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Bacterial Expression and Characterization of a cDNA for Human Liver Estrogen Sulfotransferase

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A distinct human estrogen sulfotransferase (hEST-1) cDNA has been isolated from a human liver λZap cDNA library using a PCR procedure. The enzymatically active protein has been expressed in two bacterial expression systems and the kinetic and immunologic properties of the enzyme have been characterized. The full-length cDNA for hEST-1 is 994 base pairs in length and encodes a 294 amino acid protein with a calculated molecular mass of 35,123 Da. Purified hEST-1 migrated with an apparent molecular mass of 35,000 Da during SDS–polyacrylamide gel electrophoresis. Immunoblot analysis of hEST-1 expressed in *E. coli* with a rabbit anti-hEST-1 antibody yields a band of approximately 35,000 Da. The anti-hEST-1 antibody also detects a single band in human liver and jejunum cytosol which migrates with the same molecular mass as expressed hEST-1. There was also no cross-reactivity of hEST-1 with rabbit anti-hP-PST or rabbit anti-hDHEA-ST antibodies upon immunoblot analysis. hEST-1 was expressed in bacteria and purified to homogeneity. Expressed hEST-1 activity has a significantly greater affinity for estrogen sulfation than that found for the other human STs which conjugate estrogens. hEST-1 maximally sulfates β-estradiol and estrone at concentrations of 20 nM. hEST-1 also sulfates dehydroepiandrosterone, pregnenolone, ethinylestradiol, and 1-naphthol, at significantly higher concentrations; however, cortisol, testosterone and dopamine are not sulfated. The results presented in this paper describe the expression and characterization of a human EST distinct from other human STs which sulfate estrogens. The high affinity of hEST-1 for estrogens indicates that this ST may be important in both the metabolism of estrogens and in the regulation of their activities.

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INTRODUCTION

The conjugation of steroids with sulfate is a significant process in the biotransformation of steroids and bile acids and in the regulation of their activities in human tissues. Sulfation is a major pathway involved in both the synthesis and inactivation of steroids. Large quantities of steroid sulfates, such as dehydroepiandrosterone (DHEA) sulfate and pregnenolone sulfate, are synthesized and secreted by the fetal and adult adrenal cortex [1]. These steroid sulfates may serve as precursors for the synthesis of androgens and estrogens in peripheral tissues [1, 2]. High levels of estrogenic steroid sulfates have also been reported to occur in human breast cancer tissue where the estrogen sulfates serve as precursors for the biologically active estrogens. The sulfation of steroids is also involved in their inactivation and in the protection of tissues from the effects of steroids as steroid sulfates do not readily bind and activate steroid receptors. The presence of steroid sulfation activity in a tissue, such as the liver, may therefore have an important function in regulating the levels of active steroids via inactivation of the steroid by sulfate conjugation.

Sulfation is catalyzed by a family of enzymes termed sulfotransferases (STs). The STs represent a family of enzymes involved in the conjugation of a variety of endogenous and xenobiotic compounds with a sulfonate moiety. All members of the ST family utilize 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the donor compound for the sulfonate group [3, 4]. At least

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two different human liver cytosolic STs have previously been reported to be involved in the conjugation of steroids and bile acids [5–8]. These STs are dehydroepiandrosterone-ST (hDHEA-ST) and the phenol-sulfating form of phenol sulfotransferase (hP-PST). hDHEA-ST has been purified from human liver cytosol and shown to be very similar to the adrenal form of the enzyme [7, 8]. The human liver DHEA-ST cDNA has also been cloned and the active enzyme has been expressed in both mammalian cells and in bacteria [9–11]. hDHEA-ST is capable of conjugating a number of different steroids and is responsible for the majority of bile acid sulfation in human liver [5, 7, 11]. Both purified liver hDHEA-ST and hDHEA-ST expressed in bacteria are capable of sulfating estrogens, testosterone and hydroxysteroids such as DHEA and pregnenolone; however, hDHEA-ST does not exhibit detectable cortisol sulfation activity [8, 11]. Purified human liver P-PST has been reported to sulfate estrogens such estrone (E_1) and β-estradiol (β-E_2) [6, 11]. The cDNA for hP-PST has also been expressed in bacteria. The expressed enzyme conjugates estrogens and catechol estrogens but not hydroxysteroids such as testosterone or DHEA [11]. Neither hDHEA-ST nor hP-PST sulfate steroids selectively; both of these STs have a broad substrate reactivity, which includes xenobiotics as well as endogenous compounds such as steroids.

STs which are capable of conjugating estrogens have been cloned from bovine [12], guinea pig [13] and rat tissues [14, 15]. Sequence analysis of the cDNAs for these enzymes suggests that they are distinct from the cDNAs for the hydroxysteroid STs and phenol STs (PSTs), but are members of the larger PST gene family. The presence of separate ESTs in other species suggested that a distinct EST might be present in human tissues. Previous reports of the characterization of estrogen sulfation activity in human tissues have also suggested that a human EST, separate from hP-PST and hDHEA-ST, is present. Grosso and Way [16] characterized an EST activity in human RL95-2 endometrial adenocarcinoma cells which demonstrated a high affinity for estrogens. Aksoy *et al.* [17] have also recently isolated a cDNA for a human EST using a polymerase chain reaction (PCR) procedure and expressed estrogen sulfation activity in COS-1 cells; however, the expressed protein was not purified or characterized nor was the protein identified in human tissues.

This report describes the isolation, expression, kinetic and immunologic characterization of an EST cDNA (hEST-1) from a human liver cDNA library. The active enzyme has been expressed in two bacterial systems which allows for the characterization of the kinetic properties of the enzyme without interference from other mammalian ST activities. The purified hEST activity displays a significantly greater affinity for the conjugation of estrogens than that observed for hP-PST or hDHEA-ST. Specific antibodies were raised against the pure hEST-1 protein and used to characterize expression in human tissues. Characterization of the kinetic, physical and regulatory properties of this human EST is important in understanding the metabolism of estrogenic steroids and related compounds in human tissues as well as the role of sulfation in the modulation of steroid activity.

