Cloning and expression of human liver dehydroepiandrosterone sulphotransferase

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Dehydroepiandrosterone sulphotransferase (DHEA-ST) catalyses the 3′-phosphoadenosine 5′-phosphosulphate-dependent sulphation of a wide variety of steroids in human liver and adrenal tissue and is responsible for most, if not all, of the sulphation of bile acids in human liver. This report describes the isolation, characterization and expression of a cDNA which encodes human liver DHEA-ST. The DHEA-ST cDNA, designated DHEA-ST8, was isolated from a Uni-Zap XR human liver cDNA library and is composed of 1060 bp and contains an open reading frame encoding a 285-amino-acid protein with a molecular mass of approx. 33765 Da. Translation of DHEA-ST8 *in vitro* generated a protein identical in molecular size with that of DHEA-ST. Expression of DHEA-ST8 in COS-7 cells produces an active DHEA-ST protein which is capable of sulphating DHEA, has the same molecular mass as human liver DHEA-ST and is recognized by rabbit anti-(human liver DHEA-ST) antibodies. Northern-blot analysis of human liver RNA detects the presence of three different size transcripts; however, Southern-blot analysis of human DNA suggests that only one gene may be present in the genome. These results describe the cloning of a human ST which has an important role in the sulphation of steroids and bile acids in human liver and adrenals.

INTRODUCTION

Sulphation is an important conjugation reaction in the metabolism of drugs, xenobiotics and endogenous compounds. The cytosolic sulphotransferases (STs) are the family of enzymes responsible for catalysing the transfer of the sulphonate moiety from 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to the hydroxy or amine functional group of an acceptor compound to form a sulphate ester or sulphamate, respectively [1]. One of the major roles of the STs in the metabolism of drugs and endogenous compounds is the conversion of these substances into more hydrophilic water-soluble sulphate conjugates that can be easily excreted. However, sulphation may also function in a regulatory role for many endogenous compounds, such as steroids and neurotransmitters, by altering the biological properties of these compounds [2].

In the liver, sulphation plays a major role in the biotransformation and detoxification of steroids and bile acids by producing sulphate conjugates that are easily excreted into the bile or urine [3]. The sulphation of bile acids decreases their reabsorption from the small intestine and protects tissues from the toxic detergent effects of several bile acids, such as lithocholic acid and chenodeoxycholic acid [4]. Sulphation also is an important process in the synthesis and secretion of several steroids from the human adrenal cortex. Dehydroepiandrosterone sulphate (DHEAS) is quantitatively one of the major steroids secreted from the adrenal cortex and is present at micromolar concentrations in the plasma [5]. Recent reports have indicated that DHEAS plays a role in the softening of the cervix before parturition [6,7]. Also, DHEAS has been shown to have a protective effect against obesity [8] and cardiovascular disease [9].

Several different forms of STs capable of sulphating steroids and bile acids have been isolated and characterized from rodent, guinea pig, and bovine liver or adrenal tissue [10–13]. These enzymes represent a heterogeneous family of isoenzymes that often have different, but overlapping, substrate specificities [14,15]. Three of the cDNAs encoding rat liver hydroxysteroid STs have been characterized and shown to have considerable sequence similarity to one another [16–18]. However, the purification and characterization of only one of these rat hydroxysteroid STs, STa-20, has been reported [19].

Unlike the heterogeneous family of rat steroid STs, only one form of steroid ST, dehydroepiandrosterone ST (DHEA-ST), has been purified from human liver tissue [20]. DHEA-ST has also been shown to be responsible for most, if not all, of the bile acid sulphation activity in human liver cytosol [21]. DHEA-ST is also capable of sulphating a wide variety of steroids which possess a 3′-hydroxy group, as well as oestrone and testosterone. Recently, the human adrenal form of DHEA-ST has been purified and shown to be physically, immunologically and kinetically similar to human liver DHEA-ST [22].

The present paper describes the molecular cloning, sequence analysis, molecular characterization and expression in green-monkey COS-7 cells of a cDNA coding for human liver DHEA-ST. The results of these studies show that the DHEA-ST cDNA encodes a single protein that is capable of sulphating DHEA and is physically identical with purified human liver DHEA-ST. The isolation and characterization of the cDNA for DHEA-ST provides a valuable tool for further studies of the role and regulation of this enzyme in steroid and bile-acid metabolism.

MATERIALS AND METHODS

Materials

The human liver Uni-Zap XR cDNA library and *Escherichia coli*

Abbreviations used: DHEA-ST, dehydroepiandrosterone sulphotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; DHEAS, dehydroepiandrosterone sulphate; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate; TFA, trifluoroacetic acid; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle medium; FBS, fetal-bovine serum; TEA, triethanolamine; PVDF, poly(vinylidene difluoride); poly(A)$^+$, polyadenylated.

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XL1-Blue cells were purchased from Stratagene, La Jolla, CA, U.S.A. The pGem 7ZF vector was purchased from Promega, Madison, WI, U.S.A. pSV-SPORT-1 expression vector, Dulbecco's modified Eagle medium (DMEM), OPTI-MEM serum-free medium, and Lipofectin were obtained from GIBCO–BRL, Grand Island, NY, U.S.A. Fetal-bovine serum (FBS) was purchased from Hyclone Laboratories, Logan, UT, U.S.A. COS-7 cells were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Sequenase Version 2.0 DNA sequencing kit and some restriction enzymes were purchased from United States Biochemical, Cleveland, OH, U.S.A. Restriction and DNA-modifying enzymes were purchased from New England Biolabs, Beverly, MA, U.S.A. The [α-^{35}S]dATP (3000 Ci/mmol), [α-^{32}P]dCTP (800 Ci/mmol), [^{35}S]methionine (1232.7 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A. Magnagraph nylon transfer membrane was purchased from Mircon Separation, Westbourough, MA, U.S.A. Nitrocellulose paper was obtained from Schleicher and Schuell, Keene, NH, U.S.A. Nick-translation kits were purchased from Amersham, Arlington Heights, IL, U.S.A. The MAXI-script *in vitro* transcription and ReticLysate IVT translation kits were purchased from Ambion, Austin, TX, U.S.A. Sequencing-grade trypsin was obtained from Boehringer-Mannheim, Indianapolis, IN, U.S.A. Oligonucleotides were synthesized by Midland Certified Reagent Co., Midland, TX, U.S.A. Normal human livers were obtained through the Organ Procurement Program at the University of Rochester during the removal of other organs for transplantation. All other reagents were of molecular-biology grade.

