Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors

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Haemophilia B, or factor IX deficiency, is an X-linked recessive disorder that occurs in about one in 25,000 males, and severely affected people are at risk for spontaneous bleeding into numerous organs. Bleeding can be life-threatening or lead to chronic disabilities with haemophilic arthropathy. The severity of the bleeding tendency varies among patients and is related to the concentration of functional plasma factor IX. Patients with 5–30% of the normal factor IX have mild haemophilia that may not be recognized until adulthood or after heavy trauma or surgery[1]. Therapy for acute bleeding consists of the transfusion of clotting-factor concentrates prepared from human blood and recombinant clotting factors that are currently in clinical trials. Both recombinant retroviral[2] and adenoviral[3] vectors have successfully transferred factor IX cDNA into the livers of dogs with haemophilia B. Recombinant retroviral-mediated gene transfer results in persistent yet subtherapeutic concentrations of factor IX and requires the stimulation of hepatocyte replication before vector administration. Recombinant adenoviral vectors can temporarily cure the coagulation defect in the canine haemophilia B model; however, an immune response directed against viral gene products made by the vector results in toxicity and limited gene expression[3,4]. The use of recombinant adeno-associated virus (rAAV) vectors is promising because the vector contains no viral genes and can transduce non-dividing cells[5]. The efficacy of *in vivo* transduction of non-dividing cells has been demonstrated in a wide variety of tissues[6–12]. In this report, we describe the successful transduction of the liver *in vivo* using r-AAV vectors delivered as a single administration to mice and demonstrate that persistent, curative concentrations of functional human factor IX can be achieved using wild-type-free and adenovirus-free rAAV vectors. This demonstrates the potential of treating haemophilia B by gene therapy at the natural site of factor IX production.

The recombinant pSSV9-MFG-hFIX (encoding human factor IX) and rAAV-MFG-hTH (encoding human tyrosine hydroxylase) vector preparations were determined free of contaminating infectious adenovirus to a sensitivity of one adenovirus in 1×10^9 rAAV by two assays as described in Methods. To reduce the possibility that replication-competent adenovirus might contaminate rAAV stocks, the rAAV preparations were made using an E1A$^-$ mutant adenovirus, incapable of growth without the transcomplementation of the *AdE1A* gene. The CsCl gradients used in purification physically separate the adenovirus from the rAAV particles, and this yields a minimal amount of adenovirus contamination in the isolated rAAV fraction. In addition, after purification, the rAAV stocks were subjected to heating at 56 °C for 45 min. This treatment is capable of inactivating at least nine logs of adenovirus (data not shown). The preparations were determined to be free of wild-type AAV to a sensitivity of 1 wtAAV in 1×10^9 rAAV using a PCR assay to detect both naturally occurring wild-type AAV and wild-type AAV arising from recombination between the vector and helper plasmids. A modified replication centre-assay was used to detect infectious wild-type AAV. Lastly, the vectors were titred by a dot-blot assay to detect total particles; titres of the preparations were 1.8×10^{11}/ml for rAAV-MFG-hTH and 4.2×10^{11}/ml for rAAV-MFG-hFIX. This translates into approximately 3.6×10^8 and 8.4×10^8 functional units per millilitre respectively, based on a particle-to-infectious ratio of approximately 500 (ref. 13).

The recombinant AAV vector, rAAV-MFG-hFIX, was administered into C57Bl/6 mice via a portal vein infusion after the placement of an in-dwelling catheter (Fig. 1). In the first set of experiments, mice were infused with 2.1×10^{10}, 4.2×10^{10} or 8.4×10^{10} rAAV-MFG-hFIX particles, and periodic serum samples were obtained for human factor IX determination by ELISA (Fig. 1*a*). Factor IX was detectable at low levels (10–80 ng/ml) within the first week and increased continually during the first several weeks to steady-state concentrations that ranged from 250 to 2,000 ng/ml to week 36 (the duration of the experiment). The animals administered with 8.4×10^{10} particles had somewhat higher levels of factor IX than the mice given 4.2×10^{10} and 2.1×10^{10} particles; however, the animals had overlapping protein levels. Control mice receiving no AAV or an unrelated vector had no detectable human factor IX. Similar levels of human serum factor IX were obtained in a second experiment, in which immunocompetent C57Bl/6 and immunodeficient C57Bl/6 SCID mice were infused with 4.2×10^{10} AAV particles (Fig. 1*b*). This was consistent with the results of earlier studies suggesting that factor IX was not an immunogenic protein in this immunocompetent mouse strain[3,14]. In addition, anti-AAV antibodies were detected in the C57Bl/6 mice but not in the C57Bl/6 SCID mice, three to seven weeks after transfusion.

Previous studies have demonstrated that the co-administration of recombinant adenoviruses dramatically increases AAV-mediated gene expression in the liver[15]. Our preliminary unpublished results support this observation because human factor IX expression was increased 10–100-fold, reaching serum concentrations of nearly 100,000 ng/ml, or 20-fold above the normal human plasma concentration. Interestingly, factor IX concentrations slowly decreased over a period of weeks and stabilized at levels similar to those obtained with AAV alone. This decrease is most likely related to the loss of the adenovirus. The steady level of expression that followed the transient high levels was comparable to rAAV alone, indicating that the adenovirus does not increase the number of genomes that ultimately persist.

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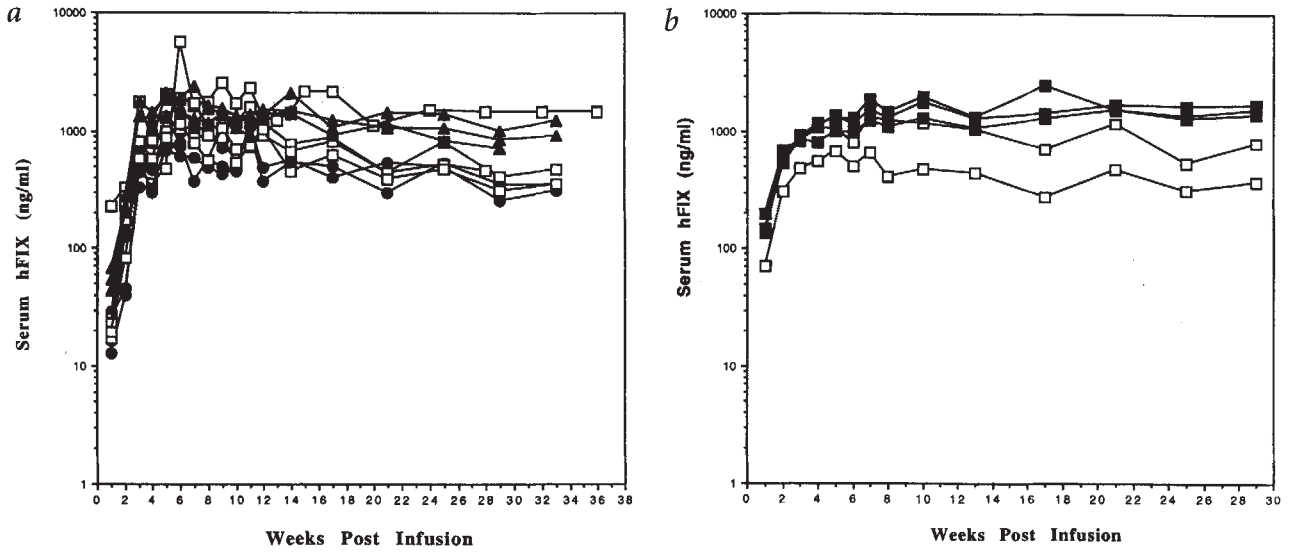

