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ARTICLE

Transduction of Hepatocytes after Neonatal Delivery of a Moloney Murine Leukemia Virus Based Retroviral Vector Results in Long-Term Expression of β-Glucuronidase in Mucopolysaccharidosis VII Dogs

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The use of Moloney murine leukemia virus (MLV)-based retroviral vectors (RV) can result in stable *in vivo* expression in the liver, but these vectors only transduce replicating hepatocytes. As newborn animals exhibit rapid growth, we evaluated the ability of MLV-based RV to transduce hepatocytes in neonatal dogs. IV injection of a β-galactosidase-expressing RV at 3 days after birth resulted in transduction of 9% of hepatocytes. Prior treatment with human hepatocyte growth factor at 2.5 mg/kg did not increase transduction. Although cells from the spleen were also transduced with moderate efficiency, cells from other organs were not. Neonatal dogs with mucopolysaccharidosis VII (MPS VII) received an IV injection of an RV containing the canine β-glucuronidase (cGUSB) cDNA. At several months after transduction, clusters of hepatocytes that expressed high levels of cGUSB were present in the liver, which probably derived from replication of transduced hepatocytes. At 6 months after transduction, serum GUSB levels were 73% that of homozygous normal dogs and were 34% of the peak values observed at 1 week. We conclude that neonatal delivery of an MLV-based RV results in stable transduction of hepatocytes in dogs. This approach could result in immediate correction in patients with an otherwise-lethal genetic deficiency.

Key Words: bromodeoxyuridine, labeling index, gene therapy, dog, β-glucuronidase, lysosomal storage disease, mucopolysaccharidosis, retroviral vector

INTRODUCTION

Use of retroviral vectors (RV) can result in stable and therapeutic levels of expression of proteins in the liver [1–3]. However, Moloney murine leukemia virus (MLV)-based RV only transduce dividing cells [4], which presents a problem for liver-directed gene therapy as hepatocytes of adult animals are normally quiescent. Indeed, one of a variety of approaches has been used to induce hepatocyte replication in adult animals to potentiate transduction of hepatocytes with an MLV-based RV. These approaches included inducing compensatory hepatocyte replication by removing or damaging part of the liver, or administration of hepatic growth factors [reviewed in 5]. Hepatocyte growth factor (HGF), a 90-kD heterodimeric protein, binds to the c-met receptor on the surface of cells and induces replication [6]. HGF has induced hepatocyte replication and facilitated RV-mediated transduction of hepatocytes in young adult rodents [3,5,7–11], and induced or augmented hepatocyte replication in young dogs [12,13].

Although efficient transduction of adult hepatocytes with MLV-based vectors required a procedure to induce replication, it was possible that neonatal hepatocytes might be more conducive to transduction without these procedures due to their rapid rate of growth. Alternatively, if baseline replication was insufficient for efficient transduction, neonatal hepatocytes might be more responsive to a relatively low dose of HGF, as previous studies have demonstrated that a small (30%) partial hepatectomy [14] or portal branch occlusion [13] markedly potentiated the

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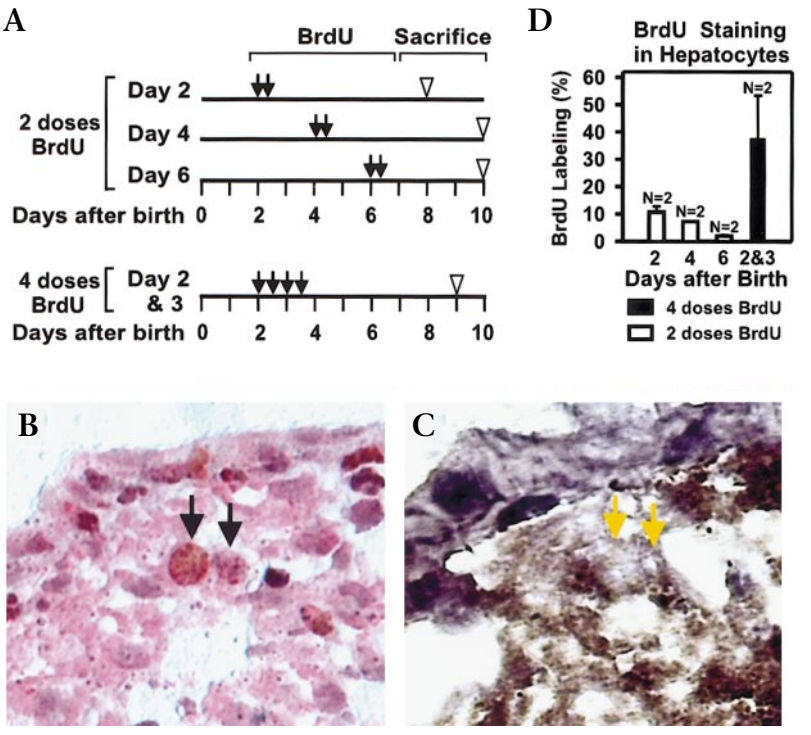

FIG. 1. Evaluation of the labeling index in neonatal dogs. (A) Method for evaluation of the labeling index. Some dogs (2 doses BrdU) were injected IP with two doses of 50 mg/kg of BrdU separated by 8 hours beginning at 2 (day 2), 4 (day 4), or 6 (day 6) days after birth, as indicated by the black arrows. Other animals (4 doses BrdU) received four doses of BrdU each separated by 12 hours beginning at 2 days after birth (day 2 and 3). Animals were sacrificed at 8 to 10 days after birth. (B) Immunocytochemistry to identify BrdU-labeled cells. A section of a liver from an animal that received 2 doses of BrdU at 2 days after birth and was sacrificed at 10 days after birth underwent immunocytochemistry to identify BrdU-labeled cells, which contain brown nuclei. The section was counterstained with eosin, which stains the cytoplasm of hepatocytes, and was very lightly counterstained with hematoxylin, which stains all nuclei faint blue. Recently replicated hepatocytes are identified with arrows. Original magnification, ×100. (C) G6Pase staining. An adjacent section to that shown in (B) underwent a histochemical stain for G6Pase, which is present in the cytoplasm of hepatocytes, and results in a brown stain. The section was counterstained with hematoxylin and eosin. The yellow arrows identify the nuclei of the same hepatocytes that were labeled with BrdU in the adjacent section. Original magnification, ×100. (D) Labeling index at different times after birth. Frozen liver sections from animals that were sacrificed as noted in (A) underwent immunocytochemistry using an anti-BrdU antibody, as shown in (B). The number of BrdU-labeled hepatocytes was divided by the total number of hepatocytes in the same field to determine the labeling index. "N" indicates the total number of animals in each group, and averages ± SEM are shown.

effect of HGF on hepatocyte replication. There are also other advantages to neonatal gene transfer for the treatment of genetic deficiencies. First, some genetic diseases are lethal shortly after birth if untreated, and neonatal gene transfer would be necessary to allow the patient to survive. Second, because neonates generally have less mature immune systems [15–19], neonatal gene transfer might result in the induction of tolerance to the therapeutic gene. Third, neonatal gene transfer with an MLV-based vector should preclude germline transduction, as germ cells do not replicate in neonatal males [20] or females [21].

In this study, the ability of an MLV-based RV to transduce neonatal canine hepatocytes was tested. We found that hepatocyte transduction was efficient at 3 days after birth without the administration of HGF. HGF did not significantly increase the percentage of transduced cells, which was likely due to the fact that hepatocyte replication was high in the neonatal period for both groups. Transduction was stable as demonstrated by the persistent serum canine β-glucuronidase (cGUSB) activity seen in dogs with mucopolysaccharidosis VII (MPS VII) that were treated with an RV containing the cGUSB cDNA. We conclude that a simple IV injection of an MLV-based RV may be effective for the neonatal treatment of genetic diseases.

RESULTS

Time Course of Hepatocyte Replication in Neonatal Dogs

Studies were performed to determine the percentage of replicating hepatocytes at various times after birth in dogs, as previous reports demonstrated that MLV-based RV only transduce dividing cells. Figure 1A diagrams the bromodeoxyuridine (BrdU)-labeling protocol that was used. BrdU is a thymidine analog that is incorporated into cells that are synthesizing DNA. Analysis of organs by immunohistochemistry for cells that contain BrdU in their nucleus after the systemic administration of BrdU can be done to determine the labeling index. Because BrdU is permanently incorporated into the DNA, animals can be given more than one dose of BrdU to increase the time interval over which labeling can occur. In addition, animals can be sacrificed several days after the administration of BrdU without the risk of labeling additional cells [22], as the half life of BrdU is very short in blood. This allowed us to wait until 8 to 10 days after birth to sacrifice the animals to allow the amount of extramedullary hematopoiesis, which complicated the quantitation of the hepatocyte labeling index in livers from younger animals, to fall to low levels in the liver. Liver sections were then evaluated for the percentage of labeled hepatocytes using anti-BrdU immunostaining.

