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Genetics

# Racial variability in the UDP-glucuronosyltransferase 1 (*UGT1A1*) promoter: A balanced polymorphism for regulation of bilirubin metabolism?

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**ABSTRACT A polymorphism in the promoter of the UDP-glucuronosyltransferase 1 (*UGT1A1*) gene has been shown to cause Gilbert syndrome, a benign form of unconjugated bilirubinemia. Promoters containing seven thymine adenine (ta) repeats have been found to be less active than the wild-type six repeats, and the serum bilirubin levels of persons homozygous or even heterozygous for seven repeats have been found to be higher than those with the wild-type six repeats. We have now examined the genotypes in persons of Asian, African, and Caucasian ancestry. Although within the Caucasian ethnic group there is a strong correlation between promoter repeat number and bilirubin level, between ethnic groups we found that this relationship to be inverse. Among people of African ancestry there are, in addition to those with six and seven repeats, also persons who have five or eight repeats. Using a reporter gene we show that there is an inverse relationship between the number of ta repeats and the activity of the promoter through the range of 5–8 ta repeats. An incidental finding was a polymorphism at nucleotide −106, tightly linked to the $(ta)_5$ haplotype. Serum bilirubin levels are influenced by many factors, both genetic and environmental. We suggest that the unstable *UGT1A1* polymorphism may serve to "fine-tune" the plasma bilirubin level within population groups, maintaining it at a high enough level to provide protection against oxidative damage, but at a level that is sufficiently low to prevent kernicterus in infants.**

Gilbert syndrome is a benign form of unconjugated hyperbilirubinemia that has long been regarded to be a manifestation of an abnormality of bilirubin glucuronide formation (1, 2). A nonsense mutation of the UDP-glucuronosyltransferase 1 (*UGT1A1*) gene was identified as a cause of a more severe defect in glucuronide formation, Crigler–Najjar syndrome in 1992 (3), but it was only in 1995 that mutations of *UGT1A1* that caused Gilbert syndrome were discovered (4, 5). A polymorphism in the promoter of the *UGT1A1* gene in which seven instead of six thymine adenine (ta) repeats exist in the promoter region accounts for most cases of this disorder. This polymorphism has been given the name *UGT1A1*28* (6). Here we use the designation $(ta)_n$, where $n$ is the number of repeats. The $(ta)_7$ mutation of the *UGT1A1* gene has been found to be associated with increased bilirubin levels in normal persons (4), in those with heterozygous β-thalassemia (7) or glucose-6-phosphate dehydrogenase (G6PD) deficiency (8), and with neonatal icterus in G6PD deficiency (9) and hereditary spherocytosis (10).

Large population studies comparing serum bilirubin values of black and white populations have shown that both black men and black women have total serum bilirubin levels that are 15–20% lower than their white counterparts (11, 12). Similarly, jaundiced black infants have lower bilirubin levels than white infants (13). Among Asian infants bilirubin levels are higher than among whites (14–16). These findings suggested that the prevalence of the $(ta)_7$ mutation might be low among people of African origin. However, we now report that contrary to expectation, the $(ta)_7$ form is more common among people of African origin than among Caucasians. Although Asians seem to have higher bilirubin levels than whites, the $(ta)_7$ is much less common in that population. Among Africans not only the $(ta)_6$ and $(ta)_7$ forms are found, but there are also promoters that contain five or eight ta repeats [$(ta)_5$ and $(ta)_8$]. When transfected into appropriate cells lines, the activity of the promoter decreases with the progressive number of repeats.

We suggest that the *UGT1A1* promoter polymorphism may provide a flexible polymorphism that maintains bilirubin levels in a range high enough to protect against oxidative damage, but not so high as to cause a high incidence of kernicterus.

## MATERIALS AND METHODS

**Subjects.** Analysis of the number of repeats in the *UGT1A1* promoter was performed on anonymous DNA samples from different ethnic groups. There were 71 Caucasians of European ancestry, 47 Asians of whom 41 were Chinese and 6 were Japanese, and 101 samples from individuals from North and Central America with varying degrees of African ancestry.

