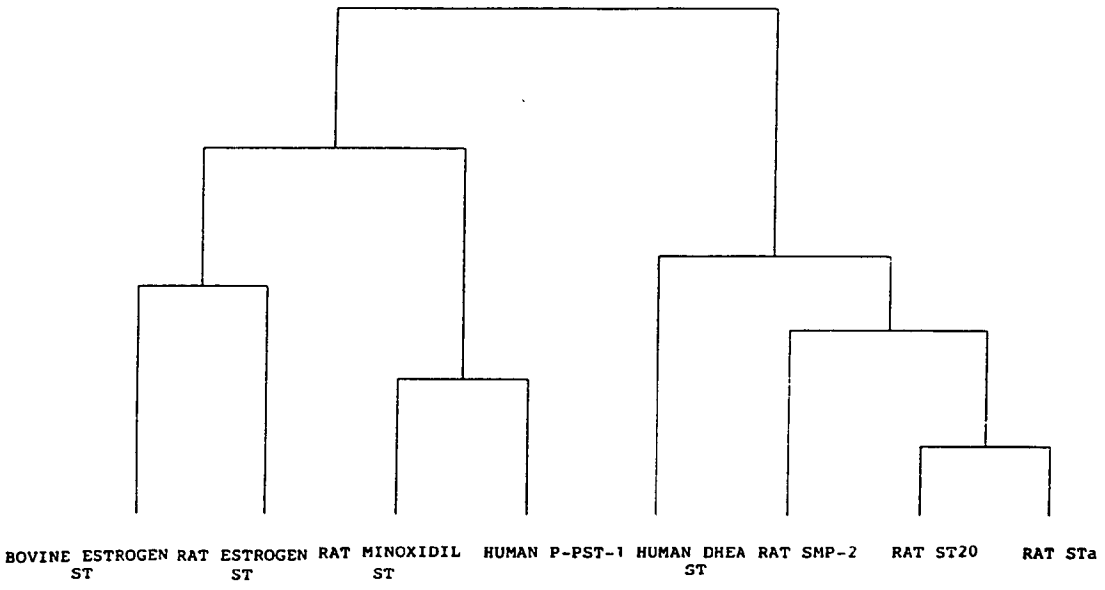

BOVINE ESTROGEN ST
RAT ESTROGEN ST
RAT MINOXIDIL ST
HUMAN P-PST-1
HUMAN DHEA ST
RAT SMP-2
RAT ST20
RAT STa

Figure 17. Consensus Sequence of Mammalian Sulfotransferases

The cDNAs encoding 8 mammalian STs were aligned and a consensus sequence calculated by the GCG programs PileUp and Pretty. The consensus is determined by finding the symbol in the column for which its comparison to all of the symbols in the column yields the greatest number of votes. A vote is cast for each symbol comparison that is over some set threshold value. If there is no coalition of votes that is larger than all of the other coalitions, or if the largest coalition is below the minimum plurality, then there is no consensus for the column, as indicated by -. Individual residues that did not vote with the winning consensus are printed. Blanks are printed at all other positions.

```
                    1                                                  50
Rat STa             .........     t          fgipk    q  cn   vk e   il
Rat ST20            .........     t        h fgisk    q  cn   vk d   il a
Rat SMP-2           .......mms    n          is qr i  ed rn   vk e   l
Human DHEA ST       .......msd    l      a   tmg rs    rk rde  i  d   vi
Human P-PST           iqdtsr  p e vk       ..i   a a  gpl.qs qa  p   l s
Rat minoxidil ST      ....sr  p vhvk       ..i   a  i gpl.qn ta  p   l s
Rat estrogen ST     t m eyyd v gd h fl     ..d r tky  ed .et la  p   l v
Bovine estrogen ST   ss k sfsd y gk g      ..y k i q  h  .ee ea  p   v v
          Consensus ME-S-P---P D--WFEGIPF  PA-KYF-ETL -NV--KFV-R --DL-ILTYP

                    51                                                 100
Rat STa                n  i       q       kwi   t     s  i t l. ....   m i
Rat ST20               n  i       q       kwi   t     s  i t  . ....   i i
Rat SMP-2              n  n       q       kwi  c f.. .gtvypd  . ....e ifrn
Human DHEA ST          n  a   l  mhs   a  kwi          s  v s  . ....  ta s
Human P-PST            t v q   ld   qg  l ekchra   m        fkap      m t k
Rat minoxidil ST       t       ld   qg kl ekcgra   a        fkcp      l t e
Rat estrogen ST      st i      d   ke  v ekckedal n    d   crne dlin ikq k
Bovine estrogen ST     t         nn  v ekckedv  n        cste h mk vkq n
          Consensus KSGT-WLSEI VC-IYTKGDP ---QSVPI-D RVPWLE--I- GVPSGY--L-

                    101                                                150
Rat STa             k kg   it         s     s         i  pr       g     ktt
Rat ST20            k kg   mt         s     s         v  pr       g     nst
Rat SMP-2           nhgg   it     i   s     s    a    m  pr       g     ntn
Human DHEA ST        ses   fs     iq        s         m  pr       g     knm
Human P-PST          tpa   l t     a    qt   dq v     va  ak  a    y h  hma
Rat minoxidil ST     tpa   l t     s    q    dq v     ia  ak  v    y n  nma
Rat estrogen ST       es  iv t    ak   a    eknc      c  ak  v    y    lim
Bovine estrogen ST   mas  iv      vk   v    eknc      s  ak  v    y   ilmvt
          Consensus EK--PRL-KS HLP-HL-PKS -F-SKAKVIY L-RN--DVLV S-Y-FWG--K

                    151                                                200
Rat STa              akk d  gt  v    k y  p        r a ls   l   f
Rat ST20             akk d  gt  v    k n  l        r a ls q w   f
Rat SMP-2             kn g  gt  f    q n  l        r g ls   w   f v
