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CLONING AND SEQUENCE ANALYSIS OF A RAT LIVER cDNA ENCODING HYDROXYSTEROID SULFOTRANSFERASE

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SUMMARY. Nothing has been known of the cDNAs encoding sulfotransferases (STs) that catalyze sulfation of steroids and xenobiotics. In the present study, a female Sprague-Dawley (SD) rat liver cDNA library was screened with rabbit anti-serum raised against hydroxysteroid ST a (STa) purified from female SD rat liver cytosol. The cDNA isolated from the library consisted of 1,028 base pairs which had an open reading frame of 852 base pairs encoding the entire rat ST subunit of 284 amino acids. The N-terminal amino acid sequences of STa and the rat liver hydroxysteroid ST, bile acid ST I, both elucidated previously by the chemical method, had a strong homology with that deduced from the cDNA. Northern blot analysis of total RNAs from female and male rat livers showed a marked sex difference (female >> male) in the expressed level of the mRNA for the predicted ST subunit protein. A remarkable sex difference (female >> male) was also observed by immuno-blot analysis in the level of the hydroxysteroid ST protein(s) cross-reacting with the anti-serum in the rat liver cytosols.

Hydroxysteroid (or alcohol) sulfotransferases (STs), together with phenol STs, play important roles not only in transformation of hydrophobic steroidal and xenobiotic alcohols and phenols into hydrophilic forms for excretion (1-2), but also in biosynthesis of steroids (3) and metabolic activation of carcinogenic polycyclic arenes with a hydroxymethyl (4-7) or N-hydroxy group (8-12). Rat liver cytosolic hydroxysteroid STs have been extensively studied mainly by Jakoby (1,13), Singer (2) and their coworkers. In the rat liver cytosol, several isozymes of hydroxysteroid ST are known to exist, which are classified into several groups depending on their properties and substrate specificities (1,2,13). However, no information has been available for the amino acid sequences of these isozymes, determined by cDNA cloning.

Recently, Barnes, et al. (14) reported the N-terminal amino acid sequence of the enzyme, bile acid ST I (BAST I, subunit Mr 30,000), isolated and purified

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from the Sprague-Dawley (SD) rat liver cytosol. They suggested BAST I to be identical with the rat liver enzyme, hydroxysteroid ST 2, isolated previously by Marcus, et al. (13). So far as we know, this was the first instance for the information on the N-terminal amino acid sequence of STs catalyzing sulfation of hydrophobic substrates, such as steroids and xenobiotics. We have also very recently reported the N-terminal amino acid sequence of a homooligomeric hydroxysteroid ST with subunit Mr of 30,500 as the second instance, which was isolated and purified from the female SD rat liver cytosol (15,16). Our enzyme, named hydroxysteroid ST a (STa), had a high sulfation activity toward dehydroepiandrosterone (DHA), a typical substrate for hydroxysteroid STs (1,2,13), but had no activity toward 4-nitrophenol, a typical substrate for phenol STs (1,2). The N-terminal amino acid sequence of our enzyme had an extremely strong homology with that of BAST I, but differed from each other only by one amino acid in the first 20 N-terminal amino acid residues. The present study was undertaken to elucidate the complete primary structures of rat liver hydroxysteroid STs for identification and classification of these isozymes at the molecular level.

The present communication deals with 1) cloning of a hydroxysteroid ST cDNA screened with rabbit anti-serum raised against STa and the deduced amino acid sequence with strong homology in N-terminal sequence to STa and BAST I, 2) marked sex difference (female >> male) in the expressed level of mRNA for this predicted hydroxysteroid ST protein in the rat livers, demonstrated by Northern blot analysis, and 3) preferential expression of the protein(s) cross-reacting with the anti-serum in female to male rat liver cytosols, demonstrated by immuno-blot analysis.

