Strong, Long-Term Transgene Expression in Rat Liver Using Chicken β-Actin Promoter Associated With Cytomegalovirus Immediate-Early Enhancer (CAG Promoter)

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For successful gene therapy in hepatic enzyme deficiencies, it is essential to use promoters that can maintain strong transcriptional activity for the long term in the liver. Using Gunn rats, a model animal for Crigler-Najjar syndrome type I, the long-term transcriptional function of the CAG promoter (a combination of chicken β-actin promoter and cytomegalovirus immediate-early enhancer) was evaluated in the rat liver. We constructed a plasmid pCAGGHUGT, containing expression cassettes of human bilirubin UDP-glucuronosyltransferase (BUGT) and hygromycin phosphotransferase, under the control of the CAG promoter and murine phosphoglycerate kinase promoter, respectively. Conditionally immortalized Gunn rat hepatocytes (IGRH), which had been established using mutant SV40 large T antigen (TS), were transfected with pCAGGHUGT. A stably transfected clone IGRHUGT, expressing a high level of BUGT, was obtained after selection with hygromycin. At 33°C, the cells doubled in number in approximately 72 h; however, at 37°C, cell proliferation stopped, indicating that the characteristic of temperature-dependent proliferation was retained in this clone. Ten million cells were injected into the spleen of syngeneic Gunn rats five times at 10-day intervals. Serum bilirubin levels were reduced by 45–50% at 70 days after the first transplantation and remained so throughout the duration of the study (120 days). These results suggested that the CAG promoter was able to maintain strong transcriptional activity in rat liver for at least 120 days.

Key words: Gunn rats; Immortalized hepatocytes; CAG promoter

INTRODUCTION

Glucuronidation of bilirubin by hepatic bilirubin-uridine diphosphate-glucuronosyltransferase (BUGT) is essential for effective excretion of bilirubin from the liver. Congenital deficiency of BUGT activity leads to Crigler-Najjar syndrome type I in humans. This autosomal recessive disorder is characterized by lifelong hyperbilirubinemia resulting in bilirubin encephalopathy at infancy or in adolescence (22). Currently, liver transplantation is the only definitive treatment for Crigler-Najjar syndrome type I. Homozygous Gunn rats having a similar metabolic defect are an authentic animal model for this syndrome (1,23). Gunn rats have been used extensively for the study of the pathophysiology of bilirubin metabolism (17,21), and for the research on liver-directed gene therapy, because therapeutic efficacy is easily evaluated by determination of serum bilirubin concentrations and excretion of bilirubin glucuronides in bile (6–8). Conditionally immortalized Gunn rat hepatocytes (IGRH) (4) were established by infecting primary Gunn rat hepatocytes with a recombinant retrovirus transferring the gene for a thermolabile mutant SV40 large T antigen (TS) (9). At a permissive temperature (32°C), the transduced cells expressed the TS T antigen, which promoted cell proliferation. At 37–39°C, the cells stopped growing and exhibited differentiated function as hepatocytes (4). Transplantation of genetically engineered IGRH cells in Gunn rats indicated that these cells were engrafted efficiently in the hepatic bed after intrasplenic injection (26). The CAG promoter was constructed by the combination of chicken β-actin promoter and cytomegalovirus immediate-early enhancer (16). This promoter expresses with high efficiency transfected
exogenous genes in various mammalian cell lines in vitro. The strong activity of the CAG promoter has been reported in rodent hepatocytes in vivo (12). In this study, a recombinant adenoviral vector expressing an exogenous gene under the control of the CAG promoter was administered intravenously into rats, resulting in a high level of transgene expression in the hepatocytes. Although these studies demonstrated the strong transcriptional activity of the CAG promoter in hepatocytes in vivo, the long-term efficacy of the CAG promoter could not be evaluated, because of the relatively short duration of gene expression by the adenoviral gene transfer system. In the present study, we examined the long-term transcriptional activity of the CAG promoter in rat hepatocytes in vivo by transplantation of Gunn rats with gene-transferred IGRH cells.

**MATERIALS AND METHODS**

**Animals**

Inbred Gunn rats and congeneric normal Wistar RHA rats were bred and maintained by T.O. at the National Children's Medical Research Center. All rats were maintained in accordance with the guidelines of the Animal Committee of the facility.

**Plasmid Construction**

The plasmid pCAGGS (16) was digested with EcoRI, blunt-ended with Klenow fragment of *E. coli* DNA polymerase, and ligated with cDNA of human BUGT (25). Then the plasmid was again digested with BamHI, and the expression cassette of hygromycin phosphotransferase gene was introduced under the control of phosphoglycerate kinase promoter (14) to generate the plasmid pCAGGHUGT (Fig. 1).

**Cloning of Immortalized Gunn Rat Hepatocytes Expressing Human BUGT**

Conditionally immortalized Gunn rat hepatocytes (IGRH) (4) were transfected with pCAGGHUGT using lipofectamine plus (GIBCO BRL, Grand Island, NY) following the manufacturer's protocol. Forty-eight hours after the transfection, 30 μg/ml of hygromycin (Wako, Osaka, Japan) was added to the media, and continued the culture until each colony was clearly identified. Sixteen colonies were picked out arbitrarily and further propagated.

**Determination of BUGT Activity**

Activity of bilirubin UGT was measured by the method described previously (13). Briefly, labeled UDP-glucuronic acid ([glucuronyl-14C(U)]) UDP; 10.55 GBq/mmol) (Dupont-NEN, Wilmington, DE) was incubated with cell homogenates, and the reaction mixtures were subjected to TLC.

