Human α-tocopherol transfer protein: cDNA cloning, expression and chromosomal localization

Makoto ARITA,* Yuji SATO,* Atsuro MIYATA,† Tadashi TANABE,† Eiichi TAKAHASHI,‡ Herbert J. KAYDEN,§ Hiroyuki ARAI*‖ and Keizo INOUE*

*Department of Health Chemistry, Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113, Japan, †Department of Pharmacology, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan, ‡Diagnostic Research Institute, Diagnostic Division, Otsuka Pharmaceutical Co. Ltd., Kagasuno, Kawauchi, Tokushima 771-01, Japan, and §Department of Medicine, New York University School of Medicine, New York, NY 10016, U.S.A.

α-Tocopherol transfer protein (αTTP), which specifically binds this vitamin and enhances its transfer between separate membranes, was previously isolated from rat liver cytosol. In the current study we demonstrated the presence of αTTP in human liver by isolating its cDNA from a human liver cDNA library. The cDNA for human αTTP predicts 278 amino acids with a calculated molecular mass of 31749, and the sequence exhibits 94% similarity with rat αTTP at the amino acid level. The recombinant human αTTP expressed in *Escherichia coli* exhibits both α-tocopherol transfer activity in an *in vitro* assay and cross-reactivity to the anti-(rat αTTP) monoclonal antibody. Northern blot analysis revealed that human αTTP is expressed in the liver like rat αTTP. The human and rat αTTPs show structural similarity with other apparently unrelated lipid-binding/transfer proteins, i.e. retinaldehyde-binding protein present in retina, and yeast SEC14 protein, which possesses phosphatidylinositol/phosphatidylcholine transfer activity. Both Southern-blot hybridization of human–hamster somatic cell hybrid lines and fluorescence *in situ* hybridization revealed a single αTTP gene corresponding to the 8q13.1–13.3 region of chromosome 8, which is identical to the locus of a recently described clinical disorder, ataxia with selective vitamin E deficiency (AVED). The relationship between αTTP and AVED will be discussed.

INTRODUCTION

α-Tocopherol, the most biologically active form of vitamin E, is widely distributed in animal tissues and may function as a lipid antioxidant [1,2]. Vitamin E occurs in nature in four different forms, α-, β-, γ- and δ-tocopherols, which differ in the number and position of methyl groups on the ring. Although the amount of dietary γ-tocopherol is many-fold higher than that of α-tocopherol, plasma and tissues are enriched in α-tocopherol compared with γ-tocopherol [3]. Therefore, a mechanism(s) for discrimination of the various forms of vitamin E in animals, including man, should exist.

It has recently been proposed that the liver is the organ responsible for discrimination of the different forms of tocopherols [3,4]. Orally administered α- and γ-tocopherol are equally well absorbed from the intestine and transported by chylomicrons in the plasma. After the uptake of chylomicrons and their remnants by the liver, only α-tocopherol is preferentially secreted in association with nascent very-low-density lipoprotein (VLDL), and then circulated in the plasma. On the other hand, γ-tocopherol taken up by the liver is eventually excreted into the bile.

It has been reported by several groups, including ours, that rat liver cytoplasm contains a protein that binds α-tocopherol and transfers it between membranes [5–9]. The transfer catalysed by this protein is preferential for α-tocopherol, and other tocopherols such as γ-tocopherol are poor substrates. We recently purified the α-tocopherol transfer protein (αTTP) from rat liver and cloned its cDNA [10,11]. An unexpected finding was its localization among rat tissues; both Western- and Northern-blot analyses revealed that αTTP is expressed exclusively in the liver in rats. Thus, αTTP most likely plays an important role primarily in the liver. The tissue distribution, as well as the substrate specificity of αTTP, strongly supports the hypothesis that it is the protein that determines the incorporation of α- or γ-tocopherol into the nascent VLDL particles during their assembly and secretion from the liver.

A group of patients with familial isolated vitamin E (FIVE) deficiency have been described; these patients have similar neurological abnormalities, no abnormality in gastrointestinal function or in lipoprotein metabolism, but have remarkably low levels of plasma α-tocopherol [12–17]. In these patients, both α- and γ-tocopherol were equally well absorbed by the intestine into chylomicrons, but both forms of tocopherol disappeared rapidly from the plasma, in contrast to the situation in a normal subject. This faster decrease in plasma α-tocopherol was attributable to impairment of the secretion of α-tocopherol in VLDL [18]. It was hypothesized that these patients may have a defective liver αTTP or none at all, leading to a lack of incorporation of α-tocopherol into nascent VLDL in the liver.

Recently, patients in Tunisia, who had neurological symptoms resembling those of Friedreich's ataxia (FA) but had a normal structure of chromosome 9 (the recognized locus of the abnormality in FA) were studied and found to have very low levels of plasma α-tocopherol. The large number of offspring and the degree of inbreeding in these patients made it possible, by

Abbreviations used: αTTP, α-tocopherol transfer protein; AVED, ataxia with selective vitamin E deficiency; CRALBP, cellular retinaldehyde-binding protein; FA, Friedreich's ataxia; FISH, fluorescence *in situ* hybridization; FIVE, familial isolated vitamin E; kb, kilobase; SEC14p, yeast SEC14 gene product; TdT, terminal deoxyribonucleotidyltransferase; VLDL, very-low-density lipoprotein.

‖ To whom correspondence should be addressed.

homozygosity mapping, to map the locus of the abnormality on chromosome 8q. This syndrome was designated ataxia with selective vitamin E deficiency (AVED), and is probably the same disorder as FIVE deficiency [19,20].

