

研究成果の刊行物・別刷

Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Iba promoter: *in vivo* model for platelet-targeting gene therapy

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ABSTRACT Platelets release several mediators that modify vascular integrity and hemostasis. In the present study, we developed a technique for efficient transgene expression in platelets *in vivo* and examined whether this targeted-gene-product delivery system using a platelet release reaction could be exploited for clinical applications. Analysis of luciferase reporter gene constructs driven by platelet-specific promoters (the GPIIb, GPIb α , and GPVI) revealed that the GPIb α promoter was the most potent in the megakaryoblastic cell line UT-7/TPO and human CD34⁺-derived megakaryocytes. Transduction of UT-7/TPO; CD34⁺-derived megakaryocytes; and c-Kit⁺, Sca1⁺, and Lin⁻ (KSL) murine hematopoietic stem cells with a simian immunodeficiency virus (SIV)-based lentiviral vector carrying *eGFP* resulted in efficient, dose-dependent expression of *eGFP*, and the GPIb α promoter seemed to bestow megakaryocytic-specific expression. Transplantation of KSL cells transduced with SIV vector containing *eGFP* into mice showed that there was preferable expression of *eGFP* in platelets driven by the GPIb α promoter [7–11% for the cytomegalovirus (CMV) promoter, 16–27% for the GPIb α promoter]. Furthermore, transplantation of *ex vivo*-transduced KSL cells by SIV vector carrying *human factorVIII* (*hFVIII*) driven by the GPIb α promoter induced the production of detectable transcripts of the *hFVIII* gene and the *hFVIII* antigen in bone marrow and spleen for at least 90 days and partially corrected the hemophilia A phenotype. Platelet-targeting gene therapy using SIV vectors appears to be promising for gene therapy approaches toward not only inherited platelet diseases but also other hemorrhagic disorders such as hemophilia A.—Ohmori, T., Mimuro, J., Takano, K., Madoiwa, S., Kashiwakura, Y., Ishiwata, A., Niimura, M., Mitomo, K., Tabata, T., Hasegawa, M., Ozawa, K., Sakata, Y. Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glyco-

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BLOOD PLATELETS, the principal cells responsible for primary hemostasis, play major roles in thrombosis, atherosclerosis, tumor metastasis, and inflammation. At the site of vascular injury, activated platelets aggregate and release several mediators that modify vascular integrity and hemostasis (1). Platelet-derived bioactive products are released by exocytosis of the three types of granules (dense-core, alpha, and lysosome) within platelets; this process is mediated by soluble NSF attachment protein receptor proteins and VAMP-3 (2). Taking advantage of the platelet-release reaction as a delivery system for a specific factor would be a reasonable approach for therapy for individuals deficient in the factor because it provides a way to enhance the local concentration of target substances at the site of vascular injury, while minimizing the influence of plasma proteins that may inhibit their activities.

In transgenic settings, platelet-targeting gene transfer has been reported to enable the storage of the targeted substance within platelets. Platelet expression of urokinase-type plasminogen activator (u-PA) using a megakaryocyte-specific platelet factor 4 promoter enabled u-PA to be stored in platelets and then released within developing thrombi when the platelets became activated (3). Furthermore, platelet-specific expression of coagulation factor VIII (FVIII) could be achieved in

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a transgenic setting with the resultant FVIII predominantly or exclusively stored in platelet granules rather than being released into the plasma (4). When transgenic mice were crossed onto a FVIII null background, whole blood clotting time was partially corrected (4). These findings have facilitated the development of methods for gene therapy that use platelets to deliver therapeutic agents to the site of vascular injury.

The present study aimed at megakaryocyte- and platelet-directed gene transfer using a platelet-specific promoter that could be directly applicable to gene therapy for not only inherited platelet disorders but also coagulation factor deficiencies such as hemophilia. Since megakaryocytes have a finite life span, hematopoietic stem cells are preferable targets for genetic transfer to establish *in vivo* long-term expression of the target protein in platelets. When retroviral vector containing the *Glycoprotein (GP) IIIa* gene driven by the GPIIb promoter was transduced into CD34⁺ cells from a Glanzmann thrombasthenia patient with defects in the GPIIIa gene, GPIIb/IIIa were detected after *in vitro* megakaryocyte differentiation (5). Furthermore, retrovirus transduction of FVIII driven by the virus promoter into human CD34⁺ hematopoietic stem cells reportedly enabled FVIII-transduced megakaryocytes to store human FVIII with von Willebrand factor (VWF; ref 6). Although the concept of platelet-targeting gene therapy is very attractive and some reports have clinical implications as described above (3–6), there have been no evaluations of whether gene transduction of hematopoietic stem cells effectively results in sufficient genetic information being given in platelets so that they synthesize enough transgene products to correct the phenotype of the hemorrhagic disorder.

In this study, we used GPIIb promoter to achieve platelet-specific gene expression, based on analyses of the promoter activities of three platelet-specific genes, *GPIIb*, *GPIIb α* , and *GPVI*, in megakaryocytes and then we assessed the gene transfer efficiency to platelets *in vivo* by transducing hematopoietic stem cells with simian immunodeficiency lentivirus vector (SIV). We also examined whether FVIII ectopically expressed in platelets corrected the hemorrhagic phenotype of FVIII-deficient mice by using the platelet release reaction.

MATERIALS AND METHODS

Mice

Hemophilic A mice with targeted destruction of exon 16 of the *FVIII* gene were kindly provided by H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA; ref. 7). C57BL/6 (B6-Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1) were purchased from Sankyo-Lab Service (Tsukuba, Japan). All animal procedures were approved by the institutional Animal Care and Concern Committee at Jichi Medical School, and animal care was performed in accordance with the guidelines of the committee (8).

Cytokines and antibodies

Recombinant human thrombopoietin (TPO) and recombinant human stem cell factor (SCF) were gifts from Kirin Brewery (Gunma, Japan). The following materials were obtained from the indicated suppliers: recombinant human interleukin (IL)-6, recombinant human soluble IL-6 receptor (sIL-6R), recombinant human basic fibroblast growth factor (b-FGF), and recombinant human Flt3-Ligand (Flt3-L; PeproTech EC, London, UK); recombinant human IL-3 (IL-3; TECHNE, Minneapolis, MN); antimouse c-Kit monoclonal antibody (MoAb; clone 2B8), antimouse Sca-1 MoAb (clone D7), antimouse Ly5 (CD45) MoAb (clone 30-F11), and antimouse Ly5.1 (CD45.1) MoAb (clone A20; BD Pharmingen, CA); antimouse GPIIb α MoAb (clone Xia.G5; Emfret Analytics, Wurzberg, Germany); and anti-human GPIIb/IIIa MoAb (clone 5B12), anti-human GPIIb α MoAb (clone AN51), and anti-human CD34 MoAb (clone BIRMA-K3; DakoCytomation, Glostrup, Denmark).

Cell culture

The human megakaryoblastic cell line UT-7/TPO was kindly provided by Dr. Norio Komatsu (Yamanashi University, Yamanashi, Japan; ref. 9). The cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 10 ng/ml of TPO. Human umbilical vein endothelial cells (HUVEC) were obtained and maintained as described previously (10). U937, a myelomonocytic cell line, and K562, an erythroleukemic cell line, were obtained from the American Type Culture Collection and were cultured in RPMI 1640 supplemented with 10% FBS. 3T3 fibroblasts were cultured with Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 10% FBS. Human aortic smooth muscle cells (SMC) were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and maintained in DMEM/F-12 with 10% FBS and 10 ng/ml of b-FGF.

Megakaryocyte differentiation

Human umbilical cord blood was obtained during normal full-term deliveries. The institutional review board of Jichi Medical School approved the study protocols, and informed consent was obtained from all donors. Human CD34⁺ cells derived from cord blood were isolated using the AutoMACS magnetic cell sorting system (Miltenyi Biotec., Auburn, CA) according to the manufacturer's instructions. The purity of isolated CD34⁺ cells was >90% (data not shown). CD34⁺ cells were expanded and differentiated into megakaryocytes with IMDM containing 1% fatty acid-free BSA, 200 μ g/ml of human iron-saturated transferrin, and 10 μ g/ml of human recombinant insulin, supplemented with 50 ng/ml of TPO and 10 ng/ml of IL-3 (11). After 14 days of culture, 75–90% of cells were positive for GPIIb/IIIa.

Construction of luciferase reporter plasmid, transient transfection, and luciferase assay

The DNA fragments for the promoter, which reportedly each had maximum promoter activity [GPIIb promoter: –554 to +33 (12), GPIIb α promoter: –254 to +330 (13), GPVI promoter: –320 to +28 (14)], were amplified by polymerase chain reaction (PCR) using human genomic DNA as template. Since the region surrounding the translation start site of GPIIb α gene at position –5 from the initiator ATG codon is important for transgene expression (15), we selected the DNA fragment just before the first ATG sequence, as reported previously (13). The oligonucleotide primer pairs used for

the cloning of the promoter sequence were as follows: 5'-CCATTCCAGAAGGTGTGAAG-3' (sense) and 5'-GTTCTCAGCCCTGTCTCG-3' (antisense) for GPVI promoter (GenBank #AF521646); 5'-CTAAAGCTTGGCTCAAGACG-3' (sense) and 5'-CTTCTTCTTCCACAACCTC-3' (antisense) for GPIIb promoter (GenBank #M33319); and 5'-GTTCTGGGATTACAGGCATGAG-3' (sense) and 5'-GAGGACCTGTGGCAAGGAG-3' (antisense) for GPIIb α promoter (GenBank #M22403).

After being subcloned into pCR-Blunt-TOPO (Invitrogen, Carlsbad, CA), the fragment was subsequently cloned into pGL3-basic, a promoterless luciferase plasmid (Promega, Madison, CA). Five hundred thousand cells (UT-7/TPO, CD34⁺-derived megakaryocytes, K562, and U937) plated in IMDM (without FBS or growth factors) or subconfluent cells (HUVEC and SMC) in each well of six-well plates were transfected with 4 μ g of plasmid DNA using DMRIE-C Reagent (Invitrogen). After 4 h, an equal volume of IMDM supplemented with 2 \times growth factors was added to each well. Cells were incubated for 48 h at 37°C, and luciferase activities were assayed according to the manufacturer's instructions (Luciferase Assay System, Promega).

Construction and production of SIV vectors

Replication-defective self-inactivating (SIN) SIV vector was created by deletion of the U3 region of 3' long terminal repeat (LTR), which contains two NF- κ B sites, three Sp-1 sites, and a TATA box, as described previously (ref. 16; Gene Bank association number: #X07805). The transduction efficiency of the SIN vector did not differ from the intact U3 sequence-containing vector (16).

The full-length *human FVIII (hFVIII)* cDNA was a generous gift from Dr. J.A. van Mourik (Blood Coagulation, Sanquin, Amsterdam, Netherlands), and the *human B domain-deleted (BDD) FVIII (hBDD-FVIII)* cDNA was generated by PCR-based mutagenesis as described previously (17). *eGFP* or *hBDD-FVIII* driven by the CMV promoter (SIV-CMV-eGFP/hFVIII) or GPIIb α promoter (SIV-GPIIb α -eGFP/hFVIII) was inserted between the LTR-containing elements of an SIV-derived vector (see Results).

