

THE GENETIC BASIS OF THE REDUCED EXPRESSION OF BILIRUBIN UDP-GLUCURONOSYLTRANSFERASE 1 IN GILBERT'S SYNDROME

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Abstract Background. People with Gilbert's syndrome have mild, chronic unconjugated hyperbilirubinemia in the absence of liver disease or overt hemolysis. Hepatic glucuronidating activity, essential for efficient biliary excretion of bilirubin, is reduced to about 30 percent of normal.

Methods. We sequenced the coding and promoter regions of the gene for bilirubin UDP-glucuronosyltransferase 1 (bilirubin/uridine diphosphoglucuronate-glucuronosyltransferase 1) — the only enzyme that contributes substantially to bilirubin glucuronidation — in 10 unrelated patients with Gilbert's syndrome, 16 members of a kindred with a history of Crigler–Najjar syndrome type II, and 55 normal subjects.

Results. The coding region of the gene for the enzyme was normal in the 10 patients with Gilbert's syndrome. These patients were homozygous for two extra bases (TA) in the TATAA element of the 5' promoter region of the gene (A(TA)₇TAA rather than the normal

A(TA)₆TAA). The presence of the longer TATAA element resulted in the reduced expression of a reporter gene, encoding firefly luciferase, in a human hepatoma cell line. The frequency of the abnormal allele was 40 percent among the normal subjects. The 3 men in the control group who were homozygous for the longer TATAA element had significantly higher serum bilirubin levels than the other 52 normal subjects ($P=0.009$). Among the kindred with a history of Crigler–Najjar syndrome type II, only the six heterozygous carriers who had a longer TATAA element on the structurally normal allele had mild hyperbilirubinemia, characteristic of Gilbert's syndrome.

Conclusions. Reduced expression of bilirubin UDP-glucuronosyltransferase 1 due to an abnormality in the promoter region of the gene for this enzyme appears to be necessary for Gilbert's syndrome but not sufficient for the complete manifestation of the syndrome. (N Engl J Med 1995;333:1171-5.)

PEOPLE with Gilbert's syndrome have mild, chronic unconjugated hyperbilirubinemia in the absence of liver disease or overt hemolysis.^{1,2} Although the syndrome is inherited, many people do not have a clear family history.³ An autosomal mode of inheritance has been proposed,⁴ and more recently, a recessive pattern of inheritance has been suggested.⁵ On the basis of serum bilirubin levels, 3 to 10 percent of the general population are estimated to have Gilbert's syndrome.⁶⁻⁸ Serum bilirubin levels fluctuate in people with Gilbert's syndrome and often fall within accepted normal limits, making it unclear whether these people constitute a distinct subpopulation⁶ or whether their bilirubin values represent the upper end of the normal distribution curve.^{7,8} Gilbert's syndrome is considered harmless in adults, although an incidental finding of hyperbilirubinemia may raise the possibility of liver disease and sometimes trigger unnecessary investigations. It is not known whether the syndrome has a role in exaggerated neonatal jaundice.

Hepatic glucuronidating activity, which is essential for efficient biliary excretion of bilirubin, is approximately 30 percent of normal in patients with Gilbert's syndrome.^{9,10} The reduced glucuronidation results in an increased proportion of bilirubin monoglucuronide in

bile.¹¹ In human liver, bilirubin glucuronidation is mediated by one specific isoform of microsomal bilirubin, UDP-glucuronosyltransferase (bilirubin/uridine diphosphoglucuronate-glucuronosyltransferase). Of the two isoforms reported,^{12,13} only bilirubin UDP-glucuronosyltransferase 1 contributes substantially to bilirubin glucuronidation.¹⁴

Genetic lesions causing an absence of enzymatic bilirubin glucuronidation result in Crigler–Najjar syndrome type I,^{2,15-21} whereas mutations causing severe deficiency of the enzyme result in Crigler–Najjar syndrome type II.²²⁻²⁴ Because mild hyperbilirubinemia is often found among relatives of patients with Crigler–Najjar syndrome, some have postulated that Gilbert's syndrome represents a heterozygous form of Crigler–Najjar syndrome.^{25,26} However, many carriers of Crigler–Najjar syndrome do not have hyperbilirubinemia,²² and the incidence of Gilbert's syndrome is much higher than that expected on the basis of the number of heterozygous carriers of Crigler–Najjar syndrome, which is 1 per 1 million births.

