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Role of liver-enriched transcription factors and nuclear receptors in regulating the human, mouse, and rat *NTCP* gene

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Jung, Diana, Bruno Hagenbuch, Michael Fried, Peter J. Meier, and Gerd A. Kullak-Ublick. Role of liver-enriched transcription factors and nuclear receptors in regulating the human, mouse, and rat *NTCP* gene. *Am J Physiol Gastrointest Liver Physiol* 286: G752–G761, 2004. First published December 30, 2003; 10.1152/ajpgi.00456.2003.—Hepatic uptake of bile acids is mediated by the Na^+-taurocholate cotransporting polypeptide (NTCP; *SLC10A1*) of the basolateral hepatocyte membrane. Several *cis*-acting elements in the rat *Ntcp* gene promoter have been characterized. However, little is known about the mechanisms that control the expression of the human or mouse NTCP/Ntcp. We, therefore, compared the transcriptional regulation of the human and mouse *NTCP/Ntcp* gene with that of the rat. By computer alignment, a sequence in the 5′-regulatory region that is conserved between species was identified near the transcription start site. Huh7 cells were transfected with luciferase constructs containing the conserved region from each species. The hepatocyte nuclear factors (HNF)1α and -4α and the retinoid X receptor/retinoic acid receptor dimer (RXRα/RARα) bound and transactivated the rat but not the human or mouse NTCP/Ntcp promoters. In contrast, activation by the CCAAT/enhancer binding protein-β was specific for human and mouse NTCP/Ntcp. The only consensus motif present in all three species was HNF3β. HNF3β formed a specific DNA-protein complex in electrophoretic mobility shift assays and inhibited NTCP/Ntcp promoter activity in cotransfection assays. Finally, a minor repressive effect of bile acids was only found for rat Ntcp. The transcriptional repressor small heterodimer partner (SHP) did not affect NTCP/Ntcp promoter activity. We conclude that *1*) the transcriptional regulation of the conserved NTCP/Ntcp 5′-regulatory region differs considerably among human, mouse, and rat; and *2*) the conserved NTCP/Ntcp regulatory region is not directly regulated by SHP. Bile acids may regulate NTCP/Ntcp indirectly by modulating the capacity of nuclear factors to activate gene expression.

organic anion transport; bile acids and salts; cholestasis; transcription factors; cytokines; liver receptor homolog; farnesoid X receptor

THE MAJOR DRIVING FORCE FOR bile excretion is the active vectorial transport of bile salts from blood into bile. In mammalian liver, the Na^+-taurocholate cotransporting polypeptide (NTCP in humans, Ntcp in rodents; gene symbol *SLC10A1/Slc10a1*) accounts for >80% of conjugated bile acid uptake across the basolateral membrane of hepatocytes (24, 36). The *NTCP/Ntcp* gene, which is highly conserved between species (16), is subject to extensive regulation under conditions such as pregnancy, cholestasis, and sepsis (24, 36, 40). Any disturbance in bile excretion leads to the accumulation of bile acids in hepatocytes and cholestatic liver damage. An early defense mechanism against the accumulation of bile acids is the downregulation of bile acid uptake systems (24, 36, 40, 48). Several models of cholestasis, such as bile acid feeding (10, 44), bile duct ligation (9, 12), or endotoxinemia (14, 31) showed downregulation of both Ntcp mRNA and protein levels in mouse and rat.

The exact molecular mechanism of decreased NTCP/Ntcp expression in cholestasis is unresolved. The rat *Ntcp* gene has been reported to be downregulated by the transcriptional repressor small heterodimer partner (SHP) 1 due to its interference with retinoid activation of the retinoid X receptor/retinoic acid receptor dimer (RXRα/RARα) (7, 8). However, the role of SHP is debatable. Bile acid feeding decreases Ntcp expression to the same degree in SHP knockout ($SHP^{-/-}$) mice as in $SHP^{+/+}$ mice, indicating that bile acids can repress the *Ntcp* gene through SHP-independent mechanisms (43). Such mechanisms could include activation of the xenobiotic pregnane X receptor (PXR), activation of the c-Jun NH_2-terminal kinase, or bile acid-mediated repression of the transcriptional activator hepatocyte nuclear factor-1α (HNF1α) (20, 43). Whereas several mechanisms that regulate the rat *Ntcp* gene have been identified (7, 8, 11, 22, 39), little is known about the regulation of the *NTCP/Ntcp* gene in human and mouse. It is unknown, for instance, whether HNF1α and RXRα/RARα have the same activating effect on human and mouse NTCP/Ntcp as on rat Ntcp (7, 8, 22).

To compare the transcriptional regulation of the human and mouse NTCP/Ntcp promoter with that of rat Ntcp, we isolated the 5′-regulatory regions of the human and mouse genes and compared sequences with the rat gene by computer alignment. A highly conserved sequence was identified that contained several *cis*-acting elements previously shown to regulate the rat *Ntcp* gene. We report the first in vitro characterization of the human and mouse NTCP/Ntcp promoters compared with the rat and show that the role of liver-enriched transcription factors and nuclear receptors in governing the transcriptional regulation of the *NTCP/Ntcp* gene differs considerably between species.

MATERIALS AND METHODS

Plasmid construction. Fragments of the 5′-region of the human, mouse, and rat *NTCP/Ntcp* genes (Table 1) were amplified from genomic DNA (PfuTurbo DNA polymerase Stratagene, Amsterdam, Netherlands) and cloned into the luciferase reporter gene vector pGL3-Basic or pGL2-Enhancer (Promega Catalys, Wallisellen, Switzerland). Rat deletional constructs were generated by PCR (Table 1) from the original Ra-Luc construct and cloned into pGL3-Basic. Site-directed mutagenesis of the rat HNF1α site was performed as described previously (18). Sequence identity of all constructs was verified by sequence analysis. Plasmid DNA was prepared using the Qiagen system (Basel, Switzerland).

