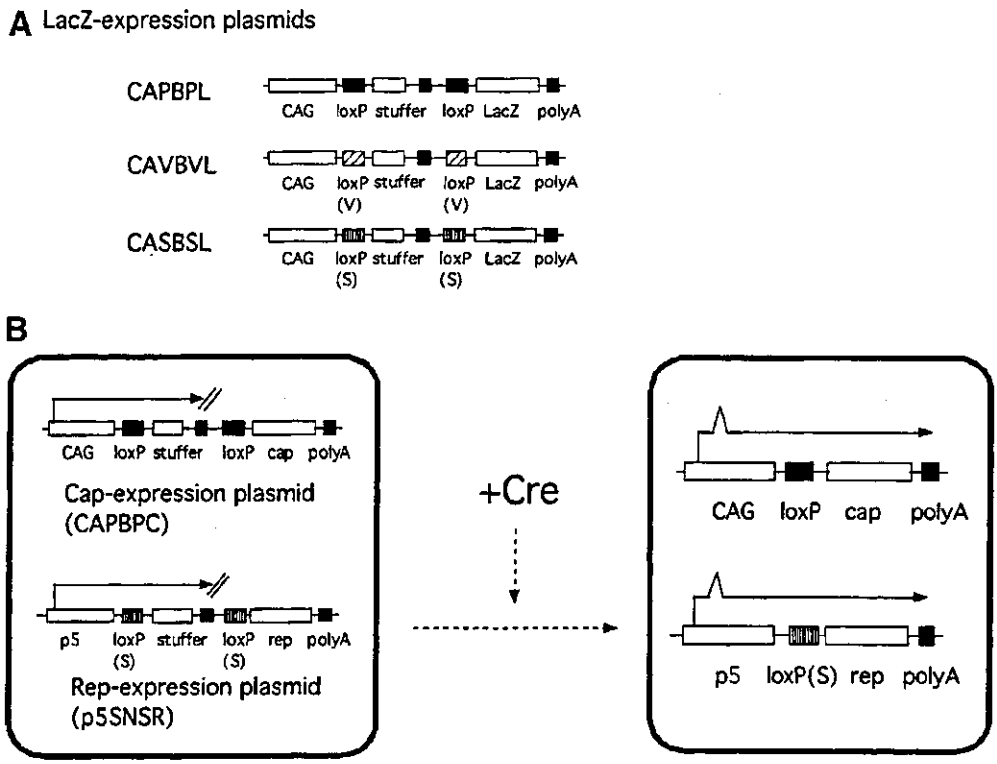

Fig. 1. Plasmid structure and the scheme of the cell line development. (**A**). To assess the recombination efficiency of the mutant loxP, three LacZ-expression plasmids are constructed. On infection with Ad-Cre, the stuffer sequences are removed with the efficiency depending on the wild-type or mutant 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.

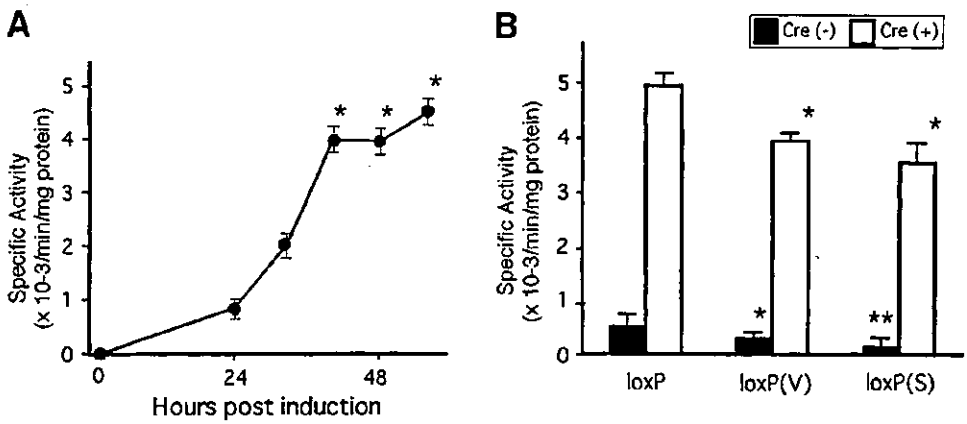

Fig. 2. Efficiency of recombination demonstrated in the wild-type and the mutant loxP. (**A**). Recombination activity of the wild-type loxP assessed at different time-points. The *asterisks* indicate the statistical significance ($p < 0.05$) of each data point against the nonindicated points. No significant 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-

