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Biochimica et Biophysica Acta 1407 (1998) 173–184

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Review

Biochemical and molecular aspects of genetic disorders of bilirubin metabolism

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Received 5 January 1998; accepted 22 June 1998

Abstract

Bilirubin, the oxidative product of heme in mammals, is excreted into the bile after its esterification with glucuronic acid to polar mono- and diconjugated derivatives. The accumulation of unconjugated and conjugated bilirubin in the serum is caused by several types of hereditary disorder. The Crigler-Najjar syndrome is caused by a defect in the gene which encodes bilirubin UDP-glucuronosyltransferase (UGT), whereas the Dubin-Johnson syndrome is characterized by a defect in the gene which encodes the canalicular bilirubin conjugate export pump of hepatocytes. Animal models such as the unconjugated hyperbilirubinemic Gunn rat, the conjugated hyperbilirubinemic GY/TR$^-$, and the Eisai hyperbilirubinemic rat, have contributed to the understanding of the molecular basis of hyperbilirubinemia in humans. Elucidation of both the structure of the *UGT1* gene complex, and the Mrp2 (cMoat) gene which encodes the canalicular conjugate export pump, has led to a greater understanding of the genetic basis of hyperbilirubinemia.

Keywords: Bilirubin; Hyperbilirubinemia; UDP-glucuronosyltransferase; Gunn rat; Crigler-Najjar syndrome; Gene therapy; Multidrug resistance protein 2; GY/TR$^-$ rat; Dubin-Johnson syndrome

Contents

Abbreviations: ABC, ATP-binding cassette; ACS, asialoglycoprotein; BR, biliverdin reductase; cMOAT, canalicular multispecific organic anion transporter (cMOAT for the human transport protein; cMoat for the rat homologue); CN, Crigler-Najjar syndrome; DJ syndrome, Dubin-Johnson syndrome; EHBR, Eisai hyperbilirubinemic rat; ER, endoplasmic reticulum; GY/TR$^-$, transport-deficient mutant Wistar rat; HO, heme oxygenase; MRP, multidrug resistance protein (MRP for the human transport protein; Mrp for the rat homologue); PB, phenobarbital; UDP-GlcUA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase

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0925-4439/98/$ – see front matter
PII: S0925-4439(98)00044-1

1. Introduction

Bilirubin is the oxidative product of the protoporphyrin portion of the heme group of proteins such as hemoglobin, myoglobin and cytochrome *P*-450. An individual with normal human metabolism, generates 250–400 mg/day of bilirubin through the breakdown of these hemoproteins [1]. Bilirubin, an organic anion with limited water solubility, is essentially unexcretable in its native form. In the liver, the bilirubin is conjugated with glucuronic acid; the resulting water-soluble bilirubin glucuronides are excreted into the bile via an ATP-dependent anion transporter (Fig. 1). Certain pathological conditions and/or hereditary disorders cause the accumulation of bilirubin and its glucuronides in the serum; this may result in jaundice. Animal models having hereditary disorders of bilirubin metabolism are important in gaining an understanding of the pathophysiology of hyperbilirubinemia in humans. In this review, we will focus on three areas of bilirubin-related research: (a) the formation of bilirubin; (b) the glucuronidation of bilirubin; (c) the excretion of bilirubin glucuronides into the bile.

