Liver receptor homologue-1 mediates species- and cell line-specific bile acid dependent negative feedback regulation of the apical sodium-dependent bile acid transporter

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Summary

Intestinal reclamation of bile salts is mediated in large part by the apical sodium dependent bile acid transporter, ASBT. The bile acid responsiveness of ASBT is controversial. Bile acid feeding in mice results in decreased expression of ASBT protein and mRNA. Mouse but not rat ASBT promoter activity was repressed in Caco-2 but not IEC-6 cells by chenodeoxycholic acid. A potential liver receptor homologue-1 (LRH-1) cis acting element was identified in the bile acid responsive region of the mouse but not rat promoter. The mouse but not rat promoter was activated by LRH-1 and this correlated with nuclear protein binding to the mouse but not rat LRH-1 element. The short heterodimer partner diminished the activity of the mouse promoter and could partially offset its activation by LRH-1. Inter-conversion of the potential LRH-1 cis-elements between the mouse and rat ASBT promoters was associated with an inter-conversion of LRH-1 and bile acid responsiveness. LRH-1 protein was found in Caco-2 cells and mouse ileum, but not IEC-6 cells or rat ileum. Bile acid response was mediated by the farnesoid X-receptor, as shown by the fact that overexpression of a dominant-negative farnesoid X-receptor eliminated the bile acid mediated down-regulation of ASBT. In addition, ASBT expression in farnesoid X-receptor null mice was unresponsive to bile acid feeding. In summary cell-line and species-specific negative feedback regulation of ASBT by bile acids is mediated by FXR via SHP-dependent repression of LRH-1 activation of the ASBT promoter.
Introduction

Reclamation of bile salts by the intestine is primarily mediated by the apical sodium dependent bile acid transporter (ASBT) located in the terminal ileum (1,2). The bile acid responsiveness of ASBT expression, which is of fundamental importance to both cholesterol metabolism and cholestatic liver disease, is a matter of on-going controversy (3). Conflicting results have been observed in a number of laboratories, where experimental methodologies and animal species have been variable (4-15). Positive (5,7,9,11,13-15), negative (4,6,8,12,15), and no (10) feedback regulation has been observed. Bile acid homeostasis has been perturbed by bile acid (4,6,8-10,12), sequestrant (4,6,8,10), or cholesterol (10,13,14) feeding; by common bile duct ligation (7,10,11,15) or diversion (5,9,11); and by genetic modification of bile acid biosynthesis (12). Investigations have been carried out in rats (4,5,7,9-11,15), mice (8,12), guinea pigs (4,6), and rabbits (13,14). Bile acid transport has been measured using intact intestine (4,6,11) and brush border membrane vesicles (5,7,9,10,15). ASBT expression has been quantified at the level of protein (9-15) and messenger RNA (8,9,11,15). The complexity of the current literature reflects the difficulties of in vivo assessment of a transport system that is part of a highly integrated and tightly regulated metabolic pathway.

The molecular mechanisms of bile acid responsiveness have been elucidated in recent elegant investigations from a number of laboratories (16). In general two systems have been characterized, those that are under positive or negative feedback regulation by bile acids. Positive feedback regulation is mediated by the direct effect of a complex of bile acids and the farnesoid X-receptor. Notable examples include
transcriptional activation of the ileal lipid binding protein (ILBP) and the canalicular bile salt excretory pump promoters by bile acids (17-21). Negative feedback regulation is more complex and involves an indirect effect. The first step is dependent upon activation of the expression of the small heterodimer partner (SHP) by a complex of bile acids and the farnesoid X-receptor. SHP then mediates down-regulation of target genes by inhibiting the activity of endogenously expressed positive trans-acting factors. For example SHP inactivates the liver receptor homologue-1 (LRH-1) and the retinoic acid receptor in the cases of cholesterol 7α-hydroxylase (22,23) and the sodium taurocholate cotransporting polypeptide (24), respectively.

The aim of the current study was to apply these new paradigms of bile acid responsiveness to the unanswered questions revolving around the regulation of the apical sodium-dependent bile acid transporter. Analysis in our own laboratory has indicated that in the rat, ileal ASBT does not respond to alterations in luminal bile acid concentrations (10). In contrast mouse studies conducted by Torchia and confirmed in our own laboratory (reported herein) reveal that ASBT is under negative feedback regulation (8,12). Recent cloning and characterization of the rat (25) and mouse (herein) ASBT promoters permits in vitro analysis of bile acid responsiveness independent of the complexities of in vivo integrated alterations in cholesterol and bile acid homeostasis.
Experimental Procedures