Fig. 1 Factor IX expression in mice. Recombinant AAV-MFG-human factor IX was delivered to the liver of 5–6-week-old-mice as a single intraportal administration of 2.1×10^{10} ($n=4$; closed circles) 4.2×10^{10} ($n=5$ in *a*; $n=2$ in *b*) open squares or 8.4×10^{10} ($n=3$; closed triangles) particles in a volume of 200 µl to (***a***) C57Bl/6 or (***b***) C57Bl/6 scid mice infused with 4.2×10^{10} particles ($n=3$; closed squares). Mice were bled at the indicated number of weeks after AAV administration and the serum was stored frozen. The expression of human factor IX was determined by an ELISA and is presented for individual animals. ***c***, Western analysis. Serum from a mouse infused with 4.2×10^{10} particles of recombinant AAV-MFG-human factor IX at seven weeks after administration was diluted and analysed by western blot analysis using a rabbit anti-human polyclonal antibody. Lanes: 1, blank; 2, naive mouse; 3–7, increasing dilutions of mouse sera 19, 38, 75, 150 and 300 pg, respectively; 8–10, human serum standard, 19, 38 and 75 pg, respectively.

The rAAV construct used in these studies contains the MuLV LTR promoter/enhancer[16]. Our experiments using an rAAV vector that contained the CMV promoter expressing the human factor IX gene resulted in very little hepatic gene expression (not shown). This may be related to the downregulation of this promoter, observed previously in the liver in retroviral vectors[17,18], adenoviral vectors[19] and transgenic mice[20].

Although there is a plethora of data demonstrating that vector-mediated expression of factor IX results in a functional enzyme[2,3], three additional studies were undertaken to demonstrate the appropriate characteristics of the recombinant protein found in the serum. First, western-blot analysis (Fig. 1*c*) from the serum of an rAAV-MFG-hFIX-transduced mouse demonstrated the presence of a prominent 71-kD protein band at a level equivalent to that seen in normal human serum, and this level was in agreement with ELISA values. Second, the factor IX protein requires the post-translational addition of γ-carboxyl groups for biological function. To estimate the fraction of γ-carboxylated factor IX, a calcium-dependent antibody directed against human factor IX[21] was used as the second antibody in an ELISA assay. Serum samples were obtained from six animals at random time points and analysed in parallel with the calcium-dependent and standard antibody (to measure

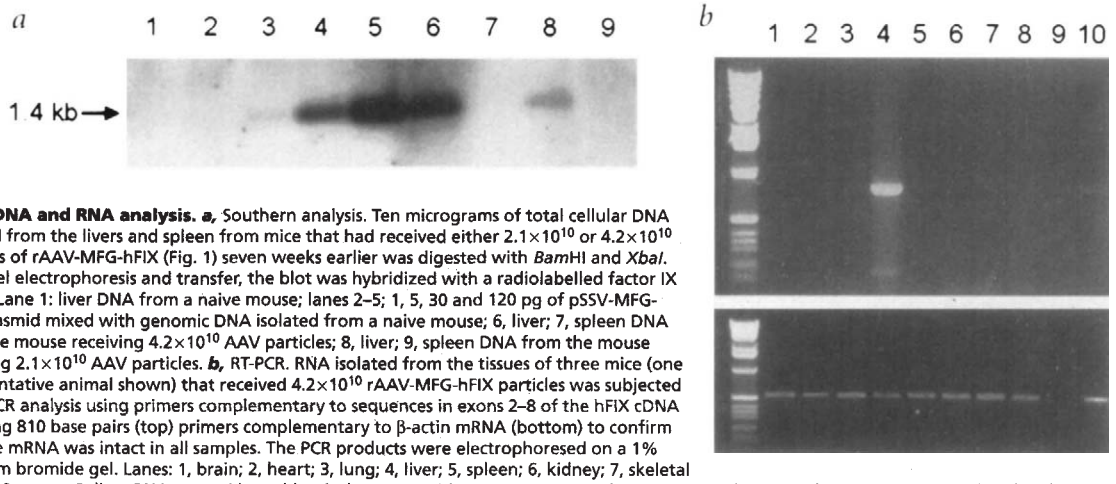

Fig. 2 DNA and RNA analysis. ***a***, Southern analysis. Ten micrograms of total cellular DNA isolated from the livers and spleen from mice that had received either 2.1×10^{10} or 4.2×10^{10} particles of rAAV-MFG-hFIX (Fig. 1) seven weeks earlier was digested with *Bam*HI and *Xba*I. After gel electrophoresis and transfer, the blot was hybridized with a radiolabelled factor IX probe. Lane 1: liver DNA from a naive mouse; lanes 2–5; 1, 5, 30 and 120 pg of pSSV-MFG-hFIX plasmid mixed with genomic DNA isolated from a naive mouse; 6, liver; 7, spleen DNA from the mouse receiving 4.2×10^{10} AAV particles; 8, liver; 9, spleen DNA from the mouse receiving 2.1×10^{10} AAV particles. ***b***, RT-PCR. RNA isolated from the tissues of three mice (one representative animal shown) that received 4.2×10^{10} rAAV-MFG-hFIX particles was subjected to RT-PCR analysis using primers complementary to sequences in exons 2–8 of the hFIX cDNA spanning 810 base pairs (top) primers complementary to β-actin mRNA (bottom) to confirm that the mRNA was intact in all samples. The PCR products were electrophoresed on a 1% ethidium bromide gel. Lanes: 1, brain; 2, heart; 3, lung; 4, liver; 5, spleen; 6, kidney; 7, skeletal muscle, 8, ovary; 9, liver RNA treated in an identical manner without reverse transcriptase; 10, positive control, DNA-based PCR using the plasmid as a template. Note: Southern analysis of the PCR products using a factor IX probe showed a strong signal in the liver but no signal in the other tissues (not shown).

total antigen). This analysis revealed that relative to human plasma, 89% +/– 5% of the recombinant human protein in the mouse serum was γ-carboxylated, confirming that most of the recombinant factor IX was post-translationally modified.

