Function and regulation of the human bile salt export pump

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Function and regulation of the human bile salt export pump

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Research

The research described in this thesis was performed at the Division of Gastroenterology and Hepatology, Department of Internal Medicine, Faculty of Medicine of the University Medical Center Groningen, the Netherlands. This group participates in the Groningen University Institute for Drug Exploration (GUIDE).

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Contents

Scope of this thesis

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The aim of the studies described in this thesis was to investigate the function and regulation of the bile salt export pump (BSEP; ABCB11): the protein that is responsible for the transport of bile salts from the liver to the bile. Just before the start of this thesis project it was shown that mutations in the gene encoding BSEP are the cause of an inherited liver disease, progressive familial intrahepatic cholestasis type 2 (PFIC-2). In chapter 2, one of these mutations is studied in detail, an aspartate to glycine change at amino acid position 482 (D482G) of this 1,321 amino acid-long protein. Although this mutation does not interfere with the transport function of BSEP, it gives rise to very low BSEP protein levels in vivo. Most likely, the cellular quality control machinery recognizes this protein as "mutant" and rapidly degrades it before it can reach its functional destination, the canalicular membrane.

Chapter 3 describes that the transcription of the human BSEP gene is regulated by its substrates, the bile salts, via the transcription factor Farnesoid X Receptor (FXR). FXR functions as a heterodimer with another transcription factor, namely the Retinoid X Receptor or RXR. Whereas bile salts are the ligands of FXR, 9-cis retinoic acid (9cRA), a vitamin A derivative, is the ligand for RXR. Chapter 4 describes the importance of vitamin A/9cRA in BSEP regulation.

FXR and bile salts regulate many genes, mostly involved in cholesterol, bile salt and lipoprotein metabolism. In recent years, also FXR-target genes have been identified that appear to be unrelated to these cellular processes. To obtain insight in the different processes that are regulated by bile salt-activated FXR, a microarray analysis was performed using the human hepatoma cell line HepG2, with and without overexpression of rat Fxr. In chapter 5, we identified fibrinogen alpha, beta and gamma as possible new FXR target genes. This finding shows that FXR regulates a broad range of different cellular processes. This is essential information for future therapies aimed at regulating the activity of FXR.

Chapter 1

Function and regulation of hepatic transporters involved in bile flow

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Division of Gastroenterology and Hepatology, University Medical Center Groningen, Groningen, The Netherlands

1.1 General introduction

The liver is regarded the chemical factory of our body. One central function is the production of bile. Bile is crucial for digestion as well as the clearance of hydrophobic toxic compounds. The liver is equipped with specialized transporter proteins that are responsible for maintaining a constant flow of bile to digestive tract. Malfunctioning of these transporters is the cause of cholestasis and jaundice. This chapter gives an overview of the function and regulation of the transporters involved in bile formation and their malfunctioning during inherited and acquired cholestatic diseases.

1.1.1 The liver

The liver is the second largest organ of the body and in human weighs approximately 1.5 kilograms.

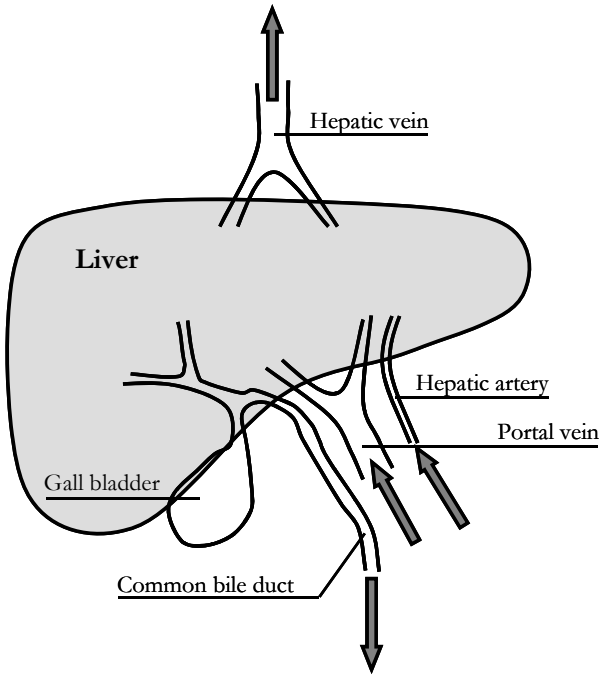

Fig. 1-1 Schematic overall view of the blood flow and bile flow from and to the liver. Nutrient-rich blood from the intestine and oxygen-rich blood enter the liver via the portal vein and hepatic artery, respectively. Blood leaves the liver via the hepatic vein. Bile, produced by the liver, is stored in the gallbladder and is secreted into the intestine via the common bile duct. Arrows indicate the flow of blood or bile.

Approximately 75% of the blood supply to the liver is accounted for by the portal vein coming from the small intestine, stomach, pancreas and spleen. The other 25% flows through the hepatic artery. The blood leaves the liver via the hepatic vein (Fig. 1-1). The liver is involved in carbohydrate metabolism, fat and lipid metabolism, protein metabolism, transformation of drugs and vitamins, and detoxification. The

liver produces bile, which helps digestion and secretion of waste products. It is collected from the hepatic bile ducts in the gall bladder and is secreted into the intestine via the common bile duct (Fig. 1-1). The liver plays a role in glyconeogenesis and glycogenolysis; it stores glycogen and releases it on demand. The liver also stores other nutrients and vitamins. It synthesizes certain plasma proteins, like albumin (major contributor to colloid osmotic pressure of plasma, transporter of hormones, bilirubin, fatty acids, etc.), fibrinogens (blood clotting) and other coagulation factors. It synthesizes lipoproteins, cholesterol and bile salts. In addition, the liver plays a role in immunological responses.

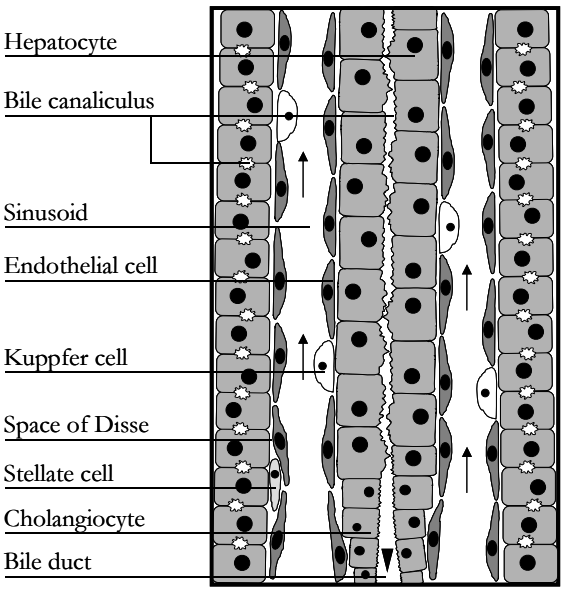

Fig. 1-2 Schematic view of the fine structure of the liver. The hepatocytes are arranged in plates. They form a physical barrier between the blood in the sinusoids and the bile in the canaliculi. The bile canaliculi are formed by the apical membranes of the hepatocyte. The sheet of endothelial cells, lining the sinusoid, is fenestrated for the passage of small molecules between blood and hepatocyte. Kuppfer cells, stellate cells, and pit cells are also present in the sinusoids. The stellate cells are located in the space of Disse. This space between the endothelial wall and the hepatocytes contains and drains tissue fluid. The bile canaliculi merge into the larger bile ducts, which are lined by the cholangiocytes. The direction of blood flow and bile flow are indicated by arrows and arrowhead, respectively.

The liver consists of different cell types: parenchymal cells (hepatocytes), endothelial cells, Kuppfer cells (macrophages), stellate cells (fat-storing and/or fibrotic cells), pit cells (natural killer cells) and oval or progenitor cells (Fig. 1-2). The vast majority of the cells in the liver are the hepatocytes. These cells carry out the primary functions of the liver: formation of bile, detoxification, and synthesis of blood proteins.

1.1.2 The liver units

There are three ways for dividing the liver in functional units: (1) the "classic" liver lobule, (2) the portal lobule, and (3) the hepatic acinus (Fig. 1-3).

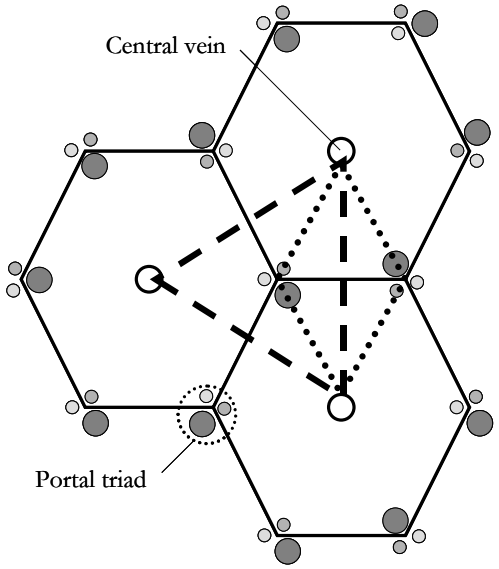

Fig. 1-3 The liver units: 1) The hexagonal shape displays the classic hepatic lobule, with the central vein in the center and the portal triads at the border. 2) The portal lobule indicated by a triangle, with the portal triad in the middle and bordered by the central veins. 3) The diamond-shaped hepatic acinus bordered by central veins and portal triads.

Traditionally, the liver was divided in "classic" liver lobules, based on macroscopic morphology. The liver lobule is a hexagonal structure surrounded by connective tissue. Branches of the central vein lay in the center of the liver lobule. Hepatocytes are arranged in layers that radiate from the central vein to the portal triads on the corners, which consist of branches of the portal vein, the hepatic artery and a bile duct. The liver lobule unit emphasizes the drainage of blood by the central vein.
The portal lobule unit places the portal triad in the center of a triangle of which the corners are branches of the central vein (Fig. 1-3) and emphasizes the blood supply by the portal vein and hepatic artery and the bile drainage by the bile duct. Bile, formed by the hepatocytes and secreted into the bile canaliculi is collected into bile ductules and drains into the bile duct of the portal triad.
The third classification is that of the hepatic acinus. The boundaries of this diamond-shaped structure are formed by two branches of the central vein and the portal triads of two adjacent liver lobules. This unit emphasizes the secretory function of the liver.

1.1.3 The hepatocyte

Hepatocytes are the main functional cells of the liver. They are responsible for the formation of bile, the metabolism of carbohydrates, proteins, fats, lipids, drugs and toxins, the synthesis of cholesterol, lipoproteins and plasmaproteins, detoxification, and storage of different substances like glycogen, fat and vitamins. Hepatocytes are especially enriched in smooth and rough endoplasmic reticulum, ribosomes, mitochondria, peroxisomes and lysosomes to adequately perform the many liver functions. Hepatocytes are polarized epithelial cells forming a physical barrier between sinusoidal blood and bile. The basolateral membrane is in contact with the sinusoidal blood and is the site for uptake of compounds. The apical membranes of adjacent cells form a lumen: the bile canaliculus. Tight junctions divide the plasma membrane in a basolateral domain and an apical domain. The vectorial transport of substances from sinusoidal blood to bile is mediated by specific transport proteins.[1,2]. A selection of these (human) proteins is shown in Fig. 1-4. Sinusoidal bile salt uptake is facilitated by NTCP and the OATP's in humans and Ntcp and Oatp's in rodents. The Na^+/K^+ATPase maintains the required electrochemical gradient. Biliary secretion of bile salts and other biliary compounds are carried out by BSEP, MDR3, MRP2, MDR1, ABCG5/G8, and FIC1 (in rodents these are Bsep, Mdr2, Mrp2, Mdr1a/1b, Abcg5/g5, and Fic1 respectively. The precise functions of these proteins are discussed later in this chapter.

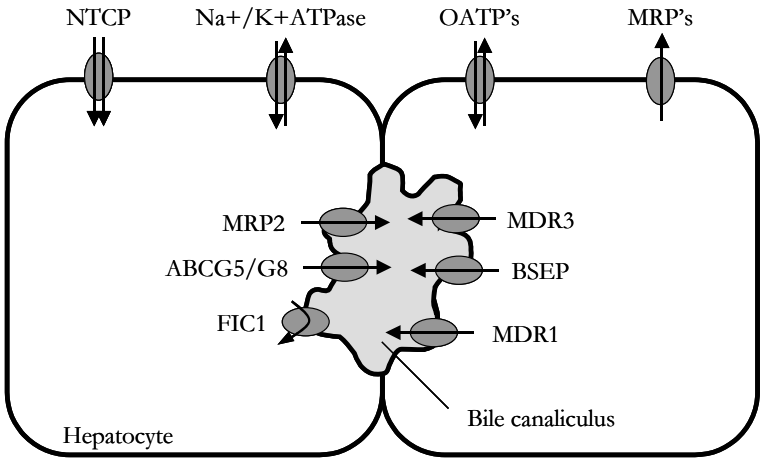

Fig. 1-4 The hepatocyte houses specific transport proteins for mediating the vectorial transport from blood to bile of various compounds. Some of these are shown here. Bile salts are taken up from the sinusoidal blood by NTCP and the OATP's. The Na+/K+ATPase maintains the required electrochemical gradient. Bile salts are secreted into the bile canaliculus by BSEP and also MRP2. Bile salt secretion triggers the release of phosphatidylcholine and cholesterol, which are supplied by MDR3 and ABCG5/G8 respectively. The best-known ABC transporter, MDR1, is involved in the elimination of bulky amphiphatic organic anions. Under cholestatic conditions, members of the ABCC family, the MRP's, are up-regulated and may function as an overflow system for bile salts.

1.1.4 Bile

The formation of bile is an important function of the liver. Bile is a body fluid, mainly containing water, electrolytes, and organic molecules including bile salts, phospholipids, cholesterol, and bilirubin. It flows through the biliary tract and via the gallbladder (except in rat) into the intestine. Bile secretion into the intestine has two main functions: (1) bile salts are important for digestion of dietary lipids and uptake of fat-soluble vitamins (A, D, E, and K); (2) it is the major route for the elimination of surplus cholesterol, waste products, bilirubin, drugs and other toxic components.

Bile secretion is an osmotic process. The major driving force for bile formation is the secretion of bile salts at the canalicular membrane of the hepatocyte, the so-called bile salt-dependent bile flow. Canalicular secretion of reduced glutathione (GSH;[3]) also contributes to bile flow, the bile salt-independent bile flow.

The biliary tract begins in the liver: the hepatocytes secrete bile into the bile canaliculi, these canaliculi merge into the bile ducts of the portal triad. The bile ducts merge into the common bile duct that allows bile to drain directly into the duodenum when it is needed for digestion. During fasting, bile is temporarily stored and concentrated in the gallbladder. Storage in the gallbladder is, however, redundant since a gallbladder is completely absent in rats and in humans it may be removed without any consequence.

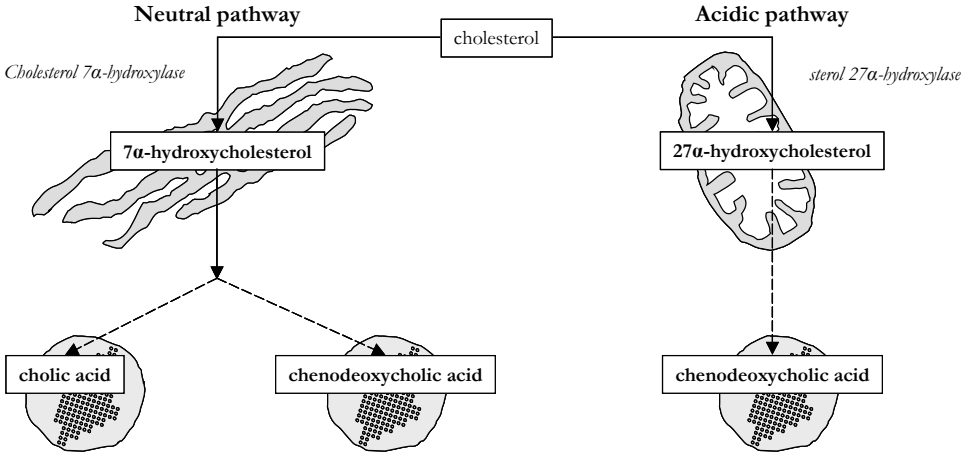

Fig. 1-5 The two pathways of bile salt synthesis: the classic pathway and the alternative pathway. The classic pathway starts with the conversion of cholesterol into 7α-hydroxycholesterol in the endoplasmic reticulum, while the alternative pathway starts in the mitochondrion with the conversion into 27α-hydroxycholesterol. Both pathways end in the peroxisomes resulting in the primary bile salts cholic acid and chenodeoxycholic acid.

1.1.5 Bile salts

Bile salts are exclusively synthesized in the liver and are formed as natural end products of cholesterol metabolism. They are amphiphatic molecules, which means that bile salts have both water-soluble (hydrophilic) and water-insoluble (hydrophobic sides) characteristics. This dual nature enables bile salts to carry out their function.

Bile salts facilitate the emulsification of lipids and subsequently help the actual digestion by lipases. In addition, bile salts help to solubilize and transport lipids by forming so-called micelles. Micelles are composed of bile salts and lipids (fatty acids, cholesterol, and monoglycerides) that are water-soluble, thus enabling transport of lipids and a great variety of other hydrophobic compounds.

In humans, the two most abundant bile salts are cholic acid (CA) and chenodeoxycholic acid (CDCA), which are referred to as primary bile acids. The primary bile acids are synthesized from cholesterol either via the classic (also called the neutral) pathway, which is also the predominant pathway in human, or the alternative (also called the acidic) pathway (Fig. 1-5[4]).

The first step of the classic pathway is established by the enzyme cholesterol 7α-hydroxylase and involves a modification of the ring structure. This reaction takes place in the endoplasmic reticulum. The first step of the alternative pathway takes place in the mitochondrion, where the enzyme sterol 27-hydroxylase modifies the sterol side chain. The classic pathway produces both cholic acid and chenodeoxycholic acid, while the alternative pathway produces predominantly chenodeoxycholic acid. Some enzymatic conversions take place in the cytosol. The final reactions in the formation of the primary bile acids occur in peroxisomes. Prior to excretion into bile, the bile acids are conjugated to taurine or glycine and are now referred to as bile salts. The ultimate fate of the conjugated bile salts is secretion into the intestine. There, approximately 95 % of the conjugated bile salts are reabsorbed and are transported back to the liver via the portal blood circulation. In the liver, they are taken up again by the hepatocytes for re-secretion into bile. This recycling process is referred to as the enterohepatic circulation of bile salts. Every day, approximately 4 grams of bile salts cycle about 6 times between liver and intestine. And although this enterohepatic circulation of bile salts is very efficient, 5% (approximately 1 gram per day) is lost each cycle via fecal secretion. This loss is compensated for by *de novo* synthesis by the liver.

As discussed before, the primary function of bile salts is their role as detergents to keep dietary fats and fat-soluble vitamins available for metabolism, and to remove hydrophobic compounds form the body, including cholesterol and toxins. In recent years, however, bile salts have been shown to perform several crucial signaling functions in the mammalian cell as well. For example, they function as highly selective mediators of gene transcription.[5] This is accomplished by binding and activation of the transcription factor Farnesoid X Receptor (FXR).[6,7] Many genes, involved in bile salt, cholesterol and lipoprotein metabolism are regulated by bile salt-activated FXR[5](see section 1.5.2). In addition, bile salts are now known to play an important role in regulation of apoptosis[8] and in intracellular signal pathways involving protein kinase C isoforms and phosphatidylinositol-3 kinase[9,10] as well.

Table 1-1. Hepatic transporters involved in bile flow. Indicated are their localization, substrates, relation to inherited diseases, regulation during acquired cholestasis, and transcriptional regulation.

Name	Loc.	Substrates	Inherited disease	Exp. Chol.	Transcriptional regulation
NTCP/Ntcp (SLC10A1/Slc10a1)	SM	Bile salts		↓	RARα,HNF1α, HNF4α(?),C/EBP, HEX
OATP-C (SLC21A6)	SM	Organic anions, Bile salts, Bilirubin (conjugates)			HNF1α
OATP-A (SLC21A3)	SM	Organic anions, Bile salts, Bulky organic cations			
OATP8 (SLC21A8)	SM	Organic anions Bile salts(?)			FXR HNF1α
OATP-B (SLC21A9)	SM	Organic anions			
Oatp1 (Slc21a1)	SM	Bile salts, Organic anions/cations		↓	HNF4α(?) HNF1α
Oatp2 (Slc21a5)	SM	Bile salts, Organic anions/cations		↓	PXR HNF1α
Oatp4 (Slc21a10)	SM	Organic anions Bile salts		↓	HNF1α
BSEP/Bsep (ABCB11/Abcb11)	CM	Bile salts	PFIC2 BRIC2	↔/↓	FXR HNF4α(?)
MRP2/Mrp2 (ABCC2/Abcc2)	CM	Bilirubin conjugates Anti-cancer drugs Bile salts	Dubin-Johnson	↓	FXR, RARα C/EBPβ PXR, CAR
MDR3/Mdr2 (ABCB4Abcb4)	CM	Phosphatidylcholine	PFIC3 ICP	↔	FXR, PPARα HNF4α(?), SP1
ABCG5/G8 Abcg5/g8	CM	Cholesterol	Sito-sterolemia		LXRα LXRβ
MDR1 / Mdr1a/1b	CM	Bulky amphiphatic organic anions Anti-cancer drugs		↔/↑	PXR SP1
FIC1/Fic1 (ATP8B1/Atp8b1)	CM	Aminophospholipids(?) Bile salts(?)	PFIC1 BRIC		

Abbreviations: SM: Sinusoidal Membrane; CM: Canalicular Membrane; PFIC: Progressive Familial Intrahepatic Cholestasis, BRIC: Benign, Recurrent Intrahepatic Cholestasis, ICP: Intrahepatic Cholestasis of Pregnancy.
↑: expression is up-regulation; ↔: expression is unaltered; ↓: expression is down-regulated

1.2 Hepatic bile salt transport proteins

The formation of bile is driven by the vectorial transport of solutes from the sinusoidal blood into the hepatocyte and from the hepatocyte into the bile canaliculus. Since hepatocytes form a physical barrier between sinusoidal blood and canalicular bile, the transport of compounds from blood to bile is carried out by specialized carrier systems. The hepatocyte houses various proteins, located in the sinusoidal and canalicular membranes, which mediate this transport (Table 1-1).

1.2.1 Basolateral or sinusoidal transport proteins

The hepatocyte is equipped with several transport proteins to extract metabolites, drugs and other compounds from the blood circulation. Sinusoidal uptake of compounds is mediated by members of the superfamily of solute carriers (SLC). SLC transport proteins are passive transporters, ion coupled transporters or exchangers (Fig. 1-6). These proteins use the electrochemical gradient to facilitate transport of solutes across the membrane. Currently, the superfamily of human solute carriers comprises 43 families and 298 genes (http://www.bioparadigms.org/slc/intro.asp). A member of a specific SLC family is assigned to that family if it has a protein sequence homology of at least 20-25 %.[11]

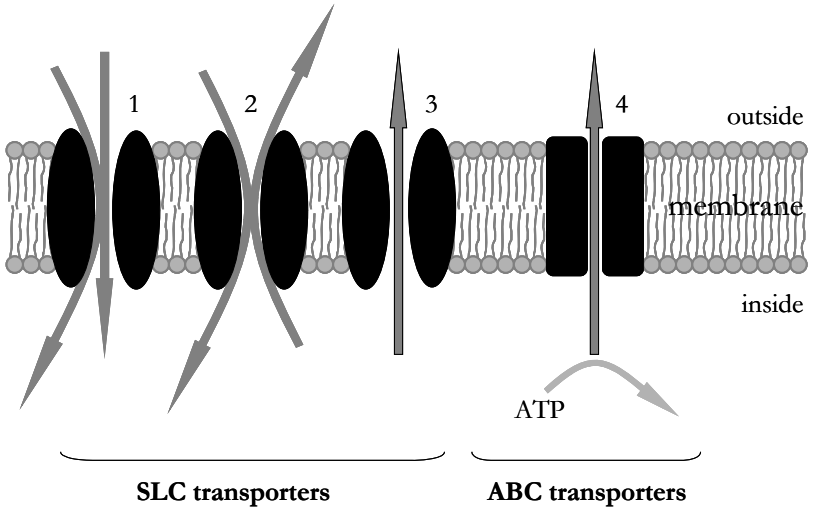

Fig. 1-6 Members of the SLC superfamily and members of the ABC transporter family are involved in the transport of compounds across the sinusoidal and apical membranes of the hepatocyte. The SLC transporter family comprises ion coupled transporters (1), exchangers (2), and passive transporters (3). ABC transporters need the energy from ATP hydrolysis for transport and are therefore active transporters (4). (Adapted from[11])

In the basolateral membrane of the hepatocyte, two families are important for bile salt uptake: (1) SLC family 10, the sodium bile salt cotransport family with 6 members;[12] and (2) SLC family 21 or O, the organic anion transport family, with 11 members.[13]
The Na^+-dependent taurocholate cotransporting polypeptide (NTCP in human, Ntcp in rodents; SLC10A1 according to SLC nomenclature) is the major hepatic bile salt

importer.[14,15] It is exclusively expressed in the liver,[15] where it is present at the basolateral membrane of the hepatocyte.[16] Its membrane topology predicts 7 membrane spanning regions, with the N-terminus outside and the C-terminus inside the cell[14]. NTCP preferentially transports conjugated bile salts[17] and are transported in a Na^+-dependent manner via cotransport of Na^+. The Na^+/K^+ATPase maintains the Na^+gradient required for this transport. Recently, it has been shown for rat Ntcp that it is also able to transport sulfobromophthalein in a Na^+-dependent manner.[18] Sulfobromophthalein is an organic anion that is used as a diagnostic tool in the assessment of hepatic function. Thus, the substrate specificity of Ntcp is possibly not as narrow as previously considered.

The Na^+-independent uptake of bile salts is mediated by members belonging to the SLC21 or SLCO family (the Organic Anion-Transporting family), also known as the OATP's. Four OATP's are expressed in human hepatocytes: OATP-C, OATP8, OATP-B, and OATP-A. The first three are (possibly) involved in bile salt uptake. OATP-C (or SLC21A6) is exclusively expressed in liver[19,20] and was initially designated as the liver-specific organic anion transporter. This protein is the major Na^+-independent bile salt importer and also transports unconjugated bilirubin and its conjugates[21]. OATP-A (SLC21A3) is expressed in many tissues, including liver, but is most important in the blood-brain barrier and transports organic anions, bile salts, and bulky organic cations.[22] OATP-8 (SLC21A8) expression is, like that of OATP-C, restricted to the liver.[23] There is some controversy whether this protein transports bile salts or not.[24] OATP-B (SLC21A9) is expressed in various tissues, with the highest expression in the liver, but there is no experimental evidence that it transports bile salts.[24]

In rodents, three Oatp's are described to be involved in the Na^+-independent uptake of bile salts: (1) Oatp1 (Slc21a1) which is expressed in liver, kidney and choroid plexus[25]; (2) Oatp2 (Slc21a5) is also expressed in liver and kidney,[25,26] and (3) Oatp-4 (Slc21a10) which is expressed only in the liver.[27]

Under cholestatic conditions when serum bile salt levels are high, the expression of members of the ABCC family is induced to protect the hepatocyte by exporting bile salts.[28-31] From this protein family, Multidrug Resistance Proteins 1, 3, and 4 are located in the sinusoidal membrane and have been shown to be capable of transporting bile salts.[32-35] Concurrently, these proteins are proposed to function as an overflow efflux system for accumulated bile salts during cholestasis.

1.2.2 Canalicular bile salt transport

Transport from the hepatocyte across the canalicular membrane occurs against a steep concentration gradient. It is predominantly mediated by members of the Adenosine Triphosphate (ATP)-binding cassette (ABC) transporter superfamily. Contrary to the SLC transporters, ABC transporters are active transporters: they couple cellular energy to the transport of substrates across membranes. In the case of ABC transporters, the energy comes from the hydrolysis of ATP (Fig. 1-6). A typical ABC transporter protein consists of two halves connected by a linker-peptide (Fig. 1-7). Each half contains a so-called nucleotide-binding domain and a transmembrane domain. The nucleotide-binding domain, located at the cytoplasmic side of the membrane, is the

place for ATP binding and hydrolysis and is characterized by the presence of the conserved Walker A and B motifs and the signature or C motif.[36] The transmembrane domain consist of 6 to 11 membrane spanning α-helices and determines the substrate specificity of the protein.[37] Up to now, 48 human ABC transporters are known and divided in seven subfamilies, designated A to G (http://nutrigene.4t.com/humanabc.htm). Members of three of these subfamilies (B, C, and G) are involved in bile formation at the canalicular membrane.

