10 Naldini L *et al*. *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; **272**: 263–267.
11 Honjo S *et al*. Experimental infection of African green monkeys and cynomolgus monkeys with a SIVAGM strain isolated from a healthy African green monkey. *J Med Primatol* 1990; **19**: 9–20.
12 Roth DA, The factor FIII transkaryotic therapy study group *et al*. Implantation of non-viral *ex vivo* genetically modified autologous dermal fibroblaststhat express B-domain deleted human factor VIII in 12 severe hemophilia A study subjects. *Blood* 2002; **100** (Suppl): 116a (abstract #430).
13 Dwarki VJ *et al*. Gene therapy for hemophilia A: production of therapeutic levels of human factor VIII *in vivo* in mice. *Proc Natl Acad Sci USA* 1995; **14**: 1023–1027.
14 Halvorsen YC *et al*. Thiazolinediones and glucocortidoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. *Metabolism* 2001; **50**: 407–413.
15 Harp JB, Franklin D, Vanderpuije AA, Gimble JM. Differential expression of signal transducers and activators of transcription during human adipogenesis. *Biochem Biophys Res Commun* 2001; **281**: 907–912.
16 Weiss GH, Rosen OM, Rubin CS. Regulation of fatty acid synthetase concentration and activity during adipocyte differentiation. *J Biol Chem* 1980; **255**: 4751–4757.
17 Mertens K *et al*. Biological activity of recombinant factor VIII variants lacking the central B-domain and the heavy-chain sequence Lys713-Arg740: discordant *in vitro* and *in vivo* activity. *Br J Haematol* 1993; **85**: 133–142.
18 Lind P *et al*. Novel forms of B-domain-deleted recombinant factor VIII molecules. Construction and biochemical characterization. *Eur J Biochem* 1995; **232**: 19–27.
19 Nakajima T *et al*. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Hum Gene Ther* 2000; **11**: 1863–1874.
20 Naito M *et al*. Defective sorting to secretory vesicles in the trans Golgi network is partly responsible for protein C deficiency: molecular mechanisms of impaired secretion of abnormal protein C R169W, R352W, and G376D. *Circ Res* 2003; **92**: 865–872.
21 Chua Jr S-C *et al*. Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* 1996; **271**: 994–996.
22 Yonemura H *et al*. Efficient production of recombinant human factor VIII by co-expression of the heavy and light chains. *Protein Eng* 1993; **6**: 669–674.

Gene Therapy (2004) 11, 1081–1086

www.nature.com/gt

RESEARCH ARTICLE

Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioral recovery in phenylketonuria mice

S Mochizuki[1,2], H Mizukami[1], T Ogura[1], S Kure[3], A Ichinohe[3], K Kojima[3], Y Matsubara[3], E Kobayahi[4], T Okada[1], A Hoshika[2], K Ozawa[1,5] and A Kume[1]

[1]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan;* [2]*Department of Pediatrics, Tokyo Medical University, Tokyo, Japan;* [3]*Department of Medical Genetics, Tohoku University Graduate School of Medicine, Sendai, Japan;* [4]*Division of Organ Replacement, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan; and* [5]*Division of Hematology, Department of Medicine, Jichi Medical School, Tochigi, Japan*

Classical phenylketonuria (PKU) is a metabolic disorder caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH). If untreated, accumulation of phenylalanine will damage the developing brain of affected individuals, leading to severe mental retardation. Here, we show that a liver-directed PAH gene transfer brought about long-term correction of hyperphenylalaninemia and behavioral improvement in a mouse model of PKU. A recombinant adeno-associated virus (AAV) vector carrying the murine PAH cDNA was constructed and administered to PAH-deficient mice (strain PAH^{enu2}) via the portal vein. Within 2 weeks of treatment, the hyperphenylalaninemic phenotype improved and completely normalized in the animals treated with higher vector doses. The therapeutic effect persisted for 40 weeks in male mice, while serum phenylalanine concentrations in female animals gradually returned to pretreatment levels. Notably, this long-term correction of hyperphenylalaninemia was associated with a reversal of hypoactivity observed in PAH^{enu2} mice. While locomotory activity over 24 h and exploratory behavior were significantly decreased in untreated PAH^{enu2} mice compared with the age-matched controls, these indices were completely normalized in 12-month-old male PKU mice with lowered serum phenylalanine. These results demonstrate that AAV-mediated liver transduction ameliorated the PKU phenotype, including central nervous system dysfunctions.
Gene Therapy (2004) **11,** 1081–1086. doi:10.1038/sj.gt.3302262; Published online 1 April 2004

Keywords: *phenylketonuria; adeno-associated virus vector; hyperphenylalaninemia; behavioral recovery*

Introduction

Classical phenylketonuria (PKU; McKusick OMIM 261600) is an autosomal recessive disorder resulting from a deficiency of the liver enzyme phenylalanine hydroxylase (PAH; EC 1. 14.16.1).[1] PAH converts phenylalanine (Phe) to tyrosine with the aid of tetrahydrobiopterin (BH_4), and a deficiency of this enzyme causes accumulation of Phe and abnormal metabolites in the body fluids. If untreated, this condition irreversibly damages the central nervous system (CNS) of the patient, resulting in severe mental retardation. Conventional therapy for PKU consists of dietary restriction of Phe, which can prevent neuronal damage if initiated very early in life. However, the strict and complicated diet is often associated with poor compliance, particularly in adolescents and young adults. Premature termination of the diet leads to declined neuropsychological function, and noncompliance in pregnant women with PKU can produce devastating defects in the offspring referred to as 'maternal PKU syndrome'. A permanent cure is therefore awaited to liberate patients from dietary restrictions, and gene therapy is an attractive novel approach to this goal.

However, previous preclinical studies of PKU gene therapy have revealed that a long-term cure of PKU is a formidable task. Generally, recombinant retroviral vectors cannot deliver the normal PAH gene to the liver at sufficient levels to overcome hyperphenylalaninemia.[2,3] Adenoviral-mediated PAH gene transfer achieved a complete reduction of serum Phe in PKU animals, but the therapeutic effects did not persist and the vector was not effectively readministered due to immune responses against the virus.[4,5] On the other hand, adeno-associated virus (AAV) vectors comprise another class of gene delivery vehicles, which have been shown to stably transduce nondividing cells such as hepatocytes, muscle fibers and neurons.[6–8]

In this study, we evaluated a recombinant AAV vector carrying the PAH gene in a mouse model of PKU (PAH^{enu2} strain).[9–11] A missense mutation (F263S) in the PAH gene was introduced into BTBR mouse strain by chemical mutagenesis, resulting in a loss of enzyme activity. Consequently, the homozygous PAH^{enu2} mice

Correspondence: Dr A Kume, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Tochigi 329-0498, Japan
Received 24 December 2003; accepted 11 February 2004; published online 1 April 2004

share many phenotypic characteristics with human PKU patients, such as profound hyperphenylalaninemia (>20 mg/dl; normal 1–2 mg/dl), behavioral disturbances and hypopigmentation. Previous work suggested that at least 10% of normal PAH activity would be required to prevent hyperphenylalaninemia in PKU mice.[4,5]

Results

Construction of the recombinant AAV vector

We first evaluated vectors derived from AAV serotypes 1 through 5. Recombinant AAV vectors containing the mouse erythropoietin (Epo) gene were infused into the mouse portal vein, and the serum Epo levels were determined. Among them, the AAV5-derived virion yielded the highest Epo concentration (unpublished results).[12,13] Next, we tested several promoters to drive the Epo gene in the context of AAV5. We found that the CAG promoter was the strongest in transgene expression in the liver (unpublished results).[14] This promoter consists of the human cytomegalovirus (CMV) immediate-early enhancer, the chicken β-actin promoter, and a chicken β-actin/rabbit β-globin composite intron.

Based on these results, we constructed an AAV vector as shown in Figure 1 (AAV5/CAG-mPAH). A recombinant AAV plasmid pAAV5/CAG-mPAH was comprised of the CAG promoter, the murine PAH cDNA and the SV40 late polyadenylation signal flanked by the AAV5 inverted terminal repeats (ITRs shown as hairpin loops in Figure 1). The vector DNA was then packaged into the AAV5 capsid through an adenovirus-free, transient transfection protocol.[15]

Correction of hyperphenylalaninemia

For liver-targeted gene transfer, the vector was injected into 5–7-week-old PAH^{enu2} mice via the portal vein. We injected male PKU mice with 3×10^{12} vector genomes (vg) ($n=3$), 1×10^{13} vg ($n=4$), 3×10^{13} vg ($n=3$) or 1×10^{14} vg ($n=3$) of AAV5/CAG-mPAH per animal. Female PKU mice were infused with 1×10^{13} vg ($n=4$), 3×10^{13} vg ($n=4$) or 1×10^{14} vg ($n=5$) per animal.

Serum Phe levels were determined prior to the infusion, biweekly until 12 weeks postinfusion, and every 4 weeks thereafter (Figure 2). Before gene transfer (week 0), all PAH-deficient mice showed profound hyperphenylalaninemia (33.7±3.4 mg/dl; range 29.3–43.5 mg/dl; $n=27$). The degree of hyperphenylalaninemia was not significantly different between males (33.2±2.6 mg/dl; $n=14$) and females (34.3±4.1 mg/dl; $n=13$). Figure 2a shows the kinetics of blood Phe in male PKU mice receiving different doses of AAV5/CAG-mPAH. A striking decrease in serum Phe was observed 2-4 weeks after gene transfer. With the lowest vector dose (3×10^{12} vg), serum Phe was only slightly lowered after 2 weeks (from 35.0±1.6 to 28.1±7.0 mg/dl; $P=0.18$ by paired *t*-test), but was significantly lowered after 4 weeks (15.6±6.9 mg/dl; $P=0.027$ by paired *t*-test). With higher vector doses (1×10^{13}, 3×10^{13} and 1×10^{14} vg), the serum Phe level was clearly lowered ($P=0.001$, 0.006 and 0.002 by paired *t*-test, respectively) to a therapeutic range (<10 mg/dl) in 2 weeks. At 4 weeks postinfusion, each cohort of male mice recorded the lowest serum Phe. In particular, it was completely normalized in the mice treated with 3×10^{13} vg (1.4±0.5 mg/dl) and 1×10^{14} vg (1.2±0.5 mg/dl) of AAV5/CAG-mPAH.