Methods

Isolation of DHEA-ST cDNA

Human liver DHEA-ST cDNAs were isolated from a human male liver Uni-Zap XR library that had been amplified once and contained approx. 1.0×10^6 independent recombinant plaque-forming units. The library was grown in the *E. coli* XL1-Blue host strain on 150 mm Petri dishes at a phage concentration of 40000–50000 plaques/plate. Duplicate nitrocellulose filters of each plate were probed with either a nick-translated full-length putative rat hydroxysteroid ST cDNA (ST2-4) or a '^{32}P-kinased' 30-mer antisense oligonucleotide that showed 90% sequence similarity to the 5'-sequence of the rat hydroxysteroid ST cDNA, STa-20, (bases 38–67) described by Ogura et al. [17]. The ST2-4 cDNA, which was isolated from a female λ-gt11 rat liver library, is composed of 1065 bp. The nucleotide sequence of the ST2-4 cDNA shows 90% sequence similarity to the nucleotide sequence of STa-20.

Approx. 500000 independent clones from the Uni-Zap XR library were screened initially, and clones that hybridized to both probes were purified by repeated cycles of dilution and rescreening [23]. Hybridization of filters with the nick-translated rat hydroxysteroid ST cDNA was performed at 55 °C overnight using 5.0×10^5 c.p.m. of the labelled cDNA (1.0×10^7 c.p.m./μg)/ml in hybridization buffer [6 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.5% SDS, 10 × Denhardt's solution and 200 μg/ml sheared salmon sperm DNA]. Hybridization of filters with the 30-mer ^{32}P-kinased oligonucleotide probe was done overnight at 45 °C using 1.0×10^6 c.p.m. of the kinased probe/ml in hybridization buffer. The filters were washed twice at 50 °C for 15 min with 3 × SSC containing 0.5% SDS, followed by a 2.5 × SSC/0.5% SDS wash and a 2 × SSC/0.5% SDS wash. The filters were then dried and exposed to X-ray film at −70 °C.

In all, 20 different full-length positive clones were isolated after four cycles of dilution and rescreening of the original positive clones. The cDNAs were isolated from the Uni-Zap XR vector in Bluescript plasmids as described by the manufacturer for further characterization.

DNA sequence analysis of DHEA-ST cDNAs

DNA sequencing was performed by the Sequenase 2.0 dideoxynucleotide chain-termination method using [α-^{35}S]dATP to label the synthesized DNA fragments. To sequence the DHEA-ST cDNAs, restriction fragments of the cDNAs were subcloned into the pGem 7ZF vector. The restriction fragments were resolved in 1.5%-agarose gels, recovered using DE81 paper and subcloned into the appropriately restriction-enzyme-digested pGEM 7ZF [23]. The largest restriction fragment, an *Eco*RI–*Xho*I fragment (889 bp), was further sequenced by shotgun sequence analysis using either *Sau*3A, *Hae*III, or *Alu*I restriction digests. Transformation of DH5αF′ cells with the restriction fragments subcloned into pGEM 7zf was performed by a $CaCl_2$ method [24]. All of the pGem 7ZF clones were sequenced using SP6 and T7 primers to allow sequence analysis of both strands of the subcloned cDNA fragments.

The sequencing reactions were resolved in 6%-polyacrylamide/urea buffer gradient gels. Sequence gels were read manually and each nucleotide was read two or three times in each strand. The DHEA-ST cDNAs and translated amino acid sequences were analysed by using the University of Wisconsin Genetics Computer Group's programs [25].

Amino-acid-composition analysis of human liver DHEA-ST

In preparation for the amino-acid-composition and amino-acid-sequence analyses, DHEA-ST was purified from human liver cytosol as described by Falany et al. [20] and then further purified by h.p.l.c. Purified DHEA-ST was applied to a Vydac C_{18} column (46 mm × 250 mm) and eluted with a linear gradient of 0–80% (v/v) acetonitrile in the presence of 0.1% (v/v) trifluoroacetic acid (TFA). Elution of protein from the C_{18} column was monitored with a Perkin–Elmer model 235 diode-array detector at wavelengths of 215 nm and 280 nm. The amino-acid-composition analysis was performed by the Protein Structure Facility of the University of Iowa using a Beckman 6300 amino acid analyser. DHEA-ST was subjected to 24, 48 and 72 h HCl hydrolysis, and the amino acid composition was calculated for a subunit molecular mass of 35000 Da. Performic acid oxidation was also carried out for the determination of cysteine and methionine residues.

Amino-acid-sequence analysis of human liver DHEA-ST

Initial attempts at amino-acid-sequence analysis of DHEA-ST determined that the N-terminal end of DHEA-ST was not available for sequencing. Therefore amino acid sequences were obtained from h.p.l.c.-purified CNBr- or trypsin-derived fragments of purified DHEA-ST [26].

For cleavage with CNBr, approx. 0.5 mg of purified DHEA-ST was resolved from contaminating proteins by SDS/PAGE, electrophoretically transferred to a hydrophobic poly(vinylidene difluoride) (PVDF) membrane and detected by staining with 0.5% Ponceau S in 0.1% acetic acid. The DHEA-ST band was excised from the filter and placed in a microcentrifuge tube containing 20 mg/ml of freshly prepared CNBr (Kodak) in 70% (v/v) formic acid. Digests were then incubated at room temp for 20 h in the dark. After 20 h, the formic acid solution was removed, the filters were washed with propan-2-ol/TFA/water

(140:1:59, by vol.) two solutions were combined and freeze-dried..

For digestion with trypsin, approx. 100 μg of h.p.l.c.-purified DHEA-ST was resuspended in 50 μl of 0.4 M ammonium bicarbonate, pH 8.0, and the pH of the mixture was adjusted to 7.5–8.0 with NaOH. Prior to digestion, DHEA-ST was reduced in the presence of 4 mM dithiothreitol (DTT) at 50 °C for 15 min and then acetylated in the presence of 8 mM iodoacetamide in the dark for another 15 min. The volume was then adjusted to 200 μl with water, 5.0 μl of freshly prepared 1.0 mg/ml trypsin in 0.1 % TFA was added, and the mixture was incubated for 24 h at 37 °C. The reaction was stopped by the addition of 5.0 μl of 10 % TFA and freeze-dried.