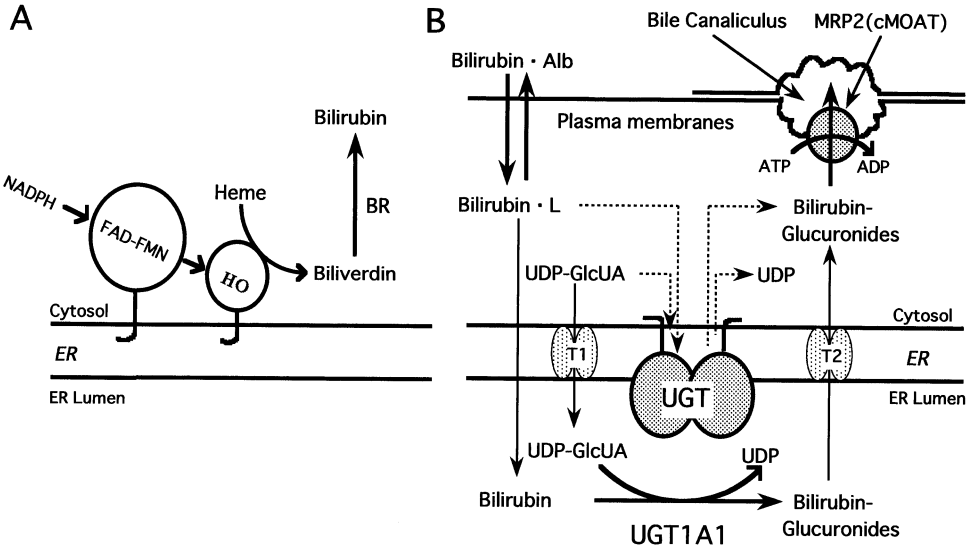

Fig. 1. Metabolic pathway from the heme group to bilirubin. Topology of UGT and MRP2 (cMOAT). (A) Formation of bilirubin from heme. FAD-FMN, NADPH-cytochrome *P*-450 reductase; HO, heme oxygenase; BR, biliverdin reductase. (B) Uptake of bilirubin, conjugation of the bilirubin molecule, and its excretion from the hepatocyte. UGT, UDP-glucuronosyltransferase; Bilirubin-Alb, bilirubin-albumin complex; Bilirubin-L, bilirubin-ligandin complex; MRP2, multidrug resistance protein 2; UDP-GlcUA, UDP-glucuronic acid. The orientation and dimer structure of UGT [30] in the membrane of the ER are depicted. T1 and T2 in the membrane of the ER are the proposed transporters of UDP-GlcUA and glucuronides, respectively. The dashed lines show the alternate pathway for glucuronidation in the ER, based on the conformational model.

2. Formation of bilirubin

The first step in the degradation of the heme group to bilirubin is cleavage of the α-methene bridge of heme to form biliverdin IX_α [2–5]. This reaction is catalyzed by microsomal heme oxygenase isoform I (Fig. 1A). O_2 and NADPH are required for the cleavage reaction. The cleavage reaction proceeds via a multistep mechanism that depends on reducing equivalents which are provided by microsomal NADPH-cytochrome *P*-450 reductase, which contains FAD and FMN as prosthetic groups [6,7]. The catalytic cycle of the heme oxygenase resembles that of the microsomal cytochrome *P*-450 mixed-function oxidase [8]. The central methene bridge of biliverdin at C-10 is reduced by NADPH-biliverdin reductase, a cytosolic enzyme, to form bilirubin. Two isomers of biliverdin, IX_α (95–97%) and IX_β (3–5%), are present in human adult bile, whereas the IX_β isomer is predominant in late fetal bile [9]. This suggests that two isoforms of biliverdin reductase are present in the liver. Biliverdin reductases with high specificity for biliverdin IX_α and biliverdin IX_β have been purified from human livers [9]. Bilirubin is an unsymmetrically substituted tetrapyrrole dicarboxylic acid. The introduction of CH_2 at C-10 of the bilirubin molecule induces a conformational change by rotation of the dipyrrinone groups about the central CH_2 group. This allows intramolecular hydrogen bonding of the propionic acid carbonyl to the amino groups of the dipyrrinone lactam and the pyrrole ring [10,11] is thought to explain the many unusual properties of bilirubin, such as its high lipid/water partition coefficient and its resistance to hepatobiliary excretion [12]. This conformation of bilirubin is hydrophobic, and has a high affinity for tissues of the central nervous system.

3. Glucuronidation of bilirubin

3.1. UGT

The UDP-glucuronosyltransferases (UGTs) are a family of microsomal membrane-bound enzymes that catalyze the conjugation of bilirubin, steroids, bile acids, and xenobiotics with UDP-glucuronic acid (UDP-GlcUA) [13–17]. The DNA sequence of UGTs contains an amino-terminal signal peptide, which is cleaved during synthesis of the polypeptide chain [14–17], and a stretch of 17 hydrophobic amino acids near the carboxy terminus, that anchors the protein to the lipid bilayer [17–19]. The stretch of 20–30 amino acids at the carboxy terminus of the UGT contains several basic residues which are responsible for retaining the UGT in the endoplasmic reticulum (ER) [20,21]. Based on identity in amino acid sequence, the UGTs are divided into two families, *UGT1* and *UGT2* [13]. Members of the *UGT1* gene family (Fig. 2A) include bilirubin- and phenol-metabolizing isoforms. They share an identical 245 amino acid carboxy terminus, whereas their N-terminal halves show a striking lack of identity (37–49%) [13,15,17,22,23]. The N-terminal half of a particular UGT determines substrate specificity for glucuronidation.

Members of the *UGT2* family include steroid-metabolizing isoforms. Comparison of the amino acid sequences of the proteins encoded by the *UGT2* gene family reveals differences in amino acid sequence throughout the length of the protein [13,15,16]. The C-terminal halves, however, are highly conserved, which may provide the binding site for the common co-substrate, UDP-GlcUA [17,24].