Human DHEA ST         kk k  ee  f   cq t  l        h g mp   ek  f   s
Human P-PST         vhpe gt ds  l k  v    s     q  q e w  srthp v
Rat minoxidil ST     hpd gt ds  l n       s     q  k e w   hthp v       i
Rat estrogen ST     sypn k  se  v k    q  p        k s w ksknsr v
Bovine estrogen ST  a pd d  qd  v k       p       tk s w ksknpq v
          Consensus LI--P-S--- --EWFMDGEV -YGSWFEHV- -W-EMRE-DN -LLLFYEDMK

                    201                                                250
Rat STa             k   gt          k       l   l ys    v    n  s  n  ek
Rat ST20            k   gt          k       l   l ys    v    d  s     mkks
Rat SMP-2           k   gt          kn g    l  ll ys    a    n  s    ik  p
Human DHEA ST       q  g t e    q   kt       ln  l ns    s    k  s     sv y
Human P-PST          npk e q   l  v rs pe  tv  mvq t   k   k    t  ttv q
Rat minoxidil ST     npk e     l    rs pe  tv s vh t   kk    c  t  tti t i
Rat estrogen ST       ir e v l i   erdpsa  lv r iq t       n  c t       t
Bovine estrogen ST    nirke m l l    r asd  lv k i  t       n  s t  tt    v
          Consensus EDTMR-IKKI CEFLG-KLEP EE-DLI-KH- SFQEMKENPM -NYSLLPEE-

                    251                                                300
Rat STa              . pgft    n  t              a    v       . ... p g
Rat ST20             . tgtg       v           s   a    v       . ... p g
Rat SMP-2            . tglk       t               a    v       . ... p g
Human DHEA ST        .vdkaq       vs              d    l      d. ... pr
Human P-PST        m hs           ma    tt      n r   ad ak      s t rs  ..
Rat minoxidil ST   m hn           t     t       n r   ah akt td  d   rc  ..
Rat estrogen ST    k          iv        pe lr r   eh  qq kd  pv  ra  *.
Bovine estrogen ST mnqk         dv            ln k   mh eqq k s t   rtki..
          Consensus IDL-VSPFMR KGT-GDWKNH FTVAQAE-FD K--QEKMAGC -LKF-PELFP
```

DISCUSSION

Cloning P-PST

The results presented in this dissertation concern the characterization of the cDNA encoding the P-PST present in human liver. This cDNA was isolated from a human liver cDNA library using a cDNA probe encoding a rat liver PST, minoxidil ST, which has been previously purified and cloned in our laboratory (Hirshey et al. 1992). Minoxidil sulfotransferase is one of at least 6 forms of PST which have been identified in rat liver cytosol (Duffel and Jakoby 1981; Mulder 1981; Borchardt and Schastten 1982; Hirshey and Falany 1990).

A number of findings supported the use of rat minoxidil ST cDNA as the probe for the human liver clone. Firstly, minoxidil ST is the major ST activity in rat liver responsible for sulfation of minoxidil, a drug used in the treatment of hypertension and alopecia (Hirshey and Falany 1990). Similarly, of the three forms of cytosolic STs identified in human liver, P-PST, M-PST, and DHEA-ST, only P-PST catalyzes the sulfation of minoxidil (Falany and Kerl 1990). Secondly, rat minoxidil ST and human P-PST display cross-immunoreactivity. Rabbit anti-rat minoxidil ST antibodies react with human P-PST (Hirshey and Falany 1990) and, conversely, rabbit anti-human PST antibodies react with rat

minoxidil ST (Heroux, Falany, and Roth 1989). Finally, cyanogen bromide generated peptides derived from both purified proteins show a high degree of similarity as previously described (Falany unpublished results).