Animal Studies

All animal studies were performed under protocols approved by the animal care and use committees of the Mount Sinai School of Medicine, Rockefeller University and the National Institutes of Health. The effect of bile acids on the expression of ASBT in the mouse ileum was determined in male C57Bl/6J mice that were fed a diet supplemented with 0.5% cholic acid or 0.5% taurocholic acid for a period of seven days. Ileal protein homogenates and brush border membrane vesicles were prepared from the distal 1/3 of mouse ileum using methods that have been previously developed for the analysis of fetal and neonatal rat ileum (26). Brush border membrane enrichment was assessed by measuring alkaline phosphatase in homogenates and membranes. Quantitative Western blot analysis of the apical sodium-dependent bile acid transporter and the ileal lipid binding protein was performed as previously described (10). Antibodies against ILBP were generously provided by Dr. Michael Crossman (Children's Medical Center, Cincinnati, OH). Beta actin was used as a loading control (Sigma Chemicals, St. Louis, MO). Northern blot analysis was used to assess changes in steady-state mRNA levels of ASBT and ILBP as previously described (10). Twenty micrograms of total RNA was analyzed from the distal one third of the small intestine from 5 separate samples in each group (chow fed and cholic acid fed). Signal intensity for 28S rRNA was used as a loading control.
Cell culture

Human Caco-2 colon epithelial cells and rat IEC-6 intestinal epithelial cells (American Type Culture Collection, Rockville, MD) were maintained in Ham’s F12 medium containing 10% fetal calf serum (FCS). The plasmid-transfected cells were cultured in Ham’s F12 medium containing 0.5% charcoal-treated FCS for 40 h before harvest for reporter gene assays. The change in media was designed to minimize the effect of bile salts found in FCS.

Cloning and characterization of the mouse ASBT promoter

A partial murine ASBT cDNA was obtained by PCR using oligonucleotide primers corresponding to amino acid sequences conserved between the ileal and liver sodium/bile acid cotransporters; the sense and antisense oligonucleotide primers corresponded to amino acids 75-81 and 261-266, respectively, of the human ASBT. Oligonucleotide primers were then designed based on this mouse sequence and the predicted exon 1 region and used to screen a mouse (strain Sv129/OLA) bacteriophage P1 library (Genome Systems; St. Louis, MO). Two mouse genomic clones (12605, 12606) containing slc10A2 sequences were obtained and mapped. The structure of the mouse slc10a2 gene was elucidated using a combination of subcloning, restriction enzyme analysis, Southern blotting, long PCR amplification, and DNA sequencing. The nucleotide sequences have been submitted to the GenBank with accession number AF266724. Specific elements of the ASBT promoter were cloned and sequenced from several representative mouse (C57Bl/6J, SJL/J and BALB/cByJ, Jackson Laboratories, Bar Harbor, ME) and rat (Long evans, SHR, Fischer-344, Taconic Laboratories, Germantown, NY) strains. Genomic DNA from each of the species was amplified with
oligonucleotide primers extending from −385 to −95 (sense 5’-GCCCTAGAAGTCTGTG-3’ antisense 5’-GCTGGGAATAATTTTAG-3’). PCR products were then sequenced directly.

For transcription studies, a Sac I fragment encompassing nucleotides -1297 to +101 (where the "A" of the ATG is +1) was subcloned into pBluescript II KS and sequenced using an ABI Prism 377 sequencer. Using this 1.4 kb ASBT 5’ sequence as a template, four fragments were PCR synthesized and subcloned into the vector pGL3-Basic (Invitrogen), forming the mouse ASBT5’ constructs pGL3-mASBT5’/1.1kb, pGL3-mASBT5’/0.7kb (no correlate in rat constructs), pGL3-mASBT5’/P2 (mP2) and pGL3-mASBT5’/P3 (mP3), which correspond to the rat ASBT5’ constructs pGL3-ASBT5’/−829/+384, pGL3-ASBT5’/−378/+118(rP2), and pGL3-ASBT5’/−208/+118(rP3), respectively (25).

**DNA oligonucleotides and site-directed mutagenesis**

To prepare ASBT 5’ mutant constructs, complimentary strands of DNA oligonucleotides, containing the appropriate sequences and the desired point mutations within the LRH-1 element, were synthesized in vitro by the Oligonucleotide Synthesis Core Facility (Mount Sinai School of Medicine, New York, NY). The site-directed point mutagenesis of mouse and rat ASBT 5’ sequences was performed by a QuikChange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA), targeted to the potential LRH-1 cis-acting element in the mouse (positioned from −336 to −327) and the corresponding sequence in the rat (positioned from −340 to −331) P2 DNA fragments, respectively (see Figure 1). The DNA oligonucleotide primers, mLRHmu and rLRHmu, were synthesized with sequences labeled as “LRH oligo sequence,” as shown in Figure...
1. Two PCR generated point mutations converted mouse original LRH-1 cis-elements into the corresponding rat sequence and vice versa (Figure 1). The resulting PCR products, pmP2-LRHmu and prP2-LRHmu, were examined for their sizes by electrophoresis, and the point mutations were confirmed by DNA sequencing.