Efficient excretion of bilirubin across the bile canaliculus requires the conversion of bilirubin to polar conjugates by esterification of the two propionic acid carboxyl groups on the bilirubin molecule. It is believed that glucuronidation occurs on the intramolecular hydrogen-bonded conformation of bilirubin [25]. In this model, the hydrogen-bonded, folded, ridge-tile conformation of bilirubin is stabilized when bound to the active site of the UGT molecule; glucuronic acid is transferred to the hydrogen-bonded carboxyl (or carboxylate) group of bilirubin. Very little is known about the relationship between the structure and function of glucuronosyltransferases. Three-dimensional analysis of the enzyme-substrate complex will throw new light on this field of research.

3.2. Topology of UGT

UGT1 isoforms in rats [26,27] and humans [28] have been demonstrated to be involved in bilirubin glucuronidation. Activity of *UGT1* isoforms in native

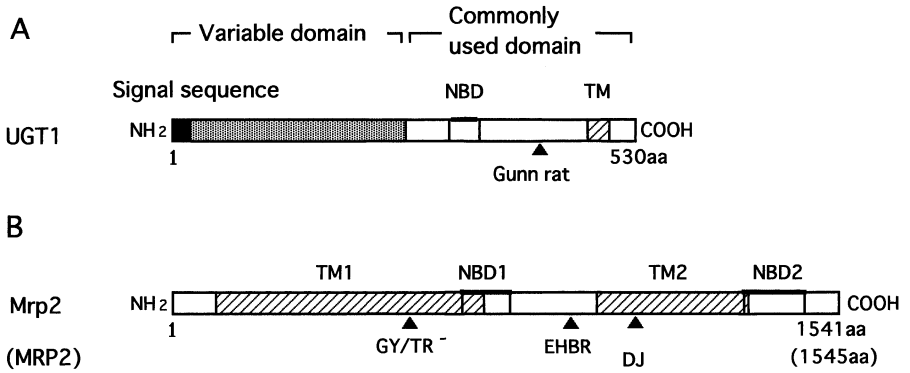

Fig. 2. Functional domains of *UGT1* and MRP2 and mutation sites. (A) Proposed primary structure of UDP-glucuronosyltransferase family 1 (*UGT1*). Studies in the Gunn rat have indicated the presence of a single base (G) deletion at either nucleotide 1239 or 1240. This results in the introduction of a premature stop codon [22,23,40]. NBD, the proposed nucleotide (UDP) binding domain [15,46]; TM, transmembrane domain [17–19]. (B) Proposed primary structure of the multidrug resistance protein 2 in the rat (Mrp2) and human (MRP2). The Mrp2 protein in the rat is composed of 1541 amino acids. The human MRP2 protein is composed of 1545 amino acids. GY/TR$^-$ indicates the location (amino acid 401) at which a stop codon is introduced by a single nucleotide deletion [87]. EHBR indicates the location of the replacement of a base, from G to A, at nucleotide 2564. This results in the introduction of a premature stop codon [88]. Dubin-Johnson syndrome (DJ) indicates the location of the replacement of a base, from C to T, at nucleotide 3496 (codon 1066, CGA to TGA; arginine to stop codon). This results in the introduction of a premature stop codon [99]. NBD1 and NBD2 indicate the two nucleotide (ATP) binding domains, and TM1 and TM2 indicate the two transmembrane domains [77,82,86,89,90].

microsomes is markedly low. In disrupted microsomes, however, the level of activity of *UGT1* isoforms increases, a phenomenon called 'latency' of glucuronidation [29–31]. It is believed that the membrane-bound UGT molecule is situated so that most of the UGT molecule lies in the lumen of the ER, where the catalytic site is located [14,19]. This hypothesized membrane topology necessitates the translocation of UDP-GlcUA from the cytosol, where it is synthesized, to the lumen of the ER [32–35]. This is the rate-limiting step of the level of UGT activity in the ER. Therefore, upon disruption of the microsomes with detergents, this activity is fully expressed. The resultant bilirubin glucuronides are transported across the membrane of the ER to the cytosole, and excreted from the hepatocyte via an ATP-dependent anion transporter into the bile (Fig. 1B). This is the so-called compartmentation model [29]. If this model is correct, this implies the presence of a translocator in the membrane of the ER, similar to MRP2 (cMOAT) located in the canalicular membrane (Fig. 1B). Such a translocator in the membrane of the ER has yet to be identified. On the other hand, the oligomer structure of the UGT molecule may itself act as a common channel for these substrates and products [30].

An alternative explanation is that such a translocational process is not essential. This would suggest that the catalytic site of UGT is located within the membrane of the ER. In this model, the so-called conformation model, the active site of the UGT enzyme lies in the ER membranes, and both UDP-GlcUA and bilirubin have direct access to the active site of the UGT enzyme (Fig. 1B); the activity of the UGT enzyme is constrained by interactions between the UGT protein and the membrane lipid bilayer [31].