Using rat minoxidil ST cDNA as the probe, five ST cDNA clones, ranging in length from 550 to 1121, were isolated from the human liver cDNA library and characterized. The longest clone, P-PST2-2, encoded a 290 residue polypeptide. The remaining clones were related in sequence to P-PST2-2; however, none of the isolated clones contained an initiation codon. Incomplete sequence information at the 5' end of clones in a library frequently occurs as a result of the procedure used to generate the cDNAs. Instead of rescreening the library, PCR was used to obtain the initiation codon and missing 5' sequence information. The full-length cDNA, P-PST-1, contains 1220 bp, and encodes a protein consisting of 295 amino acids.

Expression of enzyme activity characteristic of P-PST was evaluated in mammalian cells transfected with P-PST-1-pSV-SPORT. Cytosol from COS-7 cells transfected with the cDNA construct displayed 118- fold more ST activity toward minoxidil, when compared to background activity, and 13- fold more ST activity toward PNP than control cells. P-PST catalyzes the sulfate conjugation of micromolar concentrations of PNP; therefore, it is often chosen as the substrate in measurements of PST enzyme activity. M-PST is also capable of sulfating PNP as substrate concentrations approach the

millimolar range. Minoxidil, however, is a specific substrate for P-PST, but not M-PST or DHEA ST, the other two STs identified in human liver cytosol (Falany and Kerl 1990). The hydroxyl group in phenols, alcohols, or (N-substituted) hydroxylamines are the most common acceptor for sulfation, and purification procedures generally follow the sulfation of compounds from these categories. It is possible that conjugation at the uncommon N-oxide of minoxidil involves an unknown ST present in human liver. However, this does not appear to be the case. During the previous investigation of minoxidil sulfation by P-PST, the ratio of PNP sulfation to minoxidil sulfation remained constant during the purification procedure. Therefore, it is unlikely that an unknown ST present in human liver is involved in sulfating minoxidil at detectable levels (Falany and Kerl 1990).

Additional confirmation that the product resulting from incubation of transfected COS-7 cytosol with PNP was PNP-sulfate was provided by HPLC analysis. The radioactive product was eluted at the same time as the PNP-sulfate control, verifying that P-PST-1 encodes P-PST.

The protein encoded by P-PST-1 has a predicted molecular mass of 34,097 Da. Since the mass of P-PST as estimated by SDS-PAGE was somewhat smaller (32,000 Da), <u>in vitro</u> transcription and translation were used to verify that the protein encoded by P-PST-1 is equivalent in size to P-PST from human liver on SDS-PAGE analysis.

The immunoreactive protein expressed in COS-7 cells transfected with pSV-SPORT-P-PST-1 also displayed identical migration with P-PST from human liver cytosol. These data further support the identification of the protein encoded by P-PST-1 as P-PST. The minor discrepancy between the predicted size of P-PST from the cDNA sequence and the size of the protein determined by gel electrophoresis is most likely an artifact of the estimation of protein sizes by SDS-PAGE. Purified minoxidil ST, similarly, migrates in SDS-PAGE as a protein with a molecular mass of 35kD; however, translation of the cDNA predicts a subunit molecular mass of 33,909 Da (Hirshey et al. 1992).

Native human liver P-PST activity has an apparent molecular mass of 68 kDa as determined by size exclusion chromatography (Falany et al. 1990). The observation that the expressed protein with a subunit molecular mass of 34,097 Da is enzymatically active supports our previous hypothesis that P-PST exists as a homodimer in its active form (Falany et al. 1990). All of the mammalian STs that have been purified, with the exception of the human lung ST (Baranczykk-Kuzma 1986) and the rat liver ST purified by Borchardt and Schasteen (1982), appear to function in vivo as dimers, with subunit molecular masses from 32,000 to 38,000 (see Table I).

Human PST Gene Family

P-PST is the human enzyme that is responsible for the majority of ST activity in the liver (Heroux, Falany, and Roth 1989), and is therefore involved in the biotransformation of

many drugs and xenobiotics. It is important to investigate the complexity of the PST gene family, from a pharmacologic point of view, in order to evaluate the potential for the existence of related enzymes capable of performing analogous functions in other tissues. In view of the heterogeneity of rat liver PST activity, it was of interest to determine if homologous messages could be detected in human liver RNA that would suggest the presence of other forms of PST. However, under conditions of moderate stringency only a 1300 bp message was detected on Northern blot analysis using the P-PST2-2 probe. This finding is in close agreement with the 1220 bp P-PST-1 cDNA, and suggests that the transcript only contains a small amount of nontranslated sequence. The lack of evidence for a separate message corresponding to M-PST may reflect the low levels of expression of M-PST in human liver, or the fact that M-PST and P-PST are not homologous. However, it is possible that the messages encoding M-PST and P-PST are similar in size since the molecular masses of these STs do not differ greatly (Heroux and Roth 1988; Falany et al. 1990).