The gene transfer plasmid was transfected together with three packaging plasmids (encoding *gag-pol*, *rev*, and *VSV-G env*) into 293T cells using Lipofectamine Plus reagent (Invitrogen). After 12 h, the culture medium was replaced to start harvesting virus particles; harvesting was undertaken at 48 h and virus particles were concentrated by ultracentrifugation. Transduction units of SIV vectors carrying *eGFP* were measured by infection of 293 cells followed by measurement of eGFP expression by FACS analysis. The average infectivity of the SIV-CMV-eGFP vector was in the range of 2–5 $\times 10^8$ TU/ml. To compare viral infectivity between different promoters or targeted genes, viral particle titer was simultaneously measured by real-time quantitative reverse transcriptase (RT)-PCR. Viral RNA was isolated using QIAamp viral RNA mini kit (QIAGEN, Valencia, CA), and the isolated RNA was reverse-transcribed using SuperScript II (Invitrogen). Quantification of vector particles was performed by measuring copies of vector-specific post transcriptional regulatory element derived from the wood-chuck hepatitis virus (WPRE) sequences by real-time quantitative PCR using the QuantiTect Probe PCR system (QIAGEN). The WPRE sequence was amplified with WPRE forward primer 5'-GCTTTTCATTTCTCTCCTT-3' and WPRE reverse primer 5'-GGCCA-CAACTCCTCATAA-3'. The FAM-labeled probe sequence was 5'-ATCCTGGTTGCTGTCTC-3'. The PCR started with an initial incubation step of 15 min at 95°C. Thermal cycling consisted of 45 cycles of 94°C for 15 s, 56°C for 30 s, and 76°C for 30 s. During the annealing phase of PCR, reporter fluorescent dye from a specific probe was detected with a ABI

PRISM 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA). The standard curve of the viral titer was estimated by serial dilution of the gene transfer vector plasmid. The average virus particle titer of the SIV-based vector was in the range of 1–2 $\times 10^{10}$ TU/ml. The infectivity of SIV vectors [multiplicity of infection (MOI)] was estimated from the ratio of particle titer to SIV-CMV-eGFP.

For the transduction of UT-7/TPO and CD34⁺-derived megakaryocytes with SIV vectors, 1 $\times 10^5$ cells were resuspended in 100 μ l of culture medium containing 8 μ g/ml of polybrene. Cells were transduced with SIV vectors at various MOIs indicated in the test for 24 h. Cells were then resuspended in 300 μ l PBS containing 0.5% BSA and 2 mM EDTA, and eGFP-positive cells were subjected to FACS analysis. When CD34⁺ cells were transduced with SIV containing *eGFP* followed by differentiation of megakaryocytes *in vitro*, we could not observe sustained eGFP expression during the differentiation. This was probably due to the short life span of the eGFP-expressing differentiating cells *in vitro*. Hence, CD34⁺-derived megakaryocytes, at an indicated day after the start of differentiation, were transduced with SIV to examine the influence of megakaryopoiesis on promoter activity.

Hematopoietic stem cell isolation and viral transduction

Bone marrow cells obtained from mouse femur and tibia were depleted for cells expressing lineage cell markers B220, CD5, CD11b, Gr-1, and Ter-119 by magnetic sorting using a Lineage Cell Depletion kit (Miltenyi Biotec.) and then sorted for Sca-1⁺ and c-kit⁺ cells (KSL) by FACS (FACSAria Cell Sorter, Becton-Dickinson).

When freshly isolated KSL cells were directly infected with SIV vectors, PI-positive cells (dead cells) increased after the transduction and the efficacy of the transduction decreased (data not shown). Hence, we examined the culture conditions of KSL cells to improve SIV transduction and cell viability. When isolated KSL cells were cultured with IL-3, IL-6, and SCF, SIV transduction of the eGFP gene into KSL cells resulted in lower transduction efficiencies (25–35%: MOI of 30) and a significant increase in PI-positive cells (20–30%; data not shown). On the other hand, high proportions of eGFP positive cells were obtained by culturing the KSL cells with SCF, IL-6, sIL-6R, Flt-3L, and TPO for 3–7 days (see Results), and PI-positive cells after eGFP transduction were significantly decreased (8–12%). Hence, KSL cells were precultured with IMDM 1% fatty acid-free BSA, 200 μ g/ml of transferrin, and 10 μ g/ml of insulin, supplemented with 100 ng/ml of SCF, 10 ng/ml of TPO, 100 ng/ml of IL-6, 100 ng/ml of Flt-3L, and 400 ng/ml of sIL-6R before viral transduction in accordance with a method for human CD34⁺ expansion (18). Infection of cells with SIV was carried out in a plate coated with 50 μ g/ml of RetroNectin (TakaraBio, Tokyo, Japan). Cultured KSL cells (1 $\times 10^5$ or 1 $\times 10^6$ cells) were resuspended in 100 μ l or 1 ml of IMDM with 10% FBS. The cells were transduced with SIV vectors at various MOIs as indicated in the text for 12 h in the presence of the same cytokine combination and incubated at 37°C. Since the use of polybrene (up to 4 μ g/ml) *per se* did not significantly improve the transduction efficiencies of mouse KSL and human CD34⁺ cells (data not shown), our procedure for stem cell transduction was performed without polybrene. Cells were then resuspended in 100 μ l of PBS containing 1% BSA for stem cell transplantation or incubated for the indicated number of days for FACS analysis.

Stem cell transplantation

Bone marrow cells were obtained from B6-Ly5.1 to allow us to distinguish between donor and recipient cells. Recipient mice

(8–12 wk old B6-Ly5.2) were irradiated with a single lethal dose of 9.5 Gy (^{60}Co , Gamma Cell; Norton International, Ontario, Canada). One hundred thousand cultured KSL cells from B6-Ly5.1 without or with SIV transduction were injected together with 5×10^5 freshly isolated Ly5.2 unfractionated bone marrow cells. Since the transplantation procedure without unfractionated bone marrow cells increased mortality to 60–80%, we simultaneously transplanted KSL cells with non-transduced bone marrow cells as competitor cells. To assess the reconstitution of bone marrow, peripheral blood was drawn from the retro-orbital sinus with heparin-coated micropipettes and analyzed for the percentages of Ly5.1 (donor-derived) lymphoid and myeloid cells by flow cytometry. In our transplantation procedure, engraftment by Ly5.1 cells was 40–55% in lymphoid and myeloid cells 4 months after transplantation. For secondary transplantations, 2×10^6 unfractionated marrow cells collected from primary recipient mice 4 months after primary transplantation were used to reconstitute lethally irradiated recipients (9.5 Gy). The animals with relatively high eGFP levels after transplantation were chosen as donors for the secondary bone marrow transplantation.

Detection of the transcripts of hFVIII transgene

Detection of *hFVIII* transgene transcripts was performed by RT-PCR (19). RNA was isolated from mouse organs using an RNA isolation kit (RNeasy Protect Kit; QIAGEN). RNA samples were subjected to RT-PCR using a pair of primers for *hFVIII* (19) and an RT-PCR kit (SuperScript One-Step RT-PCR System, Invitrogen). A primer pair for mouse *GAPDH* mRNA (R&D Systems, Minneapolis, MN) was used in the control RT-PCR experiments.

Quantification of SIV vector-derived mRNA expression was examined by real-time quantitative RT, using the QuantiTect Probe RT-PCR system (QIAGEN). Mouse *GAPDH* (mGAPDH) control reagents (QIAGEN) were used to estimate the amount of RNA analyzed. A standard curve was established by analyzing duplicate aliquots of serial dilutions of SIV gene transfer vector. Fifty nanograms of purified RNA from tissues as indicated in the text were used as a template. For each sample, the amounts of WPRE sequences compared with the standard control curve were estimated, and the quantities of the WPRE sequences were determined by dividing the copy number of the WPRE sequences by those of the mGAPDH sequences.

Integration of vector sequence into genomic DNA

Genomic DNA was extracted from bone marrow cells of mice 4–5 months after transplantation using a DNA isolation kit (DNeasy Tissue Kit; QIAGEN). WPRE sequence derived from the SIV vector was amplified using real-time quantitative PCR with a QuantiTect Probe PCR system as described above. For each sample, the amounts of WPRE sequences compared with the standard control curve were estimated, and the integration of the vector sequence was calculated from dividing the copy number by the cell count corresponding to the DNA quantity.

Immunohistochemistry

Bone marrow cells attached to glass slides using a Cytospin3 (Shandon, ThermoShandon) were fixed with 3% paraformaldehyde in PBS and then permeabilized with 0.2% Triton X-100. After being blocked with 1% BSA, samples were incubated with polyclonal anti-hFVIII antibody (Ab) conjugated with biotin at 4°C for 2 h, washed with PBS, and then

incubated with streptavidin conjugated with Alexa 594 (Molecular Probes, Eugene, OR) and FITC-labeled anti-mouse GPIIb α MoAb. Immunofluorescent staining was observed and photographed using a fluorescent microscope with an attached camera.

For detection of hFVIII molecules in mouse tissues by immunohistochemistry, the spleen was fixed with 4% paraformaldehyde in PBS for 2 h at 4°C, incubated with PBS containing sucrose (10–30%), and then frozen in the presence of OCT compound in dry ice/ethanol. Sections were prepared from frozen tissues at -25°C and attached to polylysine-coated glass slides. For detection of hFVIII, tissue sections were blocked with 1% rabbit serum in PBS containing Triton-X 100 (0.1%) and incubated with sheep polyclonal anti-human FVIII Ab (Cedarlane Laboratories, Homby, Ontario, Canada) at 4°C for 16 h. After being washed with PBS, sections were incubated with biotin-conjugated rabbit anti-sheep IgG Ab followed by the avidin-biotin complex (ABC) reagents (Vectastain ABC Elite kit; Vector, Burlingame, CA) and a 3,3'-diaminobenzidine kit (Vector).

Measurement of hFVIII antigen, neutralizing antibodies, and phenotypic correction

hFVIII antigens were measured by an anti hFVIII-specific ELISA kit (Affinity Biological, Hamilton, Ontario, Canada) and compared with those in pooled human normal plasma. Two hundred and seventy microliters of whole blood were drawn from the superior vena cava of anesthetized mice using a syringe containing 30 μl of sodium citrate and 1 μM PGI $_2$ to inhibit platelet activation. When indicated, 1 μM phorbol myristyl acetate (PMA) and 50 $\mu\text{g}/\text{ml}$ of collagen were added to the blood to activate platelets. After centrifugation, the platelet-poor plasma was frozen at -80°C until assayed for FVIII antigen. Analyses of neutralizing antibodies against hFVIII developed in mice were performed by the Bethesda method as described using FVIII deficient plasma and normal pooled plasma (8). Phenotypic correction was tested in some of the transplanted mice by anesthetizing them with diethyl ether and clipping 1.5 cm of their tails. The mice were then observed for survival after 24 h.

