Hepatocyte nuclear factor 4α is a central transactivator of the mouse Ntcp gene

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Abstract

Sodium taurocholate co-transporting polypeptide (Ntcp) is the major uptake system for conjugated bile acids. Deletions of HNF1α and RXRα:RARα binding sites in the mouse 5′-flanking region corresponding to putatively central regulatory elements of rat Ntcp do not significantly reduce promoter activity. We hypothesized that HNF4α, which is increasingly recognized as a central regulator of hepatocyte function, may directly transactivate mouse Ntcp. A 1.1 kb 5′-upstream region including the mouse Ntcp promoter was cloned and compared to the rat promoter. In contrast to a moderate 3.5-fold activation of mNtcp by HNF1α, HNF4α cotransfection led to a robust 20–fold activation. Deletion analysis of mouse and rat Ntcp promoters mapped a conserved HNF4α consensus site at -345/-326 and -335/-316 bps, respectively. p-475bp mNtcpLUC is not transactivated by HNF1α but shows a 50-fold enhanced activity upon cotransfection with HNF4α. Gel mobility shift assays demonstrated a complex of the HNF4α-element formed with liver nuclear extracts which was blocked by a HNF4α specific antibody. HNF4α binding was confirmed by chromatin immunoprecipitation. Using Hepa 1-6 cells HNF4α-knockdown resulted in a significant 95% reduction in NTCP mRNA. In conclusion, mouse Ntcp is regulated by HNF4α via a conserved distal cis-element independently of HNF1α.

Keywords: Hepatocyte-enriched transcription factors, nuclear hormone receptors, transcriptional coactivator, gene regulation, bile acid transport
INTRODUCTION

The coordinated expression of a large number of genes is required to maintain essential functions of the adult liver in metabolic homeostasis including bile formation. Physiologically, bile acids and other organic anions are taken up from portal blood and secreted into bile by ATP-dependent export pumps thereby generating an osmotic gradient. The Na\(^+\)/taurocholate cotransporter (Ntcp; \textit{SLC10A1} in humans, \textit{Slc10a1} in rodents), a 56 kDa basolaterally localized membrane transporter, probably represents the main hepatocellular sodium-dependent uptake system for conjugated bile acids from sinusoidal blood in human and rodent liver (2,12,13).

Regulation of \textit{Ntcp} gene expression primarily occurs at the transcriptional level (6,20,28). Hepatocyte nuclear factor 1\(\alpha\) (HNF1\(\alpha\), TCF1) and retinoid X receptor (RXR\(\alpha\), NR2B1) : retinoic acid receptor (RAR\(\alpha\), NR1B1) heterodimer complex have so far been identified to control rat \textit{Ntcp} gene transactivation (19,28). Decreased binding activity at these two regulatory elements \textit{in vivo} occurs by either induction of inflammatory cytokines or retention of bile acids and leads to down-regulation of \textit{Ntcp} gene expression (3,7,28). In accordance with the concept of HNF1\(\alpha\) as a major regulator of the \textit{Ntcp} gene, \textit{Hnf1} knock-out mice (\textit{Tcf1/-}) exhibit a decreased Ntcp expression to less than 10\% of wild type controls (24).

In recent studies HNF4\(\alpha\) (NR2A1) has become increasingly recognized as a central regulator of hepatocyte differentiation and function (29). HNF4\(\alpha\) belongs to the nuclear hormone receptor family of transcription factors and binds DNA as a homodimer (25,29). Recently, HNF4\(\alpha\) has been found to contribute to a large
fraction of the liver transcriptome (21). In this study using combined chromatin-immunoprecipitation with a promoter microarray HNF4α has been found to bind over 40% of the active promoters in hepatocyte tissue (those occupied by RNA polymerase II, and thus likely to be transcribed).

In adult hepatocyte-specific conditional Hnf4 knock-out mice (H4LivKO) using the Cre-loxP system expression of a large number of genes whose gene products are essential for adult liver function are disrupted (15,16,18,26,27). These mice with Hnf4-null hepatocytes present with high serum bile acid levels and defects in bile acid transport including decreased expression of the Ntcp (Slc10a1) gene (15).

HNF4α and HNF1α occupy each other’s promoter as an example of a multicomponent regulatory loop in hepatocytes (11,21). However, HNF4α occupies significantly more genes in the pancreatic β-cell and the hepatocyte than does HNF1α (11). This finding does not support the theory that HNF4α necessarily mediates its effects on gene expression (including Ntcp) solely through the indirect regulation of HNF1α (11). Further information will be particularly useful in the interpretation of changes in Ntcp expression in HNF4α and HNF1α gene knockout mouse models.