Southern blot analysis was performed under conditions of high stringency in order to investigate the organization of the P-PST genomic loci. The cDNA probe detected three DNA fragments in human liver genomic DNA digested with Sac I or Pst I and one fragment in DNA digested with Hind III. These genomic fragments are consistent with the known restriction sites of P-PST-1; two Sac I sites occurred at bases 59, and 285, three Pst I sites occurred at bases 161, 928, and 977,

and there was no Hind III site. It is possible that the entire P-PST gene is encoded within the Hind III 9800 base pair fragment. The same pattern was manifested when the Southern blot was performed under conditions of moderate stringency. These findings suggest that the number of genes closely related to P-PST-1 is small and are in agreement with the small number of PSTs identified in human tissues (Falany 1991).

Data supporting the existence of a low number of human STs related to P-PST suggest that distinct differences exist between human and rat PSTs. Rats are frequently used as in vivo models in the study of drug metabolism; therefore, it is important to understand the relationship between rat and human enzymes, and to identify the rat homologue of the human enzyme.

At least six different cytosolic forms of PST have been isolated from rat liver. These enzymes share many physical characteristics such as subunit size, native molecular weight, and substrate overlap, suggesting that they are members of a gene family. Gene families evolve from a single ancestral gene by a process of duplication and divergence in order to increase the organism's chance of survival. The heterogeneity seen among the rat PSTs suggests that each of these enzymes evolved to more efficiently metabolize a specific type of endogenous or exogenous substance. For example, rat AST IV is capable of sulfating biogenic amines such as epinephrine and tyramine, and may have a role in modulating neurotransmitter

activity. AST I does not have the ability to sulfate these substances, but it does have a much higher affinity and capacity to sulfate simple phenolic compounds than does AST IV.

The lack of heterogeneity among the human PSTs suggests these two enzymes have been capable of meeting the metabolic needs of the species. The broad substrate specificity and types of functional groups capable of being sulfated by P-PST, such as peptides, hydroxyls, N-oxides, and amines, further support this concept. The human PSTs generally have a higher affinity for PAPS, with K_ms for this substrate on the order of 10 to 20 fold lower than values determined in rats, again emphasizing the efficiency of the human PSTs. Different members of a gene family are often characteristic of different tissues of the body, where they perform analogous but distinctive functions. Several of the rat PSTs are known to display hepatic specific expression (Coughtrie and Sharp 1990; Hirshey and Falany 1990), whereas human P-PST has been detected in a large number of tissues including liver, adrenal, placenta, lung, brain, platelet, and intestine (Rein, Glover, and Sandler 1981a, 1982, 1984; Reiter et al. 1983; Sundaram and Weinshilboum 1985; Baranczykk-Kuzma 1986; Heroux, Falany, and Roth 1989). This suggests that other enzyme activities are performing analogous functions in the rat, whereas in the human P-PST alone is capable of performing multiple functions. In the liver, intestine, and lung, P-PST may have a predominantly protective function, in hastening the

elimination of potentially toxic xenobiotics, whereas in the brain it may be involved in modifying neuroactive peptides.

Expression of human PST activity does not appear to be regulated by sex hormones (Falany et al. 1991). By contrast, rat PST activity has been shown to be greater in males than in females (Wu and Straub 1976; Coughtrie and Sharp 1990; Hirshey and Falany 1990). In humans, similar levels of PST activity in males and females found among many tissues again suggest that these enzymes are not subject to strict regulation.

P-PST-1 has recently been shown to sulfate estradiol (Falany unpublished observation). Estradiol sulfate has low affinity for the estrogen receptor and is, therefore, hormonally inactive. Up-regulation of the ST that inactivates estrogen in rats has been shown to occur by two physiological antiestrogens, dihydrotestosterone and progesterone, in the liver and uterus, respectively, to produce regulatory environments conducive to androgen and progesterone action (Demyan et al. 1992). P-PST may be involved in modulating estradiol activity in a similar manner. However, not subject to hormonal regulation, it may provide a specific tissue with a mechanism to inactivate low levels of estradiol.

The small number of members in the PST family also differs from the common pattern of enzyme families involved in drug metabolism which generally consists of a large number of isoenzymes with overlapping substrate specificities. For example, the cytochrome P-450 superfamily consists of more than 24 gene families, 10 of which exist in all mammals

(Nebert 1991). Similarly, at least eight forms of UDP-glucuronosyltransferase have been identified in human liver (Tephly and Burchell 1990). A major driving force for the large number of gene duplications among the families of drug metabolizing enzymes is believed to be animal-plant 'warfare' (Nebert and Gonzalez 1987). If the present human P-PST has evolved to be a highly efficient enzyme capable of eliminating phenolic and related xenobiotics ingested from the environment, further evolution may not have been necessary for the survival of the species.