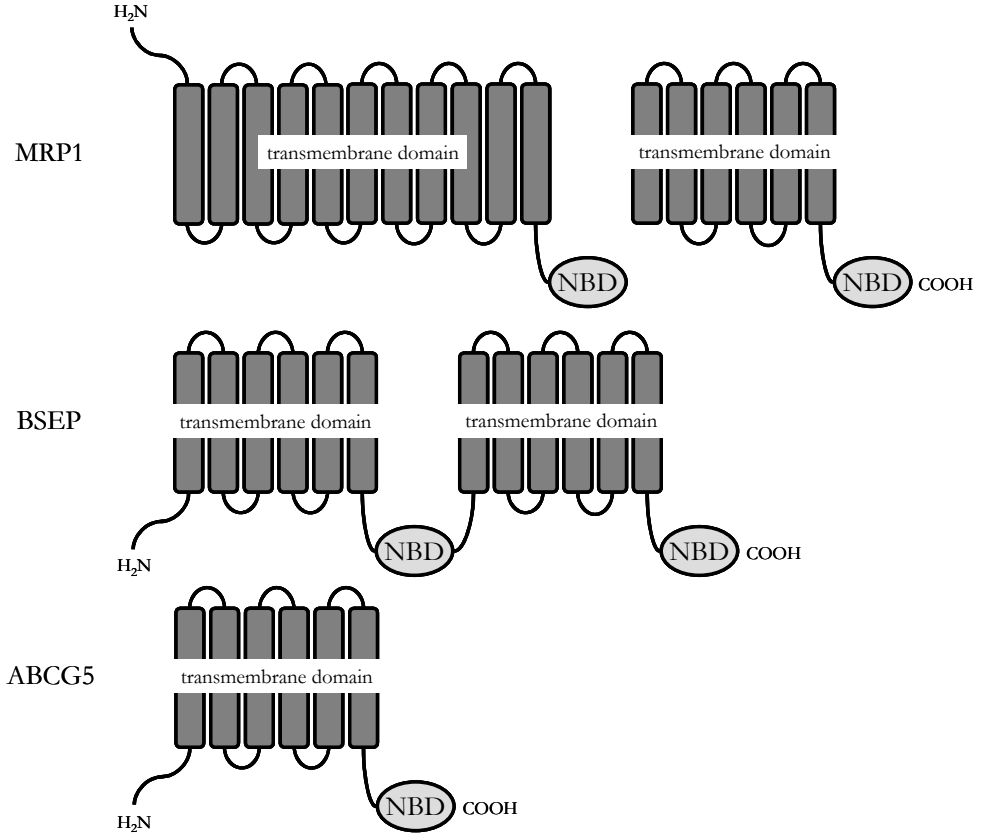

Fig. 1-7 Schematic structure of three different ABC transporters. Full transporters like MRP1 and BSEP contain two transmembrane domains and two NBD's. A half transporter like ABCG5 contains only one transmembrane domain and one NBD. The number of membrane spanning α-helices in the transmembrane domain may vary. For example, the N-terminally transmembrane domain of MRP1 contains 11 membrane-spanning α-helices. While the two transmembrane domains of BSEP contain 6 membrane spanning α-helices each.

Conjugated bile salts are excreted into bile by the Bile Salt Export Pump (BSEP in human, Bsep in rodent; *ABCB11*; previously known as sister of P-glycoprotein or SPGP).[38-40] BSEP belongs to the ABCB subfamily and is specifically expressed in the liver where it is the main determinant of bile salt-dependent bile flow. While BSEP transports monovalent bile salts across the canalicular membrane, the Multidrug

Resistance Related Protein 2 (MRP2 in human, Mrp2 in rodent; *ABCC2*) is able to mediate the transport of sulfated and glucorinated bile salts.[41,42] Besides the liver, it is also expressed in kidney and duodenum.[43] MRP2 mediates the transport of many other glutathione-, glucuronate-, and sulfate-conjugated anions, among which its primary function as transporter of conjugated bilirubin. In addition, it also transports anticancer drugs (hence its name) and reduced glutathione.[44-47] MRP2 is the main determinant of the bile salt-independent bile flow.

In response to canalicular bile salt secretion, phosphatidylcholine and cholesterol are released from the outer leaflet of the canalicular membrane and, together with bile salts, form micelles, thereby protecting the bile duct epithelium from the detergent-effects of bile salts. The Multidrug resistance 3/2 protein (MDR3 in human and Mdr2 in rodents; *ABCB4*) functions as a phospholipid flippase and mediates the transfer of phosphatidylcholine from the inner leaflet of the hepatocanalicular membrane to the outer leaflet.[48,49] It is predominantly expressed in the liver, but also in muscle, heart, and spleen.[50-52]

Surplus cholesterol is eliminated from the body via bile. On the one hand, through conversion of cholesterol to bile salts, and on the other hand, through direct transport from the hepatocyte to the bile. Two members belonging to the ABCG family, ABCG5 and ABCG8, are involved in biliary cholesterol transport.[53,54] These proteins are so-called half transporters: they contain only one transmembrane domain (consisting of 6 membrane-spanning α-helices and one NBD (Fig. 1-7). Half transporters need to form homodimers or heterodimers to be a functional transporter. ABCG5 and ABCG8 are expressed in liver and small intestine,[55] and need to be co-expressed for canalicular targeting and biliary cholesterol secretion.[56,57]

The best-known ABC transporter is a member of the ABCB subfamily: the Multidrug Resistance protein 1 or P-glycoprotein (MDR1 in human; mice and rats have two homologues: Mdr1a and Mdr1b). This transport protein was first designated as P-glycoprotein since its presence correlated with the altered drug permeability of mutant (drug resistant) Chinese hamster ovary cells.[58] Transfection of the MDR1 gene is sufficient to generate multidrug resistant cells.[59] MDR1 is expressed in the apical membranes of various tissues, such as the brain, kidney, intestine, placenta, and liver.[50,60,61] In normal human liver, MDR1 is lowly expressed and is involved in the elimination of bulky amphiphatic organic anions, including various drugs.

Besides the ABC-transporters, the canalicular membrane contains also a P-type ATPase, FIC1 or ATP8B1 that is involved in bile salt homeostasis. FIC1/ATP8B1 is expressed in liver (canalicular membrane of hepatocytes, apical membrane of cholangiocytes) and pancreas, but to a much higher level in the small intestine (brush border[62,63]). The precise function of FIC1 is not clear yet. It has been suggested that it is a translocator of amino-phospholipids.[63] The suggestion that FIC1 is involved in the transport of bile salts[62] has been contradicted by a recent study from Harris et al.[64] that suggests that FIC1 does not function as a bile salt transporter, so the precise function of this protein remains elusive. However, FIC1 does affect bile salt homeostasis. For example, loss of the murine *Atp8b1* gene[65] results in elevated serum bile salt levels. In addition, absence of human FIC1 is associated with reduced FXR, the bile salt sensor.[66,67]

1.2.3 Intracellular bile salt transporters

Bile salts taken up from the sinusoids have to be transported across the hepatocyte to the canalicular membrane. However, little is known about this intracellular transport. It is assumed that it is not vesicular-, but carrier-mediated, involving bile salt binding proteins.[68] In addition, membrane proteins are probably needed to transport bile salt intermediates during the multi-organellar processes of *de novo* bile salt synthesis. Enzymes involved in this process reside in the endoplasmic reticulum, mitochondria, cytoplasm, or peroxisomes, illustrating the need for transport systems. Little is known about the proteins involved in this process.

1.2.4 Other transporters involved in the enterohepatic circulation of bile salts

The enterohepatic circulation of bile salts requires that bile salts are reabsorbed from the intestinal lumen to the blood. The process takes place in the terminal ileum. Na+-dependent uptake of bile salts is accomplished by the ileal Apical Sodium-dependent Bile acid Transporter (ASBT) in the apical membrane of enterocytes.[69,70] In rodents, Oatp3 may add to the intestinal bile salt reabsorption through a Na+-independent process.[71] In enterocytes, bile salts bind to the Ileal Bile Acid-Binding Protein (IBABP)[72] and are transported across the basolateral membrane by the Ostα-Ostβ heterodimeric transporter.[73]

1.3 Inherited diseases associated with hepatic transport functions

In recent years, important progress has been made in our understanding of the function of hepatic transport proteins and their role in development of cholestasis. The elucidation of the genetic defect of various inherited forms of progressive cholestasis has been instrumental to show the crucial function of various transporters in bile salt homeostasis.

1.3.1 Progressive Familial Intrahepatic Cholestasis

A severe type of cholestatic liver disease is Progressive Familial Intrahepatic Cholestasis (PFIC). PFIC is inherited in an autosomal recessive manner, and becomes manifest during early childhood, and, when untreated, may result in liver failure. At least three subtypes have been recognized: PFIC 1, 2, and 3.

1.3.2 Progressive Familial Intrahepatic Cholestasis type 1

PFIC1 was first called Byler's disease, since the first PFIC patients described were descendants of the Amish Jacob Byler.[74,75] PFIC1 is characterized by jaundice, severe itching, high serum bile salt concentration, low biliary bile salt concentration, and low serum γ-glutamyl transpeptidase (γ-GT) level. It is caused by mutations in the *FIC1* or *ATP8B1* gene,[62,76] which has been mapped to chromosome 18q21-22[77]. To date, 54 different mutations in the *FIC1* gene have been documented to cause PFIC1.[78] One of these mutations, G308V, was commonly detected in patients with Byler's disease and was investigated further using a mouse model. Mice carrying this mutation display

a milder form of cholestasis compared to PFIC1 patients carrying the same mutation.[65]

Despite extensive *in vivo* and *in vitro* experiments using human and mouse FIC1/Fic1, it is still unclear what the molecular function of the FIC1 protein is. The clinical features of PFIC1 clearly indicate a role in bile formation. However, FIC1 itself seems not capable of transmembrane transport of bile salts. Recently, Chen et al.[66] showed a relationship between the loss of FIC1 and reduced activity of FXR. In addition, reduced FXR levels were also shown in a PFIC1 patient.[67] FXR is a bile salt-activated transcription factor (see below) controlling the expression of genes encoding proteins involved in bile salt biosynthesis and transmembrane transport. Reduced FXR levels would lead to reduced canalicular secretion of bile salts by the bile salt export pump (BSEP), ultimately resulting in the cholestasis. However, the mechanism that is responsible for the specific decrease in FXR levels in the absence of FIC1 remains to be elucidated.

1.3.3 Benign Recurrent Intrahepatic Cholestasis type 1

Mutations in the *ATP8B1* gene may also cause autosomal recessive inherited Benign Recurrent Intrahepatic Cholestasis type 1 or BRIC1.[62,79] While PFIC1 presents in early infancy, BRIC manifests itself during adolescence and early adulthood and is characterized by recurrent episodes of cholestasis. Deletions, frame shifts and nonsense mutation appear to lead to PFIC1, while missense mutations, which could result in suboptimal protein expression/activity, appear to lead to BRIC.[62] Intrahepatic cholestasis of pregnancy has also been associated with BRIC.[80]

1.3.4 Progressive Familial Intrahepatic Cholestasis type 2

Some non-Amish PFIC patients, who displayed characteristics of PFIC1 patients of the Amish kindred, could not be mapped to the same locus as FIC1.[81-83] In addition, morphological differences were found between these two groups of patients. PFIC1 patients have coarsely granular bile and bland intracanalicular cholestasis, while PFIC2 patients have amorphous bile and neonatal hepatitis.[82] The PFIC2 locus on chromosome 2q24[84] corresponds with the *ABCB11* gene, encoding the Bile Salt Export Pump.[85] *In vivo* experiments support these findings. Disruption of the murine *Abcb11* gene results only in mild intrahepatic cholestasis,[86] but leads to severe cholestasis when combined with cholate-feeding.[87] Several mutations have been described which lead to loss of ABCB11 expression.[88] The missense mutations lead to disturbed trafficking, decreased expression, and defective or decreased transport.[89-91] We have studied this for the aspartate to glycine mutation at position 482 that appears to result in a functional, but highly unstable and temperature-sensitive protein (chapter 3[91]). Mutations like the D482G are clinically very interesting, because they may pave the way for new therapies, aimed at the restoration of expression of mutant -but functional- proteins at the correct subcellular location.

1.3.5 Benign Recurrent Intrahepatic Cholestasis type 2

Recent studies show that the cause of benign recurrent intrahepatic cholestasis is not restricted to mutations in *FIC1* but may also result from mutations in *ABCB11* and other, yet unknown, genes. BRIC patients from 20 families were found to have no mutations in the *FIC1* gene. Subsequent sequencing of the *ABCB11* gene revealed that 11 patients (from 8 different families) contained mutations in this gene. Similar to BRIC1, BRIC2 is caused by missense mutations as well as one putative splice site mutation. Remarkably, one patient appeared to be homozygous for the E297G mutation that was previously reported to cause PFIC2. Thus, other genetic and/or environmental factors play a role in the symptoms associated with reduced BSEP function. This study also shows that there appears to be at least one more locus for autosomal recessive BRIC and PFIC with low serum GGT.[92] In addition, there are indications that single nucleotide polymorphisms in the *ABCB11* gene may be involved in intrahepatic cholestasis of pregnancy.[93]

1.3.6 Progressive Familial Intrahepatic Cholestasis type 3

The third subtype, PFIC3, is different from type 1 and 2 in that serum γ-GT level is high, biliary phospholipids are absent, serum bile salt level is normal, and the presence of extensive bile duct proliferation, cirrhosis and fibrosis.[94,95] The lack of biliary phospholipids pointed to an impaired MDR3 (*ABCB4*) function, the ABC-transporter that acts as a flippase for phospholipids. Indeed, PFIC3 patients lack MDR3 expression, due to mutations in the *MDR3* gene[94,95] *MDR3* mutations may also be a cause of intrahepatic cholestasis of pregnancy[95,96] and of cholesterol gallstone disease.[97,98]

1.3.7 Dubin-Johnson syndrome

The Dubin-Johnson syndrome is an autosomal recessive disorder with a relative benign character. This syndrome is not life threatening and no specific treatment is required. The syndrome was first described by Dubin, Johnson, Sprinz and Nelson, hence its name.[99] The only symptom of Dubin-Johnson syndrome is a benign form of jaundice throughout the patient's life. Patients with this disease lack the bilirubin transporter, MRP2 (*ABCC2*), due to mutations in the corresponding *MRP2* gene.[100,101] The secretion of conjugated bilirubin and other anionic conjugates to bile is impaired, but the transport of bile salts into bile is normal. The bilirubin(-conjugates) accumulate in liver and blood, causing the jaundice. The Dubin-Johnson syndrome is phenotypically similar to transport deficient (TR-) rats,[44] a strain that occurred naturally and lacks Mrp2 expression.[102] Several mutations in the *MRP2* gene have been described in Dubin-Johnson syndrome patients[101,103] and in (TR-) rats.[104] Some of the human *MRP2* mutations were studied in detail. Dubin-Johnson-causing mutations may result in impaired transport activity, deficient maturation and/or impaired sorting of the transporter.[105-107] The expression of another MRP isoform, MRP3 (*ABCC3*), is up-regulated when MRP2 function is impaired.[30,108] MRP3 is located in the basolateral membrane of the hepatocyt and this up-regulation may compensate for the impaired canalicular secretion of harmful compounds.

1.3.8 Sitosterolemia

Sitosterolemia is an autosomal recessive lipid disorder. It is characterized by accumulation of plant sterols (mainly sitosterol, hence the name) in blood and tissues, due to enhanced sterol absorption, reduced biliary sterol secretion and reduced cholesterol synthesis.[109,110] In 1974, this disorder was described by Bhattacharyya and Connor[111] as a lipid storage disease in two affected sisters. Other clinical features are cholesterol deposits (xanthomas) in skin, tendons, and coronary arteries, leading to premature atherosclerosis.[111-113] The disease is caused by mutations in the *ABCG5/G8* genes which are located at chromosome 2p21.[55,114,115] ABCG5 and ABCG8 are half transporters and function as a heterodimer. This explains why in PFIC1 patients, mutations in one of the genes is enough to result in Sitosterolemia. Studies performed with mice that are defective of either Abcg5 or Abcg8 confirmed these observations.[116,117] Indeed, loss of only Abcg5 is sufficient to result in symptoms of Sitosterolemia. In these mice, plant sterol concentrations in plasma are elevated.[116] Similarly, disruption of Abcg8 leads to loss of biliary cholesterol secretion, although biliary sitosterol secretion appeared to be preserved.[117]

1.4 Hepatic transporter regulation during acquired liver disease

The expression of transport proteins is also affected during acquired liver disease. These represent by far the more common causes of cholestasis. The hepatic responses to the different forms of acquired liver diseases generally serve to protect the hepatocyte against the toxic effects of accumulated bile salts and other bile compounds. Several acquired forms of liver disease can be distinguished: primary biliary cirrhosis, primary sclerosing cholangitis, inflammation-induced intrahepatic cholestasis (caused by sepsis, drugs, hormones, and alcohol), and extrahepatic biliary obstruction (caused by gallstones or tumors). Several animal models are used to study the expression of hepatic transporters during intrahepatic or extrahepatic cholestasis (for reviews: [2,118]). These models are detailed below.

1.4.1 Endotoxin-induced cholestasis

Sepsis is often associated with cholestasis. Lipopolysaccharide (LPS), a bacterial cell wall component, is the key mediator that is responsible for the development of cholestasis. It induces the release of inflammatory cytokines by the Kupffer cells in the liver, which, in turn, have major effects on protein expression in the hepatocyte.

The endotoxin-treated rodent is an animal model to study this type of acquired liver disease. In the endotoxemic rat, the bile salt transport to the bile is strongly impaired, leading to cholestasis.[119] This is associated with major effects on expression of bile salt uptake transporters in the hepatocyte. Ntcp and Oatp's are simultaneously down-regulated, while expression of the bile salt export system, Bsep, is maintained.[28,120-123] The multidrug resistance proteins are either maintained (Mdr1a and Mdr2) or elevated (Mdr1b and Mrp1).

Another characteristic of sepsis is jaundice, probably as a consequence of impaired bilirubin transport. This is indeed explained by the rodent-model: in the endotoxemic rat, the bilirubin transporter Mrp2 is down-regulated.[28,124]
The regulation of transporter expression may be different between human and rodent. For example, in both human and rat MRP2. Mrp2 expression is down-regulated, but in humans this is accomplished post-transcriptionally, while in rat it is regulated at the transcriptional level.[31,125,126] In addition, human BSEP expression is decreased in liver slices treated with LPS, while rat Bsep expression is unaltered after the same treatment.[126]

1.4.2 Estrogen-induced cholestasis

Cholestasis may also be associated with pregnancy or caused by oral contraceptives. The animal model for studying this type of cholestatic condition is 17α-ethinylestradiol (EE)-treatment of rodents. In EE-treated rats, both sinusoidal and canalicular bile salt transport systems were affected. The protein levels of Ntcp, Oatp1, Oatp2, Oatp4, Bsep, and Mrp2 were all decreased.[122,127,128] Mdr1a/b expression levels remain unchanged.[122] As in endotoxin-induced cholestasis, Na^+/K^+ATPase activity was also reduced.[129] Another important mechanism that effects efficient bile salt transport from the hepatocyte is the fact that the estrogen metabolite, estradiol-17beta-D-glucuronide, inhibits the Bsep transport activity directly. This may be an even more significant factor than alterations in expression levels.[40] Bsep has a very high transport capacity. Reduction in Bsep protein levels is therefore not necessarily associated with reduction of the canalicular bile salt secretion rate.[130]

1.4.3 Bile duct ligation

The animal models described above are models for intrahepatic cholestatic conditions. Ligation of the common bile duct is an animal model for extrahepatic obstructive cholestasis. The most common cause of extrahepatic obstructive cholestasis is the presence of gallstones. In extrahepatic obstructive cholestasis, bile ducts are blocked, leading to accumulation of bile in the liver, which in turn results in liver damage and eventually in liver failure.
Gap junctions disappear in bile duct-ligated rats,[131] leading to an altered physical barrier between portal blood and bile. Expression of Oatp1, Oatp4, Bsep, Mrp2, and Mrp6 are down-regulated,[29,121,122,127,132-134] while expression of Mdr1a, Mdr1b, Mrp3, and Mrp4 are up-regulated[29,30,122,135,136]. Mrp3 and Mrp4 are both able to transport sulfated bile salts and their up-regulation may serve as escape route for bile salts in order to protect the hepatocyte from toxic levels of bile salts.[29,33,136]

1.5 Transcriptional regulation of hepatic transport proteins

Expression of proteins involved in bile formation and homeostasis is tissue-specific with selective expression of liver-specific and intestine-specific transporters. In addition, the expression of these transporters is strictly regulated to fit the need to

maintain bile flow and formation. As discussed above, various disease conditions (sepsis, inflammation, obstruction by gallstones) and drugs/hormones effect regulation and function transporters. The mechanisms involved can be divided into (1) transcriptional regulation of genes encoding transporters (this section) and (2) various forms of regulation at the post-transcriptional (protein) level (section 1.6).

1.5.1 Tissue-specific expression of transporters

Tissue-specific expression is controlled by tissue-specific transcription factors. The liver is enriched in Hepatocyte Nuclear Factors (HNF's). Of this family of transcription factors, HNF1α and HNF4 are involved in transcription of hepatic transporters.[137,138] HNF1a is involved in transcriptional regulation of Ntcp, Oatp1, Oatp2, OATP-C, OATP8, and Oatp4.[139-141] Another hepatocyte nuclear factor, Hnf4α (NR2A1[142]) is important in hepatocyte differentiation and expression of transcription factors, including Hnf1α, as shown in mice.[143] Hnf4α null mice die during embryogensis,[143] but a study with conditional Hnf4α -/- mice shows that absence of the hepatic expression of this transcription factor, leads to reduced levels of Ntcp, Oatp1, and Mdr2, while that of Bsep was slightly increased.[144] Thus, hepatocyte nuclear factor 1α and 4α perform important roles in hepatic transporter expression.
The family of CCAAT/enhancer-binding proteins (C/EBP) are other important hepatic transcription factors (for review: [145]). C/EBPβ regulates transcription of the *MRP2* gene,[146] while an intact C/EBP element is necessary for maximal transactivation of the *Ntcp* promoter by the transcription factor Rarα.[147] *Ntcp* gene transcription is also regulated by Hex.[148] Hex is a homeobox-containing protein that is necessary for the normal development of the liver.[149] The Stimulating Protein 1 is an ubiquitously expressed transcription factor.[150] This factor is involved in the hepatic expression of MDR1, Mdr1b, and Mdr2.[151-153]

1.5.2 Modulation of transcription by NHR's

Nuclear hormone receptors (NHR's) are ligand-activated regulatory proteins and belong to the superfamily of receptors for steroid, retinoids, vitamin D and thyroid hormones (for reviews see: [154,155]). This superfamily consists of seven subfamilies, designated 0 to 6. They bind to DNA as monomers, homodimers or heterodimers. Members of this family share a common protein structure, which consists of a ligand-binding domain and a DNA-binding domain. The retinoic X receptor α (RXR, α: NR2B1, β: NR2B2) is the central dimerization partner for many of the family members. The ligands of nuclear hormone receptors are metabolites or drugs that regulate the transcription of genes involved in metabolism and/or transmembrane transport of the ligand itself. The vitamin A derivative, 9-cis retinoic acid is the natural ligand for RXR (NR2B1).[156,157] The fact that a vitamin A derivative functions as a crucial mediator of gene transcription emphasizes the importance of vitamin A for a wide range of physiological processes.
Several nuclear hormone receptors are involved in transcriptional regulation of hepatic transport proteins. The central factor in regulating bile salt homeostasis is the farnesoid X receptor (FXR; encoded by *NR1H4)*. FXR is expressed in liver, kidney,

intestine, and adrenal gland,[158] and is activated by bile salts.[6,159] Several hepatic transporters are directly regulated by this nuclear receptor together with RXR. Both human and rat *BSEP/Bsep* expression has been shown to be positively regulated by FXR.[160-162] Indeed, targeted disruption of *Fxr* in mice leads to decreased expression of *Bsep*. Cholate-feeding could not counteract this decrease.[163] The FXR/RXR heterodimer has been described as a permissive. This means that 9-cis retinoic acid activates the dimer and has an additive effect on the activation by bile salts.[164,165] However, we show in chapter 4 that for BSEP transcription this is not the case. In fact, 9-cis retinoic acid antagonized the effects of bile salts *BSEP* transcription *in vitro*. *In vivo* experiments show that Bsep transcription is especially sensitive to elevated bile salt levels in vitamin A deficient conditions. These findings described in chapter 4 emphasize the important role of vitamin A especially during cholestasis, when vitamin A absorption is compromised.

More and more genes are discovered that are regulated by FXR, including other transporter genes: *MRP2*,[166] *OATP8*,[167] and *MDR3*.[168] FXR also regulates the expression of another nuclear hormone receptor: *small heterodimer partner 1* or *SHP-1* (*NR0B2*),[163,169] which is expressed in liver, small intestine, spleen, adrenal gland, ovary, and testis.[170,171] SHP-1 is not a typical nuclear hormone receptor since it lacks a DNA-binding domain. SHP-1 performs its function through interaction with other receptors by which it inhibits transactivation mediated by these receptors.[170] For example, Shp-1 inhibits the binding of the Retinoic Acid Receptor (RAR or NR1B1) which is essential for expression of Ntcp[169,172] Shp-1 also inhibits the activity of the nuclear receptor liver receptor homologue 1 (LRH-1, NR5A2) and thereby represses the expression of the bile salt synthesis-enzyme cholesterol 7α-hydroxylase.[169] Rar is ubiquitously expressed[173] and is activated by all-trans retinoic acid.[174] Another target gene for RAR is *Mrp2*.[147]

The human *MRP2* gene promoter is transactivated by three nuclear receptors:[166] besides FXR, these are the Pregnane X Receptor (PXR; NR1I2) and the Constitutive Androstane Receptor (CAR; NR1I3). All three transcription factors activate *MRP2* transcription via an everted repeat separated by 8 bases. Thus, the response elements appear to be less factor-specific as previously assumed. Pxr is mainly expressed in liver and intestine, and its ligands are a variety of xenobiotics,[175-177] but also the more toxic bile salts like lithocholic acid.[178,179] Pxr also regulates transcription of *Oatp2*,[178,180] *MDR1*,[181] and *MRP3*[182]. CAR is predominantly expressed in liver and differs from the other nuclear hormone receptors because it transactivates the transcription of target genes in a constitutive manner, i.e. without binding of a ligand, hence the name constitutive androstane receptor.[183-185] However, a number of CAR activators, including phenobarbital, have been identified to modulate CAR regulation.[186] Therefore, CAR may be considered to be a xenobiotic sensor like PXR.[187] Murine *Mdr2* expression is regulated by the peroxisome proliferator-activated receptor alpha (PPARα; NR1C1).[188] It is expressed mainly in liver, kidney and heart,[189,190] and its ligands include fatty acids and fibrates.[191,192] PPARα interacts with another nuclear receptor, liver x receptor alpha or LXRα (NR1H3) in a yeast two-hybrid system and inhibits DNA-binding of PPARα /RXRα heterodimers.[193] LXRα is ubiquitously

expressed[194] and has been identified as a receptor for the cholesterol metabolites, oxysterols.[195] Together with the other oxysterol receptor, LXRβ (NR1H2), LXRα transactivates *Abcg5* and *Abcg8* gene expression.[196]

1.5.3 Regulation of transcription factors during cholestasis

During cholestasis the protein expression of several hepatic transport proteins is altered. Some of these alterations are caused by down-regulation or up-regulation of the expression of transcription factors. Murine Fxr mRNA and activity is down-regulated upon LPS administration.[197] For example, Ntcp and Mrp2 are down-regulated during endotoxin-induced cholestasis.[28,120,124] Studies have shown that cytokines, released in response to endotoxin, suppressed the expression of the proteins[198] by reducing the formation of RARα/RXRα complexes.[147] An important survival pathway for cells during cholestasis is represented by Nuclear Factor kappa B (NFκB). This transcription factor is activated by cytokines.[199] NFκB regulates the transcription of many genes.[199] These genes encode proteins that are involved in so-called anti-apoptotic pathways. NFκB induction due to inflammatory cholestasis may explain the increased expression of Mdr1 during cholestasis, since NFκB is involved directly in the transcription of *Mdr1b*.[200] During estrogen-induced cholestasis, down-regulation of the expression of Ntcp and Oatp's may be attributed to a reduced DNA-binding activity of Hnf1, C/EBP and Pxr.[128]

1.6 Post-translational regulation of transport proteins

The capacity for transport across the hepatic and intestinal epithelial cells is not only regulated by transcriptional processes, which primarily determine the protein levels. Especially hepatocytes may store significant amounts of transporter proteins in cytoplasmic pools. These cytoplasmic pools may be rapidly targeted to the plasma membrane to increase the transport capacity when needed. This may occur, for instance, immediately after a meal when there is a high demand for bile salt to aid in digestion. Besides acting as FXR-activators, bile salts are also involved in the short-term regulation of the bile salt transport capacity across the canalicular membrane. Taurocholate stimulate bile secretion by translocation of Bsep, but also Mdr1a/b, Mdr2, and Mrp2 from intracellular pools to the canalicular membrane.[201,202] Similar effects are described for dibutyryl-cyclic AMP, a synthetic second messenger. Cyclic-AMP also stimulates recruitment of Ntcp to the basolateral membrane.[203] The bile salt tauroursodeoxycholate is used in treatment of certain cholestatic conditions because of its known choleretic actions.[204] This bile salt activates the PKC and/or MAPK pathways, thereby enhancing the insertion of Bsep[205] and Mrp2[206] into the canalicular membrane.

Compounds may also directly inhibit the transport of endogenous substrates, thereby limiting the transport capacity. This is one of the underlying causes of drug-induced (hepato)toxicity. For instance, cholestasis may develop during treatment with cyclosporin A, rifamycin SV, rifampicin or glibenclamide. These drugs have been shown to directly inhibit the transport activity of BSEP.[207-209] Such drugs may either

selectively inhibit one transporter or have effects on other ABC-transporter family members as well.