**Determination of *UGT1A1* Promoter Genotypes.** Genomic DNA was extracted from peripheral blood leukocytes by using standard methods. Three methods were used to determine the number of repeats in the *UGT1A1* promoter. The first method used for all DNA samples consisted of PCR amplification of genomic DNA by using the $^{32}P$-labeled primers (17) as shown in Table 1. The sense primer was labeled with [$\gamma$-$^{32}P$]ATP by T4 polynucleotide kinase. The 25-μl PCR system contained 33.5 mM Tris·HCl (pH 8.8), 8.3 mM $(NH_4)_2SO_4$, 3.4 mM $MgCl_2$, 85 μg/ml of BSA, 5% DMSO, 0.2 mM dNTPs, 50 ng of each primer, 0.5 unit of *Taq* polymerase (Qiagen, Chatsworth, CA), and 50 ng genomic DNA. After 4 min of denaturation at 98°C, the samples were subjected to 30 cycles consisting of 30 sec each of 93°C, 58°C, and 72°C, followed by a final extension for 7 min at 72°C. Two microliters of the final PCR product was denatured in 2 μl of 95% formamide/20 mM EDTA/0.05% bromophenol blue/0.05% Xylene Cyanole FF and heated at 95°C for 5 min before being loaded directly on a 6% sequencing gel. The samples were subjected to electrophoresis at 50 V/cm for 4 hr. The gel was dried and exposed on AR x-ray film for 24 hr. Bands ranging in size from 96 to 102 bp representing five, six, seven, and eight ta repeats could be visualized and compared with a radiolabeled molecular weight marker, SequaMark (Research Genetics, Huntsville, AL) (Fig. 1).

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Abbreviation: ta, thymine adenine.

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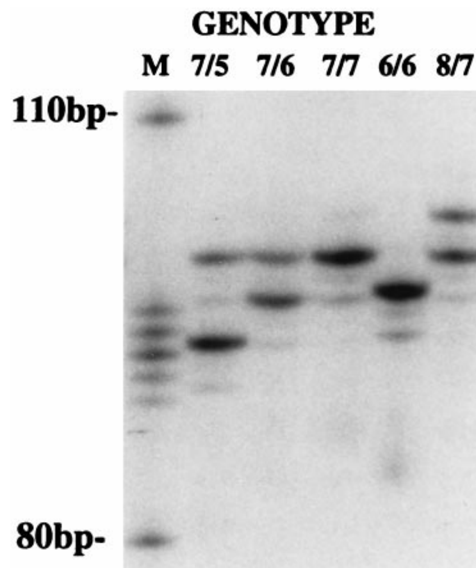


FIG. 1. Autoradiograph demonstrating the number of (ta) repeats in the *UGT1A1* promoter. M denotes the SequaMark molecular weight marker ranging in size from 80 to 110 bp with 1-bp incremental bands in the center. DNA samples from subjects with $(ta)_7/(ta)_5$, $(ta)_7/(ta)_6$, $(ta)_7/(ta)_7$, $(ta)_6/(ta)_6$, and $(ta)_8/(ta)_7$ *UGT1A1* genotypes are shown. Method is described in the text.

Additional methods of confirming the genotype of some of the DNA samples, including those with the newly described five and eight ta repeats, involved the use of automated sequencing protocols from Applied Biosystems. A PCR product was amplified with primers shown in Table 1 and sequenced with the nested primer also shown in Table 1. Alternatively, the number of ta repeats was confirmed by incorporating a HEX fluorescent-tagged antisense primer for exon 1 of the gene and a sense primer in the promoter region (Table 1) to amplify fluorescent products in the range of 334 bp for the $(ta)_5$ to 340 bp for the $(ta)_8$ repeat. PCR was performed for 25 cycles in a 50-μl system containing 25 μl *Taq* PCR Master Mix (Qiagen), which is composed of PCR buffer, nucleotides, and *Taq* polymerase; the remaining 25 μl contained 50 ng genomic DNA and 50 ng of each primer. The cycling conditions began with a 4-min 98°C denaturation followed by 25 cycles of 93°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec, and a final 7-min extension at 72°C. One-microliter aliquots of these products were separated on a fragment analysis gel and analyzed by GENE SCAN 2.1 software (Applied Biosystems) along with molecular weight markers.

