phosphate-buffered saline (overnight). We added B19 empty capsids (100 μl, 5 μg/ml) to each well (in quadruplicate) and detected binding by a mouse anti-B19 monoclonal antibody (Chemicon) followed by ^{125}I-labeled sheep anti-mouse antibody (Amersham). The radioactivity in each well was counted for 2 min with a γ counter. There was no specific binding with CDH or CTH (methanol alone, mean count of 241; CDH, mean count of 282; CTH, mean count of 244), and reduced binding to the Forssman antigen (mean count of 2088) compared to globoside (mean count of 7458).

19. B19 capsids and mouse monoclonal antibody to globoside bound to the same band on Western blotting of red cell extract, perhaps to a putative "globoprotein" [Y. Tonegawa and S. Hakomori, *Biochem. Biophys. Res. Commun.* **76**, 9 (1977)]. However, this band was removed by digestion with lipase but not by proteases, more consistent with a glycolipid moiety.

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In Vivo Gene Therapy of Hemophilia B: Sustained Partial Correction in Factor IX–Deficient Dogs

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The liver represents a model organ for gene therapy. A method has been developed for hepatic gene transfer in vivo by the direct infusion of recombinant retroviral vectors into the portal vasculature, which results in the persistent expression of exogenous genes. To determine if these technologies are applicable for the treatment of hemophilia B patients, preclinical efficacy studies were done in a hemophilia B dog model. When the canine factor IX complementary DNA was transduced directly into the hepatocytes of affected dogs in vivo, the animals constitutively expressed low levels of canine factor IX for more than 5 months. Persistent expression of the clotting factor resulted in reductions of whole blood clotting and partial thromboplastin times of the treated animals. Thus, long-term treatment of hemophilia B patients may be feasible by direct hepatic gene therapy in vivo.
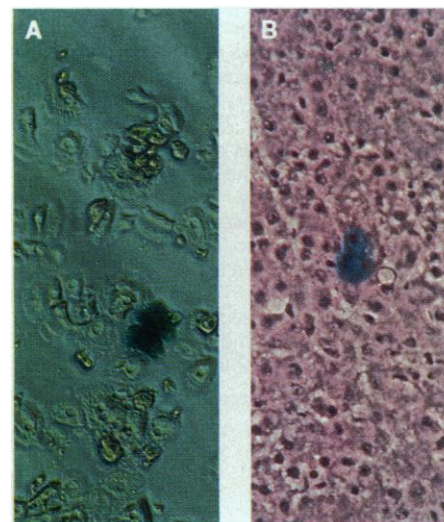
Hemophilia B is an X-linked blood coagulation disorder resulting from a deficiency of factor IX production in the liver. The disease affects about 1 in 30,000 males and can result in severe bleeding episodes that require infusion of blood products that contain factor IX (*1*). As a result of previous human protein replacement therapy, about half of hemophilia B patients are infected with human immunodeficiency virus or hepatitis viruses. A virus-free and non-thrombogenic factor IX product is now available, but because of high costs the current treatment protocols do not include prophy-

laxis and therapy is initiated after bleeding begins. A number of tissues are target organs for somatic gene therapy of hemophilia B, including fibroblasts, myoblasts, endothelial cells, keratinocytes, and hepatocytes (*2–7*).

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Because the liver is the organ of factor IX synthesis, it represents a natural target for gene replacement therapy.

We have previously reported that hepatocytes can be transduced in vivo by infusion of recombinant retroviral vectors into the portal vasculature of mice after partial hepatectomy (*8*). To determine if the same can be achieved in larger animals (such as dogs), we infused an amphotropic retroviral vector (LBGpgk) that encodes the *Escherichia coli* β-galactosidase gene (*8*) directly into the portal vasculature of normal dogs three times 1 to 3 days after partial hepatectomy (Fig. 1). Two weeks later, hepatocytes were isolated and stained with 5-bromo-4-chloro-3-imdoyl-6-D-galactopyranoside (x-Gal) (Fig. 1A). Liver sections from these animals were similarly analyzed (Fig. 1B). The proportion of stained (blue) cells in Fig. 1 represents the in vivo transduction frequency of hepatocytes and was about 1 and 0.3% in two animals. Additional tissues, including kidney and spleen, did not stain blue with x-Gal. These transduction efficiencies are similar to that previously observed in mice (*8*). Routine histologic analysis revealed no pathologic conditions in the liver.