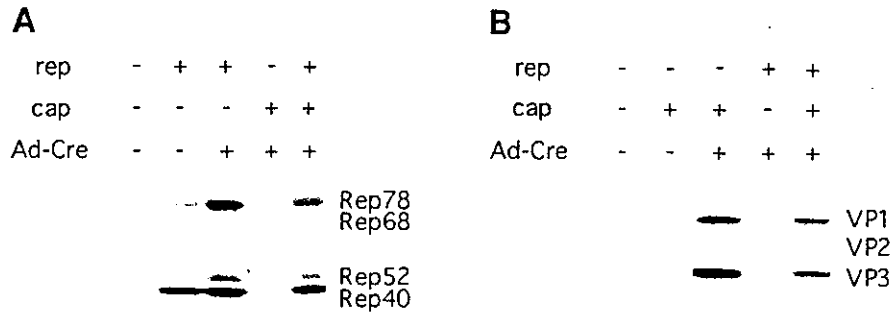

Fig. 3. *Rep* and *cap* expression profiles on treatment with Cre recombinase. One microgram of each plasmid was transfected into 293 cells in a 6-well plate. Following transfection, the cells received Ad-Cre and were lysed 48 h later. The cell lysates were analyzed by Western blotting using the monoclonal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

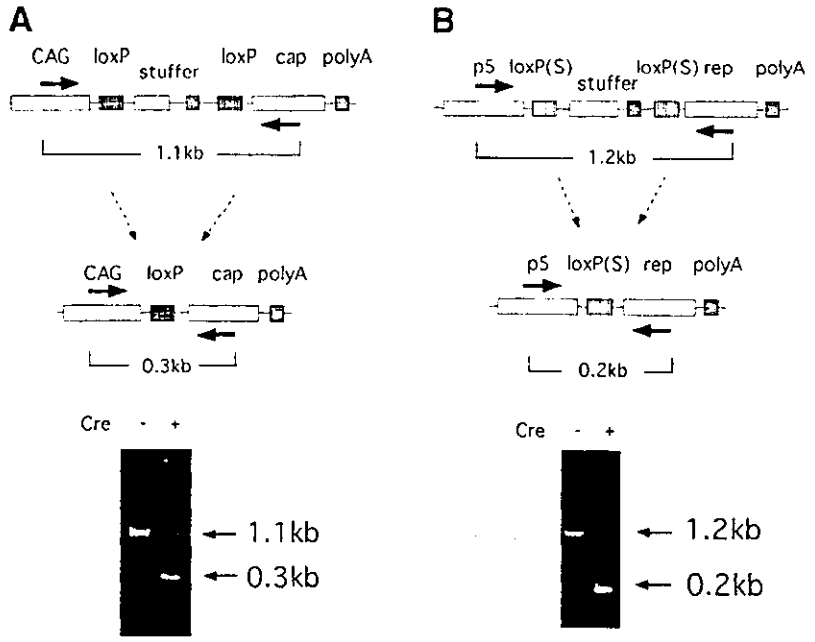

Fig. 4. The positions of the primers designed to detect both vector sequences before and after recombination are indicated by arrows. Primers corresponding to the CAG promoter and cap sequences or primers for p5 and *rep* sequences were used to amplify the vector sequences in (**A**) and (**B**), respectively. 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.

