The key transporter responsible for hepatic uptake of bile acids from portal circulation is Na\(^+\)-taurocholate cotransporting polypeptide (NTCP, \textit{SLC10A1}). This transporter is thought to be critical for the maintenance of enterohepatic recirculation of bile acids and hepatocyte function. Therefore, functionally relevant polymorphisms in this transporter would be predicted to have an important impact on bile acid homeostasis/liver function. However, little is known regarding genetic heterogeneity in NTCP. In this study, we demonstrate the presence of multiple single nucleotide polymorphisms in NTCP in populations of European, African, Chinese, and Hispanic Americans. Specifically, four nonsynonymous single nucleotide polymorphisms associated with a significant loss of transport function were identified. Cell surface biotinylation experiments indicated that the altered transport activity of T668C (Ile\(^{223}\) → Thr), a variant seen only in African Americans, was due at least in part to decreased plasma membrane expression. Similar expression patterns were observed when the variant alleles were expressed in HepG2 cells, and plasma membrane expression was assessed using immunofluorescence confocal microscopy. Interestingly, the C800T (Ser\(^{267}\) → Phe) variant, seen only in Chinese Americans, exhibited a near complete loss of function for bile acid uptake yet fully normal transport function for the nonbile acid substrate estrone sulfate, suggesting this position may be part of a region in the transporter critical and specific for bile acid substrate recognition. Accordingly, our study indicates functionally important polymorphisms in NTCP exist and that the likelihood of being carriers of such polymorphisms is dependent on ethnicity.

Bile acids, synthesized from the enzymatic catabolism of cholesterol, are the major solutes in bile, essential for the maintenance of bile flow and biliary lipid secretion (1). In addition, an important mechanism for cholesterol homeostasis occurs through its elimination in the form of bile acids. Indeed \textit{de novo} synthesis of bile acids from cholesterol is thought to account for nearly half of the daily elimination of cholesterol from the body (1). In the gastrointestinal tract, bile acids also modulate the release of pancreatic secretions and gastrointestinal peptides and activate enzymes required for the absorption of lipid-soluble vitamins (2, 3). Furthermore, their detergent properties assist solubilization of cholesterol and dietary fats in the intestine. Bile salts are efficiently reabsorbed in the small intestine and are returned to the liver via the portal circulation and resecreted into bile, thus forming an enterohepatic circuit (4). The efficient enterohepatic recirculation of bile acids is maintained by polarized expression of bile acid uptake and efflux transporters in the intestine and liver (4). Moreover, taurine or glycine conjugates of bile acids tend to be polar and hydrophilic, thus dependent on transporter proteins for cellular uptake and efflux (5).

In the liver, it is estimated that Na\(^+\)-dependent transport pathways account for greater than 80% of the hepatic uptake of conjugated bile acids such as taurocholate (6–10). The transporter responsible for the observed Na\(^+\)-dependent uptake of conjugated bile salts is Na\(^+\)-taurocholate cotransporting polypeptide (NTCP, \textit{SLC10A1}) (11–14). This bile acid uptake transporter, whose function is coupled to a sodium gradient (15), is expressed exclusively in the liver and localized to the basolateral membrane of the hepatocyte (16). The human NTCP gene encodes a 349-amino acid protein (14) and shares 77% amino acid sequence identity with rat Ntcp (17). Hagenbuch \textit{et al.} (18) demonstrated that, when \textit{Xenopus laevis} oocytes were coinjected with total rat liver mRNA and antisense oligonucleotides specific to Ntcp, the expressed Na\(^+\)-dependent taurocholate transport activity was reduced by 95%. This finding suggests a potentially central role for Ntcp in the hepatic uptake of bile acids. Accordingly, the extent of its expression or function would be predicted to significantly affect enterohepatic circulation of bile acids and directly affect cellular signaling pathways importantly involved in cholesterol homeostasis and hepatocyte function.

One potential source of altered NTCP function may be
netic heterogeneity in this transporter related to the presence of single nucleotide polymorphisms, or SNPs. Indeed, functional polymorphisms are known to exist among bile acid transporter family members. For example, mutations in the ileal Na+-dependent bile acid transporter (ISBT, SLC10A2) have been identified (19). This transporter, expressed on the apical surface of ileal enterocytes and mediating the Na+-dependent uptake of conjugated and unconjugated bile acids in the intestine (20–22), shares significant sequence homology with NTCP (23, 24). Interestingly, certain loss of function mutations in the coding region of NTCP result in a syndrome of primary bile acid malabsorption (19), characterized by severe diarrhea, malabsorption of fat, and malnutrition. Furthermore, a number of mutations in the bile salt export pump (BSEP, ABCB11), the ATP-dependent bile salt efflux transporter localized to the canicular membrane of hepatocytes (25), have been linked to progressive familial intrahepatic cholestasis type 2 (26, 27), a progressive cholestatic liver disease characterized by loss of biliary bile acid secretion and an absence of BSEP expression on the canicular membrane. However, little is known regarding genetic heterogeneity in NTCP. The only published data to date have been a report of two SNPs in the coding region of NTCP in the Japanese population, conducted as a part of a large scale SNP discovery effort but lacking any functional studies of identified SNPs (28). Clearly, loss of function mutations in NTCP, if found to be present, would significantly add to our knowledge of the genetic basis of altered bile acid absorption, cholosterol elimination, and hepatocyte function. In this report we present data that support the presence of ethnicity-dependent functionally deleterious polymorphisms in NTCP. Moreover, functional studies indicate the presence of a discrete region within NTCP that is essential for bile acid substrate recognition.