RESULTS

Comparison of luciferase reporter expression driven by platelet-specific promoters

To achieve efficient expression of genes targeted in platelets, we first compared the promoter activities of three platelet-specific genes, *GPIIb*, *GPIb α* , and *GPVI*, in megakaryocytic cells. **Figure 1A** shows a schematic diagram of the platelet-specific promoters used in this study with their unique regulatory elements. The luciferase reporter gene was used to compare promoter activities among different promoters. We used the nucleotide region in promoters that had the highest activity in previous studies (12–14). The *GPIb α* promoter directed the most powerful expression of luciferase in UT-7/TPO cells, a megakaryoblastic cell line (Fig. 1B), and its relative efficiency was even more marked in CD34 $^+$ -derived megakaryocytes (Fig. 1C). Since *GPIIb* and *GPVI* were reportedly expressed in endothelial cells (20,21), we examined whether these platelet-specific promoter activities were stimulated in

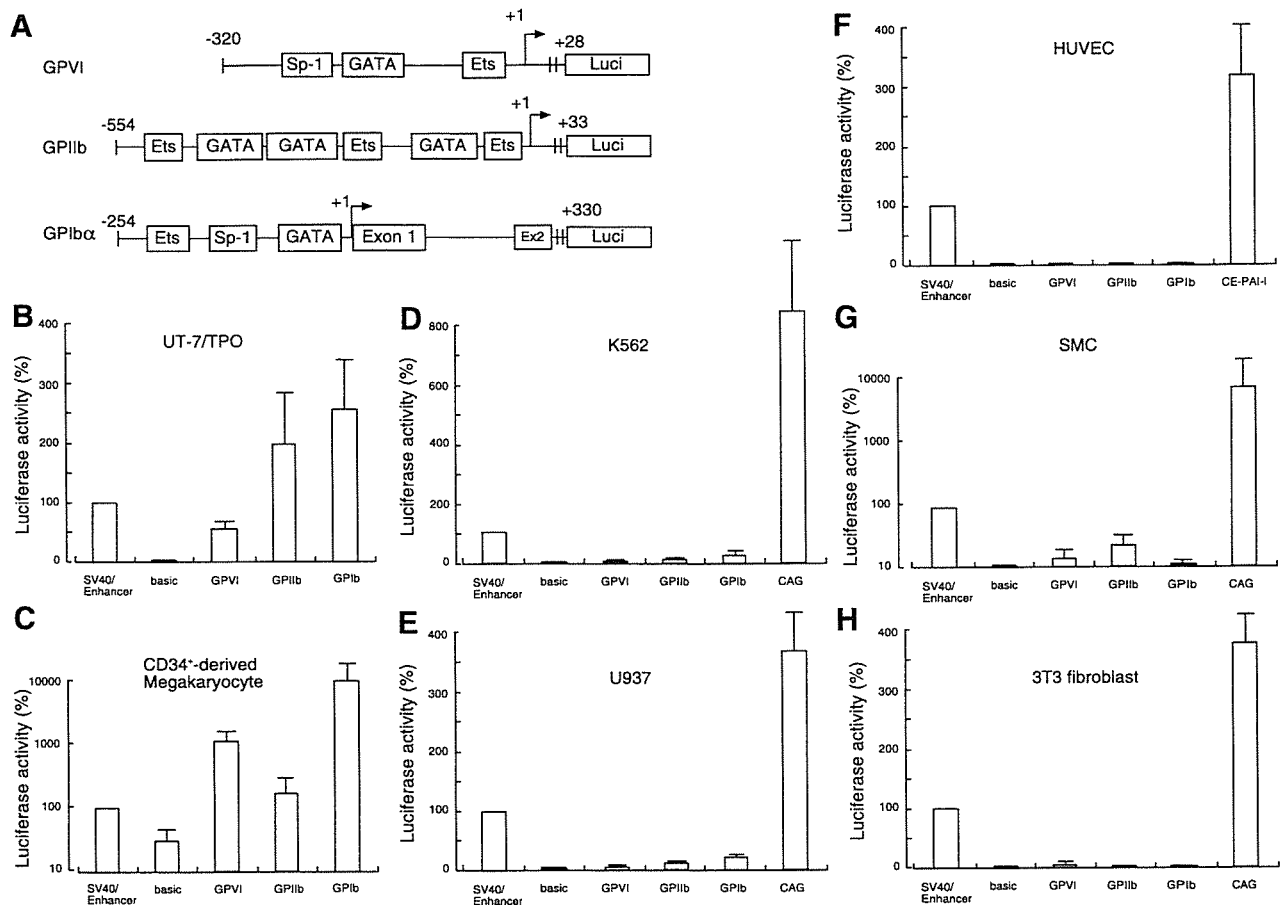


Figure 1. Comparison of platelet-specific promoter activities. *A*) A schematic presentation of platelet-specific promoters used in experiments is shown. Each construct along with a promoter-less vector (basic) or a positive control vector (SV40/Enhancer) was transfected into UT-7/TPO (*B*), CD34⁺-derived megakaryocytes (*C*), K562 (*D*), U937 (*E*), HUVECs (*F*), SMC (*G*), and 3T3 fibroblasts (*H*). Luciferase activities were measured 48 h after transfection and are shown relative to the activity driven by the SV40 promoter (SV40/Enhancer). Each experiment was carried out 5 times with duplicate samples. Columns and error bars are mean \pm SD.

endothelial cells. Under conditions in which the PAI-1 promoter (22) efficiently directed luciferase expression, the GPIIb, GPIb α , and GPVI promoters did not direct luciferase expression in HUVECs (Fig. 1). We further examined promoter specificity by using other cells including K562, an erythroleukemic cell line; U937, a myelomonocytic cell line; human aortic artery SMC and 3T3 fibroblast cell. In these cells, the platelet-specific promoters drove less reporter gene than SV40/Enhancer and CAG promoter (Fig. 1). Hence, the specificities of the platelet glycoprotein promoters did not differ, and the activity of GPIb α promoter was the strongest in megakaryocytes.

Efficient transformation of megakaryocytic cells by SIV-based vectors

Next, we constructed SIV-based lentiviral vectors containing the *eGFP* gene under the control of either the CMV promoter (SIV-CMV-eGFP), GPIb α promoter (SIV-GPIb α -eGFP), GPIIb (SIV-GPIIb-eGFP), or GPVI (SIV-GPVI-eGFP). The transgene located downstream

of the promoter was inserted between the LTR-containing elements of a SIV-derived vector (Fig. 2A). To increase gene expression in the transduced cells, a post-transcriptional regulatory element derived from woodchuck hepatitis virus (WPRES) was inserted downstream of the gene expressed (Fig. 2A). To investigate *eGFP* gene transduction of megakaryocytes, UT-7/TPO or CD34⁺-derived megakaryocytes were cultured for 24 h in the presence of various concentrations of the indicated SIV vector. SIV-GPIIb-eGFP, SIV-GPIb α -eGFP, SIV-GPVI-eGFP, and SIV-CMV-eGFP efficiently transduced the *eGFP* gene into UT-7/TPO and CD34⁺-derived megakaryocytes (Fig. 2B, C). There were no significant differences in *eGFP* expressions among cells transfected with SIV-GPIIb-eGFP and SIV-GPIb α -eGFP (Fig. 2B and C).

We next investigated whether *ex vivo* megakaryocyte differentiation affects gene expression. *eGFP* expression in CD34⁺ hematopoietic progenitor cells transduced with SIV-GPIb α -eGFP or SIV-GPVI-eGFP was <10%, whereas \approx 20% of the cells transduced with SIV-GPIIb-eGFP were positive for *eGFP* (day 0 in Fig. 2D), indicating that the GPIIb promoter worked at an

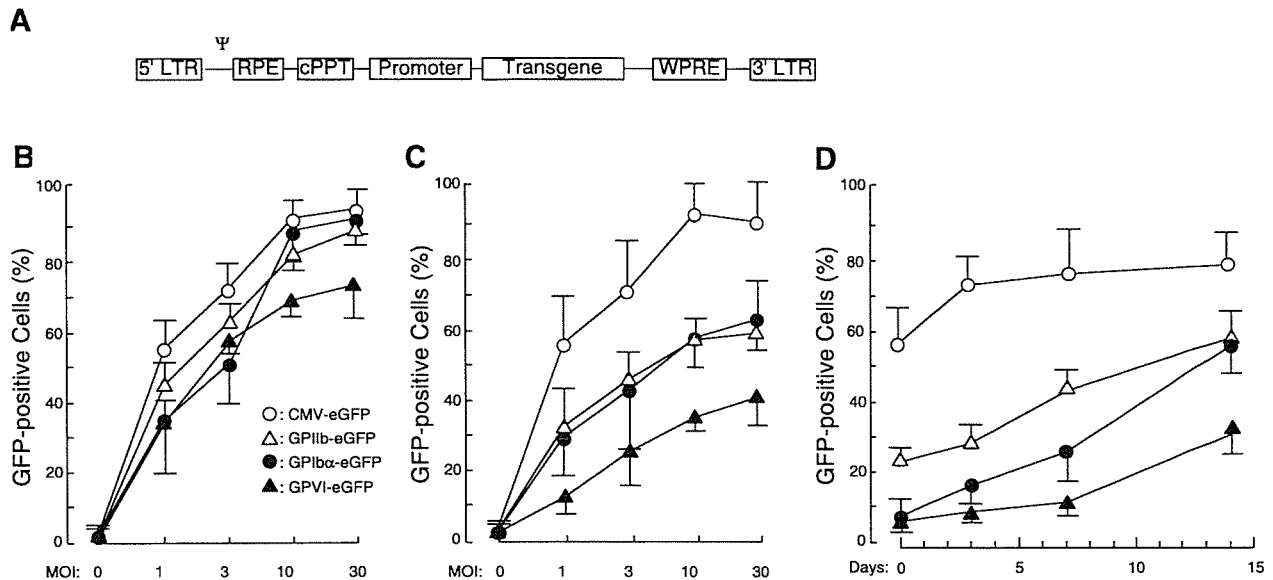


Figure 2. Expression of eGFP in UT-7/TPO cells and CD34⁺-derived megakaryocytes transduced with SIV vectors carrying the *eGFP* gene driven by the CMV, GPIIb, GPVI, or GPIb α promoter. *A*) A schematic diagram of SIV constructs used in this study is shown. UT-7/TPO (*B*) and CD34⁺-derived megakaryocytes (*C*) were infected with SIV-CMV-eGFP (open circle) SIV-GPIb α -eGFP (closed circle), SIV-GPIIb-eGFP (open triangle), or SIV-GPVI-eGFP (closed triangle) at indicated MOI. Expression of eGFP in cells was analyzed by flow cytometry. Columns and error bars are mean \pm SD ($n=3$). *D*) CD34⁺-derived megakaryocytes at day 0, 3, 7, and 14 after start of differentiation were transduced with SIV-CMV-eGFP (open circle) SIV-GPIb α -eGFP (closed circle), SIV-GPIIb-eGFP (open triangle), or SIV-GPVI-eGFP (closed triangle) at MOI of 30. Columns and error bars are mean \pm SD ($n=3$).

earlier stage of megakaryopoiesis. Furthermore, the GPIb α compared with the GPIIb promoter seemed to work during a later phase of megakaryocyte maturation, and the percentages of eGFP expression did not change after differentiation (day 14 in Fig. 2*D*). eGFP expression driven by the CMV promoter was not affected by megakaryocyte differentiation (Fig. 2*D*). We selected the GPIb α promoter as the platelet-specific promoter for *in vivo* experiments because the promoter activity of GPIb α was the strongest in differentiated megakaryocytes (Fig. 1) and the promoter drove in the later phase of megakaryopoiesis (Fig. 2).