Finally, a functional assay was performed. Factor IX clotting-activity assays from citrated plasma samples are shown in Table 1. Combined citrated plasma from two normal (naive) mice had about one-third the factor IX clotting activity of normal human plasma. Addition of one-tenth volume of normal human plasma to the normal murine plasma increased the activity to 0.41 U/ml, and the activity decreased to 0.33 U/ml after incubation with a murine monoclonal anti-human factor IX antibody. This demonstrates that 0.10 U/ml of human plasma factor IX can be distinguished in murine plasma by a clotting activity assay and that the equivalent amount of added human factor IX activity was inhibited by species-specific antibody. The antibody also inhibited a comparable amount of normal human plasma factor IX added to buffer instead of normal murine plasma (from 0.07 to <0.01 U/ml). Two distinct AAV-transduced mice showed about twice as much factor IX clotting activity as two naive mice. Activity equivalent to the amount of human factor IX antigen in the plasmas from these mice was inhibited by a murine monoclonal anti-human factor IX antibody. By immunoassay, there was no cross-reaction of murine factor IX (normal mouse plasma <0.01 U/ml) with the anti-human factor IX monoclonal antibody. The comparable human factor IX antigen levels and amount of factor IX clotting activity that was inhibited suggest that within the limits of assay variability, the specific activity of recombinant human factor IX in these mice was normal. In separate experiments, citrate was added to heparinized plasmas from the same animals, heparin was removed and factor IX

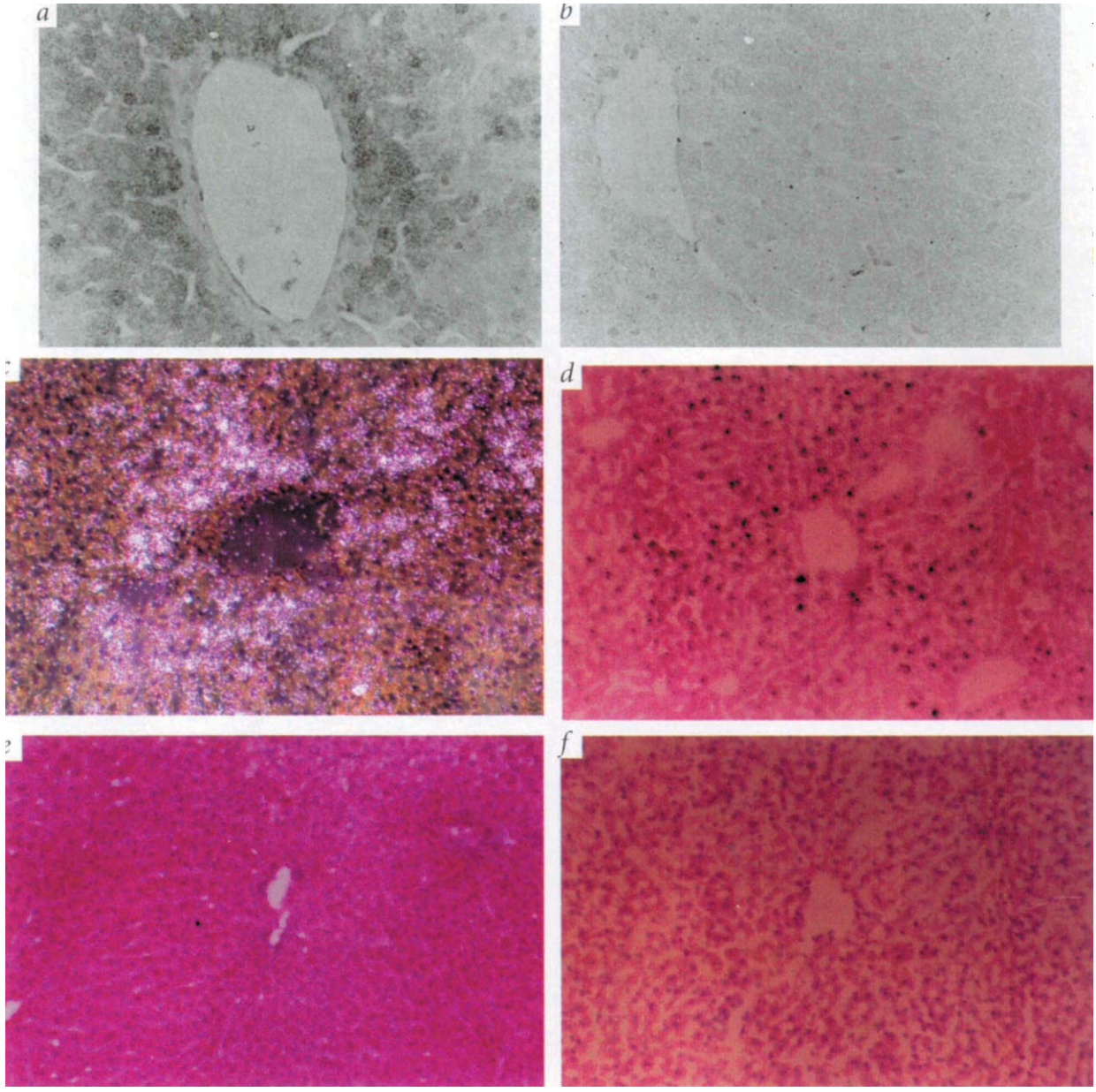

Fig. 3 Identification of transduced cells by immunohistochemistry and *in situ* hybridization. Particles of rAAV-MFG-hTH (1.8×10^{10}) were infused into six mice via a single portal vein infusion. One, three or five weeks later, the livers were analysed for TH by immunohistochemistry. The data from the three-week time point are shown: ***a***, AAV-transduced liver demonstrating TH-positive cells around a portal triad; ***b***, control naive mouse. Two mice infused with 4.2×10^{10} particles of rAAV-MFG-hFIX were sacrificed at 11 weeks, and the hFIX-mRNA signal was detected by *in situ* hybridization. Results from one animal are shown. ***c***, dark field with anti-sense probe; ***d***, light field with anti-sense probe; ***e***, light field with sense probe; ***f***, light field with anti-sense probe (control naive mouse).