MATERIALS AND METHODS

Materials

Oligonucleotide primers were synthesized in the Molecular Biology Core Facility of the Comprehensive Cancer Center at UAB. *p*-Nitrophenol, β-E_2, E_1, DHEA, dopamine, testosterone, pregnenolone, cortisol and DEAE-Sepharose CL-6B were obtained from Sigma Chemical Co. (St Louis, MO). Minoxidil was a gift from the Upjohn Co. (Kalamazoo, MI). [1,2,6,7-^{3}H]DHEA (79 Ci/mmol), [6,7-^{3}H]β-E_2 (45 Ci/mmol), [2,4,6,7-^{3}H]E_1 (96 Ci/mmol), [7-^{3}H]pregnenolone (25 Ci/mmol), [7-^{3}H]testosterone (27.7 Ci/mmol), [1,2,3-^{3}H(N)]cortisol (54.4 Ci/mmol) and [^{35}S]PAPS (2 Ci/mmol) were purchased from New England Nuclear (Boston, MA). PAPS was purchased from Dr Sanford Singer (University of Dayton, Dayton, OH). The pMAL fusion protein expression system was from New England Biolabs (Beverly, MA). Protogel and Sequagel were from National Diagnostics (Atlanta, GA). Affinity-purified goat anti-rabbit horseradish peroxidase conjugate was purchased from TAGO (Burlingame, CA). The Lumiglo Chemiluminescence Substrate kit was purchased from Kirkegaard and Perry (Gaithersburg, MD). All other chemicals were of reagent grade quality.

Isolation of human liver EST-1 cDNA

The human liver EST-1 cDNA was isolated from a human liver λZap cDNA library (Stratagene) using a PCR procedure. An oligonucleotide (5′-ATTTCTTTCACAGGATCA-3′), synthesized to a consensus sequence in the 5′-nontranslated regions of the bovine [12], guinea pig [13] and rat EST cDNAs [14], was used as the sense primer. An aliquot (1×10^7 pfu) of the human liver λZap cDNA library was used as the source of the DNA template and an oligonucleotide (5′-GTTTTCCCAGTCACGACG-3′), synthesized to a portion of the M13 universal primer, was used as the reverse primer. After 30 cycles of PCR (40°C annealing), no detectable PCR products were observed by ethidium bromide staining following agarose gel electrophoresis. An aliquot of the reaction mix was then used as the template for another 30 cycles of PCR with the same primers and a DNA fragment of approx. 1000 bp was subsequently detected. The PCR product was isolated from a low melting temperature agarose gel and subcloned into the pCRII vector (Invitrogen) for characterization and sequence analysis.

The hEST-1 cDNA was subjected to double-stranded sequencing by the dideoxynucleotide chain termination method using Sequenase 2.0 and [α-^{35}S]dATP to label the newly synthesized strands. The ^{35}S-labeled products were resolved in 6% polyacrylamide-urea gels using a buffer gradient of 0.5–2.5 × TBE (89 mM Tris–borate, 2 mM EDTA, pH 8.0). The complete cDNA sequence of hEST-1 was obtained using primer sites in the pCRII vector and oligonucleotide primers synthesized to internal sequences of hEST-1. Sequence gels were read manually and analyzed using the MacVector 4.1 sequence analysis software (Kodak/IBI).

Expression of hEST-1

The hEST-1 cDNA was expressed in the pMAL-c2 expression system (New England Biolabs) to generate pure protein for raising specific antibodies and for enzymatic characterization. This system generates a maltose binding protein (MBP) fusion protein which can be purified by affinity chromatography on an amylose resin. For insertion into pMAL-c2, a PCR procedure was used to generate a hEST-1 cDNA with a blunt 5′-end and a Hind III site at the 3′-end. An oligonucleotide primer (5′-ATGAATTCTGAACTTGACTATTATG-3′) was synthesized to the 5′-end of the coding region of the hEST-1 cDNA. Using the hEST-1 cDNA in pCRII as a template, the hEST-1 cDNA was amplified using the 5′-hEST-1 primer as a sense primer and the M13 universal primer as an antisense primer. The generated hEST-1 cDNA fragment was then treated with the Klenow fragment of DNA polymerase I and digested with Hind III which cuts in the polylinker of pCRII. The hEST-1 fragmant was purified from low melting temperature agarose and ligated into the Xmn I and Hind III sites of pMAL-c2. *E. coli* XL1-Blue cells were then transformed with the pMAL-hEST-1 vector using a calcium chloride procedure [18]. To express the MBP-hEST-1 fusion protein, XL1-Blue cells containing the pMAL-hEST-1 vector were grown in Luria broth containing 50 μg/ml ampicillin to an O.D.$_{550}$ of 0.5, then isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.3 mM and the incubation continued for 2 h. Cells were pelleted and resuspended in bacterial lysis buffer (75 mM Tris, pH 8.0, 0.25 M sucrose, 0.25 mM EDTA and 0.02 mg/ml lysozyme) and incubated on ice for 20 min. The cells were pelleted, resuspended in 5 mM phosphate, pH 7.4, containing 1.5 mM dithiothreitol (DTT) and 10 μg/ml phenylmethylsulfonylfluoride, and sonicated 3 × in 10 s bursts with 30 s cooling between each burst. The cytosolic fraction containing EST activity was recovered following centrifugation at 100,000 *g* for 1 h.

The MBP-hEST-1 fusion protein was purified by amylose affinity chromatography. The supernatant fraction was diluted to 3 mg protein/ml with 5 mM phosphate, pH 7.4, and applied to an amylose affinity column (New England Biolabs) equilibrated in the same buffer. The amylose affinity column (1.75 × 5 cm) was washed with 75 ml of 5 mM phosphate, pH 7.4, and the fusion protein was then eluted with 10 ml of 10 mM maltose in the same buffer. The yield was approx. 20 mg fusion protein/liter bacterial culture. The MBP-hEST-1 fusion protein was enzymatically active and capable of conjugating β-E_2 which facilitated purification of the enzyme. To isolate hEST-1, 2.3 mg of fusion protein was digested with 40 μg of Factor Xa (New England Biolabs) for 5 h and applied to a DEAE-Sepharose CL-6B column (1 × 5 cm) equilibrated in 5 mM phosphate, pH 7.4, with 1 mM DTT. The column was washed with 20 ml of buffer followed by 30 ml of buffer containing 100 mM NaCl. hEST-1 was then eluted with a 100 ml NaCl gradient (100–225 mM) in the phosphate buffer. hEST-1 activity eluted at approx. 150 mM NaCl. To remove residual amounts of the uncut fusion protein and the MBP, the hEST-1 fraction was passed through a second amylose column. The purified hEST-1 was then analyzed by SDS–PAGE and used for raising antibodies in rabbits and for enzymatic characterization.