The reduced serum Phe levels were stably maintained for 40 weeks. Complete correction of hyperphenylalaninemia (<2 mg/dl) persisted in the mice treated with the highest vector dose (1×10^{14} vg), and the mice receiving

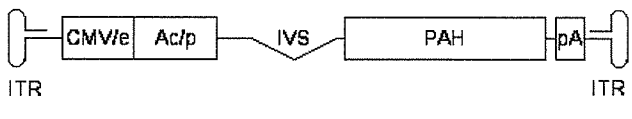


Figure 1 *Structure of the AAV5/CAG-mPAH vector. The vector consisted of a CMV immediate-early enhancer (CMV/e), the chicken β-actin promoter (Ac/p), a chicken β-actin/rabbit β-globin composite intron (IVS), the 1.4 kb murine PAH cDNA (PAH) and the SV40 late polyadenylation signal (pA) flanked by the AAV5 inverted terminal repeats (ITRs shown as hairpin loops).*

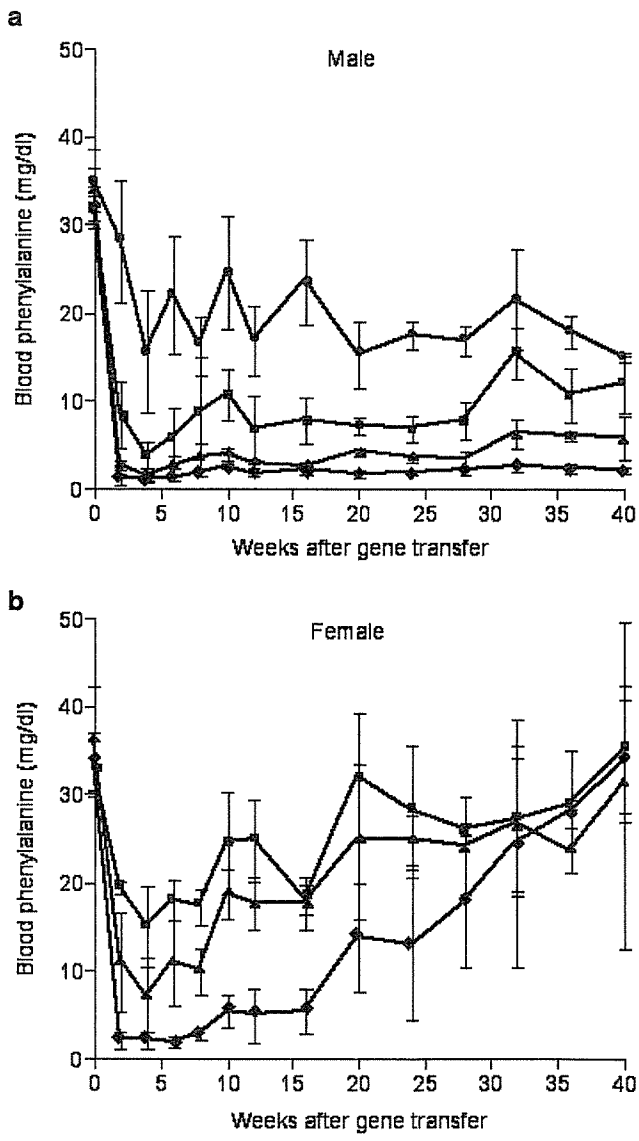


Figure 2 *Persistence of the recombinant AAV-mediated correction of hyperphenylalaninemia in male (a) and female (b) PKU mice. Serum Phe concentration was determined prior to vector infusion (week 0) and periodically after gene transfer, and represented as the mean±s.d. for each treatment group. The applied vector dose was 3×10^{12} vg (circles), 1×10^{13} vg (squares), 3×10^{13} vg (triangles) or 1×10^{14} vg (diamonds) per animal.*

the second highest dose (3×10^{13} vg) stayed in a well-controlled therapeutic range (<6 mg/dl). Mice receiving lower vector doses (3×10^{12} and 1×10^{13} vg) showed moderate correction of hyperphenylalaninemia, with significant long-term efficacy of the single AAV infusion.

Figure 2b shows the kinetics of serum Phe in female PKU mice after receiving 1×10^{13}, 3×10^{13} or 1×10^{14} vg of AAV5/CAG-mPAH. The vector administration was effective in the female PKU mice, too, but the dose–response and duration were different from the male mice; that is, about three times more vector was required for the female mice to exhibit an equivalent reduction in serum Phe (Figure 3). At 4 weeks postinfusion when the reduction was at its maximum, 1×10^{13} vg of AAV5/CAG-mPAH lowered serum Phe by 50% in the female mice, while the same level of reduction was achieved by 3×10^{12} vg in the males. Similarly, an 80% reduction was achieved by 3×10^{13} vg in the females, whereas only 1×10^{13} vg were required in the males. Complete correction of hyperphenylalaninemia was achieved by 1×10^{14} vg in the females, while it was achieved by 3×10^{13} vg as well as 1×10^{14} vg in the males. As for duration, the therapeutic effect did not persist in the female PKU mice as seen in the males. Serum Phe levels in each female cohort remained low until 8 weeks post-gene transfer, but gradually rose thereafter. With vector doses of 1×10^{13} and 3×10^{13} vg, serum Phe was greater than 20 mg/dl at 20 weeks, and returned to the pretreatment level at 40 weeks. With the highest dose (1×10^{14} vg), serum Phe was kept below 10 mg/dl until 16 weeks, then gradually increased and returned to the pretreatment level at 40 weeks.

Although we did not kill the animals for enzyme assay, previous studies on adenoviral-mediated gene transfer to PAH^{enu2} mice allowed us to estimate the PAH activity accomplished by our vector. These studies showed, in good agreement, that the threshold PAH activity to correct hyperphenylalaninemia was about 10% of normal mice.[4,5] As shown in Figure 3, male PKU mice given 3×10^{12} vg and females given 1×10^{13} vg of the vector showed 50–60% reduction in serum Phe; we speculate that these mice would express about 5% of normal PAH activity. On the other hand, male PKU mice given 3×10^{13} or 1×10^{14} vg and females given 1×10^{14} vg completely recovered from hyperphenylalaninemia, hence their liver PAH activities would be 10% of normal or greater. Male PKU mice given 1×10^{13} vg (ca. 90% reduction in serum Phe) and females given 3×10^{13} vg of AAV (ca. 80% reduction) would have 5–10% of normal PAH activity.

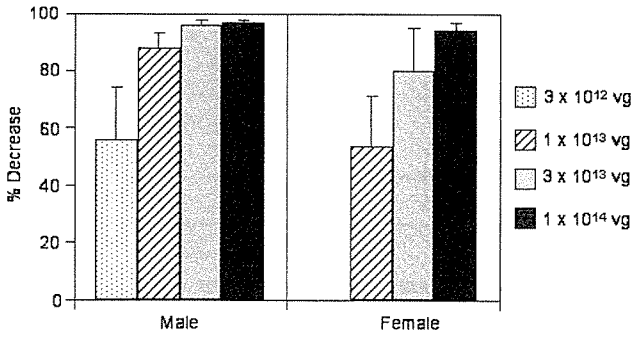


Figure 3 Vector dose-dependent reduction of serum Phe in PKU mice. Percent reduction of serum Phe was calculated by the following formula: {(serum Phe at week 0)−(serum Phe at 4 weeks)} × 100/(serum Phe at week 0). Bars represent the mean ± s.d. of % reduction of serum Phe in PKU mice treated with 1×10^{12} vg (dotted bar), 1×10^{13} vg (hatched bar), 3×10^{13} vg (gray bar), or 1×10^{14} vg of AAV5/CAG-mPAH (black bar).

Correction of hypopigmentation

Associated with extended reductions in serum Phe, hypopigmentation in the AAV-treated PKU mice was ameliorated. While the coat color of untreated mice remained grayish brown, hair darkening in the mice receiving higher vector doses was observed 2 weeks post-transduction, and the mice grew black hair in 4 weeks which was indistinguishable from that of wild-type (WT) BTBR mice (Figure 4). Male PKU mice with reduced serum Phe retained black hair throughout the observation period, while female PKU mice lost pigmentation as the therapeutic effect diminished.

Recovery from hypoactivity following PAH gene transfer

Along with persistent correction of hyperphenylalaninemia and hypopigmentation, we observed behavioral

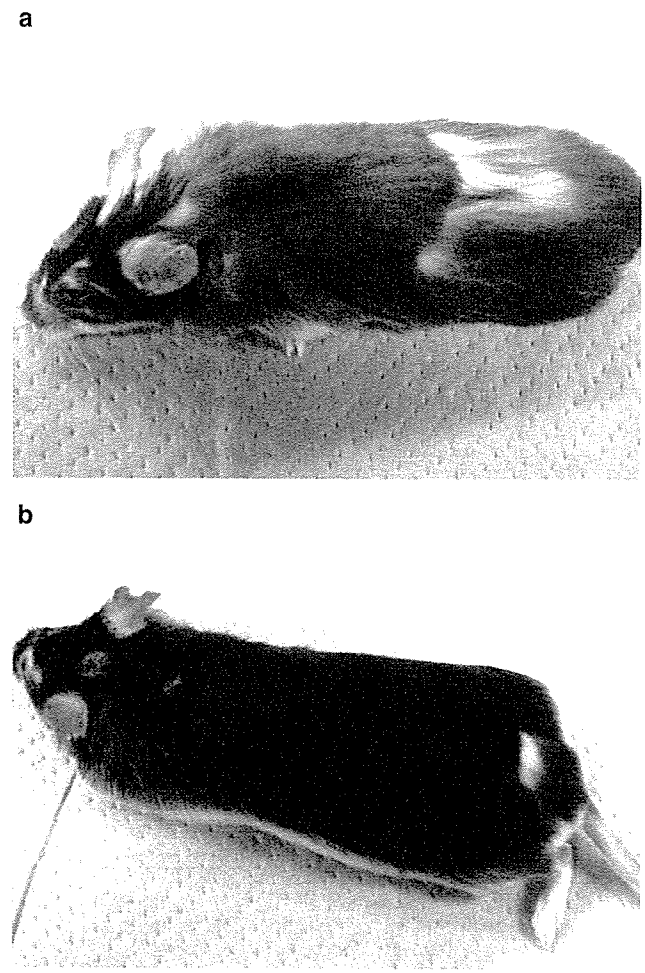


Figure 4 Correction of hypopigmentation in PKU mouse following PAH gene transfer. (a) Untreated PKU mouse showing grayish brown hair, easily distinguished from wild-type and PAH$^{+/-}$ heterozygous BTBR mice. (b) By 8 weeks after PAH gene transfer, the PKU mouse with complete correction of hyperphenylalaninemia recovered black coat color and was indistinguishable from normal BTBR mice.

recovery in AAV-treated PKU mice. Consistent with previous studies showing abnormal behavior and cognitive deficits in PAH^{enu2} mice,[9,16,17] we found that untreated PKU (PAH$^{-/-}$) mice were relatively hypoactive compared with WT (PAH$^{+/+}$) and heterozygous carrier (PAH$^{+/-}$) animals. The hypoactivity became apparent with aging, and the difference was significant among animals aged 10 months or older. Figure 5 shows the results of behavior tests on the 12-month-old animals. As for total locomotion over 24 h, the untreated PKU mice displayed about 70% of normal activity (Figure 5a, $P<0.01$ by Student's *t*-test). On the other hand, the AAV-administered male mice without hyperphenylalaninemia exhibited significantly higher 24-h locomotion than the untreated mice (Figure 5a, $P=0.001$ by Student's *t*-test). Indeed, the AAV-treated animals showed a normal activity level in this test.

Similarly, PAH gene transfer improved the PKU animals' exploratory activity in a novel environment.

