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Isolation and Expression of an Isoform of Rat Estrogen Sulfotransferase

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A new isoform of rat liver estrogen sulfotransferase (EST), rEST-6, which is distinct from the previously reported rat EST [Demyan *et al.*, *Molec. Endocrinol.* 6 (1992) 589], has been cloned, expressed, purified and characterized. A PCR procedure using oligonucleotide primers synthesized to the 5′-nontranslated and 3′-nontranslated regions of the published rEST sequence was used to isolate rEST-6 cDNA. The cloned DNA is 1000 bp in length and encodes a protein of 295 amino acids with a calculated molecular mass of 35,300 Da. rEST-6 is selectively expressed in male rats, as confirmed by Northern blot and immunoblot analyses. Northern blot analysis of male and female rat liver RNA with the rEST-6 cDNA as a probe shows a band with male RNA but not with female RNA. Similarly, immunoblot analysis of male and female rat liver cytosols with an antibody to rat EST yields a strong immunoreactive band in rat liver cytosol from male rats but not from females. Subsequent to bacterial expression and purification of rEST-6, the enzyme was analyzed kinetically and shown to sulfate estrogens but not dehydroepiandrosterone, pregnenolone, cortisol or testosterone. Maximal sulfation activity towards both β-estradiol and estrone occurred at a concentration of $1\,\mu M$ with substrate inhibition at higher concentrations. These results indicate that multiple, closely related forms of EST are present in rat liver. Analysis of the activity and regulation of these different EST enzymes is important in understanding estrogen metabolism in rats.

INTRODUCTION

Sulfation is an important process in modulating the activity, transport and excretion of hydroxysteroids and estrogens [1, 2]. Sulfate-conjugated steroids cannot activate steroid receptors and, due to the addition of the charged sulfonate group, are more water soluble and therefore more readily excreted than are the unconjugated forms. Steroid sulfates in the brain, such as dehydroepiandrosterone (DHEA) sulfate and pregnenolone sulfate, may function in modulating the activity of GABA receptors [3]. In addition, steroid sulfates may serve as transport forms for steroids; the sulfate moiety can be removed by sulfatases in the target tissue to generate the active form of the steroid [4, 5].

Multiple isoforms of cystolic sulfotransferase (ST) have been shown to conjugate steroids using 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the sulfonate donor. The sulfation of estrogens and hy-droxysteroids is catalyzed by at least three different classes of cytosolic STs. Purified rat and human hydroxysteroid STs have been shown to conjugate both estrogens and hydroxysteroids such as DHEA and pregnenolone [4, 5]. Human phenol-sulfating phenol ST (hP-PST) has been reported to sulfate estrogens but not hydroxysteroids [6, 7]. Separate estrogen STs (EST) have been purified from several species [8, 9] and distinct forms of EST have been cloned from bovine [10], rat [11] and guinea pig tissues [12]. The evaluation of the roles of the different forms of ST involved in steroid sulfation requires an understanding of the multiplicity and kinetic properties of the individual enzymes present in a given tissue.

This manuscript reports the cloning and bacterial expression of a new isoform of rat liver EST which is distinct from the previously reported rat EST [11]. The enzyme is selectively expressed in male rats and has been purified and kinetically characterized following expression in *E. coli*. Multiple isoforms of the cytosolic hydroxysteroid STs and phenol STs have been reported in rat liver [1, 13]. This report

confirms that multiple isoforms of EST are also present. Understanding the physical and kinetic properties of the different isoforms of EST is important in analyzing the roles of these enzymes in steroid metabolism.

EXPERIMENTAL

Materials

Oligonucleotide primers were synthesized in the Molecular Biology Core Facility of the Comprehensive Cancer Center at UAB. *p*-Nitrophenol, β-estradiol (E_2), estrone (E_1), DHEA, testosterone, pregnenolone, dopamine, DEAE-Sepharose CL-6B and 3′,5′-diphosphoadenosine (PAP) agarose were obtained from Sigma Chemical Co. (St Louis, MO). [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), [1,2,3-^{3}H(N)]cortisol (54 Ci/mmol), [7-^{3}H]testosterone (27.7 Ci/mmol), [7-^{3}H]pregnenolone (25 Ci/mmol) and [^{35}S]3′-phosphoadenosine-5′-phosphosulfate (PAPS) (2 Ci/mmol) were purchased from New England Nuclear (Boston, MA). PAPS was purchased from Dr Sanford Singer (University of Dayton, Dayton, OH). Minoxidil was a generous gift from the Upjohn Co. (Kalamazoo, MI). pKK233-2 was obtained from Pharmacia (Piscataway, NJ). Sequagel and Protogel were purchased from National Diagnostics (Atlanta, GA). Affinity-purified goat anti-rabbit horseradish peroxidase conjugate was purchased from Southern Biotechnology Associates (Birmingham, AL). The Lumiglo Chemiluminescence Substrate kit was obtained from Kirkegaard and Perry Labs (Gaithersburg, MD). All other chemicals were of reagent grade quality.

Isolation of rat EST cDNAs

A polymerase chain reaction (PCR) procedure was used to isolate the rat EST cDNAs. A pair of oligonucleotides (5′-CAGGAGCATCTGGACAGTAC-3′ and 5′-CTTCTACTTCTACTGAATTC-3′) were synthesized to the 5′-nontranslated and 3′-nontranslated regions of the published rat EST sequence [11], respectively. The template for the PCR reactions was obtained from a male rat λgt11 cDNA library (Stratagene). Approximately 10^7 pfu were amplified in *E. coli* Y1090 and total phage DNA was purified from plate lysates using a commercial kit (Promega). Phage DNA (0.1 μg) was denatured by incubation in boiling water for 5 min and used as the template for PCR. The PCR products were isolated from an agarose gel and subcloned into the pCRII vector (Invitrogen) for sequence analysis and characterization.