Although αTTP of rat liver origin has been extensively studied, it has not proven conclusively whether the protein exists in other species, including man. In a recent review [21], it was stated that αTTP could not be detected on biochemical studies in human liver cytosol. In the present paper, we demonstrate the expression of αTTP in human liver, by isolating its cDNA from a human liver cDNA library, and show that the chromosomal locus of the gene on chromosome 8q13.1-13.3 is identical to that of AVED.

EXPERIMENTAL

Materials

[α-^{32}P]dCTP (110 TBq/mmol) was purchased from Amersham International. A human liver λgt11 cDNA library was purchased from Clontech Laboratories Inc. Restriction endonucleases and other modifying enzymes were from either Toyobo or Takara. Monkey Moloney leukaemia virus reverse transcriptase was from Life Sciences, and terminal deoxyribonucleotidyltransferase (TdT) was from BRL. *Pyrococcus furiosus* DNA polymerase was purchased from Stratagene. Alkaline-phosphatase-conjugated goat anti-(mouse IgG) monoclonal antibody and alkaline-phosphatase colour-developing reagents were purchased from Bio-Rad. The bacterial expression vector pUCPL-cI [22] was kindly provided by Dr. Adachi (Suntory Institute for Biomedical Research, Osaka, Japan).

Screening of the cDNA library

Recombinant phages ($\sim 1 \times 10^5$) from the human liver λgt11 cDNA library were plated on *Escherichia coli* Y1090 at a density of $\sim 2 \times 10^4$ plaque-forming units (p.f.u.)/150 mm diameter plate of Luria-Bertani's agar, and were then screened with the 900 bp rat αTTP cDNA clone, λrTTP 4-2 [10]. The probe was labelled using the random primer labelling method [23]. Hybridization was carried out once at 60 °C in 6 × SSC (1 × SSC: 15 mM citrate, 0.15 M NaCl, pH 7.0), 5 × Denhardt's solution [24], 250 mg/ml salmon sperm DNA, 0.1 % (w/v) SDS, and labelled probe (1×10^6 c.p.m./ml). The filters were washed twice at room temperature for 5 min in 3 × SSC/0.1 % SDS, and then twice at 55 °C for 20 min in 1 × SSC/0.1 % SDS. Positive plaques were isolated, the purified phage DNA was digested with *Eco*RI, and then the inserts were subcloned into the pBluescript II SK(−) plasmid.

Cloning of 5′-end sequences by PCR

Amplification of the 5′ end of the human αTTP mRNA was performed as described [25], using 1 mg of total RNA obtained from an adult human liver. First-strand cDNA was synthesized using the primer 5′-TGCAGCAACGGAGAGTG-3′ (codons His-23–Asn-28, antisense). Then the poly(A) tail was appended to the 5′ end of the first-strand cDNA using TdT. Amplification of the cDNA was performed with the dT_{17}-adaptor primer 5′-TTAGCGGCCGCGAATTCT17-3′ and the gene-specific primers 5′-GAGAGTGGTCCGGTAGC-3′ (codons Leu-20–Ser-24, antisense) and 5′-CGGTAGCGCGTTGAGCT-3′ (codons Leu-17–Pro-21, antisense). The PCR products were subcloned into pBluescript II SK(−) by blunt-end ligation, and the plasmids containing human αTTP sequences were identified by hybridization to a ^{32}P-labelled oligonucleotide probe, 5′-GCTGGGATCGCGCCTCT-3′ (codons Glu-3–Gln-7).

DNA sequencing

DNA sequences were determined by the method of Sanger et al. [26] using a Sequenase DNA Sequencing kit with [α-^{32}P]dCTP, or a Taq dye primer cycle sequencing kit and an Applied Biosystems model 373A DNA sequencer. Several subclones of the cDNA were generated by serial deletions of the original clone [27].

Analysis of DNA and protein sequences

Nucleotide and deduced amino acid sequences were analysed with programs from GENETYX (Software Development, Tokyo).

Expression of human αTTP in *E. coli*

The coding region of the cDNA for human αTTP was amplified by PCR using two synthetic oligomers. The 5′ oligomer, 5′-CGGAATTCATGGCAGAGGCG-3′, contained the *Eco*RI restriction site in addition to the human αTTP sequence, and the 3′ oligomer, 5′-GCGTCGACTCATTGAATGCT-3′, contained the *Sal*I site and a complementary human αTTP sequence. The amplified cDNA fragment was subcloned into pUCP$_L$-cI and then checked by dideoxyribonucleotide sequencing. This construct was introduced into *E. coli* W3110, and the transformants were cultured in LB medium at 32 °C. At the late log phase, the temperature was shifted to 42 °C, and the culture was incubated for a further 4 h to produce human αTTP under control of the P_L promoter. The cells were collected, suspended in PBS, and disrupted by sonication. The lysate was centrifuged at 12000 *g* for 10 min, and the supernatant was collected.

Immunoblot analysis

Proteins were separated by SDS/PAGE [28] and then transferred to a nitrocellulose membrane using a Bio-Rad blotting apparatus. Immunoblotting using the monoclonal antibodies was then carried out following blocking of the nitrocellulose with 3 % (w/v) gelatin. Following incubation for 1 h at 37 °C, the nitrocellulose was washed and incubated for 1 h at room temperature in a solution containing goat anti-(mouse IgG) monoclonal antibody conjugated to alkaline phosphatase. After washing, the blot was developed by soaking in a buffer (0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8) containing 0.3 mg/ml Nitro Blue Tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate. The monoclonal antibodies (termed AT-R1 and AT-R3) were obtained as described in [10]. Two isoforms exist in rat liver, of which the isoelectric points are 5.0 and 5.1 respectively. AT-R1 reacts with both isoforms to the same extent, but AT-R3 only reacts with isoform I (p*I* 5.1).