Establishment of efficient transduction of KSL cells with SIV vector

We next optimized the transduction protocol for KSL cells by using an SIV vector containing the *eGFP* gene driven by the CMV promoter. The transduction efficiency of eGFP in cultured KSL cells reached 60–80% (Fig. 3*A*). The plateau value for transduction was observed with a MOI of 10–30. One day (24 h) after incubation with the viral vector was sufficient to achieve the efficient expression of the transduced gene (Fig. 3*B*). eGFP expression then gradually declined (Fig. 3*B*); the decrease might have been due to the reduction in cell viability, because PI-positive cells (dead cells) increased with time (data not shown). Although lentiviral vectors can express transgenes for long periods even in the absence of integration in CD34⁺ cells (23), KSL cells could not maintain eGFP expression for long

periods *in vitro*. Accordingly, we cultured KSL at an MOI of 30 for 24 h and transplanted the cells into recipient mice in the following experiments.

Preferential eGFP expression in platelets using SIV vectors harboring GPIb α promoter *in vivo*

To compare the strength and the specificity of the CMV and GPIb α promoters and to assess eGFP transduction by SIV vectors *in vivo*, KSL cells transduced with SIV-CMV-eGFP or SIV-GPIb α -eGFP were transplanted to recipient mice (Ly5.2). One hundred thousand cultured KSL cells (Ly5.1) transduced with SIV-CMV-eGFP or SIV-GPIb α -eGFP (MOI of 30) were transplanted together with 5×10^5 competitor cells (Ly5.2). When KSL cells transduced with SIV-CMV-eGFP were transplanted, eGFP expression was observed in 35–45% of CD45⁺ cells and 7–11% of platelets in peripheral blood (Fig. 4*A* and *B*). Interestingly, transduction of the SIV vector harboring the GPIb α promoter resulted in efficient gene marking to platelets (16–27%); however, only marginal eGFP expression was observed in CD45⁺ and red blood cells (Fig. 4*A*, *B*). We next analyzed bone marrow cells from transplanted mice using specific markers to identify macrophages, granulocytes, B lymphocytes, T lymphocytes, and erythroblasts. Whereas eGFP was expressed in these lineages of cells of mice that received KSL cells transduced with SIV-CMV-eGFP, the GPIb α promoter drove just marginal eGFP expression in these cell lineages, confirming the specificity of its activity in megakaryocytes and platelets *in vivo* (Fig.

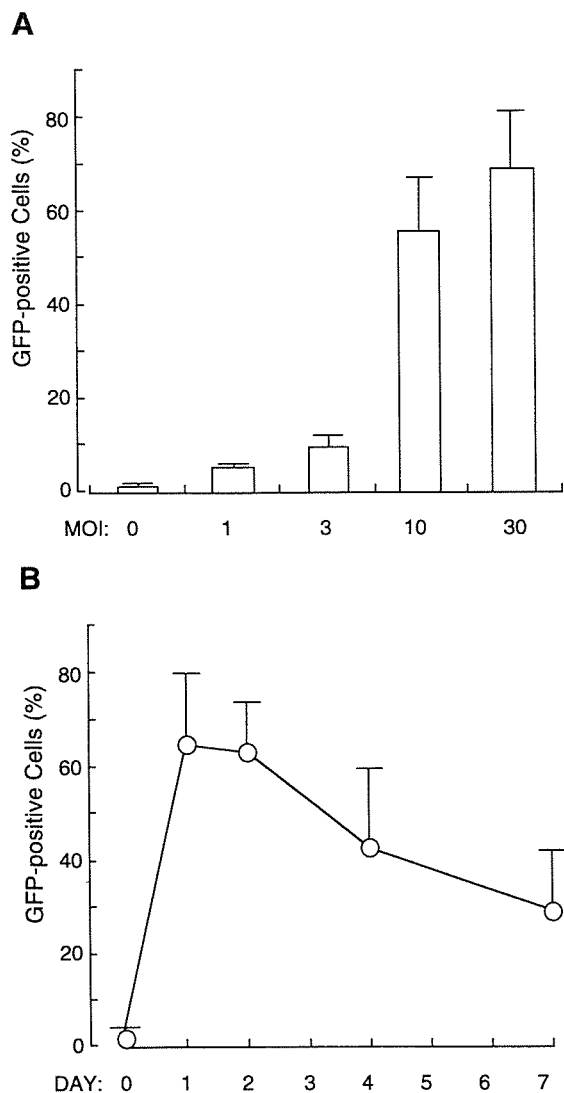


Figure 3. Transduction of KSL cells with SIV-CMV-eGFP. Cultured KSL cells were transfected with increasing concentrations of SIV-CMV-eGFP for 24 h (A) or with a fixed concentration (MOI=30) for various incubation times (B). After indicated incubations, expression of eGFP in KSL cells was analyzed by flow cytometry. Percentages of transduced cells expressing eGFP are shown. Columns and error bars are mean \pm SD ($n=3$).

4C). We next performed a second bone marrow transplantation using marrow cells obtained from mice that had been transplanted 4 months earlier. As shown in Fig. 4D, eGFP expression driven by the CMV and GPIb α promoters in hematopoietic cells was sustained after the second stem cell transplantation, indicating that the promoter maintains transgene expression during differentiation of hematopoietic stem cells.

Expression of hFVIII and phenotypic correction in hemophilia A mice transplanted with KSL cells transduced with SIV-GPIb α -hFVIII

To determine whether platelet-directed gene therapy enables sustained expression of FVIII, we constructed

two SIV-based vectors containing *hBDD-FVIII* cDNA under the control of either the CMV (SIV-CMV-hFVIII) or GPIb α promoter (SIV-GPIb α -hFVIII). We transplanted mice with 1×10^5 transduced KSL cells after lethal γ -irradiation. We first analyzed the presence of the *hFVIII* gene transcripts in organs of the transplanted recipients at 3 months after transplantation. Analyses for *hFVIII* transcripts driven by the CMV promoter revealed that the *hFVIII* gene was expressed mainly in bone marrow and to a lesser extent in the spleen (Fig. 5A, middle panel). Interestingly, *hFVIII* mRNA was predominantly found in both the spleen and bone marrow in the recipients of KSL cells transduced with SIV-GPIb α -hFVIII (Fig. 5A, lower panel). To quantify the mRNA expression concentration in each organ, vector-specific WPRE expression was measured by real-time quantitative RT-PCR. As expected, based on the results from RT-PCR, bone marrow and spleen are the major sites for mice transplanted with KSL cells transduced with SIV vectors (Fig. 5B). Furthermore, we examined vector integration into the genome of bone marrow hematopoietic cells after transplantation and detected that 0.19–2.3 vector copies/genome were integrated in cells of transplanted mice (CMV promoter: 1.07 ± 0.95 ; $n=4$; GPIb α promoter: 0.98 ± 0.62 ; $n=4$). In accordance with the data on *hFVIII* transcripts, hFVIII molecules were immunohistochemically detected in bone marrow and the spleen in both types of transduced mice (Fig. 6). Interestingly, cells expressing GPIb α concurrently expressed hFVIII in bone marrow obtained from mice transduced with SIV-GPIb α -hFVIII (Fig. 6A).

Finally, we evaluated whether platelet-specific gene transduction using SIV-GPIb α -hFVIII resulted in phenotypic correction of FVIII-deficient hemophilia A mice. The plasma FVIII antigen concentration with or without platelet activation was measured in transplanted FVIII-deficient mice at 30 and 60 days after transplantation. We detected FVIII activity in the transplanted mice in which 1–2% correction was noted in the plasma of mice transplanted with KSL cells transduced with SIV-GPIb α -hFVIII (Fig. 7A). When platelets were stimulated with collagen and PMA, the plasma FVIII concentration increased to 2–3.5% (Fig. 7A). The mortality rate after tail clipping was significantly improved in transduced mice (Fig. 7B). Furthermore, ectopically expressed hFVIII levels had not attenuated, and the appearance of inhibitor against hFVIII was not detected in mice transplanted with KSL cells transduced with SIV-GPIb α -hFVIII at day 60 after the transplantation (data not shown). We simultaneously performed transplantation experiments using SIV-CMV-hFVIII. Plasma levels of hemophilia A mice transplanted with KSL transduced with SIV-CMV-hFVIII were 3–6% after the transplantation, and phenotypic correction was also observed, as reported previously (19, 24, 25).

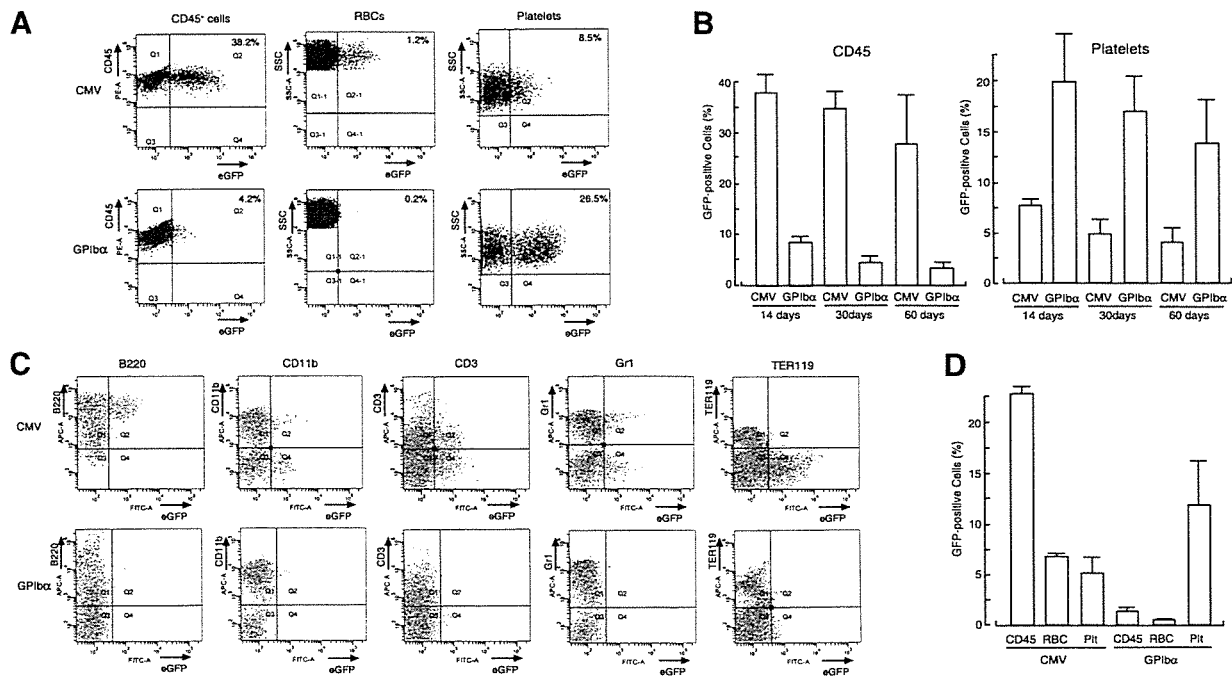
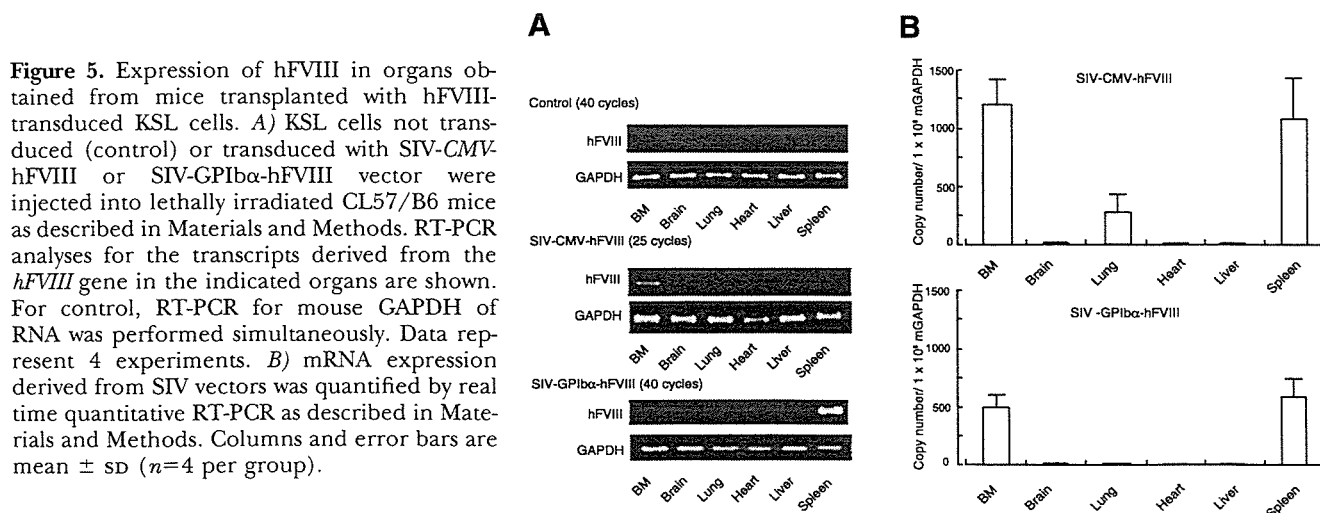


