

# Absence of a desmopressin response after therapeutic expression of factor VIII in hemophilia A dogs with liver-directed neonatal gene therapy

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**Hemophilia A (HA) is a bleeding disorder caused by factor VIII (FVIII) deficiency. FVIII replacement therapy can reduce bleeding but is expensive, inconvenient, and complicated by development of antibodies that inhibit FVIII activity in 30% of patients. Neonatal hepatic gene therapy could result in continuous secretion of FVIII into blood and might reduce immunological responses. Newborn HA mice and dogs that were injected i.v. with a retroviral vector (RV) expressing canine B domain-deleted FVIII (cFVIII) achieved plasma cFVIII activity that was  $139 \pm 22\%$  and  $116 \pm 5\%$  of values found in normal dogs, respectively, which was stable for 1.5 yr. Coagulation tests were normalized, no bleeding had occurred, and no inhibitors were detected. This is a demonstration of long-term fully therapeutic gene therapy for HA in a large animal model. Desmopressin (DDAVP; 1-deamino-[D-Arg<sup>8</sup>]vasopressin) is a drug that increases FVIII activity by inducing release of FVIII complexed with von Willebrand factor from endothelial cells. It has been unclear, however, if the FVIII is synthesized by endothelial cells or is taken up from blood. Because the plasma cFVIII in these RV-treated dogs derives primarily from transduced hepatocytes, they provided a unique opportunity to study the biology of the DDAVP response. Here we show that DDAVP did not increase plasma cFVIII levels in the RV-treated dogs, although von Willebrand factor was increased appropriately. This result suggests that the increase in FVIII in normal dogs after DDAVP is due to release of FVIII synthesized by endothelial cells.**

retroviral vector | inhibitor | B domain-deleted

**H**emophilia A (HA) is an X-linked bleeding disorder with an incidence of 1 in 5,000 males (1). Severe HA patients have  $\leq 1\%$  of normal factor VIII (FVIII) activity (normal levels are 200 ng/ml) and frequently bleed spontaneously. Patients with 1–5% of normal activity have moderately severe bleeding, and patients with 5–25% of normal activity usually bleed only with surgery or trauma. HA is generally treated with FVIII protein injections, which are expensive and inconvenient. An alternative treatment for mild HA is desmopressin (DDAVP; 1-deamino-[D-Arg<sup>8</sup>]vasopressin), which releases FVIII complexed with von Willebrand factor (VWF) from endothelial cells (2). It has been unclear, however, as to whether this stored FVIII is synthesized *de novo* in endothelial cells or taken up from blood, because both endothelial cells and hepatocytes express FVIII mRNA (3).

HA is a candidate for gene therapy, which involves transfer of a FVIII gene into cells that secrete functional protein into blood. The 7-kb FVIII cDNA encodes a 2,332-aa protein that is cleaved intracellularly to an N-terminal heavy chain (A1, A2, and B domains) and a C-terminal light chain (A3, C1, and C2 domains) (4). Because the B domain released after FVIII activation is not necessary for function, B domain-deleted (BDD) constructs of only 4.5 kb have been used in most gene therapy approaches. Stable and therapeutic levels of FVIII have been achieved in HA

mice (reviewed in refs. 5–9) by transduction of liver with gamma retroviral vectors (RVs) [ $>20\%$  of normal FVIII (10)], adenoviral vectors [ $>50\%$  of normal (11–15)], lentiviral vectors [ $>5\%$  of normal (16, 17)], hydrodynamic injection of plasmid DNA [ $>300\%$  of normal (18)], and adeno-associated virus vectors [ $2\text{--}100\%$  of normal (19–23)]. *Ex vivo* gene therapy of endothelial cells (24) or hematopoietic stem cells (25) has also resulted in  $>50\%$  of normal FVIII activity.

Gene therapy for HA has been less effective in large animals and humans than in mice. An adeno-associated virus vector resulted in  $\approx 3\%$  of normal FVIII activity in HA dogs (26). Adenoviral vectors resulted in high initial expression ( $>25\%$  of normal) in HA dogs (11, 27, 28) and normal primates (29), but expression fell over time. A helper-dependent adenoviral vector had low expression in one patient and the trial was discontinued because of inflammatory responses (9). Injection of a gamma RV (30) or implantation of *ex vivo*-transduced autologous fibroblasts (31) resulted in undetectable or low ( $<5\%$  of normal) levels of FVIII that fell over time.

A major problem with protein or gene therapy for HA is the development of inhibitors, which are antibodies that inhibit the coagulation function of FVIII. Up to 30% of HA patients develop inhibitors; development of inhibitors usually occurs in patients with inversions, large deletions, or nonsense mutations (32). Inhibitors have also developed in mice, dogs, and primates that received gene therapy, and these inhibitors have varied according to the species and strain, the dose and method of delivery, the age at the time of transfer, and the underlying mutation in the recipient.

