

# Splice-Site Mutations: A Novel Genetic Mechanism of Crigler-Najjar Syndrome Type 1

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## Summary

Crigler-Najjar syndrome type 1 (CN-1) is a recessively inherited, potentially lethal disorder characterized by severe unconjugated hyperbilirubinemia resulting from deficiency of the hepatic enzyme bilirubin-UDP-glucuronosyltransferase. In all CN-1 patients studied, structural mutations in one of the five exons of the gene (*UGT1A1*) encoding the uridinediphosphoglucuronate glucuronosyltransferase (UGT) isoform bilirubin-UGT<sub>1</sub> were implicated in the absence or inactivation of the enzyme. We report two patients in whom CN-1 is caused, instead, by mutations in the noncoding intronic region of the *UGT1A1* gene. One patient (A) was homozygous for a G→C mutation at the splice-donor site in the intron, between exon 1 and exon 2. The other patient (B) was heterozygous for an A→G shift at the splice-acceptor site in intron 3, and in the second allele a premature translation-termination codon in exon 1 was identified. Bilirubin-UGT<sub>1</sub> mRNA is difficult to obtain, since it is expressed in the liver only. To determine the effects of these splice-junction mutations, we amplified genomic DNA of the relevant splice junctions. The amplicons were expressed in COS-7 cells, and the expressed mRNAs were analyzed. In both cases, splice-site mutations led to the use of cryptic splice sites, with consequent deletions in the processed mRNA. This is the first report of intronic mutations causing CN-1 and of the determination of the consequences of these mutations on mRNA structure, by *ex vivo* expression.

## Introduction

Bilirubin, a toxic breakdown product of heme, is insoluble in water because of internal hydrogen bonding. Uridinediphosphoglucuronate glucuronosyltransferase (UGT), a family of enzymes located in the membrane of the endoplasmic reticulum (Roy Chowdhury et al. 1985), transfers the glucuronic acid moiety from uridinediphosphoglucuronate to bilirubin, as well as a wide variety of other endogenous compounds and xenobiotics. Glucuronidation converts these substrates to polar conjugates that are readily excreted in bile or urine (Dutton 1980). A specific UGT isoform mediates the glucuronidation of bilirubin (bilirubin-UGT<sub>1</sub>) (Bosma et al. 1994). UGT activity toward bilirubin is essential for the efficient biliary excretion of bilirubin. Inherited abnormalities of this enzyme (Schmidt et al. 1958; Arias et al. 1969) result in the potentially lethal Crigler-Najjar syndrome type I (Crigler and Najjar 1952), which is characterized by the accumulation of unconjugated bilirubin in the plasma.

Bilirubin-UGT<sub>1</sub>, also designated "UGT1A1," is encoded by five consecutive exons located at the 3' end of the *UGT1A* locus (Ritter et al. 1992a; Mackenzie 1997) and is expressed in the liver only. In recent studies, a large number of different Crigler-Najjar syndrome-causing mutations in the exons encoding the *UGT1A1* gene have been reported (Bosma et al. 1992a, 1992b; Ritter et al. 1992b; Moghrabi et al. 1993a, 1993b; Labrune et al. 1994; Seppen et al. 1994).

In this study, we report two cases in which intronic mutations result in bilirubin-UGT deficiency. *UGT1A1* is expressed in the liver only. Liver material of most patients is not available. Therefore, the effect of these mutations on the processing of *UGT1A1* gene transcripts was determined by *ex vivo* expression studies. We show that these *ex vivo* studies can be used to confirm the effect of intronic mutations on the structure of *UGT1A1* mRNA.

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## Subjects and Methods

### Case History

Patient A is an 18-mo-old boy who has had jaundice continuously since 48 h after birth. Serum bilirubin at 72 h after birth was 30 mg/dl (510  $\mu$ M), and phototherapy was instituted. Bile was analyzed by reverse-phase high-pressure liquid chromatography and showed that only trace amounts of bilirubin monoglucuronide were present and that >95% of the bile pigments were unconjugated bilirubin. A 2-wk course of phenobarbital therapy reduced serum bilirubin levels by 15%. Currently, while the patient is being treated by phototherapy, but without phenobarbital, the serum bilirubin levels have been 18–26 mg/dl (306–442  $\mu$ M), all of which has been found to be unconjugated, by chromatographic analysis. There is no evidence of neurotoxicity.