α-Tocopherol transfer assay

The procedure has been described previously [29]. Liposomes were prepared as follows; egg-yolk phosphatidylcholine, dicetylphosphate and butylhydroxytoluene (molar ratio, 5:0.5:0.05) were mixed with a trace amount of α-[^{3}H]tocopherol (radioactivity 1.5×10^6 c.p.m.) and *sn*-glycerol tri[carboxyl-^{14}C]oleate, as a non-exchangeable marker. After evaporation, the lipid film was suspended in SET buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris/HCl, pH 7.4) and then sonicated for 10 min under N_2 gas. A heavy membrane fraction was prepared from rat liver as described [9]. A given amount of liposomes (0.05 mol of phospholipid phosphorus) was incubated for 30 min at 37 °C with the membrane (0.05 mg of protein) in the presence or absence of a sample in 1 ml of SET buffer. The membrane was

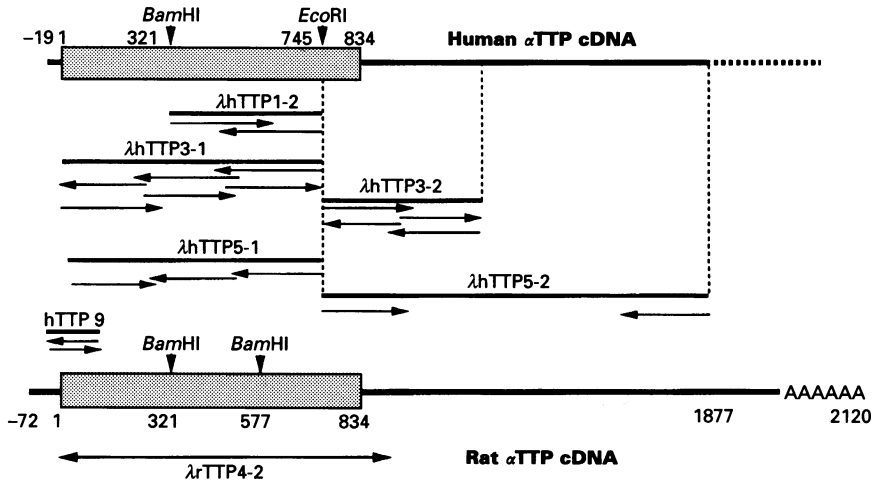

Figure 1 Sequencing strategy for human αTTP cDNA

The structure of human αTTP cDNA is shown above and that of rat αTTP cDNA below. The stippled boxes indicate the open reading frame, and the thin bars indicate the 5′- and 3′-untranslated regions. Rat αTTP cDNA fragment λrTTP4-2, which was used for screening, is indicated. hTTP9 was obtained by the method of 5′ RACE (rapid amplification of cDNA ends) [25]. Arrows indicate the direction and extent of sequencing.

precipitated by centrifugation at 15000 *g* for 15 min, and the supernatant (0.8 ml) was counted. Using this procedure, ~ 90% of the liposomes were recovered in the supernatant. The percentage transfer of α-[^{3}H]tocopherol from liposomes to the heavy membrane fraction was calculated using the following formula:

$$\left(1-\frac{^3H/^{14}C \text{ of liposomes after incubation}}{^3H/^{14}C \text{ of liposomes before incubation}}\right)\times 100\ (\%)$$

Northern blot analysis

The membrane loaded with a 2 mg spot of each of the poly(A)$^+$ RNAs from several human tissues (Clontech) was hybridized once at 42 °C in 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, 50 mM sodium phosphate (pH 6.5), 50% formamide, 250 mg/ml salmon sperm DNA, and labelled probe (2 × 10^6 c.p.m./ml). The probe was labelled by the random primer labelling method. The blot was washed twice for 5 min at room temperature in 2 × SSC, and then twice for 20 min at 65 °C in 0.1 × SSC/0.1% SDS. Autoradiography was performed at −80 °C for 3 days with an intensifying screen.

Chromosomal localization

A blot containing *Eco*RI-digested DNA samples from human–hamster hybrid cell lines as well as human and hamster controls was obtained from the BIOS Corporation. It was probed with a labelled human αTTP cDNA fragment (coding Asp-185–Glu-249), and a 5.0 kilobase (kb) genomic fragment which specifically hybridized to human αTTP but not hamster counterparts was detected. Hybridization was performed as follows; the blot was hybridized at 50 °C in the hybridization solution [10% (v/v) polyethylene glycol, 1.5 × SSPE (0.23 M NaCl, 0.02 M NaH_2PO_4, 0.003 M EDTA, 7% SDS, 200 mg/ml salmon sperm DNA), and labelled probe (2 × 10^6 c.p.m./ml)]. The final post-hybridization wash was performed in 0.1 × SSC, 0.1% SDS at 50 °C for 20 min.

Chromosomal *in situ* hybridization

Direct R-banding fluorescence *in situ* hybridization (FISH), which is based on FISH combined with replaced prometaphase R-bands [30], was applied to refine further the chromosomal localization of the human αTTP gene. DNA purified from a 10 kb EMBL3 genomic clone was nick-translated for use as a hybridization probe. For suppression of the repetitive sequences contained in this clone, we used human Cot-1 DNA (BRL) as previously described [31]. The procedures for labelling, hybridization, rinsing and detection were carried out routinely.