Considerable controversy exists between the compartmentation and conformation hypotheses [29,31]. Further investigation of both of these models should be able to provide information on the mechanism underlying the latency of glucuronidation.

3.3. Gunn rat

In 1938, Gunn described a mutant strain of Wistar rats with hereditary hyperbilirubinemia (Gunn rats) [36]. The Gunn rat is an animal model of Crigler-Najjar syndrome (CN) Type 1, and has provided valuable information on the metabolism of bilirubin, bilirubin toxicity, treatment of hyperbilirubinemia, and the molecular mechanism that underlies heredi-

tary jaundice [1]. The Gunn rat lacks hepatic UGT activity towards bilirubin, 3-methylcholanthrene-inducible phenolic substrates, and digitoxigenin-monodigitoxiside, whereas UGT activity towards other substrates, including steroids, chloramphenicol, and morphine, is normal [37,38]. These accumulative data suggest that several specific UGT isoforms are defective in the Gunn rat.

Molecular analysis of the defective UGT isoforms using antibody probes has shown that Gunn rats lack bilirubin- and phenol-metabolizing enzymes, each with a molecular mass of 53–54 kDa [39]. The cDNA of a UGT isoform designated 4-nitrophenol UGT (4NP-UGT) was isolated and sequenced from normal rats [17] and from homozygous Gunn rats [40]. Sequence analysis of the Gunn rat cDNA revealed a single base deletion in the coding region (Fig. 2A); this results in the synthesis of truncated protein. Furthermore, using the sequence of the mutant 4NP-UGT cDNA as a probe, three novel cDNAs have been isolated from a Gunn rat liver library [22,40]. The sequences of the three novel cDNAs share an identical RNA sequence at the 3′-terminal, and have a single base deletion in the same position as that of the mutant 4NP-UGT cDNA. These results suggest that these defective UGT isoforms originated from a common ancestral mutant UGT which had a truncated carboxy terminus [22,40–42]. An unstable, truncated protein with a molecular mass of 43 kDa has been detected in the liver and kidney cells of Gunn rats [42].

3.4. UGT1 gene complex

cDNA cloning of the UGT isoforms of the Gunn rat has shown that the chimeric mRNAs contain variable 5′-portions and a conserved 3′-portion. This suggests that they comprise a family of proteins, the *UGT1* family, and that these mRNAs are derived from a single gene by the use of different promoters and spliceosomes [22]. The four human UGT isoforms that belong to the *UGT1* family have identical C termini [28,43,44]. Emi et al. [23] and Ritter et al. [45] have recently isolated a large gene complex, from the rat genome and the human genome, respectively, that encodes several isoforms of UGT family 1 (Fig. 3). The *UGT1* gene complex contains several unique promoter regions which make up the first exon; each of these first exons are capable of splicing with common exons 2 through 5. This leads to different N-terminal halves, but identical C-terminal halves, of the gene products of the *UGT1* gene complex [23,45]. Although the carboxyl-terminal halves of each of these genes are identical, the first exon of each *UGT1* isoform can be regarded as a distinct gene (*UGT1A1*, *UGT1A2*, *UGT1A3*, ...) [46]. Sequence analysis of the first exons has revealed that the proteins encoded by the *UGT1* gene complex can be divided into two clusters: the bilirubin-like cluster (A1–A5), and the phenol-like cluster (A6–A9) [23]. The single base deletion responsible for the common, truncated carboxyl terminus of all of the members of the *UGT1* gene family of the Gunn rat has been identified in exon 4 (Figs. 3 and 4) [23]. Using two-dimensional SDS-PAGE and immunoblotting using isozyme-specific antibodies, each *UGT1* isoform in the Gunn rat was associated with the coincident absence of hepatic microsomes [27]. The bile of homozygous Gunn rats contains a trace amount of bilirubin mono-conjugates [47]. These results indicate that the *UGT2* isoforms, encoded by different genes, do not have significant bilirubin-glucuronidating ac-