To confirm the results generated using the hEST-1 purified after cleavage of the MBP-hEST-1 fusion protein, the hEST-1 cDNA was inserted into the bacterial expression vector pKK233-2 (Stratagene) and expressed in *E. coli* XL-1 Blue cells essentially as described previously for hP-PST and hDHEA-ST [11]. For efficient expression, the initial ATG of hEST-1 was incorporated into the Nco I site of pKK233-2 which is adjacent to the ribosome binding site. pKK233-2 was cut with Nco I and the restriction site was blunted with the Klenow fragment of DNA polymerase I [16]. The 5′-end of hEST-1 in pCRII was isolated as a 290 bp Eco RI fragment (bp 40–330), blunted and ligated into pKK233-2. The proper orientation and sequence of the 5′-end of hEST-1 were confirmed by sequence analysis. The pKK233-2-hEST-1-Eco RI plasmid was digested with Nco I and Hind III, then the 3′-Nco I–Hind III fragment of hEST-1, isolated from pCRII-hEST-1, was inserted into this site. The presence of an intact hEST-1 open-reading frame in pKK233-2 was confirmed by sequence analysis. Enzymatically active hEST-1 was expressed and purified from bacterial cytosol by DEAE-Sepharose CL-6B chromatography as described for hP-PST and for hDHEA-ST expressed in pKK233-2 [11].

Immunoblot analysis

To raise antibodies to hEST-1, pure hEST-1 protein (250 μg) was mixed with Freund's complete adjuvant and injected s.c. at several sites along the back of a female New Zealand white rabbit. Two weeks later, the rabbit received a booster injection of 250 μg hEST-1 in Freund's incomplete adjuvant in a similar manner. After 2 weeks, the rabbit was bled and the serum was

tested by immunoblot analysis for the presence of antibodies to hEST. Immunoblot analysis of expressed hEST-1 and human liver and jejunum cytosols was carried out as described previously [8, 19]. Briefly, after resolution of proteins by SDS–PAGE, proteins were electrotransferred to nitrocellulose paper. Rabbit anti-hEST-1 antibodies were diluted 1:10,000 and incubated with the nitrocellulose filter for 1 h. Goat anti-rabbit IgG horseradish peroxidase conjugate was used as the secondary antibody and immunoconjugates were visualized by chemiluminescence using a Lumiglo Chemiluminescent Substrate kit (Kirkegaard and Perry Labs). Immunoblot analysis of hEST-1 with rabbit anti-human PST antibodies [19] and rabbit anti-hDHEA-ST antibodies [8] was performed in a similar manner.

Sulfotransferase assays

Steroid sulfation was assayed as described previously [7, 8] using the tritiated steroids β-E_2, E_1, pregnenolone, DHEA, testosterone and cortisol, as sulfate acceptors. Reactions contained 50 mM Tris–HCl, pH 7.5, 7 mM $MgCl_2$, and varying concentrations of steroid substrates (0.88 nM–30 μM). Reactions were started by the addition of PAPS to a final concentration of 20 μM, in a final volume of 0.125 ml. Reactions were terminated by the addition of 4.0 ml of chloroform, followed by the addition of 0.375 ml 0.25 M Tris–HCl, pH 8.7, to alkalinize the solution. The reaction mixtures were then vortexed for 30 s and centrifuged at 600 *g* for 5 min to separate the aqueous and organic phases. Synthesis of the tritiated steroid sulfates was determined by scintillation counting of the aqueous phase. Sulfation assays using small phenols, such as *p*-nitrophenol, dopamine and minoxidil, as substrates were performed as described previously [20, 21]. The effect of varying the PAPS concentration (0–25 μM) was studied using β-E_2 as substrate at a concentration of 20 nM. The concentration of the sulfate acceptor compounds was varied to determine the concentration giving maximal sulfation activity.

Sulfation activity using non-radiolabeled steroids and related compounds was determined as described previously [11]. The reactions contained the appropriate substrates dissolved in ethanol, 7 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.4 and 20 μM [^{35}S]PAPS in a final volume of 0.125 ml. Control reactions were identical except the substrates were not added. Reactions were incubated for various times at 37°C and were terminated by spotting a 50 μl aliquot of each reaction on a silica gel F-250 TLC plate. The plate was developed in methylene chloride: MeOH: ammonium hydroxide (81:15:5, v/v/v) and the radiolabeled sulfated products were localized by autoradiography. The sulfated products were scraped into scintillation fluid and the radioactivity determined by scintillation spectroscopy.

RESULTS

Isolation and characterization of the hEST-1 cDNA

The hEST-1 cDNA was isolated from a human liver λZap cDNA library which has been used previously for the isolation of hP-PST and hDHEA-ST cDNAs [10, 21]. An oligonucleotide synthesized to a consensus sequence in the 5′-nontranslated regions of the four published EST sequences was used as the sense primer in a PCR procedure to amplify a human EST using the λZap DNA as a template. The initial amplification did not generate a detectable product, however a second amplification of the products of the first reaction generated a fragment of approx. 1050 bp. Sequence analysis of this fragment, which has been termed hEST-1, showed that hEST-1 contained a 994 bp DNA fragment which ended in a poly A tract. Figure 1 shows that hEST-1 contained an open-reading frame encoding a 294 amino acid protein with a calculated molecular mass of 35,123 Da. The amino acid sequence of hEST-1 is identical to that of a human EST cDNA reported by Aksoy *et al.* [14], although two silent nucleotides differences are present in the coding region for hEST-1.

