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Coding defect and a TATA box mutation at the bilirubin UDP-glucuronosyltransferase gene cause Crigler-Najjar type I disease

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Abstract

Mutations at the bilirubin UDP-glucuronosyltransferase (transferase) gene in a severely hyperbilirubinemic Crigler-Najjar (CN) type I individual was compared with that in a moderately hyperbilirubinemic CN II individual. The CN-I (CF) patient in this study sustained a TATA box insertional mutation which was paired with a coding defect at the second allele, unlike all coding defects previously seen in CN-I patients. The sequence of the mutant TATA box, $[A(TA)_8A]$, also seen in the CN-II patient, was compared with that at the wild-type box, $[A(TA)_7A]$. Transcriptional activity with $[A(TA)_8A]$ was 10–15% that with the wild-type box when present in the −1.7 kb upstream regulatory region (URR) of the bilirubin transferase *UGT1A1* gene which was fused to the chloramphenicol acetyl transferase reporter gene, pCAT 1.7H, and transfected into HepG2 cells. Also, a construct with a TA deletion, $[A(TA)_6A]$, was prepared and used as a control; transcriptional activity was 65% normal. The coding region defect, R336W, seen in CF (CN-I) was placed in the bilirubin transferase UGT1A1 [HUG-Br1] cDNA, and its corresponding protein was designated UGT1A1*32. The UGT1A1*32 protein supported 0–10% normal bilirubin glucuronidation when expressed in COS-1 cells. The I294T coding defect seen at the second allele in SM (CN-II) generated the UGT1A1*33 mutant protein which supported 40–55% normal activity with a normal K_m (2.5 μM) for bilirubin. The hyperbilirubinemia seen in SM decreased in response to phenobarbital treatment, unlike that seen in CF. Parents of the patients were carriers of the respective mutations uncovered in the offspring. The TATA box mutation paired with a deleterious missense mutation is, therefore, completely repressive in the CN-I patient, and is responsible for a lethal genotype/phenotype; but when homozygous, i.e. paired with itself, as previously reported in the literature, it is far less repressive and generates the mild Gilbert's phenotype.

Keywords: Crigler-Najjar types I and II; Bilirubin UDP-glucuronosyltransferase activity and gene; TATA box mutation; Compound heterozygotes

1. Introduction

The potentially neurotoxic lipophilic-like bilirubin is produced daily in high abundance from senescent red blood cells and is detoxified in humans solely through its conjugation to glucuronic acid via bilirubin UDP-glucuronosyltransferase (transferase) catalysis. The glucuronides formed have enhanced water solubility and excretability from the cell. Diseases or syndromes exist in humans reflecting three levels of

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severity. Crigler-Najjar (CN) type I patients [1] are known to contain essentially no detectable bilirubin transferase activity, suffer with a severe serum unconjugated hyperbilirubinemia (> 20 mg%), and are at risk for lethal neurotoxicity due to kernicterus. The less severely affected CN-II patients have intermediate levels of bilirubin transferase activity, manifest an intermediate level of hyperbilirubinemia (< 20 mg%), and are usually not susceptible to neurotoxicity due to bilirubin accumulation [2]. Typically, the CN-II individuals, unlike the type I population, respond to phenobarbital (PB) treatment [3] by manifesting a reduction in serum bilirubin levels. A substantial 5% of the population, classified as Gilbert's individuals [4], are often asymptomatic but have a mild hyperbilirubinemia (approx. 1.0–1.5 mg%) also due to a reduction in bilirubin transferase activity [5]. Normal serum bilirubin levels range between 0.5 and 0.75 mg%. Like CN-II patients, some Gilbert's individuals have been shown to respond to PB treatment [6]. Further, there are reports [7,8] in the literature of CN individuals with serum bilirubin levels consistent with that of the most serious type, CN-I, reaching 40 mg% who responded to PB treatment. These reports indicate that the hyperbilirubinemic phenotypes do not adhere to a consistent pattern.

The human *UGT1* complex locus [9,10] composed of at least 12 different UDP-glucuronosyltransferase (transferase) (J.W. Cho, Q.-H. Gong, C. Potter, N. Gholami and I.S. Owens, manuscript in preparation) genes, includes that for the bilirubin transferase, *UGT1A1*. Many alterations have been uncovered in the coding region of this gene [11–21] for CN individuals and for Gilbert's individuals [22,23]. We report here that a TA insertion at the TATA box element at one allele and a deleterious missense mutation at the second one are responsible for CN type I disease. The unusual TATA box element for the *UGT1A1* gene is composed of seven consecutive TA di-nucleotides $[A(TA)_7A]$ and is situated 37 nucleotides upstream from the cap site of its messenger RNA [9]. A CN-II individual also sustained this same TATA box mutation except there is a less deleterious missense mutation at the second allele. Further, this study allows us to contrast the phenobarbital response in the CN-I and CN-II patients.