EXPERIMENTAL PROCEDURES

Materials—[3H]Taurocholate (3.4 Ci/mmol, >97% purity), unlabeled taurocholate, [3H]cholate (55 Ci/mmol, >97% purity), unlabeled cholate, [3H]estrone sulfate (53 Ci/mmol, Na11032 and are as follows: exon 1, forward 5'-CAGCTCTTTCCTCCATATTGGAC-3' and reverse 5'-GCTCTTCCCCTCAATTGTGAC-3'; exon 2, forward 5'-GCTCTTCCCCTCAATTGTGAC-3' and reverse 5'-GCTCTTCCCCTCAATTGTGAC-3'; exon 3, forward 5'-GCTCTTCCCCTCAATTGTGAC-3' and reverse 5'-GCTCTTCCCCTCAATTGTGAC-3'; exon 4, forward 5'-GCTCTTCCCCTCAATTGTGAC-3' and reverse 5'-GCTCTTCCCCTCAATTGTGAC-3'; exon 5, forward 5'-GCTCTTCCCCTCAATTGTGAC-3' and reverse 5'-GCTCTTCCCCTCAATTGTGAC-3'. Using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA), PCR was carried out using ~200 ng of human liver genomic DNA for European American subjects or ~25 ng of genomic DNA (Coriell Cell Repositories) previously digested with the restriction endonuclease XhoI (New England Biolabs, Inc., Beverly, MA) for additional European Americans and all African American and Chinese American subjects consisting of DNTPs (0.25 mM each), the specific primer pair (4 μM each), 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl2, and 2.5 units of AmpliTaq® DNA polymerase (PerkinElmer Life Sciences) in a final reaction volume of 50 μL. PCR was generally carried out at 94 °C for 30 s, 55 °C for 20 s, and 72 °C for 20 s for 30 cycles with the exception of exon 2, which was carried out at 94 °C for 30 s, 58 °C for 5 s, and 72 °C for 10 s for 30 cycles. PCR products of expected sizes were completely visualized using ethidium bromide-stained 2% agarose gels and were fully sequenced in all subjects with an ABI 3130 DNA analyzer (Applied Biosystems). SNPs were identified utilizing the computer software program AlignX (Vector NTI, Version 7.0, InforMax, Inc., Frederick, MD) and confirmed by direct visualization of the sequences. A search of available SNP data bases was performed to identify any additional polymorphisms.

Wild-type and Variant NTCP Plasmid Construction—The full open reading frame of human NTCP cDNA was obtained by PCR using AmpliTaq® DNA polymerase (PerkinElmer Life Sciences) from a cDNA library synthesized from human liver mRNA using oligonucleotide primers 5'-ATGAGGCCCCCAAGGCGCTT-3' as the forward and 5'-CTAGCTGTGCAAGGAGGGA-3' as the reverse. A single PCR product of expected size was visualized on an ethidium bromide-stained 1.2% agarose gel. An aliquot of the PCR product was ligated into the pGEM®-3-His-TOPO® vector (Invitrogen). After transformation and growth in Escherichia coli, individual colonies containing the pE6V5-His-TOPO®/NTCP construct were identified. A pE6V5-His-TOPO®/NTCP with the NTCP cDNA inserted in the sense orientation downstream from the T7 promoter region was fully sequenced using an ABI 3700 DNA Analyzer (Applied Biosystems Inc.) and found to fully match the published reference sequence (GenBank™ accession number NM_003049) (14). This clone was termed NTCP®1. Site-directed mutagenesis was used to create the identified nonsynonymous allelic variants: C800T and T836C identified from Chinese American samples, A940G identified from a Hispanic American sample, and T868C identified from African American samples. The appropriate point mutations were introduced individually into wild-type NTCP (*1) packaged into pE6V5-His-TOPO® using the QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the following oligonucleotide primers: C800T, forward 5'-CACAATCCAGGCTTCTCAGCACTTCGCTGCTTCT-3' and reverse 5'-GAAGAGAAGGATTCTCTCAGCTGGTGGAGGAG-3'; T836C, forward 5'-GGGATATTGTTTTGATCTGCTCCACACTTGCGC-3' and reverse 5'-GCTCGGGGAAAGTGGTGAGG-3'; A940G, forward 5'-GAGAACATTGACAGGAAGGAG-3' and reverse 5'-GCTCTTCCCCTCAATTGTGAC-3'; T868C, forward 5'-GCCAAGACACATCTTGACAGGTCCACCTTCTCCTCCTC-3' and reverse 5'-CATACGGAGAGGGTTGTCGACCTGGCTTCTTCCCTC-3', and reverse 5'-GATTCAGGGAGGATTCTCTCAGCTGGTGGAGGAG-3'. The presence of the mutations was verified by full sequencing. Wild-type and variant NTCP were renamed the following for expression studies: wild-type, NTCP®1; C800T, NTCP®2; T836C, NTCP®3; T868C, NTCP®4; and A940G, NTCP®5.