Table 1 • Detection and inhibition of human factor IX clotting activity

Inhibitor	NMP (1.0)	NMP (0.9) +NHP (0.1)	Buffer (0.9) +NHP (0.1)	T^1MP (1.0)	T^2MP (1.0)
None (buffer)	0.35 (<0.01)	0.41 (0.10)	0.07 (0.06)	0.68 (0.18)	0.70 (0.20)
+ Antibody*	0.37	0.33	<0.01	0.46	0.49
functional human FIX	[<0.01]	[0.08]		[0.22]	[0.21]

Clotting activities and antigen levels are given in units/ml, where 1.0 is 100% is that of a plasma pool of normal human donors. Values in parentheses are human factor IX antigen levels corrected for dilution as compared to the same normal plasma pool. At the time of analysis, as determined in Fig. 2 (an independent assay), these two animals had ~20% (~1,000 ng/ml) of normal human factor IX antigen. The plasma used from naive mice were of the Balb/c strain. The functional hFIX, shown in brackets, is calculated by subtracting the FIX activity after antibody treatment from the total FIX activity before antibody addition. (Abbreviations: N=normal; M=murine; H=human; T=transduced; P=plasma.) *4 ng murine monoclonal anti-human factor IX^{35} in 5 µl was added to 45 µl of each plasma sample.

clotting activities were determined with and without a polyclonal rabbit anti-human factor IX antibody. In samples from each of the same two transduced mice assayed, the human factor IX activity was completely inhibited by the addition of the polyclonal anti-human factor IX antibody (not shown).

Immunohistochemistry and nucleic acid studies were performed to demonstrate that most of the recombinant AAV transduced the liver, and more specifically hepatocytes. To confirm the presence and estimate the number of AAV genomes in the liver, two mice (one infused with 2.1×10^{10} particles and the other infused with 4.2×10^{10} particles of MFG-hFIX) were sacrificed at week 7 when steady-state factor IX concentrations were achieved. DNA was isolated from their livers and spleens and was then analysed by Southern blot (Fig. 2*a*). Neither mouse had detectable DNA in the spleen, although there were about 1.5 and 3.7 rAAV genomes per diploid genomic equivalents in the livers of mice that received the low and high dose of AAV, respectively. Because the DNA was digested with restriction enzymes that released the internal fragment containing the hFIX cDNA, the status of the DNA (integrated vs episomal) was not determined. A puzzling and still undefined issue is the slow rise of serum factor IX over several weeks. It is possible that during this period, recombinant AAV genomes are incorporated into an appropriate form that is expressed in the nucleus. The rate-limiting step is not known, but it may be related to nuclear entry and unpackaging of the virions, conversion from a single-stranded molecule to a transcriptionally active double-stranded molecule[15,22,23], or integration[6] or concatamerization of the episomal or integrated genome[6,12,24] (Snyder *et al.*, unpublished data). Furthermore, it will be of interest to determine whether the number of transducing genomes amplifies with time and correlates with the slow rise in serum factor IX concentrations. In AAV-transduced muscle cells, β-galactosidase-positive fibers do not increase over time after three weeks[6], whereas expression of erythropoetin does increase[25] (Snyder *et al.*, unpublished data).

To determine the origin of factor IX gene expression, RNA was isolated from various organs, including heart, brain, lung, liver, spleen, muscle, kidney and ovary, of animals receiving 4.2×10^{10} particles of AAV at week 7. RT-PCR analysis revealed a strong signal in the liver but, in contrast to adenovirus[26], not in other tissues (Fig. 2*b*). Although it is not possible to exclude low-level gene expression in other tissues, most hFIX mRNA was present in liver.

An rAAV encoding the human tyrosine hydroxylase cDNA (rAAV-MFG-hTH) was used to determine the transduced cell type in the liver, where the protein may be found in fibers of sympathetic innervation but not in cells. 1.8×10^{10} particles of rAAV-MFG-TH vector were infused into the portal vein of mice. One, three and five weeks later, immunohistochemistry revealed the presence of TH protein exclusively within hepatocytes (Fig. 3*a*,*b*). At one week, there were few fields with any immunostained cells. By weeks 3 and 5, however, positive hepatocytes were found in most fields; groups of immunostained hepatocytes were found around the portal vasculature (Fig. 3*a*), while many of the immunostained cells were randomly scattered throughout the parenchyma (not shown). The TH-positive hepatocytes at three and five weeks represented a small percentage of the hepatocyte population. The low number may indicate a high level of gene expression from relatively few transduced cells. Alternatively, it could result from the elimination of some of the transduced cells by an immune response. To further establish the percentage of transduced cells in mice receiving rAAV-hFIX, *in situ* hybridization for hFIX mRNAs in two mice at 11 weeks had about 2–5% positive hepatocytes in a distribution similar to that seen for the mice receiving rAAV-TH (Figs. 3*c*–*f*). If we assume that 5% of the 10^8 hepatocytes in a mouse liver express factor IX and that, on average, the liver contains two AAV genomes per diploid cell, we estimate that there are 40 genome copies per transduced cell. The alternative is that some cells contain rAAV genomes that do not express transgene product. Nevertheless, these results, taken together, firmly establish that recombinant AAV can transduce hepatocytes *in vivo* and can supply therapeutic concentrations of clotting factor systemically.