RESULTS

Cloning of human αTTP cDNA

A fragment of the rat αTTP cDNA insert (λrTTP 4-2 [10]) was used to screen a human liver λgt11 cDNA library. Screening of ~ 100000 clones resulted in the identification of one positive clone, λhTTP 1-2 (Figure 1). Further screening of the human liver cDNA library using this clone resulted in the isolation of several nearly full-length cDNAs encoding αTTP, but substantial difficulty was encountered in the cloning of the 5′ end of the cDNA. A total of eight independent clones was isolated. Although most exceeded 2.5 kb in length, none contained sequences corresponding to the N-terminal end of human αTTP. When amplification of the 5′ end of the human αTTP mRNA by PCR was performed, several clones corresponding to the N-terminal end were obtained, one of which extended to an in-frame methionine (ATG) codon. This ATG is tentatively designated as the translation initiation codon, as it is located at the same position as the putative translation initiation codon of rat αTTP, and has a strong consensus sequence for translation initiation (GGCATGGC) [32].

The human αTTP cDNA contains an open reading frame of 834 bases, which predicts a 31749 Da protein composed of 278 amino acids (Figure 2). Alignment of the human and rat αTTPs showed that they share 94% amino acid identity (Figure 3), with the lowest degree of identity (76%) occurring within the first 50 amino acids.

```
       -10         1         10         20         30         40
 GCAGCAGCGGCGGCGGGCATGGCAGAGGCGCGATCCCAGCCCTCGGCGGGGCCGCAGCT
                 M  A  E  A  R  S  Q  P  S  A  G  P  Q  L
        50         60         70         80         90        100
CAACGCGCTACCGGACCACTCTCCGTTGCTGCAGCCGGGCCTGGCGGCGCTGCGGCGCCG
 N  A  L  P  D  H  S  P  L  L  Q  P  G  L  A  A  L  R  R  R
       110        120        130        140        150        160
GGCCCGGGAAGCTGGCGTCCCGCTCGCGCCGCTGCCGCTCACCGACTCCTTCCTGCTGCG
 A  R  E  A  G  V  P  L  A  P  L  P  L  T  D  S  F  L  L  R
       170        180        190        200        210        220
GTTCCTGCGCGCCCGGGATTTCGATCTGGACCTGGCCTGGCGGTTACTAAAAAACTATTA
 F  L  R  A  R  D  F  D  L  D  L  A  W  R  L  L  K  N  Y  Y
       230        240        250        260        270        280
TAAGTGGAGAGCAGAATGTCCAGAAATAAGTGCAGATCTACACCCTAGAAGTATTATTGG
 K  W  R  A  E  C  P  E  I  S  A  D  L  H  P  R  S  I  I  G
       290        300        310        320        330        340
CCTCCTAAAGGCTGGCTACCATGGAGTCCTGAGATCCAGGGATCCCACTGGCAGCAAAGT
 L  L  K  A  G  Y  H  G  V  L  R  S  R  D  P  T  G  S  K  V
       350        360        370        380        390        400
TCTTATTTACAGAATCGCACACTGGGACCCCAAAGTTTTTACAGCTTATGACGTATTTCG
 L  I  Y  R  I  A  H  W  D  P  K  V  F  T  A  Y  D  V  F  R
       410        420        430        440        450        460
AGTAAGTCTAATCACATCCGAGCTTATTGTACAGGAGGTAGAAACTCAGCGGAATGGAAT
 V  S  L  I  T  S  E  L  I  V  Q  E  V  E  T  Q  R  N  G  I
       470        480        490        500        510        520
CAAGGCTATCTTTGATCTGGAAGGTTGGCAGTTTTCTCATGCTTTTCAAATCACTCCATC
 K  A  I  F  D  L  E  G  W  Q  F  S  H  A  F  Q  I  T  P  S
       530        540        550        560        570        580
CGTAGCCAAGAAGATTGCTGCTGTACTTACGGATTCATTTCCATTGAAAGTTCGTGGCAT
 V  A  K  K  I  A  A  V  L  T  D  S  F  P  L  K  V  R  G  I
       590        600        610        620        630        640
CCATTTGATAAATGAACCAGTAATTTTCCATGCTGTCTTTTCCATGATCAAACCATTCCT
 H  L  I  N  E  P  V  I  F  H  A  V  F  S  M  I  K  P  F  L
       650        660        670        680        690        700
GACTGAAAAAATTAAGGAACGGATTCACATGCATGGGAACAACTACAAACAAAGCTTGCT
 T  E  K  I  K  E  R  I  H  M  H  G  N  N  Y  K  Q  S  L  L
       710        720        730        740        750        760
TCAGCATTTCCCAGACATTCTTCCTCTGGAATATGGTGGTGAAGAATTCTCCATGGAGGA
 Q  H  F  P  D  I  L  P  L  E  Y  G  G  E  E  F  S  M  E  D
       770        780        790        800        810        820
CATTTGTCAGGAATGGACAAATTTTATAATGAAGTCTGAAGATTATCTCAGCAGCATTTC
 I  C  Q  E  W  T  N  F  I  M  K  S  E  D  Y  L  S  S  I  S
       830        840        850        860        870        880
TGAGAGCATTCAATGAGAAGTTATGTCATGTGAATGGCTTCCTAACTAAAAATACATGAG
 E  S  I  Q  *
       890        900        910        920        930        940
TGATATCCAACCTGGTTAAATGAATGAAAGAAAAGGAGCAAATCTTTTAAACTAATGCTT
       950        960        970        980        990       1000
GCCTGAACTTTAAAAATGTAGAAATCTTCTGACATGAGCAACACAGGTGTTTGGAAAGAT
      1010       1020       1030       1040       1050       1060
TTTTTACTTTTTAAATGCTTTTTCTCTACTTTTGAAGTAATTAAATGTCAGTACATTTT
      1070       1080       1090       1100       1110       1120
AAGAGCATAAAAATCGGCGATTTTGTACTTGAAGAGAACATGAAAGTTATTGCTAAAGTA
      1130       1140       1150       1160       1170       1180
GCTGCTCATGTGCATCTTTGTATGTTTCTAGAAAGTTATATTTTAAACAAGTTCTCTGAT
```