We previously demonstrated that neonatal i.v. injection of a gamma RV resulted in transduction of hepatocytes, which secreted factor IX in hemophilia B mice and dogs without inhibitor development (33, 34). We therefore tested whether this large-capacity vector might allow fully therapeutic expression of FVIII to be achieved without inhibitor development after neonatal transfer in mice and dogs with HA. In addition, the hepatocyte-restricted expression achieved with this gene transfer approach provided a unique situation in which to further investigate the biology of the DDAVP response in dogs.

## Methods

**Reagents.** Reagents were obtained from Sigma-Aldrich unless otherwise stated.

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Abbreviations: FVIII, factor VIII; cFVIII, canine FVIII; hFVIII, human FVIII; VWF, von Willebrand factor; DDAVP, 1-deamino-[D-Arg<sup>8</sup>]vasopressin or desmopressin; RV, retroviral vector; Q-aPTT, quantitative activated partial thromboplastin time; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; WBCT, whole-blood clotting time; BDD, B domain-deleted; TU, transducing unit; hAAT, human  $\alpha_1$ -antitrypsin; HA, hemophilia A.

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**RV.** The plasmid pBS KS(-)-canine SQN FVIII contains a 4.5-kb canine BDD-FVIII (cFVIII) cDNA with 6 nt of 5' and no 3' untranslated sequence (19). The cFVIII cDNA was ligated into the NotI site of hAAT-WPRE-767 (35) to generate hAAT-cFVIII-WPRE-775. An amphotropic RV-packaging cell line was prepared as described (35). High-titer clones were identified by using conditioned media to infect NIH 3T3 cells and determination of cFVIII activity from infected cells by COATEST FVIII assay as described below. Large-scale preparation of RV and the assay for replication-competent retrovirus were performed as described (35). RV titer was determined after freeze/thaw once by transducing NIH 3T3 cells and determination of the RV DNA copy number in these cells 1 wk later by real-time PCR as detailed below. The RV injectate contained 0.1 unit/ml cFVIII protein.

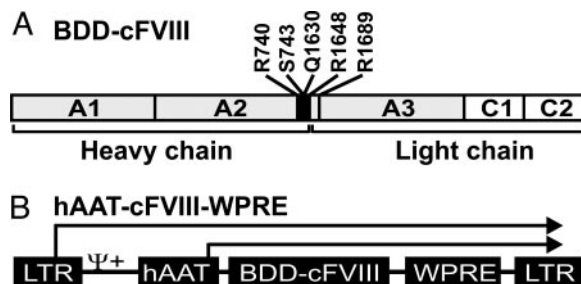
**Animal Procedures.** National Institutes of Health and Department of Agriculture guidelines for the care and use of animals in research were followed. HA mice (129 × C57BL/6) (36) were injected with RV as neonates at 2–3 d after birth via the temporal vein or as adults at 6 wk via the tail vein. Blood obtained from the retroorbital plexus with a plain capillary tube was mixed with a 1/10 volume of 3.2% sodium citrate.

Two dogs from the Chapel Hill HA colony (37) were injected i.v. with two doses of RV at 3 d after birth. H18 was male, weighed 379 g, and received two doses of RV separated by 7 h. H22 was female, weighed 409 g, and received two doses separated by 24 h. DDAVP (GensiaCicor Pharmaceuticals, Irvine, CA) was injected i.v. at 5 µg/kg into normal and RV-treated HA dogs at 3.5 yr and 8 mo of age, respectively. Plasma was tested for VWF antigen by ELISA and cFVIII activity by COATEST assay as detailed below.

**COATEST FVIII Assay.** Plasma FVIII activity was measured by a COATEST FVIII kit (DiaPharma, West Chester, OH) with bovine reagents. Samples were tested after freeze/thaw once. The assay included four steps. First, 10 µl of sample was incubated with 20 µl of a mixture of phospholipid, factor IXa, and factor X for 5 min at 37°C. Second, 10 µl of 25 mM CaCl<sub>2</sub> was added and the mixture was incubated for 5 min at 37°C. Third, 20 µl of chromogenic substrate (S-2222) and thrombin inhibitor (I-2581) were added and the mixture was incubated for 5 min at 37°C. Finally, 10 µl of 20% acetic acid was added, and the absorbance at 405 and 490 nm was measured. The dog plasma in the standards had various ratios of normal to HA plasma. Because varying the amount of plasma from different species might affect coagulation times, the samples and standards contained 0.1 µl of total mouse plasma and 0.5 µl of total dog plasma in PBS for the mouse study, or 1 µl of total dog plasma in PBS for the dog study. Samples from RV-treated animals were diluted in homologous FVIII-deficient plasma if necessary.