Infusion of recombinant adenovirus vectors into the portal vasculature results in an early and more chronic pattern of low-level liver injury as determined by liver enzyme elevation and inflammatory cell infiltrates[27]. This toxicity is the result not of an immune response directed at the viral particle but, rather, of viral antigens whose genes are present in the vector[27]. To establish whether similar toxicity occurs with rAAV, five animals injected with 4.2×10^{10} AAV-MFG-hFIX particles were monitored for serum pyruvic glutamic transaminase (SGPT), a sensitive serum marker for hepatic injury (Fig. 4). The SGPT concentrations were all within the normal range during the first nine days and similar to

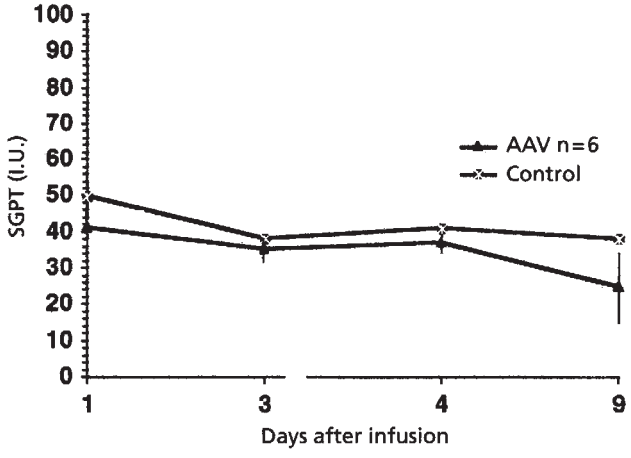

Fig. 4 Liver toxicity. Mice were infused with 4.2×10^{10} particles of rAAV-MFG-hFIX by intraportal infusion. Serum samples were obtained periodically for SGPT. Control mice (no infusion) were sampled at the same times. Mean values are presented with the standard deviations.

those found in a naive control mouse. Similarly, there was no evidence of histological infiltrates in the liver during the first week or at seven weeks after AAV administration (not shown). Thus, in contrast to first-generation adenoviral vectors, no detectable pathology or toxicity is related to the use of rAAV vectors.

The study presented here is encouraging in that a relatively safe delivery system can be used to achieve persistent and therapeutic concentrations of human plasma factor IX. In addition to delivering factor IX, rAAV vectors may be utilized for delivering other proteins both systemically and locally from the liver as a therapy for other diseases[28], including cancer[29]. Further experimentation in larger animal models may be important to determine whether this type of delivery system will be successful in human clinical trials.

Methods

Recombinant AAV vector construction. Standard cloning methods were used for rAAV vector construction. The vector plasmid pSSV9-MFG-hF9 was constructed by digestion of plasmid SSV9-MFG-S-K9F9 with *Age*I and *Bam*H1 to remove the canine factor IX sequences and insertion of the 1.6 kb *Age*I/*Bam*H1 fragment containing the human factor IX sequence from plasmid MFG-S-huFIX (human factor IX). The MFG-S-huFIX vector contains the Moloney murine leukaemia virus (MLV) 5' LTR, adjacent splice donor/acceptor sequences and the huFIX cDNA sequence precisely connected to the MLV env ATG and a poly(A)$^+$ site of bovine growth hormone from pRc/CMV (Invitrogen). The huFIX sequence was obtained from plasmid pAFFIXSVNeo[30] by PCR. The SSV9-MFG-S-K9FIX intermediate vector is derived from pXCJL-K9FIX and SSV9. The SSV9 (also called psub201) plasmid was digested with *Xba*I to remove nearly the entire AAV genome, leaving only the terminal repeats, and filled in by Klenow. The blunt-ended backbone was ligated to a 3.24-kb *Nhe*I-*Sal*I fragment (filled in by Klenow fragment) containing the Moloney murine leukaemia virus (MLV) 5' LTR, adjacent splice donor/acceptor sequences and the canine factor IX (K9FIX) cDNA sequence precisely connected to the MLV *env* ATG from plasmid MFG-S-K9FIX and the poly(A) site of bovine growth hormone from pRc/CMV (Invitrogen). For a description of MFG-S, see Dranoff *et al.*[16].

The vector plasmid pSSV9-MFG-hTH was constructed by digestion of SSV9-MFG-S-K9F9 vector backbone with *Age*I and *Bam*H1 to remove the canine factor IX sequences and insert the human tyrosine hydroxylase cDNA from plasmid pRc/TH/317 (a gift from M. Rosenberg).

Recombinant AAV vector preparation. Recombinant AAV (rAAV) vectors were prepared according to Snyder *et al.*[31] with modifications. Briefly, subconfluent 293 cells[32] were co-transfected with the vector plasmid (described above) and the AAV helper plasmid pACG 2-1 (ref. 33) using the calcium phosphate method. Cells were then infected with adenovirus Ad5dl312 (an E1A$^-$ mutant) at an MOI of 2, and infection was allowed to proceed for 60–72 h. Cells were harvested, and three freeze/thaw cycles were carried out to lyse the cells. The nucleic acid in the lysate was digested with 250 U/ml Benzonase (Nycomed) at 37 °C for 10 min and then centrifuged 1,500 g to pellet the cellular debris. The cell lysate was then fractionated by ammonium sulfate precipitation, and the rAAV virions were isolated on two sequential continuous CsCl gradients. The gradient fractions containing rAAV were dialyzed against sterile PBS, heated for 45 min at 56 °C, and stored at –80 °C.

Determination of rAAV titres. A dot-blot assay was used to determine the total particle titer[31] as follows: The rAAV stock was treated with DNaseI (50 U/ml for 30 min at 37 °C) to degrade any unencapsidated DNA, treated with proteinase K (0.25 mg/ml for 60 min at 37 °C) in the presence of 0.5% SDS and 10 mm EDTA to liberate the rAAV genomes, phenol extracted, ethanol precipitated, denatured in alkali and applied to a nylon membrane. Dilutions of the corresponding vector plasmid were used as standards to determine the rAAV virion copy number. A radioactive probe specific for the transgene (factor IX or TH) was hybridized, the filter was exposed to film and the radioactive regions of the filter were excised and counted in a scintillation counter for quantitation. Titers of total particles were 1×10^{11}–2×10^{12} per milliter, with typical preparations yielding 5 ml.

Recombinant AAV vector characterization. The AAV vector particles banded with a density of 1.42 g/ml on the CsCl gradients as determined by refractometry, which is similar to the density for wild-type AAV[13]. The rAAV preparations were stable to dialysis, and the heat treatment used to inactivate remaining adenovirus by two criteria: 1) the rAAV remained resistent to treatment with DNaseI (50 units/ml for 30 min at 37 °C), and 2) the vectors remained functional *in vitro* and *in vivo*. Thirty microlitres (approximately 1×10^{10} particles) of the vector preparation was separated on a 10% SDS-PAGE gel and stained with Coomassie R250. This analysis showed the correct virion protein composition and presence of less than 10% contaminating cellular and adenoviral proteins.