1.7 Concluding remarks

Cholestasis has various causes, for instance genetic mutations, sepsis, gallstones or drugs to name a few. But the primary clinical manifestations are the same: jaundice, pruritus, and in the end liver damage. A common cellular phenomenon in cholestatic diseases is dysfunction of transport proteins in bile formation. In the last decade much progress has been made in understanding the function and regulation of hepatic transport proteins. The knowledge gathered gives insight in the mechanism of bile formation in health and disease, and may help to further develop successful therapies for cholestatic diseases.

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Chapter 2

A progressive familial intrahepatic cholestasis type 2 mutation causes an unstable, temperature-sensitive bile salt export pump

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2.1 Abstract

Background/Aims: Progressive familial intrahepatic cholestasis type 2 (PFIC-2) patients have a defect in the hepatocanalicular bile salt secretion. The disease is caused by mutations in the bile salt export pump (BSEP). Ten different missense mutations have been described. In this study, we analysed the effect of the D482G PFIC-2 mutation on BSEP function.

Methods: Adenosine triphosphatase (ATPase) and taurocholate transport assays were performed with full-length mouse Bsep (mBsep) with and without the D482G mutation. The effect on expression and subcellular sorting was studied in HepG2 cells, stably expressing enhanced green fluorescent protein (EGFP)-tagged mBsep proteins.

Results: The D482G mutation did not significantly affect the taurocholate transport activity of mBsep, even though the bile salt-inducible ATPase activity of the mutant protein was slightly reduced. Protein expression and canalicular sorting were strongly affected by the D482G mutation. Mutant EGFP-mBsep protein was only partly glycosylated and detected in both the canalicular membrane and the cytoplasm. At 30°C, the mutant mRNA and protein levels were strongly increased, and the protein was predominantly glycosylated and efficiently targeted to the canalicular membrane.

Conclusions: These data suggest that PFIC-2 patients with the D482G mutation express a functional, but highly unstable, temperature-sensitive bile salt export pump.

2.2 Introduction

Bile salts are the main solutes in bile. They are produced from cholesterol in the hepatocyte and are secreted across the canalicular membrane in an adenosine triphosphate (ATP)-dependent manner.[1-3] They are transported from the hepatocyte to bile by the bile salt export pump (BSEP).[4] BSEP is an ATP-binding cassette transporter belonging to the ATP-binding cassette (ABC) subfamily B. It consists of two intracellular nucleotide-binding domains (NBD) and twelve transmembrane domains in two homologous halves, connected by a linker peptide. The NBD's are highly conserved and contain the Walker A and B motifs[5] that are involved in the binding and successive hydrolysis of ATP. The NBD's are located at the cytoplasmic face of the membrane and couple the hydrolysis of ATP to substrate translocation across the membrane. The membrane spanning domains are thought to determine the substrate specificity of transport.[6]

Progressive familial intrahepatic cholestasis (PFIC) is an inherited autosomal recessive liver disease mainly occurring in infants and children. Without treatment this leads to cirrhosis and death from liver failure.[7] At least three different forms of PFIC can be distinguished. PFIC type 3 is caused by mutations in the canalicular phospholipid transporter, multidrug resistance protein 3.[8] Consequently, bile of these patients does not contain phospholipids. PFIC type 1 and 2 show low concentrations of biliary bile salts. PFIC type 1 is caused by mutations in the *FIC1* gene encoding a P-type ATPase with unknown function.[9] PFIC type 2 (PFIC-2) is caused by mutations in *BSEP*.[10,11]. Several PFIC-2 related mutations have been described. Eight mutations predicted premature truncation of the protein; the remaining (10) mutations are missense changes. We investigated the effects of the PFIC-2 related missense mutation D482G, an aspartate to glycine change at position 482,[10] occurring in the first nucleotide-binding domain of *BSEP*, on the adenosine triphosphatase (ATPase) activity, transport activity, sorting, and expression of the protein. Our results show that this mutation does not cause protein malfunction nor does it block the sorting of the protein to the canalicular membrane. In fact, the D482G mutation causes a temperature-sensitive reduction of BSEP protein expression, probably caused by reduced protein stability.

2.3 Materials and Methods

Stable cell lines and culture conditions

Standard culture conditions for the human hepatoma cell line HepG2 and derivatives have been described before.[12] Ten μmol/l dibutyryl-adenosine 3',5'-cylcic monophosphate (Roche Diagnostics GmbH, Mannheim, Germany) was added to the culture medium to stimulate the formation of bile canalicular vacuoles (BCV's) by HepG2 cells. HepG2 and derivatives were grown at 30°C to increase enhanced green fluorescent protein (EGFP)-mBsep mRNA and protein expression. HepG2 cells were transfected with pEGFP-C1-mBsep$^{[WT]}$ or pEGFP-C1-mBsep$^{[D482G]}$ using the calcium phosphate precipitation method[12] and geneticin-resistant clones were selected.

Molecular biological techniques

Escherichia coli Top 10 (Invitrogen BV, Breda, the Netherlands) was used for propagation and amplification of plasmid DNA. Recombinant DNA procedures were performed as described.[13] Plasmid DNA was isolated using the EndoFree® Plasmid Maxi Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

For heterologous expression of the full-length mouse Bsep (*mBsep*) gene in Sf21 insect cells, an *Eco*RI fragment encoding the full-length *mBsep* open reading frame was excised from MSCV-SPGP-IRIS-GFP[14] and inserted into *Eco*RI-digested pFASTBAC1 (Invitrogen BV, Breda, the Netherlands). For expression of EGFP-tagged mBsep in HepG2 cells, full-length *mBsep* was amplified by polymerase chain reaction (PCR) using primers mBsep-start and mBsep-stop digested with *Hin*dIII and *Kpn*I and inserted into pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA) digested with the same enzymes. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to introduce the D482G mutation in pFASTBAC1-mBsep and pEGFP-C1-mBsep (primers used: mD482Gsense/antisense). All DNA constructs were confirmed by DNA sequencing. Details about the primers used for cloning and site-directed mutagenesis are available from the authors.

Production of mouse Bsep-enriched membrane vesicles

Sf21 insect cells were cultured at 27°C in Insect-XPRESS™ medium (Biowhittaker, Verviers, Belgium), supplemented with 5% (vol/vol) fetal bovine serum, 5 mg/ml penicillin G, 5 mg/ml streptomycin, and 10 mg/m neomycin (Invitrogen BV, Breda, the Netherlands). Recombinant mBsep bacmids and baculoviruses were generated using the Bac-to-Bac Baculovirus expression system, according to the manufacturer's instructions (Invitrogen BV, Breda, the Netherlands). The virus titer, culture time and multiplicity of infection were optimised to obtain comparable amounts of wild type and mutant mBsep proteins. Recombinant insect cells were collected and membrane vesicles were isolated as described.[15] The membrane vesicles were snap frozen in liquid nitrogen and stored at –80°C until further use.

ATPase activity

The ATPase activity of recombinant mouse Bsep was determined by a colorimetric assay essentially as described,[16] except that 2 mmol/l EGTA, 2 mmol/l dithiothreitol, 5 mmol/l sodium azide, and/or 200 μmol/l orthovanadate (inhibitor of ATPase activity)/30 μmol/l taurocholate were also added to the ATPase reaction mixture. The ATPase activities were calculated by the difference in nmol Pi produced between 10 and 20 min.

Bile salt transport assay

Transport of 0.5 μmol/l [^{3}H]-labeled taurocholate (TCA, Perkin Elmer Life Sciences, Zaventem, Belgium) was measured by a rapid filtration technique, as described.[17] In

control experiments, ATP was replaced by adenosine β,γ-methylene triphosphate (AMP-PCP, Roche Diagnostics GmbH, Mannheim, Germany).

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated as described.[12] Messenger RNA (mRNA) levels of *EGFP-mBsep* and ribosomal *18S* RNA levels were quantified using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA). Real-time RT-PCR conditions were as described.[18] Primers and probes used for *18S*, *mBsep*, and *EGFP* are available from the authors.

Western blot Analysis

Sf21 membrane fractions and total HepG2 cell lysates were separated by 7.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)[19] and analysed by Western blotting.[20] Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as standard. Polyclonal antibodies used, were raised against GFP (gift from Prof. dr. W.-H. Kunau, Bochum, Germany) and BSEP (k12[21]). Mouse anti-rabbit alkaline phosphatase (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and bromo-chloroindolyl phosphate/nitro blue tetrazolium (Roche Diagnostics GmbH, Mannheim, Germany) were used for detection according to manufacturer's protocols. Protein expression was quantified by scanning the blots using an Image Master VDS system (Pharmacia Biotech, Uppsala, Sweden).

Deglycosylation assay

For protein deglycosylation, total HepG2 protein extracts were incubated with peptide N-glycosidase F (PNGaseF, New Engeland Biolabs, Beverly, MA) according to the manufacturer's instructions.

Confocal laser scanning microscopy

For microscopical analyses, cells were cultured on coverslips and fixed using 4% paraformaldehyde. For immunofluorescence, fixed cells were treated with 1% Triton X-100 and stained for multidrug resistance-related protein 2 (MRP2, canalicular marker protein) using the monoclonal antibody M2-III6 (Alexis Biochemicals, Lausen, Switzerland), with the corresponding second antibody Alexa fluor 568 (Alexis Biochemicals, Lausen, Switzerland). Images were taken with a confocal laser scanning microscope (TCS 4D; Leica, Heidelberg, Germany) equipped with an argon/krypton laser and coupled to a Leitz DM IRB (Leica, Heidelberg, Germany) inverted microscope.

2.4 Results

Single amino acid changes in *BSEP* that cause PFIC-2 may result in inactive, missorted, and/or unstable BSEP protein. The D482G mutation is present in the first nucleotide-binding domain of BSEP, which is highly conserved between species (Fig.

2-1). Due to experimental difficulties with expression of full-length human BSEP,[22,23] we introduced this mutation in full-length mouse Bsep.

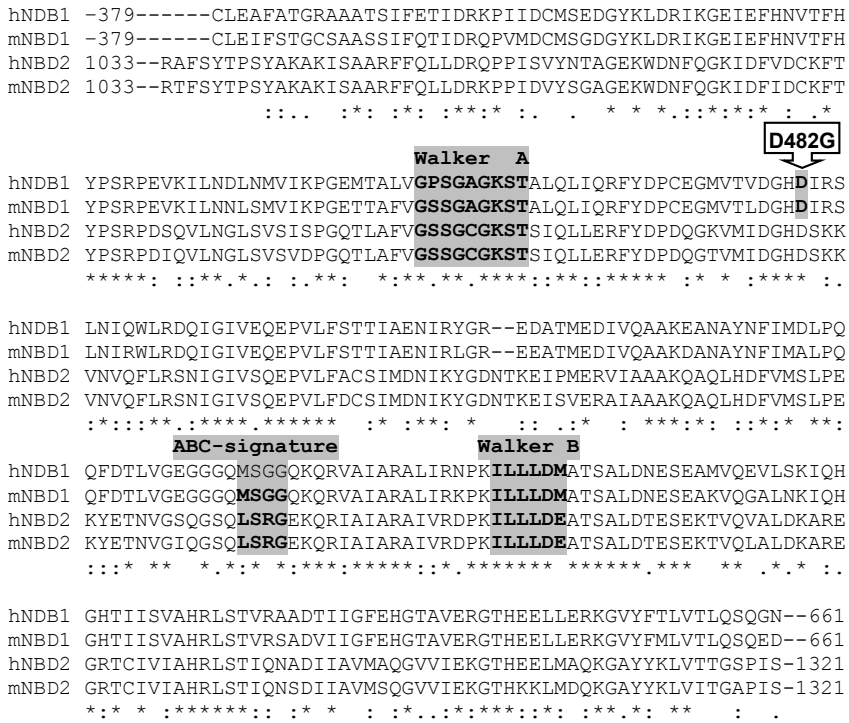

Fig. 2-1 Sequence alignment of the human and mouse BSEP NBD's. The amino acid sequences of human and mouse BSEP are highly conserved. The Walker A and B motifs and the ABC signature are shown in bold. The aspartate (D) at position 482 is conserved between human and mouse BSEP. Note that it is also conserved in the second NBD. ClustalW was used to align the NBD's of human and mouse BSEP.

The mBsep[D482G] protein is still functional as a bile salt transporter

Wild type and D482G mutant mBsep were expressed at comparable levels in Sf21 insect cells (Fig. 2-2A, insert). Membrane preparations containing mBsep[WT] (white bars) showed similar ATPase activities as control membranes (grey bars; 12.2 ± 0.85 versus 15.8 ± 0.95 nmol Pi·min^{-1}·mg^{-1} protein; Fig. 2-2a). However, upon addition of 30 µmol/l taurocholate (TCA), the ATPase activities of the mBsep[WT] vesicles were enhanced (41.8 ± 2.14 nmol Pi·min^{-1}·mg^{-1} protein), which was not observed with the control membranes (16.4 ± 1.47 nmol Pi·min^{-1}·mg^{-1} protein). Addition of orthovanadate, an inhibitor of the ABC-transporter ATPase activity, fully inhibits the TCA-dependent ATPase activity in the mBsep-containing membranes. Similar results were obtained with membrane fractions containing mBsep[D482G] (black bars). The constitutive ATPase activity of these membranes was 13.7 ± 1.47 nmol Pi·min^{-1}·mg^{-1} protein and the TCA-dependent ATPase activity (29.1 ± 1.45 nmol Pi·min^{-1}·mg^{-1}

protein) was blocked by orthovanadate. This shows that the ATPase activity of both WT and D482G mBsep are stimulated by the substrates of the transporter. The bile salt transport activity of the wild type and mutant mBsep is shown in Fig. 2-2b. Both mBsep$^{[WT]}$- and mBsep$^{[D482G]}$-vesicles show similar TCA uptake rates (32.7 ± 1.5 versus 30.5 ± 3.3 pmol TCA ·min^{-1} ·mg^{-1} protein).

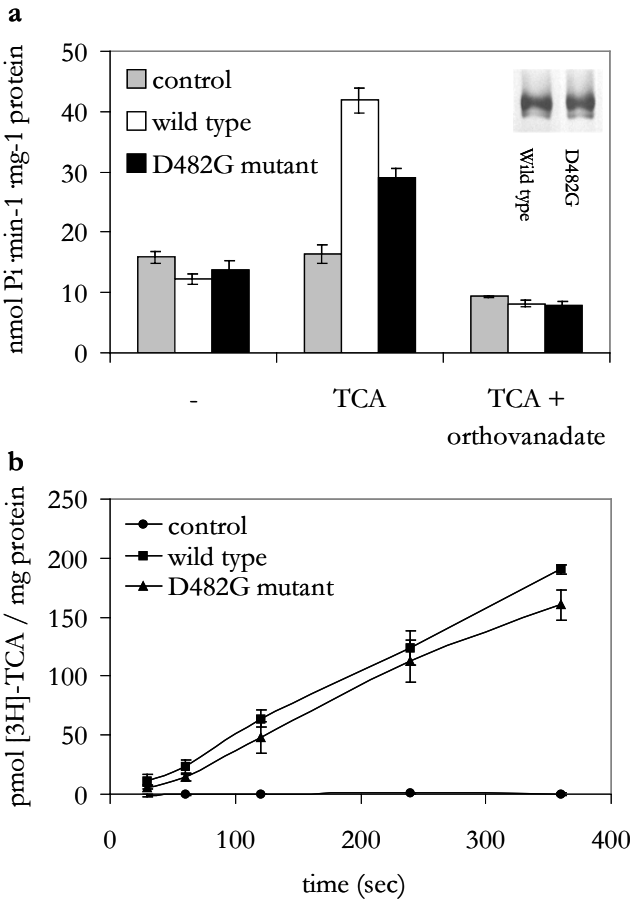

Fig. 2-2 The D482G mutation does not block the ATPase and transport activity of mBsep. (a) Membrane vesicles containing comparable levels of wild type or D482G mutant mBsep (insert) are subjected to an ATPase assay in the absence or presence of 30 μM TCA and 200 μM orthovanadate as described in Materials and Methods. The data are presented as mean ± SD of triplicate determinations in nmol liberated Pi ·min-1 ·mg-1 total protein. Membrane vesicles from uninfected insect cells served as control. (b) The same membrane vesicles were subjected to a transport assay using [3H]-labelled TCA. Samples were taken at the indicated time points. The data are presented as mean ± SD of triplicate determinations in pmol TCA/mg total protein. Membrane vesicles from uninfected insect cells served as control.

The EGFP-mBsep[D482G] is inefficiently targeted to the canalicular membrane in HepG2 cells

To study the protein stability and subcellular sorting of wild type and mutant mBsep, we generated stable HepG2 cell lines that express these proteins, N-terminally tagged to the EGFP. Geneticin-resistant clones were screened for the presence of the EGFP-signal by confocal laser scanning microscopy.

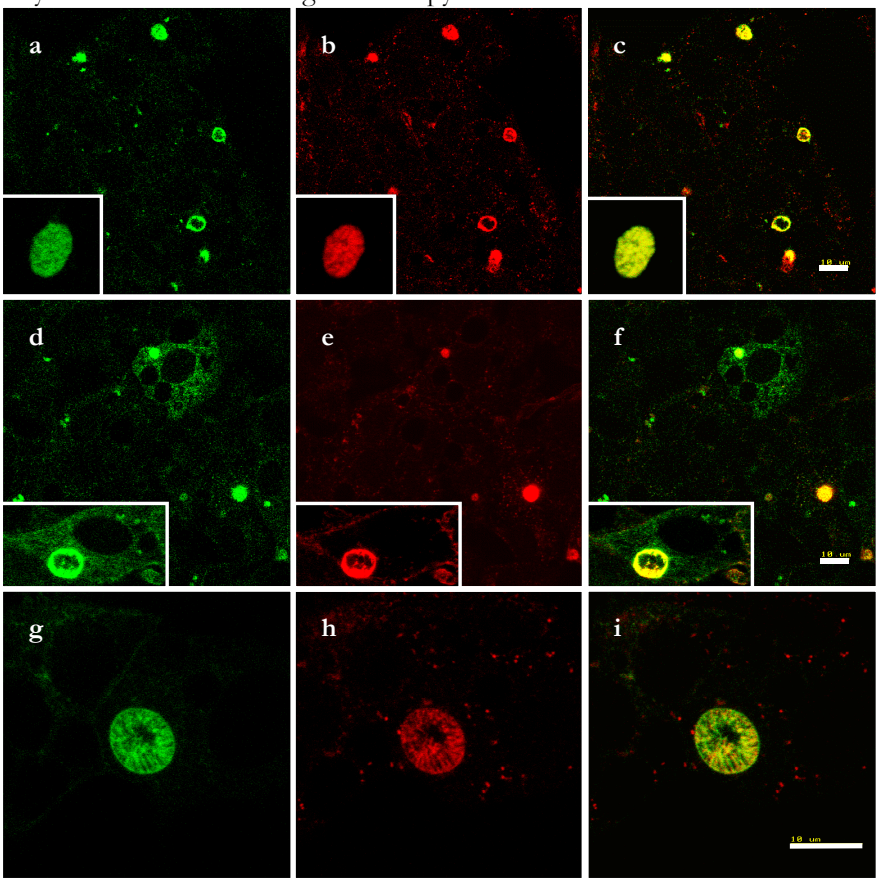

Fig. 2-3 Efficient sorting of the D482G mutant mBsep in HepG2 cells is temperature-sensitive. EGFP signal (a,d,g) is shown in green, MRP2 (canalicular) staining (b,e,h) is shown in red, and the combined images (EGFP + MRP2) are shown in c, f and i. Yellow staining indicates overlap of both signals. At 37°C, both EGFP-tagged wild type (a,c) and D482G mutant (d,f) mBsep sort to the BCV. However, significant amounts of the D482G mutant mBsep are detected intracellular (d,f). MRP2 staining (b and e) was used as marker for the BCV's. Both proteins co-localise at the canalicular membrane (c and f) in polarised HepG2 cells. When cells were grown at 30°C, the D482G mutant mBsep was solely observed in the BCV's. g, EGFP; h, MRP2 and i, merged images. Insets show higher magnifications of single BCV's (a-c) or single polarized cells (d-f). Bar = 10 µm.

As is observed with normal HepG2 cells in culture, up to 20-30% of these cells acquire a polarised phenotype, forming BCV's.[24] These BCV's contain canalicular

proteins, as shown by MRP2 staining (Figs. 2-3b, e, and h). EGFP-mBsep[WT] co-localised with MRP2 in BCV's of HepG2 cells (Figs. 2-3a-c), indicating that the N-terminal EGFP-tag does not interfere with mBsep targeting. The EGFP-mBsep[D482G] mutant protein was detected in the BCV's but, in addition, a considerable amount of protein was retained in the cytoplasm (Figs. 2-3d-f).

The D482G mutation results in unstable and immature mBsep protein

Next, we analysed the correlation between the mRNA and protein level of cells stably expressing EGFP-mBsep[WT] and EGFP-mBsep[D482G]. Western Blot analysis using the GFP antibody showed that EGFP-mBsep[WT] can be easily detected in total cell lysates as a protein of approximately 190 kDa (Fig. 2-4, lane 2). However, the EGFP-mBsep[D482G] signal at this position is hardly detectable (Fig. 2-4, lane 1). In stead, a GFP-specific protein band is present at approximately 160 kDa in these protein extracts. This might be either a degradation product or, alternatively, improperly glycosylated EGFP-mBsep protein. Treatment of EGFP-mBsep[WT]-containing extracts with PNGaseF resulted in a molecular weight shift to approximately 160 kDa (Fig. 2-4, lane 3). PNGaseF-treatment of EGFP-mBsep[D482G] resulted in the disappearance of the minor 190 kDa band. The 160 kDa band remained unchanged (not shown). This shows that the 160 kDa EGFP-mBsep[D482G] protein band is full-length, but improperly glycosylated protein. Moreover, the specific amount of EGFP-mBsep[D482G] protein appeared lower than the EGFP-mBsep[WT] protein. Therefore, we determined the specific mRNA levels of *EGFP-mBsep[WT]* and *EGFP-mBsep[D482G]* in the corresponding cell lines. In contrast to the difference in protein level, the relative amount of *EGFP-mBsep[D482G]* was 4.5-fold higher compared to the *EGFP-mBsep[WT]* mRNA level (Fig. 2-5a). These data show that high mRNA levels for *EGFP-mBsep[D482G]* do not give rise to similarly high protein levels, relative to the results obtained for *EGFP-mBsep[WT]*.

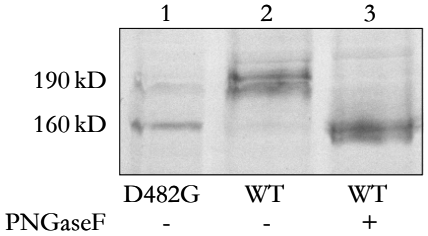

Fig. 2-4 The D482G mutation leads to low levels and incompletely glycosylated mBsep protein. Western blot analysis using a GFP antibody of approximately 160 μg of total protein extracts from EGFP-mBsep[D482G] HepG2 cells (lane 1), and EGFP-mBsep[WT] cells (lanes 2 and 3). In lane 3, the extract was treated with PNGase F.

2.4.1 Low temperature stabilizes EGFP-mBsep

Several examples exist of missense mutations that give rise to unstable temperature-sensitive proteins. The best-studied example is the ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) protein.[25] Therefore, we

cultured both the EGFP-mBsep[WT] and EGFP-mBsep[D482G] HepG2 cells at 30°C and determined the specific mRNA and protein levels. Surprisingly, we found that the relative mRNA levels for both EGFP-mBsep[WT] and EGFP-mBsep[D482G] increased approximately 12-fold in cells grown at 30°C compared to cells grown at 37°C (Fig. 2-5a). However, the 4.5-fold difference between EGFP-mBsep[D482G] and EGFP-mBsep[WT] remained despite the overall higher mRNA levels. In cells grown at 30°C, the protein level of both EGFP-mBsep[WT] and EGFP-mBsep[D482G] increased significantly when compared to cells at 37°C (Fig. 2-5b). However, the EGFP-mBsep[D482G] showed a much stronger increase in protein level than the EGFP-mBsep[WT]. Moreover, at 30°C the EGFP-mBsep[D482G] appeared predominantly in fully glycosylated form. Under these conditions, the EGFP-mBsep[D482G] protein level was approximately 5-fold higher than EGFP-mBsep[WT] as determined by densitometry. This is in good agreement with the difference in the mRNA levels, implying that both proteins show similar overall protein stability at 30°C. Confocal laser scanning microscopy revealed that at 30°C, the EGFP-mBsep[D482G] protein was efficiently targeted to the BCV's (Figs. 2-3g-i).

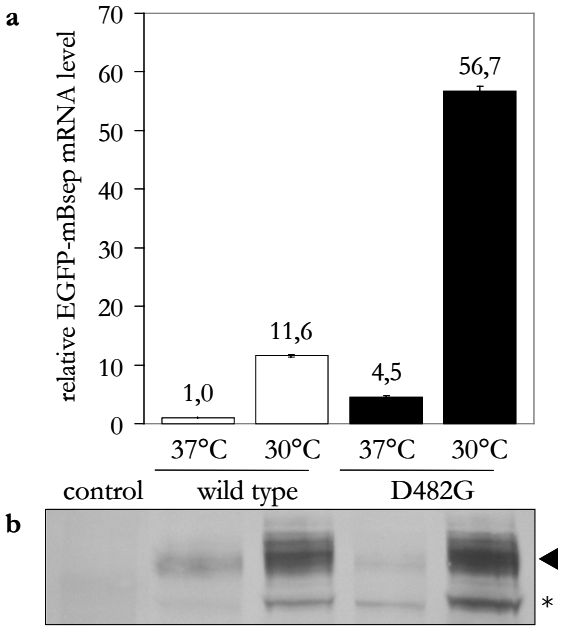

Fig. 2-5 The protein level and glycosylation of the mutant BSEP is temperature-sensitive. (a) Total RNA from HepG2 cells expressing wild type of mutant mBsep, was isolated and subjected to quantitative real-time RT-PCR. Relative *EGFP-mBsep* mRNA levels were obtained by normalising to the ribosomal *18S* RNA content. (b) Total protein lysates from HepG2 cells expressing the wild type or D482G mutant mBsep were isolated. Cells were cultured at 37 or 30°C. The parental HepG2 cells grown at 37°C were used as control. SDS-PAGE and Western blotting was performed on 100 μg total protein using a GFP antibody. Unglycosylated (*) and glycosylated (◀) EGFP-mBSEP are indicated.

2.5 Discussion

In this study, we analysed the effect of the PFIC-2 mutation, D482G, on Bsep function and intracellular expression. We found that this mutation does not abolish the ATPase activity nor the taurocholate transport activity. However, at physiological temperatures, the stability, canalicular sorting, and glycosylation of the mutant protein are disturbed. The D482G-mutant Bsep protein appears to be temperature-sensitive. At 30°C, high levels of normally glycosylated, and correctly sorted Bsep$^{[D482G]}$ were detected. In this study, we also report the remarkable finding that in transfected HepG2 cells grown at 30°C, the mRNA levels of the heterologous expressed genes are over 10-fold higher compared to the same cells grown at 37°C. Concurrently, the corresponding proteins show significantly increased levels in cells grown at 30°C.

Since the D482G mutation is present in the first NBD of BSEP, we first tested its effect on the ATPase activity. ABC transporters, like BSEP, possess an ATPase activity that is highly stimulated by the transported substrates. Concurrently, we found that the ATPase activity of full-length mouse Bsep was strongly induced by TCA. The ATPase activity of the mBsep$^{[D482G]}$-protein was also induced by TCA, be it to a lower level than the WT protein. However, the reduced bile salt activation of the ATPase activity did not affect the TCA transport kinetics of the mutant protein. Taken together, these results show that the mBsep$^{[D482G]}$ is a functional bile salt transporter with biochemical characteristics comparable to the wild type protein.

To study the effect of the D482G mutation on intracellular sorting and protein stability, we constructed stable HepG2 cell lines, expressing mBsep$^{[WT]}$ or mBsep$^{[D482G]}$ N-terminally tagged with EGFP. Detectable amounts of EGFP-mBsep$^{[D482G]}$ protein were only observed in clones with significantly (5-fold) higher mRNA levels as compared to EGFP-mBsep$^{[WT]}$. In addition, the mutant protein was improperly glycosylated suggesting accumulation of the protein in the endoplasmic reticulum. This was confirmed by confocal laser scanning microscopy analyses, in which significant amounts of the EGFP-mBsep$^{[D482G]}$ protein were detected in a fine reticulum-like pattern in the cytoplasm. In contrast, the EGFP-mBsep$^{[WT]}$ protein was observed in the BCV's and not in the cytoplasm. Collectively, our findings suggest that the D482G mutation results in low BSEP protein levels and that the mutant protein is improperly glycosylated and targeted at physiological conditions.

A comparable effect is described for the ΔF508 mutation in the ABC transporter CFTR. This mutation is the most common genetic cause of cystic fibrosis. At the molecular level, this results in increased turnover of an otherwise active CFTR. The processing and stability of this mutant protein has been shown to be temperature-sensitive.[25,26] Therefore, we cultured our stable cell lines at reduced (30°C) temperatures and found that the mRNA level for both EGFP-mBsep$^{[WT]}$ and EGFP-mBsep$^{[D482G]}$ increased approximately 12-fold compared to the level in cells grown at 37°C. This strong temperature effect on transcript levels was specific for the heterologously expressed gene and was not observed for endogenous genes, such as human *MDR1* and *MRP1* (data not shown). Recently, a similar strong temperature-dependent increase in the mRNA level of the tetracycline-dependent transactivator has been reported.[27] This effect was found to be independent of the promoter used and the site of integration in the genomic DNA of chinese hamster ovary cells.