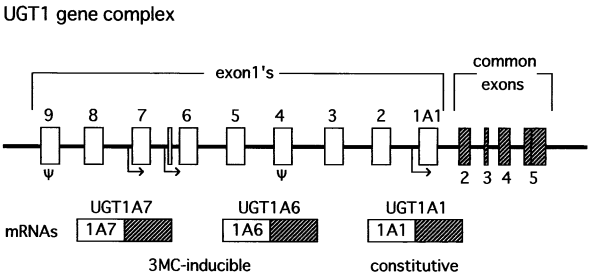

Fig. 3. The *UGT1* gene complex which encodes bilirubin and phenol UDP-glucuronosyltransferase in the rat. This gene, which spans at least 120 kb, contains at least nine first exons, which can be spliced to common exons 2 through 5 [23]. The first exons are named 1A1, 1A2, 1A3, ..., and their gene products *UGT1A1*, *UGT1A2*, *UGT1A3*, ...[46]. *UGT1A6* and *UGT1A7* are 3-methylcholanthrene inducible (3MC-inducible) forms. The *UGT1A1* is the constitutively expressed form. The shaded boxes indicate carboxyl exons 2, 3, 4, and 5. Exons 1A4 and 1A9 are pseudogenes. The *UGT1A6* first exon is composed of a non-coding exon of 153 bp and a coding exon of 756 bp [23]. At least six first exons have been identified in the human UGT1 gene complex [45,46]; an additional six first exons have been found through GenBank [46], which lie further upstream. Exons 1A2, 1A11, and 1A12 are pseudogenes. Figure cited from Seikagaku 70 (2) (1998) 105–109.

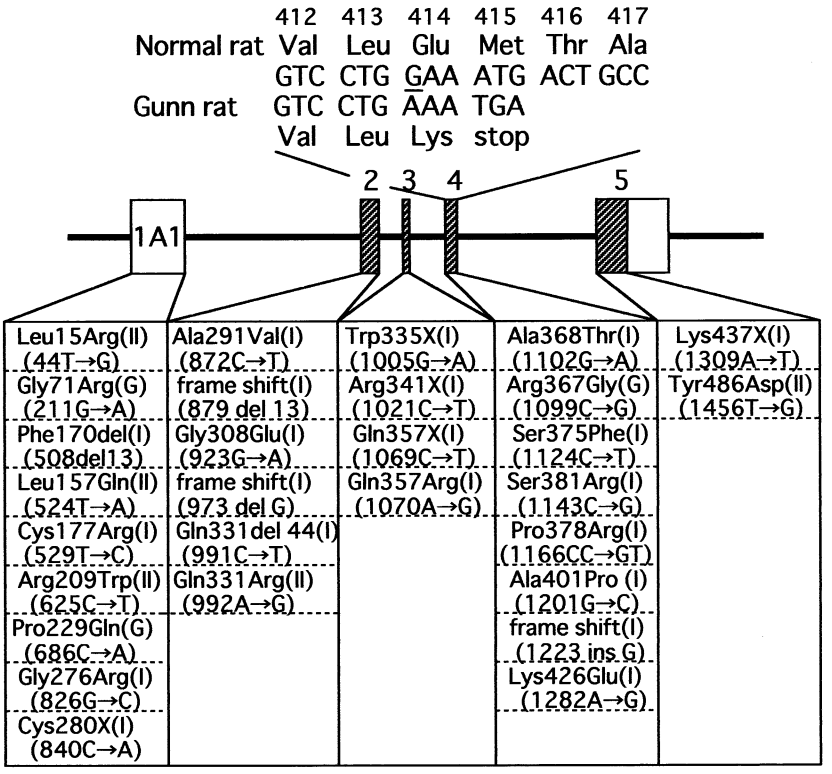

Fig. 4. Mutations which have been found in the bilirubin *UGT1* gene of the Gunn rat (upper), and in Crigler-Najjar (CN) Types 1 and 2 and Gilbert's syndrome (below). I, CN Type 1; II, CN Type 2; G, Gilbert's syndrome; X, nonsense mutation; del, deletion; ins, insertion. The 1A1 indicates the first exon; the shaded areas indicate common coding exons 2, 3, 4, and 5. Data on the human mutations were reproduced from Table 4 in Mackenzie et al. [46], with permission.

tivity. Among the hepatic *UGT1* isozymes in the rat, the enzyme encoded by *UGT1A1* is responsible for constitutive bilirubin metabolism. Administration of either clofibrate or dexamethasone increases the expression of *UGT1A1* two- to threefold [23,27]. However, the administration of phenobarbital (PB) does not increase the expression of *UGT1A1* [23,26,27]. The administration of 3MC selectively induces expression of the *UGT1A6* and *7* genes. This implies that the *UGT1A1* gene is responsible for constitutive expression, while expression of the *UGT1A6* and *7* genes requires 3MC for upregulation. The upstream regulatory region of the 1A6 exon contains the consensus sequence which encodes the binding site of an aromatic hydrocarbon (Ah) [48]. It is known that glucuronidation activity towards bilirubin increases after birth [49]. This suggests that the expression of *UGT1A1* is regulated by ontogenetic factors such as glucocorticoids [50] or thyroid hormones [51]. At present, this field is being investigated.