The complete nucleotide sequences of the EST cDNAs were determined by dideoxynucleotide chain termination DNA sequencing using [^{35}S]dATP to label the DNA fragments as described previously [14]. Electrophoresis of the DNA fragments was carried out in 8% polyacrylamide wedge gels in 8 M urea using the Sequagel system. The gels were transferred to Whatman 3MM paper, dried under vacuum and exposed to autoradiograph film for 24 h. The sequences were read manually and analyzed using the University of Wisconsin Genetic Computer group's program and the MacVector DNA Analysis program (Kodak/ IBI).

Immunological analysis

In order to generate a specific antibody to the rat ESTs, the rEST-3 cDNA was expressed in the pMAL-c2 expression system for the generation of fusion protein. Antibodies were raised to rEST-3 rather than to rEST-6 because the antibody production was done simultaneously with the complete sequencing of both rEST-3 and rEST-6, prior to sequence analysis which revealed that rEST-3 was an allele but that rEST-6 was an isoform of the previously reported rEST. The pMAL-c2 expression system generates a maltose binding protein (MBP) fusion protein which can be purified by affinity chromatography on an amylose affinity resin (New England Biolabs). For insertion into pMAL-c2, the rEST-3 cDNA in pCRII was digested with Nco I, which cuts the cDNA at base 72. The cDNA was treated with the Klenow fragment of DNA polymerase I and digested with Hind III. This procedure generated a cDNA fragment which was in-frame for translation but lacked 15 amino acids at the amino-end of the translated region. This fragment was inserted into the Xmm I and Hind III sites of pMAL-c2. *E. coli* XL1-Blue cells were transformed with the pMAL-rEST-3 vector (New England Biolabs). To express the MBP-rEST-3 fusion protein, XL1-Blue cells containing pMAL-rEST-3 were grown in Luria broth containing 50 μg/ml ampicillin to an O.D.$_{550}$ of 0.5, then induced for 2 h with 0.3 mM isopropyl-β-D-galactopyranoside. 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 re-pelleted, resuspended in 5 mM phosphate, pH 7.4, containing 1.5 mM dithiothreitol and 10 μg/ml phenolmethylsulfonylfluoride, and sonicated 3 × on ice in 10 s bursts with 30 s cooling between each burst. The cytosolic fraction was recovered following centrifugation at 100,000 *g* for 1 h.

The expressed MBP r-EST-3 fusion protein was purified by amylose affinity chromatography. The cytosolic fraction was diluted to 3 mg protein/ml and applied to an amylose affinity resin (New England Biolabs) equilibrated in 5 mM phosphate, pH 7.4. The amylose column (1.75 × 5 cm) was washed with 75 ml of 5 mM phosphate, pH 7.4, then the fusion protein was eluted with 10 ml of 10 mM maltose in the same buffer. The yield was approx. 20 mg fusion protein/ liter bacterial culture. The truncated rEST-3 fusion protein was not enzymatically active.

To raise antibodies to rEST-3, the MBP-rEST-3 fusion protein (500 µg) was mixed with Freund's complete adjuvant and injected subcutaneously at several sites along the back of a female New England white rabbit as described by Vaitukaitus et al. [15]. Two weeks later, the rabbit received a booster injection of

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rEST-3
CAGGAGCATCTGGACAGTACACCACTTGTGATGGAGACTTCTATGCCTGAATACTATGAC      60
                                   MetGluThrSerMetProGluTyrTyrAsp  10

GTTTTTGGTGATTTCCATGGATTTTTAATGGATAAACGGTTCACCAAATATTGGGAAGAC     120
ValPheGlyAspPheHisGlyPheLeuMetAspLysArgPheThrLysTyrTrpGluAsp      30

GTTGAAACATTCTTGGCAAGGCCAGATGACCTTCTCATTGTTACTTATCCTAAATCTGGC     180
ValGluThrPheLeuAlaArgProAspAspLeuLeuIleValThrTyrProLysSerGly      50

AGCACATGGATTAGTGAAATTGTGGATATGATCTATAAAGAAGGTGATGTGGAAAAATGC     240
SerThrTrpIleSerGluIleValAspMetIleTyrLysGluGlyAspValGluLysCys      70

AAGGAGGATGCACTTTTTAACAGAATACCTGACCTGGAGTGCAGAAATGAAGATCTAATA     300
LysGluAspAlaLeuPheAsnArgIleProAspLeuGluCysArgAsnGluAspLeuIle      90

AACGGAATAAAACAACTAAAAGAAAAGGAATCGCCTAGAATAGTGAAAACTCACCTGCCA     360
AsnGlyIleLysGlnLeuLysGluLysGluSerProArgIleValLysThrHisLeuPro     110

GCTAAGCTCCTTCCAGCATCATTTTGGGAAAAGAATTGCAAGATAATCTATCTTTGCCGA     420
AlaLysLeuLeuProAlaSerPheTrpGluLysAsnCysLysIleIleTyrLeuCysArg     130

AATGCCAAAGATGTCGTCGTTTCTTATTACTACTTTTTCCTGATCATGAAAAGTTATCAA     480
AsnAlaLysAspValValValSerTyrTyrTyrPhePheLeuIleMetLysSerTyrGln     150