**Coagulation Assays.** The quantitative activated partial thromboplastin time (Q-aPTT) assay described in ref. 33 was performed for the mice with 10 µl of mouse plasma, 10 µl of cFVIII-deficient dog plasma, and 80 µl of hFVIII-deficient human plasma. Standards used 10 µl of HA mouse plasma, 10–0 µl of normal dog plasma, 0–10 µl of cFVIII-deficient dog plasma, and 80 µl of hFVIII-deficient human plasma. Samples from RV-treated mice were diluted with HA mouse plasma if necessary. The tail-clip bleeding assay was performed on 4-mo-old mice as described (33), except the endpoint was 6 h, and bleeding was determined by visual inspection after blotting the tail on filter paper.

Both straight aPTT and Q-aPTT assays were performed for dog samples. In the straight aPTT assay, 100 µl of plasma from normal, HA, or RV-treated dogs was used. In the Q-aPTT assay, 40 µl of RV-treated dog plasma (diluted in cFVIII-deficient plasma if necessary) was mixed with 60 µl of PBS. Standards used 0–40 µl of normal dog plasma, 40–0 µl of cFVIII-deficient dog plasma, and 60 µl of PBS.



**Fig. 1.** RV vector. (A) BDD cFVIII cDNA. cFVIII cDNA lacks all but 22 aa of the 908-aa B domain and has a covalent linkage between S743 and Q1630. The intracellular cleavage site at R1648 and the thrombin cleavage sites at R740 and R1689 are retained. (B) hAAT-cFVIII-WPRE. This amphotropic gamma RV contains intact LTRs, an extended packaging signal ( $\psi^+$ ), the liver-specific hAAT promoter, the BDD cFVIII cDNA, and the WPRE. Arrows indicate that an RNA can initiate from the LTR or the hAAT promoter.

**Bethesda Assay.** Samples for inhibitor assays were heat-inactivated at 56°C for 1 h. For mice, 5 µl of mouse plasma, 5 µl of normal dog plasma, and 40 µl of PBS were incubated at 37°C for 2 h, and then 50 µl of hFVIII-deficient human plasma was added for the aPTT assay. Standards used 0–5 µl of normal dog plasma and 5–0 µl of cFVIII-deficient dog plasma incubated with 5 µl of heat-inactivated HA mouse plasma and 40 µl of PBS at 37°C for 2 h, and then 50 µl of hFVIII-deficient human plasma was added, and aPTT was performed. The dilution factor was 1 if 5 µl of undiluted mouse plasma was used. If necessary, samples were diluted in heat-inactivated HA mouse plasma. Samples from dogs were assayed in a similar fashion, except 5 µl of heat-inactivated RV-treated dog plasma was used. One Bethesda unit per milliliter inhibits 50% of the coagulation activity, and the limit of sensitivity was 1 Bethesda unit/ml.

**Immunoassay for VWF.** Plasma VWF antigen levels were determined by ELISA (38) using anti-human VWF antibodies (DAKO) and diluted normal dog plasma for the standards.

**Analysis of RV DNA and RNA.** The RV DNA and RNA distribution was determined as described (39). Briefly, RNA or DNA was isolated from organs, and cDNA reverse-transcribed from 0.1 µg of RNA or 100 ng of genomic DNA was analyzed by real-time PCR for the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence, with normalization to the  $\beta$ -actin sequence. RNA and DNA from nontransduced mouse or dog liver and RNA from transduced mice that did not receive reverse transcriptase were used as controls to demonstrate a lack of contamination.

## Results

**RV Expressing cFVIII.** The BDD-cFVIII cDNA (Fig. 1A) was cloned into an RV downstream of the liver-specific human  $\alpha_1$ -antitrypsin (hAAT) promoter (Fig. 1B) and used to generate an amphotropic RV. After large-scale preparation, the titer of concentrated RV was  $1\text{--}3 \times 10^8$  transducing units (TU)/ml, and there were <10 copies of replication-competent retrovirus per milliliter. NIH 3T3 cells infected with this RV at a multiplicity of infection of 1 produced  $0.3 \pm 0.002$  unit of cFVIII per 24 h per  $10^6$  cells as determined by COATEST FVIII assay.

**cFVIII Expression and Inhibitor Levels in Mice.** HA mice (129 × C57BL/6) have a disruption in exon 16 within the A3 domain of the FVIII gene and produce heavy but not light chains (40, 41). They frequently produce inhibitors after therapy with FVIII proteins or genes from mouse, dog, or human. Neonatal mice were injected i.v. with  $1 \times 10^{10}$  TU/kg RV to evaluate the effect of neonatal gene