Deletions of HNF1α and RXRα : RARα binding sites in the mouse 5'-flanking region corresponding to putatively central regulatory elements within the minimal rat Ntcp promoter did not significantly reduce promoter activity. This fact further questions the importance of these transactivators for rodent Ntcp gene expression (9,17). Based on these data, we hypothesized that HNF4α may directly
transactivate mouse and rat Ntcp through a thus far unknown element. We therefore characterize the role of HNF4α in controlling rodent Ntcp gene transactivation.

MATERIALS AND METHODS

Cloning of mouse Ntcp promoter constructs. The mouse Ntcp 5’-untranslated region including the promoter was cloned as previously described (9) and is published in Genbank (Accession number AF190698). The numbering of the mouse promoter is deduced from the rat transcription start site (Genbank L76612) as determined by Karpen et al. (19) by alignment of the mouse sequence (AF190698, with the transcription start site starting at position 975). Further numbering of the mouse promoter fragments was made relative to this site being designated as +1. Mouse Ntcp promoter fragments p-974bpNtcpLUC and p-475bpNtcpLUC in pPX2Δ2 vector were constructed as previously described (9). Additional deletion constructs were generated by PCR and cloned into the KpnI and BglII sites of pPX2Δ2. The following constructs were generated: p-350bpNtcpLUC, p-325bpNtcpLUC, p-175bpNtcpLUC, p-120bpNtcpLUC and p-69bpNtcpLUC.

Cloning of rat Ntcp promoter constructs. The rat Ntcp promoter was subcloned from pDB1.5, Genbank L76612 (19) into pGL3basic with MluI and BglII restriction sites generating a construct spanning nt –1230 to + 46 of the rat Ntcp promoter region. Further deletion constructs were generated by PCR and cloned into the MluI and BglII sites of pGL3basic thereby generating the following
constructs: p-146bprNtcpLUC, p-307bprNtcpLUC, p-368bprNtcpLUC, p-469bprNtcpLUC, p-620bprNtcpLUC, p-770bprNtcpLUC, p-930bprNtcpLUC and p-1230bprNtcpLUC. The sequence of the oligonucleotides used for PCR is available from the authors on request.

**Cell culture and transient transfections.** Hepa 1-6 and HepG2 hepatoma cells (1 x 10^5 cells/well) cells were obtained from American Tissue Culture Collection (ATCC, Manassas, VA) and grown in D-MEM or D-MEM/F12 (GibcoBRL) respectively and supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells were subcultured weekly using trypsinization followed by 1:20 dilution and plating in 100 mm petri dishes. Transfections of Ntcp promoter constructs in HepG2 cells were performed utilizing either Fugene6 (Roche, Indianapolis, IN, according to manufacturer’s instructions) or standard calcium phosphate DNA coprecipitation techniques. In cotransfection experiments, 1.8 µg/ml of each mNtcpLUC pPX2Δ2 construct was combined with 0.1-0.75 µg/ml of HNF4α- or HA-tagged PGC-1α-expression plasmids (Dr. A. Kralli, Scripps Institute, La Jolla, CA). Cointroduced pCMVβGal expression vector served as an internal control for transfection efficiency. Promoter activities are given as the mean ± SD of triplicate transfections.

**Electrophoretic mobility shift assays.** Preparation of nuclear extracts was performed as previously described (8). Protein (10 µg) was incubated on ice for 30 min with 2 x 10^4 cpm ^32^P-end-labeled oligonucleotide probe (5’-TTAGATGAGGAAGGCAAAGGCAGAAA-3’ corresponding to -352/-327 in the mNtcp promoter). For supershift assays, nuclear extracts were preincubated
for 30 min on ice with 5 µg of a polyclonal HNF4a antibody (sc-8987, Santa Cruz Biotechnology) before addition of labeled oligonucleotides. For competition assays, a 100-fold molar excess of unlabeled oligonucleotides was coincubated with the labeled probe. Separation of protein-DNA complexes was obtained by electrophoresis through a non-denaturing 6% polyacrylamide gel and quantified by phosphorimaging.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assays were performed using the Chromatin Immunoprecipitation Assay Kit from Upstate Cell Signaling Solutions according to their protocol. Briefly, mouse Hepa 1-6 cells were grown to 80-90% confluency in 10 cm dishes and crosslinked with 1% formaldehyde in tissue culture medium for 10 min at 37 °C. Cell samples were washed twice in ice-cold phosphate-buffered saline containing protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml pepstatin A). Cells were pelleted and lysed in SDS Lysis Buffer (Upstate Biotechnology Inc.). Samples were sonicated to shear DNA to a size between 200 and 1000 bp. Subsequently, an aliquot of sheared DNA was analyzed by agarose gel electrophoresis to confirm the correct size of sheared DNA length for immunoprecipitation. The chromatin samples were incubated overnight at 4 °C with HNF4α- (Santa Cruz, sc-8987) antibody. As positive and negative controls the recommended anti-acetyl Histone H4 polyclonal antibody (Upstate, #06-866) and anti-cytochrome C antibody (Santa Cruz Biotechnology, sc-8385), respectively, were used and another reaction without antibody served as a second negative control. Immune complexes were precipitated with salmon sperm DNA-bovine serum albumin-
Sepharose beads. DNA was prepared by treatment with DNase- and RNase-free proteinase K, extraction with phenol and chloroform, and ethanol precipitation. PCR was performed with primers flanking the predicted HNF4α-binding site (forward: 5’-ACAAAGCAAGGTCTCAGAGGAGGAC-3’, reverse: 5’-GCTTCTACCCCATCGGAGAAAACG-3’)

