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ISOLATION AND SEQUENCING OF RAT LIVER BILIRUBIN UDP-GLUCURONOSYLTRANSFERASE cDNA: POSSIBLE ALTERNATE SPLICING OF A COMMON PRIMARY TRANSCRIPT

Hiroshi Sato[1]*, Osamu Koiwai[2], Kazushi Tanabe[3] and Shigeo Kashiwamata[1]

Departments of [1]Perinatology and [2]Biochemistry, Institute for Developmental Research, Aichi Prefecture Colony, Kasugai Aichi 480-03, Japan

[3]Biophysics Unit, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan

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SUMMARY: A 1763-bp cDNA for rat liver bilirubin UDP-glucuronosyltransferase (UDPGT) was isolated. Bilirubin UDPGT activity was demonstrated by transfection of the pcDL1 vector carrying the cDNA into COS7 monkey kidney cells. The cDNA shares an identical 913-bp sequence (corresponding to the C-terminal 247 amino acid residues) with that for rat liver 3-methylcholanthrene-inducible 4-nitrophenol UDPGT including the locus where a -1 frameshift mutation was found in the 4-nitrophenol UDPGT cDNA from the jaundiced homozygous Gunn rat. The result suggests that both the UDPGTs are derived from a common primary-transcript and that the multiple defects of UDPGT isoenzymes observed in the homozygous Gunn rat may be produced by a single-mutated-locus after an alternative splicing of the 5' end region.

In 1938, Gunn described a mutant strain of Wistar rats (Gunn rat) that showed hereditary hyperbilirubinemia (1). The homozygous Gunn rat lacks hepatic UDP-glucuronosyltransferase (UDPGT, EC 2.4.1.17) activity towards bilirubin (2) and has been used as an animal model for human Crigler-Najjar syndrome type I (3,4). It has been also reported that the Gunn rat has genetic deficiencies of both hepatic bilirubin (Bil) and 3-methylcholanthrene (3-MC)-inducible 4-nitrophenol (4-NP) UDPGT activities (5). Recently, Iyanagi et al. (6) further clarified the genetic defect of 4-NP UDPGT in the homozygous Gunn rat liver as a -1 frameshift mutation. They also suggested that in the liver of 3-MC-untreated homozygotes, there existed another mRNA that had the same defective sequence

*To whom correspondence should be addressed.

[2]Present address: Department of Biochemistry, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan.

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as proven in the mutated 4-NP UDPGT. On the other hand, Nagai et al. (7) have demonstrated by the cross-breeding of mutant rat strains that Bil UDPGT is located on the same chromosome as 4-NP UDPGT. Based on these findings, we tried to isolate Bil UDPGT cDNA from a rat liver cDNA library using a synthetic 40-mer oligonucleotide probe that has a complementary sequence with the 4-NP UDPGT cDNA.

MATERIALS AND METHODS

Screening of cDNA library. A cDNA library from the rat liver was obtained from Clontech Lab., Inc., Palo Alto, CA. We isolated a cDNA for Bil UDPGT by screening the library with a synthetic probe covering the nucleotide sequence from positions 1,520 to 1,559 in the rat liver 3-MC-inducible 4-NP UDPGT cDNA (8). Detailed procedures for the screening of cDNAs were performed essentially by the method of Maniatis et al. (9,10).

Transfection of Bil UDPGT cDNA clone. The cDNA isolated was subcloned into the pcDL1 vector (a generous gift from Dr. T. Yokota). The cloning site in the vector has been converted from the EcoR I site to the Xba I site in our laboratory. The incubation mixture for the polymerase chain reaction (PCR) contained in a total volume of 100 µl of 60 mM Tris-HCl buffer, pH 8.8, 1 µg of cloned λ gt 11 DNA, 1 µM primers, 1.5 mM dNTPs, 10 % dimethylsulfoxide, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$ and 200 µg/ml gelatin. Transfection of the cDNA (20 µg) into monkey kidney COS7 cells (4×10^6) was performed as described by Koiwai et al. (10). Transfected cells were incubated for 72 h without treatment of chloroquine. Bil UDPGT activity was detected by thin layer chromatography according to Jackson et al. (11). The bilirubin concentration in the assay mixture was 0.5 mM. [Glucuronosyl-U-^{14}C]UDP-glucuronic acid was purchased from ICN Biochem., Inc., Irvine, CA. p-Nitrophenyl-β-D-glucuronide was obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