Therefore, it is most likely caused by increased mRNA stability. In our experiments, it was accompanied by strongly increased EGFP-mBsep[WT] protein levels and therefore may have important implications for the high level expression of heterologous (membrane) proteins in HepG2 cells and/or other mammalian cell types. At 30°C, both the mRNA and protein level of EGFP-mBsep[D482G] were approximately 5-fold higher than those for EGFP-mBsep[WT] mRNA level. This implies that both proteins show similar cellular stability at 30°C. At this low temperature, both proteins were efficiently glycosylated and sorted to the canalicular membrane.

Recently, Wang et al., 2002,[28] also reported on the molecular effect of PFIC-2 mutations on BSEP function. They found that the D482G mutation reduced the taurocholate transport activity of rat Bsep by approximately 50% and GFP-tagged rBsep[D482G] was found at the apical membrane and in the cytoplasm of Madin-Darby canine kidney (MDCK) cells. The correlation between GFP-rBsep[D482G] mRNA and protein levels was not determined in detail. Our results show little or no effect of the D482G mutation on the BSEP transport function, but a very strong effect on protein stability, maturation and/or turnover. Liver-specific protein quality control mechanism may be present in HepG2 cells but perhaps not -or to a lesser extent- in MDCK cells. Another difference between our studies and those of Wang et al., is the use of rat versus mouse Bsep. Since the proteins are highly similar to human BSEP and fully conserved at the positions of the PFIC-2 missense mutations, we feel that both are valid model proteins to study the molecular effect of this human disease. The deleterious effect of the D482G mutation could therefore be due to a combination of reduced protein stability, canalicular sorting and substrate transport defects. With the recent cloning and heterologous expression of the human *BSEP* gene in insect cells, these studies may be performed with the human BSEP in the near future.[22,23]

In conclusion, our data show that PFIC-2 patients with the D482G mutation express a functional, but highly unstable bile salt export pump. Our findings offer hopes for therapy: drugs that stabilise the protein may be able to restore the PFIC-2 defect.

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Chapter 3

Farnesoid x receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump

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3.1 Abstract

The Bile Salt Export Pump (BSEP or ABCB11) mediates the adenosine triphosphate-dependent transport of bile salts across the canalicular membrane of the hepatocyte. Mutations in the corresponding *ABCB11* gene cause progressive familial intrahepatic cholestasis type 2.

The aim of this study was to investigate the regulation of human *ABCB11* gene transcription by bile salts. First, a 1.7-kilobase human *ABCB11* promoter region was cloned. Sequence analysis for possible regulatory elements revealed a farnesoid X receptor responsive element (FXRE) at position -180. The farnesoid X receptor (FXR) functions as a heterodimer with the retinoid X receptor α (RXRα) and can be activated by the bile salt chenodeoxycholic acid (CDCA). Luciferase reporter gene assays revealed that the *ABCB11* promoter is positively controlled by FXR, RXRα and bile salts in a concentration-dependent manner. Mutation of the FXRE strongly represses the FXR-dependent induction. Second, endogenous *ABCB11* transcription regulation was studied in HepG2 cells, stably expressing the rat sodium-dependent taurocholate transporter (rNtcp). *ABCB11* expression was induced by adding bile salts to the culture medium, and this effect was maximized by combining it with cotransfection of rFxr and hRXRα. Reducing endogenous FXR levels using RNA interference fully repressed the bile salt-induced *ABCB11* expression. In conclusion, these results show that FXR is required for the bile salt-dependent transcriptional control of the human *ABCB11* gene and that the cellular amount of FXR is critical for the level of activation of *ABCB11* transcription.

3.2 Introduction

The formation of bile is an important function of the liver. Bile salts are necessary for the intestinal absorption of dietary fats and fat-soluble vitamins and the biliary elimination of surplus cholesterol and a variety of toxins. Bile salts are synthesized in the hepatocytes with cholesterol as the sole precursor. The secretion of bile components (bile salts, phospholipids) across the canalicular membrane of the hepatocyte to the bile duct is adenosine triphosphate (ATP)-dependent and is mainly performed by members of the ATP-binding cassette transporter superfamily. Impaired bile secretion results in cholestasis with accumulation of bile salts and other toxic bile components in hepatocytes and blood plasma.[1,2]

Progressive familial intrahepatic cholestasis comprises a number of inherited liver diseases of childhood that are characterized by cholestasis and jaundice leading to cirrhosis[3]. In patients with progressive familial intrahepatic cholestasis type 2, cholestasis is a result of impaired secretion of bile salts into canaliculi caused by mutations in the *ABCB11* gene.[4] The ABCB11 protein (previously named sister of P-glycoprotein or bile salt export pump) is a member of the P-glycoprotein subfamily and is the major canalicular bile salt transporter.[5]

To maintain bile salt homeostasis, both bile salt synthesis and hepatobiliary transport are strictly controlled processes. For instance, at increased bile salt concentrations in the blood, the hepatic uptake and bile salt biosynthesis are down-regulated, whereas canalicular transport into bile is up-regulated.[6] Recently, a number of bile salts have been shown to bind and strongly activate the nuclear hormone receptor farnesoid X receptor (FXR) at physiologic concentrations. [7,8,9] FXR is active in a heterodimer with the 9-cis retinoic acid receptor retinoid X receptor α (RXRα) and binds to an inverted repeat element with a 1-base pair spacing (IR-1) between the two 6-base pair half-sites.[10,11,12] Rat Fxr (rFxr) is expressed in liver, intestine, and kidney.[10]{Wang, Chen, et al. 1999 92 /id}{Wang, Chen, et al. 1999 92 /id}

FXR is now believed to be the major bile salt sensor that regulates the expression of key enzymes in the bile salt biosynthesis pathway and bile salt transporters. Bile salt-activated FXR up-regulates the intestinal bile acid-binding protein, a soluble protein that has been proposed to buffer intracellular bile salts, which may be involved in the transcellular transports of bile salts in enterocytes.[13] Conversely, it down-regulates the expression of cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting step in bile salt synthesis from cholesterol,[14] and the sodium-dependent taurocholate transporter in the sinusoidal membrane of the hepatocyte. Down-regulation occurs via the up-regulation of a transcriptional repressor, the small heterodimer partner 1.[15,16]

The central role of FXR in bile acid homeostasis is most elegantly shown by the comparative analysis of wild type and *Fxr*-null mice. These studies also show that Fxr and bile salts are involved in expression of the murine *Abcb11* gene, encoding the bile salt export pump.[17]

In this study, we investigated the transcriptional regulation of the human *ABCB11* gene by analyzing the role of its substrates, bile salts, and the nuclear receptors, FXR and RXRα. In a recent study, it was shown that the promoter region of the *ABCB11*

gene contains an FXR/RXRα responsive element and that the *ABCB11* promoter is transactivated by FXR/RXRα binding.[18] We show that FXR is required for the bile salt-induced up-regulation of human endogenous bile salt export pump expression in HepG2 cells, stably expressing the rat sodium-dependent taurocholate transporter (rNctp). Suppression of FXR expression in these cells using RNA interference inhibited the bile-salt dependent up-regulation of endogenous *ABCB11* by bile salts.

3.3 Experimental procedures

Bacterial strains and cell culture

Escherichia coli Top 10 (*endA1, recA1, hsd*R*MS, deo*R*, mcrA, lacZΔM15*, Invitrogen BV, Breda, the Netherlands) were routinely used for propagation of plasmid DNA. *E. coli* were grown in Luria Broth (Miller's modification) medium (Sigma-Aldrich, Germany), containing the appropriate selection antibiotic.

The human hepatoma cell line HepG2, and a stable derivative expressing rNtcp[19] were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air in Dulbecco's modified Eagle medium with GlutaMAX-1, 4500 mg/L D-glucose, sodium pyruvate, pyridoxine supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, 250 ng/mL fungizone and 250 μg/mL geneticin (for HepG2-rNtcp) (Invitrogen BV). The cell culture was passed twice a week.

For a time-course analysis of endogenous *ABCB11* messenger RNA (mRNA), HepG2 cells were incubated with 100 μmol/L chenodeoxycholic acid (CDCA, sodium salt; Calbiochem-Novabiochem, San Diego, CA) dissolved in phosphate-buffered saline (PBS) for different time points. Cells were harvested for RNA isolation and conventional reverse-transcription polymerase chain reaction (RT-PCR) at same cell densities.

Plasmids

Chromosomal DNA was isolated from a human blood sample using the QIAamp DNA Blood Kit (QIAGEN GmbH, Hilden, Germany). A 1,752-base pair DNA fragment containing the human *ABCB11* promoter (position -105 to -1857 relative to the translation initiation site) was amplified from chromosomal DNA using primers 5'-CAC ACT GCC CAG ATG TGT CT-3' and 5'-CCA ACC TCG GTT TTC ATC AT-3' and inserted into pGEM-T-Easy (Promega Corp., Madison, WI). The human *ABCB11* promoter was subsequently removed from this vector by digestion with *Msc*I (at –1778) and *Sal*I (multiple cloning site pGEM-T-Easy) and inserted into *Sma*I-*Xho*I digested pGL3-basic (Promega Corp.) upstream of the firefly luciferase reporter gene, resulting in pGL3-1778. A shorter promoter construct containing base pair -105 to base pair -277 (pGL3-277) was generated by PCR using pGL3-1778 as template and the primers 5'-CCC **GGT ACC** GGT TTC CCA AGC ACA CTC TG–3' and 5'-AGG A**AA GCT T**CC AAC CTC GGT TTT CAT CAT-3' and inserted into pGL3-basic, using the *Kpn*I and *Hind*III restriction sites (bold). Using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Ca), the proposed FXR responsive

element (FXRE) site of both pGL3-1778 and pGL3-277 were mutated using primers 5'-CTA TTT GCC TAA **TT**A TCA ATG T**AA** CTA AGG GCA GC-3' and 5'-GCT GCC CTT AG**T T**AC ATT GAT **AA**T TAG GCA AAT AG-3', generating pGL3-1778Mt and pGL3-277Mt (mutated base pairs in bold). All promoter constructs were checked by DNA sequencing.

Transient transfections

HepG2-rNtcp cells[19] were transfected by the calcium phosphate coprecipitation method.[20] Plasmid DNA was isolated and purified using the EndoFree Plasmid Maxi Kit (QIAGEN GmbH) according to the manufacturer's instructions.

HepG2-rNtcp cells were seeded in 6-well plates at a density of 3 x 10^5 cells per well on day 1. On day 2, the cells were transfected with a total amount of 4 μg plasmid DNA in 250 mmol/L $CaCl_2$ and 2 x HEPES buffered saline (274 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L D+glucose, 42 mmol/L HEPES, 1.6 mmol/L $Na_2HPO_4 \bullet 2H_2O$, pH 7.05. Various combinations of plasmids were used as specified in the figure legends. Concentrations of the specific plasmids used for promoter studies were 1 μg promoter construct (pGL3-derivatives), 100 ng expression vectors (pCMXrFxr, pSG5hRXRα, pCMV5) 50 ng RL-TK (transfection efficiency control; Promega Corp.), and pGEM-5 (Promega Corp.) as carrier DNA to increase the total amount of plasmid DNA to 4 μg. Concentrations of the specific plasmids used for mRNA analysis were 1 μg expression vectors (pCMXrFxr, pSG5hRXRα, pCMV5), and pGEM-5 as carrier DNA to increase the total amount of plasmid DNA to 4 μg. On day 3, approximately 20 hours after the transfection, medium was refreshed either containing 100 μmol/L CDCA dissolved in PBS or PBS alone. Cells were harvested after 24 hours for determination of luciferase activity and after 48 hours for total RNA isolation. Luciferase activity was determined by the Dual-Luciferase Reporter Assay System (Promega Corp.) as described by the manufacturer's protocol using an Anthos LUCY1 Luminometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) with a 10-second counting window. Except for experiments including pSG5hRXRα, firefly luciferase activities were corrected for transfection efficiency as determined by the Renilla luciferase activity. Cotransfection of the pSG5hRXRα vector resulted in a marked (4-8-fold) increase of the Renilla luciferase activity, which is unlikely to reflect increased transfection efficiency. This was also evident from experiments in which the pGL3-basic was used in combinations with various amounts of pSG5hRXRα. Over a concentration range of 0 to 1 μg hRXRα vector, no significant changes were observed in firefly luciferase activity, whereas the Renilla luciferase activity increased 5-fold. Therefore, the relative firefly luciferase units were used to evaluate the effect of cotransfection of rFxr and hRXRα.

RT-PCR

Total RNA from HepG2-rNtcp cells was isolated using the SV Total RNA Isolation System (Promega Corp.) according to the manufacturer's instructions. RT was

performed on 5 μg of total RNA using random primers in a final volume of 75 μL (Reverse Transcription System, Promega Corp.) as described previously.[21]
For conventional RT-PCR, 3 μL of complementary DNA was used in a final reaction volume of 50 μL containing 0.5 U Taq polymerase (Eurogentec, Seraing, Belgium) and 50 pmol of sense and antisense primers (Invitrogen BV) using a Robocycler Gradient 96 (Stratagene). The cycling program consisted of 5 minutes at 95°C followed by 22 cycles (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)), 34 cycles (*ABCB11*), or 30 cycles (*FXR*) at 95°C for 40 seconds, 58°C (*GAPDH*) or 60°C (*ABCB11, FXR*) for 40 seconds, and 72°C for 40 seconds and 5 minutes in the last cycle. Primers used for *GAPDH* were 5'-CCA TCA CCA TCT TCC AGG AG-3' (sense) and 5'-CCT GCT TCA CCA CCT TCT TG-3' (antisense) and gave a product of 576 base pairs; for *ABCB11* 5'-GGA ACC AGT GTT GTT TGC CT-3' (sense) and 5'-AAC CTG CAC CGT CTT TTC AC-3' (antisense) and gave a product of 295 base pairs; and for *FXR* 5'-GGA ATG TTG GCT GAA TGC TT-3' (sense) and 5'-GTT GCC ATT TCC GTC AAA AT-3' (antisense) and gave a product of 305 base pairs. Eight micro liters of each PCR product was loaded on a 2% (wt/vol) agarose gel and stained with ethidium bromide.
For quantitative real-time detection RT-PCR,[22,23] sense and antisense primers (Invitrogen BV) and probes (Eurogentec) for *ABCB11* and 18S were designed using Primer Express software (PE Applied Biosystems, Foster City, CA). For *ABCB11* the primers and probe used were 5'-ACA TGC TTG CGA GGA CCT TTA-3' (sense), 5'-GGA GGT TCG TGC ACC AGG TA-3' (antisense) and 5'-CCA TCC GGC AAC GCT CCA AGT CT-3' (probe), generating a 105-base pair product; for 18S 5'-CGG CTA CCA CAT CCA AGG A-3' (sense), 5'-CCA ATT ACA GGG CCT CGA AA-3' (antisense) and 5'-CGC GCA AAT TAC CCA CTC CCG A-3' (probe), generating a 109-base pair PCR fragment. Both probes were 5'-labeled with 6-carboxy-fluorescein and quenched by 6-carboxy-tetramethyl-rhodamine. For real-time PCR, 3 μL complementary DNA was used in a PCR reaction in a final volume of 50 μL containing 900 nmol/L of forward and reverse primers and 200 nmol/L of probe, 250 nmol/L $MgCl_2$, 10 nmol/L deoxynucleoside triphosphate mix, 5 μL Real-Time PCR buffer (10x), and 1.25 U Hot GoldStar (Eurogentec). Real-time detection PCR was performed on the ABI PRISM 7700 (PE Applied Biosystems) initialized by 10 minutes at 95°C to denature the complementary DNA followed by 40 PCR cycles each of 95°C for 15 seconds and 60°C for 1 minute.

RNA interference

Small interfering RNA's (saran's) specific for human FXR were designed conforming to the sequence AA(N19)TT, where AA and TT are present in the FXR open reading frame at a spacing of 19 nucleotides. Two single-stranded RNA molecules, 5'-GGG GAU GAG CUG UGU GUU GdTdT-3' (sense) and 5'-CAA CAC ACA GCU CAU CCC CdTdT-3' (sense) were synthesized (Dharmacon Research, Lafayette, CO). As control, single stranded-RNA molecules specific for the firefly luciferase gene were synthesized[24]. For annealing, 20 μmol/L of both single-stranded RNA's were incubated in annealing buffer (200 mmol/L potassium acetate, 30 mmol/L HEPES-

KOH, pH 7.4, and 2 mmol/L magnesium acetate) for 2 minutes at 90°C and cooled down to 30°C in approximately 1 hour.
HepG2-rNtcp cells were transfected with 10 µL of 20 µmol/L siRNA duplexes using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV). The next day, the transfection medium was replaced by fresh culture medium containing 100 µmol/L CDCA. After 48 hours, cells were harvested for RNA isolation and conventional RT-PCR analysis as previously described.

3.4 Results

Cloning of the *ABCB11* promoter

Using the *ABCB11* mRNA sequence AF091582[25] to search the BLAST database, the human BAC clone AC008177 was identified to contain part of the genomic *ABCB11* locus, including the known 5'-untranslated region and the possible *ABCB11* promoter region. Comparison of the 2 sequences showed the presence of a large (13,072 base pairs) intron within the 5'-untranslated region of *ABCB11*, 26 base pairs upstream of the translation initiation site.
Fig. 3-1a shows the nucleotide sequence of *ABCB11* from base pairs -1857 to +21 (relative to the translation initiation codon, excluding the 13-kilobase intron).

a

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                                        -1857 CACACTGCCCAGATGTGTCTGGCCAGCAT
-1828 GAGGGTGCAGAGGAAGACACCCTGAACTCTCCAGGGCCCTGGACAGATGGCCACTCTGTCTTCA
-1764 CTTTTCTCATCTATAAGATGAAGTTGTTGGATGATATATCAATATTTACAATGTTGTTTCTAGC
-1700 TCTAAAACTCTATGCTTTTATCTTCCCTTGGATCTTCATGGCACCAGAAGGAAAGTCTTAGGAC
-1636 CCTTGGACCTGTGAGCAGATCAGCTATAGTCCTGAGTTGACAAATTTCTCTTGGCATTTTTACC
-1572 CTTTGACTGCTGTCAATAACTTCAGCACAGCAAAGGTAGCAAAATTCTATTGGGAATCTTTTCC
-1508 CAATCAAAGCTACAGCCCCATAGCTGTGTTGCCTTTTTGGTTTTGTGTGAAGCAAAATTTTTTT
-1444 CCTGGGTCCTAATTGGTGCCAAATCCAATATTACTACATTTGCGTCAACTCAGAAGTCAACCCA
-1380 TTCAGTTTGCATAGAGGAAACATCTAGAAATCTTGCTTTTCTTTGCTGACCTTGATATATTTGA
-1316 GATTTGAACATATAATAACATATAAAATTATATGTTATAATTTTGGGTTTTATGGGCTAAGTCA
-1252 TAAACCATCTTATACATAAATTCCAATAGAGAAAAAATGGTGGATGCTGAATTTTAATAAAAAA
-1188 TTTATGACAGAGAAACCTAAAATTGAGAAAATTTGATCTTACAGTTTAATTTCTGCAAATTAAG
-1124 AAGCACTGGCCCATCAATTGCATTTCAGAGCACAGAGTGGAAGAAGGTTAGCACAGACTGGCAT
-1060 GTGGCTTCACATCTACTAGTTGTTACACCTTAGGAGGATTATTTAACCTCTCTGTGCTTCAGTT
 -996 TCCCCAACTATAAAATAGAAATGACATGATAGCACCCAACTCCTAGGGCTGTTGAGAGGCCCAA
 -932 ATGAGGTGATACAAATAACATTTTTGAAGTGGGCCAGGCACCCAACCAAGTCTTGACCAATGTT
 -868 GCCTATTATTATTCATTGCTGAAGGCTGGAGTGAGAGGCATTTAGGGAAAAGTAAGCTCAGGCA
 -804 AAGGAGAAAAAATAAGAACATTGTAGGAAAAATGGAAAGATTCACAAGAAGGGAGAGGAAGAGG
 -740 CAGCACAAATATATTGGAGGAGCTCCACATGCTTATTTGACTCAAGACCTGTTCATTTGAACCT
 -676 TTAGAAAATCGTTCATCTTTGCTTATACAGAGCTTCATCTGGTGTGTCCATGCCAGGGTGCAAG
 -612 AGTTGTCTGTGCACTCAGACTTTTGAGCAAGGCTGTTTCAAATGTTCTTTTAGGGTATTTGTCT
 -548 CCACAAAACTCTATAGCTGGGCCAGGAGCATCTGGATCCTGCAACCAGGGATTTTCCAAGAGCA
 -484 ATCTTTTATATTGAGGGGGAAAGTTTAAAGGTATTTTTTTTTTTTGTCTTGTTATGTTTTTAAG
 -420 TAACTTTTCACAACTACAGGCCTGTAAAAAATAAGGGTTGGGATAGCCTGAATTCCAGGGCTCT
 -356 TGCTGGGCCCACTCTGCTCAATTTGCCTCTCGTTCCAAGGTGAATCAGCAATTTCCAAGGCCTG
 -292 TTGACACCCTCAGAGGGTTTCCCAAGCACACTCTGTGTTTGGGGTTATTGCTCTGAGTATGTTT
                                          FXRE
 -228 CTCGTATGTCACTGAACTGTGCTTGGGCTGCCCTTAGGGACATTGATCCTTAGGCAAATAGATA
 -164 ATGTTCTTGAAAAAGTTTGAATTCTGTTCAGTGCTTTAGAATGATGAAAACCGAGGTTGGAAAA
                                                              ↑-105
 -100 GGTTGTGAAACCTTTTAACTCTCCACAGTGGAGTCCATTATTTCCTCTGGCTTCCTCAAATTCA
  -36 TATTCACAGGGTCGTTGGCTGTGGGTTGCAATTACC ATG TCT GAC TCA GTA ATT CTT
              |13072 bp intron             Met Ser Asp Ser Val Ile Leu
```

b

```
                                                    FXRE
hABCB11 -222 ATGTCACTGAACTGTGCTTGGGCTGCCCTTAGGGACATTGATCCTTAGGC
mAbcb11      ATGTCACTGAACTGTGCTAGATCTGGACTTTAGGCCATTGACCTATAAGC

hABCB11 -172 AAATAGATAATGTTCTTGAAAAAGTTTGAATTCTGTTCAGTGCTTTAGAA
mAbcb11      AAATAGATAGTGTTCTTAAAAAAGCCTGATTTCTGTTCAATGCTTTATTA