Determination of Genotypic Frequencies—Direct sequencing of PCR products was performed to determine the genotypic frequencies of the nonsynonymous polymorphisms C800T, T836C, T868C, and A940G. PCR was carried out as previously described utilizing genomic DNA digested previously with the restriction endonuclease XhoI (New England Biolabs, Inc.) from a total of 90 European American, 90 African American, 90 Hispanic American, and 100 Chinese American healthy volunteers (Coriell Cell Repositories) with primers to amplify exon 3 and exon 4 of the NTCP gene. Single PCR products of expected sizes were visualized on ethidium bromide-stained 2% agarose gels. PCR products were fully sequenced in all subjects using an ABI 3700 DNA analyzer (Applied Biosystems Inc.). Allele frequencies were calculated based upon the Hardy-Weinberg equilibrium.

Cell Culture and Virus Preparation—HeLa (American Type Culture Collection) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. For preparation of a viral stock of vif-7 virus, HeLa cells grown

2 Data bases included IMS-JST Japanese SNP (snp.imst.u-tokyo.ac.jp), dbSNP (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = snp), PharmGKB (www.pharmgkb.org), and GeneCards® (bioinfo.weizmann.ac.il/cards/).
to near confluency in 25-cm tissue culture plates were infected with 1 plaque-forming unit/10 cells. After an incubation period of 48 h at 37 °C, the infected cells were pelleted, homogenized, and recovered through centrifugation followed by titering of viral stock as described by Blakely et al. (29).

Human hepatoma (American Type Culture Collection) cells (HepG2) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. For transient transfection, cells were grown on sterile uncoated 35-mm glass-bottomed microwell dishes (MatTek, Ashland, MA) and transfected at 70–80% confluency with 4 µg of V5-tagged NTCP plasmid DNA (NTCP®-1, -2, or -3) using LipofectAMINE 2000 (Invitrogen). After 48 h, cells were analyzed by immunofluorescence confocal microscopy.

Transport Studies Using Recombinant Vaccinia Virus—HeLa cells grown in 12-well plates (0.8–10 × 10⁴ cells/well) were infected with vaccinia (vTF-7) at a multiplicity of infection of 10 plaque-forming units/cell in serum-free Opti-MEM I medium (Invitrogen) and allowed to adsorb for 30 min at 37 °C. Cells in each well were then transfected with 1 µg of wild-type or variant NTCP cDNA packaged into pEF6/V5-His-TOPO® vector (Invitrogen) along with Lipofectin® (Invitrogen) and incubated at 37 °C for 16 h. The parental plasmid lacking any insert was used as a control. Transport was then evaluated using labeled substrates as outlined previously (30). To measure the taurocholate, cholate, and estrone sulfate transport kinetics, radiolabeled substrate uptake during the linear phase (first 3 min) was assessed in the presence of various concentrations of unlabeled compound. Passive diffusion was determined by carrying out parallel experiments using the parental plasmid DNA lacking the transporter cDNA, and this value was then subtracted from the total uptake rate seen in the presence of the transporter cDNA. Michaelis-Menten-type nonlinear curve fitting was carried out to obtain estimates of the maximal uptake rate (V_max) and the concentration at which half the maximal uptake occurs (K_m).

RESULTS

NTCP Variant Critical for Bile Acid Substrate Recognition—Because of a lack of a high affinity antibody to human NTCP, we utilized a 14-amino acid epitope (V5) in the pEF6/V5-His-TOPO® vector (Invitrogen) to generate V5-tagged wild-type and variant NTCP proteins. The QuickChange® site-directed mutagenesis kit (Stratagene) was used to introduce a point mutation that converted the stop codon of NTCP to a lysine residue (TAG → AAG) utilizing the following oligonucleotide sense and antisense primers: 5'-CTGCTTCCTTGACAGAGCGAAGGGGCAATTCTCGACGAC-3' and 5'-CTGACAGATGCTTTGCTGCGTGCGTCGACGAGCGAC-3'. The presence of the mutation was verified by full sequencing. The V5 tag was generated for wild-type NTCP and each of the nonsynonymous NTCP variants, and these were used for characterization of total protein and cell surface expression.

NTCP Expression in HeLa Cells—HeLa cells transfected with wild-type NTCP cDNA tagged with the V5 epitope were scraped off plates, and the resulting suspension was centrifuged at 21,000 × g for 3 min. The cell pellet was reconstituted with HED buffer (25 mM HEPES, 1.5 mM NaCl, 8 mM KCl, 1.5 mM MgCl₂, 2.7 mM CaCl₂, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (100 µM PMSF, 1 µM aprotinin, 1 µM leupeptin, 1 µM pepstatin) and then treated with a membrane-impermeable biotinylating agent (sulfosuccinimidyl-4-(N-hydroxy-succinimidy)-butyrate, 15 mM mg/l, Pierce) at 4 °C for 1 h. Subsequently the cells were washed three times with ice-cold phosphate-buffered saline Ca²⁺/Mg²⁺ (138 mM NaCl, 2.7 mM KCl, 1.5 mM MgCl₂, 8 mM KCl, 1.5 mM CaCl₂, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (Complete, Roche Applied Science) at 4 °C for 1 h with constant agitation. Following centrifugation, 140 µl of streptavidin-agarose beads (Pierce) was added to 600 µl of cell lysate and incubated for 1 h at room temperature. Beads were washed four times with ice-cold lysis buffer, and the biotinylated proteins were released by incubation of the beads with 2% (w/v) Laemmli buffer for 30 min at room temperature. Similar to total cell lysates, samples of the biotinylated fractions (25 µl) were subjected to Western analysis for detection of immunodetectable NTCP with monoclonal anti-V5 antibody (clone 15000 dilution) and the intracellular, endoplasmic reticulum-resident protein calnexin as described previously.