We studied the genetic basis of reduced hepatic bilirubin glucuronidation in people with Gilbert's syndrome and found that a variant TATAA element (which contains two extra nucleotides, TA) in the upstream promoter region of the gene for bilirubin UDP-glucuronosyltransferase 1 is associated with the syndrome. The TATAA element is the binding site for transcription factor IID, which is important in the initiation of transcription.²⁷⁻³¹ The presence of this longer TATAA element in the promoter region of the gene for bilirubin UDP-glucuronosyltransferase 1 resulted in reduced expression of a reporter gene, encoding firefly luciferase, in a human hepatoma cell line. The presence of the longer TATAA element correlated with higher mean serum bilirubin levels in normal, healthy subjects and in

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compound heterozygous carriers of Crigler–Najjar syndrome type II.

METHODS

Patients with Gilbert's Syndrome

We studied 10 patients with Gilbert's syndrome, ranging from 15 to 54 years of age. Blood was collected from all 10 after they provided informed consent. Criteria for the diagnosis of Gilbert's syndrome included a consistent mild elevation of serum bilirubin (level, 1.2 to 5.3 mg per deciliter [20 to 90 μ mol per liter]). The bilirubin was at least 90 percent unconjugated according to van den Bergh's test and 99 percent unconjugated on the basis of high-performance liquid chromatography. Serum alanine aminotransferase and aspartate aminotransferase values were normal. Hemolysis was excluded on the basis of normal hemoglobin and haptoglobin values and reticulocyte counts. Three patients were given a 400-kcal diet for 24 hours, which doubled their serum bilirubin levels. Two patients underwent duodenal aspiration for bile-pigment analysis by high-performance liquid chromatography³²; in both, monoglucuronide made up 30 percent of bilirubin conjugates.³³

Subjects from a Kindred with Crigler–Najjar Syndrome Type II

We examined 2 patients with Crigler–Najjar syndrome type II, 10 heterozygous carriers, and 4 family members who were not carriers from a kindred with a history of the syndrome. Both patients were homozygous for a structural mutation that markedly reduced the catalytic activity of bilirubin UDP-glucuronosyltransferase.^{22,24} Four of the 10 heterozygous carriers had mild hyperbilirubinemia. All provided informed consent.

Control Subjects

We examined 55 normal subjects (28 women and 27 men; age, 21 to 55 years) with no known history of jaundice. All provided informed consent. Serum bilirubin was measured in samples collected on two different days.³⁴ In our laboratory the upper limit of normal for serum bilirubin is 1.0 mg per deciliter (17.1 μ mol per liter). For samples with a serum bilirubin level of 0.9 mg per deciliter (15.4 μ mol per liter), less than 5 percent variation is found between samples collected on two different days.

Nucleotide Sequencing of Coding and Upstream Regions of the Gene for Bilirubin UDP-Glucuronosyltransferase 1

Genomic DNA was isolated from lymphocytes and the five exons constituting the coding region of the gene for bilirubin UDP-glucuronosyltransferase 1, and their flanking intron–exon junctions were amplified by the polymerase chain reaction (PCR) and sequenced as described.¹⁵ The segment of DNA 5' to the coding region (from nucleotide –227 to nucleotide 132) was amplified with a sense primer, 5'GAGGTTCTGGAAGTACTTTGC3', and an antisense primer, 5'CCAAGCATGCTCAGCCAG3'. PCR was performed for 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, with 1.5 mmol of magnesium chloride per liter used as a buffer. Both strands of the amplified segment were sequenced with two internal primers.