The widespread expression of P-PST and its broad substrate specificity indicate that it is potentially involved in the sulfation of a number of endogenous substances. Not only is P-PST of pharmacologic interest due to its role in the sulfation of many drugs, but, as the endogenous substrates are identified, it may provide new targets for pharmacologic intervention.

Polymorphic Forms of P-PST

The high degree of variation in PST activity among the human population is thought to reflect genetic polymorphism at a single genetic locus (Weinshilboum 1990). Previous studies have indicated that at least two allelic forms of P-PST activity, P_1-PST and P_2-PST, are present in human liver cytosol based on differences in thermal stability and elution during anion-exchange chromatography (Whittemore, Pearce, and Roth 1986; Campbell, VanLoon, and Weinshilboum 1987; Falany et al. 1990). 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 NMEM, a sulfhydryl modifying reagent, and phenylglyoxal, an arginine modifying reagent, indicating that they are highly related (Falany et al. 1990). Both forms were also inhibited to similar extents by incubation with the specific P-PST inhibitor, DCNP (Whittemore, Pearce, and Roth 1986).

Although the amino acid sequence differences between the translation of P-PST-1 and the peptides derived from purified P-PST, in which the two cysteines in P-PST were reported to be glutamates, are possibly allelic differences, it is more likely that these are due to sequencing artifacts. Previous investigations concluded that there was at least one cysteine involved in the PAPS binding site (Heroux and Roth 1988; Falany et al. 1990), suggesting that replacement of both cysteines would not be likely to occur. 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 to 74) which was isolated and sequenced twice from the same liver, showed a conservative exchange of a glutamine residue for an asparagine at amino acid residue 63.

Homologous cDNA Sequences

Comparison of P-PST-1 with minoxidil ST revealed 80% homology at the nucleotide level. When the amino acid sequences encoded by these cDNAs were compared, they were found to be 80% identical and 89% similar. This high degree of

homology further implicates minoxidil ST as the rat homologue of human P-PST. However, no other sequences of rat PSTs have been reported for comparison with the human P-PST sequence.

Currently, the sequence of eight mammalian cytosolic STs cDNAs are known. In addition to P-PST-1 and rat minoxidil ST which is equivalent to rat aryl ST (Ozawa et al. 1990), the other cDNAs encode rat hydroxysteroid ST (STa) (Ogura et al. 1990), rat hydroxysteroid ST (ST20) (Ogura et al. 1989), human DHEA ST (Otterness et al. 1992), bovine estrogen ST (Nash et al. 1988), and the rat estrogen ST (Demyan et al. 1992). The protein encoded by rat SMP-2 cDNA is unknown but it is speculated to encode an enzyme related to STa (Ogura et al. 1990). It has 74% homology with STa, with an average homology of 92% in 4 regions totaling 177 amino acid residues. Due to this homology and similarities in regulation, SMP-2 was included as a hydroxysteroid ST for sequence comparisons.

Although there are regions of considerable sequence homology among all of the STs included in the comparison, two distinct groups are readily identified. The first group consists of rat STa, ST20, SMP2, and human DHEA ST. STa and ST20 are hydroxysteroid STs. The substrate spectrum of the hydroxysteroid STs includes a number of primary and secondary alcohols, but they are referred to as hydroxysteroid STs due to the physiological importance of this class of sulfates (Mulder and Jakoby 1990). DHEA ST has activity with a number of aliphatic hydroxyls present in steroids and bile acids, and low levels of activity with the phenolic hydroxyl of estrone

and estradiol (Falany, Vazquez, and Kalb 1989; Radominska et al. 1990). P-PST-1 has 39% amino acid sequence homology and 59% similarity with DHEA ST. However, P-PST-1 has significantly greater homology with the other members of the second group of enzymes identified by computer alignment. These enzymes, with high levels of activity at phenolic hydroxyls, include human P-PST-1, bovine estrogen ST, rat estrogen ST, and rat minoxidil ST.

P-PST Sequence and Functional Sites

Substrates

Due to the differences in substrate specificity between the STs, it is likely that the binding site for the sulfate acceptor, such as DHEA, dopamine, or minoxidil, would not be conserved. In contrast, PAPS is a cosubstrate in all of the known mammalian STs. Although there are a wide range of affinities of the STs for PAPS, it is likely that each possess similar sequences at the PAPS binding site.