**Mutational analysis.** Mutations in the HNF4 site of the mouse Ntcp promoter were carried out using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer’s directions. Primers used for generating the desired mutants are listed in Table 1. Mutated DNA sequences were verified by sequencing using ABI Prism machine.

**Transfection of Hepa 1-6 cells with control and HNF4 siRNAs and analysis of message levels by qPCR.** Hepa 1-6 cells were plated at a density of 5 x 10^5 cells/well in 6-well plates. Twenty-four hours later, the cells were transfected with control or m HNF4 siRNAs (siGENOME SMART pool, Dharmacon) at a final concentration of 50 nM. The SMART pool against mouse HNF4 contained a mix of 4 duplexes of siRNAs with the following sense sequence: Duplex 1: 5’-GAAGGAAGCUGUCCAAAAAUU-3’; Duplex 2: 5’-AGAGGUUCAUGGUGUUUAAAUU-3’; Duplex 3: 5’-UGUCGUUACUGGCUUUAUU-3’; Duplex 4: 5’-CUAACACGAUGGCCCUCCUCUAAUU-3’. Briefly, 5 µl control or HNF-4 of siRNA in 95 µl of OPTI-MEM and 5 µl of Trans-IT TKO in 95 µl of OPTI-MEM are incubated for 5 minutes after which they are mixed and further incubated for 20 min at room temperature. The mixture was added to cells in 6-well plates after
they have been washed once with OPTI-MEM. After a 24 hr incubation, the medium was replaced with D-MEM and the incubation continued for additional 24 hrs. The wells were then processed for total RNA isolation using TRIZOL reagent according to manufacturer’s instructions. 1 µg of total RNA was reverse-transcribed using Invitrogen’s Superscript First Strand Synthesis system for RT-PCR according to instructions. For qPCR 50 ng reverse-transcribed cDNA was used per well using Quantitect SYBRGreen PCR kit (Qiagen Corporation, CA) in a Bio-Rad Mini Opticon 3 with the following cycle parameters: 1) one cycle at 95°C for 15 min 2) 40 cycles each consisting of 1) denaturation at 95°C for 15 sec. 2) annealing at 56°C for 30 sec and 3) extension 72°C for 30 sec. 4) A final extension cycle at 72°C for 10 min. After each cycle the wells were read for emitted fluorescence and at the end of completion of PCR a melting curve analysis was included in the program. Primers were selected using Primer Express (Applied Biosystems, Norwalk, CT) to amplify 80-90 bp region using cDNA sequences for mouse HNF4, Ntcp and 36B4 in the NCBI database and are listed in Table 1. For all primers, melting curve analysis was conducted to verify that the primers resulted in a single peak of fluorescence with no primer-dimers. C_T values of each of the genes obtained were normalized by subtracation of C_T values for 36B4 (a constitutive ribosomal RNA gene). Relative expression levels were obtained using the Comparative Method as described in ABI Reference Manual using the formula 2^-ΔΔCt keeping the message level obtained in the control siRNA-treated cells at 100%.
Western blot analysis of HNF4 and HNF1 protein expression after control and HNF4 siRNA treatment. Hepa 1-6 cells were treated with control siRNA or HNF4 siRNA as described previously. Cell lysates were prepared by resuspension of cell pellet in Mammalian Protein Extraction Reagent (MPER, Pierce Chemical Company, Rockford, IL) with protease inhibitor cocktail (Sigma, St. Louis, MO), incubation on ice for 15 min followed by centrifugation at 14,000 x g for 15 min at 4°C. Western blotting of cell lysates and analysis of HNF1 and HNF4 protein levels were carried out according to procedures previously described (1). Equal amount of protein (184 µg) protein was loaded from the differentially-treated samples. Polyclonal antibodies to HNF1 (sc-8986, H-205) and HNF4 (sc6556, C-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a 1:2000 dilution. Protein levels for HNF1 and HNF4 as seen in the Western blots were quantitated using Image J (from NIH web site). As positive controls for HNF4, nuclear extract from Huh-7 cells transfected with an expression plasmid for rat HNF4 cDNA was used.
RESULTS