Functional Evaluation of the Variant TATAA Element

A fragment of the upstream region (from nucleotide –546 to nucleotide –4) of the bilirubin UDP-glucuronosyltransferase 1 gene was amplified by PCR with genomic DNA from a subject homozygous for the long TATAA element, A(TA)₇TAA, and from a subject homozygous for the normal TATAA element, A(TA)₆TAA. Amplimers were designed to introduce a *Xho*I and a *Hind*III site at the 5' and 3' ends of the amplicon (amplified product), respectively. The two amplicons were cloned in appropriate orientation in the *Xho*I and *Hind*III sites 5' to the entire coding region of firefly luciferase gene of the plasmid pXP1, which lacks a promoter region. The nucleotide sequences of both constructs were identical except for the addition of two bases in the longer TATAA box. A plasmid, pSV-lacZ (Promega, Madison, Wis.), containing the structural region of bacterial β -galactosidase driven by the promoter of the large transforming antigen of simian vi-

rus 40, was used to determine the efficiency of transfection. Cells from a well-differentiated human hepatoma cell line (HuH7) were grown to 40 percent confluence in RPMI medium containing 4 percent fetal-calf serum. The cells were cotransfected with 1.5 μ g each of the test luciferase construct and pSV-lacZ with Lipofectin (GIBCO-BRL, Gaithersburg, Md.). After the cells were harvested, luciferase activity was determined with a Promega luciferase assay system. Protein content³⁵ and *o*-aminophenol- β -galactosidase activity³⁶ were determined as described previously.

Statistical Analysis

Mean serum bilirubin values were compared by analysis of variance or a two-tailed nonparametric Wilcoxon test.³⁷ Statistical analyses were performed with Sigma Stat for Windows.

RESULTS

Patients with Gilbert's Syndrome

In four unrelated patients with Gilbert's syndrome, the nucleotide sequences of all five exons encoding the gene for bilirubin UDP-glucuronosyltransferase 1 and all intron–exon junctions were normal, indicating that the syndrome in these patients was not caused by structural mutations. To investigate whether an abnormality of the promoter region caused reduced expression of the normal enzyme, we determined the sequence of a 247-nucleotide region immediately upstream of the translation-initiation codon. Normally, an A(TA)₆TAA element is present between nucleotides –23 and –38.¹³ All four of the patients were homozygous for an additional TA in this element, resulting in the sequence A(TA)₇TAA (Fig. 1). Subsequently, we sequenced this region in six additional unrelated patients with Gilbert's syndrome, all of whom were found to be homozygous for the additional TA.

Effect of the Longer TATAA Element on Gene Expression

To determine the effect of the longer TATAA element on gene expression, a 542-base-pair (bp) region upstream of the gene, which contained A(TA)₆TAA, and a 544-bp region containing A(TA)₇TAA were each linked upstream to a firefly luciferase gene, and the construct was transfected into a human hepatoma cell line (HuH7). To assess the efficiency of transfection, a β -galactosidase expression vector, driven by a viral promoter, was cotransfected. The expression of both reporter genes was assessed in four experiments; the mean results of the four experiments are shown in Figure 2. The expression of luciferase in the presence of the longer TATAA element was only 18 to 33 percent of that recorded in the presence of the normal TATAA element. There was no significant difference in the level of expression of the cotransfected *o*-aminophenol- β -galactosidase.

Normal Subjects

The frequency of the two TATAA elements was determined in 55 normal subjects. Eight were homozygous for A(TA)₇TAA, 19 were homozygous for A(TA)₆TAA, and 28 were heterozygous. The calculated allele frequency for the longer TATAA element was 40 percent. The mean serum bilirubin levels (mean of values in blood samples obtained on two different days) were 0.5 mg per deciliter (8.3 μ mol per liter) in the subjects who