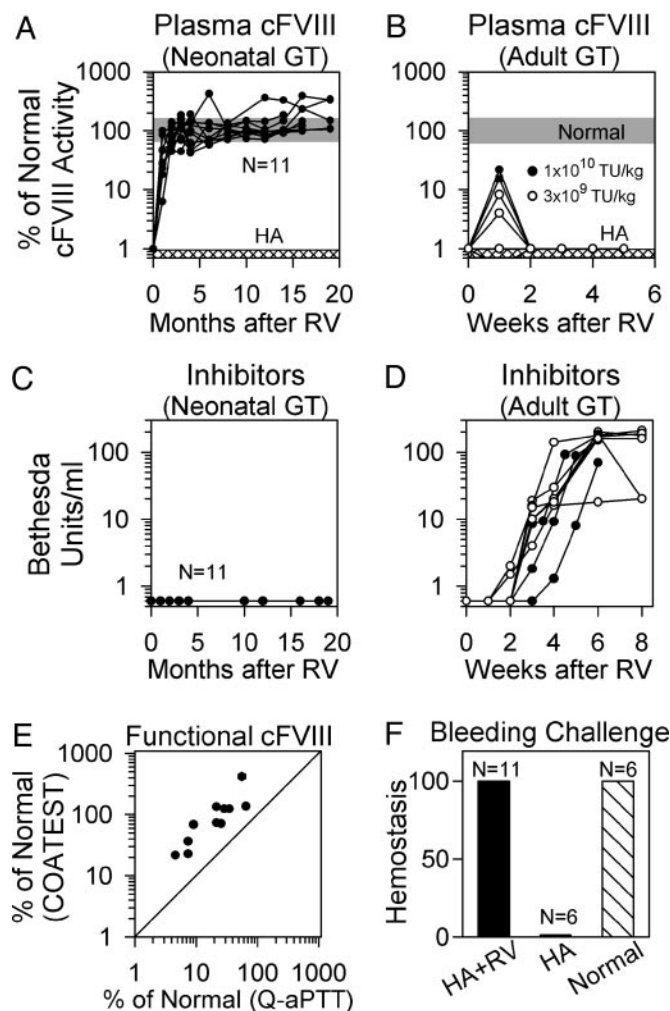
therapy. They achieved  $139 \pm 22\%$  of normal cFVIII activity in plasma by COATEST assay (Fig. 2A). The expression of cFVIII was stable for 1.5 yr, and none of 11 mice developed anti-cFVIII inhibitory antibodies (Fig. 2C). To test the ability of HA mice to develop inhibitors to cFVIII, RV was injected i.v. into 6-wk-old adult mice. cFVIII expression in these mice was low and transient (Fig. 2B) because of low levels of replicating hepatocytes in adult mice. All nine mice developed high titers of cFVIII inhibitors (Fig. 2D). The frequency of inhibitor formation in HA mice after neonatal gene therapy was significantly lower than after adult gene therapy ( $P < 0.001$ , Fisher exact test). cFVIII activity determined by Q-aPTT assay showed that RV-treated mice had  $26 \pm 6\%$  of normal activity, which was only  $26 \pm 3\%$  of that determined by COATEST assay (103% of normal) for the same samples (Fig. 2E). All HA mice that were treated with RV as newborns achieved hemostasis at 6 h after tail-clip, although untreated HA mice did not ( $P < 0.001$ , Fisher exact test) (Fig. 2F). We conclude that the cFVIII produced after neonatal gene therapy was functional *in vitro* and *in vivo*.

**RV DNA and RNA Distribution in Neonatal Mice.** The lack of antibodies in mice after neonatal delivery could be due to liver-restricted expression, because RV RNA levels in nonhepatic organs were 0.3% or less of that in liver at 6 mo after neonatal gene therapy in a previous study (39). However, because DNA transfer to nonhepatic organs was up to 10% of that in liver, and the LTR promoter can shut off over time in mice, higher expression might occur in nonhepatic cells shortly after gene transfer. Indeed, Fig. 3 shows that spleen had  $66 \pm 26\%$  and  $31 \pm 12\%$  as much DNA and RNA, respectively, as liver at 1 wk after neonatal gene transfer to C57BL/6 mice. This finding makes it unlikely that liver-restricted expression accounts for the lack of an antibody response.

**Neonatal Gene Therapy in HA Dogs.** RV transduction was performed on neonatal HA dogs from the Chapel Hill colony, which have an inversion between intron 22 and a sequence upstream of the promoter (37). These dogs express an RNA that is truncated within the C1 domain and do not usually develop inhibitory antibodies to cFVIII protein (T.C.N., unpublished data). H18 and H22 received  $\approx 0.8 \times 10^{10}$  TU/kg RV at 3 d after birth. The platelet count fell to below normal at  $78,000 \pm 12,000$  at 24 h ( $P = 0.03$  for 24 h vs. pre-gene-transfer values) and  $102,000 \pm 22,100$  at 48 h ( $P = 0.05$  for 48 h vs. pre-gene-transfer values), but returned to normal by day 4 (Fig. 4A). This fall in platelets was similar to that observed previously with a similar dose of RV (33) but did not occur when the dose was reduced to  $3 \times 10^9$  TU/kg or less (34). Liver function tests and other blood counts were not affected by the gene therapy and were similar to values reported previously.