**Nuclear protein extraction and electrophoretic mobility shift assay**

Nuclear proteins were extracted from Caco-2 and IEC-6 cells using the method as described previously (27). The protein concentration of the nuclear extracts was determined by the Bio-Rad protein assay kit as described by Bio-Rad (technical bulletin 1015), with bovine serum albumin as a standard. The protein preparations were aliquoted into vials, quick-frozen, and stored at -80°C. DNA-protein binding reactions were carried out as previously described (28) with 5 µg of nuclear protein extract and 10^6 cpm of DNA probes in 15 mM KCl, 12 mM Hepes (pH 7.9), 5 mM MgCl₂, 0.25 mM EDTA, 0.25 mM DTT and 10 % glycerol in a total volume of 10 µl at 37°C for 30 min. Following digestion with 127 units of DNase I (GIBCO/BRL) at 37°C for 30 min, samples were analyzed by electrophoresis through a 7% native polyacrylamide gel. Competition experiments were performed using specific or nonspecific unlabelled oligonucleotide probes. Supershift assays were performed using either an LRH-1 specific polyclonal antibodies or an irrelevant antibody (anti-human growth hormone).

**Transient transfection and firefly luciferase assay**

The procedures for transient transfection and firefly luciferase analysis of cells were performed as described previously (25). Briefly, transient transfection was carried
out by electroporation (0.22 kV and 0.95 μF) of 5 × 10⁶ cells with 4 μg of ASBT
5'/luciferase hybrid constructs and 0.1 μg of a quantitative control plasmid pRL-TK
containing a thymidine kinase promoter-driven Renilla luciferase gene (Promega). After
electroporation, the cells were cultured for an additional 40 h before performing dual
luciferase assays (Promega) as described by the manufacturer.

Bile acid and orphan nuclear receptor gene constructs

To examine the effect of bile acids on mouse promoter activity, mouse ASBT
5'/firefly luciferase hybrid reporter construct pGL3-mASBT5'/P2 transfected Caco-2 cells
were treated for 40h with various concentrations (0, 10, 25, 50, 75, 100 μM) of the bile
acid chenodeoxycholic acid (CDCA, Sigma, St. Louis, MO) followed by dual luciferase
assays. The effect of 100 μM CDCA on the mouse P2 and P3 constructs and the rat
ASBT 5' construct pGL3-ASBT5'/-378/+118 was assessed by transient transfection
analysis. Nuclear extracts were prepared from four separate sets of control or CDCA
treated Caco-2 cells. Western blot analysis was then performed for FXR, SHP and
LRH-1 in the linear portion of the response curve for each protein.

The plasmid constructs, pCMX-rFXR and pCMX-mSHP (generous gift from Dr.
David Mangelsdorf, University of Texas Southwestern, Dallas, TX) harbor a copy of the
rat FXR and mouse SHP genes, respectively (21). pCMX-hFXR-W469A (generous gift
from Dr. David Mangelsdorf, University of Texas Southwestern, Dallas, TX) contains a
human FXR mutant which contains a point-mutation within the AF-2 domain of the gene
(21). The resulting gene product contains bile acid and DNA binding domains, but lacks
a functional transactivation motif, essentially making it a dominant negative expression
construct. pcDNA3.1/Myc-LRH (generous gift from Dr. Alan R. Tall, Columbia University, New York, NY) directs expression of the mouse liver receptor homologue-1 gene, which is a homologue of the orphan nuclear receptor fushi tarazu F1 (Ftz-F1) from *Drosophila* (29). The effect of the orphan nuclear receptors on basal activity and bile acid mediated responses was assessed by co-transfection of these constructs with pGL3-mASBT5'/P2 as described above.

**Western analysis of ASBT expression in FXR null mice**

FXR null mice or control litter-mates were fed a diet of 1% cholic acid or normal chow for a period of five days as previously reported (30). ASBT and ILBP protein expression were measured in the distal ileum as described above.

**Statistical Analysis**

Statistical analysis was performed using InStat software (GraphPad Software, Inc. San Diego, CA). Means were compared by student's t-test or the Tukey-Kramer multiple comparisons test. Unless otherwise stated all values are mean ± standard deviation.
Results