For most animals, two doses of BrdU separated by 8 hours were injected to obtain the labeling index over an 8-hour period (day 2, day 4, and day 6). Two days after birth was chosen as the first time point to be analyzed, as this is an age by which the newborns should be stabilized

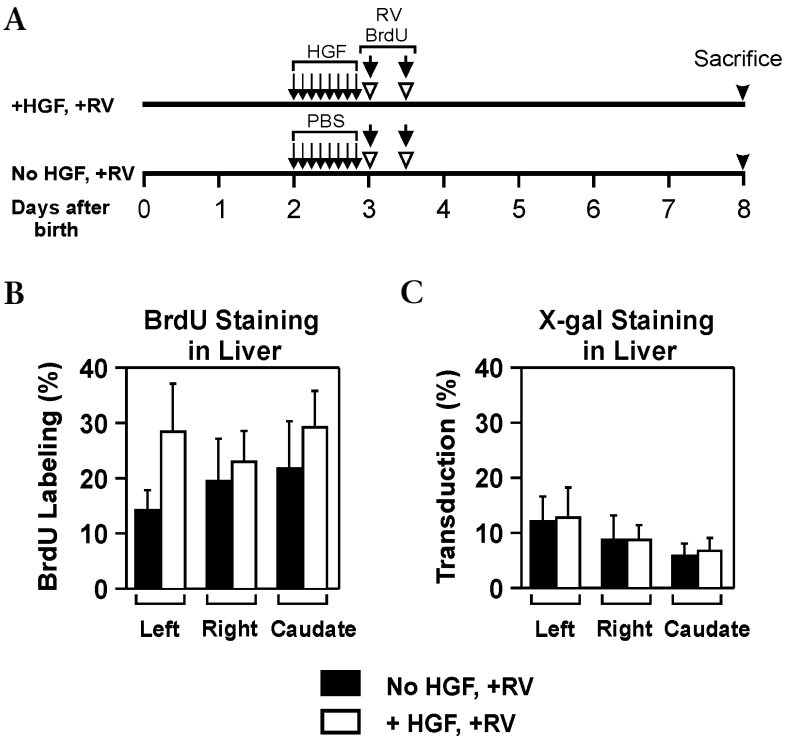

FIG. 2. Effect of HGF on hepatocyte replication and RV transduction in neonatal dogs. (A) Time course of administration of reagents. Neonatal dogs were injected IV with 8 doses of HGF between 2 and 3 days after birth for a cumulative dose of 2.5 mg/kg (+HGF, +RV; $n = 4$). Controls (No HGF, +RV; $n = 4$) received injections of PBS at the same times. For both groups, the TA7 RV was injected IV as equal divided doses at approximately 3 and 3.5 days after birth for a cumulative dose of 4.9 to 7.6 $\times$ 10^{10} bfu/kg. BrdU was injected IP at 50 mg/kg at both times that RV was injected. Animals were sacrificed at 8 days after birth. (B) BrdU labeling index of hepatocytes. Animals were treated as shown in (A). Sections of the livers underwent immunostaining for BrdU, and the percentage of labeled hepatocytes in the left, right, and caudate lobes was determined separately for each group and plotted as the average ± SEM. Four animals were in each group. The values in the two groups were not statistically different. (C) Hepatocyte transduction efficiency. Sections of livers from the same animals that were analyzed in (B) were stained with X-gal, as shown in Fig. 3A to 3H. The number of transduced hepatocytes was determined as detailed in the methods section, and plotted as the average ± SEM. The values in the two groups were not statistically different.

from the stress of birth, and would allow time for genetic tests to be performed to determine the genotype. Analysis at day 4 and day 6 was also performed, as dogs continue to exhibit rapid growth at these times. No BrdU-labeled cells were detectable in a liver from a dog that did not receive BrdU, demonstrating that staining was specific for the administration of BrdU (data not shown). In contrast, animals that received BrdU had several large cells with a large brown nucleus and eosinophilic cytoplasm. To document that these cells were hepatocytes, immediately-adjacent sections were stained for the hepatocyte-specific intracellular enzyme glucose-6-phosphatase (G6Pase). As the average diameter of hepatocytes is large at 30 μm and each section is only 8 μm, an adjacent section will contain the same hepatocyte in most cases. This confirmed that the eosin-staining cells with brown nuclei were hepatocytes (Figs. 1B and 1C). The percentage of BrdU-labeled hepatocytes was high at 11 ± 2% at day 2, and was lower at 6.1 ± 1.7% at day 4, and 2.3 ± 0.2% at day 6 (Fig. 1D). We concluded that hepatocyte replication was very high at 2 days after birth in dogs. Replication falls thereafter but remains moderately high at 6 days.

Although the labeling index was much higher at 2 days after birth than in adult animals, most cells were not replicating and would not be amenable to transduction with an MLV-based vector. Therefore we tested if additional cells would be recruited to replicate if the labeling index was performed over a longer time interval shortly after birth. Some animals (days 2 and 3; Fig. 1A) received four doses of BrdU every 12 hours over a 2-day period. This resulted in the labeling of 37.6 ± 15.7% of hepatocytes, which was 3.4-fold the value obtained when labeling was performed over an 8-hour period. This suggested that it might be possible to transduce a higher percentage of hepatocytes if RV were to be injected frequently over this period rather than as a single injection.

Effect of HGF on Replication of Canine Hepatocytes

The effect of HGF on hepatocyte replication and RV transduction in neonatal dogs was tested. For these studies, HGF was administered at 2 days after birth, and the effect on replication was evaluated at 3 days after birth, when replication was already moderately high in normal dogs. Dogs were injected with multiple doses of HGF over a 24-hour period, which was a regimen that induced hepatocyte replication in rats in our earlier studies [7] (Fig. 2A). Animals were then injected with both BrdU and RV at 24 and 36 hours after the first dose of HGF, so that the labeling index and the transduction efficiency could be assessed at the same time. At 24 hours, hepatocyte replication was high in rats in response to this regimen [7], whereas at 36 hours hepatocyte replication was high in mice in response to a slightly different regimen of HGF (C.G. and K.P.P., unpublished data). A dose of 2.5 mg/kg of HGF was used, which was 25% of the dose that was used to induce hepatocyte replication in young adult rats [7]. This dose was used because a pilot study with 10 mg/kg of HGF demonstrated toxicity, and the higher level of baseline replication in neonatal hepatocytes might have made them more responsive to a lower dose of HGF.

Quantification of the labeling index is shown in Fig. 2B, and values for this and other parameters in individual dogs are summarized in Table 1, as there was some variation in

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TABLE 1: Labeling index and transduction efficiency in neonatal dogs that were injected with PBS or HGF and transduced with TA7

Dog no.[a]	Time of PBS or HGF[b]	Time of BrdU and RV[c]	Weight[d]	BrdU labeling index (%)[e]	Dose of RV (bfu/kg)[f]	Transduction in liver by X-gal stain[g]	DNA copy number in liver[h]
PBS plus RV							
M1234	PBS at 49 to 70 h	73 and 85 h	410 g	6.0%	4.9×10^{10}	1.0%	0.043
M1236	PBS at 49 to 70 h	73 and 85 h	380 g	35.5%	6.2×10^{10}	6.0%	0.097
M1272	PBS at 57 to 78 h	81 and 93 h	210 g	16.7%	7.6×10^{10}	18.3%	0.552
M1275	PBS at 57 to 78 h	81 and 93 h	300 g	15.6%	5.3×10^{10}	10.2%	0.210
Average			325 ± 45 g	18.5 ± 6.2%	$6 \pm 0.6 \times 10^{10}$	8.9 ± 3.6%	0.226 ± 0.114
HGF at 2.5 mg/kg plus RV							
M1233	HGF at 49 to 70 h	73 and 85 h	480 g	20.2%	4.9×10^{10}	5.5%	0.048
M1237	HGF at 49 to 70 h	73 and 85 h	420 g	36.9%	5.6×10^{10}	6.9%	0.099
M1271	HGF at 57 to 78 h	81 and 93 h	230 g	35.5%	6.9×10^{10}	19.7%	0.231
M1273	HGF at 57 to 78 h	81 and 93 h	290 g	14.8%	5.5×10^{10}	6.2%	0.233
Average			355 ± 57 g	26.8 ± 5.5%	$5.7 \pm 0.4 \times 10^{10}$	9.6 ± 3.4%	0.153 ± 0.047

For each group, the average values in each of the four animals are shown at the bottom. Values in the PBS-treated and HGF-treated dogs were compared with the Student's *t*-test and were not significantly different ($P > 0.05$) for the body weight, labeling index, dose of RV, or percent transduction.

[a]The identity of specific animals.

[b]Animals were treated with 8 doses of either PBS or HGF given IV during the indicated time interval in hours after birth, as detailed in Fig. 2A.

[c]The time of administration in hours after birth of BrdU and RV, as detailed in Fig. 2A.

[d]The weight of dogs on the day when PBS or HGF was started.

[e]The labeling index for hepatocytes from the left, right, and caudate lobes was averaged for each animal.

[f]The cumulative dose of RV in bfu/kg.

[g]The average percentage of hepatocytes that were transduced for the left, right, and caudate lobe of each animal as assessed by X-gal staining.