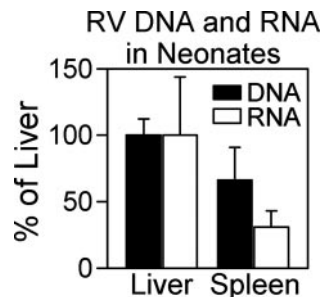
The whole-blood clotting time (WBCT) was corrected at the first time of analysis after gene transfer and has remained normal for 1.5 yr thus far (Fig. 4B). The straight aPTT fell progressively during the first 3 mo, and thereafter was usually normal for H18 and near-normal for H22 (Fig. 4C). The average COATEST cFVIII activities in plasma collected from 3 to 17 mo after gene therapy were  $129 \pm 7\%$  and  $101 \pm 4\%$  of normal for H18 and H22, respectively (Fig. 4D). The functional cFVIII activity in the Q-aPTT assay was  $61 \pm 5\%$  and  $35 \pm 5\%$  of normal for H18 and H22, respectively (Fig. 4E). Thus, the cFVIII activity by COATEST assay was 2.5-fold that observed by Q-aPTT assay for the RV-treated dogs. No bleeding episodes have occurred, and no cFVIII inhibitors were detected by the Bethesda assay (Fig. 4F).

**Liver Vector DNA After Neonatal Gene Therapy in Mice and Dogs.** Livers were obtained from three RV-treated HA mice at 12 mo after neonatal transfer. Real-time PCR demonstrated that there were  $1.9 \pm 0.5$  RV DNA copies per cell in the liver for mice whose average cFVIII levels by COATEST assay were  $106 \pm 2\%$  of normal (Fig. 5). The lower copy number in the



**Fig. 2.** Gene therapy with hAAT-cFVIII-WPRE in HA mice. (A) cFVIII activity after neonatal transduction. Neonatal HA mice were injected i.v. with  $1 \times 10^{10}$  TU/kg hAAT-cFVIII-WPRE at 2 or 3 d after birth [neonatal gene therapy (GT)], and the plasma cFVIII activity was measured by COATEST assay. Normal mice have 60–160% of normal cFVIII activity (gray shading), and HA mice have <1% of normal activity (cross-hatching). (B) cFVIII activity after adult transduction. HA mice (6-wk-old) were injected i.v. with  $1 \times 10^{10}$  TU/kg ( $n = 3$ ; ●) or  $3 \times 10^9$  TU/kg ( $n = 6$ ; ○) of hAAT-cFVIII-WPRE (adult GT). Plasma cFVIII levels were measured by COATEST FVIII assay. (C) cFVIII inhibitors after neonatal transduction. Plasma from the mice described in A was tested for anti-cFVIII inhibitory antibodies by Bethesda assay. (D) cFVIII inhibitors after adult transduction. Plasma from the mice described in B was tested for cFVIII inhibitors by the Bethesda assay. (E) Coagulation activity *in vitro*. The samples from the mice described for A were tested at 4 and 15 mo to ensure reproducibility. The average cFVIII activities from the COATEST assay are plotted vs. the average activities from the Q-aPTT assay for the same samples for individual mice. The line represents values where the percentage functional activities from each assay are the same. (F) Hemostasis assay *in vivo*. Tail-clip was performed at 4 mo after transduction in the neonatal RV-treated mice (HA + RV) described for A, and the percentage of animals that achieved hemostasis within 6 h was determined. Tail-clip was also performed on age-matched untreated HA mice and C57BL/6 normal mice.

liver observed at 1 wk after transduction in mice (Fig. 3) may be due to substantial contamination with hematopoietic cells in the liver at the early time. DNA from livers obtained at 14 mo after neonatal gene therapy contained 0.16 and 0.10 copies of RV DNA per cell in the dogs with 129% and 101% of normal cFVIII activity, respectively (Fig. 5). Thus, although mice and dogs had similar FVIII COATEST activity in plasma, mice had 14-fold more copies of RV DNA than did dogs.



**Fig. 3.** RV DNA and RNA distribution in neonatal mice. Normal C57BL/6 mice were injected i.v. with  $1 \times 10^{10}$  TU/kg hAAT-cFVIII-WPRE at 2–3 d after birth ( $n = 5$ ) and killed 1 wk later. Real-time PCR on genomic DNA and real-time RT-PCR on RNA was performed to detect RV DNA and RNA, respectively. The amounts of RV DNA and RNA are plotted as the average relative to that in the liver  $\pm$  SEM. The average RV DNA in the liver was  $0.3 \pm 0.03$  copy per cell.