MATERIALS AND METHODS

Enzyme purification and anti-serum preparation

STa was purified from the female SD rat liver as previously described (15,16). Two male New Zealand white rabbits were used for the preparation of the anti-serum raised against purified STa by the previous method (16).

cDNA library construction and screening

Total RNAs were extracted from female and male SD rat livers using the guanidine thiocyanate method (17), and poly(A)$^+$RNA was selected by oligo(dT)-cellulose chromatography (18). A female rat liver cDNA library was constructed into an EcoRI site of bacteriophage λgt11 (19). The library of 5×10^5 recombinant bacteriophages was screened with the anti-serum raised against STa in combination with horse radish peroxidase-conjugated second antibodies as described (20). Clones of positive plaques were isolated, and the phage DNAs were digested with EcoRI and separated by agarose gel electrophoresis.

Shotgun DNA sequence analysis

The largest insert (ST-20) isolated from the positive praques was sequenced by using shotgun sequence analysis (18,21) as follows: the insert was amplified in pUC19 and fragmented by sonication. The fragments were treated with S1 nuclease, repaired by DNA polymerase I (Klenow fragment), and fractionated by agarose gel electrophoresis (0.5-1kb in length). DNA sequence subcloned into a

Sma I site of M13mp9 was determined by the dideoxy chain termination method (22) using Sequenase (United States Biochemical Co., Cleveland, Ohio, USA).

Northern blot analysis

Twenty μg each of male and female SD rat liver total RNAs were electrophoresed on 1.5% agarose/formaldehyde gel (23) and transferred to a nylon membrane (24). The membrane was hybridized with [^{32}P]-labeled ST-20 cDNA, which had been prepared with a nick translation kit (Boehringer Mannheim GmbH, West Germany).

Immuno-blot analysis

Female and male SD rat liver cytosols were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a nitrocellulose membrane. The membrane was immuno-stained by using the anti-serum raised against STa as previously reported (16).

RESULTS AND DISCUSSION

Cloning and sequencing of rat liver hydroxysteroid ST

A female SD rat liver cDNA library (λgt11) was screened with the rabbit anti-serum raised against purified STa. Ninety-six positive plaques were isolated from the library. Recombinant phage DNAs were digested with EcoRI, and cDNA inserts were separated by agarose gel electrophoresis. The largest insert ST-20 was amplified in pUC19 and sequenced by shotgun sequence analysis. The strategy used to sequence the cDNA is shown in Fig. 1, and the nucleotide and deduced amino acid sequences are shown in Fig. 2. The ST-20 cDNA had 1,028 base pair nucleotides (bp), consisting of an open reading frame of 852 bp, a 37 bp non-coding region at the 5'-end upstream from the putative initiation codon ATG, and a 3'-untranslated region of 139 bp. The polyadenylation signal (25), AATAAA (at ordinates 977-982, underlined in the figure), was located by 24 nucleotides upstream from the poly(A) tract. The open reading frame encoded a polypeptide of 284 amino acid residues. The size of the cDNA accounted almost completely for the length of the messenger of 1.1kb detected by Northern blot analysis as described below.

Homology comparison of N-terminal amino acid sequences

The N-terminal amino acid sequence of the predicted protein deduced from the nucleotide sequence of the ST-20 cDNA was found to have a strong homology with those of STa and BAST I, both of which had been demonstrated by the chemical

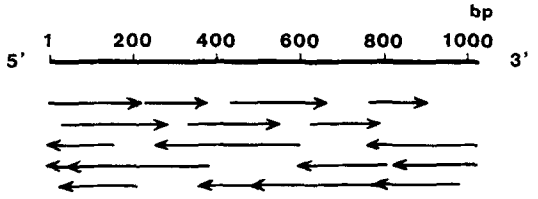

Fig. 1. Strategy for sequencing ST-20 cDNA. The axis represents the nucleotide map of the total restriction fragment in base pairs. The direction and extent of sequence determinations are shown by the horizontal arrows.