Bile acid response in the mouse

Bile acid feeding for seven days was tolerated without adverse effect. Ileal protein content (data not shown) and brush border membrane enrichment was the same in control and bile acid fed mice (alkaline phosphatase enrichment BBMV/homogenate: chow 9.4 ± 2.2, cholic acid 8.6 ± 1.3, taurocholic acid 8.5 ± 0.6; n=5 for each group, p>.2 for all comparisons). Actin was used as a loading control for both BBMV and homogenates. Equivalent loading was observed (densitometry units/mcg protein: BBMV, chow 48,400 ± 9,800, cholic acid 55,800 ± 8,600, taurocholic acid 55,027 ± 3,400; n=5 for each group and homogenates chow 64,200 ± 3,200, cholic acid 59,600 ± 1,100, taurocholic acid 50,900 ± 4,600; n=5 for each group). Both cholic and taurocholic acid feeding led to significant reductions in ASBT protein expression and significant increases in the expression of ILBP protein (ASBT expression [densitometry units/mcg BBMV] chow 82,200 ± 1,700 cholic acid 41,600 ± 2,300, taurocholic acid 12,900 ± 8,800; n=5 for each group, p<.01 for chow vs cholic acid or chow vs taurocholic acid, Figure 2 A and D: ILBP expression chow 47,200 ± 15,600 cholic acid 108,200 ± 5,100, taurocholic acid 98,900 ± 17,200; n=5 for each group, p<.01 for chow vs cholic acid or chow vs taurocholic acid, Figure 2B and 2D). Northern analysis of total RNA extracted from ileum after 7 days of cholic acid feeding revealed similar changes in ASBT and ILBP steady-state mRNA levels (phosphorimager units/mcg total RNA: ASBT control 46,900 ± 15,700, cholic acid 9,400 ± 5,800, n=5, p<.001: ILBP control 43,000 ± 8,600, cholic acid 179,900 ± 33,900, n=5, p<.001, Figure 2C and 2D). Equivalent loading was demonstrated by analysis of 28S RNA levels
Cloning and characterization of the mouse ASBT promoter

The murine slc10A2 gene is organized in 6 exons spanning approximately 24 kb of DNA sequence and its structure is identical to the human and rat SLC10A2 genes
(25,31,32). Exon/intron boundaries conform to consensus motifs; the introns all began
with a GT at the 5’ splice donor sites and ended with an AG at the 3’-splice acceptor
sites. The first exon encompasses the 5’-untranslated region and encodes amino acids
1 to 126. Exons 2 through 6 encompass the remaining coding sequence (amino acids
127 to 348). Exon 6 also encodes a long 3’ untranslated region of 2879 nucleotides and
a single polyadenylation signal (AATAAA).

One thousand one hundred base pairs of the 5’ flanking region of the mouse
ASBT promoter were cloned and sequenced (GenBank Accession AF266724). The
mouse and rat 5’ flanking sequences P2 and P3 were 88% identical (Figure 1). The
mouse 5’ flanking region was linked to the luciferase reporter construct, PGL3-basic
(Promega), and was subsequently used for transient transfection studies in Caco-2
cells. Four different constructs were transfected and all showed high level of promoter
activity (Figure 3). Unlike the rat where activities were similar, the mouse P2 construct
had significantly higher activity than P3. Promoter activity of mouse P2 but not P3 was
significantly diminished by treatment of Caco-2 cells with 100 µM chenodeoxycholic acid
(Figure 3). P2 promoter activity was inhibited in a dose dependent fashion by
chenodeoxycholic acid (Figure 4). Chenodeoxycholic acid treatment of Caco-2 cells
transfected with the human bile salt excretory pump promoter (21) revealed marked up-
regulation of promoter activity indicating that CDCA treatment did not induce nonspecific cell toxicity (data not shown).

**Molecular mechanism of negative feedback regulation of ASBT by bile acids**

The truncation studies above indicated that bile acid responsiveness was localized in the region of the mouse promoter P2 but not P3. Sequence analysis of the region of the mouse ASBT promoter between nucleotides −378 and −208, that is located upstream of P3 and is part of P2, revealed a potential LRH-1 binding site between mouse base pairs −336 and −327 (Figure 1). The same potential LRH-1 element was observed in all strains of mice that were examined. Two nucleotide differences were noted in the mouse and rat sequences in this region. Sequence analysis in this region was identical in all rat strains that were examined. In light of the role that LRH-1 plays in negative feedback regulation of cholesterol 7-alpha hydroxylase, initial efforts were directed at analysis of this element in the mouse ASBT promoter. The mouse P2 promoter activity was markedly enhanced by co-transfection of an LRH-1 expression construct (Figure 5). The enhanced activity could be partially off-set by simultaneous co-transfection with the short heterodimer partner (SHP).

Protein binding by the mouse but not rat potential LRH-1 cis-acting element was seen after electrophoretic mobility shift assay using nuclear extracts from Caco-2 cells (Figure 6A, lane 1=mouse; lane 7=rat). The band could be supershifted by an LRH-1 specific antibody (Figure 6A, lane 2), but not by human growth hormone antibody used as a control (Figure 6A, lane 3). Protein binding could be competed for with increasing concentrations of unlabelled LRH-1 oligonucleotide but not by a nonspecific
oligonucleotide (Figure 6B). Key transcription factors involved in bile acid response, SHP (35 kDa), LRH-1 (53 kDa) and FXR (75 kDa), were detected in Caco-2 nuclear extracts (Figure 6C). SHP expression was increased four-fold after treatment with CDCA (Figure 6C). LRH-1 and FXR were unchanged.