3.5. Crigler-Najjar syndrome and Gilbert's syndrome

In 1952, Crigler and Najjar described a syndrome characterized by a severe, chronic, non-hemolytic, unconjugated hyperbilirubinemia [52]. Two clinical forms of this syndrome have been described [1]. Patients with Crigler-Najjar syndrome (CN) Type 1 have an unconjugated serum bilirubin level of greater than 340 μM, while patients with CN Type 2 have an unconjugated serum bilirubin level in the range of 60–340 μM. Patients with Gilbert's syndrome have an unconjugated serum bilirubin level of 60 μM or below. Gilbert's syndrome is characterized by mild, unconjugated hyperbilirubinemia; this syndrome affects approx. 5% of the population.

The molecular bases of CN syndrome and Gilbert's syndrome have recently been characterized by enzymatic, immunochemical, and molecular genetic analyses. As a result of the elucidation of the *UGT1* gene complex in the rat [23] and humans

[45], several groups have discovered the genetic defects which cause hyperbilirubinemia (Fig. 4). Mutations in the *UGT1* gene complex among patients with CN Type 1 have been detected in common exons 2, 3, 4, and 5; these patients have a defect in all glucuronidation activities catalyzed by *UGT1*. Mutations in the first exon (1A1), such as Cys280→Stop(840C→A) [53] and Gly276Arg(826G→C) [54,55], have also been found among patients with CN Type 1; these patients have a defect in bilirubin-glucuronidating activity. This finding indicates that the protein encoded by *UGT1A1* in humans is the major bilirubin-metabolizing isoform under physiological conditions [56].

Among patients with Gilbert's syndrome, two types of genetic abnormality in the *UGT1A1* gene have been found. One is a homozygous TA insertion in the TATA box upstream from the 1A1 exon [57–60]; the other is a heterozygous missense mutation in the coding region [61]. These findings indicate that there are two inherited patterns of Gilbert's syndrome; one is autosomal recessive, and the other is autosomal dominant [62]. TA insertion in the TATA box also causes very severe hyperbilirubinemia in patients with glucose-6-phosphate dehydrogenase deficiency, an X-linked hereditary disease which causes hemolytic anemia and which occurs in high incidence [60]. Finally, the position of a particular mutation within the *UGT1* gene complex, and the effect of that mutation on glucuronidating activity towards bilirubin, may determine the type of syndrome.

A distinction between patients with CN Type 1 and CN Type 2 can be observed by measuring the bile pigments after PB treatment [63]. After PB administration to patients with CN Type 1 disease, the level of serum bilirubin does not decrease and unconjugated bilirubin predominates in the bile. After PB administration to patients with CN Type 2 disease, the concentration of serum bilirubin falls, and a decreased proportion of bilirubin unconjugates, along with an increase in biliary mono- and diglucuronides, is seen. PB-inducible UGT isoforms may be involved in bilirubin conjugation. Administration of PB to Wistar rats, however, does not significantly increase the expression of bilirubin-like, or phenol-like, isoforms [23,27]. The human *UGT1A4* isoform is inducible by PB [28], but does not exhibit significant bilirubin-glucuronidating activity [56]. The *UGT1A4* isoform in the rat is a pseudogene [23]. PB treatment of patients with CN Type 2 may increase the expression of, or stabilize, the abnormal bilirubin UGT isoform, to result in the increased production of glucuronides. The pathway through which PB regulates the expression of the human bilirubin *UGT1* gene is not yet understood. The functions of the other human bilirubin-like UGT isoforms remain to be elucidated. It has been suggested that the decrease in serum bilirubin level after PB treatment is caused by mobilization of bilirubin to the liver. This is probably caused by the induction of a cytosolic-binding protein such as ligandin, which results in the increased transport of bilirubin into the bile, in the Gunn rat [64]. Some heme may be degraded by oxidative pathways other than those described previously, such as via the cytochrome *P*-450 system.

3.6. Gene therapy of hyperbilirubinemia

Gene therapy for Crigler-Najjar disease has become an important new field of bilirubin research, and is based on the rat and human *UGT1* gene complex [23,45]. As discussed above, the Gunn rat is a good animal model for molecular analysis of human hyperbilirubinemia. A major advantage of the Gunn rat is that the bilirubin glucuronides are not excreted into the bile. This allows direct and precise quantitation of the metabolic effect of the transfer of a therapeutic gene [65]. Two types of vector are being considered for liver-directed gene therapy: viral vectors, and carrier proteins for receptor-mediated endocytosis [1]. Recombinant retroviruses and adenoviruses have been used for somatic gene therapy [66]. The rat bilirubin-like *UGT1A2* cDNA has been introduced into the liver of the Gunn rat by retrovirus-mediated gene transfer [67,68]. The results showed a significant, and stable, correction of the hyperbilirubinemia. In contrast, Choudhury et al. [65] have used adenovirus vectors, which are highly efficient in transferring foreign genes to quiescent cells. Adenoviral vectors do not integrate into the chromosomal DNA of the host, and have been able to temporarily cure a number of animal models with specific genetic diseases such as hypercholesterolenemia [69,70] and hemophilia[71]. When the recombinant adenovirus expressing human *UGT1A1* was injected into newborn Gunn rats, the protein was predominantly de-

