Transport Function and Transcriptional Regulation of a Liver-Enriched Human Organic Anion Transporting Polypeptide 2B1 Transcriptional Start Site Variant

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Abbreviations:
OATP - Organic Anion Transporting Polypeptide, HNF4α - hepatocyte nuclear factor 4 alpha, TSS - transcription start site, ChIP - Chromatin Immunoprecipitation, siRNA - short interfering ribonucleic acid
Abstract.

Human Organic Anion Transporting Polypeptide 2B1 (OATP2B1) is a membrane transporter that facilitates the cellular uptake of a number of endogenous compounds and drugs. OATP2B1 is widely expressed in tissues including the small intestine, liver, kidney, placenta, heart, skeletal muscle and platelets. Recently, it has been shown that differential promoter usage in tissues results in expression of five OATP2B1 transcriptional start site variants which utilize distinct first exons but share common subsequent exons. These variants are expected to encode either a full length (OATP2B1-FL) or shortened protein lacking 22 N-terminus amino acids (OATP2B-Short). Little is known regarding the transport activity and regulation of OATP2B1 variants with N-terminus truncation. Here, using absolute quantitative polymerase chain reaction we find the full length variant is the major form expressed in duodenum but the short variant predominates in liver. Using a transient heterologous cell expression system, we find that the transport activities of the short OATP2B1 variant towards substrates estrone sulfate and rosuvastatin are similar to the well-characterized full length variant. Transcriptional activity screening of the liver enriched OATP2B1 variant promoter identified hepatocyte nuclear factor 4 alpha (HNF4α) as a novel transacting factor. With a combination of in silico screening, promoter mutation in cell-based reporter assays, siRNA knockdown and chromatin immunoprecipitation studies, we identified a functional HNF4α binding site close to the transcription start site (-17 to -4 bp). We conclude that the major OATP2B1 protein form in liver is transport competent and its hepatic expression is regulated by HNF4α.
Introduction.

The Organic Anion Transporting Polypeptides (OATPs, gene symbol solute carrier family \textit{SLCO}) are a superfamily of transmembrane proteins. The \textit{SLCO} genes encode 12-transmembrane domain proteins capable of transporting a wide variety of amphipathic substrates in a sodium independent manner. Different OATPs have partially overlapping substrates and differing tissue expression patterns. For example, OATP1B1 and OATP1B3 and are primarily expressed on the basolateral membrane of hepatocytes, whereas others, like OATP2B1, have a much broader expression pattern (Hagenbuch and Meier, 2004). OATP2B1 is expressed in liver, placenta, small intestine, kidney, brain, skin, heart, platelets, and skeletal muscle (Hagenbuch and Meier, 2004; Knauer et al., 2010; Kullak-Ublick et al., 2001; Niessen et al., 2009; Tamai et al., 2000). A wide variety of drugs are transport substrates of OATP2B1 including benzylpenicillin (Tamai et al., 2000), bosentan (Treiber et al., 2007), ezetimibe-glucuronide (Oswald et al., 2008), fexofenadine (Nozawa et al., 2004a), glibenclamide (Satoh et al., 2005), troglitazone (Nozawa et al., 2004b), atorvastatin (Grube et al., 2006), fluvastatin (Kopplow et al., 2005), pitavastatin (Shirasaka et al., 2011), pravastatin (Shirasaka et al., 2010), and rosuvastatin (Ho et al., 2006) as well as steroid sulfate conjugates like estrone sulfate (E1S) (Tamai et al., 2001). OATP2B1 has been implicated in the pH dependent absorption of multiple drugs across the human intestine epithelial cells (Kobayashi et al., 2003; Nozawa et al., 2004a). OATP2B1, like OATP1B1 and OATP1B3, is thought to mediate the sodium independent uptake of amphiphilic organic anions into the liver (Aoki et al., 2009). Given expression in placenta and mammary gland, OATP2B1 has been connected to the uptake and supply of precursor molecules for steroid synthesis.
(Ugele et al., 2003). Previously, we have described a potential role for OATP2B1 in the skeletal muscle statin uptake and sensitization to toxicity (Knauer et al., 2010).

Originally cloned from human brain, OATP2B1 is predicted to be a 709 amino acid protein (Kullak-Ublick et al., 2001; Tamai et al., 2000). Gene regulation analysis using promoter reporters in intestinal and liver cells revealed that the constitutive expression of the originally cloned OATP2B1 protein variant was regulated by the Sp1 transcription factor (Maeda et al., 2006). Recently, Pomari and colleagues have shown that differential promoter usage in tissues results in the expression of several OATP2B1 transcription start site (TSS) variants (Fig. 1) (Pomari et al., 2009). These OATP2B1 variants utilize 5 distinct first exons and associated promoters but share common subsequent exons (exons 2 to 14). One TSS variant represents the original full length (709 amino acids) protein with translation start site within this first exon (termed exon 1b). The other variants arising from transcription initiation at exons 1a, 1c, 1d and 1e are expected to produce a shorter protein (687 amino acids) than the 1b variant, as translation is predicted to start in exon 2. Hence, the short OATP2B1 protein variants lack 22 amino acids from the N-terminus in comparison to the original full length form. To date, there is an absence of information regarding the relative expression of the OATP2B1 transporter variants in key tissues responsible for drug absorption and elimination. Moreover, the transport competency of the short OATP2B1 protein has not previously been demonstrated. In this report, we find that the short OATP2B1 transporter variant is the predominant form expressed in human liver while the major form in small intestine is the full length version. We also demonstrate liver enrichment of the short OATP2B1 variant was partly due to transcriptional regulation by hepatocyte nuclear factor 4α (HNF4α).
Importantly, we show that the short OATP2B1 protein variant has comparable transport activity to the full length form.
**Materials and Methods.**