Figure 2 shows a comparison of the translation of hEST-1 to the amino acid sequences of the human STs, hP-PST [22], hM-PST [23] and hDHEA-ST [10]. The amino acid sequence of hEST-1 is more similar to the sequences of the human PSTs than to hDHEA-ST. The sequence of hEST-1 is 71% similar and 51% identical to the sequence of hP-PST and 70% similar and 49% identical to hM-PST. hEST-1 is only 60% similar and 37% identical to hDHEA-ST. Figure 3 shows a comparison of the amino acid sequence of hEST-1 to the sequences of ESTs isolated from other species. The amino acid sequence of hEST-1 is 93% similar and 82% identical to a guinea pig EST [13] and 86% similar and 72% identical to the bovine EST sequence [12]. hEST-1 is 85% similar and 71–71% identical to the sequences of two isoforms of rat EST [15]. These comparisons indicate that hEST-1 is more closely related to the sequence of the ESTs in other species than to the human PSTs or hDHEA-ST. These results indicate that hEST is a member of the EST subfamily of the PST gene family.

Bacterial expression of hEST-1

In order to identify the protein encoded by the cDNA for hEST-1 as a functional estrogen ST, enzymatically active hEST-1 was expressed in bacteria and subsequently purified. The initial physical and immunologic characterization of hEST-1 was accomplished using the pMAL expression system for the generation of pure enzymatically active hEST-1. hEST-1 was also expressed in bacteria using the pKK233-2 expression vector which does not involve generation of a fusion protein. pKK233-2 has been used previously for the expression of hP-PST and

```
hEST                                ATTTCTTTCACAGGATCAACTAAACAGTGTACCACA   36

ATGAATTCTGAACTTGACTATTATGAAAAGTTTGAGGAAGTCCATGGGATTCTAATGTAT   96
MetAsnSerGluLeuAspTyrTyrGluLysPheGluGluValHisGlyIleLeuMetTyr   20

AAAGATTTTGTCAAATATTGGGATAATGTGGAAGCGTTCCAGGCAAGACCAGATGATCTT  156
LysAspPheValLysTyrTrpAspAsnValGluAlaPheGlnAlaArgProAspAspLeu   40

GTCATTGCCACCTACCCTAAATCTGGTACAACCTGGGTTAGTGAAATTGTGTATATGATC  216
ValIleAlaThrTyrProLysSerGlyThrThrTrpValSerGluIleValTyrMetIle   60

TATAAAGAGGGTGATGTGGAAAAGTGCAAAGAAGATGTAATTTTTAATCGAATACCTTTC  276
TyrLysGluGlyAspValGluLysCysLysGluAspValIlePheAsnArgIleProPhe   80

CTGGAATGCAGAAAAGAAAACCTCATGAATGGAGTAAAACAATTAGATGAGATGAATTCT  336
LeuGluCysArgLysGluAsnLeuMetAsnGlyValLysGlnLeuAspGluMetAsnSer  100

CCTAGAATTGTGAAGACTCATTTGCCACCTGAACTTCTTCCTGCCTCATTTTGGGAAAAG  396
ProArgIleValLysThrHisLeuProProGluLeuLeuProAlaSerPheTrpGluLys  120

GATTGTAAGATAATCTATCTTTGCCGGAATGCAAAGGATGTGGCTGTTTCCTTTTATTAT  456
AspCysLysIleIleTyrLeuCysArgAsnAlaLysAspValAlaValSerPheTyrTyr  140

TTCTTTCTAATGGTGGCTGGTCATCCAAATCCTGGATCCTTTCCAGAGTTTGTGGAGAAA  516
PhePheLeuMetValAlaGlyHisProAsnProGlySerPheProGluPheValGluLys  160

TTCATGCAAGGACAGGTTCCTTATGGTTCCTGGTATAAACATGTAAAATCTTGGTGGGAA  576
PheMetGlnGlyGlnValProTyrGlySerTrpTyrLysHisValLysSerTrpTrpGlu  180

AAGGGAAAGAGTCCACGTGTACTATTTCTTTTCTACGAAGACCTGAAAGAGGATATCAGA  636
LysGlyLysSerProArgValLeuPheLeuPheTyrGluAspLeuLysGluAspIleArg  200

AAAGAGGTGATAAAATTGATACATTTCCTGGAAAGGAAGCCATCAGAGGAGCTTGTGGAC  696
LysGluValIleLysLeuIleHisPheLeuGluArgLysProSerGluGluLeuValAsp  220

AGGATTATACATCATACTTCGTTCCAAGAGATGAAGAACAATCCATCCACAAATTACACA  756
ArgIleIleHisHisThrSerPheGlnGluMetLysAsnAsnProSerThrAsnTyrThr  240

ACACTGCCAGACGAAATTATGAATCAGAAATTGTCGCCCTTCATGAGAAAGGGAATTACA  816
ThrLeuProAspGluIleMetAsnGlnLysLeuSerProPheMetArgLysGlyIleThr  260

GGAGACTGGAAAAATCACTTTACAGTAGCCCTGAATGAAAAATTTGATAAACATTATGAG  876
GlyAspTrpLysAsnHisPheThrValAlaLeuAsnGluLysPheAspLysHisTyrGlu  280

CAGCAAATGAAGGAATCTACACTGAAGTTTCGAACTGAGATCTAAGAAGGTCTTTCTTTA  936
GlnGlnMetLysGluSerThrLeuLysPheArgThrGluIle***                  294

CTTAACATATCTGATATTAAAGATTTCTTTTCATTAAAAAAAAAAAAAAAAAAAAAAAA  994
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Fig. 1. Nucleotide sequence and translation of hEST-1. The nucleotide sequence and the derived translation of hEST-1 are numbered on the right. Nucleotides 1–18 are derived from the PCR primer.

