Successful Gene Therapy of the Gunn Rat by *In Vivo* Neonatal Hepatic Gene Transfer Using Murine Oncoretroviral Vectors

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Crigler-Najjar type 1 disease (CN1) is a rare inherited metabolic disease characterized by complete absence of hepatic UDP-glucuronosyl transferase (UGT1), resulting in high levels of unconjugated bilirubin. CN1 is an attractive candidate disease for gene therapy. Here we show that *in vivo* neonatal hepatocyte transduction using recombinant oncoretroviral vectors results in long-term and complete phenotype correction in Gunn rats, a model for CN1. Two-day-old newborn Gunn rats were injected via the temporal vein with 200 μL UGT1 or control β-galactosidase retroviral vectors. In UGT1-injected animals, bilirubinemia was normal at 6 weeks (3 μmol/L) and remained in the normal range (*i.e.,* <10 μmol/L) for more than 34 weeks. In contrast, in β-galactosidase–injected animals as well as in noninjected controls, bilirubinemia remained at a high level (*i.e.,* >100 μmol/L) during the whole experimental follow-up. Large amounts of bilirubin monoglucuronides and diglucuronides were present in the bile of treated animals. Finally, polymerase chain reaction and reverse transcription polymerase chain reaction analysis as well as Western blot confirmed the presence and expression of UGT1 almost exclusively in the liver. The estimated proportion of transduced hepatocytes was in the range of 5% to 10%. In conclusion, complete and permanent correction of hyperbilirubinemia in newborn Gunn rats using retroviral vectors can be obtained, paving the way for future gene therapy for CN1. (HEPATOLOGY **2005;42: 431-438.**)

The liver is an attractive target for gene therapy for several reasons. It is the site of many metabolic pathways and thus is involved in many inborn metabolism errors. It is a unique organ that is readily accessible via the bloodstream through a fenestrated endothelium. Finally, it is a large homogenous organ in which the effectiveness of *in vivo* liver transduction is easily monitored. Type 1 Crigler-Najjar disease (CN1) is a rare (1 in 1,000,000 births) recessive inherited genetic disorder that is an attractive candidate for gene replacement therapy. CN1 is characterized by marked accumulation of unconjugated bilirubin in the plasma. Bilirubin is a potentially toxic lipophilic waste, and hepatic conjugation to glucuronic acid (glucuronidation) is required for efficient excretion of bilirubin. Glucuronidation of bilirubin is catalyzed predominantly by 2 isoforms of the uridine diphospho-glucuronosyl transferase gene family (UGT1), the most potent isoform being UGT1A1, also known as bilirubin glucuronosyl transferase (B-UGT1; E.C. 2.4.1.17). These 2 isoforms, as well as 10 other isoforms responsible for the conjugation of various compounds, are expressed from a common locus on chromosome 2 spanning 500 kb. All 12 isoforms of the UGT1 locus are composed of 5 exons generated by alternative splicing. The four 3′ exons encoding the C terminal part of the protein are identical between all isoforms. In contrast, each isoform has a unique 5′ exon and hence a unique N terminus.[1,2] Genetic lesions in any of the four 3′ exons that encode the identical C terminal regions result in complete absence of activity of any isoform of the UGT1 family and characterize CN1 disease. Consequently, CN1 patients have profound unconjugated hyperbilirubinemia and are at risk of severe, life-threatening, bilirubin encephalopathy (kernicterus).

Abbreviations: CN1, Crigler-Najjar type 1 disease ; UGT1, UDP-glucuronosyl transferase; PCR, polymerase chain reaction; HPLC, high-pressure liquid chromatography; PEPCK, phosphoenol-pyruvate carboxy kinase; RT, reverse transcription.