Patient B is a 25-year-old man who also has been continuously icteric since birth. All of the serum bilirubin was unconjugated. No bilirubin glucuronides were detectable in bile. There was no appreciable reduction of serum bilirubin levels by phenobarbital therapy. His serum bilirubin levels were maintained at 20–24 mg/dl (340–408  $\mu$ M) by intensive phototherapy. However, during a period of noncompliance, serum bilirubin concentrations were >30 mg/dl (>510  $\mu$ M). After an episode of acute neurotoxicity, the patient began to show mild ataxia. Therefore, an orthotopic liver transplant was performed, and the patient was placed on immunosuppressive therapy. Serum bilirubin concentrations were normalized 48 h after the liver transplant and have remained normal since. Currently, the patient is asymptomatic.

### Nucleotide-Sequence Analysis

Genomic DNA was isolated from whole blood by use of the QIAamp blood kit (Qiagen) according to the manufacturer's instructions. All five exons constituting the coding region of bilirubin-UGT<sub>1</sub> and the flanking intronic regions were amplified by PCR, as described elsewhere (Bosma et al. 1992a). In patient A, the nucleotide sequence of the amplicons was determined by cycle sequencing without cloning. In patient B, the amplicons were cloned before sequencing. Sequence determination was performed on three different PCR products, for both strands.

### Minigene Construction

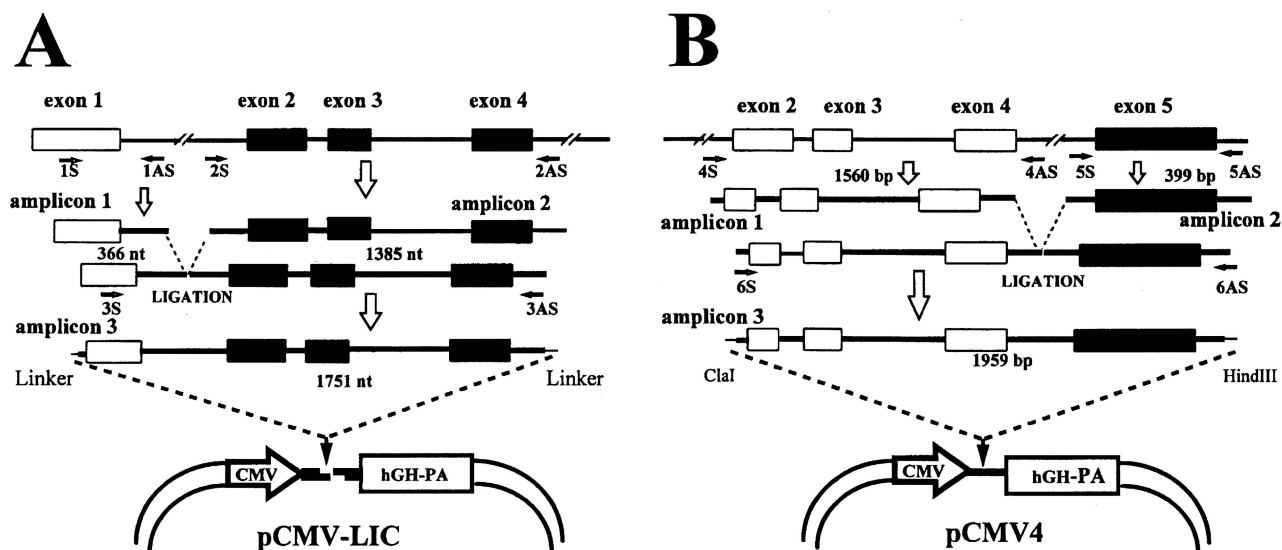
Genomic DNA samples of both patients and of several normal individuals were amplified by PCR using primer pairs as shown in figure 1. PCR was performed for 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for

1 min. Final extension was at 72°C for 5 min. The concentration of MgCl<sub>2</sub> was 1.5 mM. Amplified DNA fragments were purified by use of the Qiagen PCR purification kit according to the manufacturer's instructions.