```
                                                                      60
  hP-PST MELIQDTSRPPLEYVKGVPLIKYFAEALGPLQSFQARPDDLLISTYPKSGTTWVSQILDM
  hM-PST                                        S        N
    hEST  NSEL YYEKF  E H ILMY D VKYWDNVEA        V A          E VY
hDHEA-ST  SDDFLWFEGIAFPTM FRSETLRKVRDE.... VI DE VI L       N LAE  CL

                                                                     120
  hP-PST IYQGGDLEKCHRAPIFMRVPFLEFKAPGIPSGMETLKDTPAPRLLKTHLPLALLPQTLLD
  hM-PST           N    YV      VND  E   L       P   I S
    hEST   KE  V   KEDV  N I    CRKENLMN VKQ DEMNS  IV     PE   ASFWE
hDHEA-ST MHSK  AKWIQSV  WE S WV SEI..... YTA SESES   FSS   IQ F KSFFS

                                                                     180
  hP-PST QKVKVVYVARNAKDVAVSYYHFYHMAKVHPEPGTWDSFLEKFMVGEVSYGSWYQHVQEWW
  hM-PST             P          HR E A               A
    hEST KDC II LC         F Y FL VAG  N  SFPE V    Q Q P     K  KS
hDHEA-ST S A  I LM  PR  L  G F WKNM FIKK KS EEYF W CQ T L    FD IHG M

                                                                     240
  hP-PST ELSRTHPVLYLFYEDMKENPKREIQKILEFVGRSLPEETVDFMVQHTSFKEMKKNPMTNY
  hM-PST                                          M
    hEST  KGKSPR  F     L  DIRK VI LIH LE KPS  L  RIIH    Q   N  S
hDHEA-ST PMREEKNF L S  EL QDTG T E  CQ L KT EP ELNLILKNS  QS  E K S

                                                                     296
  hP-PST TTVPQEFMDHSISPFMRKGMAGDWKTTFTVAQNERFDADYAKKMAGCSLTFRSEL*
  hM-PST       L                                  E      S     *
    hEST   L D I NQKL        IT    NH    L  K  KH EQQ KEST K  T I*
hDHEA-ST SLLSVDYVVDK.AQLL   VS    NH     A D  KLFQE   DLPRELFPWE*
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Fig. 2. Comparison of amino acid sequence of hEST-1 with the sequences of hP-PST, hM-PST and hDHEA-ST. The sequences used in the comparison were hEST-1 (Fig. 1), hP-PST [22], hM-PST [23] and hDHEA-ST [10]. The amino acid sequences of the STs were aligned using the Pileup program of the University of Wisconsin Genetics Computer Group [28]. The initial Met residue of the hP-PST sequence was denoted as the first amino acid. Short gaps were inserted into the sequence comparisons to optimize the alignments.

hDHEA-ST [11]. The physical and kinetic properties of hEST-1 expressed in pKK233-2 and purified by anion exchange chromatography were essentially identical to those observed with hEST-1 expressed in the pMAL system and cleaved from the maltose binding protein with Factor Xa (data not shown).

During SDS–PAGE, pure hEST-1 migrated as a single band with an apparent molecular mass of 35,000 Da (Fig. 4). To identify hEST-1 in human tissues, immunoblot analysis of cytosol prepared from human liver and intestinal jejunum was carried out using the rabbit anti-hEST-1 antibody. Figure 5 shows that a protein which reacted with the rabbit anti-hEST antibody and migrated with the same molecular mass as expressed hEST-1 is present at low levels in both liver and intestine cytosol. The rabbit anti-hEST antibody did not react with either of the hPSTs or hDHEA-ST in these tissue cytosols. Additionally, expressed hEST-1 did not react with either rabbit anti-hPST [19] or rabbit anti-hDHEA-ST antibodies [8] (data not shown). Initially, the rabbit anti-hEST antibody did not detect a band in human liver cytosol using an alkaline phosphatase conjugated second antibody detection procedure which readily detects hP-PST and hDHEA-ST in human liver cytosol [8, 20]. In order to detect hEST in human liver cytosol, a chemiluminescence detection assay was utilized which was 5–10-fold more sensitive.