Table 2. *UGT1A1* promoter genotypes in three different ethnic groups

| Genotype | European | Asian | African |
|---|---|---|---|
| 6/6 | 24 | 33 | 26 |
| 6/7 | 39 | 13 | 37 |
| 7/7 | 8 | 1 | 19 |
| 7/8 | 0 | 0 | 6 |
| 8/8 | 0 | 0 | 2 |
| 6/8 | 0 | 0 | 4 |
| 7/5 | 0 | 0 | 5 |
| 6/5 | 0 | 0 | 2 |
| Total | 71 | 47 | 101 |

**Estimation of *UGT1A1* Promoter Activity.** Two sets of oligonucleotide primers (Table 1) were used to amplify the promoter from genomic DNA samples containing five, six, seven, and eight repeats. The first set of primers yielded promoters fragments 227–233 bp in length depending on the number of ta repeats; the second set of primers yielded a slightly longer promoter region containing 259–265 bp. The shorter promoter constructs extended from −17 to −245 [for $(ta)_6$] and the longer construct extended from −1 to −261 [for $(ta)_6$], numbering from the initiator ATG. The promoter regions described above were amplified in a 100-μl system using the Expand Long Template PCR system (Boehringer Mannheim), which contains a proofreading Pwo DNA polymerase for high fidelity. After amplification the PCR products were separated from the oligonucleotide primers with QIAquick PCR purification columns (Qiagen). The purified DNA was digested with the appropriate restriction enzymes listed in Table 1. The digested promoters then were cloned into pGL3 Basic Luciferase Reporter Vector (Promega) and transformed into JM 109 *Escherichia coli* bacterial cells (Promega). The clones were sequenced by using the vector primers shown in Table 1. One of the clones obtained from a subject who was homozygous for the $(ta)_8$ repeat was found to contain nine repeats, presumably a PCR error that occurred even in the presence of the high-fidelity enzyme. Plasmid purification was performed on the desired clones by using Qiagen Maxi columns according to the manufacturer's instructions.

Promoter activity was measured in Hep G2 cells and HuH 7 (18) cells. These cell lines were selected because they are human cells of hepatic origin and presumably have the capacity to conjugate bilirubin. The cells were maintained in DMEM containing 10% fetal calf serum, glutamine, penicillin, and streptomycin. Twenty-four hours before transfection, approximately $5 \times 10^5$ cells were plated into six-well plates 30 mm in diameter. Enough wells were seeded to perform all assays in quadruplicate. One microgram of plasmid DNA was trans-

Table 1. Primers

| Purpose | Primer | Cloning sites |
|---|---|---|
| Amplimers for *UGT1A1* promoter repeat analysis | 5′-GTCACGTGACACAGTCAAAC-3′ | |
| | 5′-TTTGCTCCTGCCAGAGGTT-3′ | |
| PCR primers for ABI automated sequencing | 5′-GCCAGTTCAACTGTTGTTGCC-3′ | |
| | 5′-CCACTGGGATCAACAGTATCT-3′ | |
| Nested primer for ABI automated sequencing | 5′-AGAAACCTAATAAAGCTCCACC-3′ | |
| PCR primers for ABI fluorescent fragment size analysis | 5′-GCTACCTTTGTGGACTGACAGC-3′ | |
| | HEX-5′-GTACAACGAGGCGTCAGGTGC-3′ | |
| To amplify 227- to 233-bp promoter fragment for functional studies | 5′-GTACTTGCTGTGGT*A*CCTCCAGAAT-3′ | *Kpn*I |
| | 5′-GGCGCCTTTGCTCCTGC*TC*GAGGTTC-3′ | *Xho*I |
| To amplify 259- to 265-bp promoter fragment for functional studies | 5′-**CGATAGGTACC**TGGAAGTACTTGCTGTGGTTACTCC-3′ | *Kpn*I |
| | 5′-**ATCGCAGATCT**GGCGCCTTTGCTCCTGCCAGAG-3′ | *Bgl*II |
| pGL3 vector primers for sequencing | 5′-CTAGCAAAATAGGCTGTCCC-3′ | |
| | 5′-CTTTATGTTTTTGGCGTCTTCCA-3′ | |

Italicized, positions mismatched to create restriction endonuclease digestion sites for cloning; bold, overhangs with restriction endonuclease digestion sites for cloning. ABI, Applied Biosystems.