Two PCR fragments were amplified from genomic DNA of patient A and of a normal individual, by use of primers 1S and 1AS and primers 2S and 2AS, respectively. The *Bgl*III sites present in primers 1AS and 2S were used to combine both amplicons. By ligation, primers 3S and 3AS were used to generate the minigene, which contained the 3' end of *UGT1A1* exon 1; the 5' region of intron 1; and exons 2–4; and introns 2 and 3. The minigene was cloned into the eukaryotic expression vector pCMV-LIC (PharMingen), downstream of a cytomegalovirus early region promoter. The expression vector containing the splice-acceptor-site mutation of patient B, at the 3' end of intron 3, was produced by a similar strategy. Amplicon 1—containing exons 2–4, introns 2 and 3, and the 3' flanking sequences of exon 4—was generated by use of primers 4S and 4AS, and primers 5S and 5AS were used to amplify the 3' end of intron 4 and exon 5 (amplicon 2). Primers 5S and 4AS both contained a *Bgl*III site, which was used to combine both amplicons. By ligation, the whole fragment (amplicon 3) was amplified by use of primers 6S and 6AS, which contained a *Cla*I and a *Hind*III site, respectively. The resulting minigene contained the 3' end of intron 1, exons 2–5, introns 2 and 3, and a shortened intron 4 (containing the 5' and the 3' ends of the intron) (fig. 1B). DNA sequence analysis was performed to confirm the single-base differences, at the relevant splice sites, between the minigene constructs, on the basis of normal human genomic DNA and the DNA samples extracted from the patients.

### Analysis of the Splicing of the Pre-RNA Transcribed from the Minigenes

The minigene constructs were transfected as diethylaminoethyl-dextran complexes into COS-7 cells, as described elsewhere (Bosma et al. 1994). After 5 h, the transfection medium was removed, and fresh medium was added. Cells were grown for another 24 h, before harvesting. Total RNA was isolated by use of the Qiagen kit and reverse-transcription-primed PCR (RT-PCR) was performed. The effect of the splice-donor-site mutation was studied by use of a sense primer located at nt 586–606 (5'-CCT CTC TCC TCT CAT TCA GAT-3') and an antisense primer located at nt 1138–1156 (5'-CGC CAT TGC ATA TGC TTTC-3'). For characterization of the mRNA expressed from the minigene that contained the splice-acceptor-site mutation, a sense primer located at nt 861–875 on exon 2 (5'-GAA TTT GAA GCC TAC-3') and an antisense primer located at nt 1526–1546 on exon 5 (5'-TCC GGT AGC CAT AAG



**Figure 1** Location of PCR amplimers and map of expression vectors containing bilirubin-UGT<sub>1</sub> minigenes. For patient A, primers 1S and 1AS were used to amplify the 3' domain of exon 1 and the 5' region of intron 1 of *UGT1A1*, producing amplicon 1. Primers 2S and 2AS were used to amplify the 3' end of intron 1, exons 2–4, and introns 2 and 3, producing amplicon 2. Amplicons 1 and 2 were digested with *Bgl*III and ligated, to produce amplicon 3. For patient B, the 3' end of intron 1, exons 2 and 3, and the 5' end of intron 4 were amplified by use of amplimers 4S and 4AS (amplicon 1). Amplicon 2, consisting of the 3' end of intron 4 and exon 5, was generated by use of amplimers 5S and 5AS. Amplimers 1 and 2 were ligated to produce amplicon 3. The primers used are as follows (“S” indicates sense, and “AS” indicates antisense): 1S, 5'-CCT CTC TCC TCT CAT TCA GAT-3' (exon 1); 1AS, 5'-GCT CAG CAT AGA TCT GGG GCT AG-3' (intron 1; this primer was designed to introduce a *Bgl*III site in the amplified product); 2S, 5'-AGA GGA AGA TCT CTA ATC ATA AC-3' (3' end of intron 1; this primer was designed to introduce a *Bgl*III site in the amplified product); 2AS, 5'-CGC CAT TCG ATA TGC TTTC-3' (3' end of exon 4); 3S, 5'-CTG GTT CCG GCG ACC TCT CTC TCA TTC AGAT-3' (exon 1); 3AS, 5'-CTC GCT CCG GCG ACG CCA TTG CAT ATG CTT TC-3' (3' end of exon 4); 4S, 5'-CTC TAT CTC AAA CAC GCA TGCC-3' (3' end of intron 1); 4AS, 5'-GGA AGA TCT AAC AAC GCT ATT AAA-3' (5' end of intron 4; this primer was designed to introduce a *Bgl*III site in the amplified product); 5S, 5'-GGA AGA TCT GGT AAG TCT TCT TAA GCA GCC ATG AG-3' (3' end of intron 4; this primer was designed to introduce a *Bgl*III site in the amplified product); 5AS, 5'-ACC CAC TTC TCA ATGG-3' (3' domain of exon 5); 6S, 5'-CCA TCG ATG TCT TAA GAG GAA GAT-3' (spanning 3' end of intron 1 and the 5' end of exon 2, with a *Clal* linker); and 6AS, 5'-CCC AAG CTT TCC GGT AGC CAT AAG-3' (3' domain of exon 5, with a *Hind*III linker).