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Table 1. *Oligonucleotides used for cloning and mobility shift assays*

Oligonucleotide	Sequence (5′ to 3′)
Cloning	
Human-forward	TGACAAGGGAGGAGCTCAAGTAGCACCCAG
Human-reverse	CTCCATCCTCCTGTGAAGATCTGGAAGACCACTCC
Mouse-forward	GGGTACGAGCTCCAATCGGCAGGCACAGAG
Mouse-reverse	CTCCATCCTCAGATCTAGTGAACACCACC
Rat-forward	TTGCAGGTCATGGGTGGAGCTCAGAAGCAC
Rat −12-forward	CCACAACTGTGGTTGAGCTCGTTAATC
Rat +28-forward	CAACATTTTGTCGAGCTCGTCTGTCCTGAAAGAG
Rat-reverse	CCTCTGGCAGATCTAACACGGCCCTCGCTC
Rat-SDM-HNF1α	CTGTGGTTCTGCT**GGCGCATCGCTTATTT**GCCCACAGCCACATTTTG
Mobility shift assay	
Human-HNF1α	TAGCTCAGATAGTTGCATAACCCTTCTATTTGCCCAGAG
Mouse-HNF1α	TCTGCAGGTTGCATAATCTTTTATTTGCCCACAGCAACAT
Rat-HNF1α	TTCTGCTGGTTAATCTTTTATTTGCCCACAGCCACAT
PerHNF3β	GCCCATTGTTTGTTTTAAGCC
Hu-HNF3β	TAGCTCAGATAGTTGCATAACCCTTCTATTTGCCCAGAGCTTT
Mo/Ra-HNF3β	TCTGCAGGTTGCATAATCTTTTATTTGCCCACAGCAACA
MutHNF3β	GCCCATTGGGCCATTTAAGCC
Rat-HNF4α	AGATCCGGGGCATAAGGTTATGGGCTCTTAT
Mouse-HNF4α	ATCCGGGGGAGATGAGGTTATGGGCTGTCTT
Human-HNF4α	AGGGCAGGCAGATAAGGTTCTGGGCTGTTCC

HNF, hepatocyte nuclear factor; Hu, human; Mo, mouse; Ra, rat.

Cell culture and reporter gene assay. Huh7 and chicken hepatoma (LMH) cells were cultured and transfected as described (20, 21). In case of ligand treatment, the following ligands were added 18 h after transfection: 1 μM arotinoid acid, 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid/1 μM 9-*cis* retinoic acid (9cRA), 100 μM chenodeoxycholic acid (CDCA)/1 μM 9cRA, 100 μM deoxycholic acid (DCA)/1 μM 9cRA, 100 μM cholic acid (CA)/1 μM 9cRA or DMSO, and/or ethanol as controls.

Electrophoretic mobility shift assays. Dimerized oligonucleotides (Microsynth, Balgach, Switzerland) with sequences corresponding to

Fig. 1. Analysis of the conserved 5′-regulatory region of the human, mouse, and rat *NTCP/Ntcp* gene. Using computer analysis, a highly conserved region that spans the complete 5′-untranslated region (UTR) and parts of the promoter was found in the human, mouse, and rat *NTCP/Ntcp* genes. Despite an overall sequence identity of up to 80%, major differences were found in the distribution of potential transcription factor recognition sites. Putative recognition sites are outlined. The human *NTCP* gene sequence is shown in capital letters (lower cases in mouse and rat; Ntcp indicate nonconserved nucleotides compared with the human sequence). *Start of the published NTCP/Ntcp cDNA sequences. Translated sequences are shown in bold.

the *NTCP/Ntcp* gene or a perfect HNF3β binding site (Table 1) were labeled with [γ-^{32}P]adenosine triphosphate (3,000 Ci/mmol; Amersham Pharmacia Biotechnology, Dübendorf, Switzerland) using T4 polynucleotide kinase (Stratagene). For gel mobility shift assays, 2 μl of in vitro translated HNF1α protein (TnT Quick coupled transcription/translation system; Promega Catalys) or 5 μl Huh7 or HepG2 nuclear extracts were incubated as described previously (20, 21). HNF3β (sc-6554), HNF4α (H-171), and RXRα (D-20) antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany).

Statistical analysis. Reporter gene activities are expressed as the means ± 1 SE of four to eight individual transfection experiments. All data were reproduced at least once using two different preparations of plasmid DNA.

RESULTS

Isolation of the human, mouse, and rat NTCP/Ntcp 5′-regulatory region. To identify conserved regions in the *NTCP/Ntcp* gene, we first analyzed the human *NTCP* gene sequence using the computer program rVista (http://dcode.berkeley.edu/rvista/). This program simultaneously searches the major transcription factor binding site database Transfac and uses global sequence alignment. In addition to the NTCP/Ntcp coding region, we found a second region that is highly conserved among human, mouse, and rat. This conserved region spans the complete 5′-untranslated region (UTR) as well as certain parts of the promoter, specifically nt −124 to +83 in the human gene, nt −131 to +58 in the mouse gene, and nt −53 to +135 in the rat gene (Figs. 1 and 2*A*). Analysis of the conserved region revealed 72% sequence identity between human and mouse, 65% sequence identity between human and rat, and 80% sequence identity between mouse and rat. Potential transcription factor recognition sites shown in Fig. 1 were localized using the program Mat Inspector (Genomatix Software, Munich, Germany). Despite the overall sequence identity of up to 80%, we found major differences in the distribution of potential binding sites.

To compare the transcriptional regulation of the conserved 5′-regulatory region between species, appropriate fragments were PCR amplified from genomic DNA and cloned into the luciferase reporter vector pGL3-Basic. Constructs Hu-Luc, Mo-Luc, and Ra-Luc showed sequence identity with the accession numbers AF184235, AF190698, and L76612, respectively. In transfected Huh7 hepatoma cells, all constructs showed relevant luciferase activity compared with the promoterless control vector pGL3-Basic (Fig. 2*B*).

Regulation of the NTCP/Ntcp gene by HNF1α and CEBP-β. The rat *Ntcp* gene contains a highly conserved HNF1α recognition site located within the transcription start site, previously shown to bind HNF1α (22, 39). A 4-bp insertion in the corresponding DNA sequence of the human and mouse *NTCP/Ntcp* gene results in a disrupted HNF1α binding site but creates a consensus motif for the CCAAT/enhancer binding protein-β (CEBP-β; Fig. 1). To assess the effect of HNF1α and CEBP-β on the different NTCP/Ntcp reporter constructs, expression vectors were cotransfected. Coexpressed HNF1α led to a 3.5-fold increase in rat promoter activity, whereas CEBP-β did not affect the rat Ntcp promoter (Fig. 3, *A* and *C*). Conversely,