Table 3. *UGT1A1* promoter gene frequencies (number of chromosomes) in three different ethnic groups

| Allele | European | Asian | African |
|---|---|---|---|
| 5 | 0 (0) | 0 (0) | 0.035 (7) |
| 6 | 0.613 (87) | 0.840 (79) | 0.470 (95) |
| 7 | 0.387 (55) | 0.160 (15) | 0.426 (86) |
| 8 | 0 (0) | 0 (0) | 0.069 (14) |

fected into the Hep G2 and HuH 7 cells by using SuperFect (Qiagen) according to the manufacturer's instructions. A plasmid pRL-SV40 containing Renilla luciferase driven by a simian virus 40 promoter was included as an internal control at a concentration of 0.05 μg. After incubation for 24–48 hr at 37°C under 5% $CO_2$ the cells were washed in PBS and lysed in 500 μl Passive Lysis Buffer (Promega). The lysates were cleared by centrifugation for 30 sec in a microcentrifuge, and 10–20 μl of cleared lysate was assayed for firefly luciferase and renilla luciferase with the Dual-Luciferase Reporter Assay System (Promega). Photoluminescence was measured in a Monolight 2010 single-channel luminometer.

## RESULTS

The distribution of the number of repeats in the three populations is summarized in Tables 2 and 3. The gene frequency of $(ta)_7$ in the white population was 0.387. The gene frequency of the $(ta)_7$ allele in the Asian population was only 0.16, a difference in incidence from the white population that was statistically significant, with $P = .0002$ (Fisher's exact test). Five and eight ta repeats were encountered only in persons with African ancestry, and the number of chromosomes in which there were seven or more repeats was 49.5%. The number of repeats in people of African origin was significantly greater than in the white population ($P < 0.0001$; Fisher's exact test).

The activity of promoters with five through nine repeats is shown in Fig. 2. It is apparent that the production of luciferase, the reporter product, decreases progressively as the number of repeats increases from five to eight. The $(ta)_9$ variant (which has not been encountered in nature thus far) seems to have approximately the same activity as the $(ta)_8$ form.

An incidental finding was the occurrence of a polymorphism at position −106 with respect to the start ATG codon. A cytosine is substituted for a thymine in all DNA samples from subjects with the $(ta)_5$ haplotype and in none of 23 other subjects examined.

## DISCUSSION

The steady-state serum bilirubin level is a function of numerous factors that may modify bilirubin production and excretion. Among these are the red cell mass and the red cell lifespan, which define the production of bilirubin, and the conjugation and transport of bilirubin, which define the rate of removal.

The relationship of the activity of UDP-glucuronosyltransferase, the enzyme that conjugates bilirubin, to neonatal jaundice was first shown to exist by Brown and Zuelzer in 1958 (1), and the relationship between this bilirubin-conjugating enzyme and other forms of jaundice, including Crigler–Najjar syndrome and Gilbert syndrome, has been amply documented