Deglycosylation of Total and Cell Surface-expressed NTCP—HeLa cells were grown on 6-well plates and transfected with the wild-type NTCP cDNA tagged with the V5 epitope using a protocol similar to that for transport experiments. Total and cell-surface expressed fractions of wild-type NTCP (15 µl) were released as described previously and subjected to enzymatic deglycosylation (Glyko, San Leandro, CA). Briefly 15 µl of total or cell-surface expressed NTCP®-1 was dissolved in 30 µl of deionized water. 10 µl of 5% (v/v) incubation buffer (0.25 mM sodium phosphate, pH 7.0) and 2.5 µl of denaturation solution (2% SDS and 1 M β-mercaptoethanol) were added to the proteins, gently mixed, and incubated at 100 °C for 5 min. After cooling to room temperature, 2.5 µl of detergent solution (15% Nonidet P-40) was added to the proteins. Then 1 µl each of N-glycans, sialidase A, and O-glycanase or 3 µl of water (control) was added to the proteins and subsequently incubated for 3 h at 37 °C. Samples were diluted with 2× Laemmli buffer and subjected to Western analysis by SDS-PAGE on 12% gels for detection of immunodetectable NTCP with monoclonal anti-V5 antibody (15000 dilution).

Immunofluorescence Confocal Microscopy—HepG2 cells transiently transfected with V5-tagged NTCP cDNA plasmid DNA were fixed for 10 min in ice-cold 70% methanol. After a 5-min wash in PBS, cells were permeabilized for 10 min in PBT (0.3% Triton X-100 in PBS, pH 7.4). After a 5-min wash in PBS, cells were placed in blocking buffer (2% bovine serum albumin in PBS) for 1 h at room temperature. Cells were then incubated in primary antibody (anti-V5 antibody diluted 1:500 in blocking buffer) for 2 h at room temperature. After three 5-min washes in PBS (0.05% Tween 20 in PBS, pH 7.4), cells were then incubated with secondary fluorescent dye (Texas Red-labeled goat anti-mouse whole IgG, Molecular Probes, OR). Cells were then washed two times with ice-cold PBS before three 5-min washes in PBS, cells were placed in PBS and viewed by confocal microscopy. HepG2 cells transfected with plasmid alone and cells transfected with V5-tagged NTCP wild-type plasmid DNA without incubation in primary antibody were used as two separate controls. Confocal microscopy was performed with a Zeiss Axiovert 135 inverted microscope equipped with a LSM510 laser scanning unit. A Zeiss 63 × 1.4 numerical aperture plan apochromat oil immersion objective was used for all experiments. Confocal images were obtained using single excitation (595 nm) and emission (610–630 nm Texas Red) filter sets. For low frame scanning, confocal images were obtained by scanning either laterally (top view, x-y scans) or axially (side view, x-z scans) across the cell. Image analysis and processing were performed with Zeiss LSM and Adobe Photoshop software.

Statistical Analysis—Determination of the statistical differences between various groups was determined using either Student’s t test, Mann-Whitney U test, analysis of variance (using Tukey-Kramer multiple comparison test), or Fisher’s exact test as appropriate. A p value of <0.05 was taken to be the minimum level of statistical significance.

RESULTS

Single Nucleotide Polymorphisms in NTCP—To identify coding region SNPs, initial screening PCR was performed on all five exons of NTCP from genomic DNA samples of 50 European Americans, 50 African Americans, and 50 Chinese Americans and analyzed by direct sequencing. Obtained sequences were compared with the published NTCP reference sequence (GenBank accession number NM_003049). Two nonsynonymous SNPs were identified by direct sequencing of PCR products from this initial screening of genomic DNA samples. The allelic variant C800T (Ser267 → Phe), in exon 4 of NTCP, was observed in 6 of 50 Chinese American DNA samples but was not seen in European Americans or African Americans (Fig. 1B). Another allelic variant T668C (Ile223 → Thr), in exon 3 of NTCP, was identified in 4 of 50 African American DNA samples but not seen in European Americans or Chinese Americans (Fig. 1B). Furthermore, three synonymous allelic variants

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Fig. 1. Predicted membrane topology of NTCP based on hydrophobicity analysis. Locations of the four identified nonsynonymous polymorphisms in NTCP are indicated with arrows (A). DNA sequence analysis of subjects with nonsynonymous polymorphisms in the coding regions of NTCP is shown in B.
were identified by direct sequencing. A G225A substitution (exon 1) was identified in 6 of 50 European American samples and 16 of 50 Chinese American samples but was not seen in the African American samples. A G453A substitution (exon 2) was identified in 1 of 50 African American samples but was not seen in European American or Chinese American samples. A T668C substitution (exon 3) was identified in 6 of 50 African American samples but was not seen in European American or Chinese American samples.