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Treatment of CN1 disease relies on daily prolonged phototherapy sessions, and liver transplantation is the only curative option available today. Replacement of the enzyme activity by cell or gene therapy should provide major clinical benefit to CN1 patients. The availability of an animal model of CN1, the Gunn rat, which replicates the phenotype present in CN1 patients, has made it possible to devise new therapeutic strategies for the treatment of inherited bilirubin metabolism defects.[3] The Gunn rat harbors a single nucleotide deletion (position 1242 in the bilirubin UGT cDNA) located in the 500-bp common region of the nucleotide sequence of various rat UGT isoforms and which results in a premature stop codon 115 amino acids ahead of the normal end of the protein.[4,5] Therefore, the past decade has witnessed an increasing number of studies aimed at permanently correcting hyperbilirubinemia in Gunn rats. Gene therapy using gene transfer vectors based on cationic liposomes, or viruses such as murine onco-retroviruses or SV40, achieves partial, and sometimes sustained, correction of hyperbilirubinemia in treated adult Gunn rats.[6-9] The same is true for *in utero* lentivirus-mediated gene delivery, which yields a 50% decrease in serum bilirubin level that lasts 1 year.[10] In other instances, complete correction of the conjugation defect is obtained when using highly efficient gene transfer vectors such as adenoviruses. However, induction of a cytotoxic immune response directed at corrected cells precludes long-term correction based on this strategy.[11] Attempts to circumvent this immune response by modifying the vector or the recipient animal are only partially successful.[12-15] Only very recently has the complete and permanent correction of adult Gunn rats been obtained using gutted adenoviral vectors delivered intravenously at high doses.[16] However, adenoviral vectors are toxic to humans, and their clinical use remains unsafe.

Recently, it has been reported that gene transfer to the newborn animal using murine retroviral vectors results in sustained expression of a therapeutic protein. Dogs with mucopolysaccharidosis VII are successfully treated by injection of murine retroviral vectors harboring the β-glucuronidase gene and administered at 2 to 3 days of age.[17] Subsequently, it has been shown that neonatal gene transfer with a murine retroviral vector in mice and dogs results in tolerance to a foreign therapeutic protein, human factor IX.[18]

We hypothesized that injection of high-titer murine retroviral vectors to newborn would achieve sufficient liver transduction to reverse the disease phenotype in Gunn rats. Moreover, because murine retroviral vectors integrate into the infected cell's genome, long-term correction should be observed with no induction of deleterious immune response.

Materials and Methods

Animals. Homozygous jaundiced j/j Gunn rats were obtained by mating heterozygous +/j female with homozygous j/j males. All animal experiments were conducted according to the guidelines of the French Ministère de l'Agriculture.

Two-day-old animals were bolus-injected intravenously via the temporal vein after light cold-induced anesthesia. Animals received 200 μL concentrated recombinant retroviruses. After injection, animals were left untreated for 6 weeks. For serum bilirubin determination, blood was collected from the retrorbital sinus under light gas anesthesia using isoflurane.

For bile harvest and liver biopsies, animals were anesthetized by isoflurane inhalation and laparotomized. For bile harvest, a thin polyethylene catheter was introduced into the main bile duct.

Murine Retroviral Vectors. The cDNA encoding human UGT1A1 including the ATG sequence was released from the pSVK3 plasmid[19] as a 1891bp *Eco*R1-*Xba*1 fragment containing 15 bp upstream and 276 downstream untranslated region.[19] After intermediate subcloning in phosphate-buffered saline plasmid, it was polymerase chain reaction (PCR)-cloned in the *Nco*1 site of MFG vector to place the ATG of UGT1 in frame with the *env* gene ATG of the MFG vector. The sequence was checked by sequencing. In this plasmid, the transcription of the transgene is under control of the long terminal repeat of the virus. The plasmid was transfected in 293 GPG cells.[20] The clone that produced the highest level of UGT1 murine retroviral vector was selected.

High-titer retroviral supernatants were produced by harvesting culture medium of confluent cells for three days and concentration by tangential flow filtration and ultracentrifugation. Titers were determined by infection of Te671 target cells followed by quantitative PCR evaluation of the proportion of transduced cells. It was routinely 5×10^8 transducing particles/mL.

Control β-galactosidase murine retroviral vectors were prepared from TELCeB6 AF7 cell line as previously described.[9] The titer of the β-galactosidase supernatant was 2×10^8 transducing particles/mL.