2. Materials and methods

2.1. Reagents

The sources of reagents used to carry out recombinant DNA techniques were previously reported [11,13]. The TA vector was obtained from Invitrogen (San Diego, CA). The sources of materials for the transfection of plasmid DNA into the COS-1 cells and the radiolabeling and immunoprecipitation of the expressed bilirubin transferase protein have been reported [24]. HepG2 cells were from ATCC cell culture (Rockville, MD).

2.2. Clinical profile for the CN-I (CF) and CN-II (SM) individuals

CF is an Italian female spontaneously delivered after an uneventful pregnancy. There was no maternal use of medication or antepartum illnesses. On day 2 jaundice was noted, and on day 5 total serum bilirubin concentration was 312 μM (18.1 mg/dl). Treatment with phenobarbital did not cause a decrease in serum bilirubin levels. A family history of Gilbert's syndrome – including the father but not the mother – was found [25]. Phototherapy for 18 h/day proved to be effective in reducing and maintaining bilirubinemia at an average of 12 mg/dl. Since the patient did not respond to Sn-protoporphyrin treatment or phenobarbital therapy at the age of 3.5 years and continued to have a high serum unconjugated bilirubin concentration by HPLC analysis, she was recommended for and underwent liver transplant surgery at that age. At the time of this writing the patient was doing well.

SM is also Italian and unrelated to CF. SM was hospitalized at 12 days of age because of intense jaundice first noted on day 2 in the community hospital. Gilbert's syndrome was diagnosed in the maternal grandmother [26]. Phototherapy treatment was initiated and caused a reduction in the unconjugated bilirubin level. Phenobarbital (PB) (5 mg/kg b.wt./day) was begun on day 15 and continued for 3 weeks without any evident improvement. Subsequently, an experimental trial was undertaken with intramuscular injection of Sn-protoporphyrin (2 μmol/kg b.wt.) administered when serum bilirubin concentration rose to 20 mg/dl. The Sn-derivative was interrupted

due to the appearance of erythema during phototherapy. Three months later PB was again attempted, and the positive response culminated in the definitive diagnosis of CN-II disease. Upon PB treatment the serum bilirubin levels decreased, on average, to 7–8 mg/dl.

2.3. Preparation of genomic DNA and nucleotide sequence analysis

Genomic DNA from leukocytes was prepared as already described [11]. Regions of the *UGT1A1* gene was amplified by the polymerase chain reaction (PCR), and subclones were sequenced as described [11,17]. PCR amplification of the TATA box region was carried out with sense primer PAGS1 and antisense primer PAGA1 to generate a 317 bp fragment. The sequence for PAGS1 is 5′-TTAACTTGGTGTATCGATTGGT-3′ and that for PAGA1 is 5′-GTCTTCAAGGTGTAAAA-3′. Exons in the *UGT1A1* gene were amplified with primers already described [17]. For both CF and SM, five to ten subclones representing either the non-coding or each exon/flanking region were sequenced.

2.4. Determination of inheritance of TATA box mutations

A 317 bp fragment encompassing the TATA box element of the *UGT1A1* gene of each genomic DNA sample was amplified using PCR. The sense primer, [^{32}P]PAGS1 (7.5×10^6 cpm/pmol, −83 to −62 bp 5′ of the TATA box element), and antisense primer, PAGA1 (+248–232 bp of the coding region of exon 1 of *UGT1A1*), were used. Fifty pmol of each primer were used in the reaction; the ratio of non-labeled to radiolabeled PAGS1 was 50. The products of the reactions were separated by electrophoresis through a 6% polyacrylamide gel which was exposed to X-ray film for development and print.

2.5. Construction of wild-type or mutant pUGT1A1-cDNA expression units

All missense mutants were constructed in the pSVL-based HUG-Br1-cDNA (now designated pSVL-based UGT1A1) unit as previously described [10]. Point mutations were introduced in the cDNA at amino acid positions 294 and 336 and were designated I294T (UGT1A1*33) and R336W (UGT1A1*32), respectively. The primer sets were as follows: I294T, sense 5′-GAAGCCTACACTAATGCTTC-3′ and antisense 5′-GAAGCATTAGTGTAGGCTTC-3′ and R336W, sense 5′-GACAGTCCTGTGGTGGTACA-3′ and antisense 5′-TGTACCACCACAGGACTGTC-3′. The outside primers for all were sense OP170 and antisense PXAS6 [17].

2.6. Expression of the bilirubin transferase by wild-type or mutant pUGT1A1cDNA constructs in COS-1 cells

COS-1 cells were plated in 100 mm dishes at 10^6 cells and grown to 90% confluence in 24 h in Dulbecco's modified Eagle's medium (DMEM) with HEPES buffer and 4% fetal calf serum (FCS). pUGT1A1 or each of its mutants was transfected into cells using DEAE-dextran as the carrier as described [17]. The transferase isozymes were radiolabeled during the final 4 h of the incubation as previously described by Ciotti et al. [17]. Goat anti-mouse UDP-glucuronosyltransferase IgG was added to the solubilized labeled cellular extract and processed for SDS-gel electrophoresis as described [24]; the dried gel was exposed to X-ray film for an autoradiograph. [^{35}S]Methionine incorporated into immunocomplexed protein was analyzed on SDS-polyacrylamide gels which were dried and scanned on the Fuji Phosphorimager BAS 2000; the counts were used to establish equivalent amounts of wild-type and mutant proteins for the glucuronidation reactions. The amount of bilirubin-glucuronide on TLC plates was analyzed on the Ambis Radioanalytical Imaging System Mark II as previously described [24]. X-Ray exposures of the TLC plates were developed for radiograms.