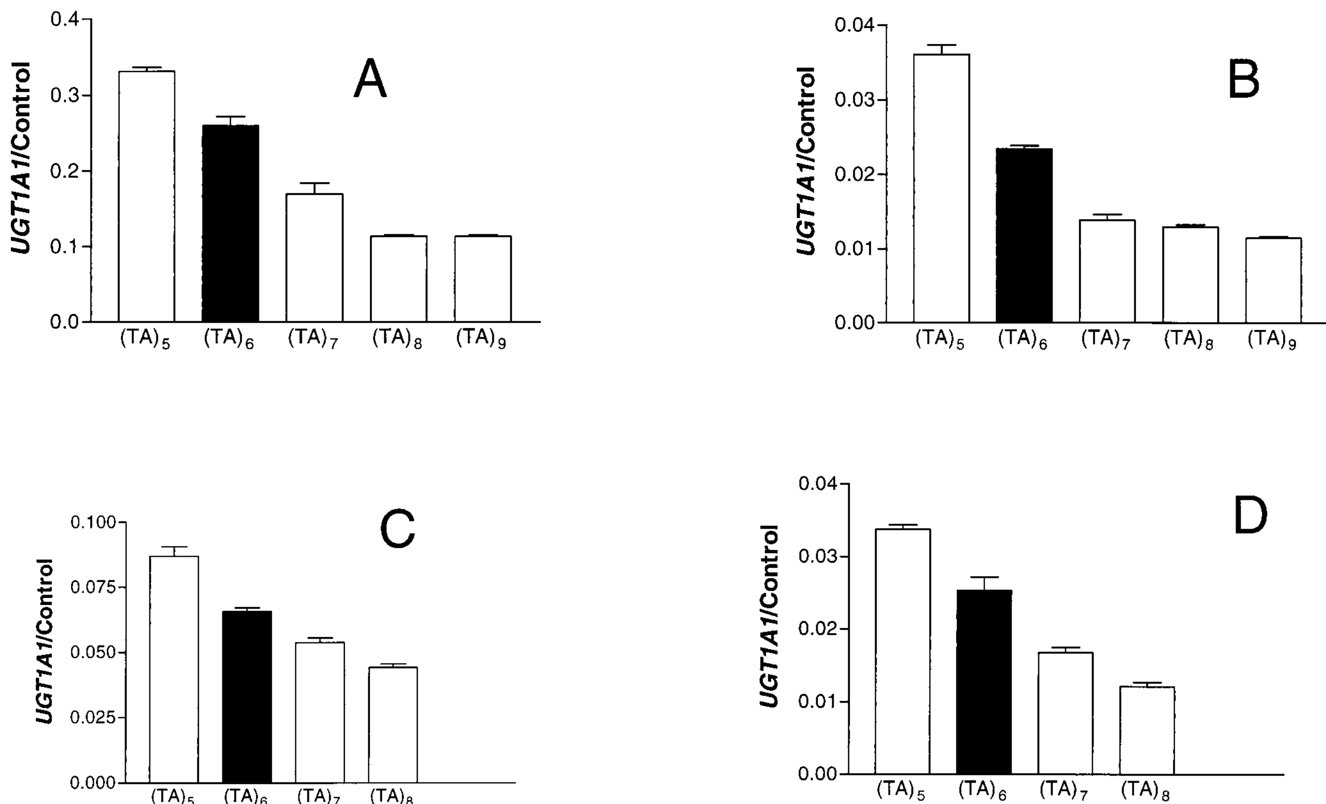


FIG. 2. The effectiveness of the *UGT1A1* promoters containing the variable (ta) repeats using a luciferase reporter. Promoter activity was analyzed in two human hepatoma cell lines: HepG2 (*A* and *C*) and HuH7 (*B* and *D*). The shorter promoter constructs (*C* and *D*) were 229 bp for the $(ta)_6$, and the longer constructs (*A* and *B*) were an additional 32 bp in length. Promoter activity with a firefly luciferase reporter gene was compared with an internal control of Renilla luciferase with an simian virus 40 promoter. Mean values of three to six replicate wells and their standard errors are shown. The short *UGT1A1* constructs in HepG2 cells (*C*) were assayed 24 hr after transfections instead of 48 hr because the cells had reached confluency. The "wild type" $(ta)_6$ is shown as a solid bar; the variants are shown as open bars. Note that the $(ta)_9$ promoter was made by mutagenesis. All other promoters are naturally occurring.

(19). Mutations of the coding region of the *UGT1A1* gene can cause the more severe Crigler–Najjar syndrome (3, 5), and heterozygotes for such mutations may manifest a dominant form of Gilbert syndrome. Among Europeans, however, the most common mutation giving rise to Gilbert syndrome is one affecting the *UGT1A1* promoter and insertion of an extra ta to create seven repeats. The gene frequency of this mutation among whites is extraordinarily high, 0.387 in the present study, similar to the frequency found in small series of studies of Caucasians published previously, namely 0.4 (4) and 0.38 (17). Based on our data one would predict a $(0.387)^2 = 0.15$ homozygote frequency. The penetrance of the $(ta)_7/(ta)_7$ genotype presumably is incomplete, because the predicted value is modestly higher than the published frequency of Gilbert syndrome in Germany, namely 12.4% among men and 4.8% of women, with a total of 8.6% for the entire group (20) and a slightly lower prevalence in another study (21).