Bilirubin Assays. Serum total and conjugated bilirubin levels were measured spectrophotometrically using the Bil-T kit from Boehringer (Boehringer Mannheim, Mannheim, Germany) at the routine biochemistry department of Nantes University hospital.

The presence of monoglucuronide and diglucuronide bilirubin conjugates in the bile was assessed by high-pressure liquid chromatography (HPLC) after alkaline methanolysis as previously described.[21]

Protein Analysis. Total microsomes (100,000*g* supernatant) were prepared from liver fragments by ultracentrifugation. For immunoblot analysis, proteins (25 μg/lane) were resolved by electrophoresis on 10% polyacrylamide gel. After electrophoretic transfer onto nitrocellulose membranes, immunoblots were incubated sequentially with 5% low-fat milk blocking solution, and the human WP1 primary antibody diluted 1:5,000. The WP1 antibody interacts with the common carboxyterminal domains of human UGT1 isoforms.[22] The secondary antibody was biotinylated conjugated anti-mouse Ig F′ab fragment that was detected by using the ECL Western blotting detection system (Amersham Biosciences, Castle Hill, Australia). Human hepatic microsomes were used as positive control.

PCR Analysis. High–molecular weight DNA was extracted by phenol-chloroform standard protocol. DNA (500 ng) was subjected to amplification by PCR using primers (5′- TCTGCTATGCTTTTGTCTGG-3′ and 5′-GGATAGTGGATTTTGGTGAA-3′) yielding a 504-bp fragment. Semiquantitative PCR amplification was performed by denaturation for 5 minutes at 94°C, followed by 32 cycles of amplification (94°C for 20 seconds; 60°C for 30 seconds; 72°C for 40 seconds), and a final extension for 10 minutes at 72°C. A control 202-bp fragment of the rat phosphoenol-pyruvate carboxy kinase *(PEPCK)* gene promoter was amplified using the 2 primers: 5′- GTCATATTTCTTCAGCTTGCG-3′ and 5′-ATAATGGTCTGGACTTCTCTG-3′. Serial dilutions of human genomic DNA in rat genomic DNA were used as a standard curve. Amplified products were separated by gel electrophoresis en 2% agarose gel, and DNA bands were revealed by ethidium bromide staining.

Real-time quantitative PCR was performed in a final reaction mixture containing 50 ng DNA, 7.5 pmol (each) of the forward and reverse primers, and Sybr Green PCR Master Mix (PE Biosystems, Warrington, UK). Reaction conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The sequence of the UGT1 forward primer was 5′-GTGATGATGCCCTTGTTTGGT and that of the reverse primer was 5′-AAACTCCACCCA-GAACACGG-3′. The Ct values of each sample were normalized for the amount of DNA using amplification of a fragment from the *PEPCK* gene promoter as an internal standard and ΔCt values were calculated. A standard curve was constructed by diluting human DNA (1 copy per haploid genome) in rat DNA, and the results are expressed in UGT1 copy number/haploid genome. The detection threshold was 0.001/copy.

RNA Analysis. Total RNA was prepared from various tissues by using the Nucleospin RNA II preparation kit (Macherey-Nagel, Hoerdt, France). Reverse transcription was carried out using an oligo dT primer and 400 ng RNA. PCR was performed using 2 μL cDNA with the same UGT1 primers as in non-quantitative PCR amplification of UGT1 DNA. As a control, we reverse transcribed and amplified a 147-bp fragment of the S6 ribosomal protein RNA.

Antibody Detection. The detection of antibodies directed against UGT1 in rats serum was carried out by indirect ELISA as previously described,[9] using purified human microsomes as target antigen.

Immunohistochemistry. We assessed the presence of β-galactosidase by immunohistochemistry as previously described.[23] After counterstaining with hematoxylin, the percentage of β-gal–positive cells was calculated in 10 fields at $\times$40 magnification.