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ORIGINAL ARTICLE

Specific detection of human coagulation factor IX in cynomolgus macaques

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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 ± 37.6 ng mL^{-1} at 1 h after the injection and gradually decreased to 1.79 ± 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 ≥6%, 2–5%, or ≤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 ≥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

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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

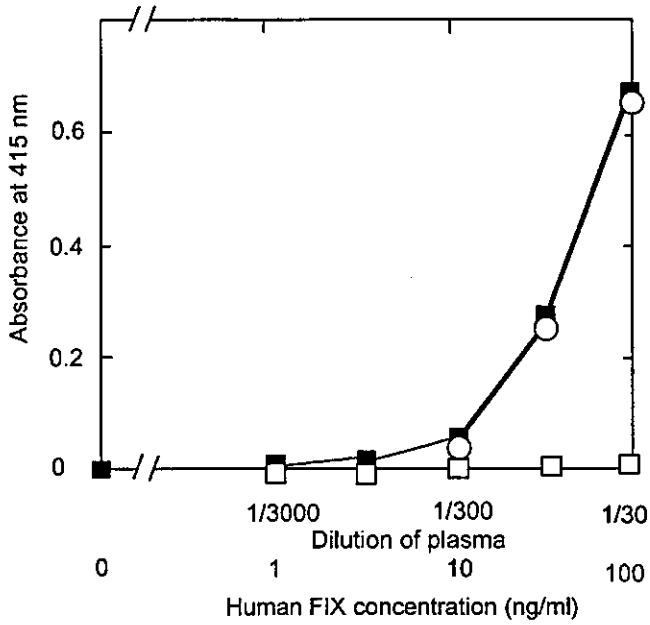

Fig. 1. Effect of macaque plasma on human factor (F)IX detection by 3A6-based enzyme immunoassay. Human plasma (■) or macaque plasma (□) diluted 30–3000-fold was incubated in microtiter plates coated with 3A6 antibody. Human plasma was diluted 30–300-fold with buffer containing 30% macaque plasma (○).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

Table 1 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

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.

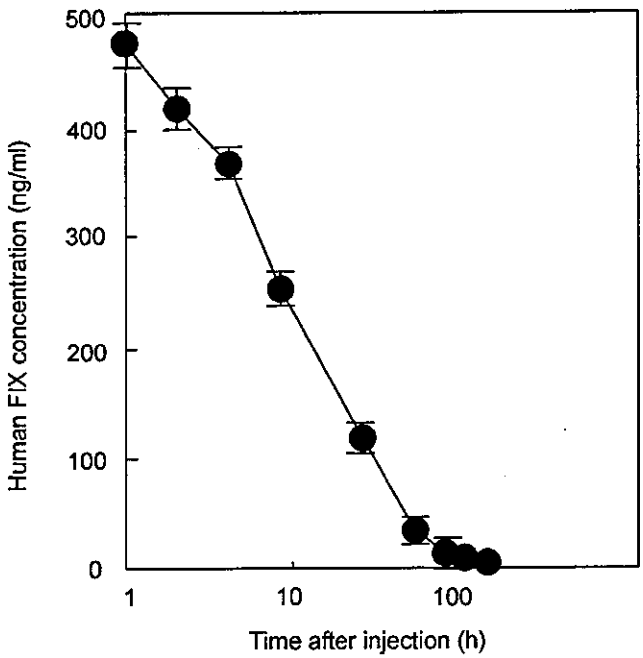

Fig. 3. Quantification of human factor (F)IX in cynomolgus macaque. FIX concentrates (10 U kg^{-1}), used to treat hemophilia patients, were injected intravenously into three cynomolgus macaques. After injection, peripheral blood was drawn and platelet-poor plasma was prepared. Human FIX levels in macaque 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