hABCB11 -123 TGATGAAAACCGAGGTTGG -105
mAbcb11      CCATGAAAACTGAACTTGG
```

c

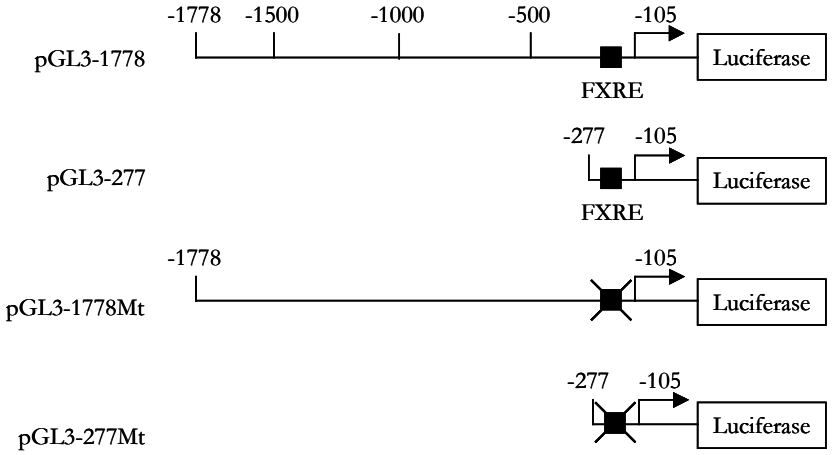

Fig. 3-1 The human *ABCB11* promoter contains an FXRE. (a) The nucleotide sequence of the human *ABCB11* locus from base pair -1857 (first base pair upstream of the start codon is numbered -1) to base pair +21. The proposed FXRE (boxed) is located at base pairs -192 to -180. A 13,072-base pair intron is located within the known 5'-untranslated region between base pairs -27 and -26. For numbering of the DNA, the 13-kilobase intron is not calculated. Position -105 indicates the 3' end of all promoter constructs. (b) Comparison of the nucleotide sequences of human *ABCB11* (AF091582, GI3873242) and murine *Abcb11* (AF303740, GI10799100) promoter region. Both contain an FXRE (boxed). (c) Schematic overview of the human *ABCB11* promoter constructs used in this study. The constructs pGL3-1778 and pGL3-277 are denoted according to the numbering of the sequence shown in a. The FXRE is indicated as a black box and which is marked with an X for the mutated constructs pGL3-1778Mt and pGL3-277Mt. Please note that all promoter constructs used end at position -105, upstream of the large intron.

Screening for possible binding sites for transcription factors using the TRANSFAC database (http://transfac.gbf.de/TRANSFAC/index.html) showed several ubiquitous and liver-specific transcriptional regulatory elements, including a putative FXR binding site at position -180 to –192 (Fig. 3-1a). The FXRE consists of an IR-1. Comparison of the human and mouse *ABCB11* promoter sequences showed that the position of the FXRE is conserved in both species and that the human FXRE contains 2 mismatches taken the consensus sequence of the FXRE, AGGTCA (Fig. 3-1b).[10,11]

Two genomic DNA fragments comprising nucleotide –1,857 to –105 or –277 to –105 were amplified by PCR and inserted into a luciferase reporter gene vector, resulting in pGL3-1778 and pGL3-277, respectively. pGL3-227 still contains the putative FXRE and only a minimum of other regulatory elements. Subsequently, the IR-1 sequence in both constructs was mutated by site-directed mutagenesis, generating pGL3-1778Mt and pGL3-277Mt, as schematically shown in Fig. 3-1c.

FXR overexpression induces human *ABCB11* promoter activity

The role of the nuclear hormone receptor FXR and its activation by bile salts on the promoter activity of human *ABCB11* was studied. HepG2-rNtcp cells were cotransfected with one of the promoter constructs and an expression vector for rFxr or a control vector. After the transfection, the cells were incubated in the presence or absence of the bile salt CDCA (100 μmol/L) for 24 hours.

Transient cotransfection of either promoter construct with the control vector did not result in a significant increase in the (relative) luciferase activities (Fig. 3-2a, white bars). In contrast, cotransfection of pGL3-1778 with the rFxr expression vector in the absence of CDCA increased the luciferase activity 48-fold. Also, the shorter construct, pGL3-277, showed a similar inducibility by rFxr (33-fold). Culturing the rFxr-cotransfected HepG2-rNtcp cells in the presence of 100 μmol/L CDCA further enhanced the luciferase activity to 175- to 231-fold for pGL3-1778 and pGL3-227, respectively.

The level of rFxr-induced *ABCB11* promoter activity was dependent on the concentration of CDCA in the culture medium. HepG2-rNtcp cells were cotransfected with the pGL3-277 and rFxr constructs and grown in the presence of variable amounts of CDCA for 24 hours. As shown in Fig. 3-2b, a positive correlation was observed between the concentration of CDCA (up to 100 μmol/L) in the growth medium and the level of luciferase activity.

FXR-RXRα co-overexpression results in maximum CDCA-induced activity of the human *ABCB11* promoter

The activity of FXR requires heterodimerization with RXRα. Therefore, we studied whether co-overexpression of rFxr together with hRXRα would further increase the CDCA-induced activity of the *ABCB11* promoter. As can be seen in Fig. 3-3, coexpression of pGL3-1778 with rFxr and hRXRα in the absence of CDCA already increased the firefly luciferase activity significantly (approximately 2.5-fold compared to rFxr alone), probably due to the presence of endogenous ligands for these nuclear receptors.[26,27] The presence of 100 μmol/L CDCA in the growth medium resulted in a further increase of 4-fold compared to rFxr and hRXRα coexpression in the absence of CDCA or rFxr alone in the presence of CDCA. The additive effect of hRXRα coexpression with rFxr on human *ABCB11* promoter activity was confirmed by analyzing endogenous *ABCB11* mRNA levels in HepG2-rNtcp cells. These data show that both FXR and RXRα are required for maximum CDCA-dependent induction of the human *ABCB11* promoter.

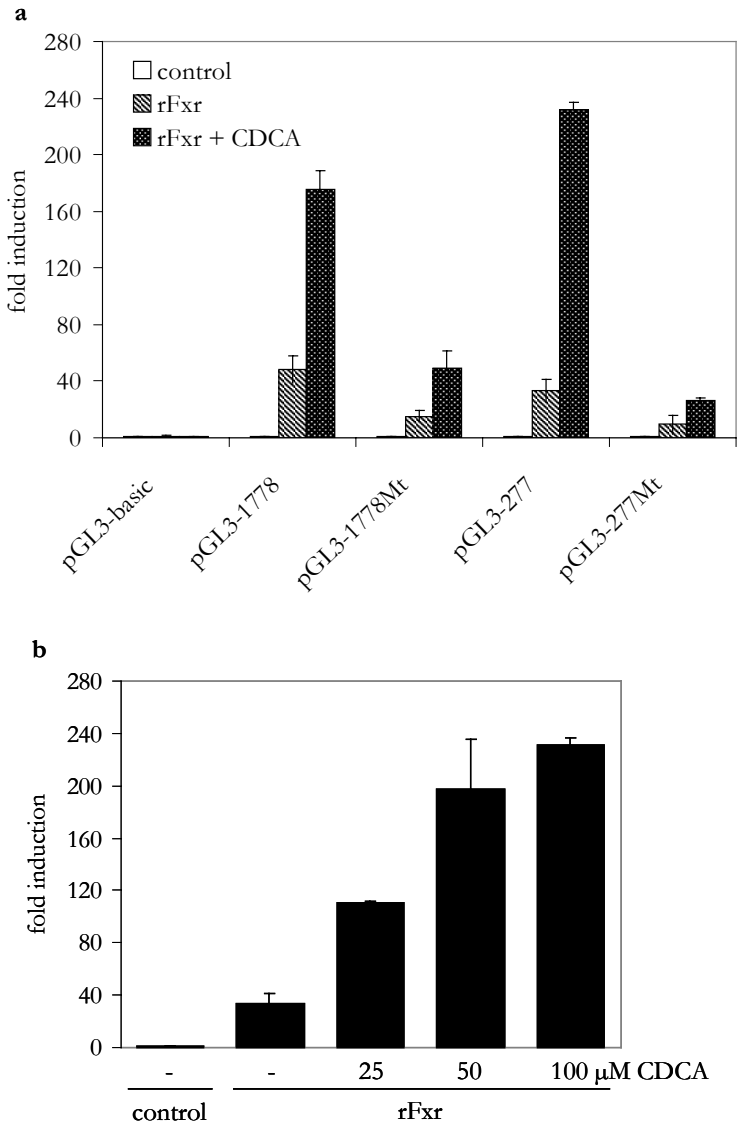

Fig. 3-2 The putative FXRE is involved in the transactivation of the human *ABCB11* promoter by rFxr and CDCA in HepG2-rNtcp cells. This transactivation is CDCA concentration-dependent. (a) HepG2-rNtcp cells were cotransfected with the indicated luciferase reporter construct and either the control vector or the rFxr expression vector and treated with PBS alone or 100 μmol/L CDCA dissolved in PBS for 24 hours. For each promoter construct, the relative firefly luciferase activity was normalized using cotransfected pRL-TK. The normalized data are presented as fold induction over control. (b) HepG2-rNtcp cells were cotransfected with pGL3-277 and either the control or rFxr expression vector. Following transfection, cells were treated with PBS (control, rFxr) or various amounts of CDCA (rFxr). After 24 hours, firefly and Renilla luciferase activities were assayed and processed as described in a.

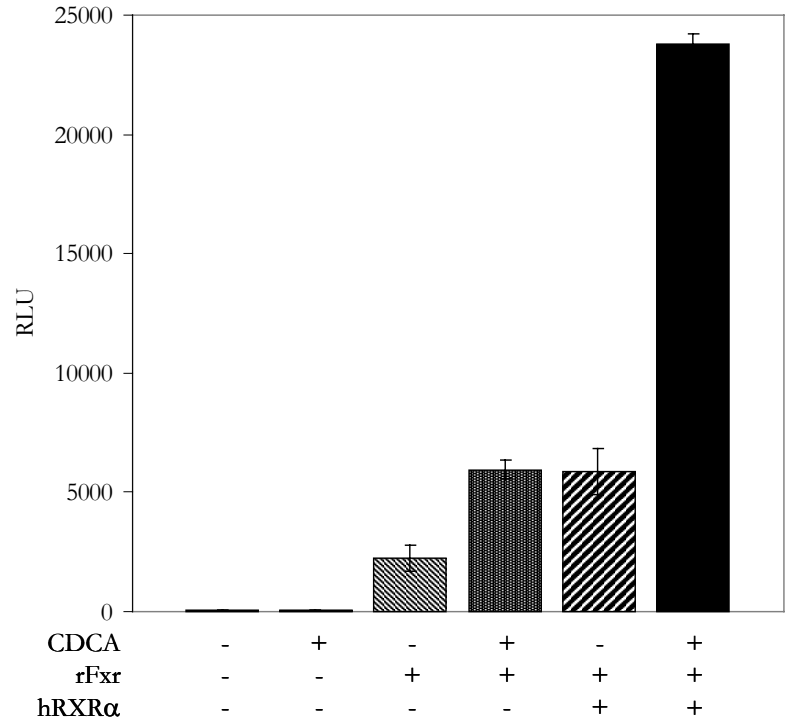

Fig. 3-3 FXR, RXRα and CDCA are required for maximum induction of the *ABCB11* promoter. HepG2-rNtcp cells were cotransfected with the pGL3-1778 and control, rFxr, and/or hRXRα expression vectors and incubated with or without 100 μmol/L CDCA for 24 hours. The results are presented in relative firefly luciferase units (RLU).

Mutating the putative FXRE reduces the FXR-CDCA inducibility of the human *ABCB11* promoter

To test whether the putative FXRE is involved in the activation of the human *ABCB11* promoter, this site was mutated by site-directed mutagenesis, resulting in pGL3-1778Mt and pGL3-277Mt. Cotransfection of the rFxr-vector with pGL3-1778Mt or pGL3-227Mt resulted in a 15- and 9-fold increase in luciferase activity, respectively, compared to cotransfection with the control vector (Fig. 3-2a). This increase was further enhanced after stimulation with 100 μmol/L CDCA to 49- and 26-fold, respectively (Fig. 3-2a). Although the promoter activities of the mutant constructs were still stimulated by rFxr and CDCA, the increase was strongly reduced compared with the wild-type promoter constructs (26-fold vs. 231-fold for rFxr+CDCA-induced expression of the short promoter construct). Taken together, these results suggest that the putative FXRE is involved in the rFxr-mediated bile salt-induced stimulation of the human *ABCB11* promoter.

FXR-RXR co-overexpression increase the endogenous *ABCB11* mRNA level in HepG2 cells

Our results show that rFxr and bile salts positively control the human *ABCB11* promoter element when present in a luciferase reporter plasmid. To determine the effect of these factors on the transcriptional control of the genomic human *ABCB11* locus, we analyzed the endogenous *ABCB11* mRNA levels in HepG2 cells under various conditions. First, we cultured HepG2 cells in the presence of 100 μmol/L CDCA and determined the level of *ABCB11* mRNA in time by conventional RT-PCR. As can be seen in Fig. 3-4, *ABCB11* mRNA was undetectable when HepG2 cells were grown in the absence of CDCA (T=0) under the specified RT-PCR conditions (see legend to Fig. 3-4 for details). The *ABCB11* mRNA gradually increased over 24 hours, after which it remained stable for an additional 72 hours. In contrast, the level of endogenous FXR did not significantly change over time.

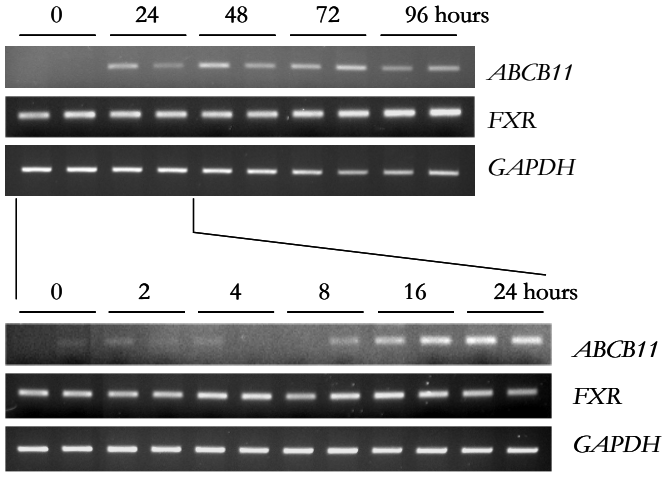

Fig. 3-4 Endogenous *ABCB11* mRNA expression is up-regulated by CDCA. HepG2 cells were treated with 100 μmol/L CDCA for different time spans (0-96 hours). Cells were harvested at comparable cell densities. Five micrograms of total RNA was transcribed into complementary DNA and subjected to conventional RT-PCR analysis of endogenous *ABCB11*, *FXR*, and *GAPDH* mRNA levels.

To determine whether the increase in *ABCB11* mRNA is controlled by the amount of FXR (and RXRα), we transfected HepG2-rNtcp cells with rFxr and/or hRXRα expression or control vector, cultured them for 48 hours in the absence or presence of CDCA, and determined the *ABCB11* mRNA level by quantitative real-time detection RT-PCR (Fig. 3-5). The relative amount of *ABCB11* mRNA in HepG2-rNtcp cells increased 3.1-fold after growth in the presence of CDCA. Transfection of rFxr or hRXRα expression vectors alone resulted in an *ABCB11* mRNA increase of 3.5- and 1.8-fold, respectively. On incubation of these cells in the presence of CDCA, these levels further increased to 24.2- and 4-fold respectively, relative to HepG2-rNtcp cells grown in the absence of CDCA. rFxr and hRXRα coexpression gave only a moderate

increase of 2.8-fold, which was maximally induced to more than 100-fold when these cells were grown in the presence of CDCA. These data confirm that, by increasing rFxr and hRXRα concentrations by cotransfection of HepG2-rNtcp cells, the level of *ABCB11* mRNA is significantly up-regulated, especially in the presence of CDCA. Notably, the level of endogenous *FXR* mRNA did not significantly change on stimulation of HepG2 cells with CDCA (Fig. 3-4). To determine whether the CDCA-dependent up-regulation of *ABCB11* mRNA is controlled by activation of FXR, we sought for a method to inhibit the synthesis of endogenous FXR in HepG2-rNtcp cells.

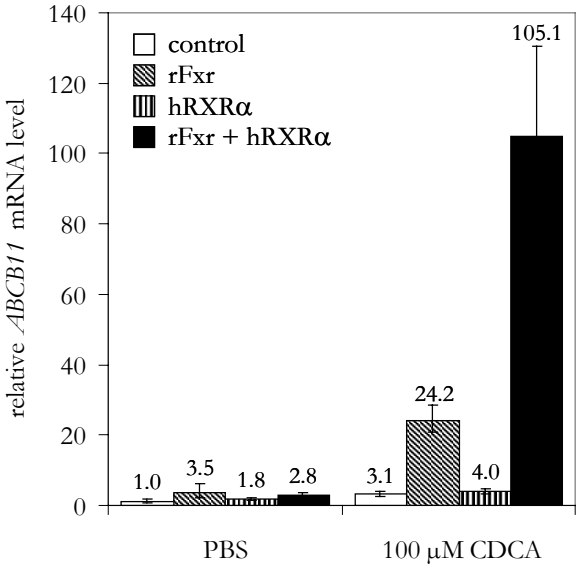

Fig. 3-5 Endogenous *ABCB11* mRNA expression is maximally up-regulated by coexpression of rFxr and hRXRα and stimulation with 100 μmol/L CDCA. HepG2-rNtcp cells were transfected with the indicated expression vectors and incubated with PBS or 100 μmol/L CDCA. After 48 hours, total RNA was isolated and subjected to quantitative real-time RT-PCR. The relative *ABCB11* mRNA level was obtained by normalizing each sample to the ribosomal RNA content using the comparative C_T method.

FXR is required for CDCA-induced up-regulation of endogenous *ABCB11* expression in HepG2 cells

RNA interference is a novel technique to suppress the expression of specific genes in cultured cells. The mediators of RNA interference are siRNA molecules. siRNA's matching a specific gene sequence, induce the selective degradation of the mRNA of this gene.[24,28-30] We developed siRNA probes to specifically suppress endogenous *FXR* mRNA levels in HepG2-rNtcp cells. After transfection using FXR-specific or control (luciferase) siRNA probes, HepG2-rNtcp cells were cultured for 48 hours in the presence of 100 μmol/L CDCA, and total RNA was isolated and analyzed by

conventional RT-PCR. As can be seen in Fig. 3-6, *FXR* and *ABCB11* mRNA levels were readily detectable in HepG2-rNtcp cells treated with the control siRNA probes and were similar to the levels observed in normally CDCA-induced cells (not shown). In contrast, cells transfected with the *FXR*-specific siRNA molecules showed significantly reduced mRNA levels of endogenous *FXR*, indicating specific suppression of the target mRNA levels. *ABCB11* mRNA levels were reduced to that observed in untreated and uninduced cells, even though *FXR* mRNA was still detectable at this time point, albeit at significantly reduced levels. These data show that FXR is required for the short-term up-regulation of *ABCB11* mRNA levels in response to elevated bile salt concentrations. Notably, a critical amount of FXR is required for effective CDCA-dependent up-regulation of *ABCB11* transcription.

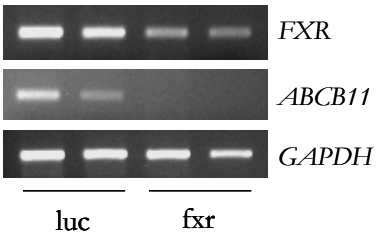

Fig. 3-6 Decreasing the endogenous *FXR* mRNA level by RNA interference results in full suppression of the CDCA-dependent induction of *ABCB11* mRNA expression. HepG2-rNtcp cells were transfected with siRNA duplexes directed against either the firefly luciferase gene (luc) as control or the human *FXR* gene (fxr) and treated with 100 μmol/L CDCA. After 48 hours, cells were harvested and RNA isolated for conventional RT-PCR analysis of *ABCB11*, *FXR*, and *GAPDH* mRNA levels.

3.5 Discussion

In this study, we determined the role of bile salts and the transcription factors FXR and RXRα on human *ABCB11* transcription encoding the liver-specific canalicular bile salt export pump (bile salt export pump or *ABCB11*). Using luciferase reporter gene assays, we show that the human *ABCB11* promoter is transactivated by the coordinate action of the bile salt CDCA, FXR, and its interacting partner RXRα. Analysis of the transcriptional control of the genomic *ABCB11* locus in HepG2-rNtcp cells confirmed the essential role of FXR in bile-salt-dependent up-regulation of human *ABCB11* transcription.

The bile salt export pump is an ATP-dependent transporter in the canalicular membrane of hepatocytes that transports bile salts against a steep concentration gradient into bile. The functional amount of ABCB11 is precisely controlled to fit the needs of the cell and the organism to maintain bile homeostasis.

To analyze the transcriptional control of the human *ABCB11* gene, we cloned a 1.7-kilobase-promoter region of the *ABCB11* gene. The promoter region seemed to contain several possible response elements for ubiquitous (activator protein 1) and liver-enriched transcription factors (CCAAT/enhancer-binding protein β, hepatocyte nuclear factor 3 β), including a putative FXRE or IR-1. The FXRE was present in the

human and murine *ABCB11* promoter regions at identical spacing from the translation initiation site, consistent with a ubiquitous role of FXR as an endogenous bile salt sensor in the mammalian liver. The FXRE sequence in the human *ABCB11* promoter does not correspond exactly to the previously described consensus sequence as found in the promoter region of intestinal bile acid-binding protein. However, a recent study demonstrated that FXR/RXRα heterodimers bind and activate a number of FXRE elements that differ from the consensus sequence at 1 or 2 positions. However, the highest affinity was observed for the consensus IR-1 sequence.[11]

This study shows that FXR, RXRα, and CDCA synergistically transactivate the human *ABCB11* promoter in HepG2-rNtcp cells. FXR alone already significantly induced the *ABCB11* promoter activity in these cells. This is probably due to the endogenous production of bile salts by HepG2 cells that may activate overexpressed FXR.[26,27] This view is supported by our finding that, in a kidney-derived cell line (Hek293), FXR required exogenously added CDCA to transactivate the *ABCB11* promoter (data not shown). Adding increasing amount of CDCA up to 100 μmol/L to the culture medium further increased the *ABCB11* promoter activity, showing that the level of activation is dependent on the concentration of bile salts. To show that the putative FXRE is indeed responsible for the FXR- and bile salt-dependent activation, the FXRE sequence was mutated. FXR and CDCA showed a strongly reduced effect on transactivation of the mutant *ABCB11* promoter constructs. However, a low but significant induction was still observed. This may be caused by a combination of factors, including the involvement of other liver-specific transcription factors, endogenous bile salts, and the excess of FXR present in the transiently transfected cells. The possible presence of another FXRE site is unlikely because other putative bile salt response elements were not found in this region. Furthermore, the mutant promoter constructs were almost silent in Hek293 cells after FXR/CDCA induction, suggesting the involvement of other liver-specific factors in activation of these constructs in HepG2-rNtcp cells (data not shown). Therefore, our data suggest that the putative FXRE indeed plays an important role in the FXR-dependent regulation of the *ABCB11* promoter activity.

Essentially the same results were recently described by Ananthanarayanan et al., who also showed that the human *ABCB11* promoter is transactivated by bile salts, FXR, and RXRα.[18] In addition, we extended our studies to the transcriptional control of the genomic *ABCB11* locus in HepG2(-rNtcp) cells. These studies were performed to (1) compare and validate our results of the *ABCB11* promoter studies to the transcriptional control of the genomic *ABCB11* locus, (2) determine the kinetics of CDCA- and FXR-dependent transactivation of endogenous *ABCB11*, (3) analyze the effect of overexpression and underexpression of FXR on *ABCB11* mRNA levels, and (4) evaluate the possible role of the large, 13-kilobase-long, intron in the 5'-untranslated region of the primary *ABCB11* transcript on CDCA/FXR-dependent regulation.

Both conventional and real-time detection RT-PCR analysis showed that the endogenous *ABCB11* mRNA level is significantly induced by adding CDCA to the medium of HepG2 cultures. Maximum induction was observed approximately 24 hours after CDCA stimulation. In the time course (up to 96 hours after CDCA

stimulation), no further change in the *FXR* mRNA levels was observed. Overexpression of rFxr (by transient transfection) alone had little effect on *ABCB11* mRNA levels, but resulted in a strong increase (25-fold) when activated by CDCA in the culture medium. The *ABCB11* mRNA levels were even further increased (more than 100-fold) by co-overexpression of rFxr and RXRα in the presence of CDCA. Reducing the level of endogenous FXR by RNA interference using siRNA molecules specific for human FXR led to a marked decrease in CDCA-induced activation of *ABCB11* transcription to uninduced levels. The successful application of RNA interference in HepG2-rNtcp cells to reduce the mRNA level of endogenous FXR opened the possibility to analyze the effect of temporary reduction of transcription of a single gene at the cellular level. It is therefore compatible with studies on gene knockout mice in which the long-term effects of gene elimination are observed. In *Fxr*-null mice, the basal level of *Abcb11* mRNA was reduced to 30% of wild-type mice and bile acid-induced up-regulation of *Abcb11* mRNA (6-fold in wt mice) was absent.[17] Our data show that reducing the level of FXR immediately results in the absence of bile salt-dependent up-regulation of the *ABCB11* transcript and that the cellular amount of FXR is the major determinant of the level of bile salt-induced *ABCB11* transcription.

Acknowledgements

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Chapter 4

Low retinol levels potentiate bile salt-induced expression of the bile salt export pump in vitro and in vivo

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4.1 Abstract

The farnesoid X receptor/retinoid X receptor α (FXR/RXRα) heterodimer regulates transcription of genes involved in bile acid homeostasis, including the *Bile Salt Export Pump* (*BSEP*) and *Small Heterodimer Partner 1*(*SHP*). FXR is activated by bile acids and RXRα by 9-cis retinoic acid (9cRA). We evaluated the role of 9cRA in the expression of *BSEP*/*Bsep* and *SHP*/*Shp in vitro* and *in vivo*.

Human *BSEP* and *SHP* expression was quantified by real time RT-PCR in HepG2-rNtcp cells with or without transfection of rFxr- and hRXRα-expression plasmids and cultured in the presence or absence of chenodeoxycholic acid (CDCA) and/or 9cRA. *BSEP* promoter activity was measured by luciferase reporter assays and FXR/RXR DNA binding by electrophoresis mobility shift assays. Vitamin A-depleted C57BL/6J mice were used to evaluate the effect on cholic acid-induced *Bsep* and *Shp* expression *in vivo*.

In vitro, 9cRA strongly antagonized the CDCA-dependent *BSEP* gene transcription, by inhibiting binding of the FXR/RXR heterodimer to the *BSEP* FXR response element. In contrast, 9cRA agonized *SHP* expression. *In vivo*, vitamin A depletion enhanced cholic acid-induced expression of *Bsep* mRNA and protein, while reducing *SHP* expression.

9cRA either agonizes (*SHP*) or antagonizes (*BSEP*) bile acid-activated transcription of FXR/RXR-target genes. Vitamin A is therefore an important determinant in regulation of bile acid transport and synthesis. In patients with obstructive cholestasis, vitamin A derivatives may be therapeutically useful to decrease *BSEP* expression, thereby reducing the hepatobiliary bile acid flux and as a result reduce the pressure in the biliary tree.

4.2 Introduction

Vitamin A or retinol is important in a number of biological processes, including reproduction, embryogenesis, visual function, growth and development. All vitamin A present in the body is acquired from the diet. It is mainly stored in the stellate cells of the liver as retinyl esters that can be converted to various biological active compounds such as retinoic acid, all-trans retinoic acid and 9-cis retinoic acid (9cRA). 9cRA is the natural ligand for the retinoid X receptor or RXR (NR2B1),[1,2] a ligand-activated transcription factor belonging to the superfamily of nuclear hormone receptors (NHRs). In fact, RXR is a central dimerization partner for several members of this superfamily, including the thyroid hormone receptor (TR), vitamin D receptor (VDR), peroxisome proliferator activated receptors (PPAR's), liver X receptor (LXR), pregnane X receptor (PXR), farnesoid X receptor (FXR) and RXR itself. The ligands for these receptors are metabolites or drugs that, through activation of the NHR, regulate the transcription of genes involved in metabolism and/or transmembrane transport of the ligand. The fact that these NHRs are active as heterodimers with RXR emphasizes the importance of vitamin A for a wide range of physiological processes.

FXR is the mammalian bile acid sensor that controls bile acid homeostasis. Consequently, FXR is crucial for cholesterol metabolism and the intestinal uptake of fat-soluble vitamins. It binds and is activated by bile acids.[3-5]. Together with RXR, it directly stimulates the expression of the hepatocanalicular *bile salt export pump (BSEP),*[6,7] the *ileal bile acid binding protein (IBABP),*[8] *Phospholipid Transfer Protein (PLTP,* [9] and the transcription factor S*mall Heterodimer Partner 1 (SHP).*[10] SHP represses the expression of *cholesterol 7a-hydroxylase,*[11] the rate-limiting enzyme in the bile acid biosynthesis, and the *sodium-dependent taurocholic acid cotransporting peptide (NTCP),*[12] the basolateral bile acid importer of the hepatocyte.

9cRA may exert 3 different effects on transcriptional regulation by NHR/RXR heterodimers; 1) it is a prerequisite for activation but does not activate by itself (non-permissive heterodimers) as observed for RXR/RAR;[13] 2) it activates transcription by itself and has an additive effect on the activation by the heterodimer partner's ligand (permissive heterodimers; RXR/PPAR[14] and RXR/LXR[15]) or 3) it may actually inhibit the transcription activation exerted by the ligand of the heterodimer partner (RXR/VDR).[16,17] FXR/RXR has been described as a permissive heterodimer in the transcriptional control of *I-BABP* and *PLTP.*[8,9]

In this study we show a target-gene selective effect of 9cRA on the FXR/RXR-controlled transcription. *In vitro*, 9cRA strongly antagonized the bile acid-induced transcription of human *BSEP*, while enhancing the transcription of *SHP*. We found that 9cRA exerted this effect on *BSEP* transcription by inhibiting the binding of the FXR/RXR protein complex to the FXR-responsive element in the *BSEP* promoter element. We confirmed these findings in *in vivo* experiments using vitamin A-deficient mice. Cholic acid feeding of these animals resulted in significantly increased *Bsep* expression compared to vitamin A-sufficient mice, whereas S*hp* mRNA levels were highest under conditions of sufficient vitamin A. We discuss our findings in relation to cholestatic disease.

4.3 Materials and methods

Cell culture and transfections

Standard culture conditions for the human hepatoma cell line HepG2-rNtcp and derivatives have been described before.[7] In some experiments, the serum concentration, and thereby the vitamin A/retinol concentration, in the medium was reduced to 1% (vol/vol) as specified in the text. HepG2-rNtcp cells were transfected with various combinations of plasmids (indicated in the text) as described before.[7] Eighteen hours after transfection, medium was refreshed and contained chenodeoxycholic acid (CDCA) and/or 9cRA in various concentrations, as described in the text. Cells were harvested after 24 hours for determination of luciferase activity and after 48 hours for total RNA isolation. HepG2-rNtcp cells stably expressing rat Fxr were obtained after cotransfecting these cells with pCMX-rFxr and pHMR272.[18] Hygromycin B-resistant clones were collected and rFxr-expressing cells were selected by RT-PCR.

Plasmids

Plasmids for overexpression of rat Fxr (pCMX-rFxr), human RXRα (pSG5-hRXRα), and the luciferase reporter vector containing the –105 to –277 base pairs *BSEP* promoter fragment (pGL3-277), control and carrier plasmids (pCMV5 and pGEM-5) were described before.[7]

Luciferase assay

The luciferase reporter assay to measure the *BSEP* promoter activity was described before.[7]

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA isolation and quantitative real-time detection RT-PCR analysis of *BSEP/Bsep*, *SHP/Shp*, Ntcp and *Rxra* mRNA levels were described before.[7,19] Details about primer- and probe-sequences are available at the authors.

Electrophoretic Mobility Shift Assay

Isolation of nuclear extracts from HepG2-rNtcp cells and the protocol for the electrophoretic mobility shift assay have been described before.[20] The FXRE probe contained the IR-1 sequence (underlined) from the *BSEP* promoter: 5'-GATCCCTTAGGGACATTGATCCTTAGG-3', and was labeled with [α-^{32}P]dATP (Amersham, Buckinghamshire, UK) using Klenow polymerase (Promega, Madison, USA).