2.7. Bilirubin transferase activity following ransfection with the wild-type or mutant pUGT1A1 constructs in COS-1 cells

Cell homogenate containing expressed wild-type or mutant enzyme was used to determine bilirubin glucuronidating activity as described [13,17] with modifications. The reaction buffers were standardized us-

ing between 300 and 600 μg cell homogenate protein as follows: for the pH 6.4 buffer, 0.1 M sodium phosphate pH 8.05 was diluted to 20 mM and adjusted to pH 6.4 with 0.65 μl of 1.0 N NaOH/300 μg protein, and for the pH 7.6 buffer 0.166 M triethanolamine pH 8.6 was diluted to 32 mM and adjusted to pH 7.6 with 1.6 μl of 1.0 N NaOH/300 μg protein. In order to compare the effect of mutations on activity, equal amounts of specific protein were required and established as described above.

2.8. Construction of mutants in the TATA box element of the UGT1A1 gene

The pCAT reporter gene was fused with either the −0.5 kb, −1.7 kb, or −2.9 kb upstream region of the wild-type *UGT1A1* gene generating constructs designated pCAT/0.5*Pst*I, pCAT/1.7*Hin*dIII, or pCAT/2.9*Xba*I, respectively. Also, the 2.9 kb fragment was inserted in the reverse orientation (pCAT2.9X(R). The wild-type TATA box element in each of the three constructs is $A(TA)_7A$. The 3′ end (*Nar*I site) of the flanking region is 36 bp downstream from the TATA box. The pCAT basic and pCAT-enhancer vector used in Fig. 7A were modified to reduce the background as seen in Fig. 7C according to Lei et al. [27]. Mutants in the pCAT/1.7 kb *Hin*dIII contained either $A(TA)_8A$ or $A(TA)_6A$. To make the mutant TATA box elements, a 317 bp fragment was amplified from the genomic DNA isolated from CF and SM using the sense primer PAGS1 and the antisense primer, PAGA1. A second more 5′ fragment which overlapped by 22 bp was amplified with sense primer 5′-UPBr13 (5′-GAGCTTTAATTGGTTGGT-3′ (−975 to −958 bp)) and antisense primer 5′-UPBr1-21 (5′-ACCAATCGATACACCAAGTTAAT-3′ (−63 to −85 bp)) to generate a 919 bp fragment. The amplified fragment overlapped the PAGS1 primed sequence by 22 bp. The two interchanged hybridizing fragments were used in a second PCR with the outside sense primer, 5′-UPBr1-3, and antisense primer, 5′-AUPBr1 (5′-CCTTTGCTCCTGCCAGAGGTTC-3′, −5 to −25 bp) nested with PAGA1 to generate a 969 bp fragment which was digested with *Stu*I and *Eco*NI and subcloned into *Stu*I/*Eco*NI-digested pCAT/1.7*Hin*dIII-based unit. The 969 bp fragment was sequenced to verify that no other changes had occurred.

2.9. Transcriptional activity generated by the pCAT constructs in the HepG2 cells

HepG2 cells were plated in 100 mm dishes at 10^6 and grown in Eagle's medium with HEPES buffer and 4% fetal calf serum (FCS) to 90% confluence in 24 h. pCAT constructs were transfected into the cells by the calcium phosphate coprecipitation method [28]. Twenty μg of each pCAT construct were mixed with 500 μl 2×HEBS buffer, pH 7.05, and while shaking the samples, 500 μl of Ca-chloroquine were added dropwise (2 M $CaCl_2$; 40 μM chloroquine). The mixture was allowed to stand at room temperature for 30 min before adding directly to the cells. After 5 h of incubation at 37°C the medium was replaced with fresh medium. Cells were harvested 72 h later and analyzed for acetylchloramphenicol activity.

2.10. Acetylchloramphenicol activity generated by wild-type and mutant pCAT/HindIII reporter gene constructs in HepG2 cells

Acetylchloramphenicol transferase activity expressed by the reporter gene in HepG2 cells was measured as described [29]. The assay conditions were optimized for cellular extract and incubation time as shown in Fig. 7A,B.

3. Results

3.1. Defects in the coding region of the CN-I and the CN-II patients

In order to determine the cause of the hyperbilirubinemia in the CN-I (CF) and the CN-II (SM) patients, we sequenced five to ten different subclones for each of the five exons of the *UGT1A1* gene [9]. Fig. 1A shows that a C to T transition changed an Arg to Trp at codon 336 (R336W) in two out of six subclones for CF. All other exons from CF were normal. The C to T transition at codon 336 was present in one out of five subclones of the DNA from the mother of CF.