CAC AAC-3') were used. Aliquots of RT-PCR from cells transfected with the normal and the mutated minigenes were analyzed by electrophoresis on 2% agarose gels.

#### Analysis of Hepatic Bilirubin-UGT<sub>1</sub> mRNA

Total RNA was isolated from the liver of patient B, as described elsewhere (Chomczynski and Sacchi 1987). RT-PCR was performed by use of the following amplimers: sense (nt 1047–1067), 5'-GAT ACT TGT TAA GTG GCT ACC-3', and antisense (nt 1386–1406), 5'-TTG TGC CTC ACA AAC TCC-3'. With agarose-gel electrophoresis, amplicons of two different sizes, representing the two alleles, were found. Both fragments were isolated and sequenced.

## Results

### Mutations in the *UGT1A* Gene

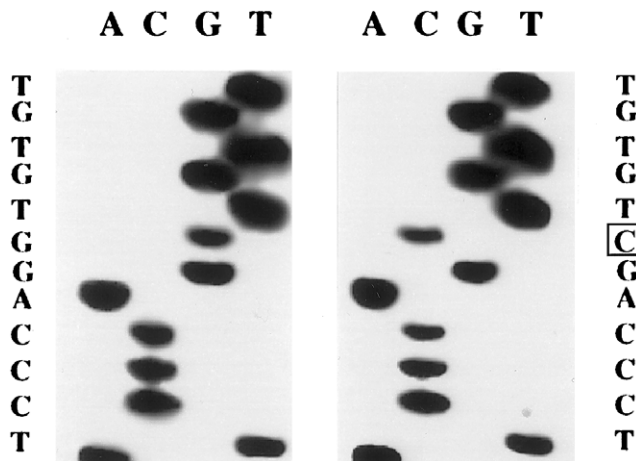
In patient A, the nucleotide sequences of the five exons composing *UGT1A1* were completely normal. This pa-

tient, however, was homozygous for a G→C mutation at the splice-donor site at the 5' end of the intron 1 between exons 1 and 2 (fig. 2). Both parents were heterozygous for this mutation.

Patient B was a compound heterozygote. One allele contained a C→T substitution in exon 1, at nt 145, creating a premature stop codon (data not shown). The proband's mother was heterozygous for this mutation. The nucleotide sequence of the entire coding region of the other allele was normal; instead, an A→G mutation was present at the splice-acceptor site in intron 3 (fig. 3). The patient's father was a heterozygous carrier of this mutation. In both patients, all other splice-donor and -acceptor sites, as well as branch points, had normal nucleotide sequences.

### Effect of the Intronic Mutations on Bilirubin-UGT<sub>1</sub> RNA Processing

To determine the consequences of the splice-donor (G→C in patient A) and the splice-acceptor (A→G in



**Figure 2** Genomic DNA sequence of the region containing the mutation in patient A (*left*) and of a normal individual (*right*). The nucleotide sequences are shown to the left and to the right of the gel. Note the G→C change at the first nucleotide of the intron (the splice-donor site) (boxed) of patient A.

patient B) mutations on the processing of the RNA transcript, COS-7 cells were transfected with normal and mutant human bilirubin-UGT<sub>1</sub> minigenes. A cDNA containing the whole structural region of bilirubin-UGT<sub>1</sub> also was transfected, as a control. The expressed RNA was extracted and amplified by RT-PCR, and its nucleotide sequence was determined.

RT-PCR, using primers directed toward nt 586–606 (primer S) and nt 1138–1156 (primer AS), of total RNA from cells transfected with the minigene containing the normal splice-donor site yielded a 570-bp (nt 586–1156) amplicon consistent with normal splicing of intron 1. In contrast, RT-PCR of RNA isolated from cells transfected with the minigene containing the G→C mutation in the splice-donor site of exon 1 produced a shorter amplicon. Sequence determination of this amplicon revealed that a cryptic splice-donor site within exon 1 was utilized, resulting in the deletion of 141 nt from the 3' end of exon 1 in the bilirubin-UGT<sub>1</sub> mRNA (fig. 4A). The sequence of the cryptic splice-donor site was as follows: GAG|GUGACU (fig. 5).