Figure 2 Nucleotides and deduced amino acid sequence of human αTTP

Nucleotide residues are numbered from the 5′ to the 3′ end from the first residue of the ATG codon encoding the initiating methionine. The deduced amino acid sequence is displayed below the nucleotide sequence as a one-letter code, starting from the methionine.

Bacterial expression of human αTTP

To confirm that human αTTP really encodes the αTTP, the human αTTP cDNA was cloned into the bacterial expression vector, pUCP$_L$-cI [22] under the control of the P$_L$ promoter. The resulting plasmid was introduced into *E. coli* W3110 competent cells. Human αTTP was expressed and cross-reactivity against anti-(rat αTTP) monoclonal antibodies was determined (Figure 4a). When immunoblotting was performed with the antibodies AT-R1 and AT-R3, raised against rat αTTP (see the Experimental section), the recombinant human αTTP cross-reacted with AT-R1 but not with AT-R3. These results indicate that the isolated human αTTP cDNA may encode a protein corresponding to rat αTTP isoform II.

α-Tocopherol transfer activity of the recombinant human αTTP was also determined. Extracts of the transfected *E.coli*

```
Human αTTP      MAEaRSqPsaGpQLNalPDHSPLLQPGLAaLRRRArE
Rat αTTP        ---m-Pg-vv-k---eq------V-----e-----q-

aGVPlAPlPLTDSFLLRFLRARDFDLDLAWRLLKNYYKWRAECPEISADLHPRSIIGLLK
e---eT-q----A------------------M------------L---------L----

AGYHGVLRSRDPTGSKVLIYRIAhWDPKVFTAYDVFRVSLITSELIVQEVETQRNGIKAI
--------------R------Sy--------------------------------V---

FDLEGWQfSHAFQITPSVAKKIAAVLTDSFPLKVRGIHLINEPVIFHAVFSMIKPFLTEK
-------i----------------V----------------------------------

IKeRIHMHGNNYKqSLLQHFPDILPLEYGGEEfSMEDICQEWTNFIMKSEDYLSSISESIQ
--g---L------s----------------N-s------------------------T--
```

Figure 3 Comparison of the deduced human and rat αTTP amino acid sequences

The amino acid sequence of human αTTP is shown above, and the corresponding rat αTTP sequence is shown below (single-letter code). Identical sequences are indicated by hyphens, and upper-case letters indicate conserved substitutions. Conserved amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E and Q; H, R and K; M, I, L and V; and F, Y and W [39].

cells showed substantial α-tocopherol transfer activity from liposomes to the heavy membrane fraction (Figure 4b).

Tissue distribution of human αTTP

A fragment of human αTTP cDNA (coding region) was hybridized to poly(A)$^+$RNAs of several human tissues (Figure 5). The results showed that αTTP is expressed in the liver, but is not

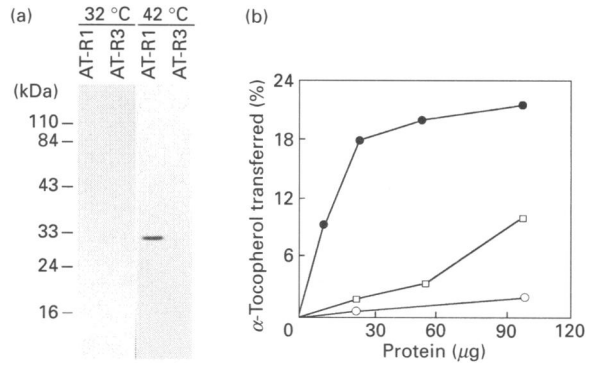

Figure 4 Expression of human αTTP cDNA

(**a**) Cross-reactivity of the recombinant human αTTP with the anti-(rat αTTP) monoclonal antibodies. *E.coli* (W3110) transformants cultured at 32 or 42 °C were disrupted with a Branson sonifier in PBS. The homogenates were centrifuged for 10 min at 12000 ***g***, and the supernatants were collected. Each supernatant (~ 50 μg of protein) was electrophoresed in a 12.5% (w/v) acrylamide gel and then transferred to a nitrocellulose membrane. Immunoblotting was performed as described in the Experimental section using the anti-(rat αTTP) monoclonal antibodies AT-R1 and AT-R3. Molecular-mass standards (kDa) are indicated on the left side. (**b**) α-Tocopherol transfer activity of the recombinant human αTTP. Transfer activity was measured in extracts of *E. coli* transformants cultured at either 32 °C (negative control, ○) or 42 °C (●). For comparison, transfer activity was also measured in the cytosol fraction of rat liver (□). Activity is expressed as the percentage of radiolabelled α-tocopherol transferred from liposomes (donor vesicles) to the heavy membrane fraction prepared from rat liver (acceptor vesicles). The procedures are described in detail in the Experimental section.