[h]The average RV DNA copy number per diploid genome in the liver based on results from real-time PCR.

the size of the animals and the results of the analyses. Littermate controls that received PBS before RV had replication of 18.5 ± 6.1% of hepatocytes at 3 days after birth when values obtained from the left, right, and caudate lobes were averaged. The higher replication observed for controls that did not receive HGF in this study, as compared with the previous study in Fig. 1, may be due to the fact that the animals in the earlier study were larger (average weight 457 ± 55 g) than in this study (average weight 325 ± 44 g), and may have exhibited slower growth of the liver. Alternatively, the longer time interval between the two doses of BrdU (12 hours instead of 8 hours) may have affected the result. It is unlikely that the injection of RV concomitantly with the BrdU affected the hepatocyte labeling index, as most stimuli for hepatocyte replication require at least 24 hours to induce replication. Administration of HGF during the 24 hours preceding the first dose of RV resulted in replication of 26.8 ± 5.5% of hepatocytes at 3 days after birth when values obtained from all three lobes were averaged. This was 1.4-fold that of the littermate controls that received PBS instead of HGF, but the difference was not significant ($P = 0.35$). We concluded that HGF did not have a dramatic effect on the percentage of replicating hepatocytes in neonatal dogs, probably because the baseline level of replication is already high.

Effect of HGF on Transduction of Canine Hepatocytes

Neonatal dogs that either did or did not receive HGF were also transduced with the amphotropic MLV-based RV TA7. This is an RV that uses the LTR promoter to express a β-galactosidase (β-gal) gene containing a nuclear localization signal for the protein product. Animals received a cumulative dose of 4.9 to 7.6×10^{10} blue forming units (bfu)/kg of TA7 given as two doses separated by 12 hours on 3 days after birth, and were sacrificed at 8 days after birth (Fig. 2A). This represents a multiplicity of infection of 7 bfu per hepatocyte, based on the assumption that the liver is 5% of the body weight and that there are 1.7×10^8 hepatocytes per gram of liver [23]. It is not clear, however, that all particles will reach the liver after an IV injection. This dose was based on the maximum volume that could be safely injected into a neonatal animal and the highest concentration of RV that we could achieve. Liver sections were then stained with X-gal to identify the transduced hepatocytes. Of 4 control dogs that did not receive any RV and were sacrificed at 8 days after birth, three had occasional small light blue dots after X-gal staining of their liver (Figs. 3A and 3B), which must represent nonspecific β-gal activity, whereas the fourth dog had no blue staining. In contrast, livers from animals that received RV had numerous eosinophilic cells with large dark blue nuclei after X-gal staining (Figs. 3C–3G). These cells are hepatocytes, as demonstrated by staining immediately adjacent sections with X-gal and the hepatocyte-specific marker G6Pase (Figs. 3G and 3H). The percent transduction was determined by counting the number of blue circles > 2 μm in diameter in a field at high power, and dividing by the number of hepatocytes in the same field. The value of

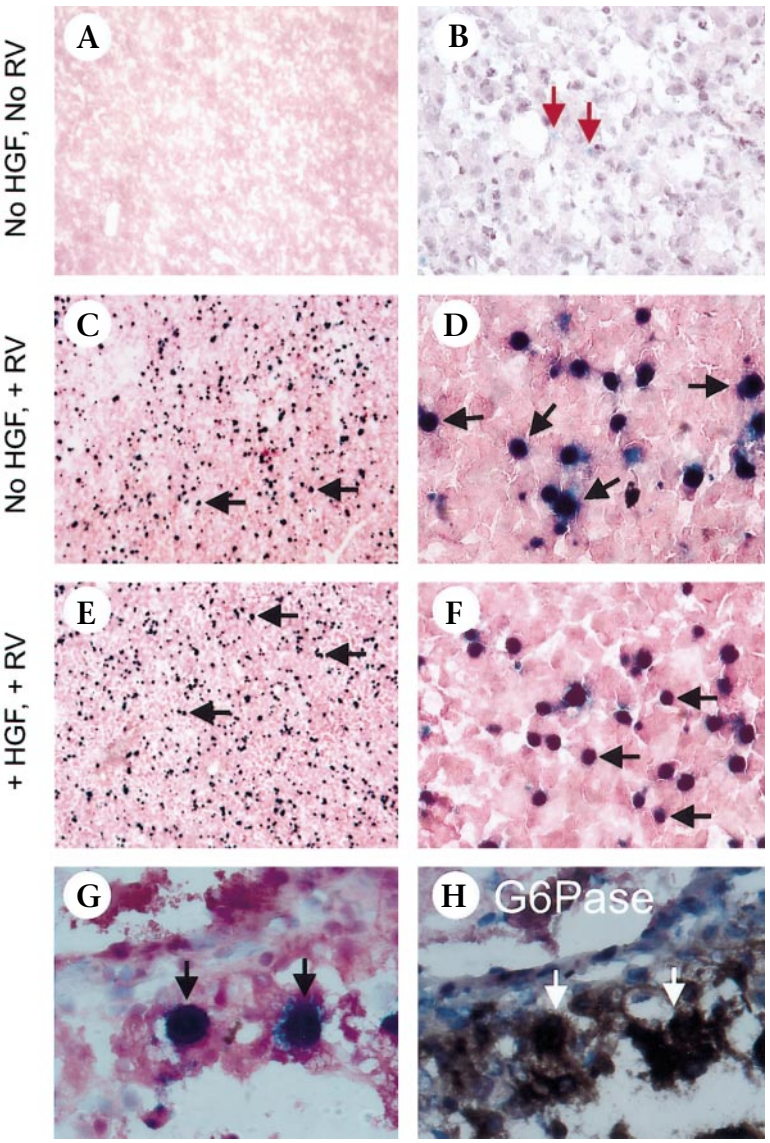

FIG. 3. β-Gal activity in livers of neonatal dogs. (A) and (B) β-Gal activity in a non-transduced control liver. Original magnification, ×10 (A) and ×60 (B). A control dog that did not receive RV was sacrificed on day 9 after birth. A liver section was stained with X-gal, counterstained with eosin, and very lightly counterstained with hematoxylin. Occasional small blue foci are identified by red arrows and are due to background staining. (C) and (D) β-Gal activity in a RV-transduced liver after PBS treatment. Original magnification, ×10 (C) and ×60 (D). This dog (M1275) was treated with PBS and RV, as detailed in Fig. 2A, and a section of the left lobe of the liver was stained with X-gal and counterstained as noted in (A) and (B). Arrows indicate the large blue nuclei of transduced hepatocytes. Dark blue nuclei were present at similar levels for sections that did not received a hematoxylin counterstain (not shown). (E) and (F) β-Gal activity in a RV-transduced liver after HGF treatment. Original magnification, ×10 (E) and ×60 (F). This dog (M1271) was treated with HGF and RV, and a section of the left lobe of the liver was stained with X-gal. Arrows indicate the nuclei of transduced hepatocytes. (G) and (H) X-gal and G6Pase staining of adjacent sections from a RV-transduced liver, respectively. A section from the liver of a dog that was treated with PBS and RV (M1272) was stained with X-gal to identify transduced cells and counterstained as noted above. The immediately adjacent section was stained for G6Pase, which is a hepatocyte-specific enzyme, and counterstained with hematoxylin and eosin. Arrows identify the same nuclei in both panels, which represent hepatocytes that were transduced. Original magnification, ×100.

0.2% was subtracted, which was the average value obtained in controls that did not receive any RV after analysis in a similar fashion.

The average transduction efficiency for animals that received RV is shown in Fig. 2C. For animals that did not receive HGF before transduction with RV, 8.9 ± 3.6% of hepatocytes were transduced when values from all three lobes were averaged. For these animals, the transduction efficiency was quite variable, ranging from 1% to 18.3% (Table 1). For animals that received 2.5 mg/kg of HGF before transduction with RV, a similar percentage of hepatocytes was transduced (9.6 ± 3.4%; $P = 0.92$ versus the no HGF group), and all animals had transduction of at least 5.5% of hepatocytes. For both groups of animals, transduction was more efficient in the left lobe than in the right or the caudate lobes. We concluded that transduction was quite efficient either with or without the administration of HGF when a high dose of RV was administered at 3 days after birth.

Evaluation of Transgene Expression in Other Organs

The biodistribution of any vector that is administered systemically is extremely important. Therefore, all organs were analyzed for transduction with the β-gal-expressing vector by homogenization and quantitation of β-gal activity. An *o*-nitrophenyl β-D-galactopyranoside (ONPG) assay confirmed that the average β-gal enzyme activity for all lobes was high in liver at 51.5 ± 22.9 mU/mg and 39.1 ± 13.5 mU/mg for PBS- and HGF-treated dogs, respectively (not significant; $P = 0.55$; Fig. 4A). Substantial transduction of spleen also occurred, as spleen samples contained 19.7 ± 6.9 and 18.7 ± 6.3 mU/mg for PBS- and HGF-treated dogs, respectively. Values in the PBS- and HGF-treated dogs were not statistically different ($P = 0.92$). Transduction was probably inefficient in kidney, lung, heart, pancreas, brain, muscle, thymus, and gonads, as β-gal activity was undetectable in these organs (<.3 mU/mg). Enzyme activity could not be reliably assessed in the large and small intestines, as some nontransduced controls had high levels of activity, which was presumably due to contamination with bacteria expressing β-gal.