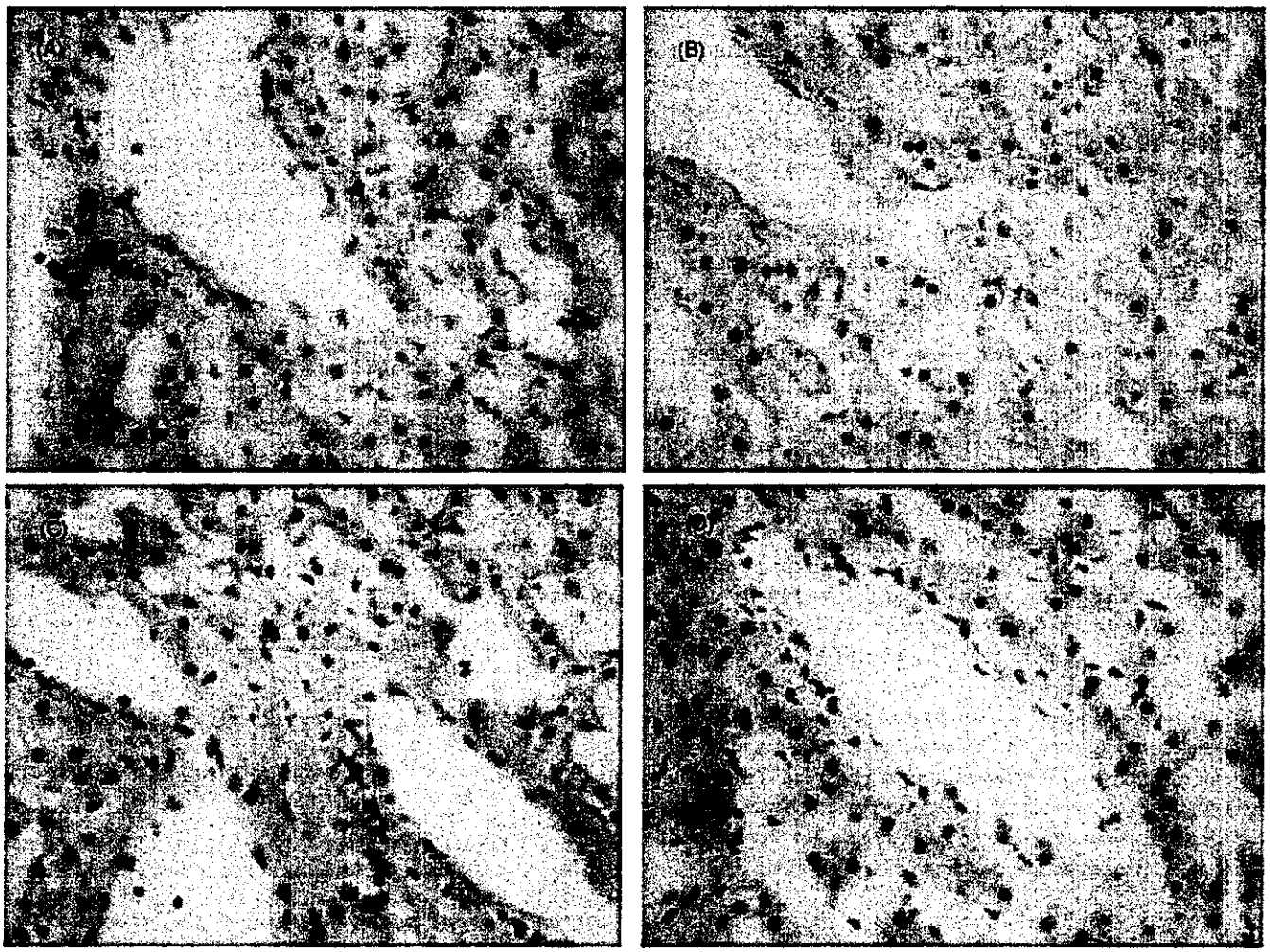

Fig. 4. Immunohistochemical study of the macaque liver. Frozen sections of the macaque liver on glass slides were incubated with monoclonal antibody 3A6 (A), normal mouse IgG (B), sheep polyclonal antibodies to human factor IX (C), or normal sheep IgG (D) and bound antibodies were detected by the avidin–biotin complex method as described in Materials and methods.

mice. However, there are obvious species differences between humans and mice, making it difficult under some circumstances to extrapolate data obtained in mice to human patients. Hemophilia B mice (FIX knock-out mice) and hemophilia B dogs have been used to study gene therapy approaches for treatment of hemophilia B [3–6]. Better animal models may be required, however, because there may be significant differences in transduction efficiency of skeletal muscles with AAV2 vectors between mice and dogs. Primates are used successfully as models in other disease applications, but there are as yet no hemophilic primates available. If one can distinguish human molecules from primate molecules *in vivo*, primates may be used for hemophilia gene therapy research, despite the fact that the genetic abnormality is not indigenous to the species.