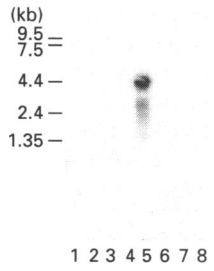

Figure 5 Northern blot analysis of αTTP in various human tissues

Poly(A)$^+$ RNA (2 μg) from human heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7) and pancreas (lane 8) was analysed with a random-primed ^{32}P- labelled human αTTP cDNA probe, as described in the Experimental section. RNA molecular-mass standards (kb) are indicated on the left side.

detected in other tissues, such as heart, brain, placenta, lung, skeletal muscle, kidney or pancreas. The cDNA probe hybridized with a liver mRNA of ~ 4.5 kb.

Similarity with other proteins

Computer searches of protein sequences revealed that the primary structure of human αTTP showed notable similarity with those of human cellular retinaldehyde-binding protein (CRALBP) and yeast SEC14p (the SEC14 gene product) (Figure 6). CRALBP specifically binds 11-*cis*-retinaldehyde and/or 11-*cis*-retinol among various forms of retinoids, and has been only found in visual organs [33,34]. SEC14p is a protein that exhibits phosphatidylinositol/phosphatidylcholine transfer activity and is required for protein secretion through the Golgi complex in yeast [35,36]. CRALBP and αTTP share 47.4% similarity, and SEC14p and αTTP share 37.4% similarity. The most conserved domains among these proteins are found in both the N-terminal (positions 49–66; Figure 6, solid line) and C-terminal (positions 211–214, Figure 6, broken line) regions.

Chromosomal localization of the human αTTP gene

To assign the chromosomal locus of human αTTP, we used a panel of a blot containing *Eco*RI-digested DNA samples from human–hamster hybrid cell lines as well as human and hamster

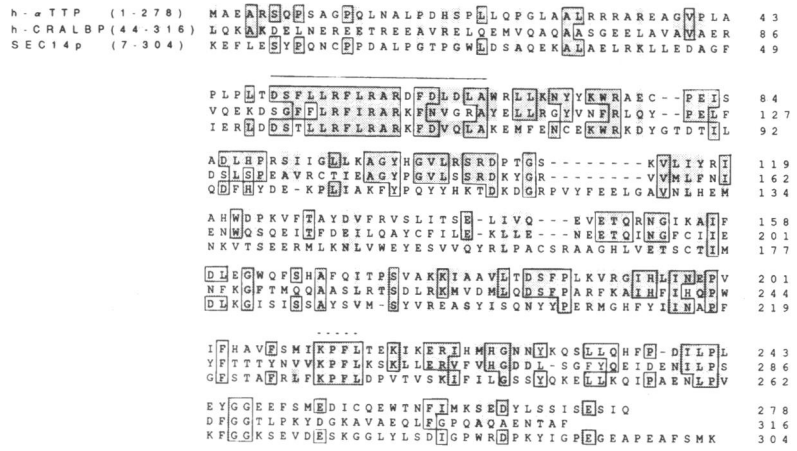

Figure 6 Alignment of human αTTP with other homologous proteins

Human αTTP has been aligned with human CRALBP (h-CRALBP) [34] and SEC14p [35]; gaps (—) have been inserted to demonstrate maximum sequence similarity. Residues identical with those of human αTTP are enclosed by solid lines, and residues showing conserved substitutions are indicated by shading. Conserved amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E and Q; H, R and K; M, I, L and V; and F, Y and W [39].

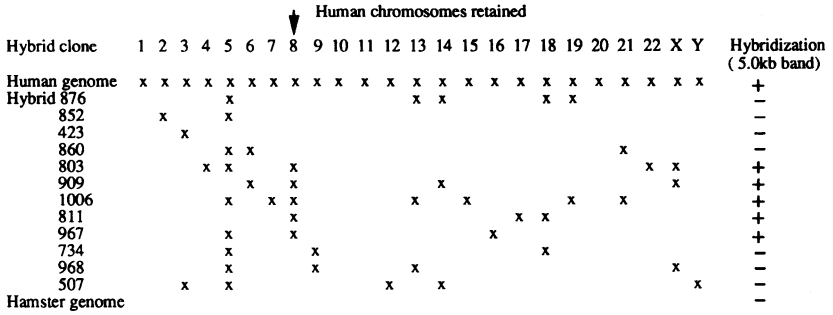

Figure 7 Chromosomal localization of human αTTP

The human αTTP cDNA probe (encoding Asp-185–Glu-249) hybridized to the membrane containing genomic DNA (*Eco*RI digest) of human–hamster hybrid cell lines. Scoring was performed as to the presence (+) or absence (—) of the human αTTP sequence in the hybrids. The results are consistent with localization to human chromosome 8, as indicated by the arrow. x, Whole human chromosomes contained in the cell line.

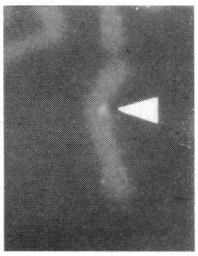

Figure 8 Regional localization of human αTTP to the 8q13.1–13.3 region of chromosome 8

A localization of a human αTTP genomic clone to human metaphase chromosomes by direct R-banding FISH [30]. The arrowhead indicates the signal on 8q13.1–13.3.

controls. A fragment of human αTTP cDNA was used as a probe for hybridization, and a 5.0 kb genomic fragment which specifically hybridized to the human αTTP gene but not hamster counterparts was detected. The result was that the human αTTP gene was detected only in the hybrid cell lines containing human chromosome 8, no hybridization being detected in other cell lines (Figure 7), indicating that the human αTTP gene is localized to chromosome 8.