Fig. 1. Retroviral vector–mediated gene transfer of canine hepatocytes in vivo. A two-thirds partial hepatectomy was performed in two, 9-week-old normal dogs (3.5 kg) by resecting the left medial, left lateral, right medial, and caudate lobes. The right lateral lobe and its segmental blood supply and biliary drainage were preserved. The distal tip of a porta-cath catheter (Access Technology, Skokie, IL) was cannulated into a splenic vein. The injection port was placed subcutaneously under the right lateral abdominal wall. The LBGpgk vector was collected from confluent packaging cells cultured in Hg DMEM and 1% Hyclone for 12 hours. About 85 ml of filtered supernatants containing 9×10^7 colony-forming units was mixed with Polybrene (20 μg/ml) and infused over 45 to 90 min through the catheter 24, 48, and 72 hours after the hepatectomy. The animals tolerated the procedure well except for occasional vomiting and transient pallor during the beginning of the first infusion. When the dogs were killed, hepatocytes were isolated, cultured (*10*), and stained with x-Gal (*8*) (**A**) (original magnification, ×200), and (**B**) liver sections were stained with x-Gal and counterstained with neutral red (*8*) (original magnification, ×400).

An amphotropic retroviral vector that encoded the canine factor IX complementary DNA (cDNA) (LX-cFIX) was constructed (9) and transduced into rat embryo 208F cells (8) to assess its ability for in vitro expression of factor IX. The media were changed daily, and canine factor IX was measured by enzyme-linked immunosorbent assay (ELISA) (8, 10) with a species-specific polyclonal antibody that was prepared as described (11). The transduced rodent cells produce 225 ng of canine factor IX antigen per 10⁶ cells per day, whereas control cells produce no detectable factor IX (12). This recombinant retroviral vector was used for infusion into the portal vasculature of four hemophilia B dogs (Table 1) from the Chapel Hill inbred strain (13). The molecular defect in these dogs is a missense mutation in the catalytic domain of factor IX that results in a complete lack of antigen in the plasma (14). A partial hepatectomy was performed in these experimental ani-

mals, followed by the infusion of the LX-cFIX retrovirus 24, 48, and 72 hours after partial hepatectomy (Table 1). Factor IX concentrations in their plasma were measured by biologic and immunoassays (Figs. 2A and 3A). The hemostatic parameters were monitored by changes in the whole blood clotting time (WBCT) (Figs. 2B and 3B) and partial thromboplastin time (PTT) (Table 1). These tests are indexes of the intrinsic pathway of clotting in which factor IX is the key component.

In dog 1, the plasma factor IX increased from undetectable amounts to a range of 2 to 6 ng/ml; these levels have been maintained constitutively for over 5 months (Fig. 2A), and there is close agreement between the biologic and immunoassay results. Most importantly, this dog had a WBCT of 15 to 20 min during the 5-month period after treatment, whereas the WBCT for untreated factor IX–deficient littermates ranged from 45 to 55 min

(Fig. 2B). A long WBCT is characteristic of a dog colony severely deficient in factor IX; normal dogs, on the other hand, have a WBCT of 6 to 8 min (Fig. 2B). In vitro addition of normal dog plasma to whole blood from hemophilia B dogs to a final concentration of 3 ng/ml reduced the WBCT to 20 min. This is in agreement with the WBCT and factor IX concentrations obtained in dog 1 after treatment (Fig. 2). The PTT for dog 1 was also shortened from 322 before treatment to 174 seconds after treatment (Table 1).

Plasma from dog 2 showed factor IX concentrations similar to that of dog 1 (Figs. 2A and 3A), whereas dog 3 had slightly greater concentrations, ranging from 3 to 10 ng/ml (Fig. 3A). Both dogs 2 and 3 had similar reductions in their WBCT, from pretreatment values of 44 to 47 min to 18 to 26 min after treatment (Fig.