Rhesus macaques are proposed to be a good primate model for studying hemophilia B gene therapy because of the amino acid sequence similarity between human FIX and macaque FIX and low immunogenicity of human FIX to rhesus macaque [13]. However, quantification of the human FIX expressed was difficult due to cross-reactivity of the rabbit anti-FIX antibodies. Rhesus macaques also developed antibodies to human FIX upon receiving viral vectors carrying the human FIX gene despite the high amino acid sequence homology [17]. A human FIX-specific EIA was developed using macaque antihuman FIX antibodies and EIA quantified human FIX levels in macaque plasma at 30 ng mL^{-1} (1% of the normal FIX level). The amino acid sequence of cynomolgus macaque FIX is identical to that of rhesus macaque FIX [14], raising the possibility that distinguishing the recombinant human molecule expressed in cynomolgus macaques *in vivo* from the endogenous macaque FIX molecule may be difficult. Thus, seven antihuman FIX mAbs available in our facilities were screened for their inability to bind to simian FIX. The 3A6 antibody did not bind to macaque FIX and an EIA was developed with this antibody to quantify human FIX in macaque plasma. The EIA was approximately 20-fold more sensitive than that used in the previous study [13], detecting human FIX at 1.79 ng mL^{-1}, or 0.06% of the normal plasma levels in cynomolgus macaques. The advantage of mAb 3A6 was also confirmed by the data that 3A6 did not react with macaque FIX in the liver by immunohistochemistry (Fig. 4).

Type 2 AAV vectors were initially considered for expression of transgenes in skeletal muscle, although there appears to be a significant difference in transduction efficiency of skeletal muscles with AAV vectors among different AAV serotypes. In mice, type 1 AAV vectors appear to be superior to other AAV serotypes, since types 3 and 5 AAV vectors are less potent than the type 1 AAV vector but have better skeletal muscle transduction efficiency than type 2 AAV vectors [18]. It remains possible that the transduction efficiency of human skeletal muscle by different AAV vectors differs from that in mice and dogs. It is also possible that the transduction efficiency of other organs, such as the liver, by AAV vectors has significant species specificity.

The cynomolgus macaque is native to southern Asia and has been used as a simian model in medical research, such as in Parkinson's disease. As reported previously, human FIX could be immunogenic to rhesus macaques transduced with adenoviral vectors or AAV vectors carrying the human FIX gene and to cynomolgus macaques that received repeated subcutaneous injections of human FIX in the presence of Freund's adjuvant [13,15]. Thus, as far as antibodies to human FIX develop in macaques during transduction with vectors carrying the human FIX gene, the long-term study for human FIX expression is impossible, but if antibody development to expressed human FIX is suppressed, macaques may mimic the human situation more closely and provide a more accurate assessment of viral vector transduction efficiencies, and we may be able to evaluate the efficacy of therapeutic FIX gene construction and select appropriate promoters, vectors, and organs for transgene expression, taking advantage of monoclonal antibody 3A6.