Results

Long-Term Correction of Serum Bilirubin Level. We used a human *UGT1A1* cDNA placed under the transcriptional control of the long terminal repeat of the retrovirus as described in Materials and Methods. We previously documented that this promoter is active in the long term after transfer in hepatocytes.[9,24] Concentrated UGT1 vectors (200 μL total volume, *i.e.,* 10^8 tp/rat or 1.5×10^{10} tp/kg) were injected via the temporal vein in 2-day-old j/j Gunn pups of both sexes (n = 9). Discrimination between icteric j/j homozygous and +/j heterozygous littermates is easily made by examination. Confirmation of genetic status was carried out in adult animals through detection of the presence or absence of the *Bst*N1 restriction site as previously described[5,25] (data not shown). Control animals included homozygous j/j littermates that were either not injected (n = 10) or were injected with the same volume of a β-galactosidase encoding murine retroviral vector (titer 2×10^8 tp/mL; *i.e.,* 0.6×10^{10} tp/kg; n = 7). Six weeks after gene transfer, serum bilirubin levels were assessed in UGT1 and control rats (Fig. 1). The mean values of serum bilirubin were similar for noninjected (69.2 $\pm$14.6 μmol/L) and control animals receiving β-galactosidase vectors (70.7 $\pm$ 11.5 μmol/L). In contrast, treated animals injected with the UGT1 vectors had low bilirubin levels (4.56 $\pm$ 2.19 μmol/L), significantly different from both other groups ($P < .0001$ using ANOVA). This value was in the normal range of wild type or +/j heterozygote littermates (*i.e.,* $<$10 μmol/L).[26] We next evaluated the long-term persistence of correction in treated animals. As shown in Fig. 2, the level of serum bilirubin in treated animals remained continuously in the normal range for at least 42 weeks,

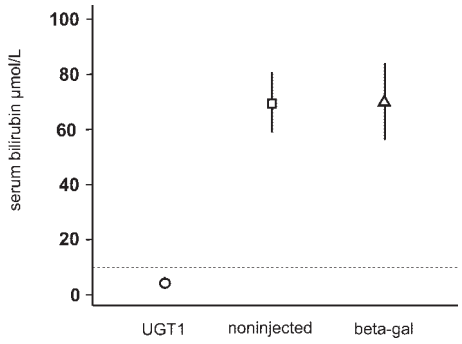

Fig. 1. Serum bilirubin levels at 6 weeks after gene transfer in experimental animals. Animals were either not injected or injected at 2 days of age with UGT1 or β-galactosidase retroviral vectors as described in Materials and Methods. Serum bilirubin levels were assessed after a 6-week recovery period. Data from each group represent the mean $\pm$ SD. UGT1-injected: n = 9; noninjected: n = 10; and β-gal-injected: n = 7. The dashed line represents the upper limit of normal serum bilirubin in rats.

whereas in nontreated controls bilirubinemia plateaued at approximately 100 μmol/L.

Bile Analysis. At 13 weeks of age, 3 animals from the treated group underwent cannulation of the main bile duct to harvest bile. The presence of bilirubin monoglucuronides and diglucuronides was analyzed by using HPLC after alkaline methanolysis. The representative chromatograms obtained in UGT1-treated rats as well as nontreated j/j Gunn rats and control wild-type Wistar rats are shown in Fig. 3. The respective proportions of the various bilirubin species in the chromatograms of the treated rats were calculated, and the results are presented in Table 1 for the animals killed at 13 weeks (rats 1-3) as well as for 2 animals that were killed at a later point (34 weeks rat 4 and 5). The mean proportion of nonconjugated bilirubin dropped in treated j/j Gunn rats. Interestingly, in treated animals, there was a predominance of bilirubin diglucuronides (mean 65.75% vs. 24.15% for monoglucuronides) in contrast to Wistar animals, which

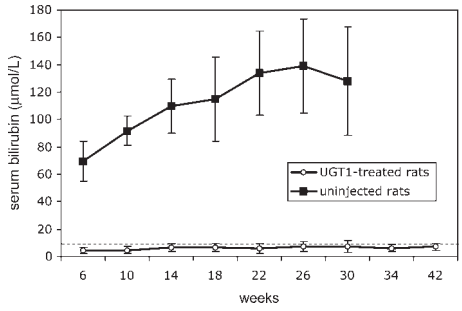

Fig. 2. Time course analysis of serum bilirubin level in UGT1-injected and noninjected homozygous j/j Gunn rats. Each value plotted represents the mean $\pm$ SD for noninjected controls (n = 10) and UGT1-injected animals (n = 7). The dashed line represents the upper limit of normal serum bilirubin in rats.