AATCCTAAATCTTTTTCTGAATTTGTGGAGAAATTTATGGAAGGGCAAGTTCCGTATGGT     540
AsnProLysSerPheSerGluPheValGluLysPheMetGluGlyGlnValProTyrGly     170

TCCTGGTATGATCATGTAAAATCTTGGTGGGAGAAGAGTAAGAATTCACGTGTTTTGTTT     600
SerTrpTyrAspHisValLysSerTrpTrpGluLysSerLysAsnSerArgValLeuPhe     190

ATGTTCTATGAGGACATGAAAGAGGATATCCGAAGAGAAGTTGTGAAGCTGATAGAGTTC     660
MetPheTyrGluAspMetLysGluAspIleArgArgGluValValLysLeuIleGluPhe     210

CTGGAGAGAGACCCATCAGCAGAGCTAGTAGACAGAATCATTCAACATACATCATTCCAG     720
LeuGluArgAspProSerAlaGluLeuValAspArgIleIleGlnHisThrSerPheGln     230

GAGATGAAGAACAATCCATGCATCAATTATTCAATGCTGCCAGAGACCATGATAGATCTA     780
GluMetLysAsnAsnProCysIleAsnTyrSerMetLeuProGluThrMetIleAspLeu     250

AAAGTATCGCCTTTCATGAGAAAGGGAATTGTAGGAGACTGGAAGAACCACTTCCCTGAA     840
LysValSerProPheMetArgLysGlyIleValGlyAspTrpLysAsnHisPheProGlu     270

GCCCTGAGGGAGAGATTTGAGGAGCACTACCAGCAGCAAATGAAGGACTGCCCTGTGAAA     900
AlaLeuArgGluArgPheGluGluHisTyrGlnGlnGlnMetLysAspCysProValLys     290

TTTAGAGCAGAGCCCTGAGACAATTCCTTGTGTCTGAAATTGGAGTAGTCTCCAATTTAT     960
PheArgAlaGluPro***                                               295

CCTTCAGTTTTTCTTGTTTTGAATTCAGTAGAAGTAGAAG    1000
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Fig. 1. Nucleotide sequence and translation of rEST-3. The nucleotide sequence and the derived translation of rEST-3 are numbered on the right. Nucleotides 1–20 and 981–1000 are derived from PCR primers. The asterisk represents the stop codon.

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500 μg MBP-rEST-3 in a similar manner. After 2 weeks, the rabbits were bled and the serum was tested by immunoblot analysis for the presence of antibodies to the rESTs.

Immunoblot analysis of the expressed rat ESTs and rat liver cytosolic fractions were carried out as described previously [16]. Briefly, after resolution of proteins by SDS–PAGE, proteins were electrotrans-

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rEST-6 .          .              .           .            .           .
CAGGAGCATCTGGACAGTACACCACTTGAAATGGAGACTTCTATGCCTGAATACTATGAA    60
                                  MetGluThrSerMet ProGluTyrTyrGlu    10


GTTTTTGGTGATTTCCATGGAGTTTTAGTGGATAAACTGTTCACCAAATATTGGGAAGAT   120
ValPheGlyAspPheHisGlyValLeuValAspLysLeuPheThrLysTyrTrpGluAsp    30


GTTGAAACATTCTCAGCAAGGCCAGATGACCTTCTCGTTGTTACTTATCCTAAATCTGGC   180
ValGluThrPheSerAlaArgProAspAspLeuLeuValValThrTyrProLysSerGly    50


AGCACATGGATTGGTGAAATTGTGGATATGATCTATAAAGAAGGTGATGTGGAAAAATGC   240
SerThrTrpIleGlyGluIleValAspMetIleTyrLysGluGlyAspValGluLysCys    70


AAGGAGGATGCAATTTTTAACAGAATACCTTACCTGGAGTGCAGAAATGAAGATCTAATA   300
LysGluAspAlaIlePheAsnArgIleProTyrLeuGluCysArgAsnGluAspLeuIle    90


AATGGAATAAAACAACTAAAGGAAAAGGAATCGCCTAGAATAGTGAAAACTCACCTGCCA   360
AsnGlyIleLysGlnLeuLysGluLysGluSerProArgIleValLysThrHisLeuPro   110


GCTAAGCTCCTTCCAGCATCATTTTGGGAAAAGAATTGCAAGATAATCTATCTTTGCCGA   420
AlaLysLeuLeuProAlaSerPheTrpGluLysAsnCysLysIleIleTyrLeuCysArg   130


AATGCCAAAGATGTCGTCGTTTCTTATTACTACTTTTTCCTGATCATAAAAAGTTATCCA   480
AsnAlaLysAspValValValSerTyrTyrTyrPhePheLeuIleIleLysSerTyrPro   150


AATCCTAAATCTTTTTCTGAATTTGTGGAGAAATTTATGGAAGGGCAAGTTCCGTATGGT   540
AsnProLysSerPheSerGluPheValGluLysPheMetGluGlyGlnValProTyrGly   170


TCCTGGTATGATCATGTAAAATCTTGGTGGGAAAAGAGTAAGAACTCACGTGTTTTGTTT   600
SerTrpTyrAspHisValLysSerTrpTrpGluLysSerLysAsnSerArgValLeuPhe   190


ATGTTCTATGAGGATATGAAAGAGGATATACGAAGAGAAGTTGTGAAGCTGATAGAGTTC   660
MetPheTyrGluAspMetLysGluAspIleArgArgGluValValLysLeuIleGluPhe   210