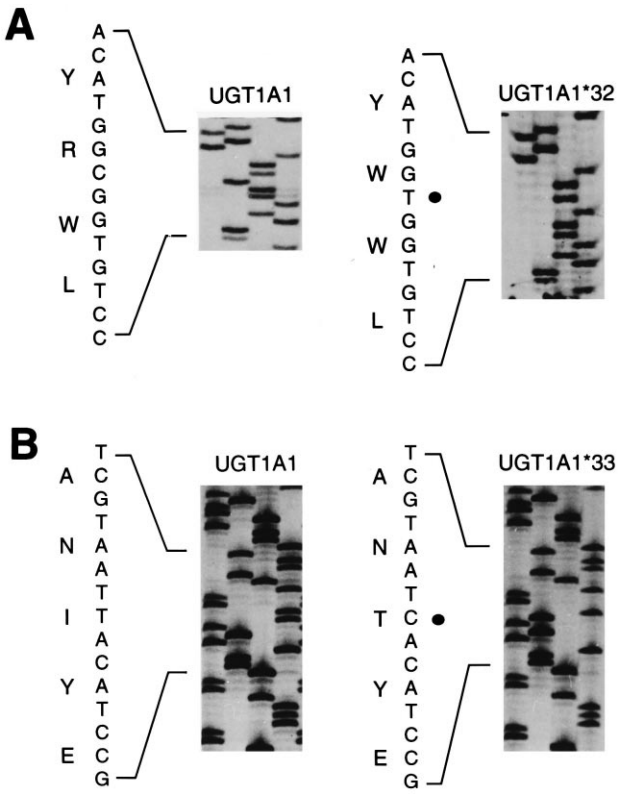

Fig. 1. Comparison of nucleotide sequences in a wild-type UGT1A1 (HUG-Br1) cDNA with that of UGT1A1*32 (R336W; CN-I) and UGT1A1*33 (I294T; CN-II) mutants. Sequencing reactions of a normal exon 3 (top left) and that from a CN-I (CF) patient code for the wild-type UGT1A1cDNA and the mutant UGT1A1*32, respectively. Also, a normal exon 2 (bottom left) is compared with that from a CN-II (SM) individual (bottom right) which codes for the UGT1A1*33 mutant. Sequencing determinations were as described in Section 2. The sequencing ladders were generated by electrophoresis through 6% denaturing polyacrylamide gels, blotted, dried, and exposed to X-ray film for the autoradiogram. A, C, G, and T represent reactions stopped with dideoxy derivatives of ATP, CTP, GTP, and TTP, respectively. The solid circle represents the substituted nucleotide in the patient genome.

In the CN-II (SM) individual (Fig. 1B), a T to C transition changed an Ile to Thr at codon 294 (I294T) in two out of five subclones for SM. All other subclones representing the *UGT1A1* gene revealed no other coding alterations. The T to C transition at codon 294 was present in one out of five subclones for the father of SM. Altered codons 294 and 336 are located in exon 2 and 3, respectively.

3.2. *Expression of wild-type and mutant bilirubin transferase proteins and the effects of mutations on glucuronidation*

Several missense mutations have been demonstrated to cause essentially a total loss of bilirubin activity in CN-I patients [11–13,17]. The effect of mutations seen in CN-II patients on bilirubin transferase activity has been documented in only one case [21]. Also, we would like to account for the difference in severity of hyperbilirubinemia in these two patients in the wake of a common mutation and differences in response to phenobarbital treatment. Expression of wild-type and mutant transferase in COS-1 cells was established by immunocomplex formation (Fig. 2). Furthermore, we quantitated the amount of specific protein produced by each construct by analysis on the phosphorimager as described above. The results were used to establish equal amounts of specific protein for glucuronidation studies.

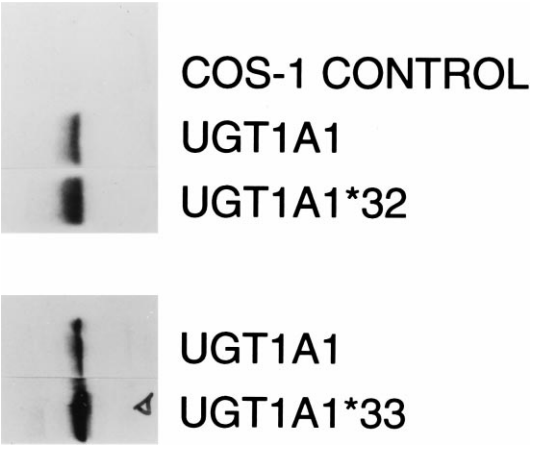

Fig. 2. Immunocomplexes of the UGT1A1 protein and its mutants, UGT1A*32 and UGT1A1*33, synthesized in COS-1 cells. Cells were transfected with either pUGT1A1, pUGT1A1*32, or pUGT1A1*33 expression units and incubated for 72 h. The proteins were radiolabeled for the final 4 h with [^{35}S]methionine, solubilized, and immunocomplexed with goat anti-mouse UDP-glucuronosyltransferase as described in Section 2. (It has been established that the HUG-Br1 (UGT1A1) cDNA encodes a 52 kDa protein in COS-1 cells which is immunocomplexed with this antibody [13,17].) Washed product was separated on a SDS-polyacrylamide gel during electrophoresis and dried; the specific bands were quantitated by scanning on a phosphorimager as described in Section 2.