To determine the regional chromosomal localization of the human αTTP gene more accurately, FISH was performed with metaphase chromosomes. We examined 100 typical R-banded (pro)metaphase plates. Of these, 18% exhibited complete twin spots on both homologues, 40% incomplete single and/or twin spots on either or both homologues, and the others (42%) no detectable spots. The signals were localized to the q13.1–q13.3 band of chromosome 8 (Figure 8). The gene could be, therefore, assigned to band 8q13.1–13.3.

DISCUSSION

The present paper reports the cloning, expression and chromosomal localization of the gene for the human αTTP. Our findings provide the first compelling evidence for the expression of αTTP in human liver and the first description of chromosomal localization of the gene. Human αTTP contains the same number of amino acids as rat αTTP and exhibits extremely high sequence similarity with it. Moreover, human αTTP is also localized in the liver, like rat αTTP. Rat liver contains two differently charged isomers (p*I*s 5.0 and 5.1) of αTTP. Utilizing an isoform-specific antibody, it was found that human αTTP encoded by the isolated cDNA corresponds to the p*I* 5.1 isomer of rat αTTP. We could not isolate a cDNA for the other isomer, even upon repeated screening of rat and human liver cDNA libraries. Thus, it seems likely that the rat p*I* 5.0 isomer is produced by post-translational modification of the p*I* 5.1 isomer in the liver. Whether or not human liver contained two (or more) isomers remains to be determined.

Although the physiological function of αTTP is not fully understood, it was postulated that this protein is involved in the discrimination of various forms of tocopherol in the liver. As mentioned above, tocopherols taken up by the intestine are incorporated into chylomicrons, and delivered to the liver as chylomicrons and chylomicron remnants. Tocopherols present in circulating low-density lipoprotein are also endocytosed to a great extent by the liver. These endocytosed tocopherols are transported to the lysosomes, where the lipoprotein vehicles are hydrolysed by lysosomal acid hydrolases, free tocopherols being released. It is postulated that these tocopherols then cross the lysosomal membrane and enter the cytoplasm of hepatocytes. Detailed studies with deuterated forms of α- and γ-tocopherol have demonstrated that α-tocopherol is preferentially repackaged by the liver and secreted into the plasma in nascent VLDL [3]. The hypothesis that the αTTP in the cytoplasm is responsible for the preferential transfer of α-tocopherol into the nascent VLDL is an attractive one, but awaits additional studies for confirmation.

VLDL is synthesized in the endoplasmic reticulum, undergoes some modification in the Golgi, and then is secreted into the plasma via exocytosis. Thus, αTTP may function in the transport of α-tocopherol from lysosomes to the organelles of the secretory pathway. In this context, it is interesting to note that human and rat αTTPs exhibit high sequence similarity with the SEC14 gene product, SEC14p. The SEC14 gene encodes the phosphatidylinositol/phosphatidylcholine transfer protein of *Saccharomyces cerevisiae*. SEC14p is found in both a cytoplasmic pool and in a stable, and apparently specific, peripheral association with the yeast Golgi complex [37]. A considerable body of evidence indicates that SEC14p plays an essential role in the Golgi secretory function, regulating the Golgi-membrane phospholipid composition such that a phosphatidylcholine content compatible with the essential secretory function of these membranes is maintained. It was also recently reported that the N-terminal 129 residues of SEC14p are sufficient to direct this protein to the Golgi complex [38]. The most conserved domains in αTTP and SEC14p are located in both the N-terminal (positions 47–66) and C-terminal (positions 211–214) regions. The former region of human αTTP has an interesting amino acid sequence; basic amino acids (arginine) and acidic amino acids (aspartate) are clustered on the N- and C-terminal sides respectively. This region, which is included in the N-terminal 129 residues of SEC14p, might be involved in the association with the Golgi complex. Although αTTP is mostly recovered from the soluble fraction of rat liver homogenates (M. Arita, Y. Sato, H. Arai and K. Inoue, unpublished work), it can be detected transiently associated with the Golgi complex in liver cells. If so, α-tocopherol bound to αTTP may be transfered to nascent VLDL at the Golgi complex. Work based on this hypothesis is in progress in our laboratory.

Patients severely affected with FIVE deficiency demonstrate the rapid disappearance of α-tocopherol from the plasma, along with an inability to discriminate between tocopherols. It is reasonable to assume that they have a genetic defect resulting in a complete lack of functionality of αTTP, attributable to either its complete absence or its inability to insert α-tocopherol into nascent VLDL. The co-localization of the gene for human αTTP and the causative gene for this genetic disorder also supports this idea.

Previously, we cloned and analysed the cDNA for rat αTTP. However, tocopherol transfer activity with the recombinant protein remained to be determined. In the present study, we have found that a recombinant protein expressed in *E. coli* shows α-tocopherol transfer activity in our *in vitro* assay. Analysis of the mutations in patients with FIVE deficiency and AVED may provide information as to whether the αTTP in the patients is functional and exhibits tocopherol transfer activity.

M.A. is a recipient of a Fellowship of the Japan Society for the Promotion of Science for Japanese Junior Scientists. H.J.K. was supported in part by grants from the U.S. Public Health Service (no. HL 30842) and the Natural Source Vitamin E Association.