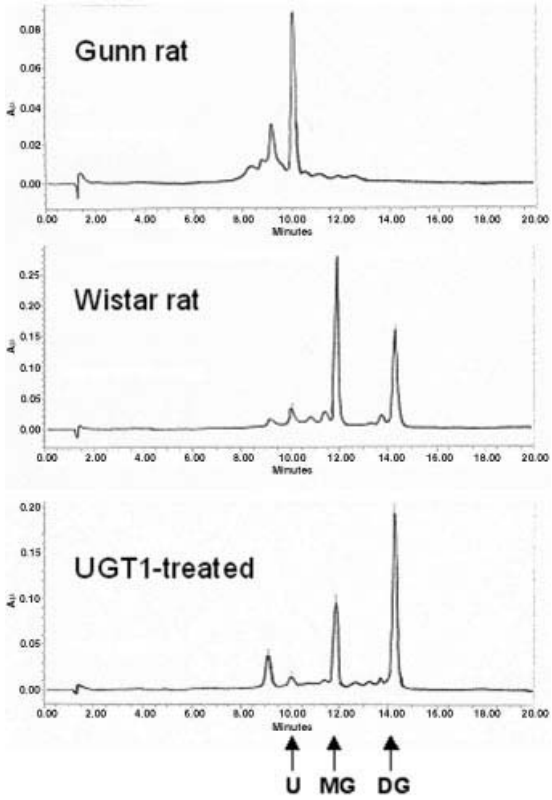

Fig. 3. HPLC analysis of rat bile. HPLC analysis was performed as described. Elution was monitored at 450 nm. Chromatograms were obtained from a j/j Gunn rat, a normal Wistar rat, or a treated j/j Gunn rat. The treated Gunn rat presented here is rat 1. U, unconjugated bilirubin; MG, bilirubin monoglucuronide; BG, bilirubin diglucuronide.

had a predominance of monoglucuronides. Such a discrepancy has also been noticed previously after *in utero* gene transfer using lentiviral vectors.[10] This may be caused by high concentrations of enzyme in a small proportion of transduced hepatocyte because of the strong activity of the viral promoter. Because UGT1 has a high affinity for monoglucuronides, the enzymatic kinetics may be shifted toward production of diglucuronides. Indeed, it has already been demonstrated that the ratio of

Table 1. Quantification of Bilirubin Glucuronides

	Unconjugated Bilirubin, %	Bilirubin Monoglucuronide, %	Bilirubin Diglucuronide, %
Rat 1	15.46	17.34	67.2
Rat 2	12.82	25.62	61.56
Rat 3	12.13	21.03	66.84
Rat 4	6.95	29.59	63.46
Rat 5	3.13	27.17	69.70
Mean	10.1	24.15	65.75
Gunn	100	0	0
Wistar	5.45	57.50	37.05
	6.95	63.02	33.89
	3.13	62.26	33.28
Mean	5.18	60.92	34.74

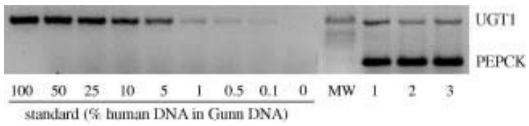

Fig. 4. Semiquantitative PCR analysis of liver DNA after *UGT1* gene transfer in homozygous j/j rats. High-molecular weight DNA was extracted from liver biopsy specimens, and the presence of the *UGT1* gene was detected by semiquantitative PCR. As a control, a 202-bp fragment from the rat *PEPCK* gene promoter was amplified. A standard curve was constructed by mixing the indicated proportions of human genomic DNA in rat genomic DNA. MW indicates molecular weight markers. Lanes 1 to 3 show rats 1 to 3.