The presence of contaminating infectious adenovirus was determined by infecting 2×10^6 293 cells (these cells supply the adenovirus E1A gene products needed by Ad5dl312) with 6×10^9 particles (20 µl) of the rAAV preparations and incubating the cells for three days at 37 °C in 5% CO_2. No cytopathic effect was detected. Additionally, an imunofluorescence assay was employed to detect infectious adenovirus as follows: 2×10^5 293 cells were incubated for three days after infection with 6×10^9 particles of rAAV-MFG-hFIX or rAAV-MFG-hTH. As a control, cells were infected with 600, 60, 6 or 0.6 adenovirus particles, which were spiked into 6×10^{10} of an rAAV stock already determined to be free of adenovirus. These cells were stained for adenovirus hexon protein in an immunofluorescence assay (Chemicon). Six adenoviral particles could be detected in the presence of 6×10^{10} control rAAV, and no signal was detected in the rAAV preparations used in the studies presented here.

The presence of contaminating wild-type AAV was determined by a PCR assay. Two hundred microlitres (approximately 6×10^{10} rAAV particles) of the rAAV stock were treated with DNaseI (100 units/ml for 30 min at 37 °C) to degrade any unencapsidated DNA, with proteinase K (0.5 mg/ml for 60 min at 37 °C) to liberate the rAAV genomes, phenol extracted twice, ethanol-precipitated and dissolved in 30 µl of water. 3 µl volume (equilivent to approximately 6×10^9 rAAV particles) was subjected to PCR along with positive and negative controls. The PCR primers were D1 (5'–ACTCCATCACTAGGGGTTCC–3'), which is in the AAV ITR, D2 (5'–GGTAATGATTAACCCGCCATGCTACTTATC–3'), also in the AAV ITR, AAV2S2 (5'–TCAGAATCTGGCGGCAACTCCC–3'), in the AAV rep gene, Splice1 (5'–TCGTCAAAAAGGCGTATCAG–3'), in the AAV splice region, CAP2 (5'–TCCCTTGTCGAGTCCGTTGA–3'), in the AAV cap gene, and CAP1 (5'–CAGAAGGAAAACAGCAAACG–3'), also in the AAV cap gene. PCR was carried out under standard conditions with Taq polymerase (Perkin-Elmer) for 25 cycles using the following primer pairs: D1+AAV2S2 to detect left end sequences in wild-type AAV virus, D2+AAV2S2 to specifically detect left end sequences in wild-type AAV arising from recombination between the vector and helper plasmids, Splice1+CAP2, a primer pair to detect internal sequences and CAP1+D2 to detect right end sequences in both wild-type AAV virus and wild-type AAV arising from recombination between the vector and helper plasmids. The products were separated on a 2% agarose gel and stained with ethidium bromide, and no bands indicative of a wild-type AAV contamination were detected to a sensitivity of one wild-type in 10^9 rAAV. The presence of contaminating infectious wild-type AAV was also determined by a modification of the Replication Center Assay (RCA) as described in Snyder *et al.*[31]. Briefly, 293 cells (5×10^4) were seeded in wells of a 24-well plate. Twenty-four hours later, all wells (now 1×10^5 cells) were co-infected with adenovirus at an MOI of 20 and 10, 5, 2.5 or 1 µl of the rAAV stock. In addition, as a control, adenovirus-infected cells were infected with 1,000, 100, 10 or 1 wtAAV particles, which were spiked into 5×10^9 of a rAAV stock already determined to be wtAAV-free. Cells were incubated for 24 h; the cell culture supernatants (containing any detached cells) were removed and diluted into 10 ml PBS. The cells were detached from the plate with trypsin and combined with their corresponding supernatants. The single-cell suspension was vacuum filtered onto nylon membranes wetted with PBS, and the membranes were incubated cell side up on Whatman filter paper saturated with 0.5 N NaOH/1.5 M NaCl for 5 min at room temperature, followed by Whatman filter paper saturated with 1 M Tris-HCl pH 7.0/2XSSC for 5 min at room temperature. The membranes were then air-dried at room temperature, probed with AAV rep and cap sequences, and exposed to film. No wild-type AAV was detected in the rAAV stock to a sensitivity of 1-in-10^9 rAAV.

Lastly, the rAAV viral preparations were tested *in vitro* by infecting 1×10^5 HeLa or 293 cells with a dilution series of the rAAV stock in the presence and absence of Ad5dl309 at an MOI of 10–20 (ref. 20) for 48 h. For rAAV-MFG-hFIX, cells were incubated in the presence of 1 µg/ml vitamin K and an ELISA was preformed (see below) to detect the presence of huFIX (R+D Systems). For rAAV-MFG-hTH, cells expressing TH were detected

immunohistologically using an anti-TH antibody (Chemicon Mab 318), followed by biotinylated goat anti-mouse (Vector) and streptavidin conjugate (Vector Elite Kit). Detection was by DAB (Biogenix), and positive cells were counted under a microscope.

Animal studies. C57Bl/6 and C57Bl/6 scid mice were obtained from Jackson Laboratory and housed under SPF conditions. Animals were treated according to the NIH Guidelines for Animal Care and the University of Washington. The AAV vectors were injected by through a portal vein catheter[26]. The 200-µl volume was infused over 30 s. Mice were periodically bled by the retro-orbital technique.

Blood analysis. Mouse serum was analysed for total huFIX antigen or γ-carboxylated containing factor IX by an ELISA assay[3] using polyclonal antisera to huFIX[21], and the serum pyruvic glutamic transaminase (SGPT) was analysed as previously described[27]. Samples for factor IX clotting activity assays were collected by periorbital capillary pipette; 90 µl of whole blood was transferred to a microfuge tube containing 10 µl of 0.35% sodium citrate and mixed within 10 s of drawing. Alternatively, the capillary pipette tubes were rinsed with heparin-sodium (1,000 U/ml). Plasmas were obtained by centrifugation and stored at –80 °C before assays. Clotting activities were based on a partial thromboplastin time assay using kaolin activation[34] with a semi-automated coagulation instrument (Coa Screener, American Labour). At least three dilutions of each sample were assayed and the dilution curves were compared to those of a normal human plasma pool (1.0 U/ml is defined as the average normal value, or 100%). For inhibition studies, plasma samples were incubated 10 min at 37 °C with one-tenth volume of dilution buffer or an antibody dilution. A murine monoclonal anti-factor IX antibody[35] gave 50% inhibition when 10 ng was added to 0.1 ml normal human plasma. A polyclonal rabbit anti-factor IX antibody fraction that bound independently of calcium[21] gave 50% inhibition when 15 ng was added.