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   1  CTGGAATCCTAACAGCACCTACACAAAGCTATTTATAATGCCAGACTATACTTGGTTT
   1                                        MetProAspTyrThrTrpPhe

  59 GAAGGAATACCTTTTCATGCCTTTGGGATTTCAAAAGAAACTTTGCAAAATGTTTGTAAT
   8 GluGlyIleProPheHisAlaPheGlyIleSerLysGluThrLeuGlnAsnValCysAsn

 119 AAGTTTGTGGTGAAAGACGAAGATTTGATTTTATTGGCTTATCCCAAGTCAGGAACGAAC
  28 LysPheValValLysAspGluAspLeuIleLeuLeuAlaTyrProLysSerGlyThrAsn

 179 TGGCTGATTGAAATTGTCTGCTTGATTCAGACCAAGGGAGATCCCAAGTGGATCCAATCT
  48 TrpLeuIleGluIleValCysLeuIleGlnThrLysGlyAspProLysTrpIleGlnSer

 239 GTGACCATCTGGGATCGCTCACCCTGGATAGAGACTGATGTAGGATATGATATATTAATC
  68 ValThrIleTrpAspArgSerProTrpIleGluThrAspValGlyTyrAspIleLeuIle

 299 AAAAAGAAAGGACCACGACTCATGACCTCCCATCTTCCCATGCATCTTTTCTCCAAGTCT
  88 LysLysLysGlyProArgLeuMetThrSerHisLeuProMetHisLeuPheSerLysSer

 359 CTCTTCAGTTCCAAGGCCAAGGTGATCTATCTCGTCAGAAATCCCAGAGATGTTCTTGTT
 108 LeuPheSerSerLysAlaLysValIleTyrLeuValArgAsnProArgAspValLeuVal

 419 TCTGGTTATTATTTCTGGGGTAATTCAACTCTTGCGAAGAAGCCAGACTCACTGGGAACT
 128 SerGlyTyrTyrPheTrpGlyAsnSerThrLeuAlaLysLysProAspSerLeuGlyThr

 479 TATGTTGAATGGTTCCTCAAAGGAAATGTTCTATATGGATCATGGTTTGAGCACATCCGT
 148 TyrValGluTrpPheLeuLysGlyAsnValLeuTyrGlySerTrpPheGluHisIleArg

 539 GCCTGGCTGTCCATGCAAGAATGGGACAACTTCTTGTTACTGTACTATGAAGACATGAAA
 168 AlaTrpLeuSerMetGlnGluTrpAspAsnPheLeuLeuLeuTyrTyrGluAspMetLys

 599 AAGGATACAATGGGAACCATAAAGAAGATATGTGACTTCCTGGGGAAAAAATTAGAGCCA
 188 LysAspThrMetGlyThrIleLysLysIleCysAspPheLeuGlyLysLysLeuGluPro

 659 GATGAGCTGGATTTGGTCCTCAAGTATAGTTCCTTCCAAGTCATGAAAGAAAATGACATG
 208 AspGluLeuAspLeuValLeuLysTyrSerSerPheGlnValMetLysGluAsnAspMet

 719 TCCAATTATAGCCTCCTGATGAAAAAATCAATTTTTACTGGTACTGGCCTCATGAGGAAA
 228 SerAsnTyrSerLeuLeuMetLysLysSerIlePheThrGlyThrGlyLeuMetArgLys

 779 GGCACTGTTGGGGACTGGAAGAATCACTTCACTGTATCCCAAGCTGAAGCCTTTGATAAA
 248 GlyThrValGlyAspTrpLysAsnHisPheThrValSerGlnAlaGluAlaPheAspLys

 839 GTGTTTCAGGAGAAAATGGCCGGTTTCCCTCCAGGGATGTTCCCATGGGAATAAAATTTC
 268 ValPheGlnGluLysMetAlaGlyPheProProGlyMetPheProTrpGlu***

 899 AAAAGTTTTAAATATTTTATGAACATTGATATTTATGTTTCTGTTGTTCTATGTCTGAAT
 959 AACTGAATGTGGTCATCGAATAAATCCTGTTCTGGTATTGTGAAAAAAAAAAAAAAAAA
1019 AAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequences of ST-20 cDNA. Both nucleotides and deduced amino acids are numbered on the left. The putative polyadenylation signal AATAAA is underlined. The stop codon (***) is located at ordinates 890-892.

method to have Pro as a common N-terminal amino acid (Fig. 3). The sequence of the first 20 N-terminal amino acid residues of the predicted protein, excluding the leading amino acid Met as illustrated in Fig. 3, differs only by two (12th and 17th) and only by one (12th) amino acid residues from those of BAST I and

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STa       PDYTWFEGIPFPAFGIPKET
BAST I    PDYTWFEGIPFPAFGISKETLQDV-NKSV-KDXDLI
ST-20   (M)PDYTWFEGIPFHAFGISKETLQNVCNKFVVKDEDLI
          1    5    10   15   20   25   30   35
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Fig. 3. Comparison of N-terminal amino acid sequences of STa, BAST I, and predicted protein deduced from ST-20 cDNA. Amino acid sequences are given in the conventional single letter code. In the sequence of BAST I, deletions are indicated by the dashes, and "X" indicates an amino acid not determined. Identities between the amino acids of the predicted protein and those of the other two enzymes are boxed.