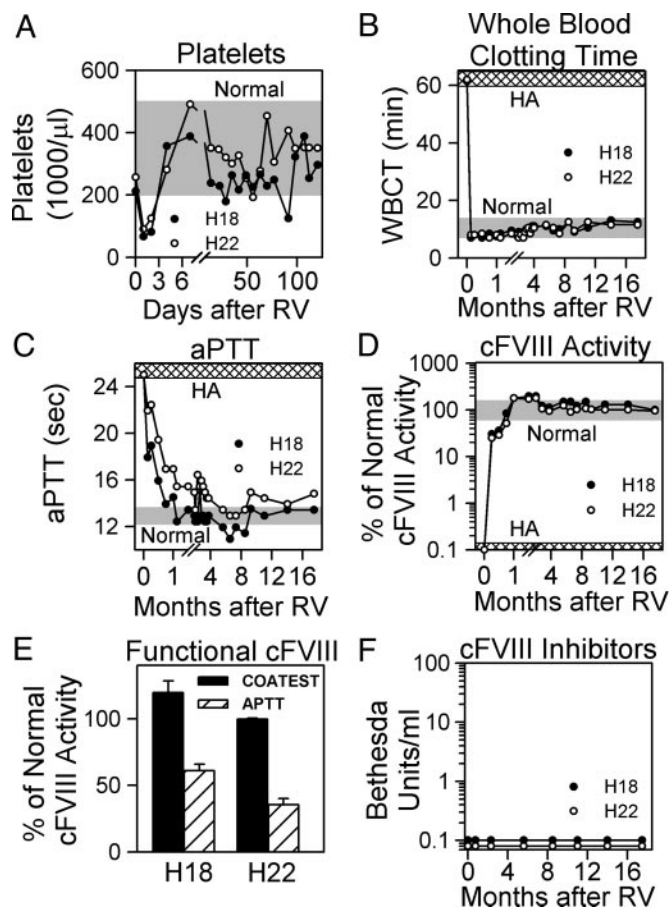
**The Effect of DDAVP on Dogs.** Administration of DDAVP to humans (42) or dogs (38) increases both VWF and FVIII within 30–60 min. In this study, DDAVP was injected i.v. at  $5 \mu\text{g}/\text{kg}$  into normal dogs ( $n = 4$ ) or RV-treated HA dogs (H18 and H22). In normal dogs, both FVIII and VWF levels increased to  $\approx 1.5$ -fold normal between 0.25 and 1 h after DDAVP (Fig. 6A). In contrast, FVIII levels in H18 did not increase after DDAVP, although VWF levels increased to 2.5-fold normal ( $P < 0.005$  for comparison of relative cFVIII to relative VWF levels at each time between 0.25 and 1 h after DDAVP, Student's *t* test) (Fig. 6B). Similarly, DDAVP had no effect on FVIII activity in H22, although VWF levels increased to 1.6-fold normal ( $P < 0.05$ , Student's *t* test) (Fig. 6C). Because plasma cFVIII in RV-treated dogs probably primarily derives from transduced hepatocytes that secrete cFVIII into blood, the increase of FVIII in normal animals is likely due to release of FVIII that is synthesized in endothelial cells, rather than taken up from the blood.

## Discussion

**Neonatal Gene Therapy Corrects HA in Mice and Dogs.** Neonatal transduction of a gamma RV resulted in stable expression of cFVIII in HA models. The plasma cFVIII activity by COATEST assay was  $139 \pm 22\%$  and  $116 \pm 5\%$  of normal in RV-treated HA mice and dogs, respectively. The COATEST cFVIII activity was 4-fold and 2.5-fold the activity determined by Q-aPTT assay in RV-treated HA mice and dogs, respectively. This discrepancy may be caused by the BDD construct, as recombinant human BDD-FVIII has  $\approx 2$ -fold more activity by COATEST assay than by aPTT assay (43). All RV-treated HA mice achieved hemostasis after bleeding challenge. Both WBCT and aPTT were normalized in the RV-treated HA dogs, and no bleeding episodes have occurred. The FVIII levels achieved in this study are  $>20$ -fold higher than the long-term expression with other vectors for HA in dogs (11, 26–29).

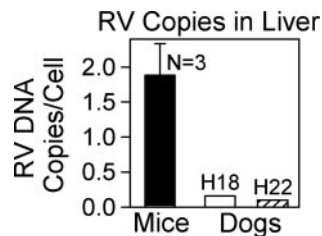
The fact that cFVIII plasma levels in dogs were almost as high as in mice was surprising because expression in C57BL/6 mice was  $\approx 10$ -fold higher than in dogs when a similar dose of RV per kg was given in our previous studies (33, 35, 39). Furthermore, in this study, mice had  $\approx 14$ -fold more RV copies per cell in liver than did dogs. Canine FVIII may not interact well with murine VWF, or may be poorly secreted in mice. It is unclear why transduction was more efficient in C57BL/6 mice than in dogs. Attempts to quantify the percentage of replicating hepatocytes in mice were not successful because of the large numbers of hematopoietic cells in livers of newborns.