Protein and nucleic acid analysis. Western-blot analysis was performed as previously described[36]. Seven weeks after intraportal infusion of the rAAV-MFG-hFIX, DNA was isolated from liver and spleen from two rAAV-treated mice and one control. DNA from naive mice with or without added pAAV-MFG-hF9 plasmid DNA was used for positive and negative controls. Ten micrograms of each DNA sample were digested for 16 h with *Bam*H1 and *Xba*I. The DNA was then electrophoresed through a 0.8% agarose gel and transferred to nylon membrane (Hybond N+, Amersham) by electroblotting. The membrane was prehybridized and then hybridized with a huFIX cDNA probe at 65 °C using Rapid-Hyb buffer (Amersham). The final stringency of wash was 0.1×SSC, 0.1% SDS at 65 °C. The probe is an 810-bp huFIX cDNA probe obtained by amplifying the DNA fragment by PCR (oligos as used in RT-PCR below) and labelling to a specific activity of 10^8 cpm/µg with [a-32P] dCTP using a random primer labelling kit (BRL). AAV genomes per diploid genomic DNA were calculated as described, using the mouse metallothonein probe as a DNA loading standard[26].

Immunohistochemistry. For detection of tyrosine hydroxylase protein, deparaffinized formal sections were incubated in diluted (1/500) primary rabbit polyclonal antibody to tyrosine hydroxylase (Eugene Tech International, Ridgefield, NJ #TE101). Detection of the primary antibody employed standard ABC techniques using biotinylated goat anti-rabbit as a secondary and elite ABC horseradish peroxidase (Vector Labs, Burlingame, CA) according to the manufacturer's recommendation. After being washed, the slides were covered with DAB substrate (with nickel chloride added) and developed for 10 min at 37 °C. Slides were washed in distilled water and counterstained with methyl green. After dehydration, the slides were cleared in Histoclear (National Diagnostics, Atlanta, GA) and mounted. Sections of human adrenal medulla were used as positive controls, while normal mouse liver sham inoculate was used as a negative control. To exclude background stain, test livers were also incubated with non-immune pooled rabbit sera instead of primary antibody.

***In situ* hybridization.** We used this technique to detect hFIX-mRNA expression in the rAAV-MFG-hFIX transduced liver cells. The fresh frozen sections were fixed in 3% PFA, followed by several washes in PBS and 2XSSC. The sections were then prehybridized with 0.1 M triethanolamine/0.25% acetic anhydride solution to reduce the background staining, followed by washes in PBS and 2XSSC, and stepwise dehydration in ethanol (70, 80, 95, 100%). The hybridization was carried out in a humid chamber at 55 °C oven for 4 h. The hybridization solution contained 1.5×10^6 cpm of riboprobe in 25 µl of hybridization buffer (40% formamide, 4XSSC, 1 mg/ml tRNA, 1mg/ml sonicated salmon sperm DNA, 4% dextran sulfate, 10 mM DTT, 5X Denhardt's solution).

The riboprobe was synthesized from a plasmid containing a 417-bp *Eco*RV-*Eco*RV fragment of the human factor IX cDNA, which was inserted into *Eco*RV site of pBKS (Stratagene). The following procedure was used to synthesize both the antisense and sense probes. First, the circular plasmid was cut with *Bss*HII to drop out the hFIX fragment flanked by both the T7 and T3 promoters. The riboprobes were then synthesized by mixing the following components in a reaction volume of 20 µl: 1X transcription buffer; 10 mM DTT; 1 µl RNAsin; 500 µM each of GTP, ATP, CTP (Promega Gemini System II); 2 µg of linearized DNA template; 70 µCi[35]S UTP (S.A.>1.100 Ci/mmol, Amersham); 5 U RNA polymerase (T3 for antisense, T7 for sense). The mixtures were incubated for 1 h at 37 °C, followed by the purification of the RNA probe by phenol extraction and ethanol precipitation.

The post-hybridization washes were performed using 50% formamide and 2XSSC at 52 °C, followed by RNAse treatment at 37 °C, and several washes in 2XSSC. Finally, the sections were dehydrated stepwise in ethanol (70, 80, 95, 100%), delipidated with xylene, air-dried, dipped in photographic emulsion (Kodak), exposed at 4 °C for three weeks, developed and counter-stained with haematoxylin-eosin. Sections were then examined and photographed by bright-field and dark-field microscopy (Zeiss).

For negative controls, adjacent sections of transduced tissue were hybridized with a single-stranded 'sense' riboprobe, or non-transduced tissue was probed with the antisense probe.

Organ RT-PCR. PCR was carried out on single-stranded cDNA obtained from RNA isolated from brain, heart, lungs, liver, spleen, kidney, muscle and ovaries. The mRNAs were isolated from tissues using Micro-FastTrack kit (Invitrogen). About 0.3 µg of RNA from each sample was reverse transcribed using a first-strand cDNA synthesis kit from Gibco-BRL. Two microlitres of the sample were amplified in a 50- or 100-µl PCR reaction performed using primers spanning an 810-bp region from exon 2 to 8 of the hFIX cDNA (5'–GATGGAGATCAGTGTGAGTCCAATCCATGT–3' and 5'–AGCCACTTACATAGCCAGATCCAAATTTGA–3'). PCR conditions were 95 °C for 1 m in, 60 °C for 2 m in and 70 °C for 3 m in for 30 cycles. Fifteen percent of the sample was analysed by gel electrophoresis. Control RT-PCR reactions using mouse β-actin primers were carried out in parallel reactions using (CLONTECH PT 1500-1) according to the manufacturer's directions.

Detection of anti-AAV antibodies. Serum (20 µl undiluted, and 1:5 and 1:25 dilutions) taken at three to seven weeks after infusion from rAAV-transduced mice was mixed with 9×10^6 of rAAV-lacZ particles and incubated for 30 min at 37 °C. Control reactions included serum from untransduced mice and anti-AAV guinea pig sera. The reactions were used to infect 1×10^6 293 cells and the infection allowed to proceed for 24 h. The cells were then stained for LacZ activity using standard procedures, and the number of blue cells were scored.