CTGGAGAGAGACCCATTAGCAGAGCTAGTAGACAAAATCATTCAACATACGTCATTCCAG   720
LeuGluArgAspProLeuAlaGluLeuValAspLysIleIleGlnHisThrSerPheGln   230


GAGATGAAGAACAATCCATGCACCAATTATTCAATGCTGCCAGAGACCATGATAGATCTA   780
GluMetLysAsnAsnProCysThrAsnTyrSerMetLeuProGluThrMetIleAspLeu   250


AAAGTATCGCCTTTCATGAGAAAGGGAATTGTAGGAGACTGGAGGAACCACTTCCCTGAA   840
LysValSerProPheMetArgLysGlyIleValGlyAspTrpArgAsnHisPheProGlu   270


GCCCTGAGGGAGAGATTTGAGGAGCACTACCAGCGGCATATGAAGGACTGCCCTGTGACG   900
AlaLeuArgGluArgPheGluGluHisTyrGlnArgHisMetLysAspCysProValThr   290


TTTAGAGCAGAGCTCTGAGACACTTCCTTGTGTCTGAAATTGGAGTAGTCTCCAATTTAT   960
PheArgAlaGluLeu***                                             295


CCTTCAGTTTTTCTTGTTTTGAATTCAGTAGAAGTAGAAG   1000
```

Fig. 2. Nucleotide sequence and translation of rEST-6. The nucleotide sequence and the derived translation of rEST-6 are numbered on the right. Nucleotides 1–20 and 981–1000 are derived from PCR primers. The asterisk represents the stop codon.

ferred to nitrocellulose paper. Primary rabbit anti-MBP-rEST-3 antibodies were diluted 1:20,000, and incubated with the blots for 1 h. Goat anti-rabbit IgG horseradish peroxidase conjugate was used as the secondary antibody and immunoconjugates were visualized by chemiluminescence (Kirkegaard and Perry Labs).

Expression of rEST-6 in E. coli

In order to characterize the enzymatic properties of rEST-6, the active enzyme was expressed in bacteria and then highly purified as has been described previously for the expression of human cytosolic STs [7]. For the expression of active rEST-6, the rEST-6 cDNA was inserted into the bacterial expression vector pKK233-3. To subclone rEST-6 into pKK233-2, an oligonucleotide primer (5'-CCACTTGACCATG-GAGACTTC-3') was synthesized which incorporated two base changes to create an Nco I restriction site at the initiating methionine of the rEST-6 cDNA. The rEST-6 cDNA was amplified by PCR using the M13 universal primer as the antisense primer and pCRII-rEST-6 as a template. The amplified DNA was completely digested with Hind III and then partially digested with Nco I. The digestion products were resolved by electrophoresis in low melting agarose and the appropriately-sized DNA fragment was isolated and subcloned into the Nco I and Hind III sites of pKK233-3. *E. coli* XL1-Blue cells made competent using a calcium chloride procedure [17] were transformed with the PKK-rEST-6 vector. Colonies were selected by ampicillin resistance, and those colonies containing the full-length Nco I-Hind III rEST-6 DNA fragment were identified by DNA sequence analysis.

Cytosol was prepared from induced *E. coli* XL1-Blue cells transformed with pKK-rEST-6 using the same method described for the preparation of the MBP-rEST-3 cytosol. Enzymatically active rEST-6 was purified from the bacterial cytosol prior to kinetic characterization because *E. coli* XL1-Blue cytosol contains enzymes that rapidly degrade PAPS. Bacterial cytosol was applied to a DEAE-Sepharose CL-6B column (1.5 × 5 cm) equilibrated in TEA buffer (10 mM triethanolamine, pH 7.5, 1.5 mM dithiothreitol, 10% glycerol). The column was rinsed with 20 ml of TEA buffer, then with 30 ml of TEA buffer containing 100 mM NaCl. The EST activity was eluted with a gradient of 100–225 mM NaCl in TEA buffer. rEST-6 was further purified by affinity chromatography on a 3',5' PAP-agarose column. The fractions from the anion-exchange column containing high levels of EST activity were concentrated and desalted by ultrafiltration. The concentrated EST activity was then applied to a 5 ml PAP-agarose affinity column and the column was washed with TEA buffer. EST activity was eluted from the column with 10 μM PAPS in TEA buffer.

Sulfotransferase assays

Steroid sulfation was assayed as described previously [18] using the tritiated steroids, β-E$_2$, E$_1$, DHEA, testosterone, pregnenolone and cortisol, as the sulfate acceptors. Reactions contained 50 mM Tris–HCl, pH 7.4, 7 mM MgCl$_2$, and varying concentrations of steroid substrates (0.1–30 μM). The reactions were started by the addition of PAPS to a final concentration of 10 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 0.5 M Tris–HCl,

```
EST-3   METSMPEYYDVFGDFHGFLMDKRFTKYWEDVETFLARPDDLLIVTYPKSGSTWISEIVDM   60
        ||||||||| |||||||| | || |||||||||| ||||||| ||||||||||| |||||
EST-6   METSMPEYYEVFGDFHGVLVDKLFTKYWEDVETFSARPDDLLVVTYPKSGSTWIGEIVDM


        IYKEGDVEKCKEDALFNRIPDLECRNEDLINGIKQLKEKESPRIVKTHLPAKLLPASFWE   120
        ||||||||||||| ||||| |||||||||||||||||||||||||||||||||||||||||
        IYKEGDVEKCKEDAIFNRIPYLECRNEDLINGIKQLKEKESPRIVKTHLPAKLLPASFWE