Transactivation of mouse Ntcp promoter by liver-enriched transcription factors

The mouse Ntcp promoter activity has been shown to be increased 3.5-fold upon co-transfection of a HNF1α-expression plasmid in luciferase assays (9). In order to investigate the potential of HNF4α to transactivate the mouse Ntcp promoter a HNF4α expression plasmid was co-transfected into HepG2 cells together with the longest mNtcp promoter construct p-974bpmNtcpLUC. To test for a linear dependency of HNF4α-induced gene transcription with the transfected amount of the HNF4α expression plasmid, overexpression of HNF4α reporter gene activity was increased in a concentration-dependent fashion between 0.1 and 0.5 µg/ml plasmid DNA (Figure 1A). In subsequent experiments a concentration of 0.25 µg HNF4α expression plasmid / ml medium was used for co-transfection experiments. Strikingly, promoter activity was up-regulated approximately 20-fold compared to basal levels (Figure 1B). This suggests the presence of a HNF4α cis-acting element in the 5' untranslated region of the mNtcp promoter. Interestingly, the minimal p-475bpmNtcpLUC promoter construct – which lacks any identified HNF1α binding site and is not transactivated by HNF1α (9) – shows a 50-fold enhanced activity upon cotransfection with HNF4α compared to basal levels (Figure 1B). These data strongly argue for a direct HNF4α-based effect on reporter gene expression independently of HNF1α.
**Basal mouse Ntcp promoter activity**

Transfection studies with mouse *Ntcp* promoter reporter constructs were performed in HepG2 cells. A series of promoter deletion constructs extending from -974bp to -69bp (p-974bpmNtcpLUC, p-475bpmNtcpLUC, p-350bpmNtcpLUC, p-325bpmNtcpLUC, p-175bpmNtcpLUC, p-69bpmNtcpLUC) or the empty reporter vector pXP2ΔLUC were transfected into HepG2 cells to determine basal promoter activities (Figure 1C). The minimal p-69bpmNtcpLUC construct exhibited background luciferase activity and the activity of the p-325bpmNtcpLUC promoter fragment was approximately 40% compared to the full-length p-974bpmNtcp promoter. Addition of a further 25 nt resulting in p-350bpmNtcpLUC increased basal promoter activity roughly five-fold (Figure 1C). These results indicate the presence of a regulatory element responsible for constitutive *Ntcp* gene expression within promoter sequence -350/-325 bp.

**Mapping the HNF4α cis-acting element in the mouse Ntcp promoter**

To identify the location of the putative HNF4α-binding site in the mouse *Ntcp* promoter, deletion constructs of the p-974bpmNtcpLUC-construct were generated as described in Materials and Methods and co-transfected in HepG2 cells with an HNF4α expressing plasmid. HNF4α conferred high reporter gene activity in constructs containing a minimal promoter sequence of 350 bp – ranging between 20- and 24-fold compared with basal activity of the full-length promoter (Figure 1C). In contrast, the truncated promoter beginning at -325 bp exhibits a
significantly reduced activation accounting for only around 25% of the p-350bpmNtcpLUC-activity. Another drop in LUC-activity was observed between -175 and -69 bp.

**HNF4α binds the mouse Ntcp promoter**

To confirm that the putative HNF4α binding site truly serves as a *cis*-acting element DNA and chromatin binding studies were performed. Firstly, a complex formation was detected after incubation of radiolabeled oligo *mNtcp* -352/-327 encompassing the newly identified site with mouse liver nuclear extract. Complex formation could be blocked using an HNF4α-specific antibody (Figure 2A).

Further corroboration came from chromatin immunoprecipitation (ChIP) assays (Figure 2B) performed on DNA from Hepa 1-6 cells. Chromatin DNA was precipitated using either anti-HNF4α antibody, anti-acetyl-Histone 4 (H4) antibody (positive control) or a cytochrome C antibody (negative control) prior to incubation with protein A Sepharose. Subsequent to purification of the precipitated DNA the sequence encompassing the HNF4α binding site was amplified using specific primers. Firstly, it was confirmed that the primer pairs gave a specific product with purified input DNA (data not shown). HNF4α antibody but not the cytochrome C antibody (as a negative control) brought down chromatin-associated DNA which when used in PCR amplified the 226-bp fragment containing the HNF4α response element. Again, this experiment confirms that HNF4α occupies the mouse *Ntcp* promoter. Respective controls verified the specificity of the signal (Figure 2B).
Mutation of the HNF4α binding site in mouse Ntcp promoter results in decreased reporter gene activity