After freeze-drying the CNBr- and trypsin-generated peptides were resuspended in water containing 0.1 % TFA and separated by reverse-phase h.p.l.c. using a linear gradient of 0–80 % (v/v) acetonitrile as described previously. Peaks absorbing at both 215 nm and 280 nm were collected and frozen at −70 °C prior to sequence analysis by the Protein Structure Facility at the University of Iowa and by Dr. B. Gurrinder at the State University of New York at Buffalo.

Northern-blot analysis of human liver RNA

Total RNA was purified from normal human liver samples using the acid guanidinium thiocyanate method of Chomczynski and Sacchi [27]. Polyadenylated [poly(A)$^+$]RNA was selected using oligo(dT)–cellulose according to the method of Davis et al. [24]. For Northern-blot analysis, aliquots of poly(A)$^+$ RNA were resolved by electrophoresis in a 1.5 %-agarose/formaldehyde denaturing gel [24]. The poly (A)$^+$ RNA was transferred to nitrocellulose membranes, baked *in vacuo* at 80 °C for 2 h, and prehybridized for 2 h at 42 °C in 50 % formamide/10 × Denhardt's solution/800 mM NaCl/10 mM Tris/HCl (pH 7.2)/1 mM EDTA (pH 8.0)/0.5 % SDS/75 μg/ml poly-(A)/50 μg/ml yeast tRNA. Filters were then hybridized at 42 °C overnight in fresh solution containing 1.0×10^6 c.p.m. of the ^{32}P-nick-translated DHEA-ST cDNA/ml. The next day the filters were washed several times with 3 × SSC, 2 × SSC and 1 × SSC at 65 °C for 10 min each. The filters were then dried and exposed to autoradiograph film for a period of 10–48 h at −70 °C with an intensifying screen.

Southern-blot analysis of human liver genomic DNA

Genomic DNA was prepared from approx. 2 g samples of normal human liver samples by the method of Strauss [28]. For Southern-blot analysis, aliquots (10 μg) of genomic DNA were exhaustively digested with the restriction enzymes *Bam*HI, *Xho*I, *Xba*I, *Hind*III and *Pst*I, and the digested genomic DNA was resolved by electrophoresis in a 0.8 %-agarose gel. After electrophoresis, the DNA was acid-treated, denatured, then transferred to a nylon membrane. Filters were then hybridized overnight at 42 °C in 50 % deionized formamide/5 × SSC/10 mM Tris-HCl (pH 7.5)/4 × Denhardt's/200 μg/ml salmon sperm DNA/200 μg/ml poly(A)$^+$, containing 1.0×10^6 c.p.m. of the ^{32}P-nick-translated DHEA-ST cDNA/ml. The next day the filters were washed for 10 min at 65 °C with 3 × SSC containing 0.5 % SDS, followed by a 10 min wash with 2 × SSC and then 1 × SSC containing 0.5 % SDS before being dried and exposed to autoradiograph film at −70 °C for 4 days.

Transcription and translation of DHEA-ST8 *in vitro*

For transcription, translation and expression of DHEA-ST8 in COS-7 cells *in vitro*, the cDNA was subcloned into the mammalian expression vector pSV-SPORT-1. To insure that the DHEA-ST cDNA would be subcloned into pSV-SPORT-1 in the proper orientation, the cDNA was first isolated from Bluescript by digestion with *Xba*I and *Kpn*I, and then subcloned into the same sites of the pGem 7ZF vector. The cDNA was then isolated from pGem 7ZF by digestion with *Eco*RI and *Hind*III and subcloned into the *Eco*RI and *Hind*III sites of pSV-SPORT-1. To determine if the DHEA-ST8 cDNA could be properly transcribed and translated *in vitro*, the pSV-SPORT-1-DHEA-ST8 and pGEM 7ZF-DHEA-ST8 DNAs were linearized with *Hind*III, and RNA transcripts were synthesized using the MAXIscript Transcription kit with RNA SP6 polymerase. The integrity and size of the generated transcripts was monitored by denaturing agarose/formaldehyde-gel electrophoresis [24].

Approx. 0.2 μg of RNA transcripts generated by transcription of DHEA-ST8 *in vitro* in both the pGEM 7ZF and pSV-SPORT-1 vectors were translated with a ReticLysate IVT kit using [^{35}S]methionine for labelling of the synthesized proteins. Aliquots of the translation reactions were resolved by SDS/PAGE in 12.5 %-polyacrylamide gels as described previously [20]. The gels were vacuum-dried on to filter paper at 65 °C and exposed to autoradiograph film overnight to detect the translated proteins labelled by [^{35}S]methionine incorporation.

Transient transfection of DHEA-ST-8 in COS-7 cells

For the expression studies, COS-7 green-monkey kidney cells were maintained in DMEM with high glucose supplemented with 10 % FBS. Transfections were carried out by a liposome-mediated transfection procedure using Lipofectin and OPTI-MEM I medium as given in the manufacturer's instructions. Approx. 8.0 μg of supercoiled pSV-SPORT-1 or pSV-SPORT-DHEA-ST8, mixed with 30 μg of Lipofectin, were used to transfect a 60 mm plate of Cos-7 cells at approx. 50 % confluency. The DNA/Lipofectin mixture was applied dropwise to each plate with mixing, and the cells were incubated at 37 °C under 5 % CO_2 for 24 h. At this time, the OPTI-MEM I medium was removed, and 3.0 ml of DMEM containing 10 % FBS was added to each plate. The cells were then incubated for 3 days. The medium was changed once after the first 24 h.

To detect the expression of DHEA-ST, cytosol from the transfected COS-7 cells was prepared for enzyme assays and immunoblot analysis. Transfected cells were washed twice with 10 mM triethanolamine buffer (TEA), pH 7.5, containing 1.5 mM DTT, 10 % glycerol and 1 mM phenylmethanesulphonyl fluoride and harvested by scraping into cold TEA buffer. Cytosol was prepared from the transfected cells and was either assayed for DHEA-ST activity was described previously [20] or resolved by SDS/PAGE for immunoblot analysis of expressed DHEA-ST with rabbit anti-(human liver DHEA-ST) antibodies [22].