**Reagents.** [³H] esterone 3-sulfate (54.26 Ci/mmol, >97% radiochemical purity) and [³H] taurocholate (4.6 Ci/mmol, >97% radiochemical purity) were purchased from PerkinElmer (Woodbridge, ON, Canada) and [³H] rosuvastatin (5 Ci/mmol, 99% radiochemical purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled rosuvastatin was obtained from Toronto Research Chemicals (North York, ON). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Cell Culture.** Human cervical adenocarcinoma HeLa (American Type Culture Collection, ATCC, Manassas, VA), human hepatocellular carcinoma HepG2 (ATCC), human colorectal adenocarcinoma Caco-2 (ATCC) and human hepatocellular carcinoma Huh-7 (Japanese Collection of Research Bioresources; http://cellbank.nihs.go.jp) cells were cultured in DMEM supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cells were plated onto 12-well plates for transport studies and 24-well plates for dual luciferase reporter assays at a density of ~1 × 10⁶ cells/mL.

**Transporter Expression Plasmids.** The expression plasmid for the full length (1b) OATP2B1 (OATP2B1-FL) is described elsewhere (Tirona et al., 2003a). The cDNA of the short variant of OATP2B1 1e (OATP2B1-Short) was obtained by PCR, using Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN), from a human
liver cDNA library using oligonucleotide primers (Table 1) and ligated into pEF6/V5-His-TOPO vector (Invitrogen).

**Adenoviral Vectors.** An adenoviral vector containing OATP2B1-Short was generated using the ViraPower Adenoviral Expression System (Invitrogen) as previously described for OATP2B1-FL (Knauer et al., 2010).

**SLCO2B1 Luciferase Reporter Plasmids.** The 5' region of the *SLCO2B1* full length promoter (1b exon) and truncated promoter (1e exon) were PCR amplified with Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) from human genomic DNA using primers listed in Table 1. The resulting *SLCO2B1* 1b promoter PCR product (-2000 to +1 bp) was digested with *Kpn* I and *Xho* I then ligated into pGL3-Basic (Promega, Madison, WI). The *SLCO2B1* 1e promoter amplicon was cloned into pCR2.1, digested with *Hind III* and *Xho I* then ligated into pGL3-Basic. Disruption of the DR1-1 site in the *SLCO2B1* 1e promoter was done by site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) using primers listed in Table 1.

**Gene Expression Analysis.** The absolute mRNA expression of each OATP2B1 TSS variant, was determined by SYBR green quantitative real-time PCR (RT-PCR), with an ABI Prism 7700 sequence detection system (Applied Biosystems, Carlsbad, CA). Total RNA from a cohort of 23 healthy human livers and 17 intestinal samples were obtained from healthy individuals undergoing diagnostic esophagogastroduodenoscopy was described elsewhere (Ho et al., 2006; Urquhart et al., 2010). Human skeletal muscle,
kidney, placenta and brain total RNA was sourced from BioChain (Hayward, CA). Total RNA was also isolated from Caco-2, HeLa, HepG2 and Huh-7 cells using TRIzol (Invitrogen). cDNA was synthesized using Multiscribe reverse transcriptase (Applied Biosystems, Carlsbad, CA) according to manufacturer’s instruction and 40 ng of cDNA was used in each PCR reaction. Primer sequences for quantitative PCR were obtained from Pomari et al., (2009) and are listed in Table 1. Standard curves of the OATP2B1 TSS variant amplicons cloned into pCR2.1 TOPO vector (Invitrogen) were generated for absolute determination of copy number. Expression was normalized to the copy number of 18S-rRNA.

**Immunoblot and Immunofluorescence Microscopy.** HeLa cells were transduced with adenovirus encoding LacZ (control), OATP2B1-FL or OATP2B1-Short. Protein samples were separated by SDS-PAGE and transferred to NuPAGE nitrocellulose membrane using the XCell SureLock Western blotting system (Invitrogen, Carlsbad, CA). Membranes were probed with custom-made rabbit polyclonal OATP2B1 antibody based on a C-terminus epitope (CSPAVEQQLLVSGPGKKPEDSRV) (Invitrogen). Anti-rabbit horseradish peroxidase–labeled antibody (Bio-Rad, Hercules, CA) was used as the secondary. The immobilized secondary antibody was detected using the ECL Plus Western Blotting Detection System (GE Healthcare) and KODAK ImageStation 4000 MM (Mandel). HeLa cells were transduced with adenovirus encoding LacZ (control), OATP2B1-FL or OATP2B1-Short on culture slides and were stained as previously described (Knauer et al., 2010). The custom-made rabbit polyclonal OATP2B1 antibody was used to localize transporter expression and anti-rabbit Alexa Fluor® 488 (Invitrogen)
was used as the secondary antibody. Images were obtained by confocal fluorescence microscopy. For the negative control, the primary antibody was omitted.