To further characterize the functional role of HNF4α on mNtcp gene expression, we studied the effects of a HNF4α binding site mutation on Ntcp promoter transactivation. Mutation of the HNF4α binding site led to a 42% decrease in activity (11.8 ± 3.3 in wild type compared to 6.9 ± 0.5 normalized luciferase activity in the mutated promoter-transfected cells; 2\textsuperscript{nd} bar vs. 3\textsuperscript{rd} bar) (Figure 3). Peroxisome-proliferator-activated receptor gamma coactivator-1α (PGC-1α) has previously been shown to serve as a coactivator for HNF4α for a number of genes using promoter studies (22,30). Consistent with this notion, when we cotransfected PGC-1α with the mouse Ntcp promoter into hepatoma cells we observed a 50% increase in activity compared to cells that were not cotransfected suggesting that PGC-1α further potentiated the effect of HNF4α on the mouse Ntcp promoter (Figure 4; 1\textsuperscript{st} bar vs 2\textsuperscript{nd} bar; 17.8 ± 0.9 in PGC-1α cotransfected cells vs 11.8 ± 3.3 normalized luciferase activity in controls). Transfection of PGC-1α alone with the promoter did not result in significant change in activity.

HNF4 siRNA treatment results in downregulation of Ntcp message

In order to see if HNF4 plays a significant role of transcription of Ntcp mRNA \emph{in vivo}, mouse-derived Hepa1-6 cells were treated with siGENOME SMARTpool siRNAs for mouse HNF4 and a control siRNA obtained from Dharmaco as described in Materials and Methods. SMARTpool consists of a mixture of
siRNAs for different parts of the message which has been shown to result in uniform downregulation of message levels for many proteins compared to treatment with a single siRNA species. As would be predicted, transfection of Hepa 1-6 cells with HNF4 siRNA led to significant downregulation of HNF4 mRNA (4.3 ± 0.6 % of control siRNA, p<0.005, Fig. 4) as measured by real time qPCR. Consistent with the data on the mNtcp promoter studies shown above, Ntcp message levels were also significantly downregulated in HNF4 siRNA-treated cells compared to control siRNA treatment (4.6 ± 1.2 % controls; p<0.001). We wanted to see if HNF4 siRNA treatment also led to downregulation of HNF4 and HNF1 proteins by Western blot analysis using specific antibodies to HNF4 and HNF1. Data in Figure 5 show that HNF4 siRNA treatment resulted in decreased HNF4 (0.4% of control) and HNF1 (26% of control) proteins consistent with the real-time PCR data. Taken together with the data on the mNtcp promoter, these observations show that HNF4 plays a significant role in transcriptional regulation of the Ntcp gene. Thus, our studies also confirm the fact that liver-specific HNF4 null mice exhibited significant downregulation of Ntcp mRNA in their livers (15).

Comparison of HNF4α transactivation of mouse and rat Ntcp promoters

To investigate if HNF4α mediates transactivation of rodent Ntcp promoters in general, several deletion constructs of the rat Ntcp promoter were also tested in the luciferase gene reporter assay using HNF4α cotransfection experiments. Similar to the mouse Ntcp promoter the promoter fragment encompassing
approximately 350 bp (p-368bprNtcpLUC) displayed a four-fold higher reporter gene activity than the slightly shorter p-307bprNtcpLUC truncation (Figure 6A). In general, activities of the rat Ntcp promoter mirrored those of the highly homologous mouse sequence. Analysis of the mouse sequence between -350 and -325 revealed a putative HNF4 binding site which is conserved in the rat promoter sequence (Figure 6B).

**DISCUSSION**

Sodium taurocholate cotransporting polypeptide (NTCP/Ntcp) is a 56 kDa polypeptide localized exclusively at the basolateral membrane of the hepatocyte and mediates Na-dependent uptake of conjugated bile acids. Previous work from our laboratory and those of others have implicated HNF1, RARα:RXRα, C/EBPβ and GR in activation of the rat/human genes (5, 19). We recently performed preliminary analysis of the mouse 1.1 kb Ntcp promoter and found that an upstream HNF1 site is involved in regulation by HNF1 (9). HNF4α (NR2A1) is a member of the nuclear receptor superfamily which is highly conserved and expressed at high levels in liver, kidney, intestine and pancreas in mammals and homologous structures in vertebrates. It can be activated in the absence of ligands but some studies have suggested that fatty acyl CoA-thioesters can act as agonists or antagonists depending on fatty acid chain length and degree of saturation (23). A large number of genes were shown to be targets of HNF4α in liver and pancreas based on a recent study using ChIP based promoter analysis (21). Most of the
target genes were also positive for ChIP using an antibody to RNA polymerase II suggesting that HNF4α is involved in initiation.