tected in the liver, with only traces being present in other tissues [65]. The levels of hepatic bilirubin UGT activity on day 2 and day 56 after injection were 80 and 27 nmol/mg liver wet weight/min, respectively. The level of hepatic bilirubin UGT activity in normal Wistar rats was 75 nmol/mg liver wet weight/min. The serum bilirubin level in these Gunn rats was 70–76% less than that in untreated Gunn rats; both bilirubin mono- and diglucuronides were excreted into the bile.

The receptor-mediated gene transfer systems have been used as an alternative method for gene therapy. Because of the high level of expression of asialoglycoprotein (ACG) in the plasma membrane of hepatocytes, ACG receptor-mediated endocytosis has been utilized for incorporation of the targeted *UGT1A1* gene [72]. The human *UGT1A1* expression vector DNA was complexed with an ACG, which itself was conjugated to a polycation. The ACG-polycation-*UGT1A1* gene complex was internalized into cells of the Gunn rat liver via ACG receptor-mediated endocytosis. Although expression of the *UGT1A1* gene was transient, bilirubin glucuronides were observed in the bile, and the serum bilirubin level was reduced by 25–35% in 2–4 weeks. Microtubular disruption by colchicine treatment permitted prolonged expression of the DNA which was endocytosed into hepatocytes. These data also support that the *UGT1A1* isoform is responsible for the formation of bilirubin glucuronides.

4. Biliary transport of bilirubin glucuronides

4.1. GY/TR$^-$ rat

Bilirubin is secreted from the liver into the bile mainly as mono- and diglucuronides. Biliary transport has been proposed to be the rate-limiting step in the overall transfer of bilirubin. Jansen et al. [73] described homozygous mutant Wistar rats, GY/TR$^-$, which displayed a mild, chronic conjugated hyperbilirubinemia. This mutant rat has an autosomal recessive defect in the hepatobiliary excretion of organic anions, including bilirubin glucuronides, bromosulfophthalein-glutathione, cysteinyl leukotrienes, and the reduced and oxidized forms of glutathione [74–76]. However, the biliary excretion of bile acids, tauro- and glycocholate, and of organic cations is normal. Using isolated bile canalicular membrane vesicles, it was demonstrated that several organic anions are transported via an ATP-dependent transporter termed cMoat [75,76]. The substrate specificity of cMoat is very similar to that of the human multidrug resistance protein (MRP), which is one of the ATP-binding cassette (ABC) transporters [77]. MRP was originally cloned and sequenced from multidrug-resistant cancer cell lines [78–80]. The MRP family consists of at least six members, designated as *MRP1* through *MRP6*. *MRP1*, *MRP2*, *MRP3*, *MRP4*, *and MRP5* are encoded by separate genes; however, the 3′ end of *MRP6* is nearly 100% identical with that of a MRP-like half-transporter, anthracycline resistance-associated (ARA) protein. This suggests that the ARA isoform is possibly derived by alternative splicing from the *MRP6* gene [80,81]. The MRP family proteins require two nucleotide-binding regions and two associated transmembrane regions to be functional [77,82], and are different in sequence and substrate specificity. MRP1 has a similar substrate specificity to that of cMoat [78,83–85]. On the basis of these data, several groups have postulated that cMoat is likely to be a liver-specific homologue of the human MRP [86–88]. The cDNA of cMoat has been isolated from rat liver cDNA libraries by polymerase chain reaction amplification using degenerate primer pairs to conserved regions of human MRP. Sequence analysis of the putative rat cMoat has identified it to be an ABC transporter with highest overall identity to human MRP2 [79,87–90], the human canalicular isoform of *MRP1*. The cMoat protein, which consists of 1541 amino acids, is located in the canalicular membrane of hepatocytes [86,90]. In the GY/TR$^-$ rats, a single base deletion in the coding region of the cMoat gene results in a truncated protein (Fig. 2B) [87]. The Eisai hyperbilirubinemic rat (EHBR) exhibits an autosomal recessive defect in the biliary excretion of non-bile acid and organic anions [76,88,91]. In this rat, the replacement of one base in the coding region results in a truncated protein [88]. The rat cMoat cDNA expressed in NIH/3T3 cells has the ability to transport glutathione conjugates in an ATP-dependent process, the most important characteristic of cMoat [92]. This transporter is predominantly expressed in hepatocytes and is located on the bile canalicular membrane. This trans-

porter is absent in both the allelic mutant rat strain, GY/TR$^-$, and EHBR. These data strongly suggest that the impaired expression of these proteins is involved in the pathogenesis of jaundice in these mutant animals. These mutant rats have aided in the characterization of the canalicular bilirubin conjugate export pump.