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1. Reiner, A.P. & Davie, E.W. Introduction to hemostasis and the vitamin K–dependent coagulation factors. in *The Metabolic Basis of Inherited Disease* Vol. 3 (eds Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D.) 3181–3221 (McGraw-Hill, New York, 1995).
2. Kay, M.A. *et al. In vivo* gene therapy of hemophilia B: sustained partial correction in factor IX-deficient dogs. *Science* **262**, 117–119 (1993).
3. Kay, M.A. et al. In vivo hepatic gene therapy: complete albeit transient correction of factor IX deficiency in hemophilia B dogs. *Proc. Natl. Acad. Sci. USA* **91**, 2353–2357 (1994).
4. Yang, Y. *et al.* Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* **91**, 4407–4411 (1994).
5. Muzyczka, N. Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr. Top. Microbiol. Immunol.* **158**, 97–129 (1992).
6. Xiao, X., Li, J. & Samulski, R.J. Efficient long-term gene transfer into muscle of immunocompetent mice by an adeno-associated virus vector. *J. Virol.* **70**, 8098–8108 (1996).
7. McCown, T.J., Xiao, X., Li, J., Breese, G.R. & Samulski, R.J. Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res.* **713**, 99–107 (1996).
8. Kaplitt, M.G. *et al.* Long-term gene transfer into porcine myocadium following selective percutaneous coronary artery infusion of an adeno-associated virus vector. *Ann. Thorac. Surg.* 62, 1669–1676 (1996).
9. Kaplitt, M.G. *et al.* Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nature Genet.* **8**, 148–154 (1994).
10. Lalwani, A.K., Walsh, B.J., Reilly, P.G., Muzyczka, N. & Mhatre, A.N. Development of *in vivo* gene therapy for hearing disorders: Introduction of adeno-associated virus into the cochlea of guinea pig. *Gene Ther.* **3**, 588–592 (1996).
11. Flotte, T.R. *et al.* Stable *in vivo* expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc. Natl. Acad. Sci. USA* **90**, 10613–10617 (1993).
12. Afione, S.A. *et al. In vivo* model of adeno-associated virus vector persistence and rescue. *J. Virol.* **70**, 3235–3241 (1996).
13. Carter, B.J. The growth cycle of adeno-associated virus. in *Handbook of Parvoviruses* Vol. 1 (ed. Tijssen, P.) 155–168 (CRC Press, Boca Raton, FL, 1990).
14. Barr, D. *et al.* Strain related variations in adenovirally mediated transgene expression from mouse hepatocytes *in vivo*: comparisons between immunocompetent and immunodeficient inbred strains. *Gene Ther.* **2**, 151–155 (1995).
15. Fisher, K.J. *et al.* Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J. Virol.* **70**, 520–532 (1996).
16. Dranoff, G. *et al.* Vaccination with irridated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulate potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA* **90**, 3539–3543 (1993).
17. Kay, M.A. *et al.* Hepatic gene therapy: persistent expression of human α_1-antitrypsin in mice after direct gene delivery *in vivo*. *Hum. Gene Ther.* **3**, 641–647 (1992).
18. Kay, M.A. *et al.* Expression of human α_1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. *Proc. Natl. Acad. Sci. USA* **89**, 89–93 (1992).
19. Guo, Z.S., Wang, L.H., Eisensmith, R.C. & Woo, S.L.C. Evaluation of promoter strength for hepatic gene expression in vivo following adenovirus-mediated gene transfer. *Gene Ther.* **3**, 802–810 (1996).
20. Baskar, J.F. *et al.* The enhancer domain of the human cytomegalovirus major immediate-early promoter determines cell type-specific -specific expression in transgenic mice. *J. Virol.* **70**, 3207–3214 (1996).
21. Bray, G.L., Weinmann, A.F. & Thompson, A.R. Calcium-specific immunoassays for fator IX: reduced levels of antigen in patients with vitamin K disorders. *J. Lab. Clin. Med.* **107**, 269–278 (1986).
22. Ferrari, F.K., Samulski, T., Shenk, T. & Samulski, R.J. Second-strand synthesis is a rate limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J. Virol.* **70**, 3227–3234 (1996).
23. Alexander, I.E., Russell, D.W. & Miller, A.D. DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. J. Virol. 68, 8282–8287 (1994).
24. McLaughlin, S.K., Collis, P., Hermonat, P.L. & Muzyczka, N. Adeno-associated virus general transduction vectors: analysis of proviral structures. *J. Virol.* **62**, 1963–1973 (1988).
25. Kessler, P.D. *et al.* Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. USA* **93**, 14082–14087 (1996).
26. Vrancken Peeters, M.T.F.D., Lieber, A., Perkins, J. & Kay, M.A. Method for multiple portal vein infusions in mice: Quantitation of adenovirus-mediated hepatic gene transfer. *Biotechniques* **20**, 278–285 (1996).
27. Lieber, A., He, C.-Y., Kirillova, I. & Kay, M.A. Recombinant adenoviruses with large deletions generated by Cre-mediated excision exhibit different biological properties compared with first-generation vectors *in vitro* and *in vivo*. *J. Virol.* **70**, 8944–8960 (1996).
28. Kay, M.A. & Woo, S.L.C. Gene therapy for metabolic disorders. *Trends Genet.* **10**, 253–257 (1994).
29. Su, H., Chang, J.C., Xu, S.M. & Kan, Y.W. Selective killing of AFP-positive hepatocellular carcinoma cells by adeno-associated virus transfer of the herpes simplex virus thymidine kinase gene. *Hum. Gene Ther.* **7**, 463–470 (1996).
30. St. Louis, D. & Verma, I.M. An alternative approach to somatic cell gene therapy. *Proc. Natl. Acad. Sci. USA* **85**, 3150–3154 (1988).
31. Snyder, R.O., Xiao, X. & Samulski, R.J. Production of recombinant adeno-associated viral vectors. in *Current Protocols in Human Genetics* Vol. 1 (eds Dracopoli, N. *et al.*) 1–24 (John Wiley & Sons, New York, 1996).
32. Graham, F.L., Smiley, J., Russell, W.C. & Nairn, R. Characterization of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**, 59–74 (1977).
33. Li, J., Samulski, R.J. & Xiao, X. Role for highly regulated *rep* gene expression in adeno-associated virus vector production. *J. Virol.* (in the press).
34. Thompson, A.R. & Counts, R.B. Removal of heparin and protamine from plasma. *J. Lab. Clin. Med.* **88**, 922–929 (1976).
35. Thompson, A.R. Monoclonal antibody to an epitope on the heavy chain of factor IX missing in three hemophilia B patients. *Blood* **62**, 1027–1034 (1983).
36. Schowalter, D.B., Tubb, J.C., Liu, M., Wilson, C.B. & Kay, M.A. Heterologous expression of adenovirus E3-gp19K in an E1a deleted adenovirus vector inhibits MHC I expression in vitro but does not prolong transgene expression *in vivo*. *Gene Ther.* **4**, 351–360 (1997).