Knowledge of a PAPS binding motif would be valuable in determining the mechanism involved in sulfation. The sequence of this motif is not known, but several of the nucleotide binding motifs, including the ATP binding motif, have been defined (Aitken 1990). The minimal energy conformation of PAPS and ATP, as determined by the molecular modeling program Alchemy III, suggests a number of structural similarities, including the orientation of the adenine ring and the phosphoribose ring (Figure 18). It is, therefore, likely that there are similarities in these two motifs. The Rossmann ATP

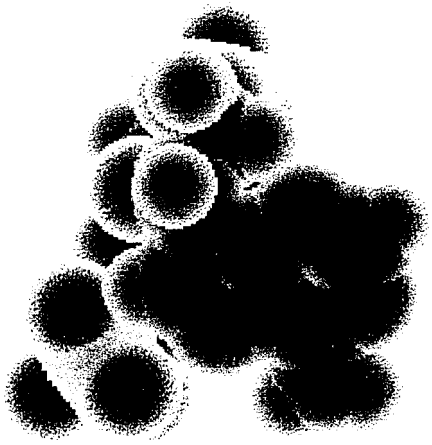
binding motif, GXGXXG, is involved in the formation of a loop or fold that enables binding of the nucleotide phosphate or pyrophosphate (Aitken 1990). Glycines are present in all known nucleotide binding domains and are thought to be important in forming this folded structure. The first glycine is in contact with the ribose ring and it is believed that a side chain at this amino acid residue would prevent proper ribose binding. The second glycine lies adjacent to the pyrophosphate (Aitken 1990). Although the pyrophosphate moiety is not present in PAPS, the phosphosulfate moiety is similar in charge and shape.

There is only one region of P-PST-1 that contains three glycines in close proximity. However, this sequence is not present in any of the other STs; therefore, it is unlikely that it is part of the PAPS binding motif. Only two regions containing two glycines in close proximity are conserved among the STs. The first region begins at residue #168 of the consensus sequence and the second region begins at residue #262. The ATP binding site is known to be located in a region of the protein containing a beta-sheet: alpha-helix: beta-sheet fold (Aitken 1990). Secondary structure predictions of P-PST-1 were made according to the methods of Chou-Fasman (1978) and Garnier-Osguthorpe-Robson (1978) employed by the GCG program Peptide Structure (Devereux, Haeberli, and Smithies 1984). None of the potential PAPS binding regions mentioned above were predicted by both models to be present in this type of fold. However, secondary structure predictions

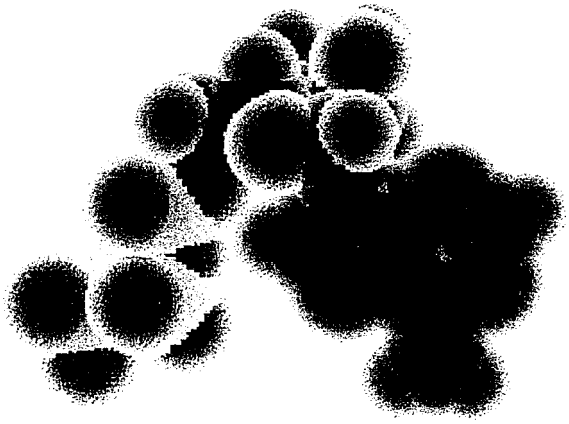
Figure 18. Molecular models comparing ATP and PAPS minimal energy conformations.

All of the mammalian STs require PAPS as the sulfate donor. A PAPS consensus sequence is not known; however, the consensus sequence of the related nucleotide, ATP, has been determined. The molecular modeling program, Alchemy III, was used to compare the minimal energy conformation of these two nucleotides in order to predict whether they would bind at similar regions. The orientation of the adenine ring is approximately constant for each nucleotide. Note that the ribose ring points away from the observer in PAPS (A), but is nearer the observer in the case of ATP (B).

A



B



are often inaccurate and these data do not preclude the possibility that these regions are involved in the PAPS binding site.

Previous experiments have suggested that the presence of at least one cysteine residue is associated with the PAPS binding site in P-PST. N-ethylmaleimide (NMEM) is a sulfhydryl modifying reagent that is a potent PST inhibitor (Heroux and Roth 1988). When P-PST or M-PST was preincubated with saturating levels of PAPS prior to addition of NMEM, enzyme inactivation was prevented, indicating the presence of a sulfhydryl moiety in the active site (Heroux and Roth 1988; Falany et al. 1990).

There are only two cysteines in P-PST-1. The cysteine at amino acid #287 is only conserved in two of the sequences encoding STs active at phenolic hydroxyls. In addition, the region surrounding this residue is not well conserved among these sequences. The cysteine at residue #70 in P-PST-1 is conserved in all four of the sequences encoding STs active at phenolic hydroxyls. In addition, there is greater homology in the region surrounding this cysteine, suggesting the functional importance of this site. This cysteine is not conserved in the hydroxysteroid STs. Interestingly, NMEM does not inhibit DHEA ST activity (Falany unpublished observation). However, the effect of NMEM on the activity of other hydroxysteroid STs has not been tested.