The MRP2/Mrp2 isoform does not transfer primary bile acids such as taurocholate or glycholate [75,90,93], but their activity is still retained in the canalicular membrane of both GY/TR$^-$ and EHBR mutant rats. Purified rat canalicular membrane vesicles exhibit ATP-dependent bile acid transport [94,95]. The putative bile acid transporter has been cloned and sequenced from rat liver mRNA, which was predominantly expressed in the liver [96]. It was further localized to the canalicular and to subcanalicular vesicles of the hepatocytes. The cloned rat cDNA, which was expressed in Sf9 cells, exhibited similar kinetics and similar substrate preference as ATP-dependent bile acid transporter in canalicular liver plasma membrane vesicles [96]. This transporter belongs to a subgroup of ABC transporter family that is called the multidrug resistance P-glycoproteins (MDRs) [77,89].

4.2. Dubin-Johnson syndrome

Both GY/TR$^-$ rats [73–75] and EHBR [76,91] are animal models of the Dubin-Johnson (DJ) syndrome of humans [97]. This syndrome is characterized by a selective abnormality in the excretion of conjugated anions into the bile canaliculus, and mild, chronic, conjugated hyperbilirubinemia. However, bile acid excretion into bile is usually normal in these patients. As with the GY/TR$^-$ and EHBR, it is likely that a truncated, non-viable protein is responsible for the impaired transport of organic anions from the liver to the bile in patients with DJ syndrome. Patients with DJ syndrome also do not express the human homologue of rat cMoat, human cMOAT [98]. Recently, the cDNA corresponding to human cMOAT in fibroblasts obtained from a patient with DJ syndrome was analyzed. One base replacement was found in the coding region of the cMOAT gene, resulting in the expression of a truncated protein (Fig. 2B) [99]. The predominant substrate of human cMOAT (MRP2) is conjugated bilirubin [100]. These data strongly demonstrate that bilirubin glucuronides are secreted into the bile canaliculus by an ATP-dependent mechanism, which is defective in the GY/TR$^-$, EHBR, and human patients with DJ syndrome. However, under physiological conditions, the overall level of bilirubin diglucuronide excreted into the bile in GY/TR$^-$ is indistinguishable from that in normal rats, despite the fact that the bilirubin concentration in the livers of GY/TR$^-$ is three times higher than in normal rats [101].

Two distinct mechanisms of bilirubin glucuronide transport have been proposed [102]. In addition to an ATP-dependent system, bile excretion is also caused by an ATP-independent system driven by the membrane potential. In GY/TR$^-$ rats, the ATP-dependent system is defective; however, the mechanism driven by the membrane potential is retained. Thus, the mild degree of conjugated hyperbilirubinemia and the presence of bilirubin glucuronides in the bile of both mutant rats and humans with hyperbilirubinemia may be explained by a membrane-potential transport system, that is, a low-affinity transport system capable of accommodating bilirubin diglucuronides [101,102]. Further study is needed to clarify the relationship between the membrane potential-dependent and ATP-dependent transport systems.

5. Conclusion

In 1995, Roy Chowdhury et al. published a review on hereditary jaundice and disorders of bilirubin metabolism, in *The Metabolic Basis of Inherited Diseases* [1]. The application of molecular biology to the study of heme-metabolizing enzymes has considerably advanced our understanding of the biological mechanisms underlying this important function. Elucidation of the structure of the rat bilirubin *UGT1* gene complex, and the rat cMoat (Mrp2) gene, has aided in determining the specific genetic defects that underlie human hyperbilirubinemias. The unconjugated hyperbilirubinemic Gunn rat, conjugated hyperbilirubinemic GY/TR$^-$, and EHBR models have greatly contributed to the understanding of the biochemical characterization of the enzymes and transporter system in these defects. The Gunn rat also serves as an experimental model for the development

of therapeutic strategies for the treatment of congenital hyperbilirubinemia in patients with Crigler-Najjar syndrome Type 1. Future identification of *cis* and *trans* elements which regulate the expression of these genes will enable us to understand the molecular mechanisms involved in the induction, tissue distribution, and ontogeny of these enzymes and transporters.

Acknowledgements

We thank Dr. Brian Burchell at the University of Dundee, UK, for helpful discussion and for reading the manuscript. This work was supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and from the Hyogo Science and Technology Association.

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