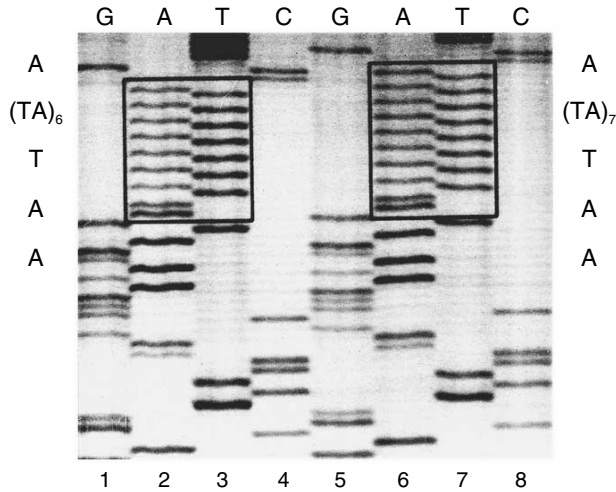


Figure 1. Length of the TATAA Element in the Promoter Region of the Gene for Bilirubin UDP-Glucuronosyltransferase 1.

The upstream region of the gene for bilirubin UDP-glucuronosyltransferase 1 was amplified with specific primers and sequenced directly. The sample on the left (lanes 1 through 4) is from a subject homozygous for the normal TATAA element (A(TA)₆TAA), and the sample on the right (lanes 5 through 8) is from a subject homozygous for the variant element (A(TA)₇TAA). Both TATAA elements are boxed.

were homozygous for A(TA)₆TAA, 0.6 mg per deciliter (10.4 μ mol per liter) in the heterozygotes, and 0.8 mg per deciliter (12.8 μ mol per liter) in the subjects who were homozygous for A(TA)₇TAA (Fig. 3). The mean serum bilirubin levels were significantly higher ($P=0.009$) in the 3 men who were homozygous for A(TA)₇TAA than in the other normal subjects (1.0 mg per deciliter [17.1 μ mol per liter] vs. 0.6 mg per deciliter in the other 52 subjects and 0.7 mg per deciliter [11.2 μ mol per liter] in the other 24 normal men), whereas the mean values in the 5 women who were homozygous for A(TA)₇TAA did not differ significantly from those in the subjects who were homozygous for A(TA)₆TAA (0.6 mg per deciliter vs. 0.5 mg per deciliter [8.3 μ mol per liter]).

Kindred with Crigler-Najjar Syndrome Type II

In a large kindred with a history of Crigler-Najjar syndrome type II,²² 2 family members with the syndrome who were homozygous for a structural mutation that reduces the catalytic activity of bilirubin UDP-glucuronosyltransferase to 4 percent of normal²⁴ were studied, as were 10 family members who were heterozygous for this mutation (carriers) and 4 family members who were not carriers (Table 1). The coding region of the second allele for the bilirubin UDP-glucuronosyltransferase 1 gene was normal in the heterozygotes. Determination of the sequence analyzed in the upstream region revealed that both patients with Crigler-Najjar syndrome type II were homozygous for A(TA)₆TAA, indicating that the structurally mutated allele contains a normal promoter. In six of the heterozygous carriers, the structurally normal allele contained the long TATAA element, A(TA)₇TAA, whereas in four

the short element was present. The six heterozygotes with the promoter abnormality had significantly higher serum bilirubin values than the four with the normal TATAA element (1.6 mg per deciliter [27.4 μ mol per liter] vs. 0.6 mg per deciliter, $P=0.01$).

DISCUSSION

We investigated the genetic mechanism of reduced bilirubin UDP-glucuronosyltransferase 1 activity in Gilbert's syndrome. The absence of any mutation in the coding region of the gene in four consecutive unrelated patients indicates that the decreased bilirubin glucuronidation is not due to a structural alteration of the enzyme. The presence of a long TATAA element, containing an extra TA, in both alleles in these four patients and in six additional patients with Gilbert's syndrome suggested the involvement of this variant promoter in the reduced expression of the enzyme. As the binding site for transcription factor IID, the TATAA element has an important role in the initiation of transcription,²⁷⁻³¹ and its mutation can result in reduced frequency and accuracy of transcription initiation.^{30,31} Our