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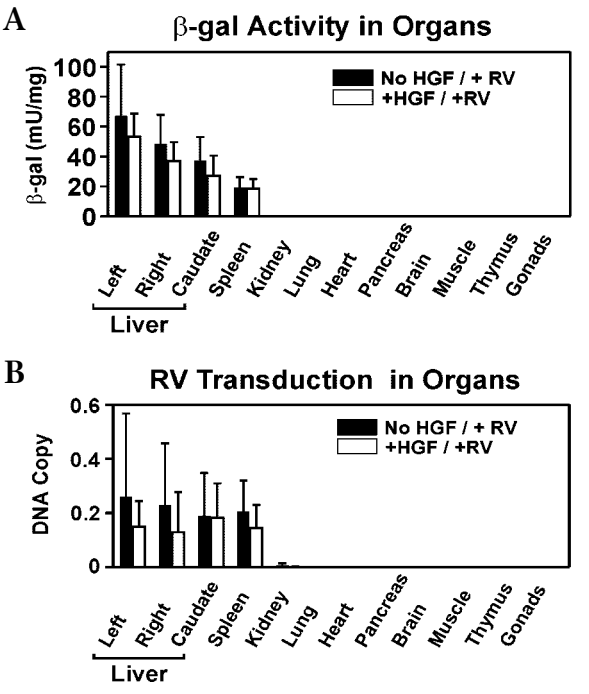

FIG. 4. Evaluation of other organs for transduction. (A) β-Gal activity in organs after neonatal injection of RV. Neonatal dogs were transduced with the β-gal-expressing TA7 RV after injection of either PBS or HGF, as outlined in Fig. 2A, and sacrificed at 8 days after birth. Mean β-gal activity of homogenates ± SEM was determined by ONPG assay, and normalized to the protein concentration in the sample. The average background activity in nontransduced dogs was determined for each organ and subtracted from the values in transduced animals. There were no statistically significant differences in the values obtained for the two groups. (B) RV DNA copy number in organs after neonatal injection of RV. DNA was isolated from organs of the same dogs that were analyzed in (A), and tested for the RV DNA copy number by real-time PCR as described in the methods section. The mean number of copies of the RV per diploid genome ± SEM is shown for each organ.

Analysis of Organs for RV DNA Sequences

DNA from livers and spleens of transduced animals was analyzed by real-time PCR to determine the RV DNA copy number. In addition, real-time PCR was performed on DNA obtained from other organs to determine if the failure to detect β-gal activity was due to inefficient transduction rather than the inability to express the RV. The sensitivity of the assay was 0.001 copies of RV per diploid genome. All organs from nontransduced animals had no detectable signal for β-gal DNA when PCR was performed for 40 cycles. The liver had 0.226 ± 0.114 and 0.153 ± 0.047 copies of RV/diploid genome for HGF-untreated and HGF-treated animals, respectively (Fig. 4B). The difference between the two groups was not statistically significant ($P = 0.57$). The spleen had 0.204 ± 0.058 and 0.144 ± 0.043 copies of RV/diploid genome for the HGF-untreated and the HGF-treated animals, respectively (no significant difference; $P = 0.40$). The DNA copy number in the spleen was 92% of that present in the liver. The RV DNA copy number was < 0.001 copies/diploid genome for lung, brain, thymus, and the gonads for both groups. For animals that did not receive HGF, the DNA copy number per diploid genome was 0.006 for kidney, 0.002 for heart, 0.005 for pancreas, and 0.002 for muscle. For animals that received HGF before RV, the DNA copy number per diploid genome was 0.002 for kidney, 0.003 for heart, 0.003 for pancreas, and 0.002 for muscle. There were no significant differences in the copy number between the two groups for any organ. The DNA copy number could not be reliably assessed in the intestines, as nontransduced controls had a variable, but frequently high, signal, which likely derived from contamination with bacteria containing the LacZ gene. We conclude that the RV DNA copy number is relatively high in liver and spleen, but that transduction of other organs is less than 2.8% of that observed in the liver.

Staining for β-Gal Activity in the Spleen

As the spleen had β-gal activity in homogenates and RV vector DNA sequences by real-time PCR, X-gal staining was carried out to evaluate the number and distribution of transduced cells. Although there were no blue cells in the spleen for animals that did not receive RV (data not shown), transduced cells were scattered throughout the spleen for animals that were injected with RV after PBS (Figs. 5A and 5B) or with RV after HGF (Figs. 5C and 5D). The location of these cells was not clear, as the delineation into the white and red pulp was not appreciated after hematoxylin and eosin staining (data not shown) in these neonatal spleens. This presumably reflects the fact that the immune system in these 8-day-old dogs is immature. Thus, the identity and location of the transduced cells in the spleen is unclear.

Long-Term Evaluation of RV Transduction after Neonatal Transfer into Dogs

The long-term evaluation of expression using the β-gal reporter gene is complicated by the fact that dogs can mount a potent cytotoxic T-lymphocyte response to β-gal [24]. Therefore we chose to follow the expression of the normal canine GUSB cDNA in MPS VII dogs to assess the longevity of expression. These dogs have a point mutation in the cGUSB gene that results in an arginine to histidine substitution at amino acid 166 and a decrease in the functional activity of GUSB [25]. We hypothesized that these dogs would be unlikely to mount an immunological response to the transgene product, as most of the epitopes of the normal protein are expressed in the mutant dogs and should result in tolerance to the protein.

A plasmid designated hAAT-cGUSB-WPRE was generated (Fig. 6A). The inclusion of the woodchuck virus post-transcriptional regulatory element (WPRE) increased the expression of GUSB in transiently transfected GUSB-deficient mouse fibroblasts by eightfold over that observed from a similar plasmid construct that did not contain the WPRE (data not shown). Therefore, hAAT-cGUSB-WPRE was chosen to generate an amphotropic RV. Large-scale

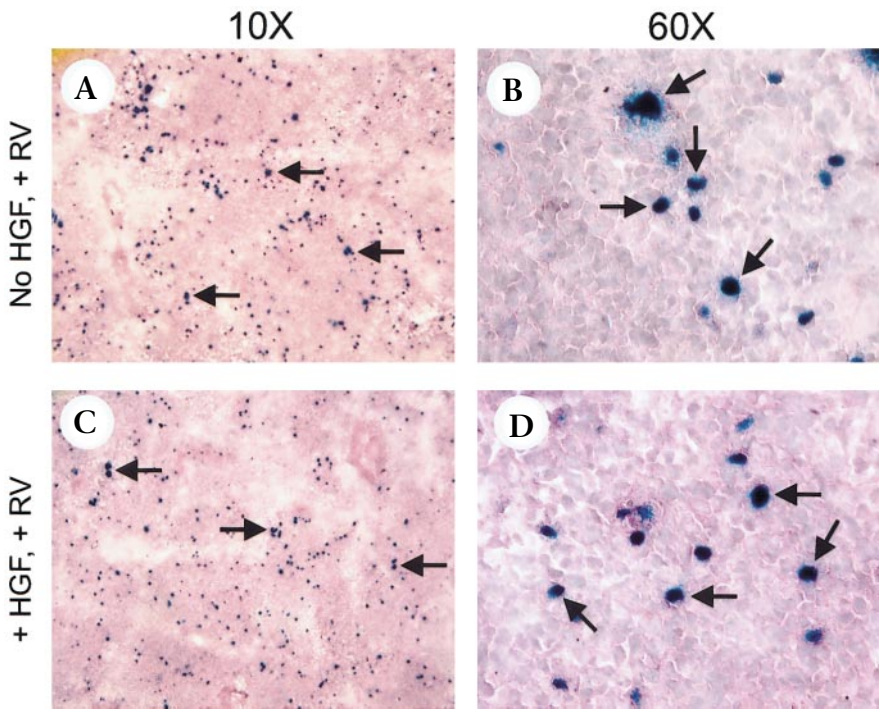

FIG. 5. β-Gal activity in spleen. (A) and (B) β-Gal activity after PBS and RV. Original magnification, ×10 (A) and ×60 (B). A neonatal dog (M1275) was transduced with the RV TA7 after the administration of PBS, as outlined in Fig. 2A. A section of spleen was stained with X-gal and counterstained with eosin, and lightly counterstained with hematoxylin, as noted in Fig. 3. Arrows identify the dark blue nuclei of transduced cells. (C) and (D) β-Gal activity after HGF and RV. Original magnification, ×10 (C) and ×60 (D). A neonatal dog (M1271) was treated with the RV TA7 after the administration of HGF, as outlined in Fig. 2A, and stained as noted in (A) and (B).

production of the RV was done at 32°C in the presence of the histone deacetylase inhibitor sodium butyrate. This increased the titer of unconcentrated RV by four-, eight-, and fourfold at 24, 48, or 72 hours, respectively, for cells grown in 5 mM sodium butyrate relative to that observed in cells that were cultured in the same medium without sodium butyrate (data not shown).