**Transport Studies.** For transport kinetic experiments, HeLa cells (human cervical cancer cell line) were grown in 12-well plates (approximately $0.8 \times 10^6$ cells/well) and 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 absorb for 30 minutes. Cells were transfected with 1 µg of transporter cDNA or parental plasmid lacking insert as control using Lipofectin (Invitrogen) and incubated at 37°C for 16h. To examine pH dependent transport of OATP2B1, HeLa cells were transduced with the adenoviruses containing the full length or short OATP2B1 variant transporter coding region in DMEM medium at a multiplicity of infection (MOI) of 100 and incubated at 37°C for 48h. Transport was evaluated in Opti-MEM I using labeled substrate as previously reported (Cvetkovic et al., 1999). Drug accumulation was determined after 5 min for rosuvastatin, 10 min for estrone 3-sulfate, and 10 min for taurocholate by washing cells three times with ice-cold PBS followed by lysis with 1% sodium dodecylsulfate. Retained cellular radioactivity was quantified by liquid scintillation spectrometry. Concentration-dependent transport by OATP2B1 variants was performed at various substrate levels (rosuvastatin 0.1 to 20 µM; estrone 3-sulfate 0.1 to 100 µM). Transporter specific uptake was determined as the difference in solute accumulation between OATP2B1 variant transfected and vector control transfected cells. Transport studies with varying pH environments were performed in Krebs-Heinseleit bicarbonate Buffer (KHB) using
rosuvastatin (100 nM), estrone 3-sulfate (100 nM), and taurocholate (150 nM) as previously reported (Urquhart et al., 2010).

**Luciferase Reporter Assays.** Reporter gene constructs were transfected into HeLa, HepG2, Huh-7 or Caco-2 cells using Lipofectamine 2000 (Invitrogen) according to the manufacture’s protocol. Cells were plated into 24 wells plates at approximately $0.6 \times 10^6$ cells per well for 24h before transfection. Each well was transfected with 250 ng of reporter construct and 2.5 ng of pRL-CMV vector (Promega) as an internal control in 400 $\mu$L Opti-MEM I. After 24h the media was changed to DMEM with 10% FBS, then cells were incubated for 24h at 37°C. Cells were then rinsed with PBS and harvested using passive lysis buffer (Promega). Luciferase activity was measured in cell extracts using Dual-Luciferase reporter assay (Promega) and detected using a Flouroskan Ascent FL luminometer (Thermo Scientific, Hudson, NH). Relative luciferase activity is reported as the ratio of firefly/Renilla luciferase activities. Nuclear receptor expression vectors were prepared as previously described (Tirona et al., 2003b) and in certain instances, 250 or 500 ng/well of each nuclear receptor expression plasmid or blank vector control plasmid were used for reporter assays.

**siRNA Knockdown of HNF4α in Huh-7 Cells.** Huh-7 cells were transfected in 24-well plates at approximately 70% confluence using DharmaFECT Duo Transfection reagent (Thermo Scientific, Ottawa, ON,) in Opti-MEM I according to the manufacture’s protocol. Each well was transfected with ON-TARGETplus SMARTpool short interfering RNA (siRNA) targeting HNF4α (Thermo Scientific) or control ON-
TARGETplus Non-targeting siRNA 1 (Thermo Scientific) to a final concentration of 25 nM in 100 μL Opti-MEM I (Popowski et al., 2005). After incubation at room temperature for 20 min, the siRNA mixtures were added to each well containing 400 μL Opti-MEM I supplemented with 10% FBS. Cells were harvested 48h after transfection and total cellular RNA was extracted using TRIzol. For luciferase reporter assays, Huh-7 cells were transfected in 24-well plates at 70% confluence with HNF4α or control siRNA (25 nM) together with 250 ng of reporter construct and 2.5 ng of pRL-CMV vector (Promega) in 100 μL Opti-MEM I. DNA and siRNA mixtures were added to each well containing 400 μL Opti-MEM supplemented with 10% FBS. Dual luciferase reporter assays were performed 48h after transfection as described above.

**Chromatin Immunoprecipitation (ChIP).** For DNA cross-linking and chromatin immunoprecipitation, the EZ ChIP Assay (Millipore, Billerica, MA) was used according to the manufacturer’s instructions. Briefly, HepG2, Huh-7, HeLa and Caco-2 cells were cultured in 10-cm dishes. DNA was cross-linked, sheared by sonication (Virsonic 100, Virtis, Gardiner, NY), and then chromatin was incubated with 2 μg anti-HNF4α C-19 (sc-6556; Santa Cruz Biotechnology, Santa Cruz, CA) antibody overnight at 4°C. Binding of HNF4α was determined by qPCR (Table 2 for primer sequences). Western Blot of total cell lysates was performed using anti-HNF4α C-19 (sc-6556; Santa Cruz Biotechnology) (1:1000) and anti-actin C-11 (sc-1615; Santa Cruz Biotechnology) (1:200).

**Statistical Analysis.** Statistical differences between group parameters was determined by unpaired T-test, 1-way ANOVA, or 2-way ANOVA, using Bonferroni’s multiple
comparison test, as appropriate (GraphPad Software Inc, San Diego, CA). A $P$ value of <0.05 was considered statistically significant.
Results.

**Tissue Expression of OATP2B1 TSS variants.** We performed variant-specific quantitative RT-PCR to determine the absolute expression of the OATP2B1 variants in tissues important in drug disposition, namely the duodenum and liver. While transcripts for the OATP2B1 1b, 1d and 1e variants were readily observed; we were not able to detect OATP2B1 1a and 1c transcripts in these tissues (not shown). In duodenum, the OATP2B1 variant 1b, encoding the full length protein variant was the most abundant transcript (Fig. 2). On average, OATP2B1 1d and 1e transcripts were 10-fold lower than 1b transcripts in the intestine. In liver, OATP2B1 1e transcript (short variant) was the predominant form followed by 1b and 1d transcripts. It is notable that for each transcript, the absolute expressions of OATP2B1 1b and 1d were similar when comparing duodenum versus liver levels. The clear exception was for the OATP2B1 1e transcript, whose expression was 100-fold greater in liver than in duodenum. We confirmed that the OATP2B1 1e transcript detected by qPCR arose from an mRNA encoding the short protein variant by long range PCR using liver cDNA template, cloning of the amplicon and sequencing.