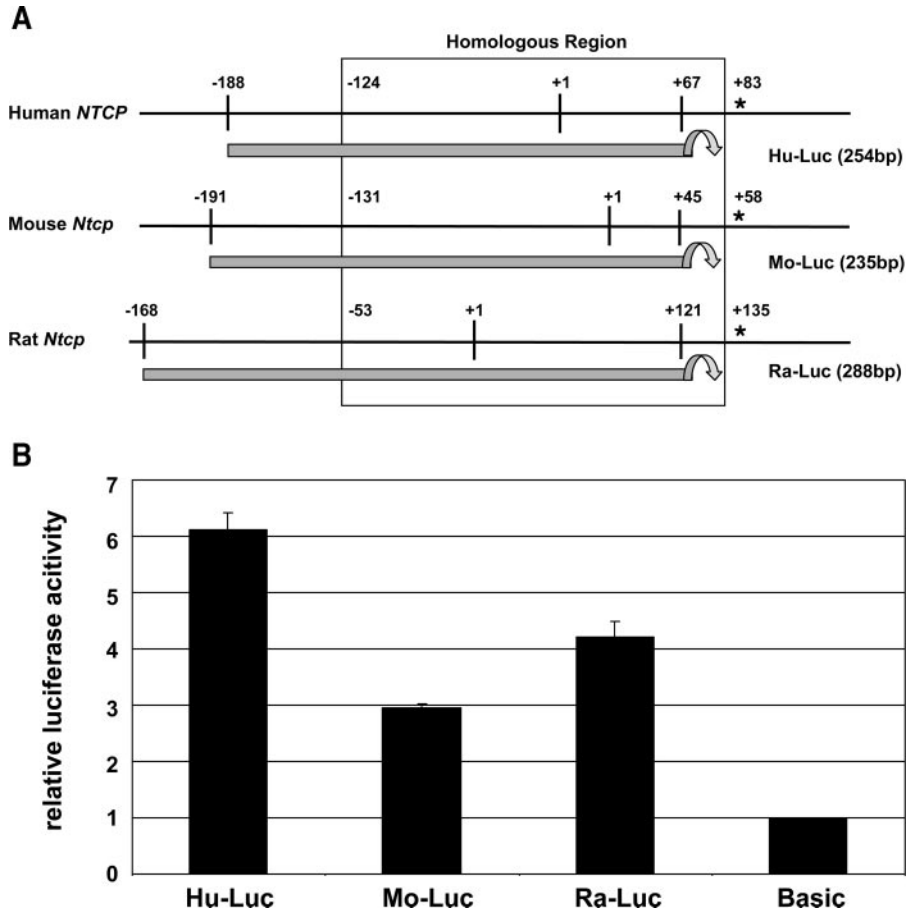

Fig. 2. Analysis of baseline human, mouse, and rat NTCP/Ntcp promoter function in Huh7 cells. *A*: design of promoter constructs used in this study. The curved arrow indicates the start of the luciferase reporter vector. *Translation start site. Nucleotide numbering is relative to the transcription initiation site (+1). The sequence conserved between species is outlined. *B*: functional analysis of the NTCP/Ntcp promoter constructs in transfected Huh7 cells. All constructs were active in this cell line compared with the promoterless pGL3-Basic vector (Basic). Hu, human; Mo, mouse; Ra, rat.

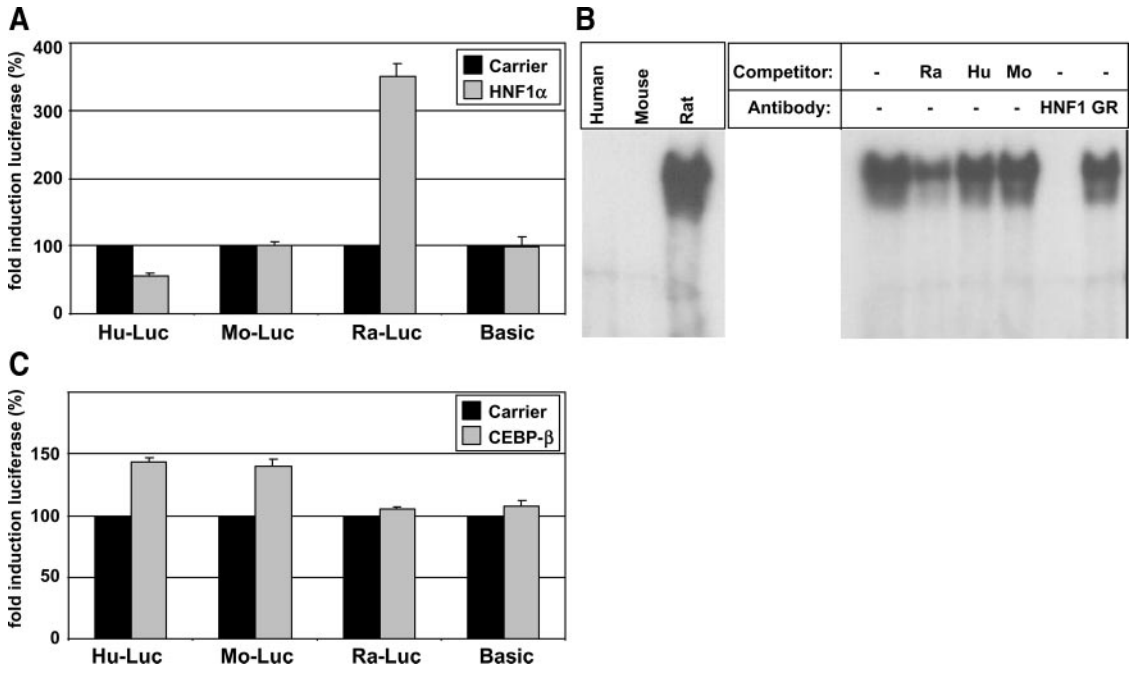

Fig. 3. Role of hepatocyte nuclear factor (HNF)1α and CCAAT/enhancer binding protein (CEBP)-β in the regulation of NTCP/Ntcp promoter function. *A*: Huh7 cells were transfected with the NTCP/Ntcp promoter constructs together with either an HNF1α expression plasmid or empty pBluescript vector (Carrier) as a control. Coexpressed HNF1α led to a 3.5-fold increase in rat Ntcp promoter activity (Ra-Luc), whereas the human and mouse NTCP/Ntcp promoter constructs were not affected. *B*: oligonucleotides derived from conserved regions of the human, mouse, or rat promoter sequences that corresponded to the HNF1α binding site in the rat gene were radioactively labeled and incubated with in vitro-translated HNF1α protein. Only the oligonucleotide derived from the rat sequence formed a DNA-protein complex. Competition experiments using the ^{32}P-labeled rat oligonucleotide and a 100-fold excess of unlabeled rat, human, or mouse oligonucleotides showed that the human and mouse oligonucleotides did not inhibit protein binding, whereas the unlabeled rat oligonucleotide inhibited the formation of a DNA-protein complex. Addition of an antibody raised against HNF1α completely abolished complex formation, whereas a control antibody against the glucocorticoid receptor (GR) had no effect. *C*: cells were cotransfected with the indicated NTCP/Ntcp promoter constructs and either a CEBP-β expression plasmid or empty pBluescript vector (Carrier). Coexpressed CEBP-β led to a moderate increase in human and mouse NTCP/Ntcp promoter activity, whereas the rat construct and the promoterless Basic vector were not affected.

the human and mouse NTCP/Ntcp constructs were not activated by HNF1α (Fig. 3*A*), whereas CEBP-β increased luciferase activity by 40% (Fig. 3*C*).