**Neonatal Gene Therapy Did Not Induce Inhibitors in HA Mice or Dogs.** Inhibitor formation is a major complication in the treatment of HA patients with FVIII protein and is a critical challenge for gene therapy. Inhibitors have generally developed after gene therapy to



**Fig. 4.** Neonatal gene therapy with hAAT-cFVIII-WPRE in HA dogs. Two HA dogs (H18 and H22) were injected i.v. at 3 d after birth with  $0.9 \times 10^{10}$  and  $0.7 \times 10^{10}$  TU/kg hAAT-cFVIII-WPRE, respectively, as detailed in *Methods*. The ranges of values in normal and HA dogs for each assay are indicated as gray and cross-hatched regions, respectively. (A) Platelets were evaluated at the indicated times after the first dose of RV. (B) The WBCT is 8–13 min for normal dogs and  $>60$  min for HA dogs. (C) aPTT. (D) Plasma cFVIII activity. Plasma cFVIII activity was determined by COATEST assay. The normal range of cFVIII is 60–160%, and HA dogs have  $<1\%$  of normal cFVIII activity. (E) Comparison of COATEST and Q-aPTT cFVIII activities in dogs. cFVIII functional activity in plasma obtained from dogs at 9–11 mo after transduction was analyzed by COATEST and Q-aPTT assays. (F) cFVIII inhibitors were tested by Bethesda assay.

adult immunocompetent HA mice. However, in this study, none of 11 HA mice developed FVIII inhibitors after neonatal gene therapy with RV, whereas all mice developed FVIII inhibitors after adult transduction. Our results in mice with cFVIII differ from a previous study that showed that  $\approx 50\%$  of HA mice produced inhibitors after neonatal transduction of a vesicular stomatitis virus G glycoprotein pseudotyped RV expressing human BDD-FVIII (10). Because the human FVIII (hFVIII) levels were low at the first time of evaluation in animals with inhibitors, inhibitor formation may have been induced by low levels of hFVIII, as low expression of human factor IX is more likely to induce antibodies than high expression in adult mice (44). Other possible reasons for this discrepancy are that: (i) the human BDD-FVIII may be more immunogenic than canine BDD-FVIII in mice; (ii) the vesicular stomatitis virus G glycoprotein envelope may induce more inflammatory responses than the amphotropic envelope; or (iii) the genetic background of the mice may be different. Further experiments are needed to evaluate this discrepancy. Liver-specific promoters may prevent an antibody response by avoiding expression in antigen-presenting cells. However, liver-restricted expression is not the mechanism of tolerance



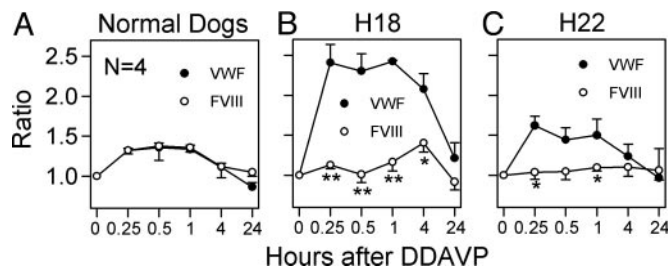
**Fig. 5.** Evaluation of RV DNA copy number in the liver after neonatal transduction in HA mice and dogs. Genomic DNA was isolated from the livers of three neonatal RV-treated mice (see Fig. 2A) at 12 mo of age (mean  $\pm$  SEM is shown), and from liver biopsy samples from H18 and H22 (see Fig. 4) at 14 mo of age. RV DNA copy numbers were determined by real-time PCR.

here because RNA levels in spleen were 31% of the level in liver in mice at 1 wk after gene transfer, which is likely due to transcription from the LTR. The low expression reported previously in spleen at 6 mo (0.3% of that in liver) after a similar gene transfer procedure (39) likely reflects shut-down of the LTR.

There was also no inhibitor formation in the RV-treated dogs in this study. This result is consistent with the hypothesis that neonatal gene therapy results in tolerance to cFVIII in HA dogs; although the small number of animals analyzed ( $n = 2$ ) and the relatively low incidence of inhibitor formation (0–50%) in dogs with a similar mutation after cFVIII protein (45) or gene (11, 26–28) therapy to adults make it impossible to be certain that this will be consistently effective. Studies using the vector expressing cFVIII and HA dogs from an inhibitor-prone lineage, or a vector expressing hFVIII in mice and dogs, followed by challenge with hFVIII protein, are needed to demonstrate true tolerance.

**DDAVP Did Not Increase FVIII Activity in RV-Treated Dogs.** FVIII is stabilized by VWF in the circulation. VWF is produced by endothelial cells and megakaryocytes and is stored in Weibel–Palade bodies in endothelial cells and  $\alpha$ -granules in platelets (4). DDAVP causes VWF and FVIII to be released from endothelial cells, and this release increases the plasma levels of both VWF and FVIII (2). However, the source of the released FVIII after DDAVP stimulation has not been determined (3). Liver is a major site of FVIII production because liver transplantation completely corrects HA (46), and hepatocytes express FVIII mRNA (47). However, end-stage liver disease does not cause a decrease in FVIII, FVIII mRNA is found in other organs, and spleen and lung transplantation can ameliorate HA (3). Expression of FVIII in other organs may be due to endothelial cells because endothelial cells express FVIII mRNA and protein *in vitro* (47) and store FVIII with VWF in Weibel–Palade bodies (48). Thus, there are two possible sources of stored FVIII in endothelial cells: uptake from blood or *de novo* synthesis by endothelial cells.