Site directed mutagenesis was performed in order to convert the mouse LRH-1 site into the rat site (P2 -> P2µ) and vice versa. Basal activity of the mouse P2 promoter was higher than the rat and the relationship was reversed after the LRH binding sites were interchanged (Figure 7). The mouse but not rat promoter could be stimulated by co-transfection of LRH-1. The response to LRH-1 was reversed after inter-conversion of the LRH-1 binding sites between the mouse and rat promoters. Mutation of the rat LRH-1 site led to binding of LRH as demonstrated by gel shift analysis (Figure 6A, lanes 10 - 12). Similarly, mutation of the mouse LRH-1 site lead to loss of LRH-1 binding (Figure 6A, lanes 4 - 6). The mouse but not rat promoter could be inhibited by the addition of 100 µM chenodeoxycholic acid and the responsiveness to bile acids was also reversed after interchange of the mouse and rat LRH-1 element sequences (Figure 7). The bile acid responsiveness of the mouse P2 promoter was not observed in IEC-6 cells treated with 100 µM CDCA (Figure 8). Basal activity of the mouse P2 promoter was lower in IEC-6 cells compared to Caco-2 cells. Expression of LRH-1 in Caco-2 and IEC-6 cells enhanced the activity of the mouse P2 promoter and conferred bile acid responsiveness in IEC-6 cells. Western analysis of nuclear extracts revealed that FXR and SHP are expressed in Caco-2 cells, IEC-6 cells, and rat and mouse ileal epithelial cells (RIE and MIE, respectively, Figure 9). Bile acid response of mouse P2 correlated with the expression of LRH-1, which was observed in Caco-2 cells.
and mouse ileum, but not IEC-6 cells or rat ileum. LRH-1 could be detected in rat liver nuclear extracts, confirming that the antibody was able to recognize LRH-1 in rat (data not shown). Overexpression of a mutant FXR that acts as a dominant negative protein abrogated the bile acid responsiveness of the mouse P2 promoter construct (Figure 10).

### ASBT expression in FXR null mice

Alkaline phosphatase enrichment in brush border membrane vesicles from the mouse terminal ileum averaged 6.2 (standard deviation 3.4) and was similar in all four groups of mice (Group 1: chow fed wild type, Group 2: chow fed FXR null mice, Group 3: cholic acid fed wild type mice, Group 4: cholic acid fed FXR null mice). ASBT expression was increased in chow fed FXR null mice relative to controls (densitometry units/mcg BBMV wild type chow 30,500 ± 5,200, FXR null chow 61,800 ± 2,800, n=3 in each group, p<01, Figure 11A and 11B). As expected cholic acid feeding in wild type mice lead to reduction in ASBT protein expression (12,600 ± 6,000, n=3, p<.01). In contrast cholic acid feeding in the FXR null mice resulted in no significant change relative to chow fed FXR null mice (61,500 ± 3,200, n=3, p<.01). Overall ASBT expression was nearly five-fold higher in cholic acid fed FXR null mice relative to wild type littermates. ILBP expression was markedly reduced in FXR null mice (groups 2 and 4, Figure 11A and B) independent of feeding status, indicating that FXR was essential for basal expression of ILBP. As expected cholic acid feeding resulted in increased expression of ILBP protein in wild type mice (densitometry units/mcg homogenate protein: wild type chow 22,300 ± 4,000, wild type cholic acid 40,200 ± 2,100, n=3, p<.01). Actin was used as a loading control and revealed similar
quantification in all four groups (densitometry units/mcg homogenate protein: wild type chow 48,600 ± 1,100, FXR null chow 48,000 ± 1,700, wild type cholic acid 48,300 ± 2,100, FXR null cholic acid 47,800 ± 400, n=3 for each group, p>.2: BBMV protein wild type chow 55,500 ± 3,600, FXR null chow 54,300 ± 8,000, wild type cholic acid 54,200 ± 4,000, FXR null cholic acid 53,100 ± 4,700, n=3 for each group, p>.2).
Discussion

Transcriptional activation of the apical sodium-dependent bile acid transporter is mediated by at least three different trans-acting factors. Hepatocyte nuclear factor-1 trans-activates the ASBT promoter and the corresponding null mice have markedly reduced ASBT expression (33). The AP-1 protein c-Jun trans-activates ASBT and expression of c-Jun correlates with the developmental-stage and region-specific expression of ASBT (25). The current studies indicate that the liver receptor homologue-1 protein is also a transcriptional activator of ASBT. LRH-1 does not appear to be essential for basal activity, but plays a crucial role in mediating bile acid responsiveness. This is supported by the enhanced activity of the mouse ASBT promoter versus that of the rat promoter, the former of which harbors a functional LRH-1 cis-acting element.