RESULTS

DNA sequence analysis of DHEA-ST8

The DHEA-ST clones were isolated by screening a human liver Uni-Zap XR library with a full-length putative rat hydroxysteroid cDNA and a 30-mer oligonucleotide synthesized to the rat hydroxysteroid ST, STa-20. In all, 20 full-length positive clones were isolated and partially sequenced to determine their relationship to one another. The 5′- and 3′-ends of the open reading frames, as well as the 3′-non-translated regions of all the cDNAs, were identical. The differences between the clones were in the size and sequence of the 5′-non-translated regions. All of the cDNAs sequenced fell into one of three groups based on the size and sequence of the 5′-non-translated region (Figure 1a).

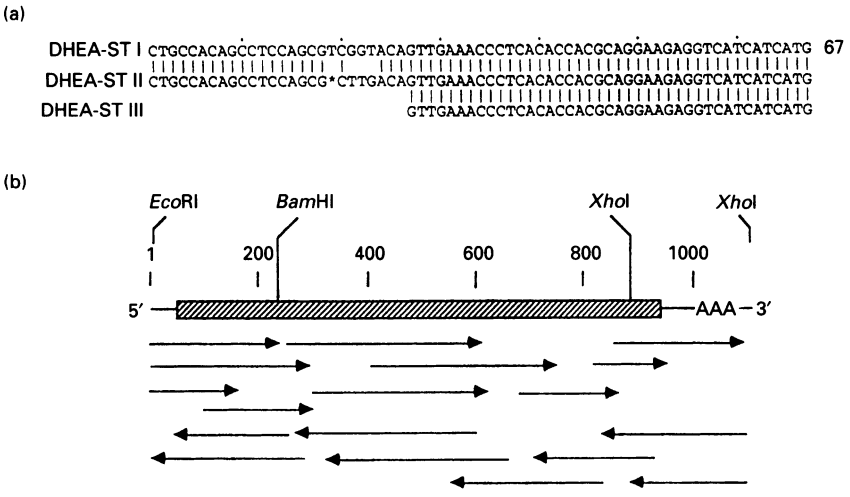

Figure 1 (a) Comparison of the 5′-non-translated region of the three different types of DHEA-ST cDNAs, DHEA-ST I, II and III, isolated from the human liver cDNA library and (b) a partial restriction map and the strategy for sequencing DHEA-ST8

(**a**) The parallel bars between the three sequences indicate nucleotides that are identical. A one-nucleotide gap has been inserted into the sequence of DHEA-ST II for optimal sequence alignment, as indicated by the asterisk (*). (**b**) The protein coding region is represented by the box with hatched lines. The direction and extent of the representative nucleotide sequences obtained from the sequence analysis of DHEA-ST8 are illustrated by the horizontal arrows.

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                GAATTCGGCACGAGGTTGAAACCCTCACACCACGCAGGAAGAGGTCATCATC

ATGTCGGACGATTTCTTATGGTTTGAAGGCATAGCTTTCCCTACTATGGGTTTCAGATCC
MetSerAspAspPheLeuTrpPheGluGlyIleAlaPheProThrMetGlyPheArgSer  20

GAAACCTTAAGAAAAGTACGTGATGAGTTCGTGATAAGGGATGAAGATGTAATAATATTG
GluThrLeuArgLysValArgAspGluPheValIleArgAspGluAspValIleIleLeu  40

ACTTACCCCAAATCAGGAACAAACTGGTTGGCTGAGATTCTCTGCCTGATGCACTCCAAG
ThrTyrProLysSerGlyThrAsnTrpLeuAlaGluIleLeuCysLeuMetHisSerLys  60

GGGGATGCCAAGTGGATCCAATCTGTGCCCATCTGGGAGCGATCACCCTGGGTAGAGAGT
GlyAspAlaLysTrpIleGlnSerValProIleTrpGluArgSerProTrpValGluSer  80
_____Pro_____

GAGATTGGGTATACAGCACTCAGTGAAAGCGAGAGTCCACGTTTATTCTCCTCCCACCTC
GluIleGlyTyrThrAlaLeuSerGluSerGluSerProArgLeuPheSerSerHisLeu 100

CCCATCCAGTTATTCCCCAAGTCTTTCTTCAGTTCCAAGGCCAAGGTGATTTATCTCATG
ProIleGlnLeuPheProLysSerPhePheSerSerLysAlaLysValIleTyrLeuMet 120
                                                        ____Asp___

AGAAATCCCAGAGATGTTTTGGTGTCTGGTTATTTTTTCTGGAAAAACATGAAGTTTATT
ArgAsnProArgAspValLeuValSerGlyTyrPhePheTrpLysAsnMetLysPheIle 140

AAGAAACCAAAGTCATGGGAAGAATATTTTGAATGGTTTTGTCAAGGAACTGTGCTATAT
LysLysProLysSerTrpGluGluTyrPheGluTrpPheCysGlnGlyThrValLeuTyr 160

GGGTCATGGTTTGACCACATTCATGGCTGGATGCCCATGAGAGAGGAGAAAAACTTCCTG
GlySerTrpPheAspHisIleHisGlyTrpMetProMetArgGluGluLysAsnPheLeu 180

TTACTGAGTTATGAGGAGCTGAAACAGGACACAGGAAGAACCATAGAGAAGATCTGTCAA
LeuLeuSerTyrGluGluLeuLysGlnAspThrGlyArgThrIleGluLysIleCysGln 200

TTCCTGGGAAAGACGTTAGAACCCGAAGAACTGAACTTAATTCTCAAGAACAGCTCCTTT
PheLeuGlyLysThrLeuGluProGluGluLeuAsnLeuIleLeuLysAsnSerSerPhe 220

CAGAGCATGAAAGAAAACAAGATGTCCAATTATTCCCTCCTGAGTGTTGATTATGTAGTG
GlnSerMetLysGluAsnLysMetSerAsnTyrSerLeuLeuSerValAspTyrValVal 240