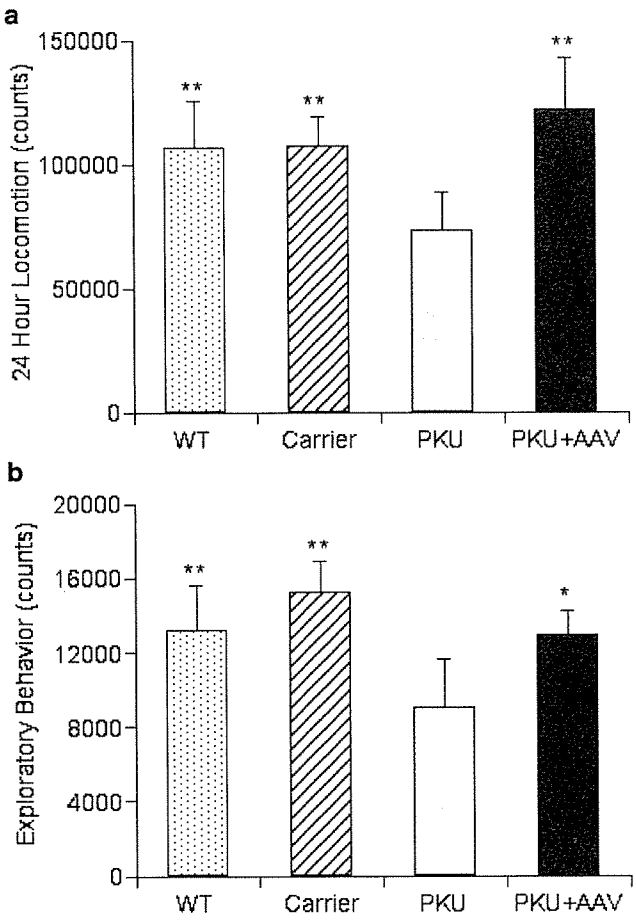


*Figure 5 Recovery from hypoactivity following PAH gene transfer. (a) Total locomotion over 24 h. Mice were placed under an infrared sensor and ambulatory activity was recorded consecutively for 24 h. Wild-type (WT), heterozygous and AAV-treated PKU mice exhibited significantly higher locomotory activity than untreated PKU mice (**$P<0.01$ by Student's t-test). (b) Exploratory behavior. Mice were placed in a novel cage under a sensor and ambulatory activity was quantified during the first 2 h in the chamber. This test showed significantly higher performance by WT, heterozygous and AAV-treated PKU mice than untreated PKU animals (**$P<0.01$ and *$P<0.05$ by Student's t-test). Bars represent the mean ± s.d. of WT mice (WT; dotted bar), heterozygous mice (Carrier; hatched bar), untreated PKU mice (PKU; gray bar), and AAV-transduced PKU mice (PKU + AAV; black bar).*

When settled in a novel cage, the untreated PKU males showed 60–70% of normal exploratory activity (Figure 5b, $P<0.01$). On the other hand, PKU mice that had recovered from hyperphenylalaninemia explored as vigorously as WT animals, and their activity level was significantly greater than that of untreated PKU mice ($P=0.015$). These results clearly indicate that the PAH gene transfer improved the CNS function of PKU mice in addition to correction of hyperphenylalaninemia.

Discussion

In this study, we demonstrated that AAV-mediated transduction of the PKU mouse liver brought about a long-term cure of the disease. A single infusion of AAV5/CAG-mPAH completely normalized the hyperphenylalaninemic phenotype in male PKU mice, and the longevity of the therapeutic effect was superior to any other gene delivery vehicle thus far. Although not thoroughly investigated, the result suggests that the transgene was transcriptionally active during observation, and that no significant immune response was elicited against the transduced hepatocytes in the animals. In addition, infusion of very large amounts of AAV did not show any toxicity in the treated mice. The vector safety and viability may be further improved by adopting recently developed purification methods, such as iodixanol gradient, affinity or ion-exchange chromatography.[18–21]

A major problem we encountered in this study was that the same AAV vector was less effective in female PKU mice. About three times more vector was required to achieve an equivalent reduction of serum Phe seen in males, and the therapeutic effect was shorter in duration. The underlying mechanisms for these female-specific phenomena are currently unknown. Davidoff *et al*[22] recently reported similar observations that AAV2- and AAV5-derived vectors less efficiently transduced livers of female mice than males. They suggested that the difference was due to an androgen-dependent pathway for augmenting hepatocyte transduction, but its mode of action is undetermined. Since precise molecular events involved in recombinant AAV-mediated transduction remain obscure, the critical step accounting for the observed sex difference is also a mystery. Androgen may augment the uptake of AAV particles into the cell or traffic them to the nucleus; alternatively, it may stabilize the AAV genome in an episomal state, or enhance vector integration into the host chromosome. Of these possibilities, the last one is less likely, because only a small fraction (<10%) of recombinant AAV genome was reportedly integrated into the mouse hepatocytes.[23] If there is an androgen-dependent mechanism to retain the AAV genome in an episomal state in the liver, lack of such machinery would allow gradual loss of the vector DNA in females, thereby transgene-derived PAH activity would descend over time as we observed. Other possibilities accounting for the lower therapeutic efficacy include transcriptional silencing and an immune response against AAV-transduced hepatocytes, although the latter is unlikely to occur only in female mice.

In genetic treatment of autosomal and acquired disorders, sex-dependent transduction raises a novel issue. Development of more efficient vectors may over-

come this problem, or other approaches can be considered. In terms of PKU, the disease-associated pathology is caused by accumulated Phe in the body fluids. Thus, it can be prevented by 'heterologous gene therapy', ie targeting tissues other than hepatocytes. Several investigators have exploited this strategy because of difficulties with liver transduction and safety concerns. Christensen *et al*[24] transduced primary keratinocytes with genes for PAH and GTP cyclohydrolase I, which is the rate-limiting enzyme in BH_4 biosynthesis. They showed that the cells cleared excess Phe in the culture medium, and suggested that engraftment of enough of these cells may function as a metabolic sink for detoxification. Harding *et al*[25] investigated the potential of skeletal muscle as a PAH-expressing organ. Using a transgenic technique, they created mice expressing PAH in the skeletal muscle but not in the liver. These mice showed hyperphenylalaninemia at baseline, but serum Phe significantly decreased when the animals were supplemented with BH_4. A similar approach to bone marrow cells was unsuccessful,[26] and careful consideration is required in translating these transgenic studies into human applications.

A novel finding in this study was that AAV infusion lead to behavioral improvement in addition to correction of hyperphenylalaninemia and hypopigmentation. To our knowledge, this is the first demonstration that a gene-based approach to PKU actually benefited CNS function. It has been reported that free amino acid and amine contents are dramatically reduced in the PAH^{enu2} mouse brain, as in untreated human PKU patients.[27,28] Presumably, the observed hypoactivity in older PKU mice was associated with the abnormal synthesis of biogenic amines, whereas the abnormality was reversed in AAV-treated PKU animals with normal serum Phe. We speculate that the behavioral recovery in these mice represents an analogous situation in which dietary restriction of Phe can improve some neuropsychiatric symptoms in untreated PKU patients. It is of particular interest whether an earlier genetic intervention can prevent irreversible neuronal defects in PKU and preserve more sophisticated CNS function such as memory. The AAV vectors and PAH^{enu2} mice will provide an attractive system to address such prompting questions.

Materials and methods

AAV vector construction

To isolate murine PAH cDNA (GenBank Accession # NM008777), liver mRNA was prepared from a C57BL/6J mouse (from Clea Japan, Tokyo, Japan) with Isogen reagent (Nippon Gene, Toyama, Japan) and an mRNA Purification kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The PAH cDNA was cloned by reverse transcriptase-directed polymerase chain reaction using a Superscript II cDNA synthesis kit (Invitrogen, Grand Island, NY, USA). The CAG promoter was derived from pCAGGS (a gift from Dr J Miyazaki, Osaka University, Osaka, Japan).[14] The AAV5 vector plasmid pAAV5LacZ and a helper plasmid 5RepCapA were generous gifts from Dr JA Chiorini (National Institutes of Health, Bethesda, MD, USA).[12] To construct a recombinant AAV5 vector plasmid for PAH expression, the expression cassette of pAAV5LacZ was replaced with the CAG promoter, the murine PAH cDNA and the SV40 late polyadenylation signal, and the plasmid was referred to as pAAV5/CAG-mPAH (Figure 1).

Recombinant AAV stocks were propagated according to an adenovirus-free, three-plasmid transfection protocol described previously.[15] Briefly, subconfluent 293 cells (4×10^8 cells per 10 trays) in Cell Factories 10 (Nunc, Roskilde, Denmark) were cotransfected with 650 μg of the vector plasmid pAAV5/CAG-mPAH, 650 μg of the AAV helper plasmid 5RepCapA and 650 μg of the adenoviral helper plasmid pLadeno1 (identical to pVAE2AE4-2 in Matsushita *et al*;[15] kindly provided by Avigen, Alameda, CA, USA) by using the calcium phosphate precipitation method for a period of 6 h. Cells were harvested 72 h after transfection and lysed by three freeze–thaw cycles. The crude viral lysate was incubated with Benzonase (Merck KGaA, Darmstadt, Germany) and centrifuged. Finally, the clear supernatant was subjected to two rounds of CsCl density-gradient ultracentrifugation for purification. The physical titer of the viral stock was determined by DNA dot blot and hybridization with the murine PAH cDNA probe, along with plasmid standards. Typically, we obtained 5×10^{13} vg of AAV5/CAG-mPAH from a culture container (10 trays).

Transduction of mouse liver

All animal experiments were carried out in accordance with our institutional guidelines. PAH^{enu2} mice were generous gifts from Dr T Shiga (University of Tsukuba, Tsukuba, Japan), and a colony was established at Jichi Medical School (Tochigi, Japan). PKU mice used for *in vivo* gene transfer were 5–7 weeks of age. Mice were anesthetized with isoflurane inhalation followed by laparotomy. A 300 μl of saline suspension containing 3×10^{12}–1×10^{14} vg of AAV5/CAG-mPAH was slowly injected into the portal vein using an insulin syringe with a 29-gauge needle (Terumo, Tokyo, Japan).

Serum Phe assay

Serum Phe was measured by an enzymatic microfluorometric assay using an Enzaplate PKU-R kit (Bayer Medical, Tokyo, Japan). Mice were tail phlebotomized and the blood was spotted onto a mass-screening grade paper filter (#545, provided by Advantec Toyo, Tokyo, Japan). A 3 mm diameter disc was punched out from the dried blood spot and placed in a 96-well plate. Phe was eluted from the disc and incubated with Phe dehydrogenase, an NAD-dependent enzyme, and resazurin. The enzyme reaction produces NADH, which in turn converts resazurin to resorufin with the aid of diaphorase. The resultant resorufin was measured on a Fluoroskan Ascent plate reader (Labsystems, Helsinki, Finland) with a 544/590 nm filter set.

Mouse behavior tests

Mice were tested at 12 months of age. To measure locomotory activity over 24 h, the home cage of the mouse was placed under an infrared sensor that detects thermal radiation from animals (Supermex; Muromachi Kikai, Tokyo, Japan).[29] Ambulation was scored by a personal computer interfaced to the sensor. Alternatively, exploratory behavior was tested by placing the mouse in a novel cage under the infrared sensor.

Ambulatory activity was quantified during the first 2 h in the chamber.

Acknowledgements

We are grateful to Dr JA Chiorini for pAAV5LacZ and 5RepCapA, Dr J Miyazaki for pCAGGS, Dr T Shiga for PAH^{enu2} mice, Avigen for pLadeno1, and Advantec Toyo for #545 filter paper. We also thank Dr Y Hakamata for technical assistance in the animal experiments. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare, Japan.