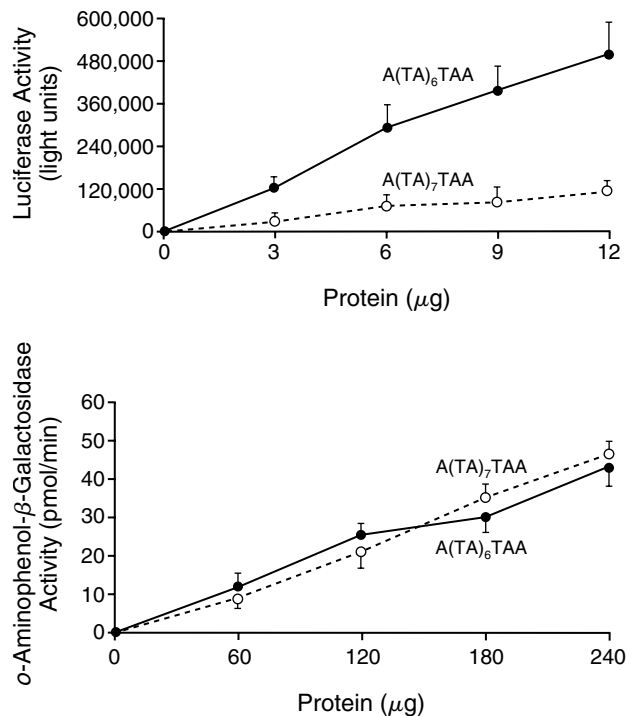


Figure 2. Functional Efficiency of Bilirubin UDP-Glucuronosyltransferase 1, According to Whether the Promoter Region of the Gene Contained the Normal or the Variant TATAA Element.

A 542-bp segment of DNA located upstream of exon 1A of the gene for bilirubin UDP-glucuronosyltransferase 1 containing the normal TATAA element (A(TA)₆TAA) and a 544-bp segment containing the variant element (A(TA)₇TAA) were cloned upstream of the coding region of the firefly luciferase gene. Each construct was cotransfected by PCR into a human hepatoma cell line (HuH7) with pSV-lacZ with use of Lipofectin. Forty-eight hours later, luciferase activity and o-aminophenol- β -galactosidase activity were assayed with the use of various amounts of lysate protein. The mean (\pm SD) results of four experiments are shown.

functional studies showed that the presence of the longer TATAA element in the upstream regulatory region of the gene reduces the expression of a reporter gene in a human hepatoma cell line. Together, these results suggest that the decreased bilirubin glucuronidating activity in Gilbert's syndrome results from reduced expression of the bilirubin glucuronidating enzyme.

All 10 patients with Gilbert's syndrome were homozygous for the longer TATAA element, suggesting that reduced expression of bilirubin UDP-glucuronosyltransferase 1 is essential for the syndrome. However, a mild reduction in the enzyme is not always sufficient for the full manifestation of the phenotype. Our results indicate that as much as 16 percent of the population should be homozygous for the long TATAA element, whereas only 3 to 10 percent of the general population have clinically diagnosed Gilbert's syndrome.⁶⁻⁸ Among the normal subjects, only men who were homozygous for the longer TATAA element had significant elevations in serum bilirubin levels, reflecting a greater bilirubin load in men per kilogram of body weight or the inhibition of enzymatic glucuronidation by androgenic steroids (or both).³⁸ This finding is consistent with the high male-to-female ratio among patients with diagnosed Gilbert's syndrome.^{4,8} The presence of other inherited or acquired factors affecting bilirubin metabolism, in addition to reduced glucuronidation, may result in the full manifestation of the syndrome. In some patients, impaired hepatic uptake of bilirubin has been found.^{33,39,40} Although hemolysis is not part of the syndrome, many patients who consult physicians may have a high bilirubin load because of a slightly reduced

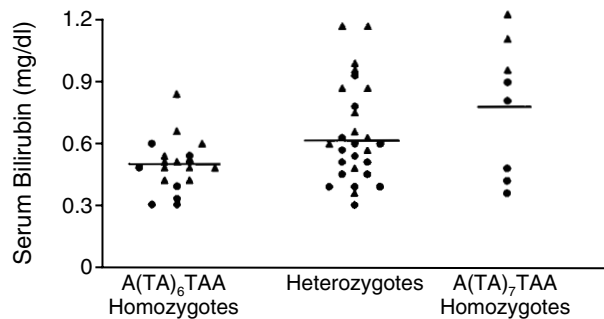


Figure 3. Correlation between Serum Bilirubin Levels and the Length of the TATAA Element in the Promoter Region of the Gene for Bilirubin UDP-Glucuronosyltransferase 1 in 55 Normal Subjects.