Absence of HNF1α binding to the human and mouse sequence was further supported by mobility shift assays. Only a rat-derived oligonucleotide (Table 1) was able to bind in vitro translated HNF1α protein (Fig. 3*B*). The specificity of the complex was confirmed by competition and supershift analyses. No binding occurred using the corresponding sequence of the human or mouse *NTCP/Ntcp* genes.

HNF3β represses NTCP/Ntcp promoter function. HNF3β represents the only transcription factor with conserved binding sites in the 5′-regulatory region of the human, mouse, and rat *NTCP/Ntcp* genes (Fig. 1). In cotransfection experiments, HNF3β decreased luciferase activity of all promoter constructs (Hu-Luc −66%, Mo-Luc −41%, Ra-Luc −64%), suggesting that HNF3β may directly repress the *NTCP/Ntcp* gene (Fig. 4*A*). To verify that HNF3β binds to the 5′-regulatory region, electrophoretic mobility shift assays were performed using labeled oligonucleotides that corresponded to the HNF3β-I binding motif of the human and rodent genes (Fig. 1) or to a perfect HNF3β binding site (perHNF3β in Table 1). In the presence of nuclear extracts from Huh7 cells, a specific DNA-protein complex was formed with both the human and rodent binding motif (Fig. 4*B*). This complex was competed off in the presence of excess unlabeled human and mouse or rat oligonucleotides, respectively. Using a labeled perHNF3β oligonucleotide, complex formation was again competed off by the human and rodent binding motifs, whereas a mutated sequence (mutHNF3) did not inhibit (Fig. 4*C*). Specificity of the DNA-protein complexes formed with both the NTCP/Ntcp-derived binding motifs as well as the perHNF3β binding motif was confirmed by supershift analyses (Fig. 4, *B* and *C*).

The nuclear receptors RXRα/RARα and HNF4α selectively activate the rat Ntcp construct. The nuclear receptor heterodimer RXRα/RARα is an important activator of the rat Ntcp gene (7, 22, 26). The RXRα/RARα response element is part of the conserved region and extends from nt −56 to −37 in the rat sequence. Computer analysis of the conserved 5′-regulatory region failed to identify an RXRα/RARα binding site in the human and mouse *NTCP/Ntcp* genes. In accordance with the computer prediction, only the rat but not the human or mouse NTCP/Ntcp constructs were induced by ligand activated RXRα/RARα (Fig. 5*A*). Of note, a putative binding site for HNF4α is located within the RXRα/RARα sequence. Coexpressed HNF4α increased the activity of the rat Ntcp promoter construct twofold, suggesting a possible functional role of the identified consensus motif. In contrast, HNF4α had no effect on the activity of the human or mouse NTCP/Ntcp constructs