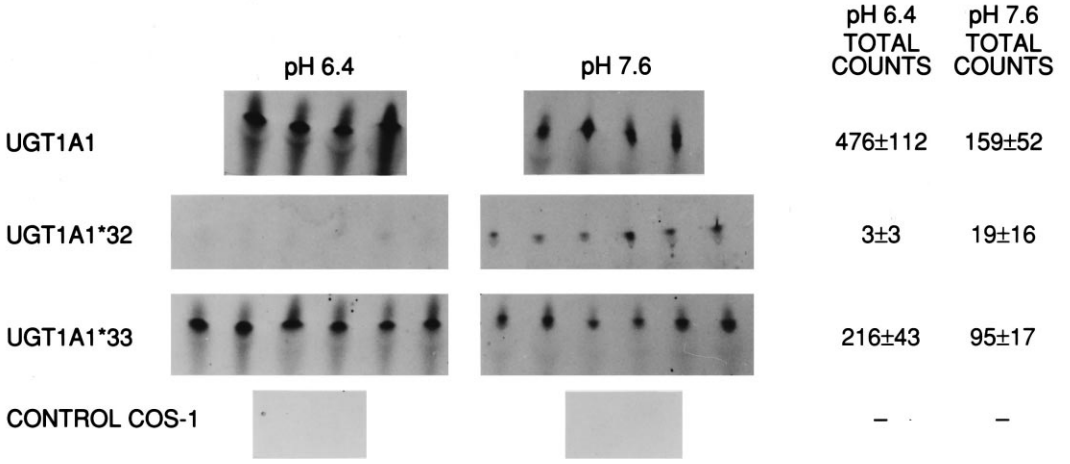

Fig. 3. Catalysis of bilirubin glucuronidation by UGT1A1, UGT1A1*32, and UGT1A1*33 at both pH 6.4 and 7.6. COS-1 cells were transfected as described in the legend to Fig. 2. The same amount of specific protein for UGT1A1, UGT1A1*32, (CN-I), and UGT1A1*33 (CN-II) was used to determine bilirubin glucuronidation at both pH 6.4 and 7.6 as described in Section 2. The assays, containing 1.4 mM [^{14}C]UDP-glucuronic acid (1.4 μCi/μmol) and 100 μM bilirubin, were incubated at 24°C for 16 h, and product was analyzed as described in Section 2. Bilirubin glucuronides separated on TLC plates were exposed to X-ray film, developed, and printed for autoradiograms. All films were exposed for 14 days.

In Fig. 3, the results are shown for bilirubin glucuronidation when carried out at both pH 6.4 and 7.6. For wild-type UGT1A1, there is 3-fold more product formed at pH 6.4 than at pH 7.6. The R336W coding mutation in the CN-I patient expressed no activity at pH 6.4, but had 12% normal activity at pH 7.6.

Under each pH condition, the I294T missense mutation in the CN-II individual caused a partial loss of activity. At pH 6.4, 45% of the activity remained, and at pH 7.6 59% remained. For the wild-type protein, the physiological significance of 2–3-fold more measurable activity at pH 6.4 compared to that at the more typical condition of pH 7.6 is not understood. The results show that the I294T mutant protein has far superior activity (overall some 50% of normal) at both pH 7.6 and 6.4 compared to the R336W mutant (0–10% normal). Hence, the missense mutation in the CN-I patient is far more debilitating than that in the CN-II patient.

Also, the I294T mutant of the UGT1A1 protein was assessed for its affinity toward bilirubin. Fig. 4 shows that the K_m for bilirubin is approx. 2.5 μM at both pH 6.4 and pH 7.6. While the V_{max} for bilirubin glucuronidation was decreased, its affinity for bilirubin was essentially normal [13].

3.3. Demonstration of mutations in the TATA box element

The unusual TATA box element upstream from the bilirubin transferase gene, *UGT1A1*, was shown [9] to contain seven consecutive TA di-nucleotides as

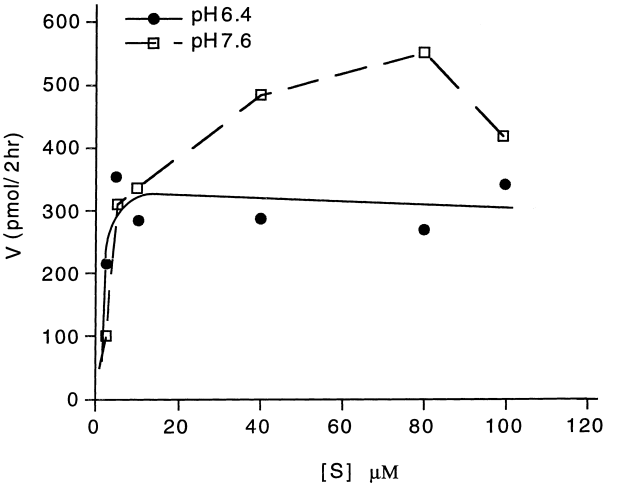

Fig. 4. Relative reaction velocity versus bilirubin concentration of the UGT1A1*33 enzyme. Glucuronidation reactions at both pH 6.4 and 7.6 were carried out with bilirubin between 2.5 and 100 μM for 2 h at 37°C and analyzed as described in the legend to Fig. 3.