The ASBT gene is under negative feedback regulation by bile acids in the mouse ileum. Our studies of ASBT protein using mouse ileal BBMV and mRNA confirmed the findings of a similar study of ASBT mRNA (8). In both studies ASBT expression was repressed after bile acid feeding. We have also observed the negative feedback regulation of ASBT in cholesterol 7-alpha hydroxylase knock-out mice, which have markedly reduced bile acid synthesis and secretion (34). The knock-out mice have enhanced expression of ASBT (12). In both the bile acid fed and cholesterol 7-alpha hydroxylase knock-out mice, regulated changes in the ileal lipid binding protein are opposite of ASBT. This is expected as the ileal lipid binding protein is positively regulated by bile acids.
Bile acid responsiveness of the ASBT gene is mediated in an indirect fashion by farnesoid X-receptor dependent activation of the short heterodimer partner and subsequent inhibition of LRH-1 activity. This paradigm has been previously shown to be relevant for a similar negative feedback regulation of cholesterol 7-alpha hydroxylase (22,23). The cell-line and species-specific nature of the negative feedback regulation of ASBT can now be explained by the presence or absence of both the LRH-1 cis- and trans-acting elements. The mouse but not rat ASBT promoter has a functional LRH-1 cis-acting element. In addition, mouse but not rat ileum expresses the LRH-1 protein. Similar differences in LRH-1 protein expression are found in Caco-2 and IEC-6 cells. It is fascinating that there has been a divergent and coordinate evolution of both the LRH-1 cis- and trans-acting elements between these two relatively closely related rodent species. The implications of the differences are profound in regard to the regulation of bile acid homeostasis. It is tempting to speculate that this difference may have evolved in conjunction with the presence (mouse) or absence (rat) of the gallbladder. Preliminary sequence analysis of both the rabbit and human promoters does not give a clear indication as to whether these promoters will be bile acid responsive, and thus functional assays will be necessary.

The physiologic relevance of the bile acid response is apparent in the FXR null mice (30). The critical role of FXR in mediating the bile acid response of the ASBT promoter is evident from our transient transfection studies using the dominant negative FXR construct, where bile acid responsiveness was lost. FXR null mice have enhanced expression of ASBT. Presumably, this is a result of derepression of the basal inhibition of ASBT expression by bile acids that normal cycle through the enterohepatic
circulation. The FXR null mice have a relatively unremarkable phenotype when fed a standard chow diet. In contrast, dietary supplementation with cholic acid is highly toxic in the FXR null mice. The reason for this is now clearer. FXR null mice that are fed bile salts have markedly elevated serum concentrations of bile salts. This is associated with a diminished capacity to excrete bile salts in the stool. The current studies indicate that ASBT is paradoxically up-regulated in the FXR null mice that are fed cholic acid. The central role of FXR in mediating the bile acid responsiveness of the mouse ASBT gene explains this anomalous effect. The current studies of ASBT protein in the FXR null mice are similar to the reported data on ASBT mRNA, which apparently did not reach statistical significance (30). Preliminary descriptions of an SHP null mouse have revealed a similar and consistent toxic effect of bile acid feeding (35). This is not surprising given the role that SHP plays in mediating the negative feedback of bile acids.

Bile acid responsiveness of ASBT may be of great importance in humans. Surgical and pharmacological methods are able to induce bile acid wasting and lead to cholesterol catabolism via enhanced conversion of cholesterol to bile acids (36-39). If the human ASBT gene is under negative feedback regulation these physiological responses to the surgical or pharmacological treatment may be diminished by adaptive up-regulation of ASBT expression. Inhibition of this adaptive response would have the potential to markedly enhance the effectiveness of these approaches to hypercholesterolemia. It is also noteworthy that in familial intrahepatic cholestasis type 1 (Byler’s disease) intestinal reclamation of bile salts is anomalously enhanced (40).
In summary, these studies have helped resolve a long-standing controversy regarding the bile acid responsiveness of the ASBT gene. Negative feedback regulation is mediated by FXR-dependent up-regulation of SHP expression. SHP in turn represses LRH-1 dependent activation of ASBT expression. The presence or absence of functional LRH-1 cis- and trans-acting element correlates with cell line and species-specific bile acid responsivity. The physiologic significance of this can be seen in the lethal effects of cholic acid feeding in FXR null mice, where paradoxical up-regulation of the bile acid transporter ASBT occurs due to failure of FXR-mediated inhibition of the LRH-1 dependent ASBT activation.
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Figure Legends

Figure 1. Sequence analysis of the mouse and rat ASBT promoter P2. Nucleotide sequence comparisons show that there is 88% identity between mouse and rat ASBT 5’ promoter P2 (genomic nucleotides –378 to +118). The two P3-contained AP-1 cis elements are conserved in both species. The LRH-1 consensus sequence is present only in the mouse P2 region. LRH-1 oligo sequence represents the sequence of the oligos used in LRH-1 site-directed mutagenesis and band shift assays, as described in “Experimental Procedures”. The exact sites of the point mutations in the LRH-1 element are marked by the arrowheads. uAP-1 = upstream AP-1; dAP-1 = downstream AP-1.

Figure 2. Analysis of the response of ASBT and ILBP to bile acid feeding in the mouse. 2A and B Western Blot Analysis. C57Bl/6J mice were fed a diet enriched with 0.5% cholic or taurocholic acid for seven days. Ileal homogenates and brush border membrane vesicles were prepared from a single mouse intestine. Proteins from five separate mice (three shown) were analyzed for each group (control [lanes 2, 5 8], cholic acid [lanes 1, 4, 7], taurocholic acid [lanes 3, 6, 9]). ASBT expression (panel A) is reduced in the cholic or taurocholic acid fed mice, while ILBP expression (panel B) is enhanced. Actin expression, used as a loading control, was equivalent in all samples.