**RESULTS**

**Establishment of Immortalized Gunn Rat Hepatocytes Overexpressing Human Bilirubin-UDP-Glucuronosyltransferase**

The conditionally immortalized Gunn rat hepatocytes (IGRH) were transfected using cationic liposome with the plasmid pCAGGHUGT, which was carrying both expression cassettes of human bilirubin-UDP-glucuronosyltransferase (UGBT) and hygromycin phosphotransferase. After the selection with 30 μg/ml hygromycin for 14 days, 16 colonies were arbitrarily selected and expanded by culturing at a permissive temperature (33°C). Activities of UGT were assayed with cell homogenates of each clone. The average UGT activity of the six clones, which were selected arbitrarily, was 8.42
nmol/min/mg (0.10–27.23 nmol/min/mg). The highest specific BUGT activity corresponded to 150% of the activity of normal rat liver homogenates. The clone "IGRHUGT," which showed the highest specific activity, was used for further analysis.

Temperature-Dependent Proliferation of IGRHUGT

To study the temperature-dependent proliferation of IGRHUGT, the cells were cultured at 33°C or 37°C, and cell counts were determined at 24-h intervals. At 33°C, the cells doubled in number in approximately 72 h (Fig. 2). On the other hand, when the cells were cultured at 37°C, their number did not increase significantly, suggesting that this clone maintained temperature-dependent proliferation characteristics similar to those of its original clone, "IGRH" (4).

Bilirubin Glucuronidation In Vitro

The activity of bilirubin glucuronidation in IGRHUGT was analyzed by detecting bilirubin glucuronides with HPLC after adding 85.0 μmol/L of free bilirubin to the culture media of the cells. IGRHUGT showed 0.142 or 0.351 nmol/10⁶ cells/h of bilirubin glucuronidation when the cells were cultured at 33°C or 37°C, respectively, indicating that the cells of IGRHUGT obtained the ability to carry out the complete glucuronidation process by the gene transfer of human BUGT (Fig. 3).

Amelioration of Hyperbilirubinemia in Gunn Rats After Transplantation of IGRHUGT

In the process of seeding the cells into the hepatic bed, 1 x 10⁷ IGRHUGT cells were injected into the spleen of syngeneic Gunn rats five times at 10-day intervals. Serum bilirubin levels in Gunn rats were 5–8 mg/dl before the transplantation. They decreased by 30% within a week, and the highest reduction (approximately 48%) was observed at 70 days after the first transplantation. At day 70, serum bilirubin levels slightly increased, but a 40–45% reduction was maintained throughout the duration of the study (120 days) (Fig. 4). These results suggested that the CAG promoter was able to maintain strong transcriptional activity in rat hepatocytes for at least 120 days in vivo.

DISCUSSION

In this article, we show long-term therapeutic effect of transplantation of syngeneic genetic-modified immortalized hepatocytes. We established conditionally immortalized hepatocytes expressing human BUGT by liposome-mediated gene transfer, followed by hygromycin selection. Rapid and prolonged amelioration of hyperbilirubinemia in Gunn rats was observed after the transplantation of the cells. These results suggest that this approach of ex vivo gene therapy may be applied to the treatment of several inherited hepatic enzyme deficiencies, such as phenylketonuria, tyrosinemia, or orni-
Figure 3. Detection of bilirubin glucuronides by HPLC analysis. Bilirubin glucuronides in the media cultured IGRHUGT or primary hepatocytes were analyzed by reverse phase HPLC using a Nova pack C18 column (Millipore-Waters, Milford, MA) (A). Peaks were detected by absorbance at 436 nm, identified by retention time using authentic bilirubin standards, and quantified by integration of the areas under the curve (B). Bilirubin glucuronides were detected at both temperatures. These results suggested that IGRHUGT was able to carry out the complete glucuronidation process. 1: IGRHUGT cultured at 33°C. 2: IGRH cultured at 37°C. 3: Primary Gunn rat hepatocytes cultured at 37°C. UCB: unconjugated bilirubin, BMG: bilirubin monoglucuronide.

This study also demonstrated that the CAG promoter (16), a chicken β-actin promoter combined with cytomegalovirus immediate-early enhancer, maintained transcriptional activity in Gunn rat liver at a high enough level to treat hyperbilirubinemia for at least 120 days (duration of the study). Although several promoters or promoter/enhancer combinations have been employed in animal experiments of liver-directed gene therapy (11,18), few experimental systems were designed to evaluate the long-term promoter activity in the liver. The transcriptional activities of several promoters or promoter/enhancer combinations have been evaluated in the rat liver using a retroviral gene transfer system (5,19). Retroviral vectors expressing human α1-antitrypsin under the control of the promoters of mouse albumin, mouse RNA polymerase II, or rat phosphoenolpyruvate carboxykinase were evaluated in regenerating the rat liver. Although these results are useful in designing and constructing recombinant retroviruses for liver-directed gene therapy, they are not optimal for evaluating the...
promoter functions. As the long terminal repeat (LTR) regions of retroviruses have strong promoter activity, interaction of the LTR promoter and an internal promoter cannot be neglected. In adenoviral systems, the activity of internal promoters can be determined on a short-term basis, but determination of long-term promoter function in the liver is not possible because of the limited duration of transgene expression by this episomal vector system and the host immune response against the transduced cells (2,27). The cell transplantation system described in this article could be very useful for testing long-term transcriptional activities of novel promoters or promoter/enhancer combinations in the liver, because it is free from the drawbacks of the retroviral or adenoviral systems.

Recently, the CAG promoter has been used widely in adenoviral constructs (10,12). An aden-associated virus vector containing the CAG promoter has been reported as well (3). As the CAG promoter exhibits long-term strong transcriptional activity in rodent liver, it should be appropriate to test novel vector systems in liver-directed gene therapy using an expression cassette containing this promoter.

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REFERENCES