To further characterize the representative allele frequencies of the nonsynonymous variants in ethnically defined populations, PCR and direct sequencing of exons 3 and 4 of NTCP were performed on genomic DNA samples, representing an additional 40 European Americans, 40 African Americans, 50 Chinese Americans, and 90 Hispanic Americans. Upon further examination of these sequences, two additional rare nonsynonymous allelic variants were identified. The allelic variant T836C (Ile279 Thr) (Fig. 1 B), in exon 4, was found in 1 of 100 Chinese American samples, while the allelic variant A940G (Lys314 Glu), also in exon 4, was found in 1 of 90 Hispanic American samples (Fig. 1 B). Polymorphic variants tended to be ethnicity-dependent. Of the synonymous variants, G225A was found with relatively high frequency in Chinese American (23%) and European American (7%) populations but was rarely seen in African Americans (Table I). Conversely, the T627C variant was found with relatively high frequency in African Americans (7.2%) but was absent among European Americans, Chinese Americans, or Hispanic Americans. The C800T variant was found with low allele frequency only in African Americans (1%). With regard to the nonsynonymous variants, T668C was found with a relatively high allele frequency in African Americans (5.5%) and a lower allele frequency in Hispanic Americans (0.5%) and was absent in European American or Chinese American populations. The C800T variant was relatively common in Chinese Americans (7.5%) but was absent in European Americans, African Americans, and Hispanic Americans. The nonsynonymous variants T836C and A940G were seen in only one subject of Chinese Americans and Hispanic Americans, respectively.

Membrane Topology of NTCP—The membrane topology of NTCP was estimated based on hydrophobicity analysis using PredictProtein and is shown in Fig. 1A. It is predicted to possess seven transmembrane-spanning domains, and previous data have indicated that the carboxyl-terminal domain is localized intracellularly (16). In Fig. 1A, exons in NTCP are represented by different colors. One variant, T668C (Ile279 Thr, NTCP*3), is predicted to be located within the sixth transmembrane domain. The variant C800T (Ser267 Phe, NTCP*2) and T836C (Ile279 Thr, NTCP*4) changes are predicted to be localized to the third putative extracellular loop. The A940G (Lys314 Glu, NTCP*5) variant is estimated to lie within the intracellular carboxyl terminus.

Functional Analysis of NTCP Variants—A panel of expression plasmids comprising five NTCP allelic variants was constructed. When expressed using the recombinant vaccinia virus system (vrf7), NTCP*1 was capable of transporting known substrates, including the conjugated bile acid taurocholate, the unconjugated bile acid cholate, and the steroid conjugate estrone sulfate (Fig. 2). To determine the relative transport efficiencies of the NTCP variants, we initially focused on taurocholate as it appeared to be the most efficiently transported substrate among the compounds tested. Under linear kinetic conditions, transport activity was markedly decreased in NTCP*2, demonstrating ~98% reduction in the total uptake of taurocholate compared with NTCP*1 (Fig. 2). In fact, uptake in NTCP*2 was only slightly greater than in vector control, which essentially showed no uptake, demonstrating that transport activity for taurocholate was almost completely abolished in this variant. The NTCP*3, -4, and -5 variants also demonstrated a significant reduction in taurocholate uptake when compared with NTCP*1 although not to the degree seen with NTCP*2.

Although NTCP is primarily considered a bile acid transporter, it has been shown to transport non-bile acid substrates such as estrone sulfate (17). We next studied the relative transport efficiencies of NTCP variants for estrone sulfate uptake. Surprisingly, estrone sulfate uptake in NTCP*2 was comparable to that seen in NTCP*1, whereas NTCP*3, -4, and -5 again demonstrated a significant reduction in uptake (Fig. 2). To determine whether the loss of taurocholate uptake activity seen with NTCP*2 extended to bile acids in general, we then evaluated variant NTCP uptake activity for the unconjugated bile acid cholate. Similar to the taurocholate data, the NTCP*2 variant demonstrated markedly reduced uptake of cholate relative to NTCP*1 (Fig. 2). Again transport in the NTCP*2 variant was only slightly greater than vector control, showing almost complete loss of activity for cholate. Furthermore the NTCP*3, -4, and -5 variants demonstrated a more modest but still significant reduction in uptake of cholate. Kinetic analysis of NTCP*2 revealed that the $K_m$ values were markedly higher and $V_{max}$ values were lower for both taurocholate and cholate, while the $K_m$ and $V_{max}$ values for estrone sulfate were essentially equivalent to those for NTCP*1 (Fig. 3). Loss of NTCP*3, -4, and -5 activity, for the most part, appeared to be related to reduced $V_{max}$ (Fig. 3).