References

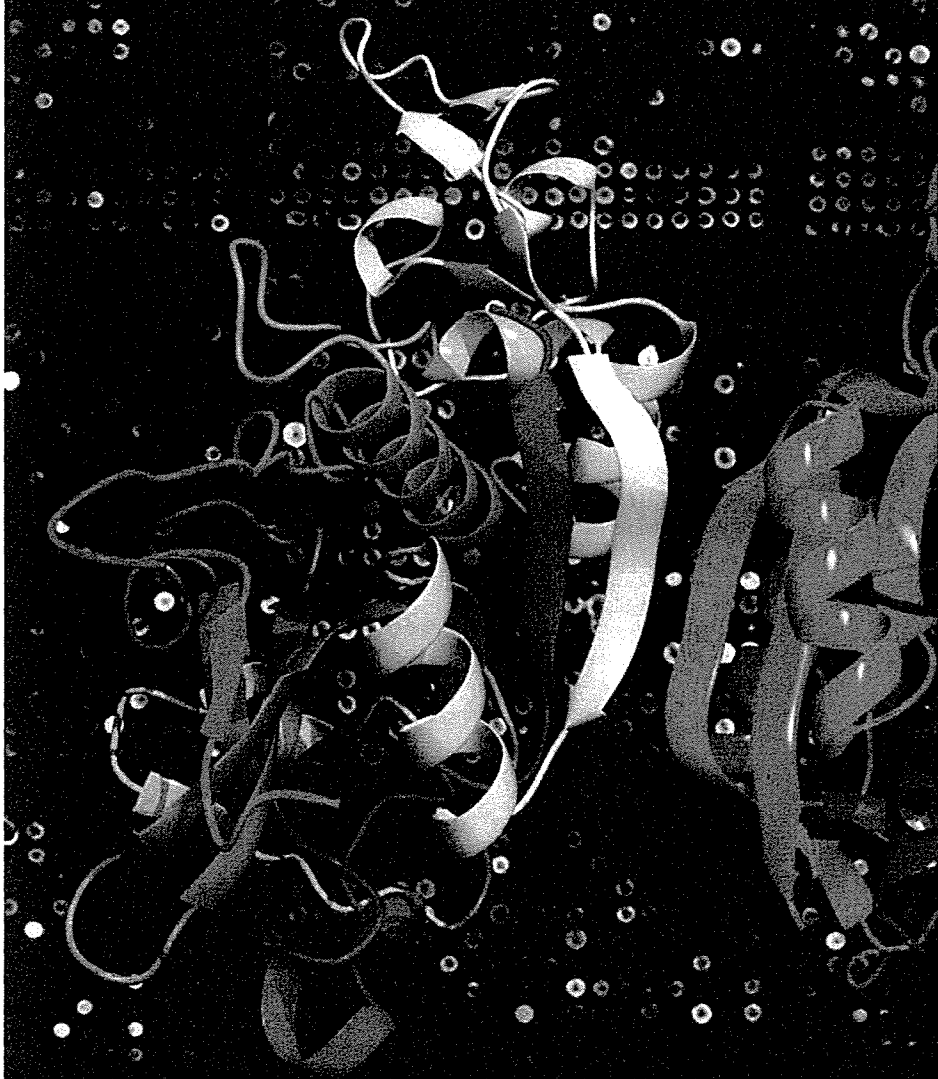
1 Scriver CR, Kaufman S. Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) *The Metabolic and Molecular Basis of Inherited Diseases*. McGraw-Hill: New York, 2001, pp 1667–1724.
2 Liu T-J, Kay MA, Darlington GJ, Woo SLC. Reconstitution of enzymatic activity in hepatocytes of phenylalanine hydroxylase-deficient mice. *Somat Cell Mol Genet* 1992; **18**: 89–96.
3 Eisensmith RC, Woo SLC. Gene therapy for phenylketonuria. *Eur J Pediatr* 1996; **155** (Suppl 1): S16–S19.
4 Fang B *et al*. Gene therapy for phenylketonuria: phenotypic correction in a genetically deficient mouse model by adenovirus-mediated hepatic gene transfer. *Gene Therapy* 1994; **1**: 247–254.
5 Nagasaki Y *et al*. Reversal of hypopigmentation in phenylketonuria mice by adenovirus-mediated gene transfer. *Pediatr Res* 1999; **45**: 465–473.
6 Nathwani AC *et al*. Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. *Blood* 2002; **100**: 1662–1669.
7 Kay MA *et al*. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; **24**: 257–261.
8 Muramatsu S *et al*. Behavioral recovery in a primate model of Parkinson's disease by triple transduction of striatal cells with adeno-associated viral vectors expressing dopamine-synthesizing enzymes. *Hum Gene Ther* 2002; **13**: 345–354.
9 Shedlovsky A, McDonald JD, Symula D, Dove WF. Mouse models of human phenylketonuria. *Genetics* 1993; **134**: 1205–1210.
10 McDonald JD, Charlton CK. Characterization of mutations at the mouse phenylalanine hydroxylase locus. *Genomics* 1997; **39**: 402–405.
11 McDonald JD *et al*. The phenylketonuria mouse model: a meeting review. *Mol Genet Metab* 2002; **76**: 256–261.
12 Chiorini JA, Kim F, Yang L, Kotin RM. Cloning and characterization of adeno-associated virus type 5. *J Virol* 1999; **73**: 1309–1319.
13 Mingozzi F *et al*. Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. *J Virol* 2002; **76**: 10497–10502.
14 Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; **108**: 193–200.
15 Matsushita T *et al*. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Therapy* 1998; **5**: 938–945.
16 Zagreda L *et al*. Cognitive deficits in a genetic mouse model of the most common biochemical cause of human mental retardation. *J Neurosci* 1999; **19**: 6175–6182.
17 Cabib S *et al*. The behavioral profile of severe mental retardation in a genetic mouse model of phenylketonuria. *Behav Genet* 2003; **33**: 301–310.
18 Hermens WT *et al*. Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Hum Gene Ther* 1999; **10**: 1885–1891.
19 Zolotukhin S *et al*. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Therapy* 1999; **6**: 973–985.
20 Auricchio A, O'Connor E, Hildinger M, Wilson JM. A single-step affinity column for purification of serotype-5 based adeno-associated viral vectors. *Mol Ther* 2001; **4**: 372–374.
21 Kaludov N, Handelman B, Chiorini JA. Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. *Hum Gene Ther* 2002; **13**: 1235–1243.
22 Davidoff AM *et al*. Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. *Blood* 2003; **102**: 480–488.
23 Nakai H *et al*. Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction *in vivo*. *J Virol* 2001; **75**: 6969–6976.
24 Christensen R, Kolvraa S, Blaese RM, Jensen TG. Development of a skin-based metabolic sink for phenylalanine by overexpression of phenylalanine hydroxylase and GTP cyclohydrolase in primary human keratinocytes. *Gene Therapy* 2000; **7**: 1971–1978.
25 Harding CO *et al*. Metabolic engineering as therapy for inborn errors of metabolism – development of mice with phenylalanine hydroxylase in muscle. *Gene Therapy* 1998; **5**: 677–683.
26 Harding CO *et al*. Expression of phenylalanine hydroxylase (PAH) in erythrogenic bone marrow does not correct hyperphenylalaninemia in *Pah^{enu2}* mice. *J Gene Med* 2003; **5**: 984–993.
27 Puglisi-Allegra S *et al*. Dramatic brain aminergic deficit in a genetic mouse model of phenylketonuria. *NeuroReport* 2000; **11**: 1361–1364.
28 Pascucci T, Ventura R, Puglisi-Allegra S, Cabib S. Deficits in brain serotonin synthesis in a genetic mouse model of phenylketonuria. *NeuroReport* 2002; **13**: 2561–2564.
29 Masuo Y, Matsumoto Y, Morita S, Noguchi J. A novel method for counting spontaneous motor activity in the rat. *Brain Res Protoc* 1997; **1**: 321–326.

The Journal of Molecular Biology Research, Protocols, Reviews, and Applications

Molecular Biotechnology

Editor-in-Chief: John M. Walker

Volume 27, Number 1, May 2004



Featured in this issue:

- Yeast-Based Screening of CED-3 Substrates
- Packaging Cell Lines for AAV Vectors
- Role of a Cysteine-Rich Region of PC6B
- Naked Plasmid DNA Transfer
- Calmodulin's Interactions
- Biopharmaceutical Safety Testing
- SNP Assay Using Exo$^+$ Polymerase

HUMANA PRESS

RESEARCH

Separate Control of Rep and Cap Expression Using Mutant and Wild-Type LoxP Sequences and Improved Packaging System for Adeno-Associated Virus Vector Production

*Hiroaki Mizukami, Takashi Okada, Yoji Ogasawara, Takashi Matsushita, Masashi Urabe, Akihiro Kume, and Keiya Ozawa**

Abstract

Adeno-associated virus (AAV) vectors are a practical choice for gene transfer, and demand for them is increasing. To cope with the necessity in the near future, we have developed a number of approaches to establish packaging cell lines for the production of AAV vectors. In our previous study, a highly regulated expression of large Rep proteins was obtained by using the Cre–loxP switching system. Therefore, in the present study, to regulate Cap expression as well, we developed an inducible expression system for both Rep and Cap proteins by using an additional set of mutant loxP sequences. The mutants possess two base alterations in the spacer region of loxP and recombine specifically with the same counterpart in the presence of Cre. By using two separate plasmids, one with mutant and the other with wild-type loxP sequences, the expression of two different proteins can be induced simultaneously by Cre recombinase. When the LacZ-encoding plasmid vector was used as a packaging model, a significant packaging titer of 2.1×10^{10} genome copies per 10-cm dish was obtained. These results indicate the importance of controlling Cap expression, in addition to Rep, to achieve an optimum production rate for AAV vectors.

Index Entries: Cre–loxP; mutant loxP; dependovirus; AAV vector; packaging cell line; 293 cells.

1. Introduction

Adeno-associated viruses (AAVs) are currently being investigated as a gene transfer vector for a variety of applications. Several diseases are thought to be prime candidates for AAV vector-medicated therapeutic intervention; clinical trials are already set out for the correction of hemophilia B *(1)*, and for Parkinson's disease in the near future *(2,3)*. However, one drawback to the use of AAV is difficulty in making large-scale preparations. To improve the process of preparation, we have developed packaging cell lines for AAV *(4,5)*. Early studies indicate that in addition to Rep, relatively large amounts of Cap proteins should be expressed to achieve a high titer of virus production, despite the fact that constitutive expression of these proteins has cytotoxic consequences *(6,7)*. Therefore, controlling the expression profiles for these proteins has vital significance. For this purpose, the Cre–loxP system is one of the best-known approaches as an induction system, and in our previous study we used loxP sequences to regulate Rep expression *(4)*. However, there was a limitation to this approach in that Cap expression could not be regulated efficiently; only Rep expression could be regulated. To control Cap expression in addition to Rep expression, we used mutant loxP sequences along with the wild type. These mutant loxP sequences are shown to recombine specifically with each

*Author to whom all correspondence and reprint requests should be addressed: Dr. Keiya Ozawa, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1, Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi, 329-0498, Japan.E-mail: kozawa@ms2.jichi.ac.jp

other, but less efficiently with wild-type sequences on treatment with Cre *(8)*. In the present study, we compared the efficiency of recombination and designed plasmids to express optimal amounts of Rep and Cap proteins on Cre treatment. By optimizing these parameters, we developed a packaging cell line with improved production rate compared with our prototype cell line.

2. Materials and Methods

2.1. Cells and Plasmids

A human embryonic kidney cell line, known as 293 cells *(9)*, was maintained as described previously *(4)*. Plasmid ploxlox (a gift from Dr. Jamey D. Marth), which contains two adjacent loxP sequences in the same direction, was used as a backbone for the wild-type loxP *(10)*. To make plas-mids with mutant loxP, the sequences corresponding to the spacer region of loxP were mutated by synthesizing oligonucleotides based on published sequence information (**Fig. 1A**) *(8)*. Briefly, the spacer region of wild-type loxP constitutes ATGTATGC; for the loxP (V) and loxP(S), the sequences correspond to 5'-ATGT GTAC-3' and 5'-AAGTATCC-3', respectively. The CAG promoter (a gift from Dr. J Miyazaki, Osaka University, Japan) *(11)*, neomycin resistance gene, blasticidin S resistance gene (Invitrogen Corp., Carlsbad, CA), bacterial LacZ sequence, and AAV sequences corresponding to *p5*, *rep*, and *cap* genes were excised and ligated to complete plasmids named CAPBPL, CAVBVL, CASBSL, CAPBPC, and p5SNSR, respectively (*see* **Fig. 1B**), using standard techniques as reported previously *(4)*.