Five neonatal MPS VII dogs were injected with 3 to 3.7 $\times$ 10^9 rfu (red forming units) /kg of the hAAT-cGUSB-WPRE RV at 2 or 3 days after birth without the administration of HGF. The average weight of the dogs at the time of transduction was 362 ± 14 g (SEM). To follow expression over time, serum was tested for GUSB activity. Although most of the GUSB produced by a cell is targeted to the lysosome, some is secreted, which results in the appearance of enzyme in the blood after transduction of hepatocytes [3]. RV-treated MPS VII dogs had an average of 568 ± 109 U/ml (SEM) of GUSB in serum at 1 week after transduction. As approximately 6000 total units of GUSB were present in the injectate, the serum GUSB derived from the injection should have approximated 200 U/ml shortly after injection and would likely have been much lower at later times due to the disappearance of the enzyme from the blood. Serum GUSB levels were 195.4 ± 46.2 U/ml at 6 months after transduction, which represents 34.4% of the peak value. The values at 6 months were 72.6% of that observed for adult homozygous normal dogs. Adult affected MPS VII dogs have very low serum GUSB activity (0.38 U/ml). We conclude that neonatal IV injection of RV can result in long-term and high-level expression of GUSB in the serum of MPS VII-affected dogs.

To determine whether the source of GUSB production was at least in part from the liver, all hAAT-cGUSB-WPRE-transduced dogs had a liver biopsy at 4 months, at which time the body weight had increased by 25-fold from the time of transduction. In addition, one dog was sacrificed at 6 months after transduction, when his body weight had increased by 39-fold. The livers and the spleen were analyzed for GUSB activity by histochemistry, and compared with positive and negative controls. After a 1-hour period of GUSB staining, enzyme activity (red) was highest in small cells that line the sinusoids of a liver from a normal dog (Fig. 7A), which are likely Kupffer cells. A liver from an untreated MPS VII dog had no detectable red in any cells after 24 hours of GUSB staining, demonstrating that it had very low levels of enzyme activity (Fig. 7B). GUSB staining of a liver that was obtained from an MPS VII dog at 4 months after RV transduction demonstrated that there were many clusters of large cells with the histochemical appearance of hepatocytes that were bright red after staining for 1 hour (Fig. 7C). Similar clusters of red cells were present in the liver of the animal that was sacrificed at 6 months after transduction (Fig. 7D). G6Pase staining of an immediately adjacent section confirmed that these red cells were hepatocytes (Fig. 7E). Because hepatocyte GUSB staining was never seen at 1 hour in liver from normal animals with similar serum levels of GUSB, we conclude that these red cells must represent transduced hepatocytes, rather than hepatocytes that took up enzyme from the blood via the mannose-6-phosphate (M6P) receptor. Longer GUSB staining of liver from RV-transduced MPS VII dogs demonstrated that most of the cells were red (data

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not shown), which is likely due to uptake of enzyme by nontransduced cells via the M6P receptor. Clusters of hepatocytes with high levels of GUSB activity were present in liver from all of the five RV-transduced MPS VII dogs at 4 months after transduction. At this time point, 2.8 ± 0.4% of hepatocytes appeared to be transduced with hAAT-cGUSB-WPRE, as determined by counting the number of bright red hepatocytes in 20 randomly chosen fields at ×40 power and dividing by the total number of hepatocytes. We conclude that transduced hepatocytes express very high levels of GUSB, and that these cells contribute to the long-term expression of GUSB in blood.

GUSB staining was also carried out on spleen from treated and control dogs. Although the internal hAAT promoter is liver-specific, the LTR of the RV could drive expression of cGUSB in non-hepatic cells. A spleen from an untreated MPS VII dog had no red cells after 24 hours of GUSB staining (data not shown), demonstrating that it had little enzyme activity. Spleen from both a normal (Figs. 7F and 7G) and the six month-old RV-treated MPS VII dog (Figs. 7H and 7I) had several regions in the red pulp that were light red after GUSB staining for 1 hour, although cells that were as intensely red as some hepatocytes of RV-transduced MPS VII dogs were rare. Because these samples had a similar appearance, it was impossible to determine if the staining in the RV-treated dog derived from *de novo* expression or from cells that took up enzyme from the blood via the M6P receptor. Further studies will be necessary to determine if RV RNA and DNA are present in the spleen at late times. A 10-hour GUSB stain resulted in red staining throughout most of the red pulp of both spleens (data not shown), which likely derives from uptake of enzyme from the blood for the RV-transduced animals.

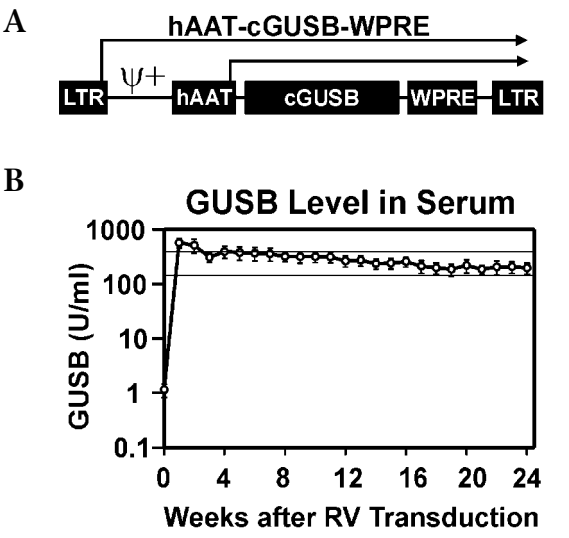

FIG. 6. Treatment of MPS VII dogs with hAAT-cGUSB-WPRE. (A) Diagram of hAAT-cGUSB-WPRE. The RV hAAT-cGUSB-WPRE expresses the canine β-glucuronidase (cGUSB) cDNA from the human α_1-antitrypsin (hAAT) promoter. It also contains long terminal repeats (LTRs) at the 5′ and 3′ ends, an extended packaging signal (ψ^+), and the woodchuck post-transcriptional regulatory element (WPRE). Arrows indicate that transcription can initiate from either the LTR or the internal hAAT promoter. (B) GUSB serum activity in hAAT-cGUSB-WPRE-treated MPS VII dogs. Four doses of 5 ml each of hAAT-cGUSB-WPRE were injected at 2 or 3 days after birth over a 12-hour period for a cumulative dose of 3.0 to 3.7 × 10^9 rfu/kg. Serum was tested for GUSB activity at the indicated number of weeks after birth, and the average ± SEM for five dogs is shown. The range of enzyme levels in homozygous normal dogs (269 U/ml ± 2 standard deviations) was 145 to 394 U/ml, as indicated by the lines on this semi-log graph. The initial value is the average of the pre-gene therapy GUSB levels for these MPS VII dogs.

DISCUSSION

In Vivo MLV-Based RV Transduction of Normal Neonatal Hepatocytes

Liver-targeted gene therapy could be used to treat many genetic disorders such as lysosomal storage diseases, hemophilia, and metabolic diseases. Neonatal gene therapy has many potential advantages, including the ability to treat an otherwise-lethal genetic disease shortly after birth, and the possibility of a reduced immunological response due to the fact that the neonatal immune system is relatively immature. In this study, staining of livers for β-gal activity 5 days after neonatal IV injection of a high-titer RV containing the β-gal gene demonstrated that 8.9 ± 3.6% of canine hepatocytes were transduced. This high level of transduction was possible because the level of hepatocyte replication is high, which is likely due to the rapid rate of growth of the entire animal, including the liver, at this stage of development. For example, the BrdU labeling index at 2 days after birth was 11.0%, while at the same time the body weight of the puppies increased 10.7 ± 1.3%. This level of transduction would be sufficient to correct many, but not all, genetic deficiencies involving proteins that are synthesized by the liver.