Total and Cell Surface Expression of NTCP Variants—To determine whether the observed differences in the transport kinetics were due to altered cell membrane surface trafficking, cell surface biotinylation experiments were carried out to capture only the cell surface-associated NTCP. Due to a lack of a

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TABLE I

| SNP       | Exon | Amino acid change | European Am | African Am | Chinese Am | Hispanic Am |
|-----------|------|-------------------|-------------|------------|------------|-------------|
| G225A     | 1    | Synonymous        | 7.0         | 1.0        | 23.0       | N/A         |
| G453A     | 2    | Synonymous        | None        | 1.0        | None       | N/A         |
| T627C     | 3    | Synonymous        | None        | 7.2        | None       | None        |
| T668C *3  | 3    | I223T             | None        | 5.5        | None       | 0.55        |
| C800T *2  | 4    | S267F             | None        | None       | 7.5        | None        |
| T836C *4  | 4    | E279T             | None        | None       | 0.5        | None        |
| A940G *5  | 4    | K314E             | None        | None       | None       | 0.55        |

$^*$ PredictProtein can be found at cubic.bioc.columbia.edu/.
high affinity antibody for NTCP, we created V5-tagged NTCP variants for Western blot analysis. The V5-tagged variants were functionally identical to the non-tagged NTCP variants (data not shown). Total cellular levels of expressed NTCP variants did not significantly differ in comparison to NTCP*1 (Fig. 4). However, cell surface biotinylation experiments revealed that the plasma membrane expression of NTCP*3 was significantly reduced in comparison to NTCP*1 (Fig. 4), whereas the surface expression of the NTCP*2, NTCP*4, and NTCP*5 variants appeared to be similar to NTCP*1. Interestingly, the cell surface-expressed NTCPs were enriched in a larger apparent molecular mass (~50-kDa) major band compared with that observed in total cell lysates (~40 kDa). The enrichment of cell surface proteins within these biotinylated fractions was evidenced by the lack of immunodetectable calnexin (an intracellular protein) in the samples. Furthermore, while decreased cell surface expression of the NTCP*3 may be one of the reasons for the observed reduction of activity in this variant, cell surface expression of NTCP*2, *4, and *5 was similar to the reference NTCP*1.

Deglycosylation of Total and Cell Surface-expressed NTCP—When expressed in HeLa cells, the cell surface-expressed NTCPs appeared to possess a larger apparent molecular mass (~50 kDa) when compared with the ~40-kDa band observed in total cell lysates. We performed deglycosylation experiments to determine whether this apparent difference was due to glycosylation of cell surface-expressed NTCP. In the total cell lysate, two bands of ~40 and ~50 kDa are readily visible, whereas the cell surface-expressed fraction appeared to be comprised primarily of the larger ~50-kDa product (Fig. 5). When subjected to enzymatic deglycosylation with N-glycosidase, O-glycosidase, and sialidase, a single band of ~37 kDa was detected in both total and cell surface-expressed fractions. This suggests that overall a significant portion of NTCP expressed in HeLa cells with this transfection method remains within intracellular spaces and that larger more highly glycosylated forms of NTCP are most abundantly localized on the cell membrane.

Plasma Membrane Localization of NTCP Variants in HepG2 Cells—Cell surface biotinylation experiments and Western analysis of total cell lysates in transfected HeLa cells suggested that the reduced function associated with the NTCP*3 variant may be due to altered trafficking of the protein to the cell surface. To further assess our findings, we performed immunofluorescence and confocal microscopy experiments in transiently transfected HepG2 cells. Moreover, unlike HeLa cells, since HepG2 is a liver-derived human cell line, membrane expression seen in this cell line may better reflect the in vivo situation. HepG2 cells were transiently transfected with V5-tagged variant NTCP plasmid DNA (*1, *2, or *3). Utilizing a secondary Texas Red-labeled antibody, we were able to demonstrate that wild-type NTCP (*1) protein was targeted to the plasma membrane of HepG2 cells when viewed laterally (x-y scans) across the cells by confocal microscopy (Fig. 6, left panel). Expression of the NTCP*2 variant in HepG2 cells showed that this protein was also properly targeted to the plasma membrane (Fig. 6, middle panel). In contrast, expression of the NTCP*3 variant in HepG2 cells showed that the majority of protein appeared to be retained intracellularly and exhibited significantly reduced plasma membrane immunofluorescence (Fig. 6, right panel). Views taken axially (x-z scans) across the cell demonstrated similar results (data not shown).

DISCUSSION

To date, little information has been available regarding the presence or functional relevance of genetic polymorphisms in NTCP despite increasing evidence supporting a crucial role of this transporter in the hepatic uptake of bile acids, thereby maintaining enterohepatic recirculation of bile acids. In this report, we describe the identification of seven SNPs in the coding region of NTCP, including four nonsynonymous polymorphisms, in populations of European Americans, African Americans, Chinese Americans, and Hispanic Americans. Although, for the most part, our data would suggest that SNPs in NTCP are relatively uncommon, certain SNPs were found to have relatively high allele frequencies but only in certain ethnic populations.