HNF4α is a transcription factor controlling a majority of hepatocyte-specific genes. We wanted to investigate if it is involved in direct regulation of NTCP/Ntcp, although it can also indirectly regulate via HNF1α. In order to map the potential HNF4 response element in mouse Ntcp promoter, we initially created nested deletion constructs from -69 to -974 bp upstream of the start site and found that significant basal activity is obtained in constructs containing at least -350 bp (Figure 1C). In order to differentiate between HNF1 and HNF4 transactivation, we examined the transactivation after cotransfection with HNF1 and HNF4 encoding plasmids. While there was significant transactivation by HNF4 in reporter constructs containing at least -475 bp, most activation was obtained in constructs less than -475 bp of the 5’-untranslated region where HNF1-induced transactivation is almost fully abolished (Figure 1B). A further deletion construct that differed only by 25 bp between -325 bp and -350 bp was then generated to finely map the binding site as shown in Figure 1C. The p-325 construct had modest activation while further addition of 25 bp led to sharp increase in HNF4 transactivation capacity.

Furthermore, we established that the murine response element is bound by HNF4 using EMSA and ChIP assays. The gelshift experiment demonstrates that the HNF4-oligonucleotide complex can be blocked by a specific antibody to HNF4 (Figure 2A). Albeit there's no supershift visible upon HNF4 antibody addition the disappearance of the complex is a clear signal for specificity together with the
specific competition experiment resulting in loss of labelled complex. To assess
direct promoter occupation at the chromatin level, we performed ChIP assays with
the mouse \( Ntcp \) promoter and a specific HNF4 antibody and demonstrate that the
region of the promoter containing -345/-326 is indeed occupied by HNF4 (Figure
2B).

Additional studies were conducted to functionally prove that HNF4 directly
transactivates \( Ntcp \) promoter. Mutation of the putative binding site in \( \text{mutNtcp} \)
promoter exhibited a significant loss of transactivation by HNF4 (Figure 3). The
reason for the inability to completely abrogate the transactivation by HNF4 in the
mutant construct may be due to the presence of yet another HNF4 binding site in
the mutated construct. However, based on TRANSFAC and MATINSPECTOR
sequence analyses no other potential HNF4\( \alpha \) site can be identified within the
respective region of the mouse \( Ntcp \) promoter. Further proof that the
transactivation by HNF4 is direct comes from the fact that the transactivation was
potentiated by cotransfection with PGC-1\( \alpha \) (Figure 3) as shown for a number of
HNF4 responsive genes.

In order to demonstrate that HNF4 siRNA had any effect on endogenous Ntcp
levels, we transfected Hepa 1-6 cells with a smartpool siRNA for HNF4 and
measured Ntcp mRNA levels in addition to protein and mRNA levels for HNF4
and HNF1. The results from this analysis shown in Figures 4 and 5 indicate that
there was significant decrease in the mRNA levels for HNF4 and, more
importantly, as expected from our hypothesis, for Ntcp. Since the HNF1 promoter
contains a binding site for HNF4 (21) it is quite to be expected, HNF1 protein levels dropped to 26% in HNF4 siRNA treated cells.

In order to study if the homologous rat Ntcp promoter also possessed a HNF4 binding site, nested deletion constructs were created and the transactivation by HNF4 was tested. As shown in Figure 6A, HNF4 cotransfection experiments had a comparable effect on rat and mouse promoters when their promoters contained > -300bp upstream of the start site. Comparison of the sequences in this region (-345/-326, Figure 6B) between rat and mouse showed the presence of a conserved binding site in both species. The rat sequence had only two changes compared to the mouse sequence suggesting high conservation of the binding site. Very recent studies from our group further corroborate the notion that HNF4 also regulates Ntcp expression in rats (4). In rats Ntcp was upregulated 1.5-fold after 48 hours of food deprivation concomitant with a 2-fold enhanced DNA binding activity of HNF4.

Based on these data, we propose that HNF4 is a direct transactivator for rodent Ntcp (Figure 7). Our findings are further supported by a liver-specific knockout of HNF4 using the Cre-lox system achieved by Hayhurst et al. (15). An analysis of a large number of genes and their expression patterns in null mice carried out in this study corroborated the data from the genomic analysis of Odom et al. (21) in that there were global alterations in expression of genes involved in multiple metabolic pathways. More specifically was the fact that the levels of Ntcp message were significantly decreased in the livers of the knockout mice compared to controls and their sera exhibited higher bile acid levels due to their decreased
uptake from portal circulation. Ntcp mRNA in HNF1 knockout mice is also reduced by more than 90% (24). However, the facts that HNF1 also regulates HNF4 expression (14) and that mutation of the HNF1 binding site in the mNtcp promoter does not result in a significant loss in promoter activity (7) strengthen the hypothesis of HNF4α acting as the central regulator of murine Ntcp expression.