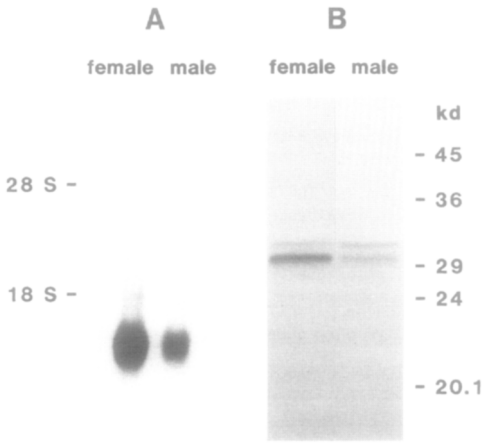

Fig. 4. Northern blot analysis of female and male total RNAs and immuno-blot analysis of female and male cytosols. (A) Twenty μg each of total RNAs prepared from female and male rat livers were separated on a agarose/formamide gel followed by Northern blot analysis. The membrane was hybridized with [^{32}P]-labeled ST-20 cDNA prepared by nick-transration as described in Methods. Positions of the 28 S and 18 S ribosomal subunits are indicated on the left side of figure. (B) Female and male rat liver cytosols were resolved by SDS-PAGE and transferred to a nitrocellulose membrane and immuno-stained by using rabbit anti-STa anti-serum. Horizontal bars on the right side of figure indicate Mr markers; ovalbumin (45,000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36,000), bovine erythrocyte carbonic anhydrase (29,000), bovine pancreas trypsinogen (24,000), and soybean trypsin inhibitor (20,100).

STa, respectively. The 12th amino acid residue, however, is identical between STa and BAST I. On the contrary, there exists a weak homology in the sequence of the 23rd to 36th amino acid residues from the N-terminal (Pro) of the predicted protein and BAST I, the latter of which includes deletion of two amino acids compared with that of the former. The homology comparison of the N-terminal amino acid sequences indicates that the N-terminal Pro of these hydroxysteroid STs is to be located next to the initiating amino acid Met. These results indicate that the initiation codon ATG for the predicted ST protein subunit is located at ordinates 38-40 in the ST-20 cDNA.

Northern- and immuno-blot analyses

Total RNAs from female and male rat livers were separated, blotted and hybridized to the ST-20 cDNA nick-translated with [^{32}P]dCTP. A 1.1kb band was detected in the female and male liver total RNAs as shown in Fig. 4A. However, there was a marked difference in intensity of the 1.1kb band between the RNAs from the animals of both sexes (female >> male), suggesting a marked sex difference in the expressed levels of the mRNA(s) related very closely to STa (Fig. 4A).

Immuno-blot analysis, carried out by using the anti-serum against STa, indicated that the female rat liver cytosol contained a much higher level of

protein(s) (Mr 30,500) cross-reacting with the polyclonal antibody than did the cytosol from the male rat liver (Fig. 4B). The liver cytosols also contained a very low level of another immunodetectable protein (Mr 32,000) with little sex difference.

The above results would coincide with the well known fact that, in the SD rat liver, the hydroxysteroid ST activity measured with DHA or cortisol is much higher in female than in male (1,2,13,26,27).

Thus, the present investigation provides the first evidence for the entire nucleotide sequence of the cDNA encoding an ST subunit in the rat liver. A further study in progress in our laboratory on the sequences of the other cDNAs from the library used in the present investigation will make it possible to identify and classify the ST isozymes readily and accurately at the molecular level although, in spite of many efforts, no crucial evidence has been provided by the classical methods used until now for the identity of these enzymes with strong homology.

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