2.2. Induction of Recombination and Demonstration of Gene Expression

Plasmids encoding *LacZ* gene with the "stuffer" sequences between the two loxP sequences (CAPBPL, CAVBVL, CASBSL) were introduced into 293 cells using a standard calcium phosphate transfection technique *(12)*. Briefly, 1 μg of plasmid was mixed with 150 μL of 0.3*M* $CaCl_2$ and 2X HBS buffer and added to a single 6-well chamber. Six hours later, the medium was replenished. To assess the efficiency of recombination, a Cre-expressing adenovirus vector (AxCANCre, a gift from Dr. I. Saito) was applied to the culture thereafter at an MOI of 1 *(13)*. At various time-points, cells were dislodged, lysed, and β-galactosidase activity was measured by orthonitrophenyl-β-galactosidase assay (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Lysates were then subjected to Western analyses, either with anti-Rep (clone 303.9, Progen, Heidelberg, Germany) or anti-Cap (clone B1, Progen, Heidelberg, Germany), as reported previously *(4)*.

2.3. Development of Clones

Seven micrograms of each plasmid were used to transfect one 10-cm dish of 293 cells using a standard calcium-phosphate method at 70% of confluence. Forty-eight hours later, the cells were replated to several dishes and exposed to the selection medium containing both 800 μg/mL of G418 and 10 μg/mL of blasticidin S. The selection medium was replenished every 3 d. After 2 to 3 wk of selection, individual clones were recovered and amplified in 12-well plates in the presence of a half concentration of the selection medium of G418 and Blasticidin S. When a clone grew to semiconfluence in a 12-well plate, it was assumed to be established and was subjected to the analysis for packaging titer. The clones were numbered according to the order of establishment.

2.4. Titration of Vector Production

Established clones were further expanded, replated in new 12-well plates, then transfected with 0.5μg of the vector plasmid containing the *LacZ* gene cassette (driven by cytomegalovirus [CMV] promoter) flanked by two ITR sequences. Six hours after transfection, the medium was replenished, and Cre-expressing adenoviruses (AxCANCre) were added at an MOI of 1. Forty-eight hours later, cells were collected, subjected to three cycles of freeze–thawing, and treated with deoxyribonuclease I (Takara Bio, Inc., Ohtsu, Japan) for 30 min at 370°C as indicated by the manufacturer. The samples were quantified using dot-blot analysis. Known copy numbers of LacZ-expressing plasmid was used as controls.

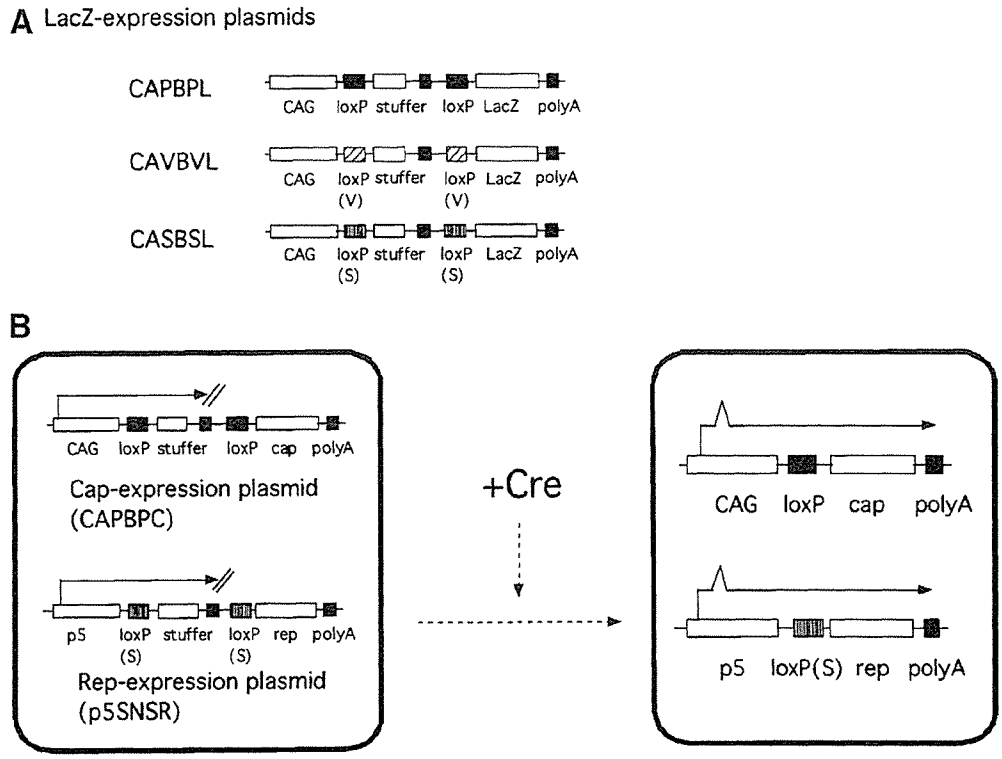


Fig. 1. Plasmid structure and the scheme of the cell line development. (**A**). To assess the recombination efficiency of the mutant loxP, three LacZ-expression plasmids are constructed. On infection with Ad-Cre, the stuffer sequences are removed with the efficiency depending on the wild-type or mutant loxP sequences, and the *LacZ* genes are driven by the CAG promoter. (**B**). The design of the cell line and the strategy for *rep* and *cap* expression are shown. To control *rep* and *cap* expression, a stuffer sequence is flanked by two loxP (wild-type or mutant) sequences. In the presence of Cre recombinase, the stuffer sequences are removed and the *cap* and *rep* genes are expressed.

2.5. Demonstration of Cre-Mediated Recombination by Polymerase Chain Reaction

Genomic deoxyribonucleic acid (DNA) was extracted from clone no. 3 by the standard techniques. Briefly, the recovered cells were treated with proteinase K, and total DNA was extracted with phenol chloroform. One microgram of total DNA was used as a template. A thermal cycler and the DNA polymerase ex-*Taq* (Takara Bio Inc., Ohtsu, Japan) were used for the PCR reaction according to the manufacturer's instructions. The forward primer sequences were 5'-TTC GGC TTC TGG CGT GTG AC-3' and 5'-TTG CGA CAT TTT GCG ACA CCA-3' for the *cap* and *rep* sequences, respectively. The reverse primer sequences were 5'-TCT GCG TAG TTG ATC GAA GCT-3' and 5'-GGG ACC TTA ATC ACA ATC TCG-3', respectively. The conditions for PCR were 940°C for 30 s, 560°C for 30 s, and 720°C for 1 min, and a total of 20 cycles of amplification were applied. The products were analyzed by agarose gel electrophoresis (0.8%) and visualized through ethidium bromide staining.

2.6. Statistical Analysis

The significance of the difference was estimated by Student's paired *t*-test.

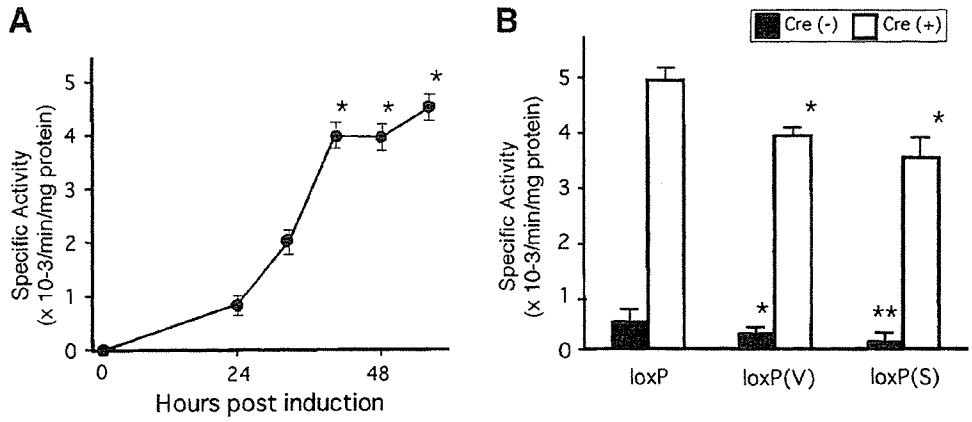


Fig. 2. Efficiency of recombination demonstrated in the wild-type and the mutant loxP. (**A**). Recombination activity of the wild-type loxP assessed at different time-points. The *asterisks* indicate the statistical significance ($p < 0.05$) of each data point against the nonindicated points. No significant differences were observed among the data points indicated by an asterisk. (**B**). Recombination efficiencies of wild-type and mutant loxP sequences. LacZ-expressing plasmids described in **Fig 1A** were used. Following transfection of the plasmids, 293 cells were infected by Ad-Cre at an MOI of 1. After 48 h, b-Gal activity was quantified. As for the baseline expression, values observed by loxP(V) were significantly lower than those with wild type as indicated by an *asterisk*. The baseline expression with loxP(S) was further significantly lower than that with loxP(V), as indicated by two *asterisks*. In terms of the expression levels following induction with Cre, values with both mutant loxPs showed significantly lower expression than those with wild type, as indicated by an *asterisk*. No differences were found in the values obtained by the mutant loxPs.

3. Results

3.1. Recombination Efficiency of Wild-Type and Mutant LoxP

To determine the optimal conditions for Cre–loxP-mediated induction of gene expression, we first examined the efficiency of recombination at various time-points. Recombination became significant at 40 h of induction by Ad-Cre and reached a plateau level thereafter (**Fig. 2A**). Therefore, we selected 48 h as a standard time-point for assessment of recombination. Then we compared the efficiency of recombination among the loxP sequences. The wild-type loxP showed the highest recombination efficiency, as assessed by LacZ expression (**Fig. 2B**). Therefore, we selected wild-type loxP for Cap expression plasmid. On the other hand, we selected the loxP (S) sequences for the Rep expression plasmids, as the basal level of expression was the lowest with this system.

3.2. Expression of Rep and Cap on Induction

Rep and Cap expression could be induced simultaneously by Cre recombinase (**Fig. 3**). Coexpression of the other protein inhibited the expression levels in both cases. The degree of Cap suppression was clearer than that of Rep suppression.

3.3. Development of Clones With Packaging Capacity

Following a period of 2–3 wk in selection medium containing both blasticidin S and G418, a total of 192 clones were chosen and amplified into 6-well culture plates. Of these, 22 clones reached semiconfluence in 10-cm dishes. These clones were further amplified, transfected with LacZ-expressing vector plasmids, and their ability to produce vector was determined. All of the 22 clones showed significant levels of vector production (**Table 1**). These LacZ-encoding vectors were capable of transducing 293 cells with similar effi-

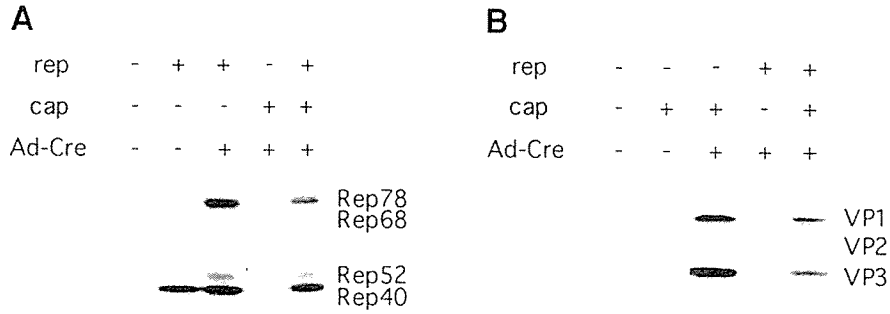


Fig. 3. *Rep* and *cap* expression profiles on treatment with Cre recombinase. One microgram of each plasmid was transfected into 293 cells in a 6-well plate. Following transfection, the cells received Ad-Cre and were lysed 48 h later. The cell lysates were analyzed by Western blotting using the monoclonal antibodies (**A**) 303.9 for *rep*, and (**B**) B1 for *cap*, respectively.

ciency to that made by the standard transfection method as assessed by conventional X-Gal staining (data not shown).