Investigations into the inhibition of M-PST and P-PST by PAPS analogs provide additional information regarding the

binding of this cosubstrate at the enzyme active site (Rens-Domiano and Roth 1987). For both PSTs, the naturally occurring product 3',5'-PAP was the most effective inhibitor. Other inhibitors included 2'5'-PAPS, 2'5'-PAP, 5'-ATP and 5'ADP, but these inhibitors were approximately 100 times less effective than PAP. Three AMP derivatives and 5'APS were approximately 1000 fold less effective than 3'5'-PAP. This reveals that there are structural requirements for binding the ribose portion of adenosine to both M-PST and P-PST that involve the groups on both the 3' and 5' positions. Positively charged amino acids may be involved in ionic interactions with the negatively charged moieties at these positions. As seen in the consensus sequence, there is conservation of a number of positively charged amino acids among the STs, several of which are close to the cysteine at residue #70 of P-PST-1. As more ST sequences are reported, there will be increased information necessary to determine the PAPS binding motif and key amino acids involved in transfer of the sulfate moiety.

Regulatory and Other Sequence Motifs

Knowledge of the human P-PST protein sequence allows for the identification of motifs that may be important for its regulation or function. The GCG program Motifs (Devereux, Haeberli, and Smithies 1984) was used to analyze the cDNA sequence. Motifs searches through the predicted amino acid sequence for the patterns that are defined in the PROSITE Dictionary of Protein Sites and Patterns. Four motifs were

detected in the amino acid sequence encoded by P-PST-1. These sites include the consensus pattern for an N-glycosylation site and N-myristoylation sites; however, it is unlikely that these sites are functional since these covalent modifications occur in the Golgi apparatus, a compartment to which the cytosolic STs are unlikely to have access.

Six casein kinase II (CK-2) phosphorylation sites were also identified in the human P-PST sequence. CK-2 is a protein serine/threonine kinase that has activity independent of calcium and cyclic nucleotides. A large number of proteins are phosphorylated by CK-2, which include proteins involved in gene expression and DNA synthesis, signaling pathways, and key enzymes of metabolic pathways (Pinna 1990). Serine is favored over threonine as the phosphorylation site, and an acidic residue must be present in a position three residues to the C-terminal side of the phosphate acceptor site. Most physiological substrates of CK-2 have at least one additional acidic residue in positions +1, +2, +4, or +5. In the P-PST sequence, only one of the six CK-2 sites has an additional acidic residue. This site also contains a basic residue on the N-terminal side of the acceptor serine, a factor known to decrease the phosphorylation rate at CK-2 sites. It is, therefore, uncertain if phosphorylation at any of these sites in vivo is involved in regulation of P-PST activity.

Protein kinase C (PKC) phosphorylation motifs were identified at four sites in P-PST. PKC exhibits a preference for the phosphorylation of serine or threonine residues close

to a C-terminal basic residue (Woodget, Gould, and Hunter 1986). There are a number of subspecies of PKCs that vary in their response to phospholipid, diacylglycerol, and calcium. Following cell disruption, most of these enzymes phosphorylate a wide variety of proteins, but due to their differential distribution in various cell types, the actual physiological substrates have been difficult to determine (Kikkawa, Kishimoto, and Nishizuka 1989). Most of the known physiological substrates are integral membrane proteins or proteins that have a close association with the membrane; therefore, it is unclear if any of the cytosolic STs are cellular substrates of PKC activity.

The endogenous substrates of P-PST are not yet known. Its ability to sulfate peptides on tyrosine residues indicates a possible involvement in cell signaling, and this may require a rapid mechanism for regulation of P-PST activity. P-PSTs may compete with a tyrosine kinase for conjugation of specific tyrosine hydroxyl residues. There is no evidence for the reversibility of tyrosine sulfation (Huttner 1987); therefore, sulfation would prohibit the substrate from undergoing future phosphorylation.

Although there is no direct evidence that phosphorylation is involved in regulation of ST activity, the presence of several phosphorylation motifs in human P-PST suggests this may be an important area to investigate in future studies of the regulation of P-PST activity. The potential CK-2 and PKC phosphorylation sites present in human P-PST were not well

conserved at similar regions in the other STs. The only potential CK-2 phosphorylation site that was present in the three other STs with activity at phenolic hydroxyls was that beginning at residue #122 of the consensus sequence. The only other sequence with a comparable PKC site was found in minoxidil ST at residue #231.

Future studies

The results of this dissertation support the concept that the predominant PST activity in human liver is provided by P-PST. The cDNA encoding P-PST will aid in investigating the catalytic mechanism of sulfate transfer, the different mechanisms by which the rat and human enzymes are regulated, the function of hepatic sulfation in drug and carcinogen metabolism, and the consequences of polymorphic forms of P-PST existing in the population.

Analysis of the P-PST sequence, previously discussed, has defined several regions that may be present at the active site of this enzyme. P-PST could be mutated by deleting regions of the sequence with restriction endonucleases and then assessing the effect on enzyme activity of the protein expressed in COS cells. Likewise, PCR could be used to introduce specific substitutions or deletions in the cDNA sequence and effects on enzyme activity similarly monitored. For example, substitutions of the glycines in potential PAPS binding site previously discussed may interfere with the fold believed to accomodate the adenine ring of PAPS, thus preventing enzyme activity.