follows: ATATATATATATATAA [A(TA)$_7$A] (Fig. 5, left ladder). Also, Fig. 5 shows a representative sequence ladder across the TATA box element that was observed for two out of five subclones for the CN-I (CF) genome and two out of five subclones for the CN-II (SM) genome. Hence, a TA insertion, [A(TA)$_8$A], was detected at one allele for both patients. Since each patient also contained a coding region defect, described above, one can conclude that each is a compound heterozygote.

3.4. Inheritance of the TATA box mutation by CF and SM

In the 6% polyacrylamide gel, as shown in Fig. 6, the PCR-amplified TATA box region of the normal individual (lane 1) generated a single 317 bp fragment. This 317 bp fragment, diagnostic for the normal TATA box, was present in every sample. The TA insertion in the TATA box generated a distinct band for the predicted 319 bp fragment at a position equivalent to 2 bp above the typical 317 bp fragment as seen for both probands (Fig. 6, CF, lane 2, and SM, lane 5). The father (lane 3) of CF and the mother (lane 7) of SM are also carriers of the TA insertion. Hence, CF inherited the R336W missense mutation and the TA insertion, [A(TA)$_8$A], from her mother and father, respectively. SM inherited the I294T coding defect and the [A(TA)$_8$A] mutant TATA box from her father and mother, respectively. There is no kindred among the two families.

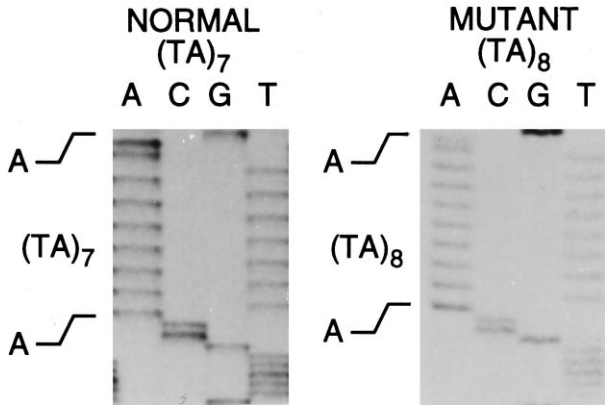

Fig. 5. Sequences surrounding the TATA box element of the gene from a normal and a defective element seen in both a CN-I (CF) and a CN-II (SM) individual. Sequence determinations were as described in Section 2. The sequencing ladders were generated and analyzed as described in the legend to Fig. 1. A normal element, A(TA)$_7$A, and the insertion mutant box, A(TA)$_8$A, were seen in both CN-I and CN-II patients. A deletion mutant TATA box, A(TA)$_6$A, was constructed to use as a control and was sequenced.

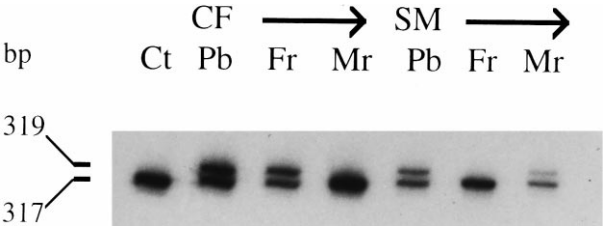

Fig. 6. Inheritance of the TA insertion at the TATA box of the bilirubin transferase gene by CF and SM. Genomic sequences surrounding the TATA box of the *UGT1A1* gene of a control (Ct), the probands (Pb), and their respective mother (Mr) and father (Fr) were amplified by PCR using the sense primer, [^{32}P]PAGS1, and the antisense primer, PAGA1, as described in Section 2. The radiolabeled product was separated on a 6% polyacrylamide gel as described in Section 2 and exposed to X-ray film which was developed and printed. The 317 bp fragment represents wild-type and the 319 fragment represents the mutant TATA box element with TA di-nucleotide insertion.

3.5. Transcriptional effects of TATA box mutations of the UGT1A1 upstream regulatory region fused to the pCAT reporter gene

Initially, we determined the minimum upstream region of the *UGT1A1* gene required to generate maximum transcriptional activity in the pCAT reporter gene assay. The upstream-reporter gene constructs were transfected into HepG2 cells as described; cells were harvested and analyzed for acetyl chloramphenicol (CAT) activity. Fig. 7A (arrow) shows that the pCAT/1.7*Hin*dIII with the −1.7 kb upstream regulatory region generated greater activity than pCAT/2.9*Xba*I with a −2.9 kb URR. The relative counts demonstrated that the 1.7 kb fragment was 2.5-fold more active than the 2.9 kb and 3.1-fold more active than the pCAT/0.5*Pst*I with a −0.5 kb URR (data not shown). Also, we established the optimal protein and time course for the CAT activity (Fig. 7B). Since the background was shown to be significant in Fig. 7A (arrow, pCAT Basic), the vectors were modified as described in Section 2 to reduce read-through as shown in Fig. 7C (arrow, pCAT Basic).