Figure 4. Effect of promoter differences on eGFP expression in blood cells *in vivo*. Cultured KSL cells were transduced with SIV-CMV-eGFP or SIV-GPIIb α -eGFP at a MOI of 30. Each irradiated mouse received 100,000 transduced cells together with 5×10^5 unfractionated whole marrow cells. **A)** Representative flow cytometry analyses of eGFP-positive cells in CD45⁺ lymphocytes and granulocytes, red blood cells (RBCs), and platelets in peripheral blood are shown. **B)** Percentages of eGFP-positive cells in CD45⁺ cells (*left*) and platelets (*right*) 14, 30, and 60 days after transplantation are shown. Columns and error bars are mean \pm SD ($n=5$ per group). **C)** 60 days post-transplantation, bone marrow cells were stained using antibodies to detect B lymphocytes (B220), T lymphocytes (CD3), granulocytes (Gr1), macrophages (CD11b), and erythroblasts (TER119). GFP-positive cells in each lineage cells are measured by flow cytometry. Data represent 3 experiments. **D)** Flow cytometric analyses of CD45⁺ cells, RBCs, and platelets in peripheral blood obtained from mice 30 days after second bone marrow transplantation. Columns and error bars mean \pm SD ($n=5$ per group).

DISCUSSION

In this study, we examined the gene transduction of platelets and megakaryocytes by using an SIV lentiviral vector harboring a platelet-specific promoter *in vivo*. Since the strategy of using platelets as potential targets for producers of transgene products has already been proposed in transgenic mice (3, 4), it was shown that it

is possible to apply this strategy to correct hemorrhagic disorders including hemophilia by efficient platelet-directed gene transduction *in vivo*. However, detailed comparisons of platelet-specific promoters and the efficiency of the transduction of transgenes *in vivo* have not previously been reported. In our system, the transduction of hematopoietic stem cells with an SIV lentiviral vector resulted in the expression of the transgene



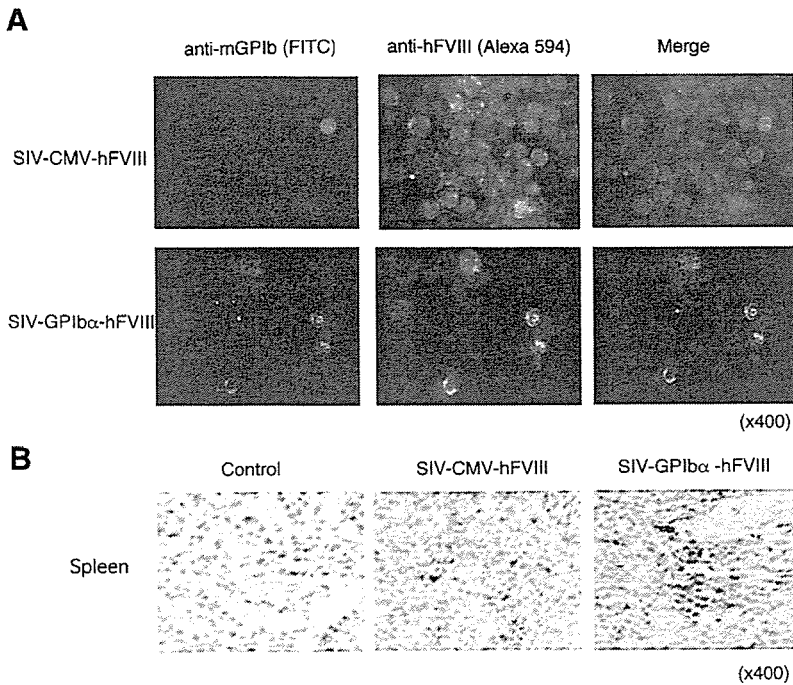


Figure 6. Expression of hFVIII in bone marrow and spleen in transplanted mice. A) Isolated bone marrow cells from mice transplanted with KSL cells transduced with SIV-CMV-hFVIII or SIV-GPIb α -hFVIII were immunostained for mouse GPIb α (left) and hFVIII (middle); 2 images are overlapped in right column, showing that in SIV-GPIb α -hFVIII-transduced bone marrow cells, GPIb α and FVIII expression overlapped. B) Immunohistochemistry for hFVIII in spleen of each transplanted mice (positive stain: brown). For control, sections of spleen obtained from mice transplanted with KSL cells without vector infection were processed simultaneously with anti-FVIII antibodies. Original magnification $\times 400$.

in $\approx 20\%$ of platelets and also resulted in a phenotypic correction of hemophilia A mice, suggesting that platelet-targeting gene therapy has the potential for further clinical applications. This is a first study to achieve a phenotypic correction of a coagulation abnormality such as hemophilia A by using platelet-directed gene transduction.

Megakaryocytes have a finite life span of ≈ 10 – 21 days (26); therefore, hematopoietic stem cells are a more practical target than megakaryocytes for genetic

transfer to establish long-term expression of a target protein in platelets. Because lentiviruses are capable of infecting certain types of quiescent cells, there has been significant interest in the application of lentivirus-derived vectors to the transduction of hematopoietic cells; indeed, it has been shown that lentiviral vectors can efficiently transduce hematopoietic stem cells (27). We used the SIV lentiviral system for efficient platelet-targeting gene transduction because of its probable safety. The SIV lentiviral system was derived from SIVagmTYO1 and is nonpathogenic to its natural host and to experimentally infected Asian macaques (16). Replication-competent virus particles were not detected in vector-infected cells, and the risk of development of replication-competent lentivirus particles in HIV carrier patients may be significantly lower than that for the HIV-based vectors (19). Accordingly, SIV vectors have a safety advantage for clinical applications of gene therapy.

Most reported studies have used the GPIIb promoter for megakaryocyte- and platelet-specific gene transduction. We used the GPIb α promoter as a platelet-specific promoter in this study because the promoter activity of GPIb α was more potent than that of GPIIb in UT-7/TPO and CD34 $^{+}$ -derived megakaryocytes. Another reason we selected this platelet-specific promoter was that the GPIb α promoter works at a late stage of megakaryopoiesis. Although the *GPIIb* gene is expressed in platelets and megakaryocytes, it is an early gene for megakaryopoiesis (28). In conditional knockout mice in which the thymidine kinase gene was driven by the GPIIb promoter, the administration of gancyclovir led to a dramatic reduction in the platelet count (29). In bone marrow, erythroid and myeloid progenitors were also affected, which indicated the presence of *GPIIb* in progenitor cells (29). Indeed, 18% of human CD34 $^{+}$

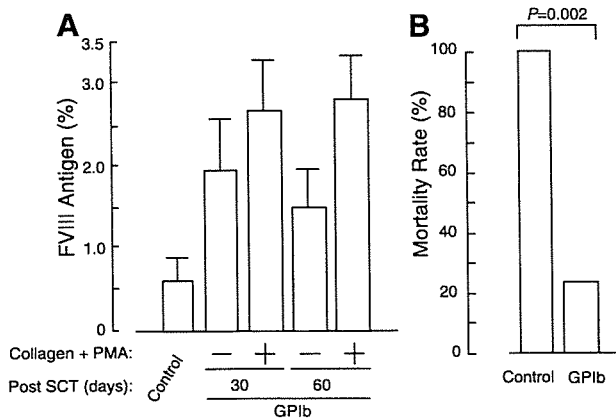


Figure 7. Phenotypic correction of hemophilia A mice by platelet-targeting gene delivery. A) Blood from FVIII-deficient mice transplanted with KSL cells transduced with SIV-GPIb α -hFVIII was stimulated without or with 50 $\mu\text{g}/\text{ml}$ of collagen and 1 μM PMA for 15 min. After centrifugation, platelet-poor plasma was obtained, and hFVIII antigen levels were measured by ELISA. Columns and error bars are mean \pm SD ($n=4$ per group). B) Mortality rate within 24 h after tail clipping in mice transplanted with KSL cells transduced with control or SIV-GPIb α -hFVIII ($n=10$ for control; $n=8$ for GPIb α). Mortality rate was statistically evaluated by a χ^2 test.

hematopoietic stem cells already expressed GPIIb, and so the appearance of GPIb was markedly delayed as compared with that of GPIIb, indicating that GPIb is a later marker of megakaryocytic maturation. Platelet-targeting gene therapy using the GPIb α promoter was therefore expected to allow more specific and restricted expression of gene products in platelets than that using the GPIIb promoter.

Another important finding here was that the eGFP gene driven by the CMV promoter showed significantly decreased expression in platelets, despite the high transduction efficiencies of CD45⁺ cells *in vivo*. Generally, the reduction of transgene expression caused by a shortened protein half-life is even more pronounced in terminally differentiated blood cells (30). The decreased expression might have been mediated by the down-regulation of the transgene during differentiation; the stability of the encoded protein is at least as relevant for the expression of a transgene as the choice of the promoter or *cis*-elements influencing RNA processing in differentiated cells (30). In this context, the use of the GPIb α promoter, which drives expression in late megakaryocyte differentiation, might be important for gene transduction of terminally differentiated anucleate platelets.