Fig. 2. Plasma factor IX concentrations and WBCTs in hemophilia B dog 1 after hepatic transduction with the LTR-cFIX retroviral vector. The plasma factor IX concentrations were determined immunologically by ELISA and by bioassay. The bioassays were performed by a modified one-stage method (16) in which a kaolin-activated canine hemophilia B plasma was used as a substrate, a limiting dilution of a normal canine plasma pool was used as reference plasma (11.5 μg/ml), and test plasmas were diluted in a 1:10 ratio. The value of 11.5 μg/ml was estimated from the ratio of the concentration of normal human factor IX activity (5.0 μg/ml) to the concentration of normal canine factor IX activity. Hemostatic testing consisted of a determination of the WBCT (with the two-tube method at 28°C with the use of silicone-coated tubes tilted every 30 s until clotted). (A) Values for the antigen concentrations of factor IX are shown in solid symbols, and the values from the bioassay are shown in open symbols. (B) The WBCTs in dog 1 (solid squares), two affected hemophilia B littermates (open squares), and the range in normal dogs (open circles). The days after infusion represent the number of days after the first retroviral infusion; day 0 represents measurements from samples obtained before any procedural manipulations.
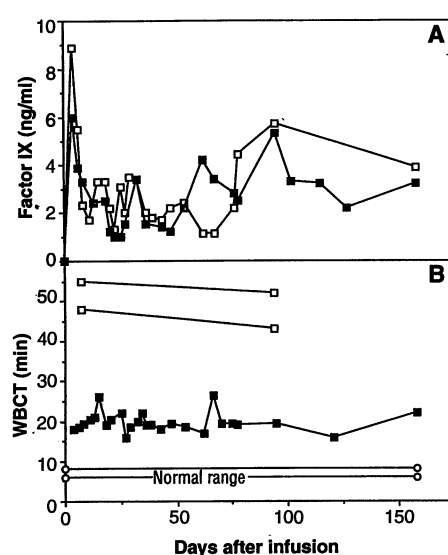
Table 1. Hemophilia B dogs used in a gene therapy protocol (23). The weight and age were recorded on the day the study was started. The methods for partial hepatectomy were essentially the same as outlined in Fig. 1. Blood samples were obtained from the animals before surgery, and hemostatic coverage was maintained with multiple infusions of normal fresh frozen plasm given immediately before and for 24 hours after the operation (57.4 to 72.7 units of factor IX per kilogram of body weight). Circulating factor IX from plasma infusions in untreated animals is cleared within 8 to 10 days (22). The PTTs (24) were obtained from two to four samples before the start of the experiments (Before) and then were analyzed 6 to 10 times for each animal on different days starting at least 11 days after the viral infusion (After). The times shown indicate nonactivated PTTs, which for normal dogs are 42 to 47 s. The standard deviations are in parentheses.

Dog*	Weight (kg)	Age (weeks)	Infusion volume (ml)	PTT (s)	
				Before	After
1	5.3	11	510	322 (15)	174 (6.5)
2	7.3	14	720	262 (0.8)	180 (7.5)
3	6.7	14	720	195 (13)	154 (8.6)

*One animal died as a result of a surgical complication and was not included.
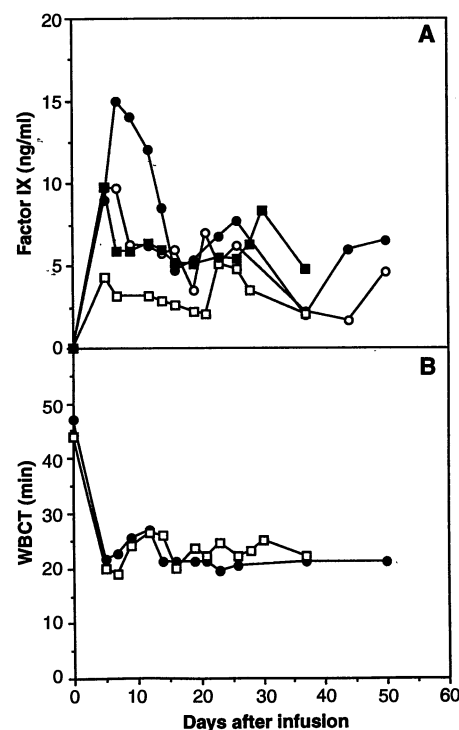