RT-PCR of the RNA isolated from the cells transfected with the *UGT1A1* miniconstruct containing the A→G mutation at the splice-acceptor site of intron 3 (as detected in patient B) also produced an amplicon shorter than that obtained from cells transfected with the corresponding normal construct. Sequence determination showed that in the mutated minigene a cryptic splice-acceptor site within exon 4 had been utilized. The context of the cryptic splice acceptor AG was as follows: UCAG|AUUGG (fig. 5). Use of this cryptic site resulted

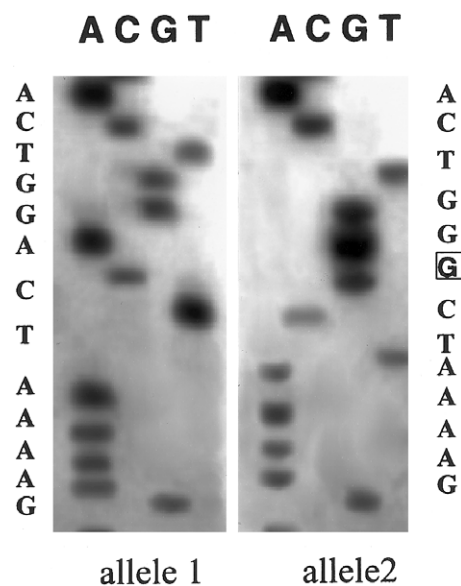
in the deletion of 107 nt from the 5' end of exon 4 in the final mRNA product (fig. 4B).

#### Sequence of Hepatic Bilirubin-UGT<sub>1</sub> mRNA

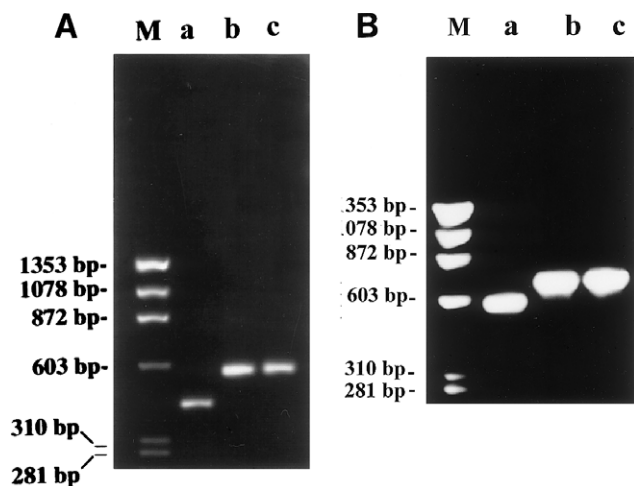
Patient B had recently received a liver transplant; thus, liver material was available for analysis. Total RNA was isolated, and RT-PCR was performed. By amplification, two fragments with different lengths were found. Both fragments were isolated and sequenced. The sequence of the longer fragment was identical to that of the normal *UGT1A1* mRNA sequence. In the shorter fragment, the first 107 nt of exon 4 were deleted (fig. 6). Thus, in the liver of this patient, the same cryptic splice site in exon 4 as that identified by *ex vivo* expression studies was used.

#### Discussion

Several UGT isoforms that are expressed from the *UGT1A* locus have identical carboxy-terminal domains, encoded by four exons located at the 3' end of the gene (Ritter et al. 1992a; Mackenzie et al. 1997). The amino-terminal domains of these isoforms are encoded by single unique exons, a series of which are located upstream of the common-region exons. Each unique-region exon is preceded by an independent promoter and transcriptional initiation site. Thus, various lengths of pre-mRNA are thought to be transcribed from *UGT1A*, by differential promoter selection (Ritter et al. 1992a). Imme-



**Figure 3** Genomic DNA sequence of the region containing the mutation in patient B. The mutant allele differs from the normal allele by a n A→G single-base substitution at the splice-acceptor site (boxed).