For the β-gal-transduced animals, the calculated RV DNA copy number in the liver (0.226 ± 0.114 copies/diploid genome), as assessed by real-time PCR, was 2.5-fold the value expected if each hepatocyte that was determined to be transduced by X-gal staining contained a single copy of the RV. This apparent discrepancy might be due to multiple transductions per cell, to transduction of non-parenchymal cells that were too small to be included in the quantitation of X-gal staining due to the size cut-off used in the analysis, or to the failure of some transduced cells to express a sufficient amount of protein to appear blue after X-gal staining. These data suggest that an individual hepatocyte probably contained only one or a few copies of the RV, although definitive proof of this hypothesis would require the isolation of individual clones, which is difficult due to the inability to propagate hepatocytes in culture. Small numbers of transductions per cell should reduce the possibility of insertional mutagenesis resulting in a cancer, as the development of cancer requires multiple mutations per cell. In this study, there

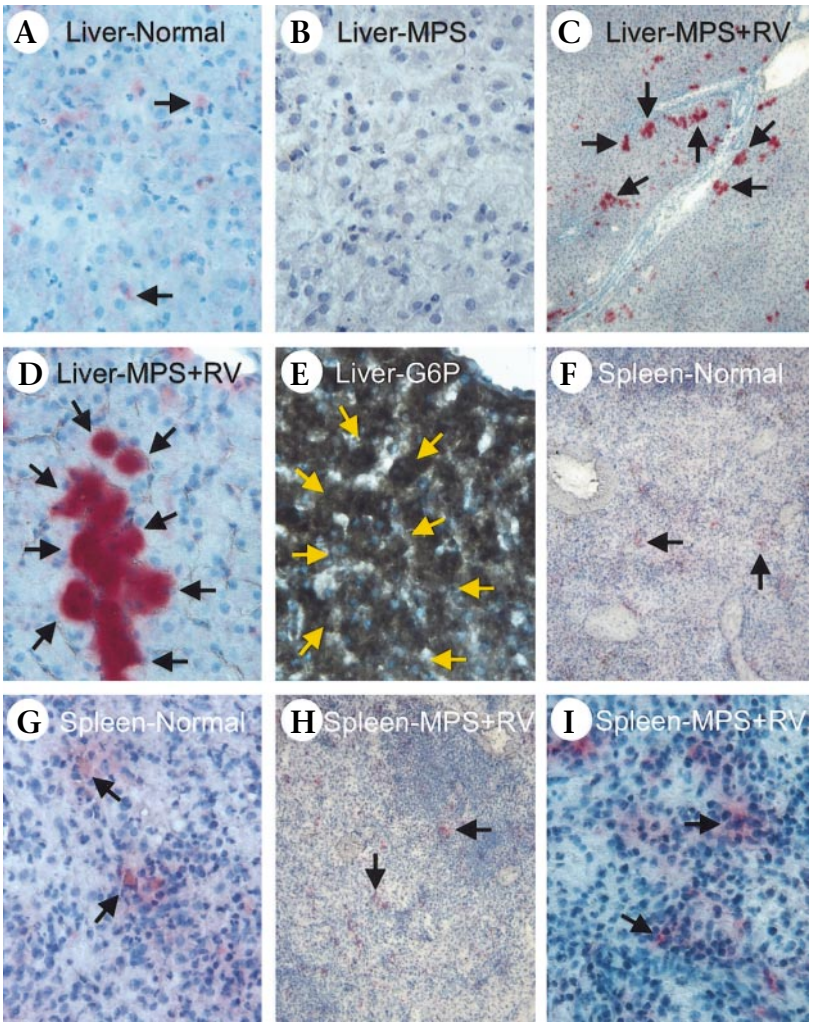

FIG. 7. Histochemical staining for GUSB activity in livers and spleens from control or hAAT-cGUSB-WPRE-transduced MPS VII dogs. Unless otherwise stated, sections were stained for GUSB enzymatic activity (red color) and counterstained with hematoxylin (blue). (A) GUSB activity in a normal liver. After 1 hour of GUSB staining, the liver of a 6-month-old homozygous normal dog had occasional small bright-red cells lining the sinusoids (black arrows), which are likely Kupffer cells. Hepatocytes had lower levels of activity, as they were uncolored. Original magnification, ×60. (B) GUSB activity in an MPS VII liver. After 24 hours of GUSB staining, there was no red staining in any cell in a liver section from a 6-month-old untreated MPS VII dog, demonstrating that little or no enzyme activity was present. Original magnification, ×60. (C) GUSB activity in a RV-transduced MPS VII liver. An MPS VII dog received hAAT-cGUSB-WPRE as a neonate as detailed in Fig. 6B, and the liver was biopsied 4 months later. After 1 hour of GUSB staining, the liver had several clusters of hepatocytes that were bright red (arrows). Original magnification, ×10. (D) and (E) GUSB and G6Pase activity in a RV-transduced MPS VII dog liver. A different MPS VII dog from that shown in (C) received hAAT-cGUSB-WPRE as a neonate and was sacrificed 6 months later. After 1 hour of GUSB staining of the liver (D), some clusters of large cells with the histochemical appearance of hepatocytes stained bright red. The section that was immediately adjacent to that shown in (D) was stained for G6Pase activity (E), which indicated that this cluster consisted of hepatocytes. The same blood vessel is present in the right upper region as a landmark in (D) and (E), and the black or yellow arrows indicate the outer edges of the same cluster of cells. Original magnification, ×60. (F) and (G) GUSB activity in a normal spleen. The spleen from a homozygous normal dog was stained for 1 hour for GUSB. Arrows indicate regions with enzyme activity. Original magnification, ×10 (F) and ×60 (G). (H) and (I) GUSB activity in a spleen from a RV-transduced MPS VII dog. An MPS VII dog received hAAT-cGUSB-WPRE as a neonate and was sacrificed 6 months later. The spleen was stained for 1 hour for GUSB activity. Original magnification, ×10 (H) and ×60 (I).

were no overtly abnormal regions of the liver from the one animal that was sacrificed at 6 months after transduction. In addition, none of the clusters of red hepatocytes were extremely large from livers that were obtained at 4 or 6 months after transduction, suggesting that none of the transduced cells had acquired markedly abnormal growth properties. However, longer follow-up in more animals will be necessary to assess the risk of this neonatal gene therapy approach for inducing cancer.

Cells in the Spleen Are Also Transduced with This Approach

In this study, cells in the spleen were also transduced after IV injection of RV into neonates. This is likely due to the fact that some splenocytes have direct contact with the blood and, thus, would bind RV, as well as the fact that and the spleen has a large number of replicating cells. Indeed, in this study, BrdU staining demonstrated that a considerable proportion of the cells in the spleen were replicating at the time of RV injection (data not shown). Transduction of cells in the spleen was observed previously in adult mice after IV injection of an MLV-based RV [3,26]. Transduction of splenocytes could be important for correcting lysosomal storage diseases, in which the spleen is a major site of pathology. However, transduction of splenocytes could have adverse consequences for gene therapy for inherited genetic deficiencies, as cytotoxic T-lymphocyte responses are reported to require transduction of antigen-presenting cells [27]. Anti-cGUSB immune responses probably did not occur in the RV-transduced MPS VII dogs in this study, as serum GUSB levels were stable and GUSB-expressing cells were still present in the liver at 4 to 6 months. However, this could simply reflect tolerance to the normal cGUSB, as the affected dogs have a missense, rather than a null, mutation and may not necessarily indicate that this neonatal approach is non-immunogenic. Additional studies are in progress to define the cell types that are transduced, and to determine if RV-transduced cells remain in the spleen at late points.

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Transduction Is Relatively Specific for the Liver and Spleen

The biodistribution of any vector that is injected IV is very important. Transduction of cell types that do not contribute to the correction of the disease could increase the risk of insertional mutagenesis, whereas transduction of germ cells could result in germline transmission. Analysis of β-gal activity in homogenates demonstrated that enzyme activity was high in liver and spleen, but was undetectable in other organs. Real-time PCR demonstrated that transduction of kidney, heart, pancreas, and muscle was detectable, but was inefficient at < 0.006 copies of RV DNA per diploid genome, which was < 2.8% that obtained for the liver. The RV DNA copy number was undetectable (< 0.001 copies/diploid genome) in lung, brain, thymus, and gonads.

The lack of or inefficient transduction of other organs may be due to the presence of an endothelial barrier which prevents the RV from contacting the parenchymal cells, some of which may be dividing in some organs in the neonatal period. In contrast, both liver and spleen have fenestrations in the endothelial barrier that would allow the RV to extravasate and directly contact the parenchymal cells. Inefficient transduction of other organs might also be due to the fact that MLV-based vectors only transduce dividing cells, and the cells that would have direct contact with the RV, such as endothelial cells, do not have a high labeling index. Indeed, there were no or only a few blue cells after X-gal staining on the luminal side of blood vessels in lung, kidney, heart, and muscle in the TA7 (β-gal vector)-transduced dogs (data not shown), suggesting that endothelial cells were not efficiently transduced. Thus, the requirement for replication for transduction with MLV-based vectors may contribute to the relative specificity for the liver and spleen in this study. In contrast, lentiviral vectors, which can clearly transduce some nondividing cells [28,29], may result in more promiscuous transduction in neonates. In addition, it is not necessarily the case that lentiviral vectors would be more efficient at transferring genes into the neonatal liver, as transduction of non-replicating hepatocytes was low in some [30,31], although not all [32], studies. Thus, the requirement of replication for transduction with an MLV-based vector may represent an advantage of this vector within the context of neonatal hepatic gene therapy.

Effect of HGF on Transduction of Neonatal Hepatocytes

When this study was initiated, it was unclear if neonatal hepatocyte replication would be sufficient to allow transduction of hepatocytes with an MLV-based RV. Therefore, we tested if injection of HGF would stimulate hepatocyte replication and transduction, as HGF effectively induces replication of hepatocytes in adult rodents. The initial trial involved three dogs from the same litter and used 10 mg/kg of HGF given over a 24-hour period. However, all dogs experienced gastrointestinal bleeding after 4 or 5 doses of HGF, and two animals died. Pathological evaluation did not show any obvious cause of bleeding. BrdU labeling in the animal that survived demonstrated that 40% of hepatocytes were replicating when BrdU was injected at 24 and 36 hours after the first dose of HGF.