bilirubin diconjugates to monoconjugates was increased when UGT1 activity was enhanced in the liver.[27] Accordingly, when partial activity is restored in a large number of hepatocytes, a predominance of monoglucuronides is observed.[6]

Liver Analysis. At the time of bile harvest (13 weeks), the liver was removed from the same animals to document the presence of transduced cells. High–molecular weight DNA extracted from the liver was subjected to semiquantitative PCR. The positive signal that was obtained in treated animals was compared to a standard curve. As shown in Fig. 4, the estimated proportion of transduced cells was in the range of 5% to 10%. This was confirmed by performing quantitative PCR experiments that revealed the presence of 0.11, 0.04, and 0.16 copies/haploid genome in the liver of rats 1, 2, and 3, respectively. We also performed western blot analysis of liver microsomes extracted from treated animals. Immunoblot detection of UGT1 revealed a single band with the same molecular weight as in control human liver (Fig. 5). In keeping with PCR experiments, the signal from rat 2 had the lowest intensity.

Hepatocyte Transduction. We were unable to perform immunohistochemistry using the WP1 antibody to assess the number of transduced hepatocytes expressing UGT1. The background level was too high as previously reported in another study.[10] However, to gain insights into the efficiency of gene delivery to hepatocytes in newborn animals using our strategy, we performed immunohistochemical detection of β-galactosidase in 3 control j/j Gunn rats that had been injected with the β-galactosidase retroviral vector.

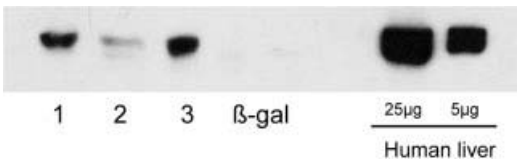

Fig. 5. Western blot analysis of rat liver after UGT1 gene transfer. Microsomes were prepared from liver fragments of 3 animals 13 weeks after gene transfer. After immunoblotting, the presence of UGT1 protein was detected in all animals. Human microsomes were used as positive control. Lanes 1 to 3 show rats 1 to 3; β-gal, control liver from a Gunn rat injected with β-galactosidase vector.