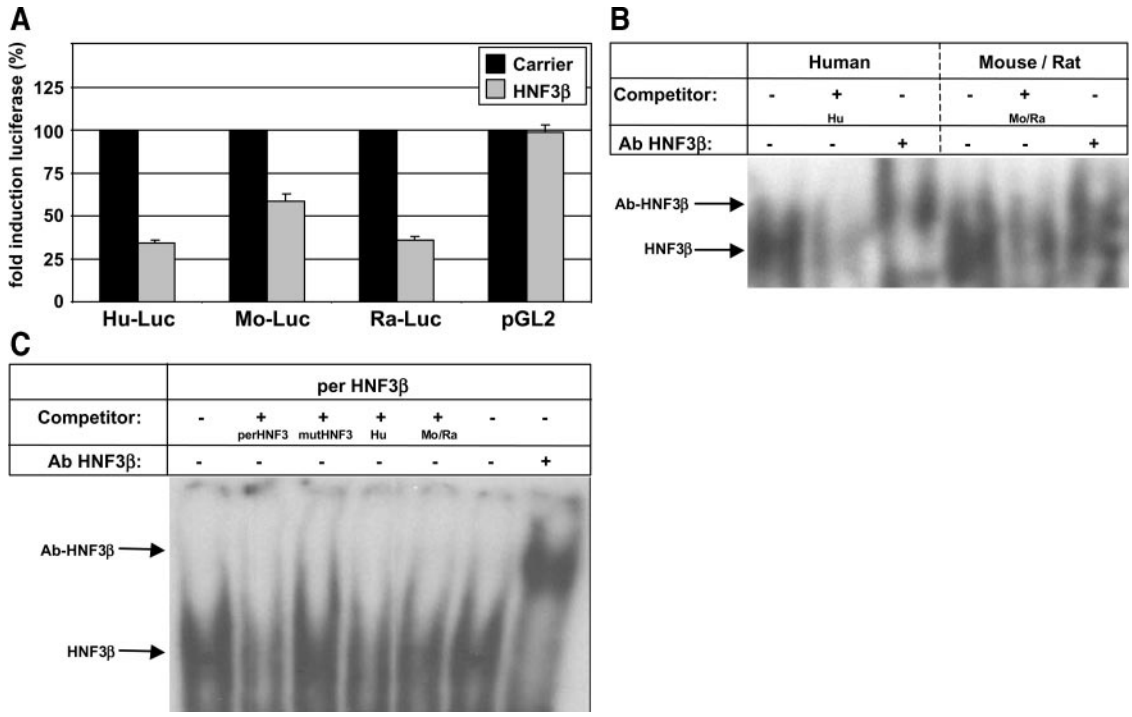

Fig. 4. HNF3β binds to the *NTCP/Ntcp* gene and represses promoter function. *A*: Huh7 cells were cotransfected with NTCP/Ntcp promoter constructs and either an HNF3β expression plasmid or pBluescript (Carrier) as a control. Coexpressed HNF3β repressed luciferase activity in all species, whereas it had no effect on the promoterless reporter vector (pGL2). *B*: HNF3β binds to a consensus motif conserved between species. Electrophoretic mobility shift assays were performed using ^{32}P-labeled oligonucleotides corresponding to the human or rodent HNF3β binding sites. The addition of nuclear extracts from Huh7 cells resulted in a DNA-protein complex (*bottom* arrow) that was competed off in the presence of excess unlabeled human (Hu) and rodent (Mo/Ra) oligonucleotides, respectively. The addition of antibody against HNF3β led to a supershift (*top* arrow). *C*: to confirm the specificity of HNF3β binding, an oligonucleotide containing a perfect consensus motif for HNF3β was incubated with nuclear extracts from Huh7 cells, resulting in a DNA-protein complex as expected (*bottom* arrow). This complex was supershifted by the addition of antibody against HNF3β (*top* arrow). The addition of excess unlabeled oligonucleotides corresponding to the sequence of the perfect consensus motif (perHNF3) or the consensus motifs in the human or rodent *NTCP/Ntcp* genes competed off DNA-protein complex formation. In contrast, an oligonucleotide containing a mutated HNF3β binding site (mutHNF3) had no effect on complex formation.

(Fig. 5*B*), despite the presence of a consensus motif at nt −43/−26 of the human sequence.

To investigate whether HNF4α activates rat Ntcp through direct binding to the putative binding site or through an indirect mechanism such as induction of HNF1α (45), two additional rat promoter deletional constructs (−12-Ra-Luc and +28-Ra-Luc) and a construct containing a mutated HNF1α binding site (mutH1-Ra-Luc) were generated (Fig. 6*A*). As shown in Fig. 6*B*, coexpressed HNF4α again increased the activity of the initial rat Ntcp promoter construct (Ra-Luc). In contrast, deletion of the HNF4α binding site in constructs −12-Ra-Luc and +28-Ra-Luc abolished this activation, whereas the full-length construct with a mutated HNF1α site was still activated (Fig. 6*B*). These data suggested that HNF4α transactivates through direct binding to the rat Ntcp promoter. To verify binding of HNF4α, electrophoretic mobility shift assays were performed using labeled oligonucleotides that corresponded to the HNF4α- RXRα/RARα binding motif of the rat *Ntcp* gene (Fig. 1, Table 1). In the presence of nuclear extracts from HepG2 cells, two specific DNA-protein complexes were formed with the rat binding motif (Fig. 6*C*). These complexes were competed off in the presence of excess unlabeled rat HNF4α oligonucleotide but not by unlabeled human or mouse oligonucleotides, respectively. Specificity of the DNA-protein complexes formed with the rat HNF4α-RXRα/RARα binding motif was confirmed by supershift analyses (Fig. 6*C*). Addition of antibody against HNF4α resulted in a supershift (*top* arrow in Fig. 6*C*) and attenuated the formation of the HNF4α DNA-protein complex (bottom arrow in Fig. 6*C*). Addition of antibody against RXRα abolished formation of the RXRα/RARα DNA-protein complex (*middle* arrow in Fig. 6*C*). These data confirmed binding of both factors to the HNF4α-RXRα/RARα response element in the rat Ntcp promoter.

Neither bile acids nor the transcriptional repressor SHP inhibit baseline NTCP/Ntcp promoter function. The rat *Ntcp* gene is thought to be repressed by bile acids via induction of the small heterodimer partner SHP through bile acid-activated farnesoid X receptor (FXR). To test this hypothesis, we used the LMH cell line, previously shown to possess conserved FXR signaling pathways (17, 21). CDCA treatment of LMH cells transfected with the different NTCP/Ntcp promoter constructs resulted in only a slight decrease in luciferase activity of the mouse and rat promoters. In contrast, the bile acid DCA decreased the activity of the rat but not the mouse promoter, indicating that only the rat promoter is in any way responsive to bile acids (Fig. 7*A*). In contrast, a promoter construct of the human *HNF1α* gene, previously shown to contain a classic "bile acid response element" (20), was markedly suppressed as

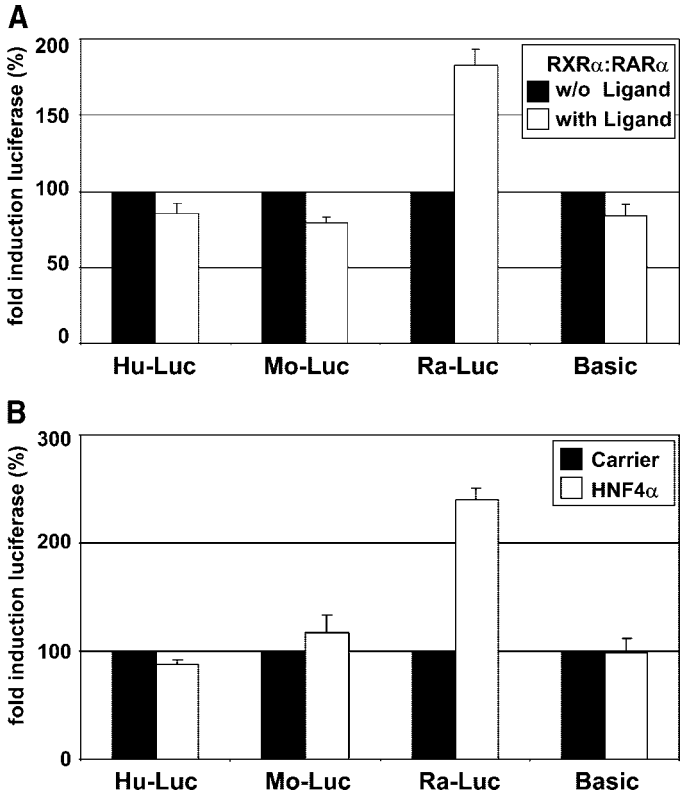

Fig. 5. Ligand-activated retinoid X receptor (RXR)α/retinoic acid receptor (RAR)α and HNF4α activate the rat Ntcp promoter construct in Huh7 cells. *A*: Huh7 cells were cotransfected with expression plasmids coding for RXRα and RARα and the indicated NTCP/Ntcp promoter constructs or the promoterless luciferase vector (Basic). Treatment with the RXRα/RARα ligands 9-*cis* retinoic acid (9cRA)/arotinoid acid {4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB)} resulted in a 2-fold activation of the rat Ntcp reporter construct, whereas neither the human and mouse NTCP/Ntcp constructs nor the control (Basic) showed any response. *B*: cotransfection of an HNF4α expression plasmid activated the rat Ntcp promoter construct 2-fold, whereas it had no effect on the human and mouse NTCP/Ntcp constructs or the Basic vector. w/o, Without.

expected (Fig. 7*A*), confirming that the bile acid-controlled gene regulatory cascade was functional in LMH cells. It is of note that the bile acid CA, which has not been described as a functional FXR ligand (29), had no effect on any promoter construct analyzed (Fig. 7*A*).