Although mouse, rat and human Ntcp/NTCP genes exhibit a similar pattern of down-regulation upon inflammatory stimuli and cholestasis (10), the underlying regulatory mechanisms are distinct. In contrast to our study, a recent study (17) by Jung et al. where they compared the regulatory profiles of rat, mouse and human ntcp/NTCP genes concluded that the rat but not the human or mouse promoter is activated by HNF4. However, their study showed that all three promoters were inhibited by HNF3β while the human and the mouse but not the rat promoter was activated modestly by C/EBPβ. The same group also showed in a separate study that glucocorticoid receptor (GR) activates human NTCP promoter which is potentiated by PGC-1α and suppressed by bile acids in an SHP-dependent manner (5). We attribute the failure of Jung et al. to see transactivation by HNF4α in the mouse promoter to their use of very short promoter constructs for all three promoters (up to -131 bp upstream of start site). Clearly our studies using promoter constructs including sequences upstream of -300 bp showed significant stimulation by HNF4 which was further strengthened by EMSA, mutational analysis and siRNA knockdown studies.
In summary, we have shown that rodent Ntcp promoters and possibly also the human counterpart is significantly transactivated directly by HNF4. Our studies are consistent with the recent liver-specific knockout studies in which the null mice showed elevated serum bile acid levels. Our data also further confirm the genomic scan analysis of Odom et al. (21) employing ChIP assay that HNF4α is a major transactivator of multiple liver genes.
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Figure Legends

Figure 1. Basal transcriptional activities of mouse Ntcp promoter and transactivation by HNF4α in luciferase assays.

Various lengths of the Ntcp gene promoter upstream of the firefly luciferase gene in pXPΔ2 were transiently transfected into HepG2 cells together with or without HNF1α- or HNF4α-expression plasmids and luciferase activity was determined 48 h later. The values were corrected for transfection efficiency and normalized to the basal activity of the full-length p-974bpNtcpLUC-construct. A: Transactivation of the full-length mouse Ntcp promoter construct by the liver-enriched transcription factor HNF4α. p-974bpNtcpLUC was transiently transfected into HepG2 cells with increasing concentrations of HNF4α expression plasmid. HNF4α concentration-dependently transactivated gene transcription between 0.1 and 0.5 µg/ml in a linear fashion. B: Full-length p-974bpNtcpLUC (shaded bars) or p-475bpNtcpLUC (white bars) was transiently transfected into HepG2 cells together with 0.25 µg HNF4α expression plasmid/ml. Data represent mean ± SD of at least three independent experiments. C: Mapping the HNF4α binding site in the 5’-UTR of mouse Ntcp. A series of 5’-deleted mouse Ntcp promoter reporter gene constructs were generated as described in Materials and Methods and co-transfected into HepG2 cells with (shaded bars) or without (white bars) an HNF4α expression plasmid. Data represent mean ± SD of at least three independent experiments.
Figure 2. HNF4α binding to mouse Ntcp promoter. A: Electrophoretic mobility shift assay representative autoradiograph. 10 μg of mouse liver nuclear extract was incubated with the 32P-end-labeled oligonucleotide spanning the putative newly identified HNF4α-binding site (see Materials and Methods) and electrophoresed through a 6% non-denaturing polyacrylamide gel. For the supershift assay nuclear extracts were pre-incubated with HNF4α-antibody (Ab) before addition of labeled oligonucleotide. SC, specific competitor DNA; NSC, non-specific competitor DNA. B: Chromatin immunoprecipitation (ChIP) assay. Chromatin was prepared from mouse Hepa1-6 cells as described in the Materials and Methods section and immunoprecipitated with anti-HNF4α antibody (HNF4), anti-acetyl-Histone 4 antibody (H4, positive control) or a cytochrome C antibody (CytC, negative control). No addition of antibody served as a further negative control (no Ab). DNA was amplified with a primer pair covering the identified HNF4α binding site in the mouse Ntcp promoter.

Figure 3. Mutation of HNF4α binding site leads to decreased mouse Ntcp promoter activity and PGC-1α cotransfection leads to potentiation of HNF4α transactivation. HNF4α binding site in mouse Ntcp promoter was mutated as described in Materials and Methods, transfected into HepG2 cells and cotransfected with HNF4α cDNA; luciferase activity was assayed, corrected for transfection efficiency and normalized to the basal activity of the full-length p-974bpNtcpLUC-construct. An expression vector encoding PGC-1α was
cotransfected with HNF4α cDNA to measure its capacity to potentiate transactivation of mouse Ntcp promoter as described in Materials and Methods.