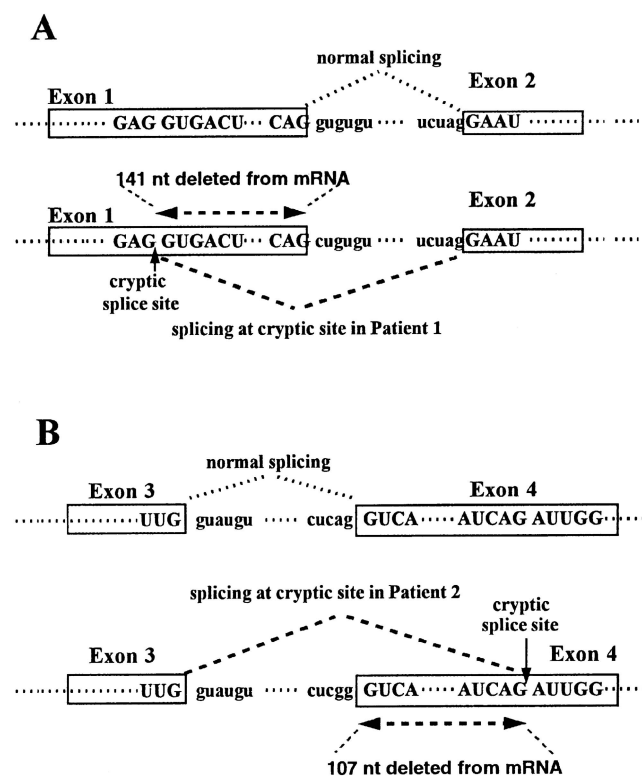
**Figure 4** A, RT-PCR amplification of mRNA from COS-7 cells transfected with minigene constructs. Lane a, Amplicon of mRNA expressed from the patient A minigene. Lane b, Amplicon expressed from the corresponding normal minigene. Lane c, Amplicon from *UGT1A1* cDNA. Lane M, Molecular size markers. Normally spliced mRNA yielded a 570-bp amplicon, whereas in patient A the deletion of 141 nt from exon 1 resulted in a 429-bp amplicon. B, RT-PCR amplification of transcripts from COS-7 cells transfected with minigene constructs. Lane a, Amplicon of the mRNA expressed from the patient B minigene (578 bp). Lane b, Amplicon from cloned *UGT1A1* cDNA (685 bp). Lane c, Amplicon expressed form a normal minigene (685 bp). Lane M, Molecular size markers. The shorter amplicon from mRNA expressed by the patient B minigene indicates the deletion of 107 nt.

diately 3' of each unique exon, there is a splice-donor site, but there is no splice-acceptor site upstream of these exons. Therefore, the exon located at the 5' end of the transcript is spliced to the first available splice-acceptor site located 5' of exon 2, and the entire stretch of DNA between the exon at the 5' end of the transcript and exon 2 is treated as an intron and is spliced out. According to the recently adopted nomenclature, the gene is named according to the transcription initiation site within the *UGT1A* locus. For example, in the case of bilirubin-UGT<sub>1</sub>, the transcription begins at the first unique exon upstream of exon 2. Therefore, this gene is termed "*UGT1A1*."

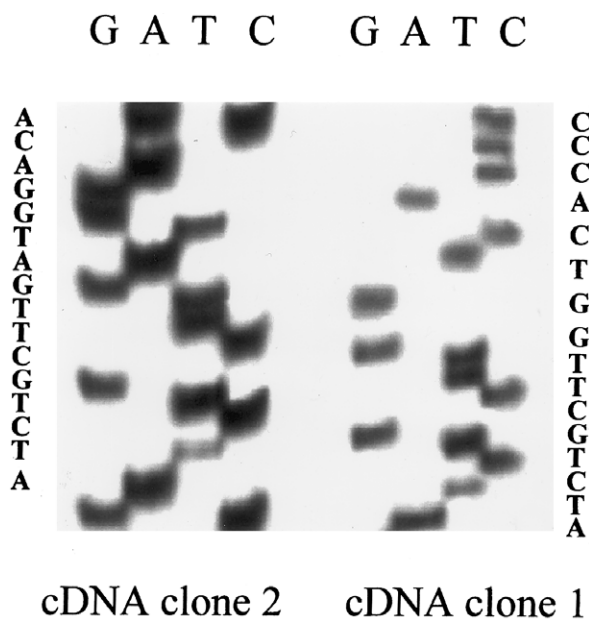
In patient A, a mutation of the splice-donor site was located immediately 3' of *UGT1A1* exon 1. This resulted in the utilization of a cryptic splice site within exon 1, causing the loss of 141 nt from the 3' end of this exon. The abnormally spliced mRNA is predicted to result in substitution of all amino acids at positions 241–486, followed by a premature stop codon. A protein with such extensive sequence abnormality may not be expressed, because of abnormal folding, or, if expressed, is predicted to be functionally inactive. Because the

*UGT1A1* exon 1 is used only in the mRNA for the bilirubin-UGT<sub>1</sub> isoform, other UGT isoforms expressed from the *UGT1A* locus are expected to be spliced normally. The virtual absence of bilirubin glucuronides in the bile of patient A is consistent with the observation that only the bilirubin-UGT<sub>1</sub> isoform contributes significantly to bilirubin glucuronidation in man (Bosma et al. 1994).