GACAAAGCACAACTTCTGAGAAAAGGTGTATCTGGGGACTGGAAAAATCACTTCACAGTG
AspLysAlaGlnLeuLeuArgLysGlyValSerGlyAspTrpLysAsnHisPheThrVal 260

GCCCAAGCTGAAGACTTTGATAAATTGTTCCAAGAGAAGATGGCAGATCTTCCTCGAGAG
AlaGlnAlaGluAspPheAspLysLeuPheGlnGluLysMetAlaAspLeuProArgGlu 280

CTGTTCCCATGGGAATAACGTCCAAAACACTCTGGATCTTATATGGAGAATGACATTGAT
LeuPheProTrpGlu*** 285

TCTCCTGTCCTTGTACATGTACCTGACTGGGGTCATTGTGTAAGACTTATTATTTTATCC
TGAAACCTTTATAAACAAACCTCTGCAAAAAAAAAAAAAAAAAAAAAACTCGAG
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Figure 2 Nucleotide sequence and translation of DHEA-ST8

The amino acid sequence is numbered to the right. Underlined regions correspond to sequences obtained from direct sequence analysis of cyanogen bromide and tryptic peptides derived from purified DHEA-ST. The stop codon (***) is located at base positions 907–909.

The DHEA-ST cDNA, DHEA-ST8, used for these studies possessed a complete open reading frame and poly(A)$^+$ tract, but contained only 25 bp of the 5′-non-translated region. The 25 bp region of DHEA-ST8 was common to all of the clones. A partial restriction map and the sequencing strategy for DHEA-ST8 are shown in Figure 1(b).

The nucleotide sequence and translation of DHEA-ST8 are shown in Figure 2. The DHEA-ST8 cDNA is 1060 bp in length and contains an open reading frame beginning at base 53, which encodes a 285-amino-acid protein. The termination codon (TAA), located at base positions 907–909, is followed by 165 nucleotides of 3′-non-translated sequence, including a 20-base poly(A)$^+$ tract. DHEA-ST8 encodes a polypeptide with a predicted subunit molecular mass of 33765 Da. This calculated molecular mass for DHEA-ST8 is slightly smaller than the previously reported subunit molecular mass of approx. 35000 Da for pure human liver DHEA-ST estimated by SDS/PAGE [20].

Amino acid sequence analysis

The amino acid sequences of several peptides derived from pure DHEA-ST were compared with the translation of DHEA-ST8. The N-terminal end of pure DHEA-ST was not available for sequence analysis; however, amino acid sequence was obtained from two tryptic fragments (5 and 16 amino acids in length) and a CNBr fragment (11 amino acids) derived from purified human liver DHEA-ST (Figure 2). Each sequence was identified in the translation of the cDNA. The 11-amino-acid peptide was identical with the translated sequence and aligned immediately after the methionine at position 274. The 5- and 16-amino-acid peptides each showed one-amino-acid difference with the translation of DHEA-ST8. The 5-amino-acid peptide was apparently derived by cleavage at the lysine in position 60. The sequence of the 16-amino-acid fragment was derived from a larger peptide, but the first ten amino acids of that peptide could not be assigned because of a smaller contaminating peptide. Overall, the three peptides showed 94% sequence identity (30 of 32 amino acids) with the translated sequence of DHEA-ST8.

The amino acid composition of pure DHEA-ST was also compared with the composition derived from the translation of DHEA-ST8. Table 1 shows the amino acid composition de-

Table 1 A comparison of the amino acid composition deduced from the DHEA-ST8 nucleotide sequences and that obtained from direct analysis of purified human liver DHEA-ST

The cDNA amino acid composition was calculated from the deduced amino acid sequence using the Wisconsin Genetics Computer Group programs [25]. Affinity-purified DHEA-ST was subjected to 24, 48 and 72 h HCl hydrolysis, and the amino acid composition was calculated for a 35 kDa subunit molecular mass. Performic acid oxidation was performed to determine the cysteine and methionine residues. Abbreviation: ND, not determined.

	Composition (mol/100 mol of protein)	
Amino acid	From cDNA	By analysis
Cys	1.05	0.97
Asx	8.42	8.41
Glx	12.28	12.66
Ser	9.12	9.42
Gly	4.56	5.19
His	1.75	1.62
Arg	4.21	3.90
Thr	3.51	4.22
Ala	3.16	3.57
Pro	4.56	6.17
Tyr	3.16	2.60
Val	4.91	5.19
Met	3.15	4.22
Ile	5.26	5.19
Leu	10.18	10.39
Phe	7.72	7.47
Lys	8.42	8.44
Trp	3.16	ND

termined by analysis of pure DHEA-ST based on a subunit molecular mass of 35000 Da and the composition obtained from the translation of DHEA-ST8. The high degree of similarity between the molar percentage values obtained for each amino acid based on the protein's subunit molecular mass, along with the amino-acid-sequence data, supports the conclusion that DHEA-ST8 does encode DHEA-ST.

Northern- and Southern-blot analysis

Northern-blot analysis of human liver poly(A)$^+$ RNA was done to determine the size and number of messages hybridizing to DHEA-ST8. Initial analysis of human liver total RNA revealed that DHEA-ST8 hybridized to three different size messages (Figure 3). To determine if all three messages were closely related or different forms of the DHEA-ST message, the smaller *Xho*I-*Xho*I (bp 890–1060) restriction fragment was used in conjunction with the *Eco*RI-*Xho*I (bp 1–889) restriction fragment to probe the poly(A)$^+$ fraction of human liver RNA. The *Xho*I-*Xho*I fragment, which contains only 22 bases of the 3′-coding region and all of the 3′-non-translated region of DHEA-ST8, would be expected to possess the least sequence similarity to the transcripts of other closely related genes. Figure 3 shows that both probes hybridized identically with the three different-size poly(A)$^+$ RNAs. The largest and most abundant message detected by DHEA-ST8 is approx. 1800 nucleotides in length. The smaller, less-abundant, messages are approx. 1300 and 1100 nucleotides in length respectively. No bands were detected in the rRNA and tRNA fractions obtained during oligo(dT)–cellulose chromatography of the total liver RNA (results not shown).