Kinetic characterization of hEST-1

hEST-1 activity, partially purified from bacterial cytosol following expression in the pKK233-2-hEST-1 vector, was used for the characterization of the kinetic properties of the enzyme. hEST-1 readily sulfated both β-E_2 and E_1 at low nanomolar concentrations. Figure 6 shows the effects of varying β-E_2 concentrations on hEST-1 activity. Maximal β-E_2 sulfation was observed at a concentration of approx. 20 nM and substrate inhibition was observed with higher β-E_2 concentrations in the reactions. Very similar results were observed when using varying concentrations of E_1 as a substrate. When comparing the ability of hEST-1 to conjugate β-E_2 and E_1 at a concentration of 20 nM, hEST-1 sulfated β-E_2 at approx. 1.2 times the rate at which it sulfated E_1 (Table 1). Both β-E_2 and E_1 are apparently conjugated at the 3-phenolic hydroxyl group since hEST did not sulfate the 17-aliphatic hydroxyl group of testosterone (Table 1). The effect of increasing PAPS concentrations in the reaction with 20 nM β-E_2 as substrate is also shown in Fig. 6. Substrate inhibition was not observed with increasing PAPS concentrations. The estimated K_m for PAPS is

```
                                                                    50
  hEST     MNSELDYY EKFEEVHGIL MYKDFVKYWD NVEAFQARPD DLVIATYPKS
rEST-6   METSMPEYY EVFGDFHGVL VDKLFTKYWE DVETFSARPD DLLVVTYPKS
  rEST   METSMPEYY DVFGDFHGFL MDKRFTKYWE DVETFLARPD DLLIVTYPKS
 gpEST  MMDSSEHDYY EYFDEFRGIL LYKQFIKYWD NVEAFQARPD DLVIAAYPKS
  bEST   MSSSKPSFS DYFGKLGGIP MYKKFIEQFH NVEEFEARPD DLVIVTYPKS

                                                                   100
  hEST   GTTWVSEIVY MIYKEGDVEK CKEDVIFNRI PFLECRKENL MNGVKQLDEM
rEST-6   GSTWIGEIVD MIYKEGDVEK CKEDAIFNRI PYLECRNEDL INGIKQLKEK
  rEST   GSTWISEIVD MIYKEGDVEK CKEDALFNRI PDLECRNEDL INGIKQLKEK
 gpEST   GTTWISEVVC MIYAEGDVKK CRQDAIFNRV PFLECRNDKM MNGVKQLEEM
  bEST   GTTWLSEIIC MIYNNGDVEK CKEDVIFNRV PYLECSTEHV MKGVKQLNEM

                                                                   150
  hEST   NSPRIVKTHL PPELLPASFW EKDCKIIYLC RNAKDVAVSF YYFFLMVAGH
rEST-6   ESPRIVKTHL PAKLLPASFW EKNCKIIYLC RNAKDVVVSY YYFFLIIKSY
  rEST   ESPRIVKTHL PAKLLPASFW EKNCKIIYLC RNAKDVVVSY YYFFLIMKSY
 gpEST   NSPRIIKTHL PPRLLPASFW EKRCKMICIC RNAKDVAVSY YYFFLMVANH
  bEST   ASPRIVKSHL PVKLLPVSFW EKNCKIIYLS RNAKDVVVSY YFLILMVTAI

                                                                   200
  hEST   PNPGSFPEFV EKFMQGQVPY GSWYKHVKSW WEKGKSPRVL FLFYEDLKED
rEST-6   PNPKSFSEFV EKFMEGQVPY GSWYDHVKSW WEKSKNSRVL FMFYEDMKED
  rEST   PNPKSFSEFV EKFMEGQVPY GSWYDHVKSW WEKSKNSRVL FMFYEDMKED
 gpEST   PDPGSFPEFV EKFMQGQVPY GSWYDHVKSW WEKSTDPRIL FIFYEDMKED
  bEST   PDPDSFQDFV EKFMDGEVPY GSWFEHTKSW WEKSKNPQVL FLFYEDMKEN

                                                                   250
  hEST   IRKEVIKLIH FLERKPSEEL VDRIIHHTSF QEMKNNPSTN YTTLPDEIMN
rEST-6   IRREVVKLIE FLERDPLAEL VDKIIQHTSF QEMKNNPCTN YSMLPETMID
  rEST   IRREVVKLIE FLERDPSAEL VDRIIQHTSF QEMKNNPCTN YSMLPETMID
 gpEST   IRKEVLKLIH FLGRKPSEEL VDKIIKHTSF QEMKNNPSTN YTMLPEEIMN
  bEST   IRKEVMKLLE FLGRKASDEL VDKIIKHTSF QEMKNNPSTN YTTLPDEVMN

                                                                   297
  hEST   QKLSPFMRKG ITGDWKNHFT VALNEKFDKH YEQQMKESTL KFRTEI*
rEST-6   LKVSPFMRKG IVGDWRNHFP EALRERFEEH YQRHMKDCPV TFRAEL*
  rEST   LKVSPFMRKG IVGDWKNHFP EALRERFEEH YQQQMKDCPV KFRAEL*
 gpEST   QKVSPFMRKG ISGDWKNHFT VALNESFDKH YQQQMKGSTL QLRTEI*
  bEST   QKVSPFMRKG DVGDWKNHFT VALNEKFDMH YEQQMKGSTL KFRTKI*
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Fig. 3. Comparison of amino acid sequence of hEST-1 with the sequences of other ESTs. The sequences used in the comparison were the guinea pig EST (gp EST) [13], hEST-1 (Fig. 1), bovine EST (bEST) [12], and two rat ESTs (rEST-3 and rEST-6) [15]. The amino acid sequences of the STs were aligned using the Pileup program of the University of Wisconsin Genetics Computer Group [28]. The initial Met residue of the gpEST sequence was denoted as the first amino acid. Short gaps were inserted into the sequence comparisons to optimize the alignments.

0.5 μM. Mg^{2+} ions have been reported to stimulate the activity of other steroid STs [6, 7], however, varying the concentration of Mg^{2+} (0–10 mM) had no effect upon the sulfation activity of hEST-1 towards either β-E_2 or E_1.