Isolation of rat Bil UDPGT cDNA. Plaque screening of the rat liver λ gt 11 cDNA library (1×10^5 independent clones) with the radiolabeled oligonucleotide probe identified four UDPGT cDNA clones. The entire nucleotide sequences of these clones were determined by the M13 dideoxy chain termination method (12). The results obtained clearly showed that the isolated clones were a new type of UDPGT cDNA. The longest clone (pSK1) contained 1,763-bp with an open reading frame of 1,593-bp. Figure 1 shows the nucleotide sequence together with its predicted amino acid sequence of the pSK1 clone. Comparison of the sequence of the clone with that of the 4-NP UDPGT cDNA revealed that both the cDNAs shared an identical sequence of 913-bp (C-terminal 247 amino acid residues from positions 285 to 531). However, there is a considerable divergence of the amino acid sequence in the N-terminal region between them.

Expression of the rat Bil UDPGT cDNA clone in COS7 cells. To confirm that this new clone was for Bil UDPGT, we examined the expression of pSK1 in mammalian cells. A cDNA fragment of 1,624-bp including an extra two bases (AT) at the 5' end of the clone was synthesized by PCR. Synthetic primers containing the Xba I site (5'AGATCTAGATGGGATTGTGTGCACCCCTTCG3' and AGATCTAGAATA