REFERENCES

1 Diplock, A. T. (1985) in Fat-Soluble Vitamines (Diplock, A. T., ed.) pp. 154–224, Technomic Publishing Co., Lancaster, PA

2 Burton, G. W. and Ingold, K. U. (1986) Acc. Chem. Res. **19**, 194–201
3 Kayden, H. J. and Traber, M. G. (1993) J. Lipid Res. **34**, 343–358
4 Traber, M. G. and Kayden, H. J. (1989) Am. J. Clin. Nutr. **49**, 517–526
5 Catignani, G. L. (1975) Biochem. Biophys. Res. Commun. **67**, 66–72
6 Mowri, H., Nakagawa, Y., Inoue K. and Nojima S. (1981) Eur. J. Biochem. **117**, 537–542
7 Murphy, D. J. and Mavis, R. D. (1981) J. Biol. Chem. **256**, 10464–10468
8 Behrens, W. A. and Madere, J. G. (1982) Nutr. Rep. Int. **25**, 107–112
9 Behrens, W. A. and Madere, R. (1982) Nutr. Res. **2**, 611–618
10 Sato, Y., Hagiwara, K., Arai, H. and Inoue, K. (1991) FEBS Lett. **288**, 41–45
11 Sato, Y., Arai, H., Miyata, A., Tokita, S., Yamamoto, K., Tanabe, T. and Inoue, K. (1993) J. Biol. Chem. **268**, 17705–17710
12 Burck, U., Goebel, H. H., Kuhlendahl, H. D., Meier, C. and Goebel, K. M. (1981) Neuropediatrics **12**, 267–278
13 Laplante, P., Vanasse, M., Michaud, J., Geoffroy, G. and Brochu, P. (1984) Can. J. Neurol. Sci. **11**, 561–564
14 Harding, A. E., Matthews, S., Jones, S., Ellis, C. J. K., Booth, I. W. and Muller, D. P. R. (1985) N. Engl. J. Med. **313**, 32–35
15 Krendel, D. A., Gilchrest, J. M., Johnson, A. O. and Bossen, E. H. (1987) Neurology **37**, 538–540
16 Yokota, T., Wada, Y., Furukawa, T., Tsukagoshi, H., Uchihara, T. and Watabiki, S. (1987) Ann. Neurol. **22**, 84–87
17 Sokol, R. J., Kayden, H. J., Bettis, D. B., Traber, M. G., Neville, H., Ringel, S., Wilson, W. B. and Stumpf, D. A. (1988) J. Lab. Clin. Med. **111**, 548–559
18 Traber, M. G., Sokol, R. J., Burton, G. W., Ingold, K. U., Papas, A. M., Huffaker, J. E. and Kayden, H. J. (1990) J. Clin. Invest. **85**, 397–407
19 Hamida, C. B., Belal, D. S., Linder, C., Reutenauer, L., Dib, C., Gyapay, G., Vingal, A., Paslier, D. L., Cohen, D., Pandolfo, M., Mokini, V., Novelli, G., Hentati, F., Hamida, M. B., Mandel, J.-L. and Koenig, M. (1993) Nature Genet. **5**, 195–200
20 Hamida, M. B., Belal, S., Sirugo, G., Hamida, C. B., Panayides, K., Ionannou, P., Beckmann, J., Mandel, J. L., Hentati, F., Koenig, M. and Middleton, L. (1993) Neurology **43**, 2179–2183
21 Traber, M. G. (1994) Free Rad. Biol. Med. **16**, 229–239
22 Kuroki, M., Murakami, M., Wakisaka, M., Ikeda, S., Oikawa, S., Oshima, T., Nakazato, H., Kosaki, G. and Matsuoka, Y. (1992) Immunol. Invest. **21**, 241–257
23 Liscoritch, M., Freese, A., Blusztajn, J. and Wurtman, R. J. (1985) Anal. Biochem. **151**, 182–187
24 Denhardt, D. T. (1966) Biochem. Biophys. Res. Commun. **23**, 641–646
25 Frohman, M. A. and Martin, G. R. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8998–9002
26 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
27 Nakayama, K. and Nakauchi, H. (1989) Trends Genet. **5**, 325
28 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
29 Bloj, R. and Zilversmit, D. B. (1977) J. Biol. Chem. **252**, 1613–1619
30 Takahashi, E., Hori, T., O'Connell, P., Leppert, M. and White, R. (1990) Hum. Genet. **86**, 14–16
31 Lichter, P., Ledbetter, S. A., Ledbetter, D. H. and Ward, D. C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6634–6638
32 Kozak, M. (1984) Nucleic Acids Res. **12**, 857–872
33 Saari, J. C. and Bredberg, D. L. (1987) J. Biol. Chem. **262**, 7618–7622
34 Crabb, J. W., Goldflam, S., Harris, S. E. and Saari, J. C. (1988) J. Biol. Chem. **263**, 18688–18692
35 Bankaitis, V. A., Malehorn, D. E., Emr, S. D. and Greene, R. (1989) J. Cell Biol. **108**, 1271–1281
36 Bankaitis, V. A., Aitken, J. R., Cleves, A. E. and Dowhan, W. (1990) Nature (London) **347**, 561–562
37 Cleves, A. E., McGee, T. P., Whitters, E. A., Champion, K. M., Aitken, J. R., Dowhan, W., Goebel, M. and Bankaitis, V. A. (1991) Cell **64**, 789–800
38 Skinner, H. B., Alb, J. G., Jr, Whitters, E. A., Helmkamp, G. M., Jr and Bankaitis, V. A. (1993) EMBO J. **12**, 4775–4784
39 Dayhoff, M. D., Schwartz, R. M. and Orcutt, B. C. (1978) in Atlas of Protein Sequence and Structure (Dayhoff, M. D., ed.), vol. 5, pp. 345–352, National Biomedical Research Foundation, Silver Spring, MD

Received 24 June 1994/24 October 1994; accepted 28 October 1994