Animals

Pregnant (two weeks post coitum) C57BL/6J mice were obtained form Harlan Nederland (Horst, the Netherlands). Food and water were available ad libitum. The mice were housed in a temperature-controlled environment with alternating 12 hours

light and dark cycles. Experimental protocols were approved by the Ethical Committee on Animal Testing of the Faculty of Medical Sciences, University of Groningen.

Experimental design

Male C57BL/6J mice were made vitamin A-deficient essentially as described by Smith, 1990.[21] In short, pregnant C57BL/6J mice received a standard laboratory chow upon arrival. At parturition (one week after arrival), the dams were divided into two groups (dam + offspring). One group received a vitamin A-deficient diet (VAD; 4148.10, Hope Farms, Woerden, the Netherlands) and the control group received control diet (4068.02, Hope Farms BV, Woerden, the Netherlands), containing 18,000 IU vitamin A/kg. After weaning (21-25 days), the male pups continued on the same diet as the mother. At the age of 13 weeks, both groups (VAD and control) were divided into two subgroups. Group 1 continued with the VAD diet and group 2 continued with the VAD diet, but now supplemented with 0.5% (wt/wt) cholic acid (CA). Group 3 continued on the control diet and group 4 continued on the control diet, but now diet supplemented with 0.5% (wt/wt) CA. One week later, the mice were weighed and killed. The livers were removed, weighed, cut into pieces, snap-frozen in liquid nitrogen, and stored at -80˚C for mRNA isolation, Western blot analysis and immunofluorescence microscopy. Vitamin A deficiency was determined by high performance liquid chromatography analysis of retinol levels in fresh liver tissue according to Academic Hospital Groningen protocols.

Liver plasma membrane isolation

Total plasma membranes were isolated from 3 to 4 pooled mouse livers using sucrose-gradient ultracentrifugation according to Meier and Boyer, 1990[22] with some modifications as described by Vos et al., 1998.[23]

Western Blot analysis

Equal amounts of liver plasma membrane proteins were separated by 7.5% SDS-PAGE[24] and analyzed for Bsep and Na^+K^+-ATPase protein expression by Western blotting.[25] Polyclonal antibodies used, were raised against Na^+K^+-ATPase (gift from Dr. W. Peters, University Medical Center, Nijmegen, the Netherlands) and BSEP (K12[23] Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as standard.

Confocal laser scanning microscopy

For microscopical analyses, frozen mouse liver tissue was cut into 4 μm-sections. After drying, the liver sections were fixed in acetone and stained for Bsep expression using K12, with the corresponding second antibody Alexa fluor 488 (Alexis Biochemicals, Lausen, Switzerland). Images were taken with a confocal laser scanning microscope (TCS 4D; Leica, Heidelberg, Germany) equipped with an argon/krypton laser and coupled to a Leitz DM IRB (Leica, Heidelberg, Germany) inverted microscope.

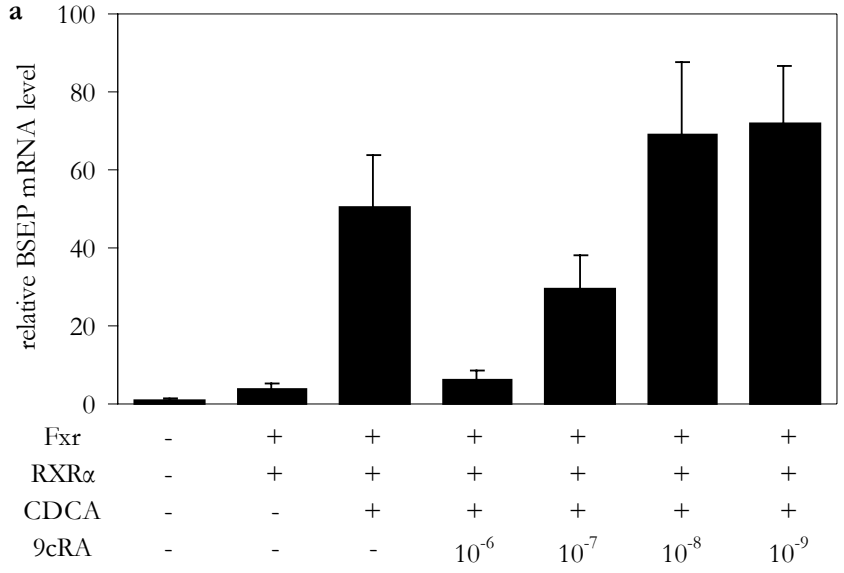
Statistics

Data are presented as means ± sd. Differences between the animal groups were determined in SPSS by one-way ANOVA with Scheffe as post hoc test and $p<0.05$.

4.4 Results

9-cis Retinoic acid antagonizes CDCA-induced expression of human *BSEP* in vitro

To study the role of 9cRA in the transcriptional regulation of the human *BSEP* gene, we transfected HepG2-rNtcp cells with expression vectors for rFxr and hRXRα and cultured these cells in the presence of CDCA with or without various concentrations of 9cRA. As observed before, CDCA alone resulted in a strong (50-fold) increase of *BSEP* mRNA level (Fig. 4-1a). Surprisingly, the bile acid induction was strongly attenuated (to 6.0-fold compared to control cells) when also 1 µmol/L 9cRA was added to the culture medium. This inhibitory effect was dose-dependent, and disappeared when 9cRA concentrations were reduced to 10 nmol/L or lower. Notably, a 9cRA-dependent elevation of *BSEP* expression above the CDCA-induced levels was never observed. The 9cRA-dependent suppression of the CDCA-induced expression of human *BSEP* was also observed in untransfected HepG2-rNtcp cells (Fig. 4-1b). Reducing the concentration of serum, which also contains 9cRA/vitamin A (± 0.5 µmol retinol/L), in the culture medium to 1% strongly increased the CDCA-induced expression of *BSEP*, which was suppressed after adding 9cRA to this medium. In HepG2-rNtcp cells stably transfected with rat Fxr, the CDCA and 9cRA regulated expression of endogenous *BSEP* was similar, but strongly amplified compared to untransfected HepG2-rNtcp cells (Fig. 4-1c).



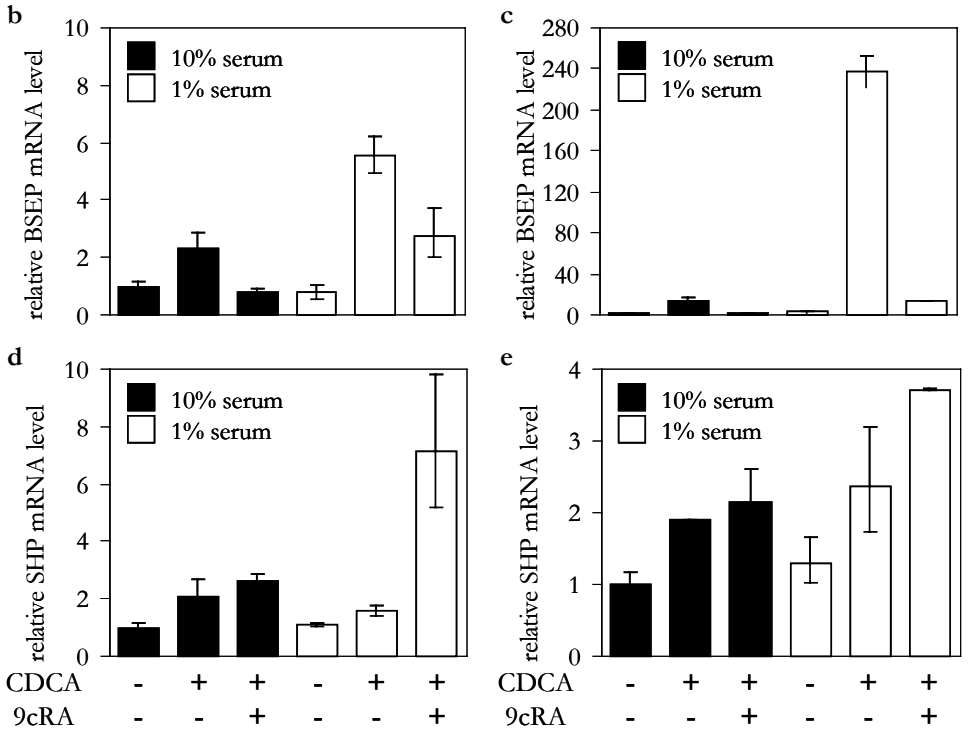


Fig. 4-19cRA inhibits CDCA-stimulated *BSEP* mRNA expression. (a) *BSEP* mRNA levels in HepG2-rNtcp cells after transfection with the indicated expression vectors for hRXRα and/or rFxr, and treatment with 100 μmol/L CDCA with or without simultaneous addition of 9cRA in the concentration range of 1μmol/L to 1 nmol/L. *BSEP* (b,c) and *SHP* (d,e) mRNA levels in HepG2-rNtcp cells (b,d) or HepG2-rNtcp cells stably expressing rFxr (c,e), cultured in 10% (vol/vol) serum (black bars) or 1% serum (vol/vol) (white bars), and incubated with 50 μmol/L CDCA or 1 μmol/L 9cRA as indicated. *BSEP* and *SHP* relative mRNA levels were determined by RT-PCR and normalized to *18S*. Values are presented as mean ± sd.

9cRA agonizes CDCA-induced expression of human *SHP*

The small heterodimer partner 1 (*SHP*) is also a well-established FXR target gene. Therefore, we determined the effect of 9cRA on the CDCA-induced expression of *SHP*. In untransfected HepG2-rNtcp cells, CDCA-induced expression of *SHP* 2.0-fold and 9cRA further increased this to 2.6-fold relative to control cells (Fig. 4-1d). The additive effect was also observed when these cells were grown in medium containing 1% serum and in HepG2-rNtcp cells stably expressing rFxr (Fig. 4-1e). Thus, 9cRA agonizes CDCA-induced expression of *SHP*, while it antagonizes *BSEP* expression.

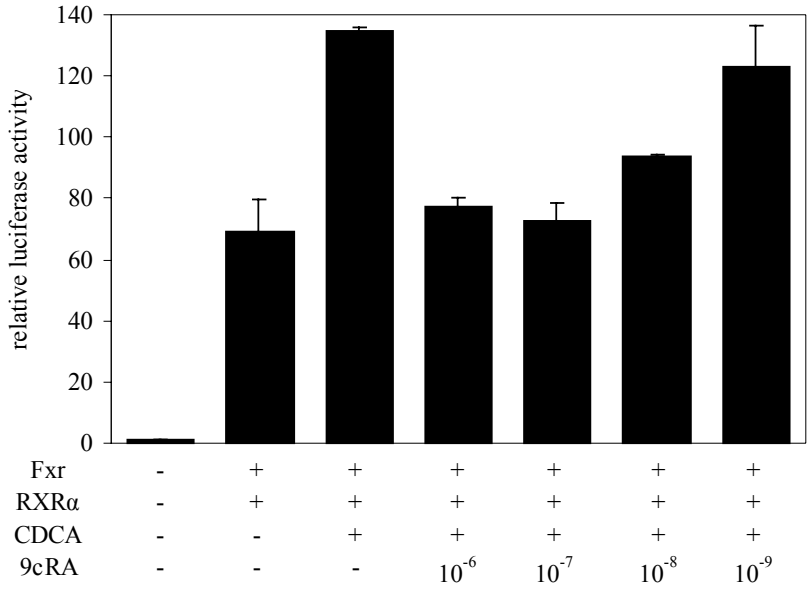


Fig. 4-2 The CDCA-induced promoter activity of *BSEP* is antagonized by 9cRA. HepG2-rNtcp cells were transfected with pGL3-277 in combination with the indicated expression vectors for hRXRα and/or rFxr, and were treated with 100 μmol/L CDCA with or without simultaneous addition of various concentrations of 9cRA (indicated as mol/L). After 24 hours, cells were harvested and luciferase activities were determined. Values are presented as mean ± sd.

9cRA reduces the CDCA-induced *BSEP* promoter activity

Luciferase reporter gene assays were performed to determine whether 9cRA acts directly on the *BSEP* promoter activity. HepG2-rNtcp cells were transfected with pGL3-277, containing the minimal *BSEP* promoter sequence including the FXR responsive element (FXRE), in combination with rFxr and hRXRα expression plasmids. When grown in the absence of CDCA and 9cRA, these cells showed significant levels of luciferase activity (69-fold induced compared to pGL3-227 alone), probably due to stimulation of the rFxr/hRXRα by bile acids produced by HepG2 cells.[26,27]. Luciferase activity was, however, super-induced by the addition of CDCA to the medium (134-fold). As observed with the endogenous *BSEP* mRNA levels, 1 μmol/L 9cRA fully inhibited the CDCA-induced luciferase activity and this effect was also dose-dependent (Fig. 4-2).

9cRA reduces FXR/RXR binding to the *BSEP*-FXR responsive element

Next, we studied whether 9cRA caused changes in the binding of the FXR/RXR heterodimer to the FXRE in the *BSEP* promoter. An electrophoretic mobility shift assay was performed using the human *BSEP* FXRE sequence and nuclear extracts

from HepG2-rNtcp cells cultured in the absence or presence of CDCA and 9cRA. In control cells (Fig. 4-3, lanes 1 and 2) and cells treated with CDCA, a clear band representing the FXR/RXR heterodimer was observed (Fig. 4-3, lane 3 and 4). In cells treated with CDCA and 9cRA this band is absent (Fig. 4-3, lanes 5 and 6), showing that 9cRA reduces binding of the endogenous human FXR/RXR heterodimer to the FXRE target sequence in the human *BSEP* promoter.

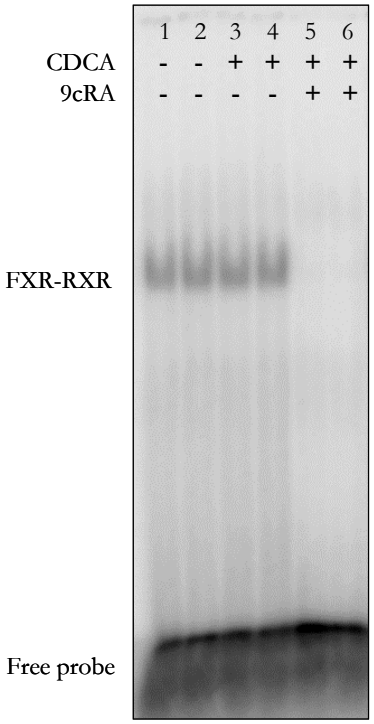


Fig. 4-3 FXR/RXR binding to the *BSEP*-FXRE oligonucleotide is lost upon 9cRA treatment. HepG2-rNtcp cells were incubated with vehicle (lanes 1 and 2), with 100 μmol/L CDCA (lanes 3 and 4), or with 100 μmol/L CDCA and 1 μmol/L 9cRA (lanes 5 and 6). Nuclear extracts were obtained and an electromobility shift assay was performed using the radioactively labeled FXRE from the *BSEP* promoter.

Vitamin A deficiency increases cholic acid-induced Bsep expression in vivo

To establish the role of vitamin A, and its derivative 9cRA, in regulating BSEP levels *in vivo*, we analyzed the effect of cholic acid-feeding in normal and vitamin A-deficient mice. From birth, male C57BL/6J mice were either fed a vitamin A-sufficient (control) or a vitamin A-deficient (VAD) diet. At 13 weeks of age, both groups were subdivided in two: one group continued on the same diet, while in the other group the diet was supplemented with 0.5% (wt/wt) CA. One week later, the mice were killed and analyzed for hepatic gene expression.

Survival of control mice was 100%, while the survival of the VAD mice was 50% (without CA) and 54% (with CA) (Table 4-1). The survivors in the VAD groups appeared healthy and showed no apparent symptoms of deficiency.
Retinol levels were undetectable in the livers (Table 4-1) and serum (data not shown) from VAD mice, whereas they were readily detectable in mice on control diets. No statistically significant differences were observed between control and VAD mice for general parameters, like body weight, liver weight, and liver to body ratio (Table 4-1), except for the mean body weight of the VAD group ($p = 0.03$ versus control) and the VAD with CA diet group ($p = 0.012$ versus VAD). The plasma bile salts of the VAD with CA diet group was significantly different compared to the control group ($p = 0.018$) and the VAD group ($p = 0.026$).

	Control n=6 Mean ± sd	**Control+CA** n=7 Mean ± sd	**VAD** n=12 Mean ± sd	**VAD+CA** n=13 Mean ± sd
Body weight (g)	26.9±1.1	25.8±.6	22.9±2.8[a]	26.6±1.1[c]
Liver weight (g)	1.0±0.2	1.2±0.1	1.0±0.2	1.1±0.2
Liver to body ratio	0.037±0.006	0.045±0.003	0.044±0.005	0.042±0.007
Plasma bile salts (µmol/L)	36.5±6.9	62.3±10.8	40.3±7.9	76.4±34.1[ac]
Survival (%)	100	100	50	54
Liver vitamin A (µmol/g)	93.6±33.8	135.9±34.2	n.d.	n.d.

Table 4-1 Animal characteristics, hepatic vitamin A level and survival percentage in male C57BL/6J mice, either fed the control or VAD diet, with or without 0.5% (wt/wt) CA supplementation. Data are presented as mean ± sd. (a: significantly different from control group; c: significantly different from VAD group; n.d.: not detectable)

Hepatic *Bsep*, *Shp*, *Ntcp* and *Rxra* mRNA expression was determined by quantitative real-time RT-PCR (Fig. 4-4) and Northern blot analysis (*Bsep*, data not shown). Both methods revealed that CA-feeding of VAD mice resulted in highest *Bsep* mRNA levels (3.4-fold increased compared to control ($p < 0.001$) whereas it led to only a 1.7-fold increase in mice on control diets (Fig. 4-4a). *Bsep* mRNA levels were not increased in mice receiving the VAD diet without CA. In contrast, *Shp* levels were highest in cholic acid-fed control mice (2.2-fold increase compared to control), slightly reduced in VAD mice (0.7-fold) but still inducible by CA-feeding (1.7-fold) (Fig. 4-4b). This was also reflected in the *Ntcp* mRNA levels that were lowest in CA-fed control mice (0.3-fold, $p = 0.007$ versus control) and not significantly changed in VAD mice (Fig. 4-4c). Importantly, *Rxra* expression was similar in all 4 groups, with only a slight, although significant, increase in the VAD+CA group (1.5-fold increase compared to control, $p = 0.007$, Fig. 4-4d).

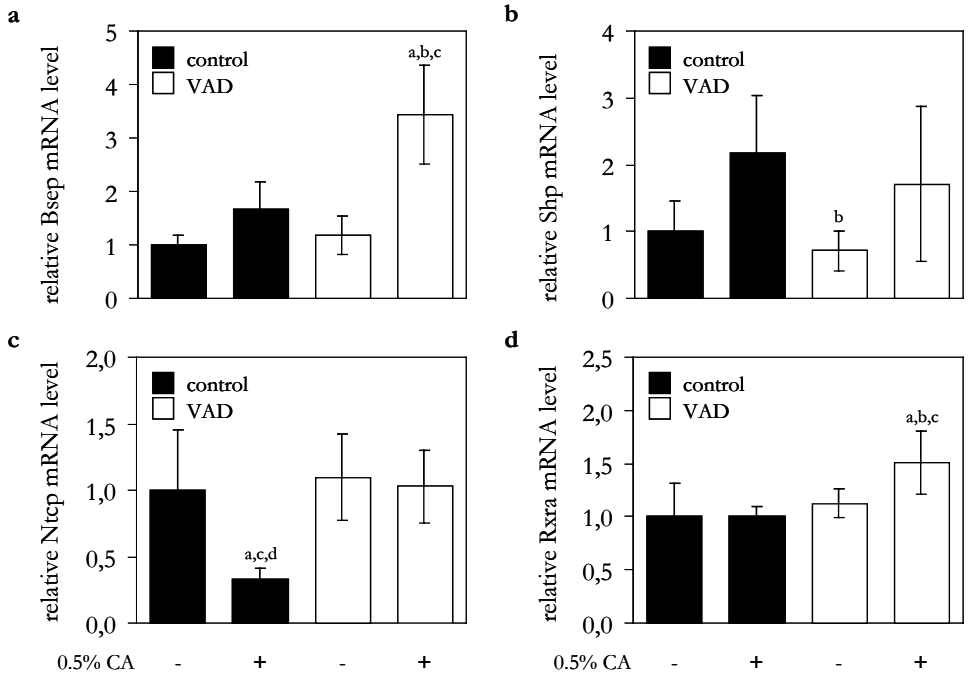


Fig. 4-4 Bsep mRNA expression is significantly increased by cholic acid-feeding in VAD mice, but not in control mice. Total RNA was isolated from liver sections of mice either fed the control diet (black bars) or VAD diet (white bars), with (+) or without (-) 0.5% (wt/wt) CA supplementation. Relative Bsep (a), Shp (b), Ntcp (c) and Rxrα (d) mRNA levels normalized to 18S were analyzed by RT-PCR. Data are presented as mean ± sd of individual mice per group. (a: significantly different from control group; b: significantly different from control+CA group; c: significantly different from VAD group; d: significantly different from VAD+CA group)

The difference in *Bsep* mRNA levels was also reflected in the Bsep protein level in liver membrane fractions (Fig. 4-5). As shown for the *Bsep* mRNA, CA-feeding in control mice and vitamin A deficiency by itself did not result in altered Bsep protein levels (Fig. 4-5). In contrast, Bsep protein levels were significantly increased in VAD mice fed a CA-containing diet (Fig. 4-5).

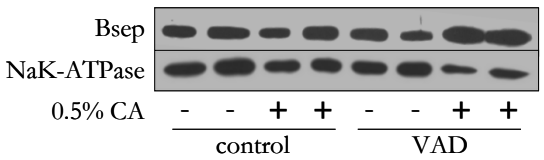


Fig. 4-5 Bsep protein expression is increased by cholic acid-feeding in VAD mice, but not in control mice. Liver plasma membranes were isolated from pools of 3 to 4 livers from mice either fed the control of VAD, supplemented with (+) or without (-) 0.5% (wt/wt) CA. Equal amounts of two independent membrane isolations were subjected to Western blot analysis, and stained for Bsep and Na+K+ATPase.

Immunofluorescence microscopical analyses of liver sections revealed a normal canalicular staining for Bsep for mice from all 4 groups, indicating that the VAD diet did not give rise to abnormal Bsep sorting in hepatocytes (Fig. 4-6).

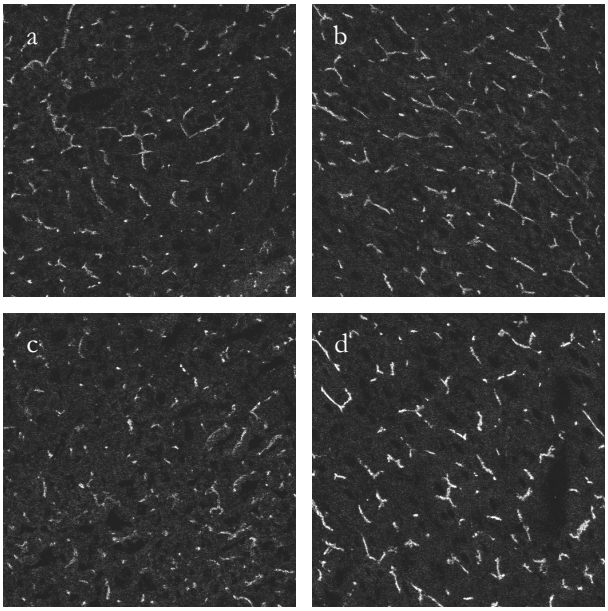


Fig. 4-6 Bsep protein expression shows a normal canalicular staining pattern in VAD mice. Liver sections from mice, fed the control diet (a), control diet supplemented with 0.5% (wt/wt) CA (b), VAD diet (c) or VAD diet supplemented with 0.5% (wt/wt) CA (d), were stained for Bsep. The protein expression pattern was analyzed using confocal laser scanning microscopy.

4.5 Discussion

In this study, we show that vitamin A and its derivative 9cRA play an important role in the transcriptional regulation of genes controlling bile acid homeostasis. *In vitro*, 9cRA antagonized the bile acid-induced expression of *BSEP* by the FXR/RXR heterodimer, while it agonized *SHP* expression. *In vivo*, vitamin A deficiency strongly increased the bile acid-induced *Bsep* mRNA and protein levels in CA-fed mice.

BSEP is specifically expressed in the liver and transports bile acids across the canalicular membrane.[28] It plays a crucial role in the enterohepatic circulation of bile acids.[29,30]. Previous studies have shown that *BSEP* gene transcription is controlled by its substrates, mediated through the bile acid sensor FXR and its heterodimer partner, RXR.[6,7]. Other FXR/RXR target genes include *SHP,*[10] *IBABP*,[8] *PLTP*,[9] *Dehydroepiandrosterone Sulfotransferase*,[31] *Apolipoprotein C-II*,[32] *Multidrug Resistance-Associated Protein 2*,[33] *Organic Anion Transporting Polypeptide 8*,[34] *Kininogen*,[35] *Bile acid-CoA synthetase* and *Bile acid-CoA: amino acid N-acetyltransferase*.[36] Expression analyses on *IBABP* and *PLTP* have shown that maximal FXR/RXR transcriptional activation was observed when both FXR- (CDCA) and RXR- (9cRA) ligands were present.[8,9] This study

revealed similar results for *SHP*, but the opposite effect was observed for *BSEP*. 9cRA strongly attenuated the bile acid-induced *BSEP* transcription by FXR/RXR.
One other example of an inhibitory effect of 9cRA on an RXR/NHR heterodimer has been described and studied in detail. In concert with RXR, the vitamin D receptor (VDR) regulates the transcription of genes involved in calcium and phosphate homeostasis.[37] Transcription of the rat osteocalcin gene was found to be activated by VDR, RXR and the VDR-ligand 1,25-dihydroxyvitamin D3, but inhibited by 9cRA, which destabilized the DNA bound RXR/VDR heterodimer.[16] Similarly, we found that the presence of 9cRA resulted in a decreased formation of the FXR/RXR-DNA complex as shown by electrophoretic mobility shift assay analysis. Our data are in line with the recent report by Kassam et al.,[38] who showed that the RXR ligands LG100268 and 9cRA inhibit the binding of *in vitro* translated FXR and RXR to the *BSEP*-FXRE. In addition, we show that 9cRA is required for maximal CDCA-induced expression of *SHP*. The molecular basis for the variable effect of 9cRA on FXR/RXR target genes is still unclear, but may be caused by differences in or close to the FXRE in the corresponding promoter elements or the selective interaction with transcription coactivators.[38]
The crucial question we wanted to address in this study was: does vitamin A play a role in *BSEP* expression *in vivo*? From the *in vitro* data we hypothesized that physiological levels of vitamin A may actually reduce bile acid-induced expression of *Bsep* in mice with a normal vitamin A status. In line with this, Wolters et al.[39] reported only minor effects on Bsep expression after taurocholic acid-feeding of wild type mice. Therefore, we fed C57BL/6J mice a vitamin A-deficient diet and analyzed its effect on mRNA levels of the Fxr-target genes *Bsep* and *Shp*. Vitamin A deficiency in mice strongly increased bile acid-dependent *Bsep* expression, whereas it reduced *Shp* expression, confirming our *in vitro* results using human HepG2 cells.
These data may have important implications for therapeutic interventions aimed at regulating canalicular bile acid secretion in cholestatic patients. Chronic liver disease, in particular chronic cholestatic liver disease, leads to vitamin A deficiency.[40,41] However, vitamin A supplementation in liver disease is a controversial issue.[42] Our data may explain this phenomenon. In chronic cholestatic liver disease, the increased intrahepatocellular bile acid concentration, in combination with a decreased vitamin A concentration, presents the optimal condition for *BSEP* induction. *NTCP*, however, remains expressed because of a lack of induction of *SHP*, the negative regulator of *NTCP*. Thus the hepatobiliary secretion of bile acids remains intact to some extend. Supplementation with vitamin A would, in theory, aggravate the cholestasis in these patients: it would reduce *BSEP* expression, activate *SHP* and thereby reduce *NTCP* expression. When viewed from the hepatocyte, vitamin A has a cytoprotective effect. However, in case of extrahepatic bile duct obstruction, persistent or elevated BSEP activity may contribute to increase intraluminal pressure in the intrahepatic cholangioles. This may result in bile infarcts and cause considerable damage of the liver parenchyma.[43] Ursodeoxycholic acid therapy may be harmful for these patients, since it further increases biliary pressure. Reducing BSEP expression through vitamin A supplementation may protect the liver in this case. Support for this hypothesis was recently provided by de Freitas et al., who showed that vitamin A supplementation in

combination with biliary obstruction results in a significant reduction of liver fibrosis.[44]

In conclusion, it is evident that vitamin A plays a crucial, but complex role in the physiological processes regulated by the nuclear hormone receptors. Vitamin A is required for optimal activation of RXR/RAR-, RXR/TR-, RXR/LXR- and RXR/PPAR-target genes. In contrast, high levels of vitamin A attenuate, and low levels stimulate, bile acid-induced transcription of *BSEP*. These observations show that 9cRA is an important molecular switch and that its effect needs to be determined for every individual RXR/NHR target gene and cannot be extrapolated from data obtained from studies on other target genes. Finally, the clinical role of vitamin A repletion in chronic liver disease needs to be redefined.

Acknowledgements

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Chapter 5

Identification of a new FXR target gene: fibrinogen Bβ

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5.1 Abstract

Bile salts act as detergents and keep hydrophobic compounds in solution in the gastrointestinal tract. They are also potent regulators of gene transcription. They bind and activate the Farnesoid X Receptor (FXR), which regulates transcription of genes encoding enzymes and transmembrane transporters involved in bile salt and cholesterol metabolism. FXR is also known to regulate bile salt/cholesterol-unrelated genes. In this study, we performed a transcriptome analysis to determine the genome-wide effect of FXR-activation and to identify possible new FXR target genes.

Native and ratFxr-overexpressing HepG2-rNtcp cells were cultured in the absence or presence of the bile salt chenodeoxycholic acid. Changes in mRNA levels were analyzed using microarrays and real time RT-PCR.

Besides known non-bile salt/cholesterol-related FXR-target genes like *kininogen, apolipoprotein AV, apoliprotein C-II* and *SLC21A88*, *Fibrinogen Bβ* was identified as a novel gene positively regulated by FXR and chenodeoxycholic acid. Analysis of the 5' regions of the human, mouse, and rat *Fibrinogen Bβ* gene revealed a highly conserved inverted repeat element separated by 9 nucleotides that might be involved in the FXR-dependent regulation.

In conclusion, this study reveals a novel, positively regulated FXR-target gene, *Fibrinogen Bβ,* which encodes for a plasma glycoprotein that is involved in blood clotting.

5.2 Introduction

Bile salts are the main solutes in bile and function as stimulators of fat digestion. They also facilitate the biliary secretion of hydrophobic compounds such as cholesterol en toxins. In recent years, bile salts have been shown to perform many other physiological functions. For example, they are mediators of apoptosis,[1] activators of protein kinase C[2] and phosphatidylinositol-3 kinase.[3] Most importantly, bile salts play a crucial role as regulators of gene transcription, by means of binding and activation of the transcription factor, farnesoid X receptor (FXR).[4,5]

FXR belongs to the nuclear hormone receptor superfamily of ligand-regulated transcription factors. It is active as a heterodimer with the retinoic acid receptor (RXRα).[6] RXRα is activated by the vitamin A derivative 9-cis retinoic acid.[7] The activated FXR/RXR heterodimer binds to a specific DNA sequence, the FXR responsive element (FXRE), in the promoter region of target genes. This FXRE consists of an inverted repeat element of two 6 base pair motif, AGGTCA, with a 1-base pair spacing (IR-).[6,8]

FXR was first identified as a receptor for farnesol metabolites, and is expressed in liver, gut, adrenal gland and kidney.[6] Recent data show that this nuclear receptor is the mammalian bile salt sensor that plays an important role in maintaining bile salt and cholesterol homeostasis. In accordance with this role, several FXR-target genes have been identified that are involved in bile salt and cholesterol metabolism. These include (1) the *bile salt export pump* (*BSEP, ABCB11*),[9] the major hepatic bile salt exporter;[10] (2) the *intestinal bile acid-binding protein*,[11] which is an intestinal protein that binds bile salts with high affinity in the cytosol of enterocytes and (3) the transcription factor *small heterodimer partner 1*.[12] Small heterodimer partner 1 in its turn represses transcription of *cytochrome P450 7a1*,[12] the rate-limiting enzyme of bile salt synthesis and the *sodium-dependent taurocholate cotransporting protein (Ntcp*), the major hepatic bile salt importer. a*polipoprotein A-1* and the *phospholipid transfer protein* are both involved in HDL-cholesterol metabolism and are also regulated by FXR.[13,14].

Recent studies have shown that FXR is also involved in the transcription of non-bile salt and cholesterol-related genes. These include a*polipoprotein AV*[15] and a*poliprotein C-II*,[16] which are both involved in triglyceride metabolism. Also the *organic anion transporting polypeptide 8* (*OATP8* or *SLC21A8*),[17] transporter of organic anions and xenobiotics, and *Kininogen (KNG)*,[18] which is involved in anti-coagulation fall into this latter category. *KNG* transcription shows the most pronounced response to CDCA-activated FXR of all FXR-target genes studied thus far.

In the present study, we performed a transcriptome analysis on human HepG2 cells stably expressing rat Fxr to determine the effect of activated Fxr on approximately 18,000 human genes with known functions. In addition to the known FXR target genes indicated above, we show that also *Fibrinogen Bβ* (*FGB*) gene transcription is positively regulated by bile salts and FXR. The genes for human, rat and mouse *FGB* contain a sequence in their promoter elements that closely resembles a FXRE. However, in contrast to the IR-1, the inverted repeat sequences in the *FGB* promoter elements are separated by 9 nucleotides. This IR-9 sequence might be a novel FXRE involved in FXR-dependent regulation of *FGB*. These results provide evidence for an

increasing number of genes unrelated to bile salt or cholesterol metabolism that are regulated by FXR. Since FXR is considered a drug target for the treatment of cholestatic conditions, possible unwanted effects may result from the changed expression of these other FXR-target genes.

5.3 Materials and methods

Cell culture

The stably transfected human hepatoma cell line HepG2, expressing the rat sodium-dependent taurocholate cotransporting protein (HepG2-rNtcp)[19] were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air, in DMEM with GlutaMAX-1, 4500 mg/L D-glucose, sodium pyruvate, pyridoxine supplemented with 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 250 ng/ml fungizone and 250 μg/ml geneticin (Invitrogen BV, Breda, The Netherlands). The cell culture was passed twice a week.

Stable transfection

Plasmid DNA was isolated and purified using the EndoFree® Plasmid Maxi Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. HepG2-rNtcp cells [19] were transfected by the calcium phosphate co-precipitation method[20] using an expression vector for rat Fxr, pCMXrFxr and the hygromycin B-selection plasmid, pHMR272.[21] Upon selection on hygromycin B-resistance, the rFxr-expressing cells were further cultured in normal HepG2-rNtcp medium.