Although it was possible that the dogs had an alternative cause for the hemorrhage, subsequent dogs were treated with a dose that was 25% of the original dose tested. HGF at 2.5 mg/kg had no obvious toxicity, and resulted in replication of 26.9 ± 5.5% and transduction of 9.6 ± 3.4% (n = 4) of hepatocytes. Neither of these values was statistically higher than in the littermate controls that received PBS before the injection of the same dose of BrdU and RV. We conclude that prior administration of HGF did not have a marked effect on replication or transduction of canine hepatocytes at 3 days after birth. Although it remains possible that a statistically significant effect might be observed if more animals were evaluated, or if replication was analyzed during different time intervals relative to the first dose of HGF, transduction was reasonably efficient without the administration of HGF. Because HGF might have some toxicity at 2.5 mg/kg that was not evident in this study and has not yet received approval for clinical use, the simple injection of RV without pretreatment with HGF would likely be preferable for gene therapy to treat genetic diseases. If a higher transduction efficiency is necessary for a particular disease, multiple injections over a 2- or 3-day period could further increase the percentage of transduced cells, as the number of labeled cells increased when BrdU labeling was performed over a longer time period. HGF or some other method of inducing hepatocyte replication may still need to be used to achieve transduction in older animals, whose baseline levels of replication are much lower. Because many patients with genetic diseases are not diagnosed in the neonatal period and would thus not be treatable with this neonatal approach, future studies will test the transduction efficiency in older dogs either with or without HGF.

Expression from a RV Is Persistent after Neonatal Gene Transfer

The neonatal gene transfer approach described here resulted in long-term expression, as five MPS VII dogs treated as neonates with IV injection of hAAT-cGUSB-WPRE maintained serum GUSB activity that is 73% that of homozygous normal dogs at 6 months. Persistence of high serum GUSB activity occurred despite the fact that the dogs underwent a 39-fold increase in body weight from 0.36 ± 0.01 kg at the time of injection to 14.0 ± 1.4 kg six months later. The major source of long-term expression is likely the liver, as evaluation of livers at 4 to 6 months after injection demonstrated that there were many clusters of hepatocytes with very high levels of GUSB activity. These probably represent transduced hepatocytes rather than hepatocytes that had taken up enzyme via the M6P recep-

tor, as normal dogs do not have such high levels of enzyme activity in hepatocytes, although they have serum GUSB activity that is similar to the RV-treated MPS VII dogs. The clusters of transduced cells are likely derived from transduced hepatocytes that replicated as part of normal liver growth. These data are consistent with a model in which the differentiated hepatocyte gives rise to new hepatocytes after birth, which seems to be the mechanism for generating new hepatocytes in rodents [33,34]. It is unclear if the spleen still contained transduced cells at 6 months, as the GUSB staining that was observed in RV-treated MPS VII dogs was diffuse, suggesting that it might be due to uptake of enzyme in nontransduced cells via the M6P receptor. Further studies will be done to determine the RV DNA and RNA levels in the spleen of the RV-transduced MPS VII dogs.

Although serum GUSB activity remained high at 6 months after gene transfer, the level was only 34.4% of that present at 1 week. This may reflect clearance of enzyme that was present in the injectate with the RV, the death of transduced splenocytes or non-parenchymal cells of the liver that were replaced with non-transduced cells, attenuation or shut-down of expression, or a decrease in the percentage of hepatocytes that were transduced. The remaining four RV-transduced MPS VII dogs will be followed for a longer period of time to determine expression stability.

Evaluation of the clinical effects of this gene therapy approach for MPS VII will require evaluation of enzyme levels in all organs, pathological analysis of all organs, and numerous specialized analyses of organs that are routinely affected in the disease such as the skeleton, heart, eye, and brain. These studies are in progress and will be reported once the animals reach an age at which the clinical manifestations are invariably present in the affected dogs.

Implications for Gene Therapy

This study demonstrates that an MLV-based RV can deliver genes to neonatal hepatocytes, thus resulting in high-level expression that persists for at least 6 months. There are two major advantages to treatment of neonates. First, neonatal gene therapy will be essential for otherwise-lethal genetic defects such as severe urea cycle disorders, and will reduce the time that a patient is symptomatic for nonlethal disorders such as hemophilia. Second, neonatal gene therapy may allow the patient to become tolerant to the transgene, as immune responses are generally less developed in neonates than in adults [15–19]. As shown here, RV can result in long-term expression due to the ability to integrate into the chromosome and be maintained in all daughter cells. In contrast, AAV or adenoviral vectors, which do not routinely integrate, might be lost rapidly over time as the hepatocyte replicates during normal animal growth. Although these results are encouraging, more information will be needed before this approach could be considered for the treatment of human patients with genetic diseases. As discussed above, insertional mutagenesis by MLV-based vectors could lead to the development of cancer in the liver, spleen, or, less likely, other organs. Although to our knowledge there are no reports of tumor formation in animals or humans that have been treated with IV injection of a replication-incompetent RV, monkeys that received bone marrow-derived cells that were transduced with RCR developed lymphoma [35]. It will be necessary to follow the animals for several years to determine if the risk of insertional mutagenesis is acceptably low.

Materials and Methods

Reagents. Reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated. Human HGF was purified from the conditioned medium of 293-N3S cells (Microbix Biosystems, Toronto, Ontario) that were infected with the adenoviral vector Ad.CMV.HGF [5] and characterized as described [7].

Retroviral vectors. The amphotropic RV designated TA7 that expressed the *E. coli* β-galactosidase (β-gal) with a nuclear localization signal [36] was generously provided by Francois Cosset (Ecole Normale Superieure, Lyon, France). This amphotropic packaging line was derived from human HT1080 cells and generates a human serum-resistant vector. For large-scale production, 80 15-cm diameter plates at > 90% cell confluence were incubated at 32°C for 24 hours with 20 ml per plate of fresh Dulbecco's modified Eagle's medium (D-MEM) with high glucose (Gibco BRL, Grand Island, NY) and 2% supplemented calf serum (Hyclone Laboratories, Logan UT). Conditioned medium was collected daily from the same cells for 3 days. Approximately 1 liter was concentrated 100-fold with an M14S-260-01P ultrafiltration device with molecular weight cutoff of 400 kD (Spectrum, Laguna Hill, CA) for approximately 3 hours, then the void volume was collected from a Sepharose 4B column as described to remove low molecular weight proteins [37]. The vector was frozen at –70°C in a solution containing 25 mM Tris (pH 7.4), 60 mM NaCl, 50 mg/ml lactose, 5 mg/ml bovine serum albumin, and 1 mg/ml arginine [38]. Titer was determined after freezing and thawing once by infection of NIH 3T3 cells followed by staining for β-gal activity [5] 2 days later. The final titer ranged from 0.5 to 3.6×10^9 bfu/ml. For injection into dogs, Polybrene was added to a final concentration of 8 μg/ml just before injection. The RV was tested for the presence of replication-competent retrovirus (RCR) by a vector rescue assay [39] on the unconcentrated conditioned medium. This involved infection of MDZ cells that were transduced with a β-gal expressing RV, and testing the supernatant obtained after five passages of these infected cells for its ability to infect naive murine MD7 cells. The sensitivity of the assay was 0.1 RCR/ml.

The RV hAAT-cGUSB-WPRE-781 (referred to here as hAAT-cGUSB-WPRE) was generated as follows. A 591-nt fragment containing nt 1093 to 1684 (GenBank acc. no. J04514 [40]) of the woodchuck post-transcriptional regulatory element (WPRE) in the *Cla*I site of Bluescript II (Bluescript II SK+ WPRE-B11; generously provided by Tom Hope, Salk Institute, San Diego, CA) was released after restriction with *Not*I and *Xho*I. hAAT-hFX-514 [1], an LNL6-based RV that contained the MLV long terminal repeats (LTRs) and nt –347 to +56 of the human α_1-antitrypsin (hAAT) promoter, was digested with *Not*I and *Xho*I to remove some other elements, which were replaced with the WPRE to generate hAAT-WPRE-767. The 2199-bp canine β-glucuronidase (cGUSB) cDNA (GenBank acc. no. GI2425090 [25]) was ligated as an *Eco*RI fragment into pcDNA3.1+ to generate pcDNA3.1-cGUSB-772. After *Not*I restriction, a cGUSB cDNA containing 15 nt of 5′ untranslated sequence, 1956 nt of coding sequence, and 184 nt of 3′ untranslated sequence was ligated into the *Not*I site of hAAT-WPRE-767 to generate hAAT-cGUSB-WPRE-781. An amphotropic packaging cell line with the hAAT-cGUSB-WPRE was made as described [3]. Briefly, this involved transfection of the ecotropic GP+E86 packaging cells [41] followed by infection of the amphotropic packaging cells GP+AM12 [42] at a multiplicity of

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infection of 1 to 8. As no selectable marker was present, infected GP+AM12 cells were plated at a low density to obtain individual colonies. Approximately 400 colonies were screened for the ability of their supernatant to confer GUSB activity upon GUSB-deficient murine fibroblast 3521 cells [43]. Large-scale production was as for the TA7 vector, except that 5 mM sodium butyrate was added to the medium. The injectate contained 154 to 387 U/ml of GUSB activity.