        KNCKIIYLCRNAKDVVVSYYYFFLIMKSYQNPKSFSEFVEKFMEGQVPYGSWYDHVKSWW   180
        |||||||||||||||||||||||| ||| ||||||||||||||||||||||||||||||||
        KNCKIIYLCRNAKDVVVSYYYFFLIIKSYPNPKSFSEFVEKFMEGQVPYGSWYDHVKSWW


        EKSKNSRVLFMFYEDMKEDIRREVVKLIEFLERDPSAELVDRIIQHTSFQEMKNNPCINY   240
        |||||||||||||||||||||||||||||||||||||||| ||||| ||||||||||||| ||
        EKSKNSRVLFMFYEDMKEDIRREVVKLIEFLERDPLAELVDKIIQHTSFQEMKNNPCTNY


        SMLPETMIDLKVSPFMRKGIVGDWKNHFPEALRERFEEHYQQQMKDCPVKFRAEP*   296
        |||||||||||||||||||||||||| |||||||||||||||||  |||||| |||| |
        SMLPETMIDLKVSPFMRKGIVGDWRNHFPEALRERFEEHYQRHMKDCPVTFRAEL*
```
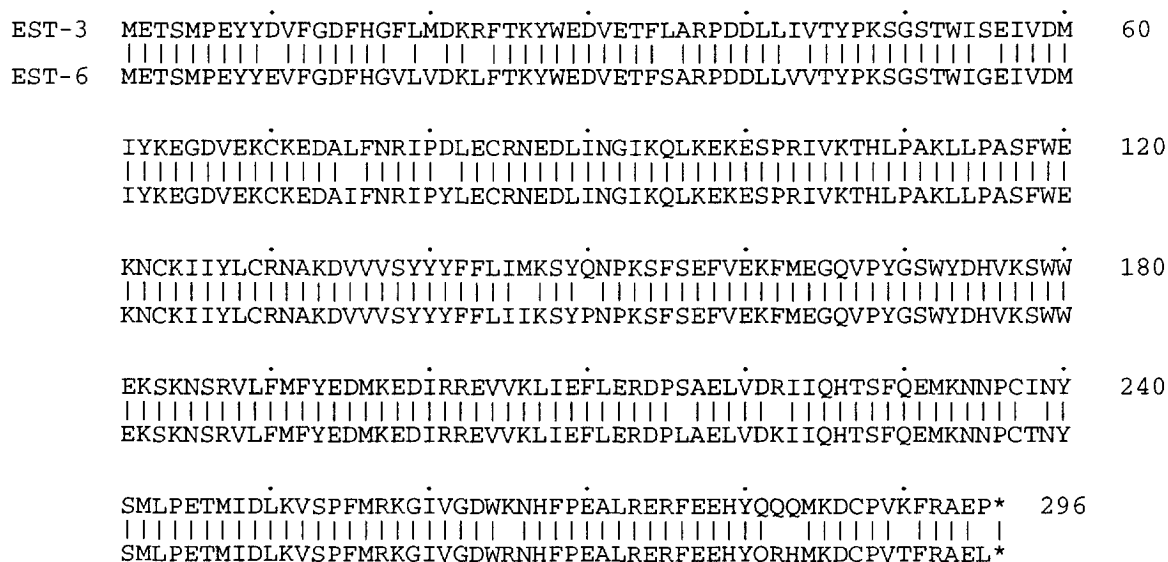
Fig. 3. Comparison of the amino acid sequences of rEST-3 and rEST-6. The derived amino acid sequences of rEST-3 and rEST-6 are compared and identical amino acids are shown by a vertical line. The asterisks denote stop codons.

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 non-radiolabeled steroids and small phenols, such as *p*-nitrophenol, dopamine and minoxidil, were performed as described previously [19, 20].

Northern blot analysis

Total RNA was isolated from liver samples of 125–150 g male and female Sprague–Dawley rats using an acid guanidinium thiocyanate extraction method [21]. The RNA was resolved by agarose gel electrophoresis in the presence of formaldehyde, visualized by brief staining in ethidium bromide, and then transferred to nitrocellulose paper. The nitrocellulose paper was then heated *in vacuo* and prehybridized in 50% deionized formamide with 0.8 M NaCl, 10 mM Tris–HCl, pH 7.2, 1 mM EDTA, 0.5% SDS, 50 μg/ml poly A+, 100 μg/ml yeast tRNA and 10 × Denhardt's solution. [^{32}P]-Labeled rEST-6 cDNA was generated by an oligolabeling procedure (Pharmacia). The nitrocellulose was hybridized at 42°C in fresh prehybridization buffer containing 2 × 10^6 dpm/ml of [^{32}P]DNA. The filter was washed 2 × for 30 min at 65°C in 3 × SSC containing 0.5% SDS, then for 30 min in 3 × SSC alone. Autoradiography was performed at -70°C with an intensifying screen.