Microarray

For the microarray analysis, HepG2-rNtcp and HepG2-rNtcp-rFxr cells were cultured in the presence or absence of 100 μmol/L CDCA (sodium salt, Calbiochem-Novabiochem, San Diego, CA) dissolved in phosphate-buffered saline (PBS) for three days. Total RNA was isolated as described[22] and labeled using the Agilent Fluorescent Direct Label Kit according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA). The control samples (HepG2-rNtcp+PBS) were labeled with Cyanine-5 and the experimental samples (HepG2-rNtcp+CDCA and HepG2-rNtcp-rFxr + CDCA) were labeled with Cyanine-3. The labeled cDNA's were hybridized on Human 1A Oligo Arrays, using the Agilent Oligonucleotide Microarray Hybridization Kit (Agilent Technologies, Palo Alto, CA). The experiment was done in duplo. The arrays were collected and analyzed using the Agilent Microarray Scanner System and Feature Extraction Software (Agilent Technologies, Palo Alto, CA). Genes meeting the criteria (average of cyanine-3 and cyanine-5 normalized signals > 8 and P-value of the Log Ratio < 0.01) were used for further analysis.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA isolation and quantitative real-time detection RT-PCR analysis of *BSEP/Bsep*, *FGB/Fgb*, *SLC21A8*, and *KNG* mRNA levels were described before.[22,23] Details about primer- and probe-sequences are available at the authors.

Animals

Male C57BL/6J mice (Harlan Nederland, Horst, the Netherlands) were housed in a temperature-controlled environment with alternating 12 hours light and dark cycles. Food and water were available ad libitum. Experimental protocols were approved by the Ethical Committee on Animal Testing of the Faculty of Medical Sciences, University of Groningen.

Experimental design

At the age of 13 weeks, the mice were divided into groups. Group 1 continued with their normal diet, while group 2 received their diet supplemented with 0.5% (wt/wt) cholic acid (CA). One week later, the mice were weighed and killed. The livers were removed, weighed, cut into pieces, snap-frozen in liquid nitrogen, and stored at -80°C for mRNA isolation.

Statistic

Data are presented as means ± sd. Differences between the two animal groups were determined in SPSS by a Mann-Whitney U-test and $p<0.05$.

5.4 Results

Generation of rFxr-expressing HepG2-rNtcp cells

FXR is a potent regulator of transcription of genes involved in bile salt and cholesterol metabolism. As such, it is regarded an important target for controlling bile salt/cholesterol homeostasis in humans.

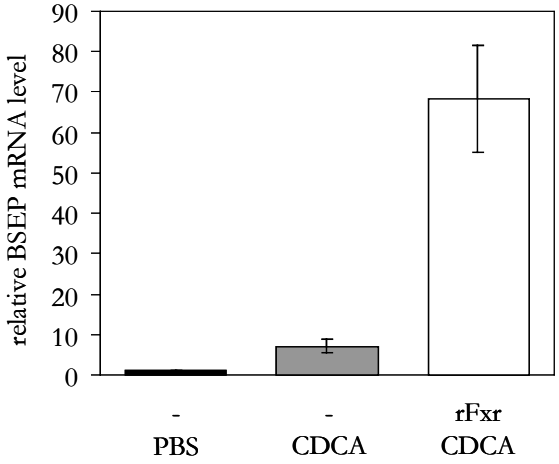

Fig. 5-1 CDCA-induced BSEP expression is strongly increased in HepG2-rNtcp cells stably transfected with rat Fxr. HepG2-rNtcp cells were incubated with vehicle (PBS, black bar) or 100 μM CDCA (gray bar) and HepG2-rNtcp-rFxr cells were incubated with 100 μM CDCA (white bar). Total RNA was isolated and BSEP mRNA levels were determined by real time RT-PCR. The level of 18S was determined as control.

In recent years, novel FXR-target genes have been identified that are not involved in bile salt/cholesterol metabolism. These genes may cause unwanted or unexpected side effects during FXR-targeted therapy. We therefore determined the effect of activated FXR on the transcription of approximately 18,000 human genes with the aim to characterize FXR-controlled processes unrelated to bile salt/cholesterol homeostasis. We generated HepG2-rNtcp cells that stably express rat Fxr for the identification of new FXR- and CDCA-regulated genes. *BSEP* mRNA expression served as a positive control for the functionality of rFxr in this cell line. Both native and rFxr-expressing cells were treated with 100 μM CDCA and compared to the native cells treated with vehicle. As expected, *BSEP* expression was significantly increased by both CDCA and rFxr, showing 7-fold increased mRNA levels in CDCA-treated HepG2-rNtcp and a 68-fold increase in CDCA-treated HepG2-rNtcp-rFxr cells, respectively, compared to HepG2-rNtcp cells grown in the absence of CDCA (Fig. 5-1).

Selection of significant up-regulation or down-regulation of genes in microarray

Next, the same cDNA samples were analyzed using the Agilent Human 1A Oligo Arrays to identify novel FXR target genes. Four individual microarray hybridization experiments were performed. cDNA from control cells (group *C*) were labeled with Cy5 and used as control in all 4 experiments. cDNA from CDCA-treated HepG2-rNtcp (*CDCA*) or HepG2-rNtcp-rFxr (*FXR/CDCA*) cells were labeled with Cy3. Two arrays were hybridized with the combination *C* versus *CDCA* (chip 1a and b). The other two with the combination *C* versus *FXR/CDCA* (chip 2a and b). The rational behind this setup was that possible rFxr-unrelated CDCA effects on transcription could be excluded, and that limited regulation of FXR-target genes would be strongly enhanced in the rFxr-transfected cells. Criteria were set to evaluate the significance of changed expression levels under the 3 different conditions. These parameters were: (1) The CDCA-induced expression (*C* versus *CDCA*) should be at least 1.5; (2) the average of both (Cy3 and Cy5) normalized (log) signal intensities should be > 8. This limit is arbitrary and signals with an intensity of ≤ 8 are assumed to be unreliable; (3) the P value for significance was < 0.01. Four hundred thirty five (435) transcripts met these criteria on both a and b chips and were included for the final analysis: The rFxr-induced expression (*CDCA* versus *FXR/CDCA)* should be at least 1.2.

Positively regulated genes by CDCA-activated rFxr

BSEP expression, though used to validate our experimental setup (Fig. 5-1), did not meet the microarray analysis parameters. This was, however, not unexpected since HepG2 cells are well known to express *BSEP* only at very low levels.[22] Even the 68-fold increase of *BSEP* in the CDCA-treated HepG2-rNtcp-rFxr cells remained below the detection limits of the microarray 2. Two other established FXR-target genes, *SLC21A8*[17]) and *KNG*,[18] were expressed to significant levels and were selected to determine their expression differences between the two arrays (bold in Table 5-1). *KNG* shows the strongest relative increase to CDCA-treatment, i.e. 5.5-fold in

HepG2-rNtcp cells and 7.9-fold in HepG2-rNtcp-rFxr cells. *SLC21A8* mRNA expression levels were 2.2-fold increased in HepG2-rNtcp cells and 3.0-fold in HepG2-rNtcp-rFxr cells. The additive effect of rFxr for both *SLC21A88* and *KNG*, is a 1.4-fold increase according to microarray analysis.

Name	CDCA mean±sd	FXR/CDCA mean±sd	CDCA vs. FXR/CDCA
FGB	2.68±0.87	5.72±2.02	2.1
KNG	5.49±2.06	7.91±2.50	1.4
FGG	1.99±0.22	2.79±0.49	1.4
SLC21A8	2.16±0.33	2.98±0.78	1.4
SURF6	2.07±0.79	2.77±0.16	1.3
FGA	1.41	1.87±0.29	1.3
FLJ20671	2.31±0.02	3.01±0.05	1.3
LOC89953	3.70±0.97	4.77±0.92	1.3
RCN1	1.57±0.24	1.98±0.15	1.3
TMOD4	3.14±0.92	3.87±0.53	1.2
I_1152006	2.91±0.06	3.56±0.04	1.2
CAPN5	3.10±0.42	3.77±0.17	1.2
PPAP2A	3.33±0.54	4.05±0.26	1.2

Table 5-1: List of human genes of which the expression is increased at least 1.2-fold by CDCA and rFxr in HepG2-Ntcp cells. Agilent Human 1A Oligo arrays were hybridized with cDNA obtained from HepG2-rNtcp cells and HepG2-rNtcp-rFxr cells treated with CDCA (CDCA and FXR/CDCA respectively). The mRNA expression levels of both CDCA-treated cell lines were compared to those in HepG2-rNtcp cells treated with the vehicle, PBS (group C). Only the genes that meet the criteria as described in experimental procedures were analyzed for the effect of the presence of rFxr. Note: mRNA expression levels for FGA in CDCA column did not meet all criteria, but are included for comparison to FGB and FGG.

To more accurately quantify the relative increase caused by CDCA and rFxr separately, RT-PCR was performed for *SLC21A8* (Fig. 5-2a) and *KNG* (Fig. 5-2b). In CDCA-treated HepG2-rNtcp cells, the *SLC21A8* and *KNG* mRNA levels were increased with 4.9-fold and 20.6-fold, respectively. These values further increased to 10.2-fold and 33.7-fold, respectively, in the CDCA-treated HepG2-rNtcp-rFxr cells. According to this analysis the rFxr-specific effect for *SLC21A8* is 2.0-fold and for *KNG* 1.6-fold, whereas for *BSEP* a rFxr effect of 9.7 was detected (Fig. 5-1).

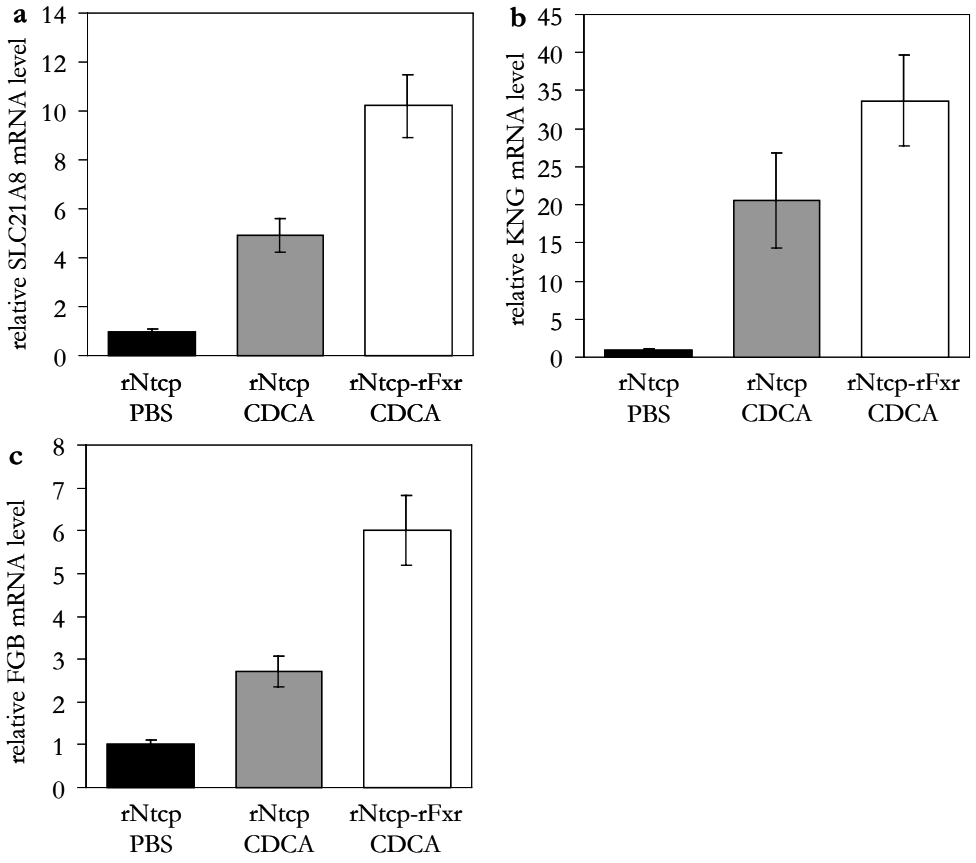

Fig. 5-2 Human *FGB* expression is increased by CDCA and rFxr in HepG2-Ntcp cells. HepG2-rNtcp cells were incubated with vehicle (PBS, black bar) or 100 μM CDCA (gray bar) and HepG2-rNtcp-rFxr cells were incubated with 100 μM CDCA (white bar). Total RNA was isolated and *SLC21A8* (a), *KNG* (b), and *FGB* (c) mRNA levels were determined by real time RT-PCR. The level of *18S* was determined as control.

Human *Fibrinogen Bβ* is a putative FXR-target gene

Table 5-1 shows that only few additional genes confirmed to our set criteria of 1.5-fold up-regulation upon CDCA-treatment of HepG2-rNtcp cells *and* a further significant increase of at least 1.2-fold in CDCA-treated HepG2-rNtcp-rFxr cells. The most eye-catching genes from this shortlist are that of Fibrinogen Aα, Bβ and gamma (*FGA*, *FGB* and *FGG*). Together, these genes encode the plasmaprotein Fibrinogen. The *FGB* gene shows the strongest response to rFxr from all detected genes in the microarray experiment. The *FGB* mRNA was increased 2.7-fold and 5.7-fold in CDCA-treated HepG2-Ntcp and HepG2-Ntcp-Fxr cells, respectively. Thus, the rFxr-effect for *FGB* amounts to 2.1-fold. Though significantly below the 9.7-fold observed for *BSEP* by RT-PCR, it tops all genes detected in the microarray experiment, including *KNG* and *SLC21A88*. The rFxr-effect was confirmed by RT-PCR analysis

for *FGB*, in which a 2.7-fold and 6.0-fold increase was detected in CDCA-treated HepG2-Ntcp and HepG2-Ntcp-Fxr cells, respectively. In addition, the expression of *FGA* and *FGG* was also significantly increased by CDCA and rFxr (Table 5-1). For *FGG* the CDCA-effect was 2.0-fold and the additional rFxr-effect 1.4-fold. Although *FGA* (in italic in table 5-1) did not meet our preset criterion of 1.5 (the CDCA-effect was just 1.4-fold), we added this gene to the list of up-regulated genes, because of its relation to *FGB* The additional rFxr effect for *FGA* was 1.3-fold.. Thus, all three genes encoding the 3 subunits of Fibrinogen show enhanced expression upon CDCA-activation of rFxr in HepG2-Ntcp cells.

Fibrinogen Bβ mRNA expression in cholate-fed mice

To determine whether FXR-controlled expression of *FGB* also occurs *in vivo* we studied hepatic expression in mice fed a bile salt-containing diet and compared these to mice fed normal chow. Male C57BL/6J mice were fed a 0.5% cholate diet during one week. Total RNA from the livers was isolated and *Fgb* and *Bsep* mRNA expression levels were quantified using RT-PCR (Fig. 5-3). The *Fgb* mRNA level was 1.3-fold ($p = 0.073$) increased in cholate-fed animals compared to control mice, whereas *Bsep* mRNA levels were 1.7-fold ($p = 0.001$) higher in the cholate-fed animals.

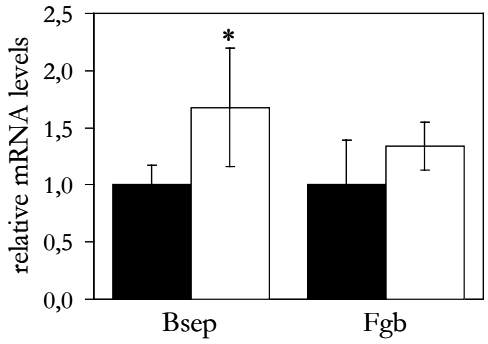

Fig. 5-3 The effect of cholate-feeding on hepatic *Fgb* expression in mouse. C57BL/6J mice were fed either a control diet (black bars) or a 0.5% CA-supplemented diet (white bar) for one week. Total RNA was isolated from their livers and *Bsep* and *Fgb* mRNA levels were determined by real time RT-PCR. The level of *18S* was determined as control. Data are presented as mean ± sd. (*: significantly different from control diet, $p < 0.05$)

Identification of a putative FXRE in the human *Fibrinogen Bβ* promoter region

The positive regulation of the *FGB* gene by CDCA and rFxr suggest that FXR is directly involved in the regulation of this gene. This implies that FXR binds the *FGB* promoter element. Concurrently, we screened the *FGB* promoter region from human (gi:31400), mouse (gi:38077445) and rat (gi:204100) for candidate FXRE's. All three sequences show a high degree of identity/similarity in the region immediately upstream of the transcription start site (Fig. 5-4). In this region we identified a highly conserved element (from nucleotide –94 to –74 in the human *FGB* promoter; +1 is

the transcription initiation site), which may function as an FXRE. In human and rat, it consists of a perfect inverted repeat of two 6 base pair-motifs, GGTTCA and GTAACC, which are separated by 9 nucleotides (which would than be a IR-9). In the 5'-flanking region of the mouse *Fgb* gene, there is one nucleotide difference compared to the human and rat sequence; AGTTCA and TGAACC.

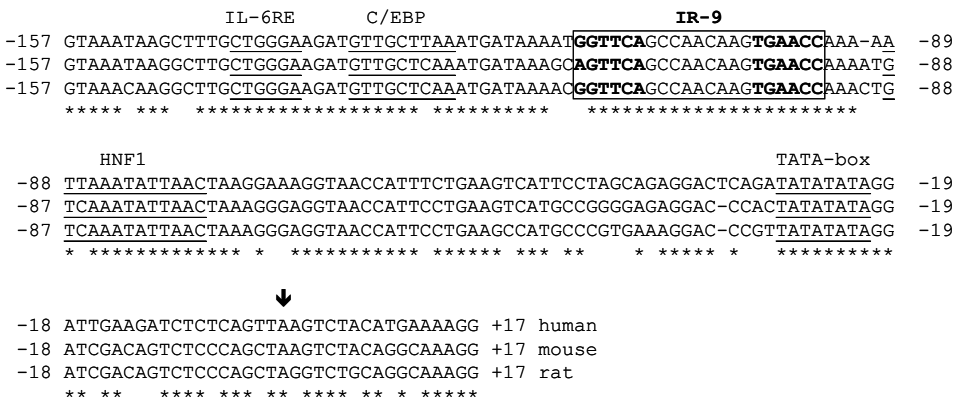

Fig. 5-4 The promoter region of mammalian *FGB* genes contain a putative FXRE. Sequences of the 5'-flanking regions of the human, mouse, and rat *FGB* genes are shown. The conserved nucleotides are indicated by *. The site of transcription initiation is designated as +1 (🡻). The regulatory sequences IL-6RE, C/EBP, HNF1, and TATA-box, established by previous studies, are underlined. The proposed IR-9 is boxed, the inverted repeat element is shown in bold.

5.5 Discussion

In this study we show that Farnesoid X receptor (FXR) directly regulates the transcription of genes encoding human Fibrinogen. This is another example of FXR regulating the transcription of genes that appear unrelated to bile salt and/or cholesterol homeostasis. These effects need to be considered in future therapies aiming at manipulating bile salt and/or cholesterol levels through FXR.

FXR is generally viewed as the bile salt sensor that regulates transcription of key genes involved in bile salt homeostasis. It may activate transcription of target genes directly after binding to FXR-response elements in their promoter region, or repress transcription indirectly through up-regulation of the transcriptional repressor, small heterodimer partner (SHP-1). Genes involved in bile salt homeostasis that are directly or indirectly regulated by FXR include the ones for the rate-limiting enzyme in bile salt biosynthesis, *cytochrome p450 7a1*, the hepatic bile salt transporters *Ntcp* (uptake) and *BSEP* (secretion), and *intestinal bile acid binding protein*. It is therefore an important pharmacological target to control the enterohepatic circulation of bile salts. Recent studies have shown that FXR also directly regulates genes that are not linked to bile salt and cholesterol metabolism. We performed microarray analyses to reveal more of these non-bile salt and cholesterol related genes as targets for FXR. HepG2-rNtcp cells and their rat Fxr-expressing derivatives, the HepG2-rNtcp-rFxr cells, were

treated with CDCA. This treatment resulted in increased mRNA expression levels of already established FXR-regulated genes, like *SLC21A8* and *KNG*. *BSEP* expression remained undetectable in the microarray experiments, even though its expression is 68-fold increased in the CDCA treated HepG2-rNtcp-rFxr cells. *BSEP* expression is known to be low in HepG2 cells. The limited sensitivity of microarray experiments is clearly a drawback of this technique and biases the results towards highly expressed genes. Physiological important FXR-target genes may therefore escape identification using immortalized cells like HepG2 that show strongly reduced basal mRNA levels of subsets of genes, including *BSEP* and *NTCP*. However their accessibility towards genetic manipulation, here stable rFxr-overexpression, does result in relevant data about genes that are (still) expressed at detectable levels in such cells.

The gene that showed the highest increase upon FXR-activation was *Fibrinogen Bβ* (*FGB*). FGB is a subunit of Fibrinogen that in addition consists of Fibrinogen Aα (FGA) and γ (FGG). Fibrinogen is a plasma glycoprotein synthesized in the liver and plays a role in blood clotting. The *FGA*, *FGB* and *FGG* genes are located in a cluster on chromosome 4.[24] Upon vascular injury, it is cleaved by thrombin to form fibrin. This is achieved by coordinate transcriptional regulation of all three genes, in which interleukin-6 (IL-6) plays an important role.[25] In addition, several cleavage products of fibrinogen and fibrin are involved in cell adhesion, vasoconstriction, chemotactic and mitogenic activities.[26] The promoter regions of the human, mouse and rat *FGB* genes are highly conserved between position –157 and position +17 relative to transcriptional start site. Previous studies have shown that several regulatory elements are located within this region.[27] For example, the relevance of an IL-6 responsive element, a C/EBP motif and an HNF-1 binding site has been demonstrated.[28-30] Our analysis of this region revealed a highly conserved and new element between nucleotides –94 and –74. This element consists of a perfect inverted repeat separated by 9 base pairs (GGTTCA-N_9-GTAACC). We hypothesize that this inverted repeat may be a novel FXR responsive element. The consensus FXRE is an inverted repeat of the AGGTCA motif with a 1 base pair spacing (IR-1). However, many exceptions on this consensus have been described that also bind FXR, resulting in increased transcription of the downstream gene. The rat *dehydroepiandrosterone sulfotransferase* promoter contains an inverted repeat with no spacing (IR-0[31]), the human *multidrug resistance-related protein 2* promoter contains an everted repeat with a 8 base pair spacing (ER-8[32]), and human *apolipoprotein-AV* contains an inverted repeat with an 8 base pair spacing.[15] Since the IR-9 in the *FGB* promoter is conserved between species, we consider this sequence as a putative FXRE. A previous study already identified the positions –94 and –93 in the human *FGB* promoter as a positive cis-acting element.[30]

The IR-9 is present in the 5'-flanking region of both rat and mouse *Fgb*. Therefore, we performed experiments with freshly isolated primary rat hepatocytes, exposed them to CDCA and determined the effect of *Fgb* gene transcription. Unfortunately, *Fgb* mRNA levels strongly increase during the isolation procedure of hepatocytes, which returns to basal levels only 2 to 3 days after seeding the cells. During this period, expression of the bile salt uptake transporter, *Ntcp*, is reduced to below 10% of its normal level in hepatocytes. Consequently, we have not been able to determine the effect of bile salts on the expression of *Fgb* in rat hepatocytes. In addition, we have

analyzed the expression of *Fgb* in livers of mice fed a diet containing 0.5% cholate for 1 week. The *Fgb* mRNA expression showed a tendency to be increased in livers of the CA-fed animals (1.3-fold) compared to animals fed control chow, however the difference was not significant (P=0.073), primarily due to small number of animals analyzed (control diet: n=6; cholate diet n=7). The relatively low induction level of *Fgb* does, however, not mean that this might not be physiological relevant. Also expression of *Bsep* was only increased 1.7-fold and this has previously been shown to result in a significant increase of hepatic bile salt secretion.[33]

No candidate FXRE's were detected in the promoter elements of the *FGA* and *FGG* genes even though the expression of both genes were increased by FXR and CDCA. The increased expression of *FGA* and *FGG* may however be caused by FGB, since it is known that overexpression of one of the *Fibrinogen* genes may stimulate expression of the other two genes.[34]

Cholestatic liver diseases, like primary biliary cirrhosis and primary sclerosing cholangitis, are known to be associated with preserved clothing function or even a hypercoagulable state.[35,36] This may explain the high incidence of portal vein thrombosis in primary biliary cirrhosis.[35] The hypercoagulable state may in part offset the decreased synthesis of clothing factors during advanced liver cirrhosis. The study of Pihusch *et al.*, showed increased Fibrinogen levels in primary biliary cirrhosis and primary sclerosing cholangitis patients.[36] Our findings suggest that in cholestatic liver diseases fibrinogen synthesis is stimulated by bile salts, thus counteracting a decreased synthesis due to cirrhosis. This may aid the preservation of blood clothing in cholestatic liver disease.

In conclusion, our results show that *FGB* transcription is positively regulated by FXR and bile salts. As such, it is another example showing that FXR transcriptional regulation is not restricted to genes involved in bile salt and cholesterol metabolism.

Note:

During finalizing this thesis, Anisfeld et al.[37] also reported that FXR activates *FGB* gene transcription. Our results are largely in line with their study. In addition, they performed a promoter deletion analysis to locate the FXR response element (FXRE) in the human *FGB* promoter. They show that the region -2281/-1700 is required for FXR-induced transcription after activation by the synthetic ligand GW4064. This region does not contain a sequence that conforms to the currently known FXRE's. Future experiments need to reveal which sequence in the *FGB* promoter directly binds FXR.

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Summary

Introduction

The liver is the second largest organ of the body and plays an important role in the metabolism of carbohydrates, fats, and proteins. It is the main producer of plasma proteins, like albumin and coagulation factors. It is also the organ involved in cleaning the body from toxic and waste products, eliminating a wide variety of these compounds predominantly via bile and feces. Bile is not only an escape route, but is also necessary for efficient digestion and absorption of dietary fats and fat-soluble vitamins. The driving force for bile formation is the hepatic synthesis and secretion of bile salts. After delivery into the intestine, bile salts are efficiently reabsorbed and returned to the liver. This shuttle of bile salts between the liver and intestine is called the enterohepatic circulation of bile salts. Every cycle, a small portion (~ 5%) of bile salts is lost via feces. *De novo* bile salt synthesis from cholesterol takes place in the liver parenchymal cells (hepatocytes) and substitutes for the fecal loss. Improper functioning of the enterohepatic circulation may lead to cholestasis, i.e. high serum levels of bile salts, and liver disease.

The hepatocytes form a physical barrier between blood and bile. Bile salts and most other compounds cannot pass this barrier without the help of specialized transport systems. The hepatocyte houses various specific transport proteins located at the cell membrane, mediating the vectorial transport of compounds from blood to bile. For example, reabsorbed bile salts are taken up from the blood by members of the "solute carriers" superfamily. For secretion into bile, a steep bile salt concentration gradient must be overcome. This transport requires ATP hydrolysis and is accomplished by the Bile Salt Export Pump (also called ABCB11).

Classically, bile salts are considered a detergent that is required to keep hydrophobic compounds, such as cholesterol or vitamins, in solution to aid in their excretion or absorption. Recent research has, however, shown that bile salts are also important signaling molecules. For instance, bile salts have been shown to regulate their own synthesis rate, as well as the transporters active in the enterohepatic circulation. They can bind to, and activate the transcription factor Farnesoid X Receptor (FXR), which in its turn regulates transcription of the genes that produce rate-limiting enzymes in the bile salt biosynthetic pathway, as well as the bile salt transporters. The Bile Salt Export Pump, bile salts and FXR are the central topics of this thesis. In **chapter 1**, an overview is given of the current knowledge of hepatic transport proteins, their roles in inherited and acquired liver disease, and their function and regulation under normal and pathophysiological conditions.

The D482G mutation results in a functional but unstable BSEP protein

The inherited liver disease, Progressive Familial Intrahepatic Cholestasis type 2 (PFIC2), is characterized by jaundice, itching, low biliary bile salt level, high serum bile salts level, but normal serum γ-glutamyltransferase (γ-GT) levels. These features imply that defective transport of bile salts from the hepatocyte into bile is the basis for this type of cholestasis. It has been shown that PFIC2 patients have mutations in the

ABCB11 gene, which encodes for the hepatocanalicular bile salt export pump (BSEP). One of these PFIC2-causing mutations is studied in detail in **chapter 2**. The mutant protein harbors a glycine (G) residue at amino acid position 482 in stead of an aspartate (D), which is present at this position in the normal (wild type) protein. The rest of the 1,320 amino acids of both proteins are identical. We examined what the effect this specific mutation (D482G) is on bile salt transport activity, canalicular sorting and protein expression of BSEP. To study the effect on protein activity we expressed the murine Bsep protein in insect cells and performed ATPase and transport assays. Surprisingly, the mutation, which is located in one of the two ATP-binding domains, did not abolish the ATPase nor the transport of the bile salt, taurocholate. To study the effect on protein sorting and expression, we expressed murine Bsep tagged with the Green Fluorescent Protein (GFP) in human hepatoma cells (HepG2). We observed that at physiological temperatures (37^{o}C) the sorting as well as the protein expression were strongly effected. The GFP-Bsep$^{[D482G]}$ was not fully glycosylated and significant amounts of the protein were detected in the cytoplasm. In addition, the amount of GFP-Bsep$^{[D482G]}$ protein present was significantly lower compared to GFP-Bsep$^{[WT]}$ whereas the corresponding mRNA levels were 4 to 5-fold higher for GFP-Bsep$^{[D482G]}$ in the obtained stably transfected HepG2 cell lines. Lowering the culture temperature to 30^{o}C increased the GFP-Bsep$^{[D482G]}$ protein level to 4 to 5-fold compared to GFP-Bsep$^{[WT]}$, and resulted in efficiently targeted and fully glycosylated protein. These data demonstrated that the D482G mutation results in a still functional, but highly unstable BSEP protein. Therefore, PFIC2 patients, carrying this specific mutation, may benefit from therapies aimed at stabilization of the protein.