The ability of expressed hEST-1 to sulfate a number of estrogenic compounds, hydroxysteroids and xenobiotics was also tested. Expressed hEST-1 was capable of conjugating DHEA, pregnenolone, 1-naphthol, diethylstilbestrol, ethinylestradiol and equalenin (Table 1). The rate of sulfation of these compounds was determined at the concentration of the individual compounds which displayed maximum sulfation activity. No activity was observed when testosterone, cortisol or dopamine were tested as substrates. Figure 7 shows the sulfation of increasing concentrations of ethinylestradiol and DHEA with expressed hEST-1. Although ethinylestradiol was sulfated more rapidly than was β-E_2, maximum sulfation activity was observed at 1 μM and substrate inhibition was observed at higher concentrations. Figure 6 also shows that the sulfation of DHEA was still increasing at 30 μM, the highest concentration tested due to the limited solubility of DHEA. Similar results were observed with pregnenolone as a substrate.

DISCUSSION

This paper describes the cloning, expression and characterization of a human liver ST, termed hEST-1, capable of efficiently sulfating estrogens at low nanomolar concentrations. The affinity of hEST-1 for

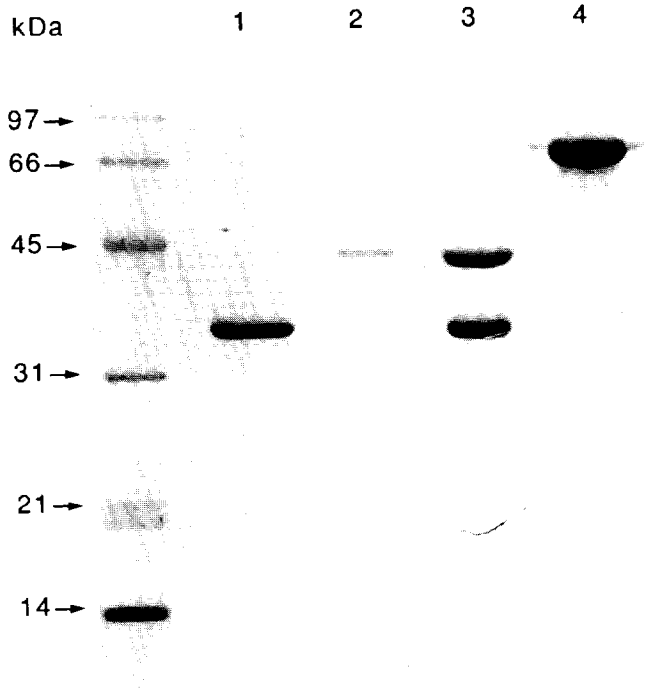

Fig. 4. SDS–polyacrylamide gel of hEST-1. Proteins were resolved by SDS–PAGE in a 12.5% gel, stained with Coomassie blue, and destained. Lane 1, 2.5 μg purified hEST-1; lane 2, 0.5 μg MBP; lane 3, 5 μg MBP-hEST-1 fusion protein after enzymatic cleavage with Factor Xa; lane 4, 5 μg MBP-hEST-1 fusion protein.

the sulfation of β-E_2 and E_1 is greater than that reported for the sulfation of any other substrate by a human ST. With the cloning and characterization of hEST-1, there have now been identified four forms of cytosolic ST which are present in human tissues. These are the phenol-sulfating form of PST, P-PST; the monoamine-sulfating form of PST, M-PST; dehydroepiandrosterone-ST, DHEA-ST; and EST. Three of these STs (hP-PST, hDHEA-ST and hEST-1) are involved in the sulfation of estrogens and hDHEA-ST is also capable of conjugating hydroxysteroids and bile acids [5–7, 11]. It has been reported that partially purified hM-PST does not sulfate estrogens but this observation has not been confirmed using the cloned or purified enzyme [6].

The investigation of estrogen sulfation in human tissues has been complicated by the presence of multiple human STs capable of conjugating estrogens with different kinetic properties [11]. Identification of an EST activity in human tissues with characteristics similar of those of hEST-1 is important because purification of an EST activity with these properties from human liver has not been reported. Grosso and Way [16] have characterized an EST activity in cytosol prepared from human RL95-2 endometrial adenocarcinoma cells with kinetic properties very similar to those of hEST-1. The RL95-2 EST activity had maximal E_1 sulfation activity at 15–20 nM and a K_m for PAPS of 0.6 μM. Based upon its kinetic properties and substrate specificities, the EST activity found in RL95-2 endometrial carcinoma cells is most likely due to the presence of hEST-1 in these cells.

Aksoy *et al.* [17] have recently reported the cloning of a human liver EST cDNA which has an identical translated sequence to that of hEST-1. The EST cDNA was isolated by a RT-PCR procedure and used to express EST activity in COS-1 cells which maximally sulfates estrone at a concentration of 50 nM. The isolation or identification of a similar EST protein in human tissues was not reported nor were the immunologic properties of the protein reported. The immunologic detection of hEST in human liver and intestinal cytosols in this report confirms the expression of hEST in human tissues. The low levels of EST protein in human liver cytosol may be responsible for the inability of Aksoy *et al.* [17] and our laboratory to isolate the hEST cDNA from human liver cDNA libraries using a rat EST cDNA as a probe.

Bernier *et al.* [24] have reported the cloning and expression of a putative human placental EST; however, the amino acid sequence of this ST is identical to that reported for human M-PST [23, 25] only 65.9, 68.6 and 68.2% similar to the rat [14], bovine [12] and

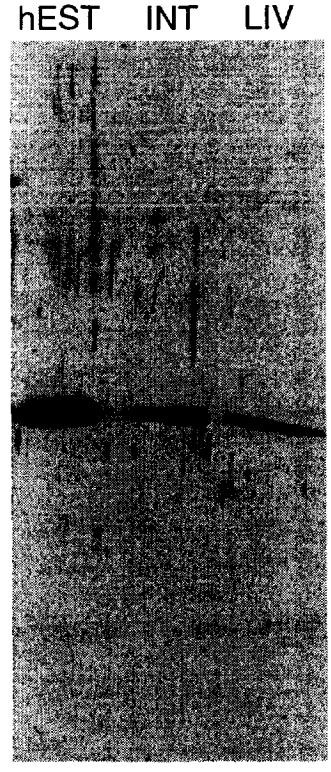

Fig. 5. Immunoblot analysis of hEST-1. Proteins were resolved by SDS–PAGE in a 12.5% gel and transferred to nitrocellulose. After incubation with a 1/20,000 dilution of the rabbit anti-hEST-1 IgG, the blot was developed as described in Methods using a chemiluminescence procedure with a 15 min exposure. The lanes contained: hEST (expressed hEST); INT, human intestinal jejunum (300 μg); LIV, human liver cytosol (300 μg).

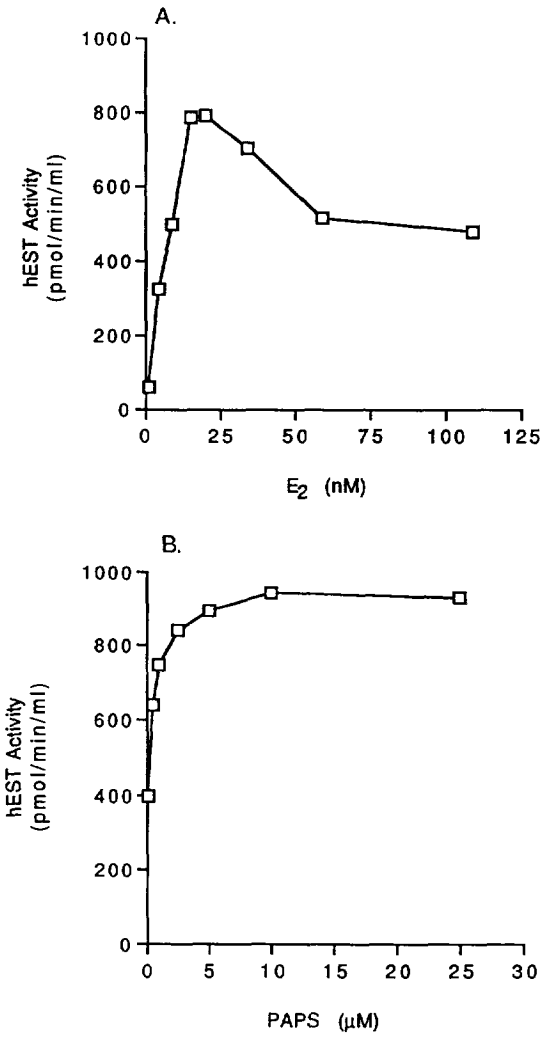

Fig. 6. β-Estradiol sulfation by expressed hEST-1. (A) The effect of increasing the β-E_2 concentration on hEST-1 activity. hEST-1 was purified by DEAE-Sepharose CL-6B chromatography after bacterial expression using the pKK233-2 expression system. Activity was assayed with varying concentrations of β-estradiol in the presence of 10 μM PAPS. (B) The effect of increasing the PAPS concentration on hEST-1 activity. Activity was assayed with varying concentrations of PAPS in the reaction at a β-E_2 concentration of 20 nM.