Our strategy of platelet-directed gene transduction has potential for not only inherited platelet disorders (such as Glanzmann's thrombasthenia and Bernard-Sourlier syndrome) but also other hemorrhagic disorders. Hemophilia A is an X chromosome-linked bleeding disorder caused by defects in the *FVIII* gene and affecting \approx 1:5000 males (31). Hemophilia is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well vary over in a broad range (5–100%; ref 31). Although sustained therapeutic expression of FVIII has been achieved in preclinical studies using a wide range of gene transfer technologies targeted at different tissues (32), emergence of neutralizing Ab often limits their clinical applications (33). The targeting of hematopoietic stem cells is not an exception. Although lentiviral FVIII gene transduction of hematopoietic stem cells is able to produce therapeutic levels of FVIII (19, 24, 25, 34), the emergence of neutralizing antibodies to FVIII has resulted in decreased levels of FVIII activity (34). Platelet-directed gene therapy for hemophilia A has a possible advantage for therapeutic applications, because the use of the platelet-specific system may limit the development of inhibitors by preventing the expression of FVIII in antigen presenting cells. Furthermore, 10–30% of populations with hemophilia A develop inhibitors to infusion products, which leads to the disruption of coagulation and severe bleeding (31). Under these conditions, platelet-directed gene therapy of hemophilia A is very attractive because platelets could specifically store the protein in the bloodstream and then specifically release it at sites of thrombus formation, thereby minimizing the influence of any circulating inhibitors. For further clinical application, the long-

term observations are required to substantiate long-term *in vivo* gene expression because our observation periods were limited in this study.

During the course of this study, the therapeutic expression of GPIIb/IIIa in GPIIIa-deficient mice using HIV-lentivirus vector containing GPIIIa cDNA under the control of the GPIIb promoter was reported (35). That study used a heterogeneous population of bone marrow cells as a source for stem cell transplantation and gene transduction. We demonstrated efficient transduction of KSL murine hematopoietic cells by a SIV vector harboring the GPIb α promoter and phenotypic correction of hemophilia A mice. Primitive KSL cells are a nearly homogeneous population, and a single KSL cell frequently can provide long-term multilineage engraftment of lethally irradiated mice (36). Targeting of primitive hematopoietic stem cells is thought to be a safer approach, because the number of transduced cells needed for reconstitution is much lower than that needed when using a heterogeneous bone marrow population. The development of leukemia in two children with severe combined immunodeficiency disease who were transplanted with retroviral vector-transduced bone marrow cells caused renewed concern about the risks associated with the integration of proviral sequences into chromosomal DNA (37). One way to possibly reduce the risks of insertional mutagenesis would be to use transduction protocols that minimize the total number of genetically modified cells (38). From this aspect, our procedure using KSL cells transduced with SIV lentiviral system is a practical approach for platelet-specific gene modification in clinical applications.

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REGULAR ARTICLE

Phenotype correction of hemophilia A mice with adeno-associated virus vectors carrying the B domain-deleted canine factor VIII gene[☆]

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Abstract Adeno-associated virus (AAV) vectors carrying the B domain-deleted canine FVIII (BDD cFVIII) gene utilizing the β -actin minimum promoter (167b) pseudotyped with serotype 1 (AAV1- β -actin-cFVIII) and serotype 8 (AAV8- β -actin-cFVIII) were developed to express cFVIII in hemophilia A mice. FVIII clotting activities measured by the APTT method increased in hemophilia A mice with intramuscular injection of AAV1- β -actin-cFVIII in a dose-dependent manner. Therapeutic FVIII levels (2.9–1.0%) in hemophilia A mice with the AAV1- β -actin-cFVIII dose of 1×10^{12} gc/body were achieved, suggesting partial correction of the phenotype with AAV1- β -actin-cFVIII vectors. FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8- β -actin-cFVIII also were increased dose-dependently, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8- β -actin-cFVIII doses of $1-3 \times 10^{11}$ gc/body and supernormal FVIII levels (180–670%) in hemophilia A mice with the AAV8- β -actin-cFVIII dose of 1×10^{12} gc/body. Transduction of the liver with AAV8- β -actin-cFVIII is superior to transduction of skeletal

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muscles with AAV1cFVIII regarding the FVIII production and antibody formation. These data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have a potential for hemophilia A gene therapy.

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Introduction

Hemophilia A is an inherited X-linked life-threatening bleeding disorder caused by abnormalities in the factor VIII (FVIII) gene that lead to deficiency of FVIII and bleeding diathesis. Hemophilia is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well be in a wide range (5–100%) without strict gene regulation [1–3]. Gene therapy is expected to provide an alternative to current FVIII supplemental therapy because it may be able to prevent lethal intracranial bleeding episodes and provide a good quality of life without bleeding. Among a variety of vectors, Adeno-associated virus (AAV) vectors are thought to be ideal for transfer of therapeutic genes since they are derived from non-pathogenic viruses and have been demonstrated to provide sustained transgene expression in non-dividing cells with little toxicity, although delivery of the FVIII gene using AAV vectors is limited by their small packaging capacity [4].

The dual AAV vector system that utilized two AAV2 vectors separately carrying the FVIII heavy chain gene and the FVIII light chain gene was successful for expressing functional FVIII molecules and correction of phenotypes of hemophilia A mice [5]. A recent report has shown that construction of single AAV vectors carrying the 4.5-kb B domain-deleted (BDD) canine FVIII (cFVIII) gene can be packaged in AAV vectors using the 543-base (b) DNA fragments composed of the insulin-like growth factor binding protein (IGBP) promoter, an enhancer element, and an intron, although the packaging efficiency for incorporation of the cFVIII gene into the AAV vectors was low [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact, minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy, whereas no liver dysfunction was observed upon injection of the same vector into the skeletal muscles in the same series of clinical trials [7,8]. Although the precise mechanisms of these phenom-

ena have not yet been elucidated, the T cell response to viral capsid was thought to be one of the causes of the liver injuries [8]. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs.

To explore the possibility that skeletal muscles transduced with AAV vectors could produce FVIII resulting in increase of FVIII levels in the circulation, we packaged the BDD FVIII gene in AAV vectors using the same promoter and compare production of FVIII in the skeletal muscles and in the liver that were transduced with AAV vectors. We developed AAV vectors carrying the BDD cFVIII gene utilizing the β -actin minimum promoter (167b) and tried to express canine FVIII in hemophilia A mice. Recent studies on recombinant AAV vectors have shown that AAV serotypes have tropism, suggesting that a specific AAV serotype vector can be used for gene delivery to certain organs [3,6,9]. AAV serotype 1 (AAV1) may be the best AAV serotype for transduction of skeletal muscles and the AAV serotype 8 (AAV8) is superior to other AAV serotypes for transduction of the liver [6,9], thus in this study, we constructed AAV1 and AAV8 vectors carrying the BDD cFVIII gene and studied efficacies of these vectors for FVIII transgene expression, long term transgene expression, and neutralizing antibody formation to the transgene products in the hemophilia A mice.

Materials and methods

Vector construction

The full-length human FVIII (hFVIII) cDNA was a generous gift from Dr. J.A. van Mourik (Blood Coagulation, Sanquin, Amsterdam, Netherlands) and the human B domain deleted (BDD) FVIII (hFVIII) cDNA was generated by PCR-based mutagenesis as described [10,11]. The canine FVIII (cFVIII) cDNA was a generous gift from Dr. Brownlee (Chemical Pathology Unit, University of Oxford, UK) and the BDD canFVIII cDNA also was generated by PCR-based mutagenesis. The intervening amino acid sequence of the heavy chain and the light chain of BDD cFVIII was RSFS⁷⁴³-Q¹⁶³⁰NPPVSK. The CAG promoter is a chimeric promoter, composed of the CMV enhancer, the chicken β -actin promoter,

and an intron, was derived from pCAGGS [12]. The chicken β -actin minimum promoter (-155~+12, 167 b) was generated by PCR, cloned in pCR2.1 TOPO (Invitrogen), and sequenced. Plasmid vector p1.1c, composed of the CMV promoter, human growth hormone gene intron 1, and the SV40 polyadenylation signal sequences, was kindly supplied by Avigen Inc. The DNA fragments spanning the CMV promoter and the human growth hormone intron of p1.1c were replaced with the CAG promoter, the phosphoglycerokinase 1 (PGK1) promoter, or the β -actin minimum promoter DNA fragments to make plasmid p1.1CAG, p1.1PGK1, or p1.1 β -actin, respectively. The DNA fragments encoding the BDD hFVIII cDNA or the BDD cFVIII cDNA were cloned in the downstream of the respective promoter sequences of these plasmids to make p1.1CMV-hFVIII, p1.1CAG-hFVIII, p1.1PGK1-hFVIII, p1.1 β -actin-hFVIII, and p1.1 β -actin-cFVIII, respectively. The Lac Z gene was cloned in the downstream of the β -actin promoter to make plasmid p1.1 β -actin Lac Z. DNA fragments spanning the promoter, the LacZ gene, and the polyadenylation signal sequence of pAAV2 Lac Z (Stratagene) were replaced with DNA fragments spanning the β -actin promoter, the BDD cFVIII cDNA, and the SV40 polyadenylation signal sequences of p1.1 β -actin-cFVIII to make the gene transfer vector pAAV2- β -actin-cFVIII in which these DNA fragments were flanked by ITR sequences of AAV serotype 2 (AAV2) as described previously [9,13]. The gene transfer vector pAAV2- β -actin-Lac Z equipped with AAV2 ITRs was also constructed. The chimeric packaging plasmid for AAV8 capsid pseudotyping was a generous gift from Dr. James M. Wilson (Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, PA) [6]. The packaging plasmid composed of the AAV2 rep gene and the cap gene derived from AAV1 for AAV1 capsid pseudotyping was described previously [9].

AAV vector production

Viral vectors were packaged with AAV1 or AAV8 capsid by pseudotyping. The FVIII gene or the Lac Z gene located in the downstream of the β -actin minimum promoter and flanked by AAV2 ITRs was packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, kindly supplied by Avigen Inc., with the chimeric packaging plasmid, the adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA), and gene transfer plasmid vectors (pAAV2- β -actin-cFVIII or pAAV2- β -actin-Lac Z) as described previously [9,13]. For virus vector purification, the DNase (Benzonase, Merck Japan, Tokyo, Japan)-treated virus particle

containing samples were subjected to two rounds of iodixanol-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM EDTA at 21 °C as described [9]. Titration of recombinant AAV vectors was carried out by quantitative dot-blot hybridization using the 32 P-labeled probes [9,13].

Analysis of the β -actin minimum promoter activity

Expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1 β -actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII by the calcium phosphate coprecipitation method was studied to show that the β -actin minimum promoter had an enough FVIII expression activity. After incubation with the DNA containing media for 6 h, HEK 293 cells were incubated further in DMEM/HAM F-12 media supplemented with 10% fetal bovine serum for 48 h at 37 °C in the presence of 5% CO₂. FVIII clotting activities in the conditioned media of HEK 293 cells harvested after 48 h incubation were quantified by the activated partial thromboplastin time (APTT) method using FVIII deficient plasma. FVIII activities were expressed as the percentages of normal control plasma.

Animal experiments

FVIII-deficient mice (Hemophilia A mice) with targeted destruction of exon 16 of the FVIII gene were previously reported by Bi et al. [14] and generously given to us by Dr. H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA). J1 ES cells were used for targeted destruction of the FVIII gene and blastocysts derived from C57BL/6 mice were used to generate chimaeras [14]. C57BL/6 wild-type mice were purchased from SLC Inc. Mice were maintained in a standard lighting condition in a clean room. All surgical procedures were carried out in accordance with guidelines approved by the institutional Animal Care and Concern Committee at Jichi Medical School [15]. Male hemophilia A mice and male wild-type C57BL/6 mice were used in the experiments. Blood was drawn from the cervical vein plexus of mice and mixed with 1 / 10 volume of 3.8% sodium citrate, and then platelet-poor plasma was prepared by centrifugation. AAV8 vectors were injected intravenously into the cervical vein plexus while AAV1 vectors were injected directly to the skeletal muscles of lower extremities of mice under anesthesia with isoflurane [15]. Cyclophosphamide (100 μ g/body/day, SIGMA-ALDRICH Japan, Tokyo, Japan) and tachrolimus (12.5 μ g/body/day, Fujisawa Pharmaceuticals Co., Tokyo, Japan) were

given (s.c.) to mice daily after vector injection as the immunosuppressant.