Southern-blot analysis of human liver DNA was performed to investigate the number and size of genomic DNA fragments which hybridize to DHEA-ST8. Figure 4 shows that, using high-

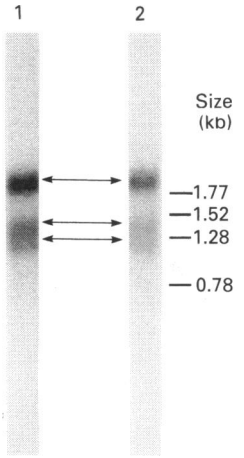

Figure 3 Northern-blot analysis of human liver poly(A)$^+$ RNA using DHEA-ST8 cDNA as a probe

Aliquots (2 μg) of the poly(A)$^+$ fraction of human RNA purified from the liver of a 49-year-old woman were resolved in a 1.5% agarose/formaldehyde denaturing gel. The RNA was transferred to a nitrocellulose membrane, the membrane was cut in half, and probed with restriction fragments of nick-translated DHEA-ST8 as described under 'Methods'. Lane 1 contains poly(A)$^+$ RNA hybridized with the [^{32}P]nick-translated *Xho*I–*Xho*I (175 bp) restriction fragment. Lane 2 contains poly(A)$^+$ RNA hybridized with the ^{32}P-nick-translated *Eco*RI–*Xho*I (889 bp) restriction fragment. The migration of RNA size markers (Gibco–BRL) is indicated at the right. The 1.8, 1.3 and 1.1 kb messages detected by the DHEA-ST cDNA are indicated by arrows to the left.

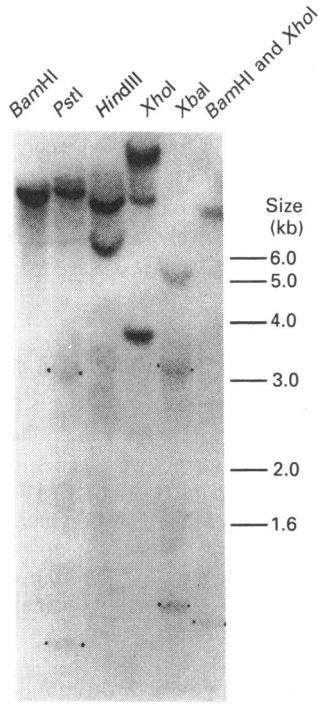

Figure 4 Southern-blot analysis of human genomic DNA using DHEA-ST8 as a probe

Aliquots (10 μg) of human genomic DNA were exhaustively digested with the following restriction enzymes: *Bam*HI, *Pst*I, *Hin*dIII, *Xho*I, *Xba*I and *Bam*HI/*Xho*I. The digested DNA was then electrophoresed in a 0.8%-agarose gel as described under 'Methods.' Bands smaller than 600 bp would not have been resolved during the electrophoresis of the restriction-enzyme-digested genomic DNA. DNA fragments were transferred to a nylon membrane and probed with ^{32}P-nick-translated DHEA-ST8. The migration of DNA size markers (Gibco–BRL) is indicated to the right. Two dots were added to either side of those smaller reproducible bands that did not develop as well as the larger bands.

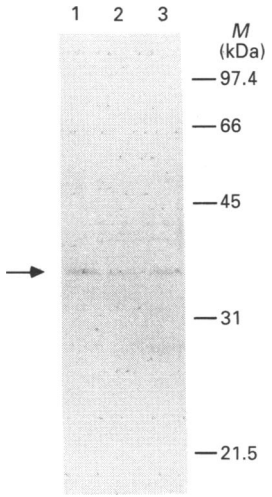

Figure 5 Translation of DHEA-ST8 *in vitro*

The transcription and translation of the DHEA-ST8 *in vitro* was done as described in the Materials and methods section. Aliquots of the translation reactions were resolved in a 12.5%-polyacrylamide gel. The gel was dried and exposed overnight to autoradiograph film to detect the [^{35}S]methionine-labelled products. Lanes 1 and 2 contain translation products of pSV-SPORT-1-DHEA-ST8. Lane 3 contains translation products of pGEM 7ZF-DHEA-ST8. Abbreviation: *M*, molecular mass.

stringency hybridization conditions, DHEA-ST8 hybridized to only one to three fragments of genomic DNA which had been digested with one of several different restriction enzymes. The greatest number of fragments which hybridized to DHEA-ST8 was present in the *Pst*I-digested genomic DNA. The sizes (bp) of the DNA fragments detected by DHEA-ST8 are as follows: *Bam*HI, 7400; *Pst*I, 8000, 7600, 3300, 1090; *Hin*dIII, 7200, 6000; *Xho*I, 7500, 3900; *Xba*I, 5200, 3300, 1300; *Bam*HI/*Xho*I, 7000, 1200. The number and size of fragments detected by hybridization with DHEA-ST8 is relatively small, suggesting the presence of a small number of related genes.

Transcription and translation of DHEA-ST8 *in vitro*

Transcription and translation of DHEA-ST8 *in vitro* was done to determine the size and integrity of the polypeptide translated by the cDNA. RNA transcripts were generated from DHEA-ST8 that had been subcloned into both pGEM 7ZF and pSV-SPORT-1 vectors using T7 and SP6 RNA polymerases respectively. The RNA transcripts were translated *in vitro* using a rabbit reticulocyte-lysate system and [^{35}S]methionine to label the translation products. Figure 5 shows that the major translated product detected with the transcripts generated by both vectors had a subunit molecular mass of approx. 35000 Da as determined by SDS/PAGE and co-migrated with immunoreactive DHEA-ST in human liver cytosol during SDS/PAGE; however, insufficient amounts of protein were translated by this system for detection of the translation product with the rabbit anti-DHEA-ST antibodies.