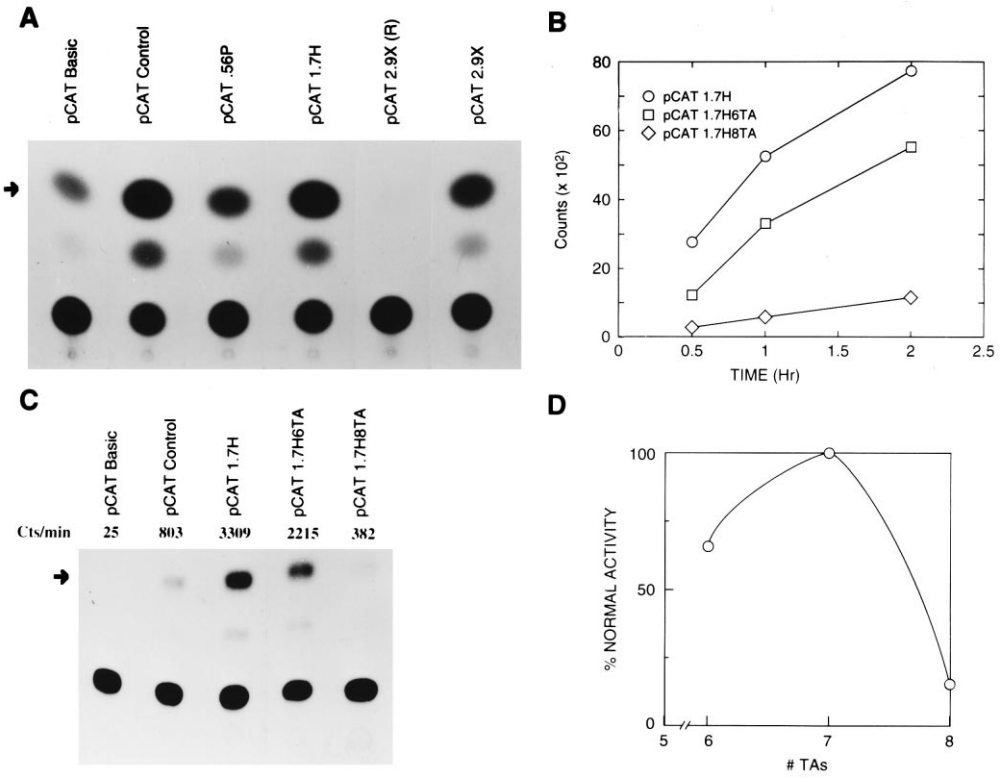

Fig. 7. Effect of the wild-type TATA box, $A(TA)_7A$, versus the mutant boxes, $A(TA)_8A$ and $A(TA)_6A$, on transcriptional activity assessed with the *UGT1A1* promoter region-CAT reporter gene constructs transfected into HepG2 cells. (A) Transcriptional activity, measured by product (arrow) generated by pCAT basic reporter gene or the pCAT/enhancer control, is compared with that by pCAT basic constructs which contained either the −0.56 bp, −1.7 bp or −2.9 bp upstream flanking region of the *UGT1A1* gene. The −2.9X (R) is in the reversed orientation. (B) Time course of transcriptional activity estimated by CAT product supported by the pCAT basic vector with either the wild-type TATA box (ATA_7A) designated pCAT1.7H, the TA deletion mutant (ATA_6A) box designated pCAT1.7H6TA, and the TA insertion mutant (ATA_8A) box designated pCAT1.78TA in the −1.7 bp upstream flanking region of the *UGT1A1* gene. Product is denoted with the arrow. (C) Product (arrow) of CAT activity is evaluated as counts/min generated with the same experimental constructs used in B. The plot in D represents the relative effectiveness of the number of TAs in the TATA box element on transcriptional activity contained in the −1.7 bp upstream region/pCAT construct.

The results in Fig. 7C show that transcriptional activity with the $A(TA)_8A$ promoter element is 89% reduced and that for the $A(TA)_6A$ is 33% reduced. For three experiments the range of inhibition was 85–89%.

Fig. 7D shows that seven TAs are the optimal number to generate the highest transcriptional activity in vitro. Although we expected to find deletions at the TATA box, 20 randomly selected individual DNA samples examined contained no mutation of any type at the TATA box. This compares to a report of 51% frequency (28/55 individuals) of heterozygotes with the insertional mutation at the TATA box in the population [23].