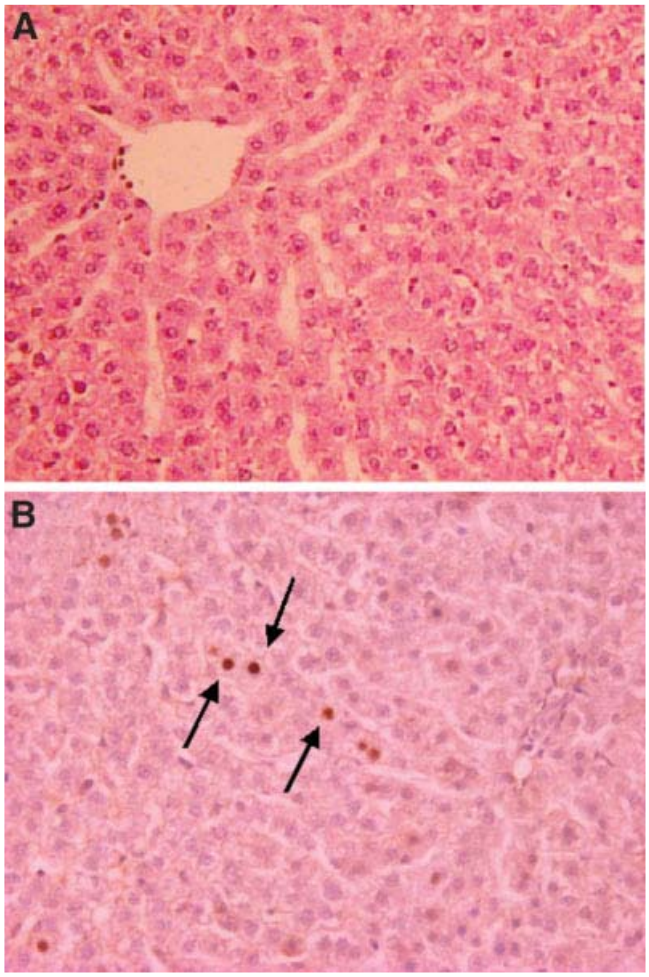

Fig. 6. Immunohistochemical detection of transduced hepatocytes in β-galactosidase–injected animals. The presence of β-galactosidase–positive hepatocytes was detected by immunohistochemistry at 2 weeks of age. (A) Nontransduced control liver. (B) Liver from a β-galactosidase–injected animal. Because of the presence of a nuclear localization signal, β-galactosidase is concentrated in the nucleus, and positive cells appear with a brown nucleus (arrows). Hematoxylin counterstained. Original magnification ×220.

Immunohistochemistry performed 2 weeks after injection showed that 2% ± 0.78% of hepatocytes expressed β-galactosidase (Fig. 6). Because there is a 2.5-fold difference in the amount of viral particles injected in β-galactosidase and UGT1-treated rats (0.6×10^{10} tp/kg vs. 1.5×10^{10} tp/kg, respectively), and because transduction of hepatocytes is proportional to the amount of particles injected,[28] we expected that the transduction level after UGT1 vector injection would be in the range of 5%. This figure is in accordance with the results of the PCR experiments. Because murine retroviral vectors are able to in-

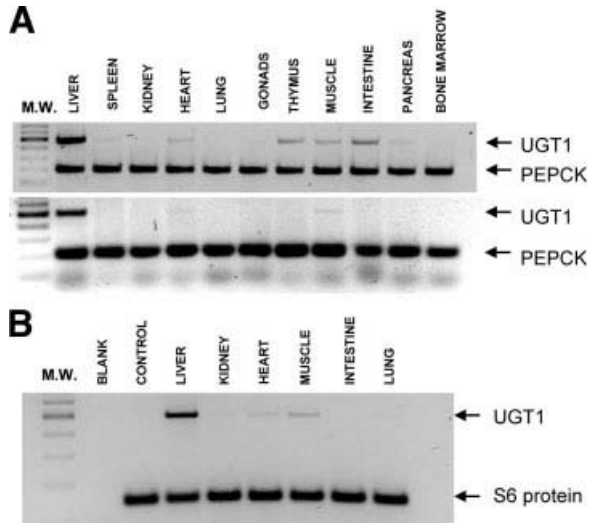

Fig. 7. Determination of transgene distribution and expression by PCR and RT-PCR. (A) DNA was extracted from various tissues of 2 treated animals killed 32 weeks after treatment. The presence of the UGT1 transgene was detected by PCR amplification as described in Materials and Methods. As a control, a 202-bp fragment from the rat PEPCK gene promoter was amplified. (B) Total RNA was extracted from the tissues that were positive in PCR experiments and subjected to RT-PCR. RNA extracted from liver of a nontreated j/+ Gunn littermate was used as a control (control). A no-DNA template PCR-negative control was also performed (blank). The quality of RNA was checked by amplification of a fragment of S6 ribosomal protein.

tegrate the transgene into the infected cell's genome, the proportion of transduced cells is constant with time even during growth. This results in a constant proportion of transduced hepatocytes over time for more than 1 year, as we have previously described,[24] and we expect a definitive correction with our strategy.

Absence of Immune Response. In a previous study performed in adult Gunn rats and using murine retroviral vectors delivered to the regenerating liver, we observed induction of a immune response that precluded long-term correction in treated animals.[9] This was mainly attributable to cytotoxic elimination of transduced cells, but the presence of antibodies directed to human UGT1 protein were also detected. Therefore, we verified in our animals that no immune response had occurred by evaluating the presence of anti-UGT1 antibodies in the serum of treated animals by indirect ELISA. At all times during the follow-up of the animals (up to 42 weeks), serum were consistently negative (data not shown).