3.4. Stability of the Clones Developed

The established clones were further amplified and their stability was assessed at different time-points. As shown in **Table 1**, among the 22 clones developed, 6 continued to amplify for 2 wk. All of the expanded clones showed a significant packaging capacity. Of these, four clones tolerated additional expansion. These clones kept the growth speed of 293 cells, whereas the clones developed later (the higher numbered clones) tended to become slow growing. Of these, clone no. three maintained a significant packaging capacity throughout this period in terms of production of the AAV vector.

3.5. Detection of Cre-Mediated Recombination Events

Results of the analysis of clone no. 3 are shown in **Fig. 4**. PCR detection resulted in the amplification of the 1.1 and 1.2 kb for cap- and rep-expressing sequences within the untreated cells. Following Cre administration, shorter truncated sequences with 0.3 and 0.2 kb were amplified, suggesting simultaneous recombination events.

Table 1
The Actual Titer of the Clones Obtained in This Study

Clone no.	0 Wk[a]	2 Wk	4 Wk
3	20.9[b]	11.6	15.0
8	15.3	6.7	1.3
10	11.3	4.4	0.4
11	0.7	—	—
12	31.5	2.4	2.8
15	2.5	—	—
17	5.2	—	—
22	2.9	—	—
25	3.4	—	—
27	7.5	—	—
28	15.0	12.5	—
31	1.6	—	—
33	6.7	—	—
34	5.5	—	—
35	7.7	—	—
36	2.6	—	—
38	10.6	—	—
40	2.9	—	—
41	5.8	—	—
44	10.9	11.1	—
57	4.4	—	—
60	1.8	—	—

[a]The time-point started when a clone grew to semiconfluence within a 10-cm dish. At that time, the clone was challenged for packaging titer. Clone no. 3 retained a significant packaging capacity throughout the study.

[b]Titers of AAV vector per 10-cm dish ($\times 10^9$)

4. Discussion

In this study, we extended our previous findings *(4,5)* to regulate the expression of both *rep* and *cap* simultaneously, leading to development of a novel packaging cell line for the production of AAV vectors. Numerous attempts have been made to establish packaging cell lines, and sev-

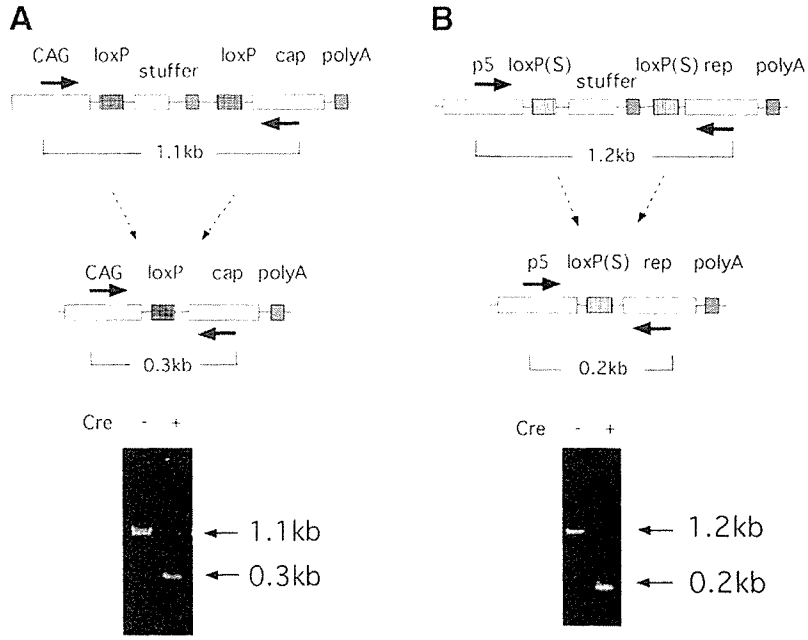


Fig. 4. The positions of the primers designed to detect both vector sequences before and after recombination are indicated by arrows. Primers corresponding to the CAG promoter and cap sequences or primers for p5 and *rep* sequences were used to amplify the vector sequences in (**A**) and (**B**), respectively. In both cases, a smaller number of cycles (20 cycles for this experiment) were used to avoid plateau-level amplifications.

eral promising lines with high production rates have been reported ***(14,15)***. Our strategy is advantageous in that there is a potential to optimize the condition for expressing Rep and Cap independently, as the genes of interest are encoded into two separate plasmids. In our previous study, we developed a cell line using Cre/loxP system to regulate Rep expression ***(4)***. Our present study showed that the simultaneous regulation of Cap expression in addition to Rep resulted in the improvement of production rate by 30-fold. The ideal amounts for the optimal production of vector are examined using two different plasmids encoding for *rep* and *cap* ***(16)*** by transient transfection, and the result indicates that a large amount of Cap and relatively small amount of Rep are suitable for the maximum production rate. To accomplish these conditions, we used a stronger promoter for *cap* and a weaker one for *rep*. Also, the choice of loxP sequences from a panel of mutant and wild-type sequences merits discussion in terms of recombination efficiency and leakage. Recombination efficiencies of the mutant and wild-type loxP in our system were comparable to those reported earlier ***(8)***. In addition to the recombination efficiency, the degree of "leakage" is also important to avoid toxicity, especially for Rep. As demonstrated in **Fig. 2B**, wild-type loxP showed the highest leakage despite the fact that it recombines most efficiently. As Rep proteins are highly toxic, the baseline expression must be kept as low as possible. Also, it is known that Rep expression need not be high even after induction. For these reasons, loxP (S) seemed to be the appropriate choice for the *rep*-expressing plasmids. Because low-level expression of Rep proteins is optimal for efficient vector production, a weak native promoter (p5) was used for the Rep-expression plasmid. On the other hand, because the expression of a large amount of Cap protein is required, a strong CAG

promoter was selected in combination with wild-type loxP sequences for the Cap-expression plasmid. CAG promoters have been shown to be strong and versatile in various systems *(17)*.

In **Fig. 3**, we observed a reduction in Rep or Cap expression in the presence of the other protein. Especially, Cap expression was suppressed by the presence of Rep. This may reflect the interference of both proteins, especially at the time of overexpression.

Although we tried to obtain a suitable condition for vector production, there is a possibility that the other factors might influence the optimal conditions. Of particular concern are the choice of producer cell type and promoters. For the prototype, we used 293 cells for a number of reasons. First of all, as 293 cells are derived from nonmalignant cells, they are appropriate for production of clinical-grade vectors. Also, we have several experiences developing cell lines based on 293 cells. The cells are easy to handle, and can be introduced efficiently into genes by conventional transfection methods. Regarding the promoter to drive *rep* and *cap* genes, there is a wide range of choice. Our use of heterologous promoters may contribute to high titers of vector production *(16)*.

The stability of the cells is also an important aspect from a practical point of view. In this study, we followed virus production for 4 wk after development. This period is sufficient for the cells in a large-scale culture. We numbered the clones according to the order of establishment. As a matter of fact, for the clones that developed later, there was a tendency not to survive long enough to assess their capability. Actually, clones later than no. 60 could not be expanded sufficiently to assess their long-term potential. Although the precise mechanisms are not clear, these properties in growth may be related to the leaky expression of Rep and Cap before induction with Cre. It is likely that only clones with optimum conditions can continue to grow during the course of large-scale expansion.

The use of AAV vectors is moving toward clinical applications. The refinement of the vector production system is vital to meet the anticipated increase in demand.

Acknowledgments

The authors are grateful to Drs. I. Saito and Y. Kanegae for valuable advice concerning mutant loxP sequences and for Cre-expressing adenovirus vectors. Also, we thank RIKEN DNA bank (Tsukuba, Japan) for distributing adenovirus vectors, and Avigen, Inc. for supplying plasmids for AAV vector production. This work was supported in part by grants from the Ministry of Health, Labor and Welfare of Japan and Grants-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology.

References

1 Kay, M. A., Manno, C. S., Ragni, M. V., et al. (2000) Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet.* **24,** 257–261.

2 During, M. J., Kaplitt, M. G., Stern, M. B., and Eidelberg, D. (2001) Subthalamic GAD gene transfer in Parkinson disease patients who are candidates for deep brain stimulation. *Hum. Gene Ther.* **12,** 1589–1591.

3 Muramatsu, S., Fujimoto, K., Ikeguchi, K., et al. (2002) Behavioral recovery in a primate model of Parkinson's disease by triple transduction of striatal cells with adeno-associated viral vectors expressing dopamine-synthesizing enzymes. *Hum. Gene Ther.* **13,** 345–354.

4 Ogasawara, Y., Mizukami, H., Urabe, M., et al. (1999) Highly regulated expression of adeno-associated virus large Rep proteins in stable 293 cell lines using the Cre/loxP switching system. *J. Gen. Virol.* **80,** 2477–2480.

5 Okada, T., Mizukami, H., Urabe, M., et al. (2001) Development and characterization of an antisense-mediated prepackaging cell line for adeno-associated virus vector production. *Biochem. Biophys. Res. Commun.* **288,** 62–68.

6 Holscher, C., Horer, M., Kleinschmidt, J. A., Zentgraf, H., Burkle, A., and Heilbronn, R. (1994) Cell lines inducibly expressing the adeno-associated virus (AAV) rep gene: requirements for productive replication of rep-negative AAV mutants. *J. Virol.* **68,** 7169–7177.

7 Yang, Q., Chen, F., and Trempe, J. P. (1994) Characterization of cell lines that inducibly express the adeno-associated virus Rep proteins. *J. Virol.* **68,** 4847–4856.

8 Lee, G. and Saito, I. (1998) Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene* **216,** 55–65.

9 Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line trans-

formed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–74.

10 Orban, P. C., Chui, D., and Marth, J. D. (1992) Tissue- and site-specific DNA recombination of transgenic mice. *Proc. Natl. Acad. Sci. USA* **89,** 6861–6865.

11 Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108,** 193–199.

12 Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978) Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as a donor. *Cell* **14,** 725–731.

13 Kanegae, Y., Lee, G., Sato, Y., et al. (1995) Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res.* **23,** 6816–3821.

14 Gao, G. P., Qu, G., Faust, L. Z., et al. (1998) High-titer adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus. *Hum. Gene Ther.* **9,** 2353–2362.

15 Inoue, N. and Russell, D. W. (1998) Packaging cells based on inducible gene amplification for the production of adeno-associated virus vectors. *J. Virol.* **72,** 7024–7031.

16 Ogasawara, Y., Urabe, M., Kogure, K., et al. (1999) Efficient production of adeno-associated virus vectors using split-type helper plasmids. *Jpn. J. Cancer Res.* **90,** 476–483.