Variant-specific quantitative RT-PCR of OATP2B1 variants was also performed in samples of kidney, brain, placenta, and skeletal muscle. Like liver and intestine, transcripts for OATP2B1 1b, 1d, and 1e were observed, but OATP2B1 1a and 1c transcripts were not detected in these tissues (Table 3). Similar to duodenum, the OATP2B1 1b variant was the most abundant transcript found in kidney, brain, placenta and skeletal muscle. Again we noted, the absolute expressions of OATP2B1 1b transcripts (full length form) in kidney, placenta and skeletal muscle were similar when
compared to duodenum and liver levels. Of the tissues analyzed, brain shows highest
expression of the OATP2B1 1b transcript, encoding the full length protein. On average,
OATP2B1 1d and 1e transcripts were over 10 to 100-fold lower than 1b transcripts in the
brain, placenta and skeletal muscle, exhibiting a comparable expression pattern to that of
intestine. However, in kidney the expression of the OATP2B1 1e transcript was 10-fold
higher than duodenum, brain, placenta, and skeletal muscle and only 2 fold lower then
the OATP2B1 1b transcript. These findings demonstrate 3 different expression patterns
of OATP2B1 protein variants. Liver primarily expresses the shorter OATP2B1 protein,
kidney expresses similar levels of full length and short OATP2B1, while the other tissues
analyzed predominantly express full length OATP2B1.

Transport Function of OATP2B1-Short. Many groups have studied the transport
function of OATP2B1-FL but to our knowledge the transport function of the short variant
has not been previously assessed. Here, we examined the transport function of the
OATP2B1-Short variant in comparison to OATP2B1-FL using three substrates: estrone
3-sulfate, rosuvastatin, and taurocholate. Both OATP2B1-FL and OATP2B1-Short show
time- and concentration-dependent uptake kinetics for estrone 3-sulfate and rosuvastatin
(Fig. 3C-F). The apparent affinities ($K_m$) for both substrates were not significantly
different between OATP2B1-FL and OATP2B1-Short (Table 4). The maximal velocity
($V_{max}$) of the OATP2B1-Short was slightly higher for both rosuvastatin and estrone 3-
sulfate; however, the differences did not reach statistical significance (Table 4). Transport
clearance (CL) of the OATP2B1-Short was also slightly higher for both rosuvastatin and
estrone 3-sulfate but it also did not reach statistical significance. Heterologous expression
of OATP2B1-FL and OATP2B1-Short in HeLa cells, using recombinant adenoviruses and protein detection with an OATP2B1 antibody directed towards a common C-terminus epitope, showed that both variants products are expressed at comparable levels as determined by densitometric analysis of the Western Blot (actin normalized relative expression: LacZ (100%), OATP2B1-FL (396%) and OATP2B1-Short (432%)). Both OATP2B1 variants traffic to the cell surface as revealed by indirect immunofluorescence (Fig 3A and B). Interestingly, basal OATP2B1 expression was detected by immunoblot (Fig. 3A) and immunofluorescence under higher gain/exposure setting (not shown) while quantitative PCR measured very low levels of the full length OATP2B1 transcript (Table 3) in HeLa cells which may account for some uptake of estrone 3-sulfate and rosuvastatin in vector control transfected cells (Fig. 3C and D). These findings indicate that the SLC02B1 1e promoter transcript produces a short transporter variant with similar transport activity as the previously characterized full length OATP2B1 protein.

We further examined the pH dependent transport of the OATP2B1-Short variant since previous studies have demonstrated OATP2B1-FL transports certain substrates, including estrone 3-sulfate and taurocholate, in a pH dependent manner (Kobayashi et al., 2003; Nozawa et al., 2004a). For both the full length and the short OATP2B1 variants, we observed that rosuvastatin transport was stimulated with decreasing pH (Fig 4A). Rosuvastatin transport by OATP2B1-Short is significantly greater than OATP2B1-FL at low pH (5.5 and 6.0) but not at higher pH (6.5 and 7.2) (Fig. 4A). In contrast, estrone 3-sulfate transport activities for both OATP2B1-FL and OATP2B1-Short do not appear to be pH-dependent (Fig. 4B). Despite that a previous report had demonstrated stimulation of taurocholate uptake by OATP2B1 by low pH (Nozawa et al., 2004a), we did not
observe OATP2B1-dependent taurocholate transport under any experimental condition (Fig. 4C). These results suggest that amino acids in the predicted intracellular N-terminus of OATP2B1 modulates substrate-specific, pH-dependent membrane transport.