Dissemination of the Transgene. Preferential hepatic transduction was confirmed by performing PCR as well as reverse transcription (RT)-PCR experiments in animals that were killed 32 weeks after treatment. As shown in Fig. 7A, PCR showed a positive UGT1 signal in heart, thymus, muscle, and intestine from 1 animal. However, tissues from a second animal were negative. Importantly, we never detected germline transmission of the transgene. RT-PCR showed that the liver was the major site of UGT1 transcription when multiple tissues were transduced (Fig. 7B). Densitometric analysis of the signals in Fig. 7B indicated that muscle and heart accounted for 1.5% and 8.2% of the signal present in the liver, respectively. We did not find transcription from the intestine. Therefore, in treated animals, liver contributed to at least 90% of UGT1 activity, indicating that bilirubin conjugation in extra hepatic tissues was weak or absent.

Discussion

We demonstrate that neonatal *in vivo* gene delivery to the liver using retroviral vectors results in complete cure of hyperbilirubinemia in Gunn rats. This correction is accompanied by secretion of bilirubin glucuronides in the bile, confirming successful hepatocyte transduction. Moreover, the liver is the main site of expression of the transgene. In addition, gene correction is sustained, and no deleterious immune response prevents long-term transgene expression.

The liver is freely accessible to gene transfer vectors via the bloodstream because fenestrated endothelium of the liver sinusoids makes extravasation of gene transfer vectors possible.[29] In contrast, other tissues such as muscles harbor a continuous endothelium and therefore are less susceptible to gene delivery via the bloodstream. This explains why we observed an almost exclusive liver transduction in our animals, with few transgene detected in muscle intestine and heart. Therefore, many recessive inherited liver metabolic disorders could potentially be cured by bloodborne liver gene therapy, as already documented for type VII mucopolysaccharidosis.[17] CN1 is an ideally suited disease for liver gene therapy: (1) It is a serious, life-threatening disease that can be cured only by liver transplantation. Shortage of liver grafts means other therapeutic strategies are needed for affected children. (2) Precise gene control is not required to achieve clinical benefit. (3) Partial transduction of liver cells should be sufficient for biological cure because bilirubin in excess may be taken up and disposed of by a limited number of hepatocytes. This was suggested by the results of the first clinical trial of hepatocyte transplantation in CN1 patients.[30] Restoration of 5.5% of enzyme activity after transplantation of allogenic hepatocytes resulted in a 50% reduction in serum bilirubin. (4) In CN1, the liver parenchyma is completely normal throughout the course of the disease, making hepatocytes accessible to gene transfer vectors. For all these reasons, CN1 appears as a valid candidate for gene therapy.

Among various gene transfer vectors that have been devised, retroviral vectors are attractive tools for gene

therapy of metabolic inherited diseases. In young animals in which liver growth is present, hepatocyte division makes transduction with oncoretroviral vectors possible, as shown here and in a previous study.[17] In contrast to other tissues (*e.g.*, bone marrow[31]), the long terminal repeat of retroviral vector is active for more than 1 year after *in vivo* gene delivery to the liver.[9,24] Using integrative vectors, transgene integration in the infected cell's genome ensures that permanent expression is achieved, whatever the subsequent number of divisions of the corrected cell. This is a major requirement if gene therapy is to be applicable to young children. The major drawback of integrating vectors is insertional mutagenesis, as exemplified in a recent gene therapy trial for severe X-linked immunodeficiency.[32] However, it must be stressed that, in this previous trial, specific features such as high multiplicity of infection, as well as selective growth advantage conferred by gene transfer, may have amplified the risk of tumor formation. In no preclinical studies involving small as well as large animals has liver-directed *in vivo* gene transfer resulted in uncontrolled proliferation of target cells. Thus, we believe that the risk of malignant transformation after *in vivo* gene delivery to the liver is low and does not compromise the risk/benefit ratio for severe genetic diseases. Finally, retroviral delivery to the newborn can induce tolerance to the transgene product.[18] Accordingly, we never detected immune response to UGT1 gene product or to β-galactosidase in our experimental animals (not shown). This is in contrast to the strong immune response that we observed before in adult rats after retroviral delivery of the same transgenes.[9]

In conclusion, we believe that our study demonstrates that *in vivo* gene delivery to the liver using retroviral vectors in young CN1 patients is a valid therapeutic strategy that deserves further consideration for future clinical application.

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