Fig. 3. Plasma factor IX concentrations and WBCTs in hemophilia B dogs 2 and 3 after hepatic transduction with the LTR-cFIX retroviral vector. The hemostatic characterizations were as described (Fig. 2). (A) The values for the antigen concentrations are in solid symbols. Dog 2, squares; dog 3, circles. Values from the bioassay are in open symbols. (B) Whole blood clotting times. Dog 2, open squares; dog 3, solid circles. The study on dog 2 terminated on day 42 as multiple transfusions were given for hemorrhage. Dog 3 developed a minor bleeding episode that required one infusion of normal dog plasma on day 26, which accounts for the hiatus in data for days 27 to 36. Previous studies (22) have shown that by 10 days after infusion exogenous factor IX is undetectable by bioassay.

3B). The PTTs in dogs 2 and 3 were also decreased significantly (Table 1). To further establish the biological activity of plasma factor IX, we treated samples from dogs 1 and 3 with barium sulfate to remove γ-carboxylated proteins, including factor IX (15). The barium sulfate–treated plasmas of treated dogs had a prolonged PTT that was similar to the pretreatment PTT values.

Our study demonstrates the feasibility of in vivo retroviral-mediated gene transfer into the liver of a large animal, which results in phenotypic improvement of a deficiency syndrome. The factor IX antigen amounts achieved after gene transfer were only about 0.1% of the endogenous concentration of factor IX in normal animals, which demonstrates that the constitutive expression of a relatively small quantity of the factor IX protein is sufficient to cause a reduction in the WBCT and a shortening of the PTT. Our data indicate that the WBCT is extremely sensitive to changes in factor IX concentration in the hemophilia B dogs. In moderate and mild human hemophilia patients with shortened WBCT and factor levels in the 3 to 8% range, there is still a risk of hemorrhage, although both the frequency and the severity of episodes are considerably less than those of severely affected individuals (16). For future human applications, however, increased circulating factor IX levels must first be achieved. This may be accomplished by developing methods that lead to greater efficiencies of hepatocyte transduction in vivo and by creating expression vectors with stronger promoters. These reservations notwithstanding, our results illustrate the efficacy of in vivo gene therapy of hemophilia B and other metabolic disorders secondary to hepatic deficiencies.

Note added in proof: Plasma factor IX concentrations and WBCTs remained at the same values at 9 months after treatment for dog 1 and 6 months after treatment for dog 3.

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9. In the construction of the LX-cFIX retroviral vector, the 1.4-kb cFIX cDNA was cloned by reverse transcriptase–polymerase chain reaction (RT-PCR) amplification of canine liver polyadenylate-containing RNA. The 5′ primer was synthesized to contain the 5′-GCCACC-3′ Kozak sequence [M. Kozak, *Nucleic Acids Res.* **15**, 8125 (1987)]. The sequence of the cDNA was determined and found to be identical to the published sequence (14). The cDNA was cloned into a modified LNCX retroviral vector (17), with its *neo* gene and cytomegalovirus promoter removed by Bcl I–Bam HI digestion and religation. The cloned DNA was transfected into GP+E-86 ecotropic packaging cells (18), and supernatants were used to infect amphotropic GP+AM12 cells (19). The single clone that gave the greatest amount of cFIX production in transduced 208F cells was titered (20) by limiting dilution PCR (21) and found to have a concentration of 2×10^6 colony-forming units per milliliter.
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23. All experimental procedures were in accordance with institutional guidelines at the Baylor College of Medicine and the University of North Carolina. Preanesthetic agents included atropine sulfate (0.24 mg per kilogram of body weight) and sodium thiamylal (0.1 to 0.2 g/kg). Maintenance anesthesia was 2% halothane. The postoperative analgesic was butorphanol (0.5 mg) administered intravenously as needed every 4 to 6 hours during the first 24 hours. The animals were treated prophylactically with antibiotics (cefadroxil, 22 mg/kg) for several days before and after the operation. Before retroviral infusion, the animals were given diphenhydramine (10 mg) intramuscularly, and some animals were sedated with acepromazine (5 mg) intravenously.
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