**Transactivator Screen of the SLCO2B1 1e Promoter.** The full length form of OATP2B1 appears to be ubiquitously expressed in many tissues and the constitutive expression of OATP2B1-FL is under regulatory control by the Sp1 transcription factor (Maeda et al., 2006). However, regulatory control mechanisms of the other OATP2B1 TSS variants have not been previously assessed. We have focused on the OATP2B1 1e TSS variant because it shows liver specific expression unlike the OATP2B1 1b variant found ubiquitously (Pomari et al., 2009) (Fig. 2 and Table 3). The SLCO2B1 1e promoter was cloned from genomic DNA (-2250 to +78) and ligated into pGL3-basic luciferase reporter. The +1 position was considered to be the transcription start site of exon 1e described by Pomari and colleagues (GeneBank no. FM209054) (Pomari et al., 2009). We screened the ability of a variety of nuclear receptors (HNF4α, constitutive androstane receptor, liver X receptor, pregnane X receptor, small heterodimer partner 1, peroxisome proliferator activated receptors α and γ, liver receptor homolog 1 and vitamin D receptor) to activate the SLCO2B1 1e promoter using co-transfection and luciferase reporter assays in HeLa cells (Fig. 5A). Of the nuclear receptors analyzed, we found that HNF4α strongly transactivated the SLCO2B1 1e (-2250/+78) promoter. Interestingly, we found that HNF4α did not transactivate the SLCO2B1 1b promoter (Fig. 5B).
Deletion-Mutation Analysis of *SLCO2B1* 1e Promoter Activity. Reporter assays were performed in liver (HepG2 and Huh-7), colon (Caco-2) and cervical carcinoma (HeLa) cell lines to evaluate the roles of cellular milieu and *cis*-acting factors to *SLCO2B1* 1e promoter activity. For this purpose, *SLCO2B1* 1e promoter constructs were created by sequentially deleting the 5' upstream segments (-2250 to +78, -1000 to +78, -500 to +78 and -200 to +78). We observed reporter activity in three cell lines (HepG2, Huh-7 and Caco-2) for all *SLCO2B1* 1e deletion constructs (Fig. 6A). In comparison, *SLCO2B1* 1b reporter activity was significantly lower than of *SLCO2B1* 1e. The pattern of reporter activity was similar for all cell lines except for HeLa cells, which displayed no significant activity for all reporter constructs. Reporter activity decreased upon the deletion of -2250 to -1000 region, suggesting the presence of positive regulatory sequences. While deletion of the -1000 to -500 revealed a large increase in reporter activity in three cell lines (HepG2, Huh-7 and Caco-2) suggesting that the deleted segment contains negative regulatory sequences. Even the shortest construct (-200/+78) retained reporter activity in HepG2, Huh-7 and Caco-2 suggesting the presence of an important positive regulatory sequence in this region.

Given that OATP2B1 le transcripts are prominently expressed in liver and that *SLCO2B1* 1e luciferase reporter activities were higher in hepatic and intestinal cell lines in comparison to cervical carcinoma cells, we suspected a role for liver enriched transcription factors such as HNF4α in regulating gene expression. Hence, we performed *in silico* analysis of transcription factor binding sites within the 2.5 Kb fragment of the *SLCO2B1* 1e gene promoter using the NUBIsan algorithm (Podvinec et al., 2002). Several direct repeats with one base pair spacing (DR1), known to be a potential binding
site for HNF4α (Bolotin et al., 2010), were predicted which we have termed DR1-1 (AGGGCAaAGTCCA) located at position -17 to -4, a DR1-2 (AGGCCTcAGACCT) located at -954 to -941 and DR1-3 (AGAGCAaGGGCCA) located at -2149 to -2136. On the basis that the -200/+78 construct had retained significant activity in liver and intestinal cells, we hypothesized that the DR1-1 site would represent a functional HNF4α binding site. Guided by the position weight matrix for HNF4α-binding sequence motifs (Bolotin et al., 2010), the DR1-1 site was mutated at three key base pairs (Fig. 6B). Furthermore, we deleted three base pairs in DR1-1 to disrupt the HNF4α motif spacing (Fig. 6B). Reporter assays were performed in HeLa cells transfected with HNF4α and the DR1-1 mutation constructs (Fig. 6C). It is notable that in contrast to HepG2, Huh-7 and Caco-2 cells, HeLa cells do not express native HNF4α (Fig. 6D). Disruption of the DR1-1 response element by mutation or deletion significantly decreased the transcriptional activation of the SLC02B1 1e promoter by HNF4α, suggesting that the DR1-1 site is a functional HNF4α binding site (Fig. 6C). These studies demonstrate that the SLC02B1 1e promoter contains a functional HNF4α-binding sequence near the 1e transcription start site.

Analysis of SLC02B1 1e Gene Expression and Promoter Activity after HNF4α Knockdown with siRNA. We found that Huh-7 cells extensively express both OATP2B1 1b and 1e transcripts (Table 3). To further determine whether SLC02B1 1e expression was regulated HNF4α, we assessed the consequences of HNF4α knockdown on transporter variant levels in Huh-7 cells. Transient HNF4α knockdown with siRNA resulted in a significant 38 ± 15 % reduction in the expression of HNF4α mRNA (Fig.
7A). This was accompanied by a significant 40 ± 17 % decrease in OATP2B1 1e transcript levels but no change in OATP2B1 1b expression. In parallel experiments, reporter assays were performed in Huh-7 cells to evaluate the effect of HNF4α knockdown on SLC02B1 1e promoter activity (Fig. 7B). siRNA knockdown of HNF4α significantly decreased the transcriptional activation of SLC02B1 1e promoter constructs of various lengths, including the (-200/+78) reporter containing the DR1-1 binding site. Collectively, these results suggest that HNF4α expression affects OATP2B1 1e variant levels through a transcriptional mechanism.