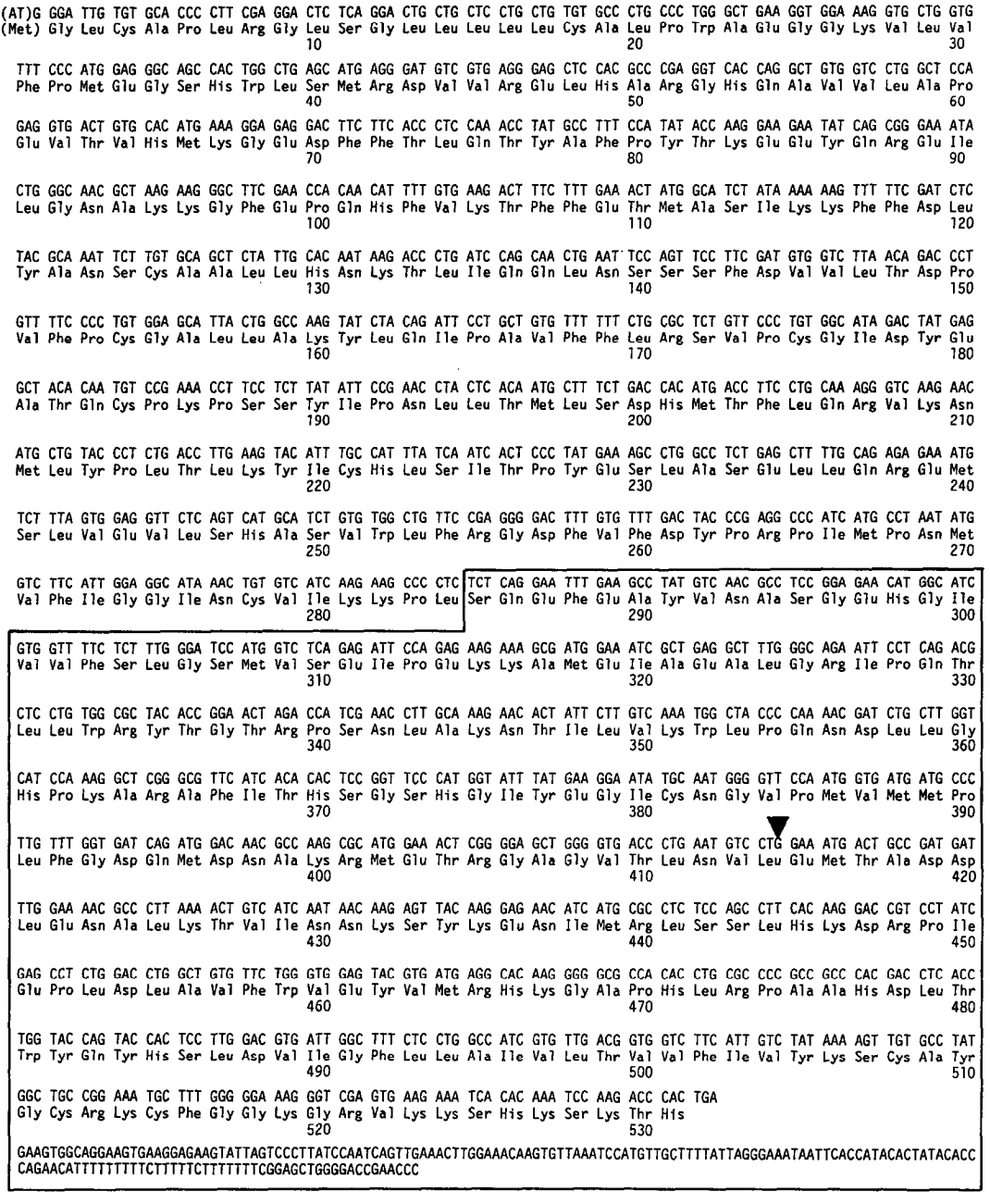

Fig. 1. Nucleotide sequence of bilirubin UDPGT cDNA and its predicted amino acid sequence. The nucleotides are numbered on the right-hand side. The nucleotide sequence identical with that of the 3-MC-inducible 4-NP UDPGT cDNA is indicated by a closed line. An arrowhead points to the locus where one base deletion occurs in the 4-NP UDPGT cDNA of the homozygous Gunn rat. Parentheses indicate the 5' end two nucleotides and N-terminal methionine that have been introduced by PCR.

CTTCTCCTTCACTTCC) were utilized for production of the cDNA fragment. The synthesized cDNA fragment was inserted into the Xba I site of the pcDL1 expression vector in the correct and opposite orientations, and the constructed

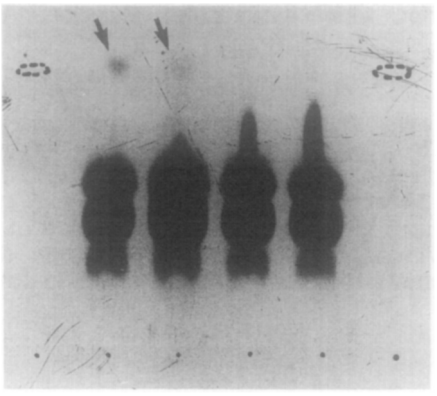

Fig. 2. Glucuronidation of bilirubin by Bil UDPGT expressed in COS7 cells - autoradiography of a thin layer chromatogram. A and B, transfected by pcDL1 containing the cDNA in the correct orientation (duplicate experiments); C, transfected by pcDL1 with the cDNA inserted in the opposite orientation; D, non-transfected COS7 cells; S, authentic 4-NP glucuronide (dotted circles). The reaction mixture (11) contained the homogenate of COS7 cells equivalent to 70 μg protein and 9.25 kBq [glucuronosyl-U-^{14}C]UDP-glucuronic acid. Arrows, enzymically formed bilirubin conjugate with UDP-[glucuronosyl-U-^{14}C]glucuronic acid.

vectors were transfected into COS7 cells. Figure 2 shows Bil UDPGT activity detected by autoradiography on a thin layer chromatogram. Only the pcDL1 vector carrying the cDNA fragment in the correct orientation expressed the enzyme activity in COS7 cells. Gross estimation of the activity showed that Bil UDPGT expressed in COS7 cells was comparable to that in rat hepatic cells.

DISCUSSION

In this paper, we presented the nucleotide sequence of a cDNA clone encoding rat liver Bil UDPGT (Fig. 1). Although the isolated cDNA was lacking in two bases (AT) at the 5' end of the full length open reading frame that compared with the 4-NP UDPGT cDNA, the cDNA fragment synthesized by PCR to introduce the initiation codon at the 5' end successfully expressed Bil UDPGT activity in COS7 cells (Fig. 2). The cDNA for Bil UDPGT shares an identical 913-bp sequence (C-terminal 247 amino acid residues) with that for 4-NP UDPGT. Interestingly, this region includes the locus where a single base deletion occurs in the 4-NP UDPGT cDNA from the homozygous jaundiced Gunn rat (6). Both Bil and 4-NP UDPGTs are located on the same chromosome (7) and have genetic defects in the jaundiced Gunn rat (5,6). On the basis of these facts, we suppose that Bil and 4-NP UDPGT mRNAs are derived from a common primary-transcript. After an alternative splicing of the 5' end region of the transcript, the respective mature mRNAs may be produced. Thus, the multiple defects of UDPGT isoenzymes observed in the jaundiced Gunn rat may be caused by sharing a common 3' end

region on the transcript, which has a point mutation. We are now performing investigations that may prove our hypothesis.

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