In patient B, the splice-acceptor mutation in intron 3 resulted in the utilization of a cryptic splice site within exon 4, causing the loss of 107 nt from the 5' end of this exon. The abnormally spliced mRNA is predicted to result in the substitution of nine amino acids, at positions 262–270, followed by a premature stop codon. This would delete the highly conserved region of the carboxy-terminal domain of the enzyme, which is thought to be critical for binding of the donor substrate, UDP-glucuronic acid, thereby inactivating the enzyme. Since exon 4 is used in all expression products of the *UGT1A* locus, this lesion is expected to affect several UGT isoforms that share exons 2–5 (Bosma et al. 1992b; Ritter et al. 1992b). The structural mutation identified



**Figure 5** Predicted splicing mechanism using cryptic splice sites, owing to mutations at splice-donor site G→C in patient A and splice-acceptor site A→G in patient B, and utilization of cryptic splice sites. In patient A, 141 nt are deleted from upstream of exon 1 (A). In patient B, 107 nt are deleted from downstream of exon 4 (B).



**Figure 6** Sequence analysis of cDNA generated from hepatic bilirubin-UGT<sub>1</sub> mRNA from patient B. The RT-PCR products were cloned before sequence analysis. The sequences derived from two clones are shown, indicating the mutant sequence with the deletion of 107 nt (*left*) and the normal sequence (*right*), representing the mRNA expressed from the other allele.

in this patient, however, was present in the unique exon 1 of the *UGT1A1* gene. Thus, all other UGT isoforms derived from the allele containing the structural mutation are expected to be normal. In this patient, no bilirubin glucuronidating activity was present, again confirming that bilirubin-UGT<sub>1</sub> is the only isoform contributing to bilirubin glucuronidation in human liver.

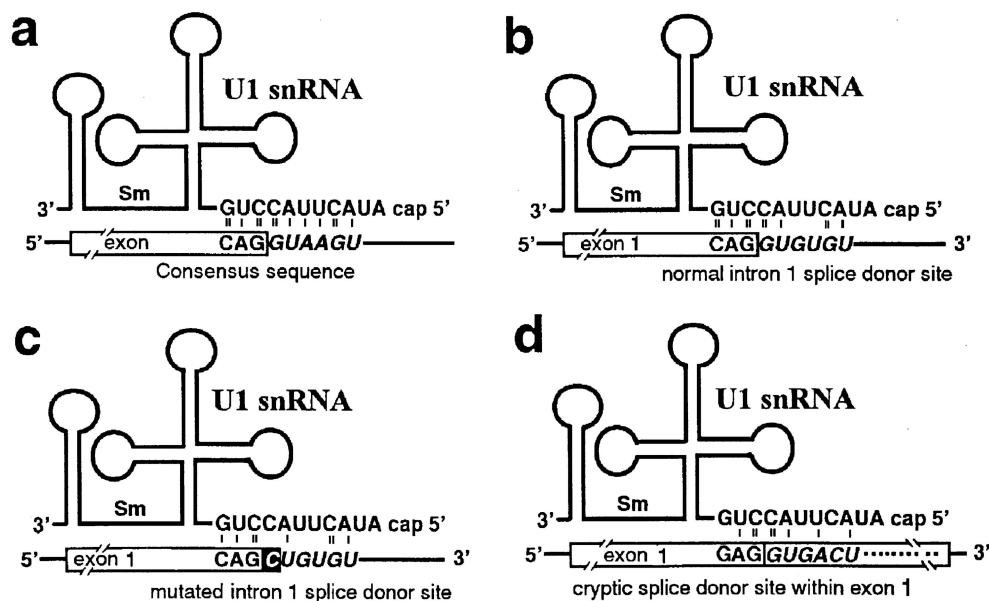
Many instances in which a single-base substitution at a splice junction resulted in abnormal splicing have been reported (Krawczak et al. 1992). However, utilization of a novel cryptic splice site, as a result of a mutation at the normal splice site, has been found much less frequently. Examples of human disease caused by such mutations include hemophilia A and B, thalassemia B, and Laron syndrome (Orkin et al. 1982, 1984; Goldsmith et al. 1983; Treisman et al. 1983; Yamada et al. 1995).