Expression of DHEA-ST in COS-7 cells

To establish that DHEA-ST8 encodes a functional DHEA-ST enzyme, DHEA-ST activity was transiently expressed in COS-7 cells using DHEA-ST8 subcloned into the mammalian expression vector, pSV-SPORT-1. Cytosolic extracts from both pSV-SPORT-1-DHEA-ST8-transfected and pSV-SPORT-1-transfected cells were tested for expression of DHEA-ST activity and immunoreactivity. COS-7 cells that were not transfected with either vector possessed only very low levels of DHEA-ST activity similar to the control pSV-SPORT-1 transfected cells. Cytosol from pSV-SPORT-1-DHEA-ST8 transfected COS cells showed a 61-fold increase (8.53 ± 0.90 pmol of DHEA sulphated/min per mg of protein versus 0.14 ± 0.14 pmol of DHEA sulphated/min per mg) in DHEA-ST activity as compared with pSV-SPORT-transfected cells. Figure 6 shows that immunoblot analysis of cytosol from pSV-SPORT-DHEA-ST8-transfected COS cells with rabbit anti-DHEA-ST antibodies detected the presence of a 35000 Da immunoreactive protein. The expressed protein also migrated at a molecular mass identical with that of immunoreactive DHEA-ST in human liver cytosol. Fractions from control cells transfected with pSV-SPORT-1 alone did not contain immunoreactive DHEA-ST.

Comparison of DHEA-ST8 to rat hydroxysteroid ST cDNAs

Figure 7 illustrates a comparison of the deduced amino acid sequence of DHEA-ST8 with the amino acid sequences of the rat hydroxysteroid ST cDNAs, STa-20, STa-40 and SMP-2. The amino acid sequence of DHEA-ST8 has a 63% sequence similarity with the deduced amino acid sequence of STa-20 and a 62% similarity with the sequence of STa-40, an isoenzyme of STa-20 [17,18]. A comparison of the rat hydroxysteroid ST amino acid sequences showed that the sequence of STa-40 was 90% similar to that of STa-20, but only 74% similar to the sequence of the senescence marker protein, SMP-2 [16]. Although the enzymic properties of SMP-2 have not been reported, it has been hypothesized that SMP-2 is a hydroxysteroid ST, owing to its strong sequence similarity to the STa cDNAs [18]. DHEA-ST shared the least amino acid similarity (60%) to SMP-2, even though a three-amino-acid gap was introduced into the SMP-2 sequence to obtain optimal sequence alignment.

DISCUSSION

The cDNA encoding DHEA-ST has been cloned, sequenced, translated *in vitro* and expressed *in vivo* in mammalian COS-7 cells. The cDNA contained an open reading frame of 855 nucleotides which encodes a 285-amino-acid protein. The deduced amino acid sequence of the DHEA-ST cDNA was 95% identical with the sequence obtained from peptides generated from affinity-purified human liver DHEA-ST. The differences between the deduced amino acid sequence of the cDNA and that of the pure protein may have been due either to errors in the protein sequencing or the existence of allelic forms of DHEA-ST in the different livers used for the purification of DHEA-ST and synthesis of the cDNA library. Results from the expression studies in COS-7 cells indicate that the DHEA-ST cDNA encodes a functional protein that is capable of catalysing the sulphation of DHEA, co-migrates with human liver DHEA-ST during SDS/PAGE and is recognized by rabbit anti-(human DHEA-ST) antibodies [20].

During these studies, 20 full-length clones were isolated from a human liver Uni-Zap XR cDNA library. These clones were identical on the basis of sequence analysis. Other cDNAs that were similar or related to the DHEA-ST cDNAs were not found during the screening of the human liver cDNA library. These results, along with the isolation and characterization of only a single steroid/bile acid ST, DHEA-ST, from human tissues

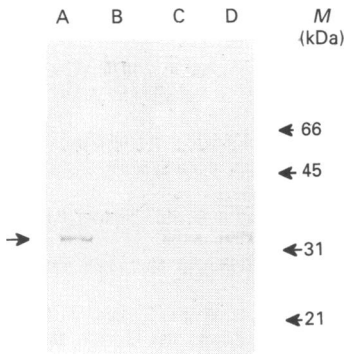

Figure 6 Western-blot analysis of COS-7 cells transfected with DHEA-ST8

Cytosol from human liver or DHEA-ST8 transfected COS-7 cells was subjected to SDS/PAGE on a SDS/15%-polyacrylamide gel, electrophoretically transferred to nitrocellulose and incubated with a 1:10000 dilution of rabbit anti-(human liver DHEA-ST IgG) antibodies as described previously [21]. Lanes A and D contain 20 μg and 10 μg of human liver cytosol respectively, prepared as described previously [19]. Lane B contains COS-7 cytosol (60 μg) of cells transfected with pSV-SPORT-1. Lane C contains COS-7 cytosol (60 μg) from cells transfected with pSV-SPORT-1-DHEA-ST8. The migration of molecular mass (*M*) standards (Bio-Rad) are indicated in the right margin, and the migration of immunoreactive DHEA-ST in human liver cytosol is indicated by the arrow to the left.

```
DHEAST   MSDDFLWFEGIAFPTMGFRSETLRKVRDEFVIRDEDVIILTYPKSGTNWLAEILCLMHSK   60
SMP2     -MS-YN-----P--AISYQR-I-EDI-NK--VKE--LL-----------N--V--IQT-
STA-20    MP-YT-----P-HAF-ISK---QN-CNK--VK---L-L-A---------I--V--IQT-
STA-40    MP-YT-----P--AF-IPK---QN-CNK--VKE--L-L-----------I--V--IQT-

DHEAST   GDAKWIQSVPIWERSPWVESEIGYTALSESESPRLFSSHLPIQLFPKSFFSSKAKVIYLM  120
SMP2     --P------C-FGTVY-DEIEW-FRNNHGGPRLIT( )-----H--S---------A----
STA-20   --P------T--D----I-TDV--DI-IKKKG---MT----MH--S--L----------V
STA-40   --P------T--D----I-TDL--DM-IKKKG---IT----MH--S-L----------I

DHEAST   RNPRDVLVSGYFFWKNMKFIKKPKSWEEYFEWFCQGTVLYGSWFDHIHGWMPMREEKNFL  180
SMP2     -----I--------G-TNLV-N-G-LGT-----L--N--F----E-VR--LS---WD---
STA-20   ----------Y--G-STLA---D-LGT-V---LK-N-------E--RA-LS-Q-WD---
STA-40   ----------Y--GKTTLA---D-LGT-V---LK-Y-P-----E--RA-LS---LD---

DHEAST   LLSYEELKQDTGRTIEKICQFLGKTLEPEELNLILKNSSFQSMKENKMSNYSLLSVDYVV  240
SMP2     V-Y--DM-K--MG--K---D----N-G-D--D-L--Y----A----N------IKE-PIL
STA-20   --Y--DM-K--MG--K---D----K---D--D-V--Y----V----D-------MKKSIF
STA-40   --Y--DM-K--MG--K---D----K---D--D-V--Y----V----N----N-MEKELIL

DHEAST   DKAQLLRKGVSGDWKNHFTVAQAEDFDKLFQEKMADLPRELFPWE*                286
SMP2     TGLK-M---TT-------------A---V------GF-PGM----*
STA-20   TGTG-M---TV---------S---A---V------GF-PGM----*
STA-40   PGFTFM-N-TT-------------A---V------GF-PGM---D*
```