2C Northern Blot analysis. C57Bl/6J mice were fed a standard chow diet or one enriched with 0.5% cholic acid for seven days. Total RNA was prepared from the distal one-third of the small intestine. Samples were prepared from five chow fed [lanes 1, 3, 5, 7, and 9] and five cholic acid fed [lanes 2, 4, 6, 8, and 10] mice. Steady-state ASBT
mRNA levels are reduced in the cholic acid fed mice, while ILBP expression is increased. Equivalent loading is demonstrated by the signal intensity for 28S rRNA.

**Graphic depiction of overall changes.** The percent change of ASBT or ILBP protein and mRNA in response to 7 days of cholic acid feeding is depicted. Clearly, ASBT is under negative-feedback regulation at the level of mRNA and protein, while ILBP is under positive-feedback by bile acid feeding.

**Figure 3. Transient transfection analysis of truncations of the mouse ASBT promoter.** To study transcriptional activities of mouse ASBT (mASBT) 5’ sequences, dual-luciferase assays were performed 40 h after transfection of Caco-2 cells with various ASBT5’/luciferase hybrid constructs as described in "Experimental procedures". The firefly luciferase reporter data were normalized to thymidine kinase promoter-driven Renilla luciferase activities. With the exception of pGL3-mASBT5’/P3 (P3), the promoter activity increased with the shortening of the 5’ sequence, and the highest reporter gene expression was observed in pGL3-mASBT5’/P2 (P2) construct. A significant decrease of the reporter gene expression was detected after chenodeoxycholic acid (BA) treatment of the cells transfected with P2, but not P3, hybrid constructs. Activity of pGL3 basic, containing no promoter sequence, was 2% of the P3 construct (data not shown).

**Figure 4. Dose-dependent inhibition of the mouse ASBT promoter by chenodeoxycholic acid.** mP2 hybrid construct-transfected Caco-2 cells were treated for 40h with CDCA at the doses as indicated in the figure. Results showed that the
luciferase expression, as an indication of mP2 promoter activity, was decreased as the concentration of bile acid was increased in the media.

Figure 5. LRH-1 induction and SHP inhibition of the mouse ASBT promoter. Caco-2 cells were co-transfected with mP2 plus SHP (+SHP), LRH-1 (+LRH) or both (+LRH/SHP) plasmid constructs, followed by dual luciferase assays. SHP or LRH-1 alone was sufficient to inhibit or enhance the promoter activity, in comparison with the control (mP2). SHP could significantly offset LRH-1 induced up-regulation of mP2 transcriptional activity.

Figure 6. Analysis of the proteins involved in the bile acid responsiveness of the mouse ASBT gene promoter. A. Band shift analysis of LRH-1 cis-trans interactions. Preparation of Caco-2 nuclear extract and LRH-1 DNA oligonucleotides are described in “Experimental procedures”. Electrophoretic mobility shift assays with Caco-2 nuclear extract and radiolabeled mouse LRH-1 DNA oligonucleotide (mLRH) detected a single band (lane 1) that was also observed with the corresponding mutated rat (rLRHmu) oligonucleotide (lane 10). The LRH-1 trans-acting factor binding to the native mouse (lane 2) and mutated rat (lane 11) LRH-1 cis elements was supershifted with an LRH-1 antibody (arrow [note N = no antibody, L = LRH-1 antibody, H = nonspecific control antibody, anti-human growth hormone]). No supershifting was observed when a nonspecific antibody, anti-human growth hormone (H) was utilized (lanes 3 and 12). No protein binding was observed with the mutated mouse LRH-1 site (mLRHmu; lanes 4-6) or the native (rLRH) rat LRH-1 site (rLRH; lanes 7-9). B. EMSA
analysis of the specificity of LRH-1 binding. LRH-1 binding could be competed for by the LRH-1 unlabelled oligonucleotide (right panel), but not a nonspecific oligonucleotide (left panel). C. Western blot analysis of FXR, SHP and LRH-1 proteins from nuclear extracts of Caco-2 cells untreated (lanes 1, 3, 5 and 7) or treated with CDCA (lanes 2, 4, 6 and 8). Four separate experimental studies are shown. SHP expression is clearly increased after CDCA treatment, while FXR and LRH-1 are unchanged. Blots were probed with histone H1 antibody to demonstrate equivalent sample loading.