To further elucidate the role of SHP, Huh7 cells were cotransfected with the NTCP/Ntcp constructs and an expression plasmid coding for SHP. As a positive control, we again employed the HNF1α construct, which is transcriptionally repressed by SHP (20). As shown in Fig. 7*B*, baseline promoter function of all three NTCP/Ntcp constructs was not affected by SHP. Because one mechanism by which SHP represses transcription is through decreased transactivation of the target gene by the liver receptor homolog 1 [LRH; also called fetal transcription factor (FTF) in humans] (2, 13, 28), we studied whether cotransfection of LRH or FTF activates the NTCP/Ntcp constructs. Coexpression of LRH or FTF had no detectable effect on NTCP/Ntcp promoter activity (data not shown). Taken together, these results indicate that neither CDCA nor SHP repress the *NTCP/Ntcp* gene promoter via a classic bile acid response element, as shown for certain bile acid-synthesizing enzyme genes as well as for the human *HNF1α* gene (6, 13, 20, 28, 47). Decreased expression of Ntcp in rat models of cholestasis is probably not attributable to a direct repressive effect of bile acids but rather to indirect effects such as cytokine-mediated inhibition of RXRα/RARα or bile acid-mediated repression of HNF1α and HNF4α (5, 7, 20).

DISCUSSION

This study reports the initial characterization of the 5′-regulatory region of the human and mouse *NTCP/Ntcp* gene, which codes for the major bile salt uptake system of mammalian liver. A conserved region with a sequence identity of up to 80% among the human, mouse, and rat *NTCP/Ntcp* genes spans part of the promoter and the complete 5′-untranslated region. Sequence alignment of this conserved region revealed major differences in the distribution of potential transcription factor binding sites (Fig. 1). The key findings of our study can be summarized as follows: *1*) the conserved region in the promoter of the rat *Ntcp* gene, but not of human or mouse NTCP/Ntcp, is directly transactivated by HNF1α and HNF4α; *2*) human and mouse NTCP/Ntcp are activated by CEBP-β; *3*) the only transcription factor that binds to a conserved motif in all three species is HNF3β, which represses NTCP/Ntcp promoter activity in cotransfection assays; *4*) the previously described RXRα/RARα binding site in the rat Ntcp promoter is not present at the corresponding position in the human or mouse promoter; and *5*) baseline function of the conserved NTCP/Ntcp promoter region in all three species is not repressed by the bile acid CDCA and is not influenced by coexpression of SHP.

To examine the regulation of the *NTCP/Ntcp* gene, we first studied HNF1α, a known regulator of basolateral bile acid transporter genes (35). The rat *Ntcp* gene contains a highly conserved HNF1α response element, previously shown to bind HNF1α (22). In this study, we showed that coexpression of HNF1α increases rat Ntcp promoter activity, confirming its role as a transcriptional activator (Fig. 3). However, neither the human nor the mouse NTCP/Ntcp promoter constructs were activated by HNF1α. Mobility shift assays indicated that the 4-bp insertion in the human and mouse NTCP/Ntcp promoter region disrupts the HNF1α binding site present in the rat, explaining the lack of activation. In place of the HNF1α site, a consensus motif for the liver-enriched transcription factor CEBP-β is present in the human and mouse *NTCP/Ntcp* gene. Both human and mouse constructs are activated by CEBP-β in cotransfection assays, whereas the rat Ntcp construct shows no induction (Fig. 3*C*). Members of the CEBP family have been implicated in regulating the differentiation of certain mammalian cells, including adipocytes and hepatocytes (1, 30, 34). CEBP-β could, therefore, contribute to the liver-specific expression of the *NTCP/Ntcp* gene in human and mouse.

Notwithstanding these data, the HNF1α knockout mice reported by Shih et al. (35), were shown to have almost absent Ntcp expression, suggesting that HNF1α binds at a different site in the mouse *Ntcp* gene. Generally, HNF1α can activate gene expression either through binding to a site near the transcription start site, as shown for the *SLCO1B1* (protein name OATP1B1, formerly called OATP-C/OATP2), *ASBT* (*SLC10A2*), and *NPT1* genes (3, 18, 19, 35), or through binding to sites that function as enhancers and are often located within intronic sequences, as shown for the *aldolase B*, *HNF4*, and