Bile salts and FXR regulate BSEP transcription

In **chapter 3** the relationship between bile salts, FXR and the expression of the human *ABCB11* gene (encoding BSEP) is described. HepG2 cells were incubated with the bile salt chenodeoxycholic acid (CDCA), which resulted in a 2-3 fold increased expression of *ABCB11*. The promoters of human and mouse *ABCB11* were analyzed for regulatory elements and a candidate responsive element for the transcription factor FXR was found. We cloned the promoter region of the human *ABCB11* gene containing this element and demonstrated that bile salts and FXR positively regulate *ABCB11* promoter activity, leading to increased *ABCB11* mRNA levels. Mutation of the FXR responsive element confirmed that this region in the *ABCB11* promoter is responsible for bile salt-induced transcription of the *ABCB11* gene. In addition, we showed that bile salts were unable to increase *ABCB11* transcription, when the amount of endogenous FXR was reduced by RNA interference. We conclude that *ABCB11* gene transcription is positively regulated by bile salts through activation of FXR. The level of *ABCB11* transcription activation is dependent of the cellular amount of this transcription factor.

Vitamin A is important for BSEP expression

Bile salts facilitate the uptake of fat-soluble vitamins, including vitamin A, D, E and K. The liver is a rich source of vitamin A, where it is stored in the hepatic stellate cells.

Vitamin A may be converted into 9-cis retinoic acid (9cRA) which is activating ligand for the transcription factor retinoid X receptor (RXR). RXR is another member of the superfamily of nuclear hormone receptors (NHRs) and functions as a key-regulator of gene transcription. It is a central dimerization partner for several NHRs, including FXR. 9cRA-activated RXR is considered to be a permissive partner of FXR, since both NHRs and both ligands are required for maximal induction of transcription of target genes, such as *I-BABP* and *PLTP*. In **chapter 4** we investigate whether this is also true for *ABCB11* gene transcription. Surprisingly, 9cRA reduced the bile salt-induced *ABCB11* mRNA expression in human hepatoma cells almost to control level. This effect of 9cRA is exerted via a decrease in binding of the FXR/RXR heterodimer to the FXR responsive element of the *ABCB11* promoter. To determine whether the inhibition of *ABCB11* expression by 9cRA plays a role *in vivo*, we generated vitamin A-deficient (VAD) mice and determine the effect of bile salt feeding compared to control (normal vitamin A) mice. In VAD mice , Bsep (*Abcb11*) mRNA and protein expression was significantly more increased by bile salts (cholate) compare to bile salt-fed control mice. We conclude that the vitamin A derivative 9cRA indeed plays an active role in the bile salt-dependent regulation of human and mouse *ABCB11*/*Abcb11* transcription. The physiological rational behind this mechanism may be that at low vitamin A levels, bile salt secretion is increased to aid in the intestinal absorption of this vitamin. The treatment of cholestatic patients with vitamin A-containing supplements, however, may need further exploration, since this may result in extra inhibition of BSEP expression, whereas the therapeutic goal would be the opposite.

FXR is involved in transcription of non-bile salt related genes

In the past few years, several genes have been shown to be transcriptionally regulated by FXR and bile salts. Most of them are involved in bile salt, cholesterol, and lipoprotein metabolism. FXR-target genes are however not restricted to these metabolic pathways, exemplified by the identification of Kininogen as a FXR-target. To get insight in genome-wide effects of bile salt-activated FXR, we performed a transcriptome analysis. This search for novel FXR and bile salt regulated genes is described in **chapter 5**. We generated a human hepatoma cell line, which stably overexpresses rat Fxr (HepG2-rFxr). These and the native cells were treated with the bile salt, chenodeoxycholic acid and mRNA expression of over 13.000 human genes were analyzed using microarrays. A novel candidate FXR-target gene was identified from this analysis, namely fibrinogen beta. Expression of this gene was induced in HepG2 cells by CDCA alone and superinduced in CDCA-treated HepG2-rFxr cells. Together with fibrinogen alpha and gamma, this protein forms fibrinogen, a plasma protein. It is synthesized in the liver and is involved in blood coagulation and possibly in the formation of liver fibrosis. Screening the conserved promoter regions of human, rat and murine fibrinogen beta for possible regulatory elements revealed a candidate FXR responsive element. Additional studies are necessary to firmly establish that this element is indeed responsible for the FXR and bile salt-dependent regulation of fibrinogen beta gene transcription.

Conclusions and perspectives

During the past decade, important progress has been made in our understanding of the pathophysiology of cholestasis. Inherited disorders have been explained at the molecular level and were shown to be the result of mutations in enzymes involved in bile salt biosynthesis or transmembrane transporters involved in bile formation. Acquired cholestasis, for instance due to inflammation, is linked to disregulation of these proteins. The challenge of future research is to use this knowledge to develop successful therapies for cholestatic patients. The studies described in this thesis initiate such studies. We showed that a mutation in BSEP may cause PFIC-2 even though the protein product is a normally active bile salt export pump. The cause of the disease is due to a specific mutation in the *BSEP* gene that strongly reduces the BSEP protein level, most likely due to increased protein degradation. This phenomenon has also been described for several other inherited diseases. An important focus of research is therefore to define conditions or drug therapies that will maintain or increase the protein level of the mutant, but active protein in these patients.

Novel targets to treat cholestasis are the transcription factors that regulate the levels of enzymes and transporters involved in the synthesis and enterohepatic circulation of bile salts. RXR and FXR are key players in this process. Endogenous and synthetic ligands for these transcription factors have been identified. Recent research, however, shows that the activation of FXR-target genes is ligand-dependent. In other words, a specific target gene may be activated by the endogenous ligand, CDCA, but not by the synthetic ligand GW4064. In addition, our own research shows that the ligand for RXR, 9-cis retinoic acid, in combination with bile salt-activated FXR, may simultaneously stimulate and inhibit expression of a subset of FXR-target genes. Therapies to treat cholestatic disease aimed at modulating the activity of these transcription factors therefore requires an in depth study of the effect of the (combination of the) individual ligands on human gene transcription.

In the meantime, it should be noted that in a significant number of PFIC patients the genetic defect still has not been elucidated. Therefore, novel genes/proteins that are essential for bile salt homeostasis may be identified in the near future.

Altogether, many challenges await us still to further understand the (molecular) causes of cholestasis and to develop successful therapies for this disease.

Samenvatting

Achtergrond

Op de huid na is de lever het grootste orgaan van ons lichaam. De menselijke lever is ongeveer 1,5 kilo zwaar. Het is eigenlijk de chemische fabriek van ons lichaam. De functies van de lever zijn dan ook heel divers, maar kunnen grofweg in drieën worden opgesplitst: de vorming van gal, dat nodig is voor de spijsvertering, het onschadelijk maken en uitscheiden van lichaamseigen afvalstoffen en lichaamsvreemde stoffen (bijvoorbeeld medicijnen en giftige stoffen) en de stofwisseling van koolhydraten, vetten, eiwitten en hormonen.

De cellen die deze functies uitoefenen zijn de hepatocyten. De lever bestaat voor het grootste deel uit deze cellen. Hepatocyten werken als een soort filter. Ze nemen allerlei stoffen op uit het bloed, en scheiden andere stoffen uit. Deze stoffen gaan òf (terug) naar het bloed òf naar de gal. Dit kan meestal niet zomaar, omdat elke lichaamscel door een zogenaamd celmembraan wordt omgeven. De celmembraan vormt een barrière voor veel stoffen. Om toch deze stoffen te kunnen opnemen en uitscheiden, maakt de hepatocyt gebruik van eiwitten die in de celmembraan zitten en die deze stoffen over de membraan kunnen transporteren: de transporteiwitten. Een hepatocyt grenst zowel aan een bloedvaatje, de zogenaamde sinusoïde, als aan een galkanaaltje, dat de gal afvoert naar de galblaas. Het gedeelte van de celmembraan dat grenst aan het bloed wordt de sinusoïdale membraan genoemd, en het gedeelte van de celmembraan dat aan het gal grenst, wordt de canaliculaire membraan genoemd. Via de sinusoïdale membraan worden stoffen opgenomen uit het bloed, maar ook afgegeven aan het bloed. Via de canaliculaire membraan worden stoffen uitgescheiden naar de gal. Elk transporteiwit is gespecialiseerd in het transporteren van bepaalde stoffen en zit in één van de membranen. Als deze transportprocessen niet goed functioneren, kan dit resulteren in (lever)ziekte. Hierbij ontstaat vaak een stapeling van bepaalde stoffen in het bloed en de lever die niet goed naar de gal getransporteerd worden. Een bekend voorbeeld hiervan is geelzucht, waarbij het transport van bilirubine naar de gal is verstoord. Door de hoge concentraties bilirubine in het bloed ontstaat de gele (huids)kleur. Dit is echter een relatief onschuldige conditie omdat bilirubine zelf geen toxische effecten heeft. Andere galcomponenten, zoals galzouten of fosfolipiden, zijn wel zeer schadelijk als deze niet goed naar de gal worden uitgescheiden. In dit proefschrift is vooral het belang en de regulatie van galzouttransport naar de gal geanalyseerd. **Hoofdstuk 1** beschrijft de huidige stand van zaken wat betreft de kennis over transporteiwitten van de hepatocyt die betrokken zijn bij galvorming. Speciale aandacht wordt gegeven aan de eiwitten die galzouten transporteren en de mechanismen die de hoeveelheid en activiteit van deze eiwitten bepalen.

Eén van deze eiwitten wordt uitgebreider bestudeerd in de volgende drie hoofdstukken: de Bile Salt Export Pump, afgekort BSEP. Deze galzoutexportpomp komt alleen in de lever voor en zit in de canaliculaire membraan. Daar is dit eiwit verantwoordelijk voor het transport van galzouten uit de hepatocyt naar de gal. Gal bestaat voor het grootste deel uit water en voor het overige uit opgeloste stoffen, waaronder de galzouten. Galzouten spelen een belangrijke rol bij de vertering van vettige voedingsstoffen. Ze functioneren als een soort zeep: ze zorgen er namelijk

voor dat deze stoffen in de darmen in oplossing blijven, zodat ze beter opgenomen kunnen worden door de darmcellen. Hiermee zijn ze essentieel voor de uitscheiding van "giftige" verbindingen. Tegelijkertijd zijn ze ook belangrijk voor de opname van vetachtige voedingsstoffen in de darm, zoals vitaminen (A, D, E en K). De lever is de enige plaats in het lichaam waar galzouten gemaakt worden. Dit is een ingewikkeld proces waarbij cholesterol wordt omgezet in galzouten. Vervolgens worden ze in een galkanaaltje uitgescheiden en komen ze uiteindelijk terecht in hun opslagplaats, de galblaas. Wanneer galzouten nodig zijn voor de vertering van vettige voedingsstoffen worden ze met de gal uitgescheiden in de darm. Ons lichaam gaat heel zuinig om met de galzouten. Het merendeel wordt dan ook weer opgenomen door de darmcellen en worden via het bloed weer teruggevoerd naar de lever. De hepatocyten nemen de galzouten uit het bloed op om ze weer te kunnen uitscheiden in het galkanaaltje. De route die de galzouten afleggen tussen darmen en lever wordt de enterohepatische kringloop van galzouten genoemd (entero = darmen; hepatisch = lever). Deze kringloop wordt in stand gehouden door transporteiwitten in de darm en in de lever, waaronder BSEP. Wanneer deze kringloop wordt verstoord, bijvoorbeeld door een defect in BSEP, is er sprake van cholestase.

De D482G-mutatie leidt tot een functioneel maar instabiel BSEP-eiwit

Kinderen met de erfelijke leverziekte "Progressive Familial Intrahepatic Cholestasis" type 2 (PFIC2) hebben vanaf hun geboorte cholestase, die vervolgens alleen maar erger wordt.. Bij PFIC2 kunnen er geen galzouten van de hepatocyt naar het galkanaaltje getransporteerd worden, omdat door fouten of mutaties in het gen van BSEP er geen (goed) eiwit gemaakt kan worden. Het gevolg is dat galzouten zich ophopen in de levercellen en in het bloed. Een belangrijk symptoom van cholestase is jeuk. Bij deze erfelijke vorm van cholestase uit dit zich echter ook in groeiachterstand van deze patiëntjes, omdat voedingsstoffen niet goed opgenomen kunnen worden door het tekort aan galzouten in de gal. Bovendien raakt hun lever door de giftige werking van de overmaat aan galzouten in de levercellen zodanig beschadigd dat de lever getransplanteerd moet worden. Er zijn verschillende mutaties van het BSEP-gen bekend en in **hoofdstuk 2** onderzoeken we één van die mutaties: de D482G-mutatie. Deze code betekent dat van de 1321 aminozuren waar het BSEP-eiwit uit bestaat er 1 anders is dan normaal. Op positie 482 is een glycine (G) aanwezig in plaats van het normale aspartaat (D). We hebben in de eerste plaats gekeken of een BSEP-eiwit met deze mutatie nog wel galzouten kan transporteren. Dat was het geval, dus dit kan niet de reden zijn waarom deze patiëntjes cholestase krijgen. Vervolgens bestudeerden we de vorming van het eiwit zelf. Door de D482G-mutatie bleek er veel minder BSEP-eiwit gevormd te worden en dit beetje komt ook nog niet eens allemaal op de goede plek (canaliculaire membraan) terecht. In het laboratorium hebben we kunnen vaststellen dat bij een lage temperatuur het D482G-BSEP-eiwit wel goed gevormd wordt. Dit alles betekent dat de D482G-mutatie niet leidt tot functieverlies van BSEP, want het eiwit kan nog wel galzouten transporteren. Echter, door deze ene mutatie wordt er te weinig van dit transporteiwit aangemaakt en dat is dus onvoldoende om

galzouten efficiënt naar de gal te transporteren. Waarschijnlijk wordt het eiwit herkend als "fout" en daardoor snel afgebroken.
Deze studie heeft aangetoond dat het mutante eiwit wel zijn normale functie kan uitvoeren. In de toekomst zou het dus mogelijk kunnen zijn dat er therapieën worden ontwikkeld die gericht zijn om de versnelde afbraak van het mutante eiwit tegen te gaan. Dit is niet geheel ondenkbaar, aangezien ditzelfde mechanisme is beschreven voor verschillende andere erfelijke aandoeningen, onder andere bij taaislijmziekte. Het is wellicht mogelijk om deze verschillende aandoeningen met eenzelfde medicijn/therapie te behandelen.

Galzouten reguleren de expressie van BSEP met behulp van FXR

Tot voor kort werden galzouten alleen gezien als "oplosmiddel" voor vetachtige verbindingen die daarmee efficiënt uitgescheiden of juist opgenomen kunnen worden. Echter, in de afgelopen jaren is gebleken dat galzouten ook zeer belangrijke signaalmoleculen zijn. Zo reguleren ze zelf dat de enterohepatische kringloop in stand gehouden wordt door voortdurend de synthese van galzouten en het transport ervan in lever en darm op elkaar af te stemmen. De synthese wordt verhoogd als de concentraties van galzouten in de lever laag zijn. Transport van galzouten naar de gal wordt verhoogd als de concentraties ervan in de lever verhoogd zijn. Het moleculaire mechanisme van deze regulatie blijkt voornamelijk te liggen in het aansturen van gentranscriptie. Galzouten zijn liganden van de transcriptiefactor Farnesoid X receptor of FXR. FXR dat geactiveerd is door galzouten reguleert de transcriptie van genen die coderen voor eiwitten die galzouten transporteren en enzymen die betrokken zijn bij de synthese van galzouten. In **hoofdstuk 3** wordt aangetoond dat galzouten ook de expressie van humaan BSEP reguleren. Deze activeren FXR, die samen met de transcriptiefactor Retinoic X Receptor of RXR de transcriptie van BSEP verhoogt. FXR en RXR binden als zogenaamde heterodimeer samen aan een specifieke DNA-sequentie, de FXRE, in de promoter van het BSEP-gen. De door galzouten gestimuleerde expressie van BSEP wordt tenietgedaan als de FXRE in de BSEP promoter is gemuteerd. Als met behulp van de techniek van RNA-interferentie de hoeveelheid FXR wordt verlaagd, is eveneens de galzout-geïnduceerde expressie van BSEP afwezig. Deze resultaten laten zien dat ook bij de mens, de galzoutexportpomp BSEP wordt gereguleerd door activering van FXR door galzouten.

Vitamine A is belangrijk voor de expressie van BSEP

Als vervolg op hoofdstuk 3 hebben we in **hoofdstuk 4** bepaald of 9-cis retinolzuur (9cRA), dat een ligand is van RXR, ook een rol speelt bij de regulatie van de transcriptie van het BSEP-gen. 9cRA wordt gevormd uit vitamine A. Vitamine A is een vetachtige vitamine en galzouten zijn belangrijk voor de opname ervan in de darm. Vitamine A is belangrijk voor verschillende processen in ons lichaam, bijvoorbeeld het gezichtsvermogen. Een bekend gevolg van tekort aan vitamine A is nachtblindheid. Deze vitamine is ook belangrijk voor de groei en ontwikkeling van het

embryo en de voortplanting. Van verschillende genen die door FXR en RXR gereguleerd worden is bekend dat de aanwezigheid van beide liganden, galzouten en 9cRA, zorgt voor de sterkste expressie. Wij hebben ontdekt dat dit niet het geval is voor BSEP. Sterker nog: 9cRA lijkt juist een remmende werking te hebben. Dus wanneer beide transcriptiefactoren én beide liganden aanwezig zijn wordt er minder BSEP gemaakt dan met alleen galzouten en beide transcriptiefactoren. We hebben dit aangetoond zowel in *in vitro* experimenten, met humane cellijnen, als in *in vivo* experimenten met muizen. De muizen kregen 12 weken voer waarin geen vitamine A zat. Vervolgens kregen ze 1 week lang ook nog galzouten toegevoegd aan hun voer. In totaal werden 4 groepen muizen vergeleken: muizen met een tekort aan vitamine A (VAD-muizen) die 1 week wel of geen galzouten in het voer kregen en controlemuizen (géén tekort aan vitamine A) die eveneens 1 week wel of geen galzouten in het voer kregen. Zoals verwacht werd in de levers van de VAD-muizen met galzouten in de voeding de hoogste BSEP-expressie waargenomen. Deze resultaten laten zien dat vitamine A een belangrijke factor is in de regulatie van galzouthomeostase. Patiënten met cholestase hebben vaak ook een laag vitamine A-gehalte. De cholestase kan een gevolg zijn van een te lage BSEP-expressie. Het behandelen van deze patiënten met vitamine A-houdende preparaten kan echter de expressie van BSEP nog verder verlagen en daarmee de cholestase verergeren.

FXR beïnvloedt ook de expressie van niet-galzoutgerelateerde genen

Van verschillende eiwitten is al bekend dat hun expressie gereguleerd wordt door galzouten en FXR (samen met RXR en 9cRA). Toch komen er nog steeds nieuwe bij. In **hoofdstuk 5** passen we een nieuwe techniek toe, de microarray-technologie, op zoek naar eiwitten die ook door galzouten en FXR gereguleerd worden. De microarray-technologie maakt gebruik van een chip waarop alle genen van alle eiwitten van, bijvoorbeeld, de mens zitten. Met deze technologie kun je bestuderen wat er met de expressie van alle genen gebeurt, wanneer er bijvoorbeeld meer galzouten en FXR aanwezig zijn. Dit hebben we dan ook gedaan en we hebben ontdekt dat mogelijk Fibrinogeen Bβ (FGB) door galzouten en FXR wordt gereguleerd. In tegenstelling tot de meeste andere FXR-targetgenen lijkt dit eiwit geen rol te spelen in de galzouthuishouding. FGB vormt samen met Fibrinogeen Aα (FGA) en Fibrinogeen γ (FGG) het eiwit Fibrinogeen. De expressie van de FGA en FGG wordt, zij het in minder mate dan FGB, ook verhoogd door galzouten en FXR. Fibrinogeen wordt in de lever gemaakt en is belangrijk voor de bloedstolling. Het is mogelijk dat Fibrinogeen een rol speelt bij littekenvorming (fibrose) in de lever. Fibrose is een reactie van de lever op weefselschade, bijvoorbeeld door de giftige effecten van een overmaat aan galzouten in de lever bij cholestase. De effecten van galzouten en FXR op de expressie van deze drie eiwitten moeten echter nog nader bestudeerd worden om vast te kunnen stellen dat hun expressie ook werkelijk door galzouten en FXR beïnvloed wordt.

Conclusies en perspectieven

De afgelopen jaren is onze kennis van de cellulaire processen die cholestase veroorzaken sterk toegenomen. Erfelijke aandoeningen die resulteren in chronische cholestase zijn tot op moleculair niveau uitgezocht: ze zijn het resultaat van mutaties in eiwitten die een rol spelen in de galzouthuishouding, zoals enzymen in de lever die galzouten maken en eiwitten in lever- en darmcellen die galzouten de cel in of uit transporteren. Cholestase kan ook vele andere, niet-genetische, oorzaken hebben, bijvoorbeeld een ontsteking in de lever. Het blijkt dat dan de hoeveelheid galzoutvormende enzymen en/of de galzouttransporters niet goed is gereguleerd. De wetenschappelijke uitdaging is nu om deze kennis te gebruiken voor de ontwikkeling van nieuwe behandelmethoden voor patiënten met cholestase. Het werk beschreven in dit proefschrift geeft hiervoor een eerste aanzet. We hebben laten zien dat sommige PFIC2-patiënten een mutatie in het BSEP-gen hebben die de functie van deze transporter niet aantast. Toch hebben deze patiënten cholestase. Dit wordt waarschijnlijk veroorzaakt doordat het mutante BSEP-eiwit in de levercel herkend wordt als "fout" en versneld wordt afgebroken. Hierdoor kan er niet voldoende galzouttransport naar de gal plaatsvinden en ontstaat er cholestase. Verschillende andere erfelijke ziekten (bijvoorbeeld taaislijmziekte) blijken een vergelijkbare oorzaak te hebben; er is te weinig van het mutante, maar wel functionele, eiwit. Een belangrijk speerpunt van toekomstig onderzoek is dan ook om behandelmethoden te ontwikkelen die de hoeveelheid van het mutante eiwit naar een voldoende niveau brengen.

Sinds een aantal jaren is er ook een volledig nieuwe groep van eiwitten geïdentificeerd waarop behandeling van cholestase gericht kan zijn. Dit zijn de transcriptiefactoren die de hoeveelheid galzoutproducerende enzymen en galzouttransporters reguleren. FXR en RXR zijn hiervoor de belangrijkste "targets". Verschillende natuurlijke en synthetische liganden die deze transcriptiefactoren activeren zijn al voorhanden. Uit recent onderzoek blijkt echter dat het effect van FXR op de regulatie van eiwitten afhankelijk is van het gebruikte ligand. Met andere woorden, het natuurlijke ligand van FXR, het galzout CDCA, leidt tot een verhoging van de expressie van een bepaald FXR-"target"eiwit, terwijl dit niet gebeurt met het synthetische ligand GW4064. Bovendien laten wij in dit proefschrift zien dat het ligand van RXR, het vitamine A-derivaat 9-cis retinolzuur, in combinatie met FXR en galzouten op hetzelfde moment de expressie van een aantal FXR-"target"-genen verhoogt terwijl dat van andere FXR-"target"-genen wordt verlaagd. Het is daarom van belang om de effecten van de verschillende (en ook combinaties van) liganden grondig te onderzoeken alvorens men een behandeling kan starten die is gebaseerd op de manipulatie van deze transcriptiefactoren.

Ondertussen moeten we ons ook realiseren dat er nog steeds een groep patiënten met PFIC is waarvan we de genetische oorzaak niet weten. Dit betekent dat er nog steeds nieuwe genen/eiwitten ontdekt zullen worden die een rol spelen in de galzouthuishouding. Ook deze nieuwe factoren kunnen van groot belang zijn bij het ontstaan van cholestase als gevolg van niet-genetische componenten zoals ontsteking, medicijnen, alcohol, drugs, enzovoort. Daarom is het belangrijk om naar deze onbekende genen/eiwitten te blijven zoeken.

Concluderend kan gesteld worden dat er nu verschillende eiwitten bekend zijn waarop nieuwe behandelmethoden voor patiënten met cholestase gericht kunnen zijn. De toekomst moet uitwijzen of hiermee efficiënte en veilige therapieën ontwikkeld kunnen worden. Met andere woorden, er is nog genoeg onderzoek te doen om meer inzicht te krijgen in de (moleculaire) oorzaken van en behandelmethoden voor cholestase.

Dankwoord

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Jacqueline

Curriculum vitae

Curriculum vitae

Jacqueline Regina Maria Plass is geboren (3 oktober 1974) en getogen in Enschede. Na het behalen van haar gymnasiumdiploma aan het Jacobuscollege in Enschede, begon ze in september 1993 met de studie Biologie aan de Rijksuniversiteit Groningen. Haar afstudeeronderzoeken verrichte ze achtereenvolgens bij de vakgroep Dierfysiologie (RUG) onder begeleiding van prof. dr. P.G.M. Luiten en dr. G.I. de Jong (onderwerp: ultrastructuur van hersencapillairen bij slechte doorbloeding) en de vakgroep Fysiologische Chemie (RUG) onder begeleiding van dr. J.W. Kok (onderwerp: differentiatie van HT29-D4 cellen). In augustus 1998 studeerde ze af als medisch bioloog. In mei 1999 begon ze als assistent in opleiding bij de vakgroep Maag-, Darm- en Leverziekten aan het NWO onderzoeksproject 902-23-253. Ze werd hierbij begeleid door prof. dr. P.L.M. Jansen, prof. dr. M. Müller en dr. O. Mol. In de loop van het onderzoek werden de begeleidingstaken van prof. dr. M. Müller en dr. O. Mol door dr. K.N. Faber overgenomen. De resultaten van het onderzoek zijn te lezen in dit proefschrift.

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- **Jacqueline R.M. Plass**, Olaf Mol, Janette Heegsma, Mariska Geuken, Joost de Bruin, Geeske Elling, Michael Müller, Klaas Nico Faber, and Peter L. M. Jansen; A progressive familial intrahepatic cholestasis type 2 mutation causes an unstable, temperature-sensitive bile salt export pump; *Journal of Hepatology 2004; 40: 24-30*
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- **Jacqueline R.M. Plass**, Mariska Geuken, Janette Heegsma, Duncan van Rijsbergen, Juul F.W. Baller, Folkert Kuipers, Peter L.M. Jansen and Klaas Nico Faber; Low retinol levels potentiate bile salt-induced expression of the bile salt export pump in vitro and in vivo