In this study, the effect of DDAVP on HA dogs after neonatal hepatic gene therapy with an RV expressing cFVIII was tested. This gene transfer approach resulted in high expression in hepatocytes without detectable expression in endothelial cells, as assessed by histochemical staining at 1 wk after gene transfer in dogs (ref. 35 and L.X. and K.P.P., unpublished data). We hypothesized that if DDAVP increases FVIII levels in this dog model, it would indicate that the FVIII increase is due to release of FVIII that is taken up from blood by endothelial cells. Alternatively, if no increase in



**Fig. 6.** The effect of DDAVP on VWF antigen and FVIII activity in dogs. DDAVP was injected i.v. at 5  $\mu$ g/kg into dogs. VWF antigen (●) and FVIII COATEST activity (○) levels at 0, 0.25, 0.5, 1, 4, and 24 h after DDAVP were determined. The ratio of the activity after DDAVP to that before DDAVP was determined for each time, and the mean ratio  $\pm$  SEM is shown. (A) Effect of DDAVP in normal dogs. Four normal dogs were injected with DDAVP. The baseline levels of VWF antigen and FVIII activity in these dogs were  $120 \pm 7\%$  and  $89 \pm 19\%$  of normal, respectively. (B and C) Effect of DDAVP after neonatal gene therapy. Two separate doses of DDAVP were given with an interval of 1 wk to RV-treated HA dogs. The baseline levels of VWF antigen were 23.5% and 61% of normal for H18 and H22, respectively. The baseline levels of cFVIII activity just before DDAVP treatment were 119% and 90% of normal for H18 and H22, respectively. The ratio to baseline VWF levels was compared with the ratio to baseline FVIII levels at each time by using Student's *t* test (\*\*,  $P = 0.0005$ – $0.005$ ; \*,  $P = 0.005$ – $0.05$ ).

FVIII occurs after DDAVP stimulation, it would suggest that the FVIII increase in normal dogs is due to release of protein that is synthesized by endothelial cells. DDAVP increased both FVIII and VWF levels in normal dogs to  $\approx 1.5$ -fold normal at 15–60 min after stimulation, which was consistent with a previous study (38). However, in RV-treated HA dogs, FVIII levels did not change, although VWF levels increased to 2.5- or 1.6-fold normal. This result suggests that the FVIII increase in normal dogs after DDAVP administration is probably due to release of FVIII that is synthesized by endothelial cells, although it is possible that our result is affected by the use of a BDD construct. Our data demonstrate that DDAVP will not be effective at increasing FVIII activity in patients that receive liver-directed gene therapy with BDD-hFVIII and only achieve partial correction. Such patients would need to be treated with factor replacement if bleeding episodes occur.

**Implications for Gene Therapy.** This study demonstrated that neonatal gene therapy with a gamma RV resulted in fully therapeutic levels of cFVIII in HA mice and dogs without inhibitor formation. This approach may ultimately be used to reduce the bleeding manifestations in humans with HA. However, it will be necessary to demonstrate that neonatal RV-mediated gene therapy has a very low risk of insertional mutagenesis or germ-line transmission in large animals before using this approach in humans, and addition of suicide or insulator elements may be necessary. In addition, it will be important to determine whether neonatal tolerance is effective at preventing antibody formation in primates before using this approach in humans that are at high risk of inhibitor formation.

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- Lozier, J. N. & Kessler, C. M. (2000) in *Hematology: Basic Principles and Practice*, eds Hoffman, R., Benz, E. J., Jr., Shattil, S. J., Furie, B., Cohen, H. J., Silberstein, L. E. & McGlave, P. (Churchill Livingstone, New York), 3rd Ed., pp. 1883–1904.
- Borchiellini, A., Fijnvandraat, K., ten Cate, J. W., Pajkrt, D., van Deventer, S. J., Pasterkamp, G., Meijer-Huizinga, F., Zwart-Huink, L., Voorberg, J. & Mourik, J. A. (1996) *Blood* **88**, 2951–2958.

- Kaufmann, J. E. & Vischer, U. M. (2003) *J. Thromb. Haemostasis* **1**, 682–689.
- Kaufman, R. J. & Antonarakis, S. E. (2000) in *Hematology: Basic Principles and Practice*, eds Hoffman, R., Benz, E. J., Jr., Shattil, S. J., Furie, B., Cohen, H. J., Silberstein, L. E. & McGlave, P. (Churchill Livingstone, New York), 3rd Ed., pp. 1850–1868.
- Lozier, J. (2004) *Semin. Hematol.* **41**, 287–296.
- Nathwani, A. C. & Davidoff, A. M. (2004) *Haemophilia* **10**, 309–318.