**HNF4α Binding to SLC02B1 1e Promoter.** To confirm that HNF4α binds to the DR1-1 response element, chromatin immunoprecipitation (ChIP) was performed with a HNF4α antibody in HeLa, HepG2, Huh-7 and Caco-2 cells. HNF4α-bound DNA was used as template for real-time PCR using primers designed to cover sequences along the SLC02B1 1e promoter covering 2.2 Kb upstream and the three predicted DR1 response elements (Fig. 8A). The analysis revealed significant binding of HNF4α to the proximal region of the SLC02B1 1e promoter near the DR1-1 (-4 bp) response element in HepG2, Huh-7 and Caco-2 cells but not HeLa cells (Fig. 8B). Analysis of the other DR1 response elements does not demonstrate significant HNF4α binding in HepG2, Huh-7 and Caco-2 cells. HeLa cells do not natively express HNF4α, so predictably we did not detect binding of HNF4α to the SLC02B1 1e promoter. Results from the reporter assays and ChIP taken together, reveal that HNF4α binds to the functional DR1-1 site in the proximal region of the SLC02B1 1e promoter to control expression of the truncated OATP2B1 variant.
Discussion.

In the present study, we characterized the transport function and transcriptional regulation of OATP2B1 1e variant. The SLCO2B1 1e variant encoding OATP2B1-Short was cloned from a human liver cDNA library. Heterologous expression of OATP2B1-Short (1e) in HeLa cells resulted in cell surface expression and functional transport activity. We found no pronounced differences in transport activity between the OATP2B1-FL and OATP2B1-Short protein for two typical OATP substrates: rosuvastatin and estrone 3-sulfate. However, we did see a significant increase in rosuvastatin uptake by OATP2B1-Short, but not for OATP2B1-FL at low pH. Reporter assays, siRNA knockdown and chromatin immunoprecipitation revealed a functional HNF4α binding motif in the proximal region of the SLCO2B1 1e promoter.

The expression of the alternative OATP2B1 variants in intestine and kidney were reported previously (Pomari et al., 2009). We confirm that the OATP2B1 exon 1b transcription variant has broad expression pattern consistent with previous reports and regulation by a constitutive nuclear receptor Sp1 (Maeda et al., 2006; Pomari et al., 2009). Quantitative PCR revealed that expression of the OATP2B1 1b full length variant was highest in the brain, the tissue of first cloning (Tamai et al., 2000). We confirm the liver enriched expression of the OATP2B1 exon 1e variants using absolute quantitative PCR, as was previously suggested using semi-quantitative PCR (Pomari et al., 2009). Interestingly we report 100-fold variation in the hepatic mRNA expression of the OATP2B1 1e variant between individuals.

We demonstrate that the SLCO2B1 1e variant encodes a shortened protein capable of trafficking to the cell surface and has functional transport activity. While there was a
non-significant trend towards higher transport efficiency for OATP2B1-Short in comparison to OATP2B1-FL during studies with concentration-dependent uptake, clear differences were observed in the transport efficiencies among the variants at low pH. Other studies have also documented the pH dependent transport of rosuvastatin, fluvastatin, pravastatin, atorvastatin, cerivastatin, and pitavastatin by OATP2B1 (Kobayashi et al., 2003; Varma et al., 2011). Previous studies have demonstrated pH dependent transport of estrone 3-sulfate by OATP2B1; however, we did not detect a significant difference in the pH dependent transport of estrone 3-sulfate (Kis et al., 2010; Kobayashi et al., 2003; Nozawa et al., 2004a; Sai et al., 2006). It has been recently shown that the transport of estrone 3-sulfate by OATP2B1 is mediated by high- and low-affinity components, with only the low-affinity component being pH sensitive (Shirasaka et al., 2012). The concentration of estrone 3-sulfate used in our study (100 nM) was low, likely explaining why we did not see pH dependent transport by the low-affinity transport component (K_m 29.9 μM) (Shirasaka et al., 2012). Previous studies have demonstrated transport of the bile acid taurocholate by OATP2B1 at low pH (<pH 6.5) but not at physiological pH of (7.4 or 7.5) (Kullak-Ublick et al., 2001; Nozawa et al., 2004a), however, we did not detect transporter-specific taurocholate uptake at any pH. It has been suggested that an inward proton gradient acts as the driving force for the OATP2B1 mediated intestinal absorption and cellular uptake of substrates (Nozawa et al., 2004a; Sai et al., 2006; Varma et al., 2011). The pH sensitivity of OATP2B1 has been linked to a conserved histidine in transmembrane domain three (Leuthold et al., 2009). Here, we propose a role for the amino acids in the predicted intracellular N-terminus in modulating substrate-specific, pH-dependent membrane transport of OATP2B1.
In this study, the liver specific nuclear receptor, HNF4α, was found to stimulate transcription of the *SLCO2B1* exon 1e promoter. Moreover, a functional HNF4α binding motif was found in the proximal region of the *SLCO2B1* 1e gene promoter very close to the transcription start site. Indeed, others have recently identified this HNF4α binding motif in the *SLCO2B1* exon 1e promoter using the ChIP-Seq method (Fang et al., 2012). HNF4α is expressed highly in metabolic tissues like the liver, kidney and intestine (Bookout et al., 2006). HNF4α has been shown to regulate key drug metabolism genes including Cytochromes P450 (*CYP* 3A4, *CYP3A5*, *CYP2D6*, *CYP2C9*, and *CYP2C19*) as well as drug transporter genes such as *ABCC2*, *ABCB1*, *SLC22A1* (OCT1) and *SLC22A7* (OAT2) (Jover et al., 2001; Jover et al., 2009; Kamiyama et al., 2007; Kawashima et al., 2006; Popowski et al., 2005; Saborowski et al., 2006; Tirona et al., 2003b). Like other transcriptional targets, we suspect that HNF4α-mediated regulation of OATP2B1 1e variant expression can be influenced by a variety of different factors including genetics, gender, environmental factors and diet (Hwang-Verslues and Sladek, 2010).