Figure 7 A comparison of the deduced amino acid sequence of DHEA-ST8 with the amino acid sequence of the rat hydroxysteroid STs, STa-20, STa-40 and SMP-2 [16–18]

The broken line indicates amino acids of the rat hydroxysteroid STs that are identical with those of DHEA-ST8. A three-amino-acid gap (parentheses) was inserted into the sequence of SMP-2 for optimical sequence alignment with the other rat hydroxysteroid STs and DHEA-ST8. An asterisk marks the end of each sequence.

indicates that humans may be different from other species in that a single enzyme may be responsible for the majority of the steroid and bile acid sulphation [20–22].

Enzymically active DHEA-ST is apparently a 70 kDa homodimer composed of two identical 35 kDa subunits [19]. The polypeptide encoded by DHEA-ST8 has a calculated molecular mass of 33765 Da; however, both the *in vitro*- and *in vivo*-expressed DHEA-ST8 proteins migrated with a molecular mass of approx. 35 kDa during SDS/PAGE. The discrepancies between the predicted size of the polypeptide encoded by DHEA-ST8 and the subunit molecular mass of pure human liver DHEA-ST may be an artifact of the SDS/PAGE procedure. Also, the expression of DHEA-ST activity after transfection of COS-7 cells with DHEA-ST8 supports our previous findings that the functional enzyme is apparently a homodimer.

Northern-blot analysis of human liver RNA revealed that three transcripts hybridized to DHEA-ST8 (Figure 3). The hybridization of all three transcripts to the 3′-non-translated region of DHEA-ST8 indicates that the transcripts are probably derived from the same, or very closely related, genes. The 1100-nucleotide transcript is most likely the mRNA that encodes DHEA-ST, since it is slightly larger than DHEA-ST8 (1060 bp), and no other cDNAs larger than the 1100-nucleotide transcript were isolated from the human liver library. The two larger transcripts may be precursors to the shorter transcript which have not been fully processed, may have been generated by different transcriptional start sites or may have more extensive 3′ non-translated regions, owing to different sites of polyadenylation. If the three transcripts are generated by polyadenylation at different sites, then the *Xho*I-*Xho*-I fragment of DHEA-ST8 would hybridize strongly to the larger transcripts, as seen in Figure 3. We have previously reported that two different-length transcripts of rat liver minoxidil ST are apparently generated by polyadenylation at different sites [29]. DHEA-ST8 was also found to hybridize to three similar transcripts during Northern-blot analysis of human adult and fetal adrenal RNA (K. A. Comer, C. R. Parker and C. N. Falany, unpublished work). The DHEA-ST activity in human adrenals is physically, kinetically and immunologically identical with the liver form of the enzyme [22].

The possibility that all three of the mRNAs that hybridized to the DHEA-ST8 are derived from the same gene is supported by the fact that, in each digest, only one to three fragments of the restriction-enzyme-digested human genomic DNA hybridized to DHEA-ST8. Also, the number and size of the hybridizing fragments is quite different from that of other conjugating enzymes, such as the cytochrome *P*-450 family of enzymes, which occur in multiple forms [30]. These results are consistent with the isolation of only a single form of DHEA-ST from human and adrenal tissue [20–22]. However, the isolation and characterization of the genomic clone for DHEA-ST will be necessary to understand further the processing of the DHEA-ST mRNA and the heterogeneity of steroid STs in human tissues.

In contrast with the situation in humans, at least six different hydroxysteroid ST activities have been purified from rat liver cytosol [10,11,14]. Recently, the nucleotide and deduced amino acid sequences of three rat hydroxysteroid STs have been reported [16–18]. A comparison of the translated amino acid sequence of DHEA-ST8 with the amino acid sequences of the rat hydroxysteroid STs shows that there is considerable similarity (60–63%) between the human and rat sequences. We have also demonstrated immunocross-reactivity between DHEA-ST and the rat steroid STs [31]. DHEA-ST8 had the greatest similarity (63%) with the STa sequence, and these enzymes have biochemical and structural properties similar to those of human liver DHEA-ST. Both enzymes are very reactive with DHEA, demonstrate similar patterns of substrate inhibition and possess similar K_m values for DHEA; however, DHEA-ST is different from the rat hydroxysteroid STs in that it is not capable of sulphating cortisol [14,19]. Although the deduced amino acid sequence of SMP-2 also shares a relatively high degree of similarity to DHEA-ST (60%), no information has been reported about its enzymic properties. It may be concluded from the similar biochemical properties and the high degree of similarity between human and rat amino acid sequences that these genes were apparently derived from a common ancestral gene.

In addition to the rat hydroxysteroid cDNAs, the isolation of a cDNA encoding bovine placental oestrogen ST has been reported [32]. The bovine oestrogen ST is capable of sulphating the phenolic hydroxy group of oestrogens, but is not reactive with 4-nitrophenol, DHEA or bile acids [11,33]. The deduced amino acid sequence of the bovine oestrogen ST has been shown to have a relatively low level of similarity to the amino acid

sequences of the rat hydroxysteroid STs (48%) as well as to the amino acid sequence of DHEA-ST (38%). Also, recent data from our laboratory indicates that DHEA-ST has relatively little amino acid similarity to the human P-form of phenol ST (36%) (T. W. Wilbom and C. N. Falany, unpublished work).

Isolation and characterization of the cDNA for DHEA-ST provides a valuable tool to study the role of sulphation in steroid and bile-acid metabolism. In addition, further characterization of the biochemical and kinetic properties of expressed DHEA-ST will aid in understanding the role of this enzyme in adrenal and liver metabolism, as well as in the development and design of steroidal drugs.

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