Figure 7. The upstream LRH-1 cis element mediates the effect of bile acids on the ASBT promoter activity. Mouse P2, but not rat P2, promoter activity, as shown by luciferase activity, was significantly inhibited by treatment of cells with 100 µM CDCA (P2 + CDCA), compared to the untreated control (P2). Overexpression of LRH-1 (P2 + LRH-1) enhanced mouse P2, but not rat P2 transcriptional activity. Rat P2 failed to respond to addition of CDCA or introduction of exogenous LRH-1. To further study the role of LRH-1 in ASBT regulation, the cis element in mP2 and rP2 were point mutated as described in “Experimental procedures”, resulting in loss and gain of the LRH-1 consensus sequence within mouse and rat P2 (P2µ)(Figure 2). Luciferase assays showed that not only the basal promoter activities interchanged between the two P2 mutants (P2µ) compared to the normal counterparts (P2), but also reactions to CDCA (P2µ + CDCA) and LRH-1 (P2µ + LRH) shifted to rat P2µ, and mouse P2µ lost these characteristics.
Figure 8. **Cell line specificity of bile acid response.** Caco-2 or IEC-6 cells were co-transfected with mP2, mP2 plus CDCA (+CDCA), LRH-1 (+LRH) or both (+LRH/CDCA), followed by dual luciferase assays. Basal mP2 activity was reduced in IEC-6 cells compared to Caco-2 cells. Addition of CDCA reduced mP2 activity in Caco-2 cells but not IEC-6 cells. LRH-1 induced mP2 activity in both Caco-2 and IEC-6 cells. This enhanced promoter activity was partially repressed by treatment with CDCA in both Caco-2 and IEC-6 cells.

Figure 9. **LRH-1 expression correlates with bile acid responsiveness.** Nuclear extracts were prepared from Caco-2 and IEC-6 cells and from rat (RIE) and mouse (MIE) ileum. Western blot analysis reveals that FXR and SHP are expressed in all of the nuclear extracts. In contrast, LRH-1 is expressed only in Caco-2 cells and mouse ileum. The expression of LRH-1 correlates with bile acid responsiveness. Equivalent loading of nuclear proteins is indicated by the signal intensity for histone H1.

Figure 10. **FXR mutant (FXRµ) blocks the CDCA inhibitory effect on mP2 promoter activity.** Under bile acid-reduced condition, Caco-2 cells transfected with mP2 and FXR (+FXR) or mP2 plus pCMX-hFXR-W469A (+FXRµ) had no significant changes in the reporter gene expression, in comparison with those transfected with mP2 alone (mP2). mP2-transfected cells expressed significantly lower luciferase activities following treatment with 100 µM CDCA (+CDCA) or with CDCA plus FXR plasmid co-tranfection (+CDCA/FXR); whereas, treatment with CDCA and pCMX-hFXR-W469A co-transfection (+CDCA/FXRµ) did not significantly affect the mP2-driven reporter gene.
expression. This indicates that the FXR mutant acted as a dominant negative factor in the CDCA initiated pathway leading to the down-regulation of ASBT promoter activity. These results also suggest that bile acid is required for the FXR inhibitory effect of ASBT expression.

**Figure 11. Regulation of ASBT and ILBP by bile acids in FXR null mice.** FXR null mice or control litter-mates were fed a diet of 1% cholic acid or normal chow for a period of five days. Group 1: chow fed wild type mice, Group 2: chow fed FXR null mice, Group 3: cholic acid fed wild type mice, Group 4: cholic acid fed FXR null mice. Western blot analysis of ASBT and ILBP in ileal brush border membrane vesicles and protein homogenates, respectively, are shown in panels A and B. Three separate samples of each group are shown. Graphic depiction of the quantitation of ASBT and ILBP expression is seen in panel C. ASBT expression is increased in FXR null mice fed standard chow (group 1 versus group 2). Cholic acid feeding in wild type mice results in the expected reduction in ASBT expression (group 3). In contrast, ASBT expression was four-fold higher in cholic acid fed FXR null mice relative to wild type littermates (i.e. group 4 versus 3). ILBP expression was markedly reduced in the FXR null mice (groups 2 and 4). Cholic acid feeding led to a significant increase in ILBP expression in the wild type mice (group 3 versus group 1).
Figure 1
Figure 2

D

% of control

ASBT  ILBP

mRNA  protein

Figure 2
Figure 3

Mouse ASBT Promoter Construct

Luciferase Activity

- 1.1 kb
- 0.7 kb
- P2
- P2 + BA
- P3
- P3 + BA
Figure 4
Figure 5

 Luciferase Activity

Transfection

mP2 +SHP +LRH +LRH/SHP
Figure 6A
Figure 6B
Figure 6C

1 2 3 4 5 6 7 8

FXR

SHP

LRH

Histone H1
Figure 7
Figure 8

Luciferase Activity

Transfection

mP2 + CDCA + LRH + LRH/CDCA

Caco-2
IEC-6
Figure 9
Figure 10
A

Group: 1 2 3 4 1 2 3 4 1 2 3 4

ASBT

Actin

B

ILBP

Actin
Figure 11

![Graph showing signal intensity (units/BMV) for ASBT and ILBP proteins across different groups.]

- Group 1
- Group 2
- Group 3
- Group 4
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