Expression of large quantities of P-PST in bacterial or yeast expression systems may allow production of sufficient enzyme for protein crystallization. The crystallization would allow determination of the tertiary structure of P-PST by X-ray diffraction techniques. Kinetic studies of human P-PST have concluded that catalysis proceeds by a sequentially ordered mechanism in which PAPS is the leading substrate (Whittemore, Pearce, and Roth 1986); therefore, it may be possible to co-crystallize P-PST with PAPS in order to determine the manner in which they bind. This would define the PAPS binding site on P-PST and provide key information in determining the sulfation reaction mechanism.

A number of differences exist between the rat P-PSTs and the human P-PST. Expression of the rat enzymes are influenced by the sex hormones, whereas in humans sex hormones do not affect activity. The rat enzymes also display more tissue specific expression than the human enzyme. The cDNAs could be used to screen rat and human genomic libraries to isolate the genomic clones. The genomic clones would allow investigation of the regulatory sequences present that result in differences in enzyme expression.

The ability of P-PST to be expressed in cell culture in the absence of many other hepatic enzymes, allows the pharmacologic substrates of P-PST to be more clearly defined. Studies with the expressed protein will also assist in clarifying differences in xenobiotic sulfation that exist between humans and commonly used laboratory animals such as

rats. PST activity has long been known to be involved in the bioactivation of certain compounds to carcinogens in several species including rats (Miller 1978; Miller et al. 1985). The sulfate esters of these compounds rearrange to form reactive electrophiles and thereby form adducts with DNA. N-hydroxy N-acetyl aminofluorene (N-OH-AAF) is one of the protypical carcinogens that is activated upon sulfation in rats, apparently by AST IV; however, in human liver cytosol, N-OH-AAF is apparently not sulfated (Falany 1990; Chou, Lang, and Kadlubar 1991). It is not known if other carcinogens that are activated upon sulfation, such as 1'-hydroxysafrole, are substrates for human liver P-PST. COS-7 cells transfected with an expression vector containing P-PST-1 could be incubated with labeled 1'-hydroxysafrole or other carcinogens, the DNA could be isolated, and the presence of DNA adducts assessed.

The P-PST cDNA will also assist in the characterization of the polymorphic forms of the enzyme and the determination of associated pharmacological consequences. Previous studies have suggested the presence of two allelic forms of P-PST in humans (Whittemore, Pearce, and Roth 1986; Campbell, VanLoon, and Weinshilboum 1987; Falany et al. 1990). An investigation of the heritability of P-PST suggested that genetic polymorphism is responsible for the genetic control of enzyme activity (Weinshilboum 1990). It further indicated the existence of a single P-PST genetic locus with alleles for high and low levels of activity that had gene frequencies of 0.2 and 0.8, respectively. The existence of rapid and slow

sulfators does not appear to have serious implications from a drug toxicity standpoint in that, although frequently present, the percentage of a drug being excreted as a sulfate conjugate is generally low. However, when drug activity depends on sulfate activation, as in the case of minoxidil, the effectiveness of the drug may depend on the rate at which the active agent is formed before it is metabolized by competing enzymes or eliminated. The rapidity of sulfation of procarcinogens to reactive nucleophiles may also affect susceptiblity to certain types of cancer.

To determine the nature of the allelic P-PST variants, cDNAs encoding P_1-PST and P_2-PST could be obtained from the liver samples previously shown to contain these two forms of enzyme activity by isolating RNA from these livers and constructing cDNA libraries. Sequence analysis of the two cDNAs would define the nature of the allelic variations. In order to distinguish the two genotypes, a PCR procedure could be designed using primers surrounding the polymorphic locus and genomic DNA which has been isolated from the same two livers, as the template. The product of the PCR reactions could be separated by denaturing gradient gel electrophoresis (DGGE), which is capable of detecting even single base changes in DNA fragments. Altered base pairing of the two sequences will affect the melting behavior of the fragments, resulting in detectable differences in DNA migration.

Genomic DNA from patients beginning treatment for alopecia with minoxidil can be categorized by PCR according to

sulfator phenotype, and responsiveness to minoxidil treatment can be monitored over a period of time. This may determine if there is a relationship between the existence of an allelic form of P-PST and minoxidil responsiveness.

Epidemiologic studies may reveal an increased risk of developing certain diseases, or susceptibility to a specific carcinogen associated with one of the allelic forms of P-PST. The ability of P-PST to sulfate endogenous peptides suggests that important physiological as well as pharmacological roles exist for this enzyme. Knowledge of phenotypic groups of sulfators may provide information as to the endogenous substrates for P-PST.

Characterization of P-PST at the molecular level provides important insight into the structure and heterogeneity of this enzyme. Future application of this information will increase our understanding of the role of sulfation in normal cellular function, as well as in drug and xenobiotic metabolism.

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