Eight subjects were homozygous for the short TATAA element (A(TA)₆TAA), 19 were homozygous for the long TATAA element (A(TA)₇TAA), and 28 were heterozygous. Each point represents the mean serum bilirubin value for a subject as determined independently on two different days. Circles denote female subjects, and triangles male subjects. The mean values in all three groups are shown. Analysis of variance showed that the mean serum bilirubin values differed significantly between groups ($P=0.012$); uncorrected P values: $P=0.033$ for the comparison of A(TA)₆TAA homozygotes with heterozygotes; $P=0.008$ for the comparison of A(TA)₆TAA homozygotes with A(TA)₇TAA homozygotes; and $P=0.164$ for the comparison of heterozygotes with A(TA)₇TAA homozygotes. To convert values for serum bilirubin to micromoles per liter, multiply by 17.1.

Table 1. Association of the Length of the TATAA Element Present in the Alleles for Bilirubin UDP-Glucuronosyltransferase 1 with Serum Bilirubin Levels in a Kindred with a History of Crigler-Najjar Syndrome Type II.

ALLELE A		ALLELE B		NO. OF SUBJECTS	SERUM BILIRUBIN (mg/dl)*
LENGTH OF TATAA ELEMENT IN PROMOTER REGION	CODING-REGION STATUS	LENGTH OF TATAA ELEMENT IN PROMOTER REGION	CODING-REGION STATUS		
A(TA) ₆ TAA	Mutated	A(TA) ₆ TAA	Mutated	2†	16.0
A(TA) ₆ TAA	Mutated	A(TA) ₇ TAA	Normal	6	1.6±0.8‡
A(TA) ₆ TAA	Mutated	A(TA) ₆ TAA	Normal	4	0.6±0.1
A(TA) ₆ TAA	Normal	A(TA) ₇ TAA	Normal	3	0.5±0.1
A(TA) ₆ TAA	Normal	A(TA) ₆ TAA	Normal	1	0.2

*Plus-minus values are means ±SD. To convert values to micromoles per liter, multiply by 17.1.

†These two subjects had Crigler-Najjar syndrome type II.

‡ $P=0.01$ for the comparison with the heterozygous carriers of Crigler-Najjar syndrome type II who were homozygous for A(TA)₆TAA.

erythrocyte life span.⁴¹ Fasting may also increase the bilirubin load,^{42,43} and the resulting hyperbilirubinemia may be exaggerated in patients with Gilbert's syndrome⁴⁴ because of the reduced expression of the glucuronidating enzyme.

Gilbert's syndrome runs in families,³ although only one family member may have jaundice.² Both autosomal dominant³ and autosomal recessive⁵ modes of inheritance have been proposed. Because homozygosity for A(TA)₇TAA appears to be a requirement for the syndrome, our findings suggest an autosomal recessive mode of inheritance, whereas the high frequency of the structurally mutated allele may explain the appearance of a pseudodominant pattern of inheritance in some instances.

Our results also help to explain the high incidence of mild hyperbilirubinemia in relatives of patients with Crigler-Najjar syndrome. Heterozygous carriers of Crigler-Najjar syndrome have one structurally normal allele and would be expected to have bilirubin glucuronidating activity that is at least 50 percent of normal, so that normal serum bilirubin levels would be maintained. However, when this structurally normal allele contains the longer TATAA element, the decreased expression of bilirubin UDP-glucuronosyltransferase 1 results in hyperbilirubinemia.

In summary, reduced expression of bilirubin UDP-glucuronosyltransferase 1 due to an abnormality in the promoter region of the gene appears to be necessary for Gilbert's syndrome but is not sufficient for the complete manifestation of the condition.

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