```
                                                       50
rEST-6   METSMPEYY EVFGDFHGVL VDKLFTKYWE DVETFSARPD DLLVVTYPKS
  rEST   --------- D-------F- M--R------ -----L---- ---I------
 gpEST   M-DS-EHD-- -Y-DE-R-I- LY-Q-I---D N--A-Q---- --VIAA----
  bEST   -SS-K-SFS DY--KLG-IP MY-K-IEQFH N--E-E---- --VI------

                                                      100
rEST-6   GSTWIGEIVD MIYKEGDVEK CKEDAIFNRI PYLECRNEDL INGIKQLKEK
  rEST   -----S---- ---------- -----L---- -D-------- ----------
 gpEST   -T---S-V-C ---A----K- -RQ------V -F-----DKM M--V---E-M
  bEST   -T--LS--IC ---NN----- ----V----V -----ST-HV MK-V---N-M

                                                      150
rEST-6   ESPRIVKTHL PAKLLPASFW EKNCKIIYLC RNAKDVVVSY YYFFLIIKSY
  rEST   --------- ---------- ---------- ---------- ------M---
 gpEST   N----I---- -PR------- --R--M-CI- ------A--- -----MVANH
  bEST   A------S-- -V----V--- ---------S ---------- -FLI-MVTAI

                                                      200
rEST-6   PNPKSFSEFV EKFMEGQVPY GSWYDHVKSW WEKSKNSRVL FMFYEDMKED
  rEST   --------- ---------- ---------- ---------- ----------
 gpEST   -D-G--P--- ----Q----- ---------- ----TDP-I- -I--------
  bEST   -D-D--QD-- ----D-E--- ---FE-T--- ------PQ-- -L-------N

                                                      250
rEST-6   IRREVVKLIE FLERDPLAEL VDKIIQHTSF QEMKNNPCTN YSMLPETMID
  rEST   --------- ------S--- --R------- ---------- ----------
 gpEST   --K--L---H --G-K-SE-- -----K---- -------S-- -T----EIMN
  bEST   --K--M--L- --G-KASD-- -----K---- -------S-- -TT--DEVMN