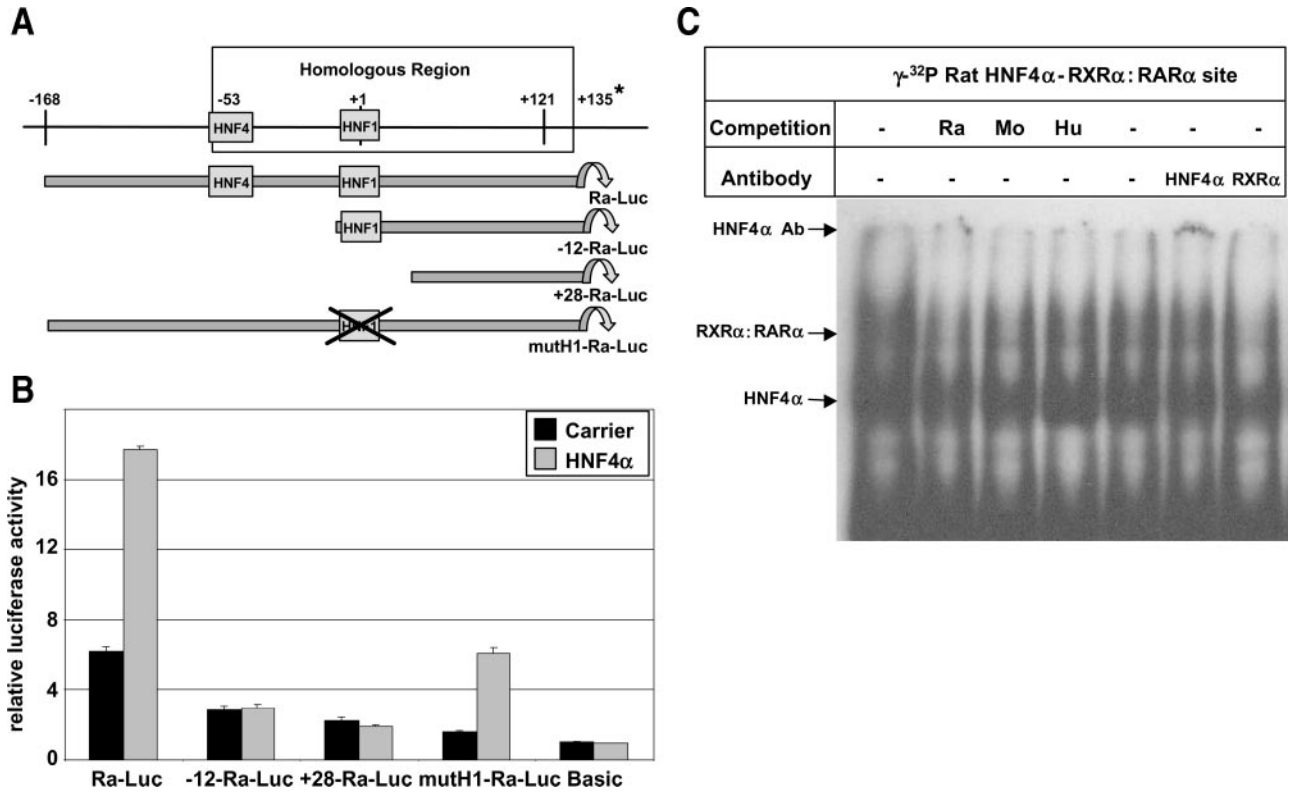

Fig. 6. HNF4α binds to the rat *Ntcp* gene. *A*: design of rat deletional constructs (−12-Ra-Luc and +28-Ra-Luc) and a construct with a mutated HNF1α binding site (mutH1-Ra-Luc). Nucleotide +1 denotes the transcription start site, the asterisk symbolizes the translation start site of the rat *Ntcp* gene. *B*: Huh7 cells were cotransfected with the rat Ntcp promoter constructs and either an HNF4α expression plasmid or pBluescript (Carrier) as a control. Coexpressed HNF4α activated rat Ntcp promoter constructs containing the HNF4α binding site (Ra-Luc, mutH1-Ra-Luc), whereas it had no effect on constructs lacking the HNF4α binding site (−12-Ra-Luc and +28-Ra-Luc) or on the promoterless reporter vector (Basic). *C*: HNF4α binds to the HNF4α consensus motif of the rat *Ntcp* gene. Electrophoretic mobility shift assays were performed using ^{32}P-labeled oligonucleotides corresponding to the rat HNF4α-RXRα/RARα binding site. The addition of nuclear extracts from HepG2 cells resulted in 2 DNA-protein complexes (HNF4α, RXRα/RARα) that were competed off in the presence of excess unlabeled rat (Ra) oligonucleotide but not by oligonucleotides derived from the corresponding sequences in the human or mouse NTCP/Ntcp promoters. The addition of antibody against HNF4α led to a supershift (*top* arrow). Addition of antibody against RXRα abolished formation of the RXRα/RARα DNA-protein complex (*middle* arrow).

NPT1 genes (3, 15, 27). Using a computer approach, we screened the proximal 1,000 bp of the promoter and all intronic sequences of the human and mouse *NCTP/Ntcp* genes for potential HNF1α binding sites. Although no HNF1α binding site was found within the promoter regions, we localized highly conserved motifs within intronic sequences (introns 1, 2, and 4 in human NTCP, intron 2 in mouse Ntcp). The exact role of these intronic HNF1α binding sites remains to be investigated.

Of note, the HNF1α$^{-/-}$ strain generated by Pontoglio et al. (32) in our hands had almost normal Ntcp expression in Western blot analysis (data not shown). In contrast, expression of the apical sodium-dependent bile acid transporter (Asbt, *Slc10a2*) was strongly decreased (data not shown). In addition, we found that luciferase constructs of the rat Ntcp promoter containing the 5′-UTR but no HNF1α binding site were still active (Fig. 6*B*), suggesting that HNF1α is not critical for baseline promoter activity. In view of these discrepancies, the exact role of HNF1α in regulating the *NTCP/Ntcp* gene remains to be elucidated.

HNF3β was the only factor with conserved binding sites in all species (Fig. 1). Coexpressed HNF3β repressed the NTCP/Ntcp promoter constructs (Fig. 4*A*), and binding of HNF3β to the HNF3β-I consensus motif (Fig. 1) was verified by mobility shift assays (Fig. 4, *B* and *C*). HNF3β is an essential transcription factor during embryonic development and is thought to be a genetic initiator of the hepatic differentiation program (37, 46). However, the function of HNF3β in adult liver is not fully understood. Ntcp mRNA levels are substantially reduced in transgenic mouse hepatocytes overexpressing HNF3β, and serum bile acid levels are increased 50-fold (33, 38). In humans, increased expression of HNF3β in hepatocellular carcinomas is associated with decreased NTCP expression in Northern blot and immunofluorescence analyses (23, 41). These results suggest that HNF3β may repress *NTCP/Ntcp* gene expression in vivo.

Several studies (25, 40) have suggested a repressive effect of bile salts on the *NTCP/Ntcp* gene in view of the consistent decrease in NTCP/Ntcp expression that is found in cholestasis. In the case of the rat *Ntcp* gene, the repressive effect of bile acids has been proposed to involve inhibition of retinoid activation of the nuclear receptor dimer RXRα/RARα by the transcriptional repressor SHP (8). The RXRα/RARα binding element in the rat *Ntcp* gene is part of the conserved