17. Kiwaki, K., Kanegae, Y., Saito, I., et al. (1996) Correction of ornithine transcarbamylase deficiency in adult spf(ash) mice and in OTC-deficient human hepatocytes with recombinant adenoviruses bearing the CAG promoter. *Hum. Gene Ther.* **7,** 821–830.

Journal of Thrombosis and Haemostasis, **2**: 275–280

ORIGINAL ARTICLE

Specific detection of human coagulation factor IX in cynomolgus macaques

J. MIMURO, H. MIZUKAMI,* F. ONO,† S. MADOIWA, K. TERAO,† A. YOSHIOKA,‡ K. OZAWA* and Y. SAKATA

*Divisions of Cell and Molecular Medicine and *Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi-ken, Japan; †Tsukuba Primate Center of National Institute of Infectious Diseases, Tsukuba, Ibaraki-ken, Japan; and ‡Department of Pediatrics, Nara Medical University, Kashihara, Nara-ken, Japan*

To cite this article: Mimuro J, Mizukami H, Ono F, Madoiwa S, Terao K, Yoshioka A, Ozawa K, Sakata Y. Specific detection of human coagulation factor IX in cynomolgus macaques. *J Thromb Haemost* 2004; **2**: 275–80.

Summary. After screening for species-specific antihuman factor (F)IX monoclonal antibodies, we found that antibody 3A6 did not bind to cynomolgus FIX. The 3A6 epitope was found to include Ala262 of human FIX. The 3A6 antibody was used as a catching antibody in an enzyme immunoassay (EIA) for specific detection of human FIX in cynomolgus macaque plasma. No significant increase of substrate hydrolysis was observed when EIA buffer containing cynomolgus macaque plasma was subjected to the 3A6-based EIA. Addition of up to 30% cynomolgus macaque plasma or canine plasma to the assay did not alter detection of human FIX. Three cynomolgus macaques were injected with human FIX ($10\,U\,kg^{-1}$; i.v.) and the circulating human FIX was quantified in the macaque plasma. The FIX level in the circulation increased to $470 \pm 37.6\,ng\,mL^{-1}$ at 1 h after the injection and gradually decreased to $1.79 \pm 1.1\,ng\,mL^{-1}$ by day 5, which is approximately 0.06% of the normal human plasma FIX concentration. These data suggest that the cynomolgus macaque can be used as a primate model for studying hemophilia B gene therapy by transduction of macaque organs with vectors to express human FIX *in vivo* and detection of human FIX using the 3A6 monoclonal antibody.

Keywords: cynomolgus macaque, factor IX, hemophilia B.

Introduction

Hemophilia B is an X-linked, hereditary life-long bleeding disorder caused by genetic abnormality of the coagulation factor (F)IX gene. The genetic abnormalities result in deficiency of FIX, which in turn creates a bleeding diathesis, such as life-threatening intracranial bleeding and bleeding in joints and muscles. Hemophilias occur as mild, moderate, or severe, depending on the patient blood FIX level of $\geq$6%, 2–5%, or $\leq$1%. Although recombinant FIX products are available in the USA, current standard therapy in Japan is still intravenous (i.v.) injection of heat-treated and monoclonal antibody (mAb)-purified FIX concentrates from plasma. Aside from certain specific situations, such as preoperative factor coverage, i.v. infusion of FIX concentrates is usually used to treat acute bleeding episodes and prophylactic FIX i.v. infusion is not recommended in Japan.

Notably, maintenance of blood FIX levels of $\geq$2% of the normal plasma FIX concentration may result in significant clinical improvement. Unfortunately, in the past, infection with hepatitis B and C viruses or human immunodeficiency virus (HIV) in hemophilia patients was a tragic result of contaminated blood-derived commercial products. After introduction of heat treatment, detergent treatment, and/or mAb isolation of FIX, the risk of viral infection by commercial products was significantly reduced, but not eliminated. Recombinant FIX products are now commercially available in the USA, but also may not be completely free of pathological substances such as prions or as yet unknown viruses. In this context, gene therapy is being explored as the next generation therapy for hemophilia [1,2]. To develop gene therapy technology, a good animal model is necessary. Hemophilia B mice (FIX knock-out mice) and hemophilia B dogs are available and have been used to study gene therapy approaches for hemophilia B. Based upon promising results gathered in these animals after receiving intramuscular injection of type 2 AAV vectors carrying the FIX gene [3–6], clinical trials for hemophilia B by transduction of skeletal muscles with these vectors were initiated, but have had limited success [7,8]. Vector doses of 1.8×10^{12} vector genome (vg) kg^{-1} yielded plasma FIX levels of >1% in mice, whereas the same vector dose yielded circulating FIX levels of 0.2–0.4% in dogs. In humans, no significant increase of FIX levels was observed with the same vector dose. One possible explanation of the differences in these results is that the transduction

Correspondence: Yoichi Sakata, Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical School, Tochigi-ken 329-0498, Japan.
Tel.: +81 285 58 7398; fax: +81 285 44 7817; e-mail: yoisaka@jichi.ac.jp

Received 8 May 2003, accepted 17 October 2003

efficiency of skeletal muscles by type 2 AAV vectors in humans is different from that in these animals. Since this vector dose was the highest dose used in the clinical trial, the possibility that vector doses were not high enough for FIX expression in hemophilia B patients was most likely [8]. In this regard, a primate model may be required to mimic more closely the human situation. Anti-FIX mAbs were screened for their inability to bind simian FIX. One antibody was found and forms the basis for an enzyme immunoassay (EIA) and quantification of human FIX in primates down to 1.7 ng mL^{-1}, or 0.06% of the normal plasma FIX concentration.

Materials and methods

Murine monoclonal antibodies

Murine mAbs raised against human FIX were developed by standard procedures. Quantification of human FIX with mAb 3A6 was reported previously [9]. JKIX-1 is a murine mAb that binds to human FIX in the presence of calcium [10]. JKIX-1 was labeled with NHS-biotin (Vector Labs, Burlingame, CA, USA) according to the manufacturer's instructions.

Enzyme immunoassay and Western blotting for FIX

Microtiter plates were coated with 3A6 in PBS (1 μg mL^{-1}) for 16 h at 4 °C. After blocking with 5% casein in PBS, samples containing FIX (wild-type human FIX or FIX mutants; see below) were incubated in Tris-buffered saline pH 7.4 (TBS) containing 1% casein and 0.1% Triton X-100 at 37 °C for 2 h. After washing with TBS containing 0.1% Triton X-100, bound FIX antigen was detected with sheep antihuman FIX polyclonal antibody (Cedarlane Labs Ltd, Hornby, Ontario, Canada) followed by horseradish peroxidase (HRP)-labeled antisheep IgG (Vector Labs). JKIX-1 based EIA was also carried out in a similar manner using buffer containing 5 mM $CaCl_2$ except during the plate-coating step. Western blotting for FIX was carried out as described previously [11] except for detection of HRP-labeled antibodies bound to polyvinylidene fluoride membrane using chemiluminescent reagent ECL + (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). For quantifying human FIX in cynomolgus macaque, macaque plasma diluted in TBS containing Triton X-100 (0.1%) and casein (1%) was added to 3A6-coated microtiter plates and 3A6-bound FIX was detected by biotin-labeled JKIX-1 followed by HRP-conjugated streptavidin (Vector Labs).

Expression of human FIX and mutant FIX in CHO-K1 cells

Human FIX cDNA was a generous gift from Dr G. G. Brownlee (Chemical Pathology Unit, University of Oxford, Oxford, UK). Human FIX cDNA was directionally cloned into the pcDNA3 expression vector (Invitrogen Japan, Tokyo, Japan) to make plasmid pcDNAFIXWT. This was subjected to site-directed mutagenesis to construct pcDNAFIX/G226D/V227A (G226 to D and V227 to A), pcDNAFIX/A262T (A262 to T), and pcDNAFIX/L321S (L321 to S), for expression of FIX mutants with the indicated amino acid substitutions. To determine the epitope of mAb 3A6, FIX mutants were expressed in CHO-K1 cells cultivated in HAMF-12 media in the presence of vitamin K (10 μg mL^{-1}). CHO-K1 cells (5×10^6) in 0.8 mL of Dulbecco's PBS were incubated with 20 μg of pcDNAFIX/WT, pcDNAFIX/G226D/V227A, pcDNAFIX/A262T, or pcDNAFIX/L321S, on ice for 15 min and subjected to electroporation at 300 V (25 μF) using a Gene Pulser (BioRad Labs, Hercules, CA, USA). To make stable transfectants that express wild-type human FIX and FIX mutants, cells were cultured in the presence of geneticin (250 μg mL^{-1}) (Gibco-Invitrogen Japan, Tokyo, Japan). Cloned geneticin-resistant cell lines were selected for FIX expression by the JKIX-1-based EIA and selected clones were cultured in the presence of vitamin K (10 μg mL^{-1}). Coagulation activities of recombinant wild-type FIX and FIX mutants expressed in CHO-K1 cells were determined using FIX-deficient plasma (Dade Behring, Marburg, Germany) and an automated coagulometer model CA-500 (Sysmex, Tokyo, Japan). Recombinant FIX antigen concentration in the conditioned medium was determined with the two different EIAs as described above.

Detection of human FIX in cynomolgus macaque

Human plasma was mixed with increasing concentrations of cynomolgus macaque plasma in EIA buffer and subjected to the EIA as described to see if macaque FIX inhibited human FIX binding to 3A6. Human FIX concentrates (10 U kg^{-1}) (Christmassin M®; Mitsubishi Pharma Co., Tokyo, Japan) were injected intravenously into three cynomolgus macaques. After injection, peripheral blood was drawn and citrated plasma was collected at indicated time periods. Concentrations of human FIX in cynomolgus macaque plasma were determined using 3A6 as a capture antibody with detection by biotin-conjugated JKFIX-1 as described above.

Binding of mAb 3A6 to deglycosylated FIX and canine FIX

Conditioned media of Chinese hamster ovary (CHO) cells secreting wild-type FIX and mutant FIX A262T were incubated in the absence or presence of N-glycosidase F (10 U mL^{-1}) (Roche Diagnostics GmbH, Mannheim, Germany) and analyzed for binding of 3A6 to deglycosylated wild-type FIX and deglycosylated FIX A262T by Western blotting [12]. We also studied the effect of canine FIX on binding of 3A6 to human FIX by the EIA. Human plasma (1 : 100 dilution) in the buffer containing increasing concentrations (0–30%) of canine plasma obtained from a beagle dog was subjected to the 3A6-based EIA as described above to see if the presence of canine FIX decreased binding of human FIX to mAb 3A6.

Immunohistochemical study of the cynomolgus macaque liver

A small part of the liver was obtained from a cynomolgus macaque under anesthesia with ketamine hydrochloride. Biopsy specimens were fixed with 4% paraformaldehyde in PBS at 4 °C

for 2 h, 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 liver tissues at −25 °C, attached to poly lysine-coated glass slides and subjected to immunohistochemistry by the standard ABC method using mouse mAb 3A6, sheep polyclonal antibodies to human FIX, control IgG (normal sheep IgG, normal mouse IgG), biotin-conjugated second antibodies and streptavidin–biotin complex reagents (Vectarstain ABC Elite kit; Vector Labs), and diaminobenzidine (DAB). Sections were counterstained with Meyer's hematoxylin solution to visualize nuclei.