Splicing of nuclear pre-mRNAs occurs through several steps. The three sequence elements that direct this RNA processing include the two sequence elements at the intron borders and the branch site upstream of the 3' splice site. Initially, the 5' splice site is cleaved, and the 5' end of the intron is joined to the 2' hydroxyl of a residue in the branch-site region of the intron. This is followed by excision of the lariat intron and ligation of the exons. The 5' splice site of a pre-mRNA is recognized by U1 small nuclear ribonucleoprotein particles, through base-pairing with the 5' end of U1 small nuclear RNA

(snRNA) (Lerner et al. 1980; Rogers and Wall 1980). Figure 7a shows the consensus sequence 5'-C(or A)AG GU(A)(or G) AGU-3', which is complementary to the first 9 nt of the 5' end of the U1 snRNA (in fig. 7, the intronic bases are shown in italics). Base pairing of the snRNA and the pre-mRNA is essential for splicing of the pre-mRNA (Steitz et al. 1988). Natural 5' splice-site sequences match the consensus sequence to various extents and usually deviate from this consensus sequence at two or three positions. The GU dinucleotide at the cleavage site is present in almost all splice-donor sites, and the G residue located 5' downstream of the cleavage site is found in 85% of cases (Steitz et al. 1988).

The G→C mutation at the splice-donor site in patient A (fig. 7b and c) increases the mismatch with the U1 snRNA, thereby weakening the binding. In particular, the mutation of the critical first G residue of the intron inactivates this splice site. This results in the use of the cryptic splice site within exon 1 (fig. 7d), which is not used normally, because its binding to the U1 snRNA is much weaker. However, U1 binding affinity is not the only criterion for 5' splice-site selection. In some situations, splicing does not occur, despite the binding of U1 snRNA to a high-affinity binding sequence. For the cryptic site to be used, it must be present within a permissive context.

In patient B, the premature stop codon present in exon 1 is predicted to truncate the bilirubin-UGT expressed from this allele. This should reduce the bilirubin-UGT activity by 50%. However, since Crigler-Najjar syndrome type 1 (CN-1; MIM 218800) is inherited as a recessive condition, a 50% reduction of the enzyme activity is not expected to produce a hyperbilirubinemic phenotype. This suggested that another genetic lesion present in the other allele prevented the expression of functional *UGT1A1* from that allele. The presence of the A→G mutation at the 3' splice-acceptor site in intron 3 explains the loss of expression of functional *UGT1A1*, from the second allele. The splice-acceptor-site mutation causes a mismatch with the 3' end of the U1 snRNA. Such mutations can cause skipping of the succeeding exon or, as was seen in this case, the use of a cryptic splice site, present within the exon, downstream of the splice-acceptor site. The cryptic site is located probably by a scanning mechanism that proceeds downstream from the branch site and locates an AG pair (Steitz et al. 1988). The AG sequence is conserved in 100% of splice-acceptor sites. The C residue 5' of the AG sequence and the G residue 3' of the AG sequence are conserved in 80% and 60% of acceptor sites, respectively. The normal splice acceptor had the sequence CAGG, whereas the cryptic site had the sequence CAGA. Thus, the cryptic acceptor site appears to be competent but is not utilized normally, because of the stronger normal splice site located upstream of it.



**Figure 7** 5' Splice-site sequences indicating the possible base-pairing interactions of the first 6 nt of splice-site sequences with U1 snRNA. *a*, Consensus sequence. *b*, Normal intron 1 splice-donor site. *c*, Mutant intron 1 splice-donor site. *d*, Cryptic splice-donor site within *UGT1A1* exon 1.

These two cases constitute the first report of CN-1 caused by splice-site mutations. Our results show that the effect of the splice-site mutation on the splicing of the pre-mRNA can be predicted on the basis of *ex vivo* expression of minigene constructs. In the liver of patient B, the same cryptic site as that identified by *ex vivo* expression studies was used. This indicates that this *ex vivo* expression method indeed can be used to determine the effect of splice-site mutation on RNA processing, without performance of a liver biopsy.

## Acknowledgments

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