We have found 100-fold difference in OATP2B1 1e expression among individuals. It is interesting to speculate that interindividual differences in the tissue expression of OATP2B1 1e variant contributes to variable drug response. In principle, conditions that alter HNF4α expression and activity, such as diabetes and liver diseases, may impact on OATP2B1 1e expression. Factors enhancing HNF4α activity would increase expression of the OATP2B1 1e variant and could increase the hepatic and renal exposure OATP2B1 substrates. Increased HNF4α activity in the intestine would lead to upregulation of OATP2B1 1e and enhanced intestinal absorption of substrate drugs.
In this report, we assessed the transport function and regulation of the liver-enriched OATP2B1 1e transcription start site variant. The encoded OATP2B1-Short protein is a functional membrane transporter for estrone 3-sulfate and rosvastatin. Analysis of the SLCO2B1 1e TSS variant promoter revealed a DR1 binding motif capable of binding HNF4α to control the liver specific expression of the short OATP2B1 1e variant. These findings indicate that differential regulation of OATP2B1 splice variant expression in tissues could contribute to variation in drug response.
Acknowledgments.

We thank Edna F. Choo (Genentech) for preparing the luciferase reporter construct of the \textit{SLCO2B1} 1b promoter. We thank Rich H. Ho and Brenda F. Leake (Vanderbilt University) for assisting with some of the transport experiments.
Authorship Contributions.

Participated in research design: Knauer, Tirona

Conducted experiments: Knauer, Girdwood

Performed data analysis: Knauer, Girdwood

Wrote or contributed to writing manuscript: Knauer, Kim, Tirona
References.


Footnotes.

These studies were supported by scholarships (M.J.K.) and an operating grant (R.G.T) from the Canadian Institutes of Health Research [MOP-86522].
Figure Legends.

Figure 1. OATP2B1 Transcription start site variants. (A) Exon/intron structure of the beginning of the *SLCO2B1* gene. Exons are shown as boxes and introns as lines. The grey shading indicated the translated regions of the exons. (B) The N-terminal amino acid sequence of the OATP2B1 protein encoded using different translation start sites encoded in exon 1b or exon 2. (C) Topology of OATP2B1, with the black shows the 22 amino acids that are lacking in the truncated variant.

Figure 2. Tissue specific expression and quantitation of alternative OATP2B1 first exon transcripts in 23 liver and 17 intestinal samples. Absolute quantification was done by quantitative PCR using sense primers for the specific first exon and a common antisense primer located within exon 3. ***significantly different expression compared to other TSS variants (p<0.001).

Figure 3. Function of OATP2B1 transcription start site variants. (A) Western blot showing expression of OATP2B1 full length (FL) and short variants in HeLa cells after adenoviral overexpression. (B) Cellular localization of full length or short variant OATP2B1 in HeLa cells in HeLa cells after adenoviral overexpression by confocal immunofluorescence microscopy. OATP2B1 is shown in green and the nucleus in blue. Intracellular accumulation of [3H]rosuvastatin (C, E) or [3H]estrone 3-sulfate (D,F) in HeLa cells transiently transfected with OATP2B1-FL or OATP2B1-Short. Time-
dependent transport of \[^{3}H\]rosuvastatin (100 nM) (C) and \[^{3}H\]estrone 3-sulfate (100 nM) (D) by OATP2B1-FL or OATP2B1-Short. Concentration-dependent transport of \[^{3}H\]rosuvastatin (5 min) (E) and \[^{3}H\]estrone 3-sulfate (10 min) (F) by OATP2B1-FL or OATP2B1-Short. OATP2B1-specific transport was determined after correction for background transport activity in control adenovirus transduced cells. Results are presented fmol per μg protein per min ± SEM (n=3).

**Figure 4.** pH dependent transport function of OATP2B1 transcription start site variants. pH dependent transport of \[^{3}H\]rosuvastatin (100 nM, 5 mins) (A), \[^{3}H\]estrone 3-sulfate (100 nM, 10 mins) (B) or \[^{3}H\]taurocholate (150 nM, 10 mins) (C) in HeLa cells transduced with OATP2B1-FL (full length), OATP2B1-Short (truncated) or control LacZ expressing recombinant adenovirus. Results are presented fmol per μg protein per min ± SEM (n=3-4). *transport significantly different from control LacZ transduced cells (p<0.05). †transport significantly different between OATP2B1-FL and OATP2B1-Short transduced cells (p<0.05).

**Figure 5.** Nuclear receptor screen for transactivation of the *SLCO2B1* 1e promoter responsible for producing the truncated protein variant. (A) Dual luciferase reporter assay in HeLa cells transfected with nuclear receptor expression plasmids and *SLCO2B1* 1e promoter (-2250 to +78) or control pGL3-Basic vector to determine if they were able to transactivate the *SLCO2B1* 1e promoter. ***significantly different response from vector control transfected cells (p<0.001) (B) Transcription activation of the *SLCO2B1* 1e promoter and not the 1b promoter in HeLa cells transfected with HNF4α and *SLCO2B1*
1b or 1e promoter constructs. Results are presented as relative luciferase activity fold of pGL3-Basic control ± SEM. ***significantly different response from cells not transfected with HNF4α expression plasmid (p<0.001).