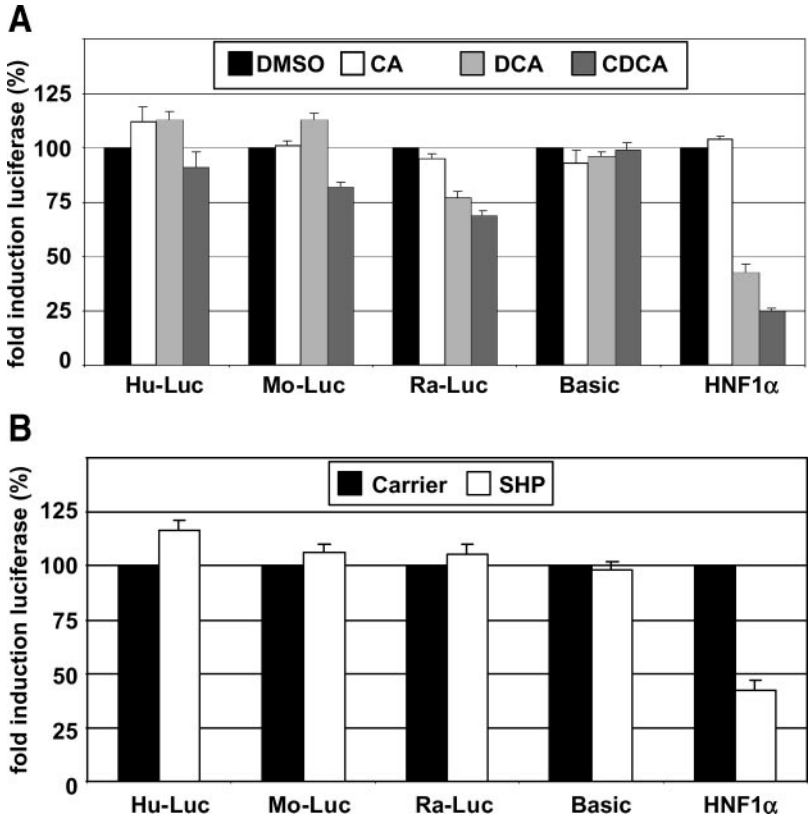

Fig. 7. Effect of bile salts and small heterodimer partner (SHP) on NTCP/Ntcp promoter function. *A*: LMH cells transfected with the indicated NTCP/Ntcp reporter constructs were treated with cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA; 100 μM), or DMSO as a control. CDCA treatment resulted in a 30% decrease, and DCA treatment resulted in a 25% decrease in rat Ntcp luciferase reporter activity, whereas the human and mouse constructs and the promoterless luciferase vector (Basic) did not show a comparable response. As a control, a promoter construct of the human *HNF1α* gene was shown to be repressed by CDCA and DCA as expected. The bile acid CA, which is not an farnesoid X receptor ligand, did not change promoter activity. *B*: Huh7 cells were cotransfected with the indicated NTCP/Ntcp promoter constructs and either an expression plasmid coding for the nuclear receptor SHP or pBluescript vector (Carrier). Coexpressed SHP did not affect NTCP/Ntcp promoter activity in any species, whereas the SHP-responsive promoter of the human *HNF1α* gene was repressed as expected.

region (Fig. 1). In the human and mouse genes, the binding site is disrupted by nucleotide exchanges (Fig. 1). Accordingly, only the rat Ntcp reporter construct is activated by retinoid ligand treatment, whereas the human and mouse promoters show no response (Fig. 5*A*). This probably represents a major difference in the regulation of the rat compared with the human and mouse *NTCP/Ntcp* genes, because the repressive effects of bile acids, cholestasis, and cytokines on Ntcp expression have been largely attributed to decreased transactivation by RXRα/RARα (7, 8, 26). Clearly, binding of RXRα/RARα to a sequence that is not part of the conserved 5′-regulatory region cannot be excluded for the human and mouse genes. The nucleotide sequence spanning the RXRα/RARα site in the rat promoter also represents an HNF4α binding site, as shown by cotransfection and mobility shift assays in this study (Fig. 6). One can only speculate as to whether, in vivo, both factors, RXRα/RARα and HNF4α, are corequisite for rat *Ntcp* gene expression or whether they have distinct functions under different physiological conditions.

Although bile acids were previously reported to inhibit retinoid activation of RXRα/RARα in the rat through an SHP-mediated mechanism, a direct suppressive effect of SHP on the Ntcp promoter has not been shown in an in vitro system. We, therefore, tested the hypothesis that SHP directly represses NTCP/Ntcp promoter activity. As an assay system, we employed LMH cells that possess a conserved FXR signaling pathway and have been used extensively as a model for ligand-dependent activation of endogenously expressed nuclear receptors (17, 18, 21). Using the LMH cell culture system, we did not find relevant repression of NTCP/Ntcp promoter activity by treatment of cells with the FXR ligands CDCA and DCA (Fig. 7*A*). In addition to the lack of repression by bile acids, overexpression of the nuclear receptor SHP was also without effect on the NTCP/Ntcp constructs (Fig. 6*B*). In contrast, a promoter construct of the *HNF1α* gene, previously shown to be negatively regulated by bile acids through an SHP-mediated pathway (20), was repressed by both bile acids (CDCA, DCA) and SHP as expected. The lack of a repressive effect of cotransfected SHP on the NTCP/Ntcp constructs is in agreement with in vivo studies in $SHP^{-/-}$ mice. CA feeding represses Ntcp expression to the same degree in $SHP^{-/-}$ mice as in wild-type $SHP^{+/+}$ mice, indicating that the repressive effect of bile acids on Ntcp expression is not directly mediated by SHP (43). It is likely that other mechanisms are responsible for the negative regulation of NTCP/Ntcp by bile acids. One such mechanism could be the induction of cytokines by bile acids (4), because several transcription factors such as ligand-activated RXRα/RARα, HNF1α, and HNF4α, which are important for the expression of rat Ntcp and other liver-specific genes, are suppressed by cytokines (39, 42). Because the rat Ntcp promoter binds and is transactivated by HNF4α (Figs. 1, 5*B*, and 6), decreased expression of rat Ntcp in cholestasis could be attributable to the known repressive effect of bile acids and cytokines on nuclear HNF4α levels (20, 42, 47). Moreover, bile acids block the association of HNF4α with its transcriptional coactivators, thereby inhibiting the transactivation of target genes of HNF4α (5). The latter mechanism has been shown to be a major pathway by which bile acids repress the cholesterol 7α-hydroxylase gene (5).

In summary, this study shows that the liver-enriched transcription factors HNF1α, CEBP-β, HNF3β, and HNF4α and the nuclear receptor dimer RXRα/RARα have an important but species-specific function in the regulation of the *NTCP/Ntcp* gene. The transcriptional regulation of *NTCP/Ntcp* thus differs among human, mouse, and rat. Of note, the conserved 5′-regulatory region of the *NTCP/Ntcp* gene does not possess a direct bile acid response element, suggesting that bile acids regulate NTCP/Ntcp expression through indirect mechanisms.

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