                                                      296
rEST-6   LKVSPFMRKG IVGDWRNHFP EALRERFEEH YQRHMKDCPV TFRAEL*
  rEST   --------- -----K---- ---------- --QQ------ K-----*
 gpEST   Q-------- -S---K---T V--N-S-DK- --QQ--GSTL QL-T-I*
  bEST   Q-------- D----K---T V--N-K-DM- -EQQ--GSTL K--T-I*
```

Fig. 4. Comparison of amino acid sequence of rEST-6 with the published amino acid sequences of the guinea pig [12], bovine [10], and rat [11] ESTs. The amino acid sequences of the ESTs were aligned and the initial methionine of the guinea pig sequence was denoted as the first amino acid. Only the amino acids which differ from the sequence of rEST-6 are shown. Identical amino acids to the sequence of rEST-6 are represented by a dash. The asterisks denote stop codons.

RESULTS

A male rat liver λgt11 cDNA library was amplified by PCR with oligonucleotide primers designed to amplify the cDNA of a previously published isoform of rat liver EST [11]. Following amplification, two different but related cDNAs were identified by sequence analysis. The two different rat EST cDNA sequences were termed EST-3 and EST-6. Figures 1 and 2 show the nucleotide sequence and translation of rEST-3 and rEST-6, respectively. rEST-3 was 1000 bp in length and possessed an open-reading frame of 885 nucleotides encoding a protein of 295 amino acids with a calculated molecular mass of 35,440 Da. The translated rEST-3 sequence contained three amino acid differences when compared to the published sequence of a previously reported male rat liver EST [11]. These differences were at amino acids 150, Q for P; amino acid 238, I for T; and amino acid 295, P for L.

rEST-6 was 1000 bp in length and also encoded a 295 amino acid protein (Fig. 2). The calculated

Fig. 6. Immunoblot of rEST-6 with anti-MRP-rEST-3 antibody. Proteins were resolved by SDS–PAGE in a 12.5% gel and transferred to nitrocellulose. After incubation with a 1/20,000 dilution of anti-MBP-rEST-3 antibody, the blot was developed as described in Experimental. Lane (A), 50 μg female rat liver cytosol; lane (B), 0.5 μg rEST-6 PAP-agarose fraction 3; lane (C), 2 μg rEST-6 PAP-agarose fraction 4; lane (D), 50 μg male rat liver cytosol.

molecular mass of the translated protein encoded by rEST-6 was 35,300 Da. The nucleotide sequence of rEST-6 was 96.7% identical to the nucleotide sequence of rEST-3. A comparison of the translated proteins showed they were 93.5% identical and 96.0% similar in amino acid sequence (Fig. 3). Nine of the 19 amino acid differences between the two sequences were conservative substitutions. Figure 4 shows a comparison of the sequence of rEST-6 with the published EST amino acid sequences of the guinea pig [12], bovine [10] and rat enzymes [11]. rEST-6 is 67.9% identical and 84.5% similar to the guinea pig EST sequence and 63.7% identical and 80.1% similar to the bovine EST, rEST-6 was 97.3% similar and 94.5% identical to a previously reported rat EST sequence.

Expression of rEST-6

Enzymatically active rEST-6 was purified by DEAE-Sepharose CL-6B and PAP-agarose affinity chromatography after expression in E. coli XL1-Blue cells using rEST-6 inserted into the pKK233-2

Fig. 5. SDS–polyacrylamide gel of rEST-6. Proteins were resolved by SDS–PAGE in a 12.5% gel, stained with Coomassie blue and destained. Lane (A), molecular weight markers; lane (B), 5 μg rEST-6 after PAP-agarose column chromatography.
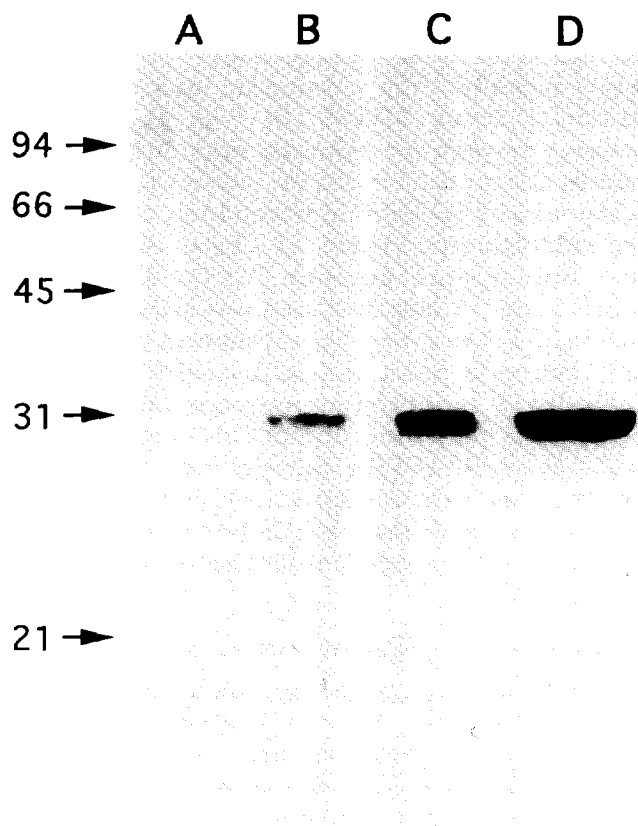
bacterial expression vector. EST activity co-eluted through both purification procedures with a protein which migrated with a molecular mass of approx. 31,000 Da during SDS–PAGE (Fig. 5). The discrepancy between the molecular mass of the protein translated from rEST-6 and the estimation of its molecular mass by SDS–PAGE is most likely an artifact of the electrophoretic system, as similar discrepancies have been reported for other STs [14, 22, 23]. The purified rEST-6 protein reacted with the rabbit anti-MBP-rEST-3 antibody (Fig. 6); however, rEST-6 did not react with rabbit anti-rat minoxidil (PST)-ST antibodies or rabbit anti-rat hydroxysteroid-ST antibodies (data not shown). Figure 6 also shows that the rabbit anti-MBP-rEST-3 antibody readily detected a protein in male rat liver cytosol which migrated with the same moleculr mass as rEST-6. In contrast, no immunoreactive protein was detectable in female rat liver cytosol.

Northern blot analysis of rat liver RNA

To further investigate the expression of EST in male and female rat liver, Northern blot analysis of RNA isolated from both sexes was carried out. Total RNA was isolated from the livers of male and female rats and analyzed by Northern blot analysis using the rEST-6 cDNA as a probe. Figure 7 shows that the rEST-6 cDNA readily hybridized to a band of approx. 1400 nucleotides in RNA prepared from male rat liver. No detectabale hybridization was observed with the RNA prepared from female rat liver. The male-specific expression of EST in rat liver is in agreement with the previously reported sexual dimorphism of ST expression in rodents [1, 11, 13, 24]. The multiple bands may be due to the presence of multiple forms of rEST or different sites of polyadenylation in the rEST messages [14, 22, 23].

Kinetic analysis of expressed rEST-6

The ability to express and purify rEST-6 from bacterial cytosol provided active enzyme for the initial characterization of its reactivity with steroids and of its kinetic properties. rEST-6 was capable of sulfating both β-E_2 and E_1 but no activity was observed with DHEA, testosterone, pregnenolone or cortisol. The effect of varying the concentrations of β-E_2 and E_1 on EST activity was tested. Figure 8 shows that maximal activity was observed with a β-E_2 concentration of approx. 1 μM and above that concentration substrate inhibition was observed. E_1 sulfation by rEST-6 also showed maximal activity at a concentration of 1 μM. The specific activity of rEST-6 was approx. 2-fold greater with β-E_2 as substrate than with E_1. Other STs which are capable of conjugating estrogens have shown a partial dependence on Mg^{2+} ions for maximal activity [6, 18]. However, varying the Mg^{2+} concentration (0–10 mM) in the β-E_2 sulfation reactions did not affect the sulfation activity of rEST-6.