**Figure 6.** A functional HNF4α-binding motif in the proximal region of the *SLCO2B1* 1e promoter. (A) Dual luciferase reporter assay in HepG2, Huh-7, Caco-2, and HeLa cells transfected with various constructs of the *SLCO2B1* 1e promoter (-2250 to +78, -1000 to +78, -500 to +78 and -200 to +78) or *SLCO2B1* 1b promoter (-2000 bp to +1). Results are presented as relative luciferase activity fold of pGL3-Basic control ± SEM (n=3-4). (B) Sequences of the proximal DR1-1 HNF4α-binding motif with a three base pair deletion (Del) or mutation of three key base pairs (Mut) based on the HNF4α position weight matrix (Bolotin et al., 2010). (C) Dual luciferase reporter assay in HeLa cells transfected with *SLCO2B1* 1e promoter constructs (WT, DR1-1 Del or DR1-1 Mut) and HNF4α or control pEF vector. Predicted HNF4α binding sites are shown in white along the 1e promoter constructs, the DR1-1 HNF4α-binding motif is shaded to indicate the binding motif disrupted by deletion or mutation. Results are presented as relative luciferase activity fold of pGL3-Basic control ± SEM (n=5), *p<0.05 versus wild type HNF4α binding site construct. (D) Western blot showing HNF4α expression in in HeLa, HepG2, Huh-7 and Caco-2 cells.

**Figure 7.** Effect of HNF4α siRNA knockdown in Huh-7 cells on OATP2B1 transcription start site variant expression and *SLCO2B1* promoter activity. (A) Relative mRNA expression of HNF4α, OATP2B1-FL (full length) and OATP2B1-Short (truncated) in
Huh-7 cells 48h after HNF4α or control siRNA transfection. Results are presented as relative mRNA expression in comparison to control siRNA ± SEM. * p<0.05 relative to control siRNA. (B) Dual luciferase reporter assay in Huh-7 cells transfected with wild type SLCO2B1 1e promoter constructs and HNF4α or control siRNA. Results are presented as relative luciferase activity in comparison to pGL3-Basic control transfected with control siRNA ± SEM (n=4-5), *p<0.05 versus similar construct transfected with control siRNA.

**Figure 8.** Chromatin Immunoprecipitation. (A) Diagram of the 2.3Kb SLCO2B1 1e promoter with the location of DR1 sites and primer pairs (arrows) used for qPCR. ChIP using an anti-HNF4α antibody in HeLa (B), HepG2 (C), Huh-7 (D) and Caco-2 (E) cells. Quantification was done by qPCR on HNF4α antibody immunoprecipitated DNA (bound fraction), control IgG immunoprecipitated DNA (background fraction) and input DNA with four sets of primers located along the SLCO2B1 1e promoter. Results are presented as the difference between the recoveries in the bound and background fractions. *p<0.05 versus all other primer pairs
### Table 1. Primer Sequences

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<td>Cloning</td>
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<tr>
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<td>5′-CAAGACAGCTCACAATCGGAATCTTCTGG-3′</td>
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<td></td>
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Table 2. Primer Sequences used for qPCR after ChIP

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Table 3. Absolute mRNA Expression of OATP2B1 TSS Variants.

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<th>1d (short)</th>
<th>1e (short)</th>
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<td>Kidney</td>
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<td>$8.59 \times 10^{-6}$</td>
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<td>$1.21 \times 10^{-7}$</td>
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<td>ND</td>
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<tr>
<td>Sk. Muscle</td>
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<td>$3.43 \times 10^{-7}$</td>
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<td>Huh-7</td>
<td>$8.22 \times 10^{-4}$</td>
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ND - not detected
Table 4. Transport Kinetics of OATP2B1 Transcription Start Site Variants.

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<td>OATP2B1-FL</td>
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<td>OATP2B1-Short</td>
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<td>57.7 ± 14.9</td>
<td>722.7 ± 160.1</td>
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</table>
Figure 1
Figure 2

Absolute mRNA Expression of OATP2B1 TSS Variants (Copies OATP2B1/Copies of 18S)

Small Intestine Duodenum

Liver
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

A

Bar graph showing relative mRNA expression levels of HNF4α, 1b (FL), and 1c (Short) with control siRNA and HNF4α siRNA.

B

Bar graph showing relative luciferase activity (fold of vector control) with control siRNA and HNF4α siRNA for different promoters.
**Figure 8**

(A) Schematic diagram showing the organization of the gene with exons labeled as DR1-3, DR1-2, and DR1-1. Exon 1e is indicated.

(B) Graph showing the binding profile of HeLa cells. The x-axis represents the distance from exon 1e TSS (bp), and the y-axis shows the bound-to-background ratio (% input).

(C) Graph showing the binding profile of HepG2 cells. The x-axis represents the distance from exon 1e TSS (bp), and the y-axis shows the bound-to-background ratio (% input).

(D) Graph showing the binding profile of Huh-7 cells. The x-axis represents the distance from exon 1e TSS (bp), and the y-axis shows the bound-to-background ratio (% input).

(E) Graph showing the binding profile of Caco-2 cells. The x-axis represents the distance from exon 1e TSS (bp), and the y-axis shows the bound-to-background ratio (% input).