guinea pig [13] EST sequences, respectively. Bernier *et al.* [24] reported low levels of EST activity following transfection of SW-13 human adrenocortical cells with this cDNA. The kinetic characteristics of this enzyme were not reported. The transfected SW-13 cells also showed increases in DHEA and pregnenolone sulfation activities as compared to controls; however, human adrenocortical cells express high levels of hDHEA-ST [8, 26]. The authors did not report the activity of the expressed enzyme with prototypical PST substrates or immunologically characterize their expressed protein to further identify the product encoded by their cDNA, and in all likelihood have not reported a human ST distinct from those previously reported.

Forbes-Bamforth and Coughtrie [27] have reported the partial purification of an EST activity in human liver cytosol which sulfates E_1 and ethinylestradiol. The kinetic properties or substrate reactivity of this EST activity with other PST substrates was not reported. This EST activity coelutes with a protein which reacts with a rabbit anti-rat EST antibody which also recognizes both human P-PST and M-PST. The rabbit anti-hEST-1 antibody described in this report does not react with the human PSTs. Since hP-PST has been reported to sulfate estrogens, the EST activity described by Forbes-Bamforth and Coughtrie [27] may represent an isoform of hP-PST.

Characterization of expressed and purified hEST-1 showed that the enzyme efficiently sulfated β-E_2 and E_1 at concentrations approx. 50-fold lower than it conjugated other estrogenic compounds. The xenobiotic estrogens, equilenin and ethinylestradiol, were sulfated over 3-fold more rapidly than β-E_2; however, both of these estrogens were conjugated most efficiently at a concentration of 1 μM. These estrogens are also substrates for sulfation by hP-PST and hDHEA-ST, although higher concentrations are required for maximal activity [11]. Because of the involvement of several different STs with different kinetic properties in the conjugation of estrogens and xenobiotic estrogenic compounds in human tissues, analysis of estrogen sulfation in a human tissue will therefore require careful kinetic and immunologic characterization of the STs involved. Characterization of the individual cloned and expressed STs and the availability of specific antibodies to the STs will greatly aid in these studies.

Although hP-PST and hDHEA-ST are capable of conjugating estrogens at micromolar concentrations, these concentrations are much greater those required for binding to estrogen receptors. Thus, hP-PST and

Table 1. Substrate specificity of hEST-1

	hEST-1 activity	
Substrates	(%)	[Substrate]
β-Estradiol	100	(20 nM)
Estrone	85	(20 nM)
Pregnenolone	53	(30 μM)
Testosterone	0	—
Dehydroepiandrosterone	74	(30 μM)
Cortisol	0	—
1-Naphthol	53	(1 μM)
Dopamine	0	—
Diethylstilbestrol	60	(10 μM)
Ethinylestradiol	355	(1 μM)
Equilenin	324	(1 μM)

Sulfation assays were run as described in the text using hEST-1 expressed in the pKK233-2 vector and partially purified by DEAE-Sepharose CL-6B chromatography. β-Estradiol sulfation activity was 610 pmol/min/mg. The sulfation of the test compounds is expressed relative to the sulfation of β-estradiol. Substrate concentrations giving maximal sulfation rates are in parentheses.

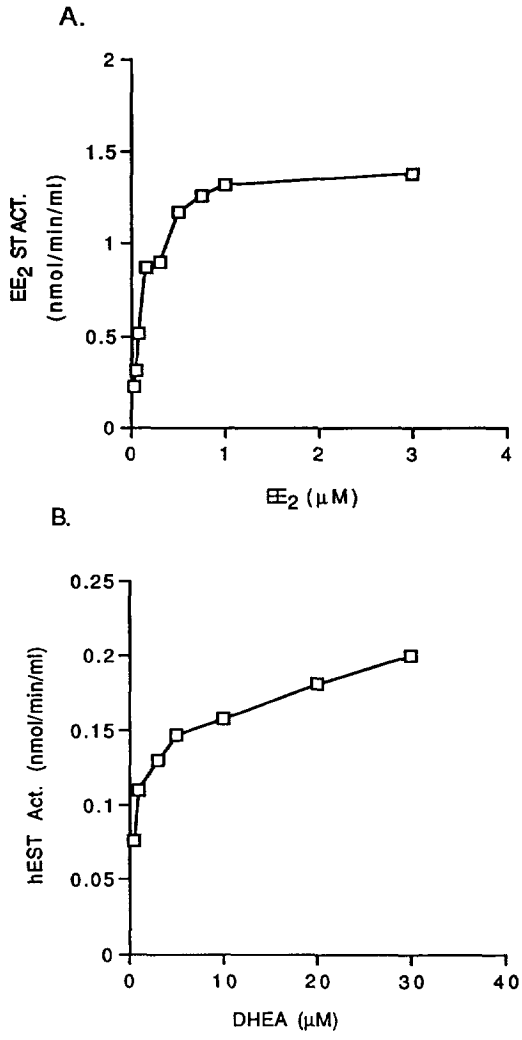

Fig. 7. Ethinylestradiol and DHEA sulfation by expressed hEST-1. (A) The effect of increasing the concentrations of ethinylestradiol (A) and DHEA (B) on their sulfation by expressed hEST-1. hEST-1 was purified by DEAE-Sepharose CL-6B chromatography after bacterial expression using the pKK233-2 expression system. Sulfation activity was assayed with varying concentrations of ethinylestradiol and equilenin in the presence of 20 μM PAPS as described in Methods.

hDHEA-ST may not play a role in modulating tissue responsiveness to estrogens, although they may be involved in the metabolism and excretion of those compounds. The significantly greater affinity of hEST-1 for β-E_2 and E_1 sulfation suggests that the enzyme may be involved in modulating estrogen levels at nanomolar concentrations where the estrogens may be functionally interacting with the estrogen receptor. Estrogen sulfates are incapable of activating the estrogen receptor. Thus, high levels of hEST-1 in a tissue may provide a mechanism for decreasing the responsiveness of the tissue to estrogens by sulfating the estrogens and rendering them functionally inactive with respect to receptor binding. Thus, further elucidation of the role of hEST-1 in estrogen sulfation may be important in understanding the effects of modulating the levels of estrogens or therapeutic estrogenic drugs on the response of hormonally sensitive tissues and tumors.

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