Analysis of cFVIII expression in mice

FVIII activities were measured by the activated partial thromboplastin time (APTT) method used for determination of plasma FVIII activities of hemophilia patients utilizing human FVIII-deficient plasma as described [15]. Antigen levels of cFVIII in mouse plasma were determined by ELISA (Asserachrom FVIII:C: Ag, Diagnostica Stago, Parsippany, NJ) as described [6]. Analyses of neutralizing antibodies against cFVIII developed in mice were performed by the Bethesda method as described using FVIII deficient plasma and normal canine plasma. Detection of the transcripts of cFVIII transgene was performed by RT-PCR [10,11]. RNA was isolated from the mouse organs using an RNA isolation kit (RNeasy Protect kit; Qiagen Inc., Valencia, CA). DNase I (Amplification grade, Invitrogen, Carlsbad, CA)-treated and heat-treated RNA samples were subjected to RT-PCR using a pair of primers (sense: 5'-GTTGGAGCACAACT-GACTTCC-3', antisense: 5'-CAATTGAGAAGGTGTCAT-CATACTC-3') for cFVIII and an RT-PCR kit (SuperScript One-Step RT-PCR System, Invitrogen). PCR amplification (25–30 cycles) for cFVIII was performed as described [10,11]. A primer pair for mouse GAPDH mRNA (R&D Systems, Inc., Minneapolis, MN) was used instead of cFVIII primers in the control RT-PCR experiments. For detection of cFVIII molecules in mouse tissues by immunohistochemistry, the skeletal muscles and the liver were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4 °C, incubated with PBS containing sucrose (10–30%), and then frozen in the presence of OCT compound in dry ice/ethanol. Sections were prepared from frozen tissues at –25 °C and attached to poly-lysine coated glass slides. For detection of FVIII, tissue sections were blocked with 1% rabbit serum in PBS containing Triton-X 100 (0.1%) and incubated with sheep polyclonal anti-human FVIII antibodies (Cedarlane Laboratories Ltd, Hombly, Ontario, Canada) at 4 °C for 16 h [10,11]. After washing in PBS, sections were incubated with biotin-conjugated rabbit anti-sheep IgG antibody followed by the ABC reagents (Vectastain ABC Elite kit; Vector, Burlingame, CA) and a DAB kit (Vector).

Detection of β -galactosidase in mouse tissues

To analyze Lac Z gene expression in mice injected with AAV- β -actin-Lac Z, mouse tissues were fixed with 2% paraformaldehyde in PBS for 5 min, washed with PBS, incubated with PBS containing sucrose

(10–30%), and frozen with OCT compound (Tissue-Tek; Miles, Inc., Elkhart, IN) in dry ice/ethanol. Sections, prepared from frozen tissues at –25 °C and attached to poly-lysine coated glass slides, were incubated in PBS containing 1 mg/ml X-gal, 2 mM MgCl₂, 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 0.01% Na deoxycholate, 0.1% Triton X-100 at 25 °C for 1 h [10]. Some sections further were processed for the Feulgen reaction (red purple) to visualize nuclei.

Results

Expression of FVIII by the β -actin minimum promoter

We studied expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1 β -actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII. FVIII clotting activities detected in the conditioned medium of HEK293 cells are shown in Fig. 1. Expression of FVIII driven by the β -actin minimum promoter (167 b) in the 293 cells was approximately 1/3–1/2 of that by the CMV promoter (1 kb) or the CAG promoter (1.7 kb). Although the β -actin minimum promoter is weaker than the CMV promoter and the CAG promoter, it

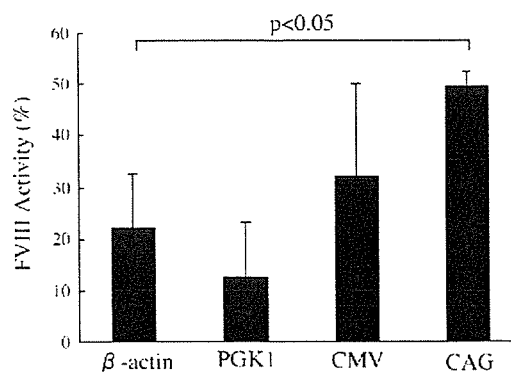


Figure 1 FVIII expression by the β -actin minimum promoter in vitro. FVIII clotting activities expressed in the conditioned media of HEK 293 cells transfected with p1.1CMV-FVIII, p1.1 CAG-FVIII, p1.1 PGK1-FVIII, or p1.1 β -actin-FVIII after 48 h incubation are shown. FVIII clotting activities in the conditioned media were quantified by the APTT method using FVIII deficient plasma and FVIII clotting activities were expressed as the percentages of normal control plasma. There were no FVIII activities in the conditioned media of HEK 293 cells with mock transfection. There was a significant difference of FVIII activity levels in the conditioned media of HEK 293 cells transfected with p1.1 CAG-FVIII and those in the conditioned media of HEK 293 cells transfected with p1.1 β -actin-FVIII ($n=4$, Student's t -test, $p<0.05$).

was stronger than the PGK1 promoter (515 b) by 1.2-fold in terms of FVIII expression activity. Since the β -actin minimum promoter was stronger than the PGK1 promoter and was short enough to construct 5.1-kb AAV vectors carrying the BDD FVIII cDNA, we used the β -actin minimum promoter to produce AAV vectors carrying the BDD FVIII gene.

Expression of Lac Z gene by the β -actin minimum promoter in vivo

To confirm that the β -actin minimum promoter can express a transgene in vivo, AAV vectors carrying the Lac Z gene located in the downstream of the β -actin minimum promoter (AAV1- β -actin-Lac Z, AAV8- β -actin-LacZ) were injected to wild-type mice and expression of the Lac Z gene was studied by X-gal staining. When AAV1- β -actin-Lac Z was injected to the skeletal muscles of lower extremities of wild-type mice, Lac Z gene expression was observed in muscle fibers as shown in Fig. 2A. No apparent Lac Z gene expression was observed in other organs in the AAV1- β -actin-Lac Z injected mice (not shown), suggesting that transgene expression in other organs was minimum. Lac Z gene

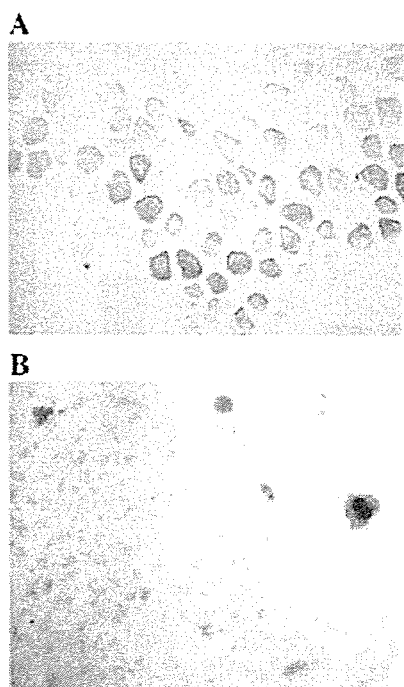


Figure 2 Expression of the Lac Z gene in mice transduced with AAV vectors carrying the Lac Z gene located downstream of the β -actin minimum promoter. X-gal staining of the skeletal muscles of mice with intramuscular injection of AAV1- β -actin-Lac Z (A) and of the liver of mice with intravenous injection of AAV8- β -actin-Lac Z (B) is shown.

expression of mice with intravenous injection of AAV8- β -actin-Lac Z mainly was observed in the liver as shown in Fig. 2B. Lac Z gene expression also was observed in other organs including the heart, lung, and skeletal muscles in accordance with the previous report [16]. The liver could be transduced with intravenously injected AAV8- β -actin-Lac Z almost as efficiently as intraportally injected vectors (not shown).

Expression of FVIII by AAV vectors carrying the BDD cFVIII gene

AAV1- β -actin-FVIII vectors were injected into skeletal muscles of hemophilia A mice and AAV8- β -actin-FVIII vectors were intravenously injected into the cervical vein plexus of hemophilia A mice. FVIII clotting activities of citrated plasma drawn from mice were measured by the APTT method using FVIII-deficient human plasma.

FVIII clotting activities in mouse plasma increased on days 14 and 28 after AAV1 vector injection. The increase of FVIII clotting activities on day 28 after injection was dose-dependent. The FVIII activity levels in peripheral blood increased to $2.9 \pm 1.0\%$ in hemophilia A mice with the AAV1- β -actin-cFVIII dose of 1×10^{12} gc/body (Fig. 3), suggesting partial correction of the phenotype with AAV1- β -actin-cFVIII vectors. After these periods, FVIII activities decreased to the basal levels of mice before vector injection. FVIII antigen levels increased in parallel with levels of FVIII activity, confirming expression of cFVIII transgene in mice (not shown). Analyses for antibody against transgene products showed that neutralizing antibodies developed in 4 out of 6 tested mice by week 12 after vector injection, although the antibody titers were not high (Table 1). The RT-PCR analysis and the immunohistochemistry study suggested the presence of the transgene transcripts and products in the vector-injected muscles, suggesting that decrease of FVIII levels may be accounted for by the presence of neutralizing antibody to cFVIII.

FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8- β -actin-cFVIII also were increased dose-dependently on day 28, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8- β -actin-cFVIII doses of $1-3 \times 10^{11}$ gc/body and supernormal FVIII levels (180–670%) were achieved in hemophilia A mice with the AAV8- β -actin-cFVIII dose of 1×10^{12} gc/body (Fig. 4). These data on AAV8 vector-transduced FVIII expression were almost comparable with the results of the previous study using the single AAV8 vector carrying the BDD cFVIII gene [6], suggesting that β -actin minimum promoter almost

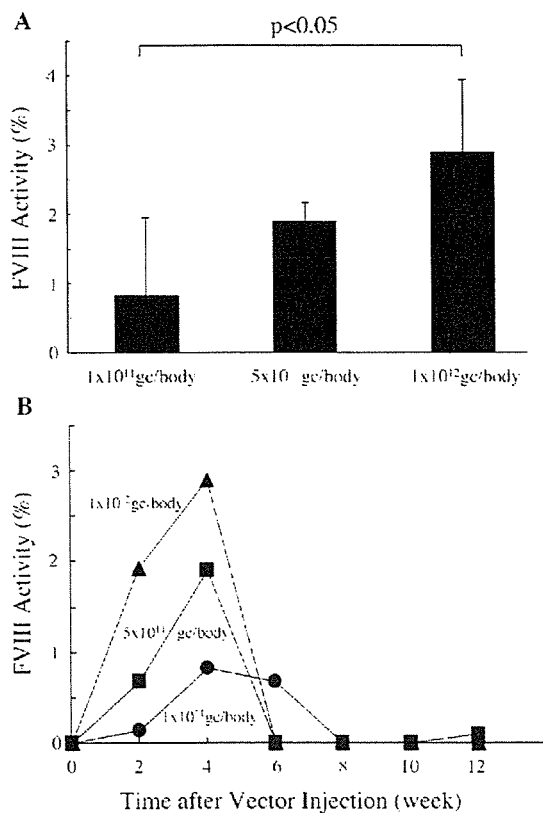


Figure 3 FVIII levels in plasma of hemophilia A mice after intramuscular injection of AAV1- β -actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$) on day 28 after intramuscular injection of AAV1- β -actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice with injection of AAV1- β -actin-cFVIII (circles, 1×10^{11} gc/body; squares, 5×10^{11} gc/body; triangles, 1×10^{12} gc/body) are shown in panel B.

worked as efficiently as the chimeric IGBP promoter complexes. High-level expression of FVIII in the vector-injected hemophilia A mice was sustained for more than 12 weeks. No apparent neutralizing antibody developed during the 12-week period after vector injection (Table 1). FVIII antigen levels also increased in parallel with FVIII activity levels, confirming expression of the cFVIII transgene in mice (not shown). The antigen levels of cFVIII determined by the ELISA for human FVIII were approximately 1/5 of the FVIII activity levels

Table 1 Neutralizing antibodies against cFVIII developed in hemophilia A mice

	Inhibitor positive mouse	Bethesda units/mL
AAV1cFVIII	4/6 (66.7%)	9.4 ± 9.5^a
AAV8cFVIII	0/9 (0%)	Not detected

^a Neutralizing antibodies detected by week 12 after vector injection.