Because repeated sequences are intrinsically unstable and tend to lengthen and shorten as a result of unequal crossing-over in meiosis, it is not surprising that other repeat patterns are found, although none have been reported previously. In the African population the $(ta)_5$ and $(ta)_8$ repeats achieve polymorphic frequencies of 0.035 and 0.069, respectively. Because the samples studied were anonymous and no clinical data were available we cannot be certain of the clinical effects of these promoter variants. However, the activities of promoter constructs of the type used here previously have been shown to be well correlated with bilirubin levels (4). It seems reasonable, therefore, to conclude that individuals carrying the $(ta)_8$ mutation would have a proclivity for the development of jaundice, whereas the $(ta)_5$ mutation, showing significantly higher activity than the wild-type $(ta)_6$ promoter, would be associated with low serum bilirubin levels.

The frequency of promoters with decreased activity was found to be highest in the African population, lowest in the Asian population, and intermediate in the European population. These findings are paradoxical in that, within the white population, in any case, longer promoter repeats are associated with higher bilirubin levels (4), whereas between populations the reverse is the case. The differences that we have observed may be because of genetic drift and have no selective basis. However, one can speculate on the reason for racial differences in *UGT1A1* promoter efficiency. Severe hyperbilirubinemia is a serious medical problem in newborn infants. Untreated, it may lead to kernicterus, a fatal or permanently disabling neurologic disorder. For this reason, even relatively mild defects in bilirubin conjugation generally have been considered to be disease states. With the recognition that bilirubin could serve as a powerful antioxidant, however, it has been proposed that under physiologic circumstances bilirubin may provide protection against oxidative damage (22–25), and there is evidence that it may play such a role (26–28). Thus, there may have been selection, in evolution, for a genotype that prevents hyperbilirubinemia leading to kernicterus, but that maintains bilirubin levels to protect against oxidative damage.

The less-efficient *UGT1A1* promoters in Africans would have predicted higher average bilirubin levels. That bilirubin levels are, in reality, lower suggest that among Africans or in their environment there are factors that would tend to lower the bilirubin levels. In Africa light exposure might be such a factor because it contributes to the degradation of bilirubin; the greater pigmentation of the skin does not appear to be a barrier to the degradation of bilirubin by light. Phototherapy is equally effective in black and white infants (13). However, persons of African descent living in North America also have lower serum bilirubin levels than do their Caucasian neighbors. It is therefore very likely that there are genetic differences between Africans and Europeans with respect to the metabolism of bilirubin other than the *UGT1A1* promoter polymorphisms. One of these is that Africans have lower hemoglobin levels and therefore presumably a smaller red cell mass than do Europeans (29–32). Given the same red cell lifespan, they would produce less bilirubin. The data available regarding bilirubin levels among Asians are limited, but all those available indicate that at least among infants the bilirubin levels are higher than those in Caucasians (14–16). As in the case of Africans, the cause of the difference is not clear, but it has been shown that the red cell lifespan of Japanese infants is less than that of Caucasian infants (15).

Thus, although within an ethnic group there is a strong correlation between promoter repeat number and bilirubin level, there is an inverse relationship between promoter strength and bilirubin levels between ethnic groups. The *UGT1A1* promoter polymorphism is particularly well suited to provide fine-tuning of bilirubin levels, bringing them to a level high enough to provide the defense against oxidants needed by the population, without allowing the incidence of kernicterus to rise to a level that is evolutionarily unacceptable. Because it is apparently an unstable repeating sequence, the population frequency can be changed not only by selection of existing genotypes but by relatively rapid creation of new ones. We suggest that evolutionary pressures may have counteracted largely undefined genetic and environmental pressures to select the number of repeats required to maintain serum bilirubin levels in an optimal range. That differences in bilirubin levels still exist between racial groups, despite changes in the number of promoter repeats, implies that alterations in bilirubin metabolism must have occurred sufficiently recently for full compensation not to have been achieved.

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