4. Discussion

We have shown that a TA di-nucleotide insertional mutation at the expansive TATA box element, $A(TA)_7A$, of the bilirubin transferase gene, *UGT1A1*, exists in the compound heterozygous state in both a CN-I and a CN-II hyperbilirubinemic patient. The potentially lethal CN-I disease has been documented in reports [11–17] to be an autosomal recessive disease requiring a deleterious mutation in both alleles of the *UGT1A1* bilirubin transferase gene to account for essentially a total loss of bilirubin transferase activity in patients. All such mutations in the high-risk CN-I population were previously found in the coding region of the bilirubin transfer-

ase gene. In this study, the TA insertion destroyed 85–89% transcriptional activity as measured in vitro, presumably, reflecting in vivo transcription at one allele in both the CN-I and II patients. The missense mutation substituting R336W in the CN-I patient expressed no detectable activity at pH 6.4 and 12% normal activity at pH 7.6. In the CN-I patient, it is predicted that the UGT1A1*32 (R336W) protein is essentially totally inactive and that there is more than 85–89% loss of transcriptional activity by the $A(TA)_8A$ box mutation to create the severe hyperbilirubinemic condition of CF. Hence, it is likely that both mutations are completely deleterious in the CN-I individual.

The CN-II disease is far less severe due to residual bilirubin glucuronidating activity [2]; here we show that this is most likely due to a mutation that partially inactivates the bilirubin transferase encoded at the second allele. The missense mutation substituting I294T in the CN-II individual exhibited some 50% normal activity at pH 6.4 and 7.6, respectively, and normal affinity ($K_m = 2.5$ μM) for bilirubin. For the CN-II patient, it is predicted that the substantially more active UGT1A1*33 (I294T) protein paired with greater than 85% loss of transcriptional activity at the allele with the mutant TATA box are responsible for the less threatening hyperbilirubinemic phenotype of SM.

Repetitive treatment of CF with phenobarbital up to 3.5 years of age failed to elicit a decrease in serum bilirubin levels. Hence, this patient received a liver transplant at that age. Since the hyperbilirubinemia seen in SM, and not that of CF, did respond to phenobarbital treatment, it is predicted that increased synthesis of the partially active protein, I294T, by phenobarbital is responsible for bringing about the decrease in serum bilirubin in SM. The drug had the potential to increase, at best, the synthesis of an essentially inactive R336W protein in the CN-I patient. Based on the outcome of the drug treatment in the two patients, it does not appear that the TATA box mutation responded to phenobarbital.

Although Gilbert's individuals have been reported [6] to respond to phenobarbital treatment, the genotypes of the responders are unknown. Genotypes for Gilbert's are shown to include missense mutations [22] as well. This population of individuals was reported in a much earlier study [5] to have from 10 to 33% bilirubin conjugating activity remaining and, presumably, that same amount of the transferase protein.

Although the effects of the TA insertion on in vitro transcription were similar between this study and that for the Gilbert's case [23], the controlled HepG2 cell culture conditions in the absence of significant amounts of the bilirubin transferase protein may not reflect actual in vivo transcriptional conditions/effects seen in the patients. The benign effect of the homozygous TA insertional mutation in Gilbert's individuals [23] and the evidently complete repression of transcription in the CN-I patient of this study suggest, however, that the effect of this regulatory mutation in vivo is quite variable and allows for the full range of hyperbilirubinemic phenotypes. This mutation may be analogous to the variable [30,31] and unpredictable [32] transcriptional/translational effects of the CGG-repeat expansion in the 5′ upstream region of the *FMR*1 gene among patients with the fragile X syndrome. Frequently, the resulting CpG island is hypermethylated. While methylated versus unmethylated DNA in the 5′ upstream region harboring the 200-fold CGG-repeat expansion generated only slight differences in mRNA levels, there was a complete lack of CAT activity with the introduction of the repeats [30–32] as measured in cell culture conditions. Hence, this regulatory model for the mutation at the *FMR1* gene leading to fragile X syndrome inexplicably exhibited repressed CAT activity based on mRNA levels. A consideration is whether the mutant TATA box in the CN patients is affected by the level of the bilirubin isozyme (estimably 50% of normal but with defective activity), whereas Gilbert's individuals have, estimably, 10–33% normal enzyme levels. Further, it is not obvious that cellular unconjugated bilirubin concentrations could affect the level of enzyme/activity in the patients studied.

Since it is possible that a TA deletion could also occur in this unusually long TATA box element, the transcriptional activity of $A(TA)_6A$ was examined. The TA deletion proved to be less deleterious; a 35% decrease in transcription activity compared to $>85\%$ for the insertional mutation. It is possible that the number of TAs present at the TATA box affects the binding/alignment of the transcriptional

machinery and the transcription start site at the *UGT1A1* gene in the DNA molecule with intrinsic helical structures for consideration.

Although the homozygous TA insertion defines the mild Gilbert's syndrome [23], this study points to the most dire outcome of this mutation in the population. Individuals who are carriers (51%) [23] of the TA mutation, like CF's father, do have the potential to generate offspring with a lethal genotype. Hence, this regulatory mutation, mild in Gilbert's and deleterious in the CN-I and CN-II individuals in this study, is more complicated than the missense mutations and does warrant consideration as an issue for genetic counseling.

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