When the transport function of the four nonsynonymous NTCP variants was assessed in an in vitro system utilizing a recombinant vaccinia system, NTCP*2, a variant only seen in Chinese Americans, was shown to have near complete loss of activity for bile acid uptake. However, for the non-bile acid substrate estrone sulfate, NTCP*2 exhibited uptake transport equivalent to NTCP*1. This finding suggests C800T (Ser267 → Phe) may be localized to a critical position for bile acid binding/recognition. Upon analysis of NTCP*2 protein expression utilizing a V5 tag, the total and cell surface-expressed fractions of NTCP*2 were equivalent to that of wild-type NTCP*1, indicating that the profound loss of bile acid uptake activity was not related to protein instability or mistrafficking. On the other hand, NTCP*3, a variant only seen in African Americans, demonstrated a global reduction in transport activity with all substrates tested. When protein expression of NTCP*3 was evaluated, the total expressed fraction of the NTCP*3 variant was similar to that of wild-type NTCP*1. However, the cell surface-
**Fig. 3.** Transport kinetics of wild-type and variant NTCP for taurocholate, cholate, and estrone sulfate. Data are expressed as mean ± S.E. (n = 4). Kinetic parameters were obtained by non-linear curve fitting.
allelic variants in HepG2 cells. Immunofluorescent Texas Red-labeled secondary antibody was used to detect V5-tagged NTCP variants. Biotinylated plasma membrane proteins were subjected to SDS-PAGE and transferred onto nitrocellulose. Blots were probed with anti-V5 antibody (top panels) and then stripped and probed with anti-calnexin antibody (bottom panels). Immunoblots for total and cell surface proteins were exposed to x-ray film for identical periods of time. VC, vector-only control.

Fig. 5. Deglycosylation of NTCP*1 in HeLa cells. Expression of V5-tagged NTCP in total cell lysates reveals two major bands with apparent molecular masses of ~40 and ~50 kDa, whereas the single major band in the cell surface-expressed fraction is ~50 kDa. Subsequent to enzymatic deglycosylation, a single band of ~37 kDa is observed in the total and cell surface-expressed fraction.

Fig. 6. Immunofluorescence and confocal microscopy of NTCP allelic variants in HepG2 cells. Immunofluorescent Texas Red-labeled secondary antibody was used to detect V5-tagged NTCP variants. NTCP*1 (left panel) was targeted to the cell surface in transiently transfected HepG2 cells as demonstrated by lateral (x-y scan) confocal imaging across the cell. Similar to NTCP*1, NTCP*2 (middle panel) also appeared to be appropriately targeted to the plasma membrane. In contrast, NTCP*3 (right panel) showed significant intracellular retention of the protein and markedly reduced immunofluorescence on the cell membrane.

expressed fraction of this variant was significantly reduced compared with NTCP*1, indicating that this SNP may alter the cell surface trafficking of NTCP.

Since NTCP is exclusively expressed in the liver, to obtain further insights to the true in vivo relevance of NTCP polymorphisms in terms of plasma membrane expression, immunofluorescence and confocal microscopy experiments were carried out using a human liver-derived cell line (HepG2) transiently transfected with wild-type or variant NTCP (*1, *2, or *3) plasmid DNA. The extent of NTCP*1 and NTCP*2 expression in the plasma membrane of transfected HepG2 cells appeared to be similar (Fig. 6). In contrast, much of the NTCP*3 variant protein appeared to be retained intracellularly, and the extent of localization to the plasma membrane in these cells was significantly diminished (Fig. 6). Accordingly, these data further support the notion that the apparent reduction in the transport function associated with this variant may be due to mutation-induced alteration in trafficking of the transporter to the cell surface.

Among the polymorphisms identified, Ser267 → Phe (NTCP*2) and Ile223 → Thr (NTCP*3) may be more clinically relevant since Ile227 → Thr (NTCP*4) or Lys314 → Glu (NTCP*5) was seen only once from a total of 370 DNA samples tested. The T668C (Ile223 → Thr) variant encodes a change from the hydrophobic amino acid isoleucine to the polar amino acid threonine, predicted to lie within the putative sixth transmembrane-spanning domain (Fig. 1A). Upon further comparison of amino acid sequences of NTCP and the related family member ISBT from human and other mammalian species (Fig. 7A), it is clear that this amino acid change occurs in a conserved region of related transporters. Interestingly, the C800T (Ser267 → Phe) polymorphism, predicted to be located on extracellular loop 3 (Fig. 1A), was associated with a profound reduction in transport activity for taurocholate and cholate relative to the wild-type allele. The Ser267 → Phe (NTCP*2) amino acid substitution is predicted to lie within an area of the protein that could be considered a signature motif for all known NTCP and ISBT transporters (Fig. 7A). While prediction of transporter structure based on a hydropathy plot (Fig. 1A) may not be reflective of the true three-dimensional structure, the presence of such a conserved domain in a putative extracellular loop along with the observation that estrone sulfate uptake was unaffected strongly suggests a specific and important role of this region to bile acid substrate-transporter interaction.