**Figure 4. Real-time PCR analysis of message levels for HNF4α and Ntcp in Hepa 1-6 cells after treatment with control and HNF4α siRNA.** Hepa 1-6 cells were treated with control siRNA and HNF4α siRNA with Trans-IT TKO (Mirus Bio Corporation, Madison, WI). Forty eight hours later, total RNA was isolated using Trizol (Invitrogen Corp, Carlsbad, CA) reagent according to manufacturer’s instructions. 1 µg of RNA was reverse-transcribed using Superscript First Strand Synthesis System for RT-PCR (Invitrogen). 50 ng of cDNA per well was used for real time PCR in a BioRad Mini Opticon-3 (Bio-Rad, Redmond, CA) with Quantitect SYBR Green kit as described in Materials and Methods. Relative expression levels were calculated as $2^{-\Delta\Delta Ct}$ using 36B4 as a normalization control by the comparative Method according to ABI Protocol.

**Figure 5. Western blot analysis of HNF1α and HNF4α protein levels in Hepa 1-6 cells after treatment with control siRNA and HNF4α siRNA.** Hepa 1-6 cells were transfected with control or mHNF4α siRNA (siGENOME SMART pool, Dharmacon, Colorado) using Trans-IT TKO (Mirus Bio Corporation, Madison, WI) as described in Materials and Methods. 48 hrs later cell lysates were prepared and probed by Western blotting using HNF1α (Panel A) and HNF4α (Panel B) antibodies. Equal amount of protein (184 µg) was loaded in all the lanes. The blots were reprobed for beta-actin using an anti-actin monoclonal
antibody (AC-120, Sigma, St.Louis, MO) to verify equal amount of proteins in all
the lanes (data not shown). Molecular weight standards are shown on the left hand
side and bands corresponding to HNF1α and HNF4α shown by the arrows. As
positive control for HNF-4 nuclear extract from Huh-7 cells transfected with an
expression plasmid for rat HNF-4 cDNA was included in Panel B. Panel C shows
quantitative analysis of a representative blot using Image J according to the
instructions for the software.

Figure 6. Comparison of HNF4α transactivation between mouse and rat
Ntcp. A: A series of 5’-deleted rat Ntcp promoter reporter gene constructs were
generated as described in Materials and Methods and co-transfected into HepG2
cells with an HNF4α expression plasmid. Luciferase activity was determined 48 h
later. The values were corrected for transfection efficiency. The numbers denote
the respective promoter fragment sizes in bp. B: Alignment of mouse and rat Ntcp
promoter around the HNF4α binding site. The major transcription start site of rat
Ntcp is designated +1 (Karpen et al., 1996, (19)) and the mouse sequence is
numbered accordingly after BLAST alignment.

Figure 7. Schematic depiction of the rat and mouse Ntcp promoters with
known binding sites for transcription factors described in this and earlier
studies. Top line represents the rat and the bottom line the mouse Ntcp promoter.
The corresponding HNF1α and RARα:RXRα sites are absent in the mouse
promoter compared to the rat ortholog whereas a C/EBP-β binding site is present
in the mouse promoter. HNF3 denotes a putative binding site for HNF3β. Numbering is relative to the transcription initiation site based on the rat promoter. The proposed distal HNF4α binding site in the rat *Ntcp* promoter is depicted in grey.
## Table 1

**Primers for mutational analysis of HNF4α site in mouse Ntcp promoter and for quantitative PCR analysis**

(Gene accession numbers: mNtcp NM_011387, mHNF4α NM_008261)

<table>
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### a) Mutational analysis (-353/323):

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### b) Quantitative PCR:

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• mutated sequence indicated in lower case.
Figure 2

A

Oligo -352/-326

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<th></th>
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B

H4, HNF4, CyC, no Ab

HNF4 α
Figure 3

- p-974bpNtcpLUC + HNF4 + PGC-1
- p-974bpNtcpLUC + HNF4
- p-974bp mut mNtcpLUC + HNF4
- p-974bpNtcpLUC + PGC-1
- p-974bpNtcpLUC
- pXP-2 LUC

normalized luciferase activity
Figure 4

![Graph showing relative expression of HNF4 and NTCP with comparison between Control siRNA and HNF4 siRNA treatments.](image)

* p < 0.01
Figure 5

A. [Western blot image showing HNF1 protein levels under control and HNF4 siRNA conditions]

B. [Western blot image showing HNF4 protein levels under control, positive control, and HNF4 siRNA conditions]

C. [Bar graph showing relative expression levels of HNF1 and HNF4 under control and HNF4 siRNA conditions]
Figure 6

A

p-930
p-469
p-368
p-307
p-146
control

normalized luciferase activity

B

-345
TTAGATGAGGAAGGCAAAGGCAGAAAC
-316
mouse Ntcp

-335
TTAGAGGAGGAAAACAAAGGCAAAAC
-316
rat Ntcp

-316

### Figure 7

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