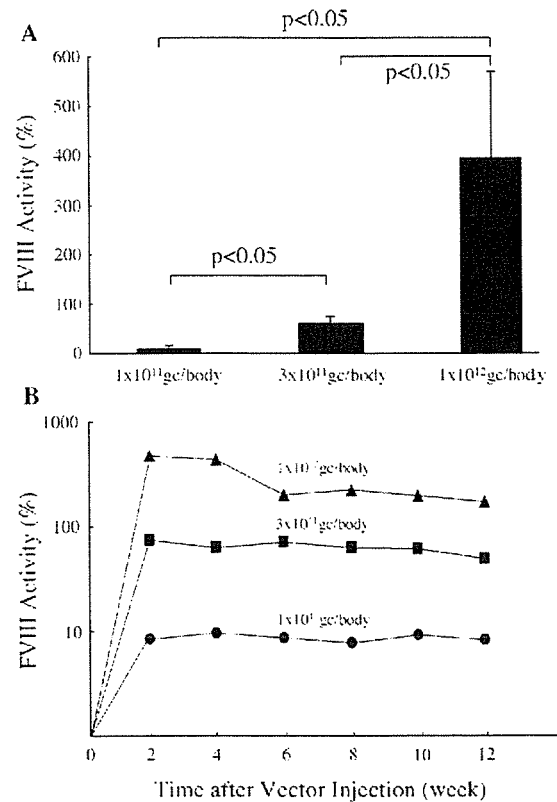


Figure 4 FVIII levels in plasma of hemophilia A mice after intravenous injection of AAV8- β -actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$, each group) on day 28 after intravenous injection of AAV8- β -actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice ($n=4$, each group) with injection of AAV8- β -actin-cFVIII (circles, 1×10^{11} gc/body; squares, 3×10^{11} gc/body; triangles, 1×10^{12} gc/body) are shown in panel B.

determined by the APTT method. Analyses for cFVIII transcripts suggested that the cFVIII gene mainly was expressed in the liver (Fig. 5) together

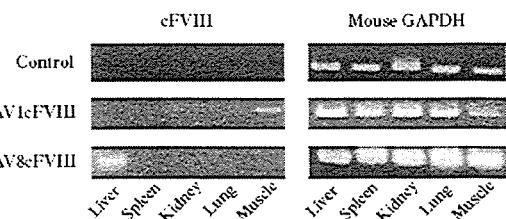


Figure 5 Analysis for cFVIII transcripts in mice. The RT-PCR analyses for the transcripts derived from the cFVIII gene (cFVIII) of RNA isolated from hemophilia A mouse organs without (control) or with intramuscular injection of AAV1- β -actin-cFVIII vectors (AAV1cFVIII) or intravenous injection of AAV8- β -actin-cFVIII vectors (AAV8cFVIII) are shown. For the control, the RT-PCR analysis for mouse GAPDH (Mouse GAPDH) of RNA isolated from hemophilia A mice with or without injection of AAV- β -actin-cFVIII vectors was performed simultaneously.

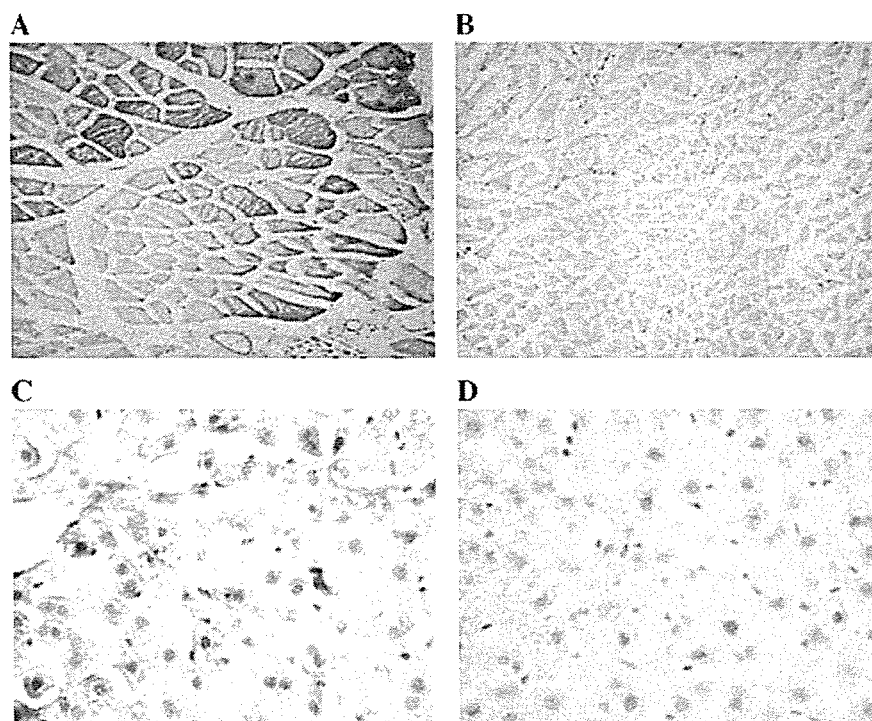


Figure 6 Immunohistochemical Analysis for cFVIII transgene products. Immunohistochemistry for cFVIII of the skeletal muscles of hemophilia A mice with intramuscular injection of AAV1- β -actin-cFVIII vectors (A) and the liver of hemophilia A mice with intravenous injection of AAV8- β -actin-cFVIII vectors (C) is shown (positive stain: brown). For the control, sections of the skeletal muscles (B) and the liver (D) obtained from hemophilia A mice without vector injection were processed simultaneously with anti-FVIII antibodies.

with the traceable expression in the heart, lung, and spleen (not shown). In accordance with the data on cFVIII transcripts, cFVIII molecules were immunohistochemically detected in the skeletal muscles of AAV1- β -actin-cFVIII injected mice and in the liver of mice with intravenous injection of AAV8- β -actin-cFVIII (Fig. 6).

Discussion

Because of the size and nature of the FVIII gene (cDNA), there were difficulties in hemophilia A gene therapy compared with gene therapy for hemophilia B. These difficulties were solved by efforts of many investigators that allowed use of a modified FVIII gene such as BDD FVIII cDNA, improved vector systems, and new strategies. Based upon these studies, a few clinical trials of hemophilia A gene therapy were conducted [17–19]. Increase of FVIII activities in the circulation and clinical improvements were observed in patients who received vector injection or transplantation of genetically modified cells. However, long-term expression of FVIII from the transgenes was not achieved in these studies. Thus, reexami-

nation of the vector systems, the target organs for transduction, and the promoters may be required.

The recombinant AAV vectors are thought to be one of the better vectors in terms of its capability to transduce non-dividing cells and long-term transgene expression, although delivery of the FVIII gene using AAV vectors were limited by its small packaging capacity [4]. The dual AAV vector system utilizing separate AAV2 vectors independently carrying the FVIII heavy chain gene and the FVIII light chain gene could express functionally active FVIII [5]. However, there was an imbalance in the expression levels of the FVIII heavy chain and FVIII light chain, suggesting that over-expressed free FVIII light chain molecules might be more immunogenic than the native molecules. The BDD FVIII gene could be packaged in AAV2 or AAV8 vectors in the previous studies and these vectors could efficiently transduce the liver with intraportal injection of the vectors [6]. Transduction of the liver with peripheral vein injection of AAV8 vectors was as efficient as portal vein injection of vectors, although that of AAV2 vectors was not [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ, so FVIII

synthesis in hepatocytes and its subsequent secretion into the circulation may be warranted. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs. AAV1 vector-based transduction of the skeletal muscles has beneficial characteristics of removing the transgenes. This is the first report of sufficient expression of FVIII in the skeletal muscles transduced with AAV vectors and suggests that skeletal muscle-directed FVIII expression has a potential for hemophilia A gene therapy.

Compared with synthesis and secretion of FVIII into the circulation from the liver, transport of sufficient FVIII into the circulation from the skeletal muscle fibers is not assured. Based upon our data, it is apparent that transduction of the liver with AAV8- β -actin-cFVIII is superior to transduction of skeletal muscles with AAV1- β -actin-cFVIII regarding FVIII production. The difference between FVIII levels in the peripheral blood of these vector-injected mice may be due to how the transduced cells secrete FVIII molecules into the circulation. Hepatocytes actively secrete a variety of molecules including FVIII into the circulation. Since recombinant cFVIII is in a BDD form, its expression in and secretion from hepatocytes is expected to be better than native FVIII [20], accounting for the high cFVIII expression in mice with intravenous injection of AAV8 vectors carrying the cFVIII gene though cFVIII expressing hepatocytes were not abundant. Although muscle fibers are surrounded by capillaries, transport of recombinant FVIII molecules from muscle fibers to capillaries would not be as efficient as that from hepatocytes.

In terms of the immune reaction to transgene products, muscle stem cells have been shown to function as antigen-presenting cells, suggesting that expression of the transgene by the ubiquitous promoter in the skeletal muscles might lead to development of antibodies against the transgene products if there is no immune tolerance to the transgene products [21]. This was confirmed by Wang et al. [22]. Neutralizing antibody formation was observed in 66.7% of mice with AAV1cFVIII injection even with administration of immunosuppressant, while it was not observed in mice with AAV8- β -actin cFVIII injection by week 12 after vector injection, supporting the potential advantage of AAV8 vector-based transduction of the liver over the muscle-directed transduction by AAV1 vectors.

Each vector system has advantages and disadvantages in these respects. We may need to confirm the results obtained in hemophilia mice using dogs and non-human primates that genetically are more close to humans because there may be differences in transduction efficiency of various serotypes between mice and humans [23]. Taken together, we may need to perform a comparative study using another animal models such as hemophilic dogs and non-human primates that are more genetically close to humans than mice to address these questions. Additionally, use of tissue-specific promoters to minimize neutralizing antibody formation may be a better strategy for expressing transgenes in a tissue- and organ-specific manner. These experiments will be performed in future studies.

In conclusion, our data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have the potential for hemophilia A gene therapy. Our present studies have provided important insight about selecting the appropriate target for delivery of the therapeutic genes and the vector system for the hemophilia A gene therapy.

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