The clinical implication of a near complete loss of bile acid transport function in NTCP, as seen with the C800T variant (NTCP*2), is unclear. A complete disruption of hepatic conjugated bile acid uptake would be predicted to markedly elevate serum bile acid levels in humans. One clinical phenotype of such marked hypercholanemia may be significant pruritus, although the role of bile acids as a pruritogen has not been clearly defined (31). Hepatocellular damage would not be expected to be a primary consequence of this defect because intracellular hepatocyte bile acid levels would likely be below normal or normal. However, cholestasis might develop if the extent of de novo bile acid synthesis was inadequate to maintain bile flow. In a report by Shneider et al. (32), in two patients who presented with pruritus and marked hypercholanemia, no mutations in the coding regions of NTCP were identified. Moreover NTCP mRNA and protein expression levels in the two subjects were noted to be similar to controls (32). This suggests that hypercholanemia as a syndrome does exist, although the true etiology of hypercholanemia in those patients remains unresolved.
It should be noted that although we were able to identify the NTCP*2 SNP from a panel of Chinese DNA samples, because these DNA samples from the Coriell Cell Repositories are anonymized, we were not able to contact those subjects for potential clinical studies. Additional considerations for assessing the clinical relevance of NTCP*2 in Chinese American subjects in terms of a clinical study relates to sample size. Given its allele frequency of only 7.5%, it is likely that at least 500–1000 subjects of Chinese descent will need to be genotyped if a sufficient number of subjects homozygous for this polymorphism are to be defined. As noted previously, none of the Chinese American Coriell Cell Repositories DNA samples (n =1100) genotyped in this study were homozygous for the mutation. In addition, if serum bile acids are to be used as a potential phenotypic marker of NTCP mutations, careful considerations must be given to diet, timing of plasma sampling in terms of fasting or postprandial states, and number of plasma samples to be obtained. Moreover such studies will require consent from pregenotyped subjects. To screen and recruit a sufficient number of Chinese American subjects for such a study may require a multicenter-type approach. Also frequencies of the NTCP*2 allele in other Asian populations such as Japanese, Koreans, Southeast Asians, and Indians will need to be defined if a similar clinical approach is to be applied to Asians in general.

However, the importance of bile acid uptake transporters in normal human physiology can be deduced from mutations found in a related family member, ISBT. Localized to the apical membrane of the ileal enterocyte, ISBT mediates the Na+/H+ -dependent uptake of conjugated and unconjugated bile acids in the intestine (20). Oelkers et al. (19) identified two missense mutations in the ISBT gene from a family with primary bile acid malabsorption, an intestinal disorder associated with congenital diarrhea, steatorrhea, disruption of enterohepatic circulation of bile acids, and reduced plasma cholesterol levels. Either of two mutations resulting in nonconservative amino acid changes in the ISBT protein, Leu243 → Pro (*) and Thr262 → Met when expressed individually or as a double mutant in COS cells, resulted in abolished transport activity for all bile acids tested, including the conjugated bile acids taurocholate, glycodeoxycholate, and glycochenodeoxycholate and the unconjugated bile acid cholate (19). Moreover total and cell surface protein expression of these variants in COS cells was equivalent to that of the wild-type allele, suggesting a loss of intrinsic transport activity and not transporter expression (19). Of relevance to the findings in this report, organization of the NTCP gene is almost identical to the ISBT gene (33). Upon further comparison of the amino acid sequences of NTCP and ISBT from various mammalian species such as human, mouse, rat, and rabbit, we note that the ISBT Leu243 → Pro and Thr262 → Met mutations identified from patients with primary bile acid malabsorption and the NTCP Ser267 → Phe (NTCP*2) variant identified in the current report lie in an area of the protein that is highly conserved between the homologous transporters (Fig. 7B). The close proximity of these mutations coupled with our data showing a near complete loss of bile acid uptake by NTCP*2 but fully normal uptake activity for the non-bile acid substrate estrone sulfate suggest this conserved region is critical for bile acid substrate recognition and that subjects who are homozygous for this mutation may exhibit a clinically significant phenotype.

It should be noted that unlike other hepatic uptake transporters such as organic anion-transporting polypeptide C (OATP-C) (34), polymorphisms in NTCP are relatively rare, suggesting evolutionary conservation. In fact, in a screen of 370 subjects, only four nonsynonymous SNPs were found. To our

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4 See locus.umdnj.edu/nigms/.

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Fig. 7. Amino acid sequence comparison of the homologous transporters NTCP and ISBT in various mammalian species. The location of the variant encoded amino acid changes are noted with arrows (A). The location of the C800T (Ser267 → Phe, NTCP*2) variant is shown in relation to the two known ISBT mutations associated with primary bile acid malabsorption syndrome, Leu243 → Pro (*) and Thr262 → Met (**) (B).
knowledge, there is no animal model for evaluating the role of NTCP in normal physiology. Further insight into the normal physiologic functions of NTCP and clinical manifestations associated with the loss of NTCP function may be gained from the creation of an Ntcp knock-out mouse model. Our current findings would suggest, however, that patients of Asian or African descent who present with hypercholanemia of unexplained etiology or ill defined cholestatic liver disease should be further investigated for mutations in NTCP.

In conclusion, we report the identification and in vitro functional characterization of novel functionally relevant polymorphisms in NTCP. To our knowledge, this represents the first detailed examination of polymorphisms in a hepatic bile acid uptake transporter and creates the framework for further investigations of the consequences of NTCP polymorphisms in vivo. We suggest that there is a naturally occurring single nucleotide polymorphism in NTCP that leads to a near complete loss of bile acid transport activity via loss of substrate specificity for bile acids. This has the potential to be associated with a clinically severe phenotype in subjects of Asian descent. Furthermore, we show that other ethnicity-dependent functional variants with a clinically severe phenotype in subjects of Asian descent. Clearly additional studies, especially among those with hypercholanemia or cholestasis, associated with the loss of NTCP function may be gained from the creation of an Ntcp knock-out mouse model. Our current findings would suggest, however, that patients of Asian or African descent who present with hypercholanemia of unexplained etiology or ill defined cholestatic liver disease should be further investigated for mutations in NTCP.

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