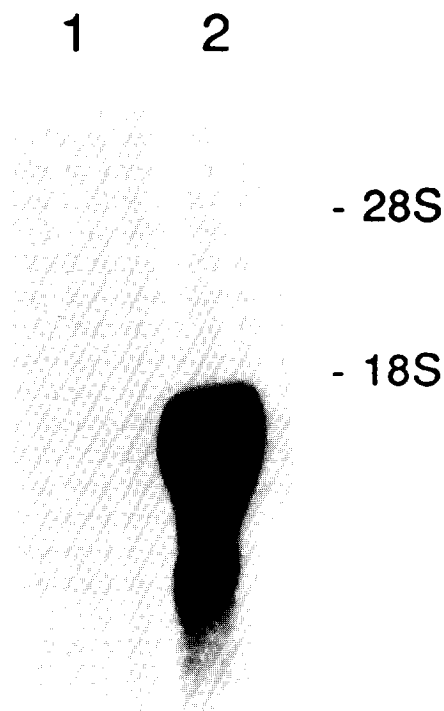
1   2

Fig. 7. Northern blot analysis of male and female rat liver RNA with rEST-6 cDNA as a probe. Total RNA samples from male and female adult rat liver were resolved in a 1.5% agarose–formaldehyde gel. The RNA was transferred to nitrocellulose and hybridized with [^{32}P]-labeled rEST-6. The autoradiograph of the blot is shown. The RNA fractions were as follows: 30 μg female rat liver total RNA (lane 1) and 30 μg male rat liver total RNA (lane 2). The migration of ribosomal RNA is indicated on the right.
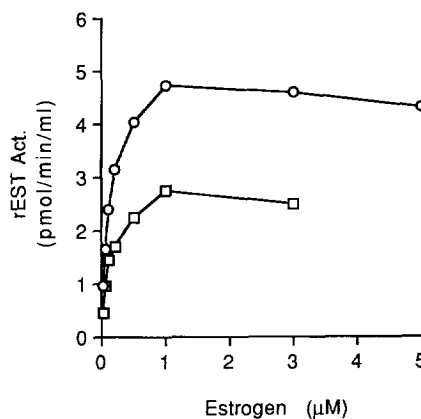

Fig. 8. Sulfation of β-E_2 and E_1 by expressed rEST-6. The effect of increasing β-E_2 and E_1 concentration on rEST-6 activity is shown. rEST-6 was expressed in bacteria and partially purified by DEAE-Sepharose CL-6B chromatography. rEST-6 activity was assayed with varying concentrations of β-E_2 and E_1 (0.025–5 μM) in the presence of 300 μM PAPS.

DISCUSSION

This paper describes the cloning, bacterial expression, purification and immunological and kinetic characterization of a new isoform of rat liver EST, rEST-6. Although three other mammalian ESTs have been expressed in mammalian cells, to date there has been no kinetic characterization of the expressed EST activities other than to report that they conjugate estrogens [11, 12]. Expression of high levels of rEST-6 activity in a bacterial expression system and subsequent purification of rEST-6 have allowed us to begin the examination of the kinetic characteristics of this important enzyme.

The enzymatic characterization of rEST-6 demonstrates that this ST sulfates only estrogens, such as β-E_2 and E_1, and does not sulfate DHEA, testosterone, pregnenolone or cortisol. Thus, rEST-6 is more specific for estrogen sulfation than are the PSTs and hydroxysteroid STs which may sulfate estrogens but only as part of a much broader spectrum of substrates [7]. Maximal sulfation activity towards both β-E_2 and E_1 occurred at a substrate concentration of about 1 μM. The maximal sulfation activity of β-E_2 by rEST-6 occurs at a lower concentration (1 μM) than the maximal activities for either hP-PST (6 μM) or hDHEA-ST (20 μM), which also sulfate estrogens [7]. Substrate inhibition has been reported for steroid sulfation by other purified and expressed STs [7, 18] and was observed for both β-E_2 and E_1 sulfation by rEST-6 at concentrations greater than 1 μM. Unlike other STs capable of sulfating estrogens, rEST-6 showed no dependence upon the presence of Mg^{2+} ions in the reaction mixture. Thus, rEST-6 sulfates estrogens in a manner different from other STs and, due to its higher affinity for estrogens, may play a distinct role in estrogen metabolism.

Sequence comparison of rEST-6 to rEST-3, as well as to the rat liver EST cloned and sequenced by Demyan et al. [11], indicates that rEST-3 and rEST-6 are isoforms rather than alleles of rat EST; however, rEST-3 and the EST reported by Demyan et al. [11] are apparently allelic forms of the same enzyme. Three amino acid differences were noted between these sequences. These differences may have arisen during the PCR amplification of rEST-3 or represent minor allelic differences between different sources of rat liver mRNA. Immunological comparison of rEST-6 to rEST-3 confirms that these isoforms are cross-reactive, as rEST-6 reacts strongly with a polyclonal antibody raised to rEST-3. The characterization of these rat ESTs confirms that there is a multiplicity of forms of EST present in rats, similar to the pattern described by Oeda et al. [12] for the guinea pig in which four individual but immunologically cross-reactive isoforms of EST occur. The multiplicity of closely related STs is apparently the norm in rats, which have multiple forms of phenol-sulfating STs as well as hydroxysteroid STs.

The rat EST described by Demyan et al. [11] shows very distinct sex differences, occurring in liver cytosol of young adult male rats but not at all in cytosol prepared from female rat liver, as determined by immunoblot analysis. Similarly, immunoblot analysis of male and female rat liver cytosols with the antibody to rEST-3 yields a strong immunoreactive band of about 31 kDa in male rat liver cytosol but not in female rat liver cytosol. These results confirm that there is selective sexual expression of EST activity in male vs female rats [1, 11, 13]. It cannot be determined from immunoblot analysis which isoform or isoforms of EST are present in male rat liver, as they are immunologically cross-reactive. Northern blot analysis of RNA isolated from male and female rat livers with rEST-6 cDNA as a probe correlates with the immunoblot analysis; the rEST-6 cDNA also hybridizes with the other rat EST cDNAs. The rEST-6 cDNA hybridizes to a band in male rat liver RNA but does not hybridize to female rat liver RNA. Both immunoblot and Northern blot analyses confirm that there are sexual differences in the levels of EST expression in rat liver. Whether the different EST activities are selectively expressed in other tissues, and whether one isoform is selectively expressed over another, has not yet been determined.

The identification of rEST-6, an ST which is relatively specific for the sulfation of estrogens, adds to the number of isoforms of cytosolic ST reportedly present in rat liver. Characterization of the physical, regulatory and kinetic properties of the individual ST isoforms is important in determining the functions of these enzymes in steroid and drug metabolism. To this end, the ability to express and purify the individual ST isoforms will be a powerful tool in their characterization, as has been demonstrated with rEST-6.

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