Animal procedures. Affected and control dogs were derived from the breeding of heterozygous mucopolysaccharidosis VII (MPS VII) mixed breed (German shepherd dog and beagle) dogs in the animal colony of the School of Veterinary Medicine, University of Pennsylvania. NIH and USDA guidelines for the care and use of animals in research were followed. The animals were housed at 21°C with *ad libitum* food and water, 12-hour light cycles, and 12–15 air changes per hour. Puppies were tested shortly after birth for their GUSB genotype as described [44]. The time of birth was 14 hours or less after they were found to have been born, as pregnant mothers were checked at least twice a day. Dogs that were evaluated for their labeling index received 50 mg/kg per injection of 5-bromo-2′deoxyuridine (BrdU; 1 to 2 ml of a 10 mg/ml solution) injected intraperitoneally (IP) with a 22-gauge needle at the times indicated in the figure legends. Dogs that received HGF (or PBS) and RV had a 26-gauge external jugular (EJ) catheter placed just before the first injection. For the injection of HGF, puppies received 8 doses of 0.31 mg/kg per dose in PBS (0.25 to 0.5 ml of a 0.35 mg/ml solution) intravenously (IV) every 3 hours via the EJ catheter for a cumulative dose of 2.5 mg/kg. Controls received a similar volume of PBS IV at the same times. RV was injected IV via the EJ catheter as 5 ml per dose given over 5 minutes. BrdU was given by IP injection at the same times that the RV was injected. While the catheter was in place, the puppies were tube fed with Nurturall puppy balanced milk replacer (Veterinary Products Laboratory, Phoenix, AZ) at 1 cc/100 g of body weight every 4 hours and stimulated to urinate and defecate. The catheter was removed after the final IV injection and the puppies were returned to their mother. For liver biopsies, animals were premedicated with an intramuscular injection of 0.05 mg/kg of atropine and 0.1 mg/kg of oxymorphone, and an IV injection of 6 mg/kg of propofol. An endotracheal tube was placed and anesthesia was induced with 3.5% and maintained with 2.0% isoflurane/O_2. The animal was placed in dorsal recumbency. Using sterile technique, a ventral midline abdominal incision was made through the skin a few centimeters caudal to the xiphoid and was extended through the musculature and into the abdominal cavity. The left liver lobe was isolated and interlocking sutures of absorbable polydioxanone monofilament were loosely placed across the area of the wedge biopsy, tightened, and tied. The biopsy specimen was removed by incision a few mm from the suture line. The parenchyma was observed for hemorrhage and blood vessels were ligated with absorbable suture. The body wall was closed with 2-0 nylon in an interrupted pattern. The subcutaneous tissue was closed with 2-0 vicryl in a continuous pattern. The skin was closed with 2-0 nylon in a simple interrupted pattern.

Euthanasia was performed with 80 mg/kg of sodium pentobarbital in accordance with the American Veterinary Medical Association guidelines. Organs were harvested and processed as described below.

BrdU immunostaining and quantification of the labeling index. Organs were immersed in optimal cutting temperature (OCT) compound (Bayer Crop, Mishawka IN) and frozen. BrdU immunostaining was performed on 8 μm frozen sections of organs as described [5]. Slides were incubated with a horseradish peroxidase (HRP)-coupled anti-goat/sheep IgG at a 1:200 dilution, and the brown color was developed with 3,3′-diaminobenzidine. To quantitate the percentage of hepatocytes that replicated during a particular interval, the number of labeled hepatocytes was divided by the total number of hepatocytes (134 hepatocytes at ×40 magnification) in 20 different randomly chosen fields, and an average obtained. Hepatocytes were identified by staining an adjacent section for G6Pase enzymatic activity as described [45].

Staining for β-gal activity and quantification of the transduction efficiency. Frozen sections (8 μm) of organs were fixed with 1.25% glutaraldehyde in PBS for 10 minutes at 4°C and stained for β-gal activity with X-gal as described [5]. For the liver, the percentage of transduced hepatocytes was determined by counting the number of blue circles that were > 2 μm and subtracting the average number of blue circles > 2 μm in livers from non-transduced dogs that were stained with X-gal and analyzed in a similar fashion, and dividing by the number of hepatocytes in the same area. Statistical analyses between two groups of animals were performed with the program QuattroPro from Corel Corp. (Ottowa, Ontario) using the Student's *t*-test.

Quantitation of β-gal activity in organs. β-Gal activity was assayed using a kit from Promega (Madison, WI). Briefly, pieces of tissue of approximately 8 mm^3 were homogenized in 300 μl of 1× lysis buffer and centrifuged at 4°C in a microfuge at 13,000*g* for 10 minutes. The protein concentration in the supernatant was determined by a Bradford assay kit (BioRad Laboratories, Hercules, CA). An ONPG assay to quantitate β-gal activity was performed for 60 minutes at 37°C on a 96-well ELISA plate after mixing 50 μl of sample, 50 μl of 1× lysis buffer, and 100 μl of a solution containing 200 mM sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml ONPG. The change in OD at 420 nm was used to determine the β-gal activity after comparison with a standard curve of purified enzyme. One unit of enzyme was defined as the amount that hydrolyzed 1 μmole of ONPG to *o*-nitrophenol per minute at 37°C. If necessary, samples were diluted to give values that fell in the linear portion of the standard curve. The background activity was defined as the average activity in mU/mg observed in homogenates from the same organ of 2 to 4 animals of the same age that did not receive any RV. This value was subtracted from the values of experimental animals.

GUSB activity. Serum was collected and frozen at –70°C until assayed for enzyme activity. The GUSB assay used 4-methylumbelliferyl β-D-glucuronide as the substrate and measurement of fluorescence as described [46]. Samples were diluted to give values that were within the linear range of the standard curve. One unit was defined as the amount of enzyme that released 1 nmole of 4-methylumbelliferone per hour at 37°C. GUSB histochemistry was performed as described using naphthol-AS-BI-β-D-glucuronic acid [46] except for the fact that the frozen sections were fixed for 20 minutes at room temperature instead of at 4°C, and in most cases were only stained for 1 hour without a preincubation step. These changes from the published protocol reduced the enzyme activity.

Real-time PCR. DNA from RV-transduced or control dog organs, or from the liver of a male lox-Piga-lac mouse with 1 copy of the β-gal gene per diploid genome [47], was obtained after homogenization in guanidinium and extraction with phenol as described [3]. DNA with 1 copy of the β-gal gene per diploid genome was mixed with DNA from a nontransduced dog to create standards with 0.5 copies of the β-gal gene per diploid genome or less. To evaluate the RV copy number in the transduced dogs, PCR was performed with real-time TaqMan technology. For Taqman probes, the fluorescent reporter dye 6-carboxyfluorescein (FAM; emission maximum 518 nm) was covalently linked to the 5′ end of the oligonucleotide, and the quenching dye (6-carboxytetramethylrhodamine (TAMRA), emission maximum 582 nm) was attached to a linker-arm-modified nucleotide at the 3′-end (Applied Biosystems, Rockville, MD). For detection of the *lacZ* gene, oligonucleotides 5′-TACTGTCGTCGTCCCCTCAAA-3′ and 5′-TAACAACCCGTCGGATTCTTC-3′ were used for amplification, and the TaqMan probe was 5′-TATCCCATTACGGTCAATCCGCCG-3′ [48]. To detect the canine β-actin gene for normalization purposes, the oligonucleotides 5′-CTCCATCATGAAGTGTGACGTT-3′ and 5′-ATCTCCTTCTGCATCCTGTCAG-3′ were used for amplification, and the TaqMan probe was 5′-CAAGGACCTCTATGCCAACACAGTGCT-3′ [49]. Each PCR mixture contained 100 ng of DNA, 3 mM $MgCl_2$, 0.25 mM dATP, dGTP, and dCTP, 0.5 mM dUTP, 200 nM of each primer or probe, 0.25 units of AmpErase UNG, 1× TaqMan buffer A, and 0.125 units of AmpliTaq Gold DNA Polymerase (TaqMan PCR Core Reagent Kit, Applied Biosystems, Rockville, MD) in 25 μl. The samples were placed in MicroAmp Optical 96-well reaction plates with optical caps. Amplification was performed in duplicate after incubation at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C using the GeneAmpR 5700 Sequence Detection System. The C_T values corresponded to the cycle numbers at which the fluorescence reached the threshold. The C_T for β-actin was subtracted from the C_T for β-gal, and the copy number determined after comparison with the standard curve as recommended by the manufacturers.

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