Results

After screening for a species-specific mAb that recognizes solely human FIX, we found that mAb 3A6 could distinguish human FIX from macaque FIX. We developed an EIA for human FIX using 3A6 and biotin-labeled JKIX-1. The assay is sensitive to 1 ng mL^{-1} purified human FIX and detects FIX antigen in human plasma at $1:3 \times 10^3$ dilution. No increase in substrate hydrolysis was observed when macaque plasma or canine plasma was added to the microtiter plates instead of human plasma. Furthermore, addition of cynomolgus macaque plasma to human plasma did not influence the substrate hydrolysis of human plasma containing samples (Fig. 1), nor did the presence of canine plasma in the buffer influence data of the EIA for human FIX.

Macaque FIX is highly homologous to human FIX with 97% amino acid similarity [13]. Since 3A6 binding is not dependent on divalent cations and binds to the catalytic domain of human FIX after RVV XCP treatment under reducing conditions on Western blotting (not shown), the 3A6 epitope was deemed to be probably a linear sequence. The amino acid sequence of cynomolgus macaque FIX is identical to rhesus macaque FIX [14]. Amino acid residues Gly226, Val227, Ala262, or Leu321 of human FIX were targeted as residues in the 3A6 epitope based upon the sequence similarity among human FIX, macaque FIX, and mouse FIX. Thus, these residues were substituted with Asp, Ala, Thr, or Ser, as these are the corresponding residues in macaque FIX. Because Gly226 and Val227 are positioned sequentially, site-directed mutagenesis was carried out to substitute these two residues with Asp and Ala in the same molecule. Conditioned media of stably transfected CHO-K1 cells were harvested and binding of 3A6 to wild-type FIX and FIX mutants was analyzed by the 3A6-based EIA, the JKIX-1-based EIA, and by Western blotting. As shown in Table 1 and Fig. 2, 3A6 bound to wild-type FIX, to FIX G226D/V227A and to FIX L321S, but failed to bind to FIX A262T. Human FIX does not have an oligosaccharide side chain at Asn260, but macaque FIX may have N-linked carbohydrates at this position because of formation of the N-linked glycosylation consensus sequence Asn-X-Thr. Mutant FIX A262T could also have an extra oligosaccharide side chain at Asn260. To study the possibility that the potential extra oligosaccharides linked to Asn260 of FIX A262T directly interfere with binding of mAb 3A6 to FIX A262T, the conditioned media of CHO cells secreting FIX A262T were treated with N-glycosidase F which can remove N-linked oligosaccharide side chains, including complex type carbohydrates, from glycoprotein and analyzed for 3A6 binding by Western blotting. As shown in Fig. 2B, 3A6 bound to wild-type FIX and deglycosylated FIX (lower molecular weight form) but did not bind to FIX A262T even after removal of carbohydrate side chains. Thus, the potential glycosylation at Asn260 may not be essential for no cross-reactivity of mAb 3A6 to FIX A262T and to macaque FIX, although it

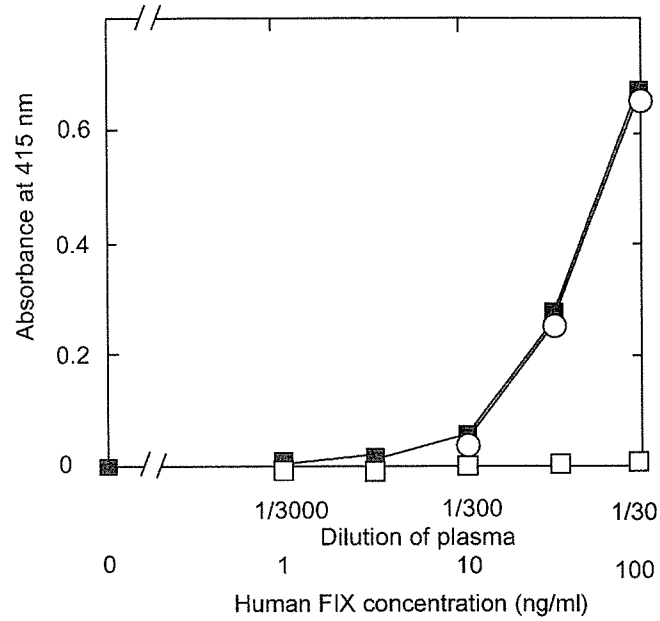


Fig. 1. Effect of macaque plasma on human factor (F)IX detection by 3A6-based enzyme immunoassay. Human plasma (■) or macaque plasma (□) diluted 30–3000-fold was incubated in microtiter plates coated with 3A6 antibody. Human plasma was diluted 30–300-fold with buffer containing 30% macaque plasma (○). Bound FIX was detected by biotin-labeled JKIX-1 followed by horseradish peroxidase-conjugated streptavidin as described in Methods.

Table 1 Concentration and activity of recombinant human factor (F)IX expressed in CHO-K1 cells

	Wild- type FIX	FIX G226D/V227A	FIX A262T	FIX L321S
3A6-based EIA, %	17.7	7.6	0	8.8
JKIX-1-based EIA, %	16.5	7.5	5.0	9.5
Clotting activity, %	16.3	1.8	3.4	2.9
Specific activity	0.99	0.24	0.68	0.30

Recombinant FIX antigen determined by enzyme immunoassay (EIA) is expressed as a percentage of the FIX concentration of control plasma. Specific activities are calculated by dividing the clotting activity of the conditioned medium by the antigen concentration determined by JKIX-1-based EIA.

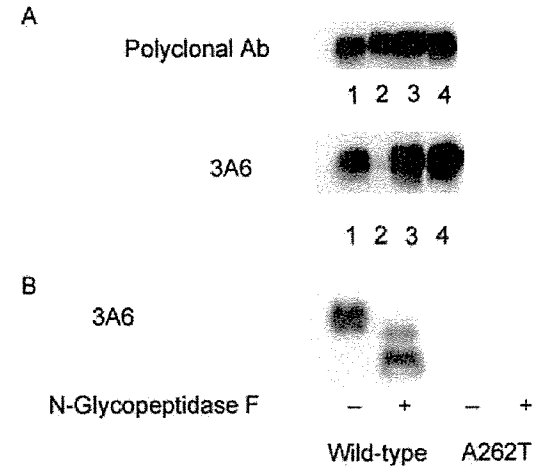


Fig. 2. Western blot analysis of factor (F)IX mutants. (A) Conditioned media of CHO-K1 cells secreting FIX G226D/V227A (lane 1), FIX A262T (lane 2), FIX L321S (lane 3) and wild-type FIX (lane 4) were transferred to polyvinylidene fluoride (PVDF) membrane after SDS–PAGE under reducing conditions and incubated with either polyclonal antibodies to human FIX or monoclonal antibody 3A6. Bound antibodies were detected with horseradish peroxidase (HRP)-labeled antisheep IgG or HRP-labeled antimouse IgG, respectively, followed by chemiluminescent reagents, as described in Materials and methods. (B) Chinese hamster ovary (CHO) cell-conditioned media containing wild-type FIX (wild-type) or FIX A262T (A262T) incubated in the absence (–) or presence (+) of N-glycopeptidase F were transferred to PVDF membrane after SDS–PAGE and analyzed for binding of 3A6 to recombinant FIX as above.

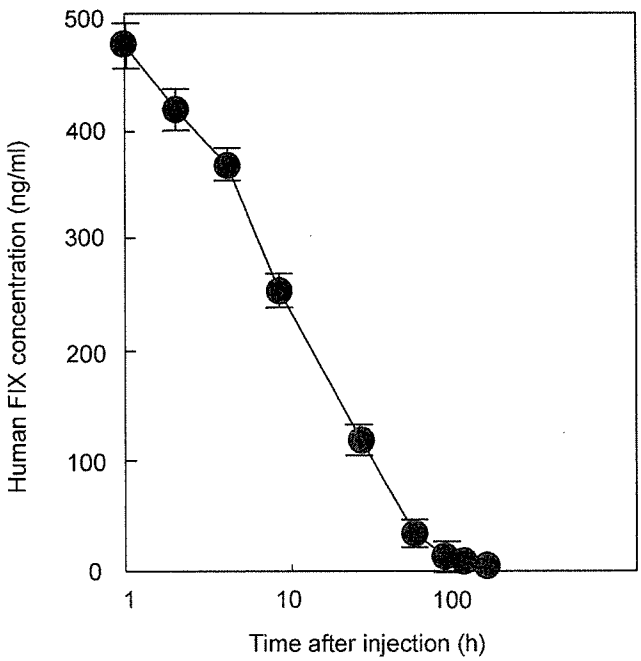


Fig. 3. Quantification of human factor (F)IX in cynomolgus macaque. FIX concentrates (10 U kg^{-1}), used to treat hemophilia patients, were injected intravenously into three cynomolgus macaques. After injection, peripheral blood was drawn and platelet-poor plasma was prepared. Human FIX levels in macaque plasma was determined by enzyme immunoassay using 3A6 and biotin-labeled JKIX-1. Data represent mean ± SD ($n = 3$).

may affect the interaction of 3A6 with FIX A262T and with macaque FIX indirectly. The presence of canine plasma in the buffer did not decrease human FIX binding to 3A6 in the EIA (not shown), supporting this possibility. The coagulation activities of the FIX mutants were also determined to explore the possibility that these FIX mutants developed severe conformational changes in the catalytic domain by these amino acid substitutions. The specific activities (clotting activity of the conditioned medium divided by the antigen concentration determined by JKIX-1-based EIA) of recombinant wild-type FIX, FIX G226D/V227A, FIX A262T, and FIX L321S were 0.98, 0.24, 0.68, and 0.3, respectively (Table 1). These observations demonstrated that the amino acid substitutions did affect coagulation activity. However, FIX A262T did retain about 70% of the activity relative to wild-type FIX, suggesting that conformational effects were minimized and that loss of 3A6 binding to FIX A262T was due primarily to epitope alteration.

In order to detect and quantify human FIX in the cynomolgus macaque, 10 U kg^{-1} human FIX was injected intravenously into three animals and the concentration of human FIX in their plasma was quantified with the 3A6-based EIA (Fig. 3). Human FIX plasma levels increased to 470 ± 37.6 ng mL^{-1} by 1 h after the injection, then gradually decreased with a half-life of 10 h. When FIX is injected intravenously into hemophilia B patients, circulating FIX levels decrease rapidly (α phase $t_{1/2}$, 4–5 h) then slowly (β phase $t_{1/2}$, 23–28 h) [15]. The rapid decrease of FIX is though to be due to distribution into extravascular space. Since human FIX levels in macaques at early time points after injection were not measured, our data may not accurately discriminate the rapid-phase and the slow-phase disappearance of human FIX in macaques. The difference between $t_{1/2}$ of intravenously injected human FIX in macaques in our experiments and that in the previous study [16] may be accounted for by the difference in FIX doses. On day 4, plasma human FIX levels fell to 4.2 ± 2.8 ng mL^{-1} (0.14% of the normal human FIX concentration) and were reduced further to 1.79 ± 1.1 ng mL^{-1} (0.06% of the normal human FIX concentraion) on day 5, indicating that the 3A6-based EIA was able to detect human FIX levels as low as 0.1% of the normal human FIX concentration.

To confirm that mAb 3A6 does not bind to cynomolgus macaque FIX *in vivo*, an immunohistochemical study was carried out. As shown in Fig. 4, positive immunostaining was observed when the frozen sections of the macaque liver were incubated with sheep polyclonal antibodies to human FIX. However, no immunostaining was observed in the liver sections incubated with 3A6.

Discussion

A wide variety of disorders are caused by genetic abnormality, giving rise to the potential of gene therapy as the next generation of therapeutics for many diseases. To establish gene therapy technology, a good animal model is required. Advances in developmental biotechnology have allowed us to create a variety of mouse disease models, transgenic mice and knock-out