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References

1 Kay MA, High K. Gene therapy for the hemophilias. *Proc Natl Acad Sci USA* 1999; **96**: 9973–5.
2 High KA. Gene transfer as an approach to treating hemophilia. *Circ Res* 2001; **88**: 137–44.
3 Kay MA, Rothenberg S, Landen CN, Bellinger DA, Leland F, Toman C, Finegold M, Thompson AR, Read MS, Brinkhous KM. *In vivo* gene therapy of hemophilia B: sustained partial correction in factor IX-deficient dogs. *Science* 1993; **262**: 117–9.
4 Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, Nagy D, Gown AM, Winther B, Meuse L, Cohen LK, Thompson AR, Kay MA. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat Genet* 1997; **16**: 270–6.
5 Herzog RW, Yang EY, Couto LB, Hagstrom JN, Elwell D, Fields PA, Burton M, Bellinger DA, Read MS, Brinkhous KM, Podsakoff GM, Nichols TC, Kurtzman GJ, High KA. Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. *Nat Med* 1999; **5**: 56–63.
6 Snyder RO, Miao C, Meuse L, Tubb J, Donahue BA, Lin HF, Stafford DW, Patel S, Thompson AR, Nichols T, Read MS, Bellinger DA, Brinkhous KM, Kay MA. Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. *Nat Med* 1999; **5**: 64–70.
7 Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, McClelland A, Glader B, Chew AJ, Tai SJ, Herzog RW, Arruda V, Johnson F, Scallan C, Skarsgard E, Flake AW, High KA. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; **24**: 257–61.
8 Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, Jen Tai S, Ragni MV, Thompson A, Ozelo M, Couto LB, Leonard DGB, Johnson FA, McClelland A, Scallan C, Skarsgard E, Flake AW, Kay MA, High HA, Glader B. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 2003; **101**: 2963–72.
9 Yoshioka A, Giddings JC, Fujimura TY, Bloom AL. Immunoassays of factor IX antigen using monoclonal antibodies. *Br J Hematol* 1985; **59**: 265–75.
10 Sugo T, Mizuguchi J, Kamikubo Y, Matsuda M. Anti-human factor IX monoclonal antibodies specific for calcium ion-induced conformations. *Thromb Res* 1990; **58**: 603–14.
11 Mimuro J, Sakata Y, Wakabayashi K, Matsuda M. Level of protein C determined by combined assays during disseminated intravascular coagulation and oral anticoagulation. *Blood* 1987; **69**: 1704–11.
12 Sugo T, Nakamikawa C, Takano H, Mimuro J, Yamaguchi S, Mosesson MW, Meh DA, DiOrio JP, Takahashi N, Takahashi H, Nagai K, Matsuda M. Fibrinogen Niigata with impaired fibrin assembly: an inherited dysfibrinogen with a Bβ Asn-160 to Ser substitution associated with extra glycosylation at Bβ Asn-158. *Blood* 1999; **94**: 3806–13.
13 Lozier JN, Metzger ME, Donahue RE, Morgan RA. The rhesus macaque as an animal model for hemophilia B gene therapy. *Blood* 1999; **93**: 1875–81.
14 Tomokiyo K, Teshima K, Nakatomi Y, Wtanabe T, Mizuguchi J, Nozaki C, Nakagaki T, Miyamoto S, Funatsu A, Iwanaga S. Induction of acquired factor IX inhibitors in cynomolgus monkey (*Macaca fascicularis*): a new primate model of hemophilia B. *Thromb Res* 2001; **102**: 363–74.
15 Zauber PN, Levin J. Factor IX levels in patients with hemophilia B (Christmas disease) following transfusion with concentrates of factor IX or fresh frozen plasma. *Medicine* 1977; **56**: 213–24.
16 MacCarthy K, Stewart P, Sigman J, Read M, Keith JC Jr, Brinkhous KM, Nichols TC, Schaub RG. Pharmacokinetics of recombinant factor IX after inravenous and subcutaneous administration in dogs and cynomolgus monkey. *Thromb Haemost* 2002; **87**: 824–30.
17 Nathwani AC, Davidoff AD, Hanawa H, Hu Y, Hoffer FA, Nikanorov A, Slaughter C, Ng C, Zhou J, Lozier JN, Mandrell TD, Vanin EF, Nienhuis W. 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**: 1662–9.
18 Chao H, Liu Y, Rabinowitz J, Li C, Samulski RJ, Walsh CE. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Mol Ther* 2000; **2**: 619–23.

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Short Communication

The adenovirus E1A and E1B19K genes provide a helper function for transfection-based adeno-associated virus vector production

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Although the adenoviral E1, E2A, E4 and VA RNA regions are required for efficient adeno-associated virus (AAV) vector production, the role that the individual E1 genes (*E1A*, *E1B19K*, *E1B55K* and *protein IX*) play in AAV vector production has not been clearly determined. E1 mutants were analysed for their ability to mediate AAV vector production in HeLa or KB cells, when cotransfected with plasmids encoding all other packaging functions. Disruption of *E1A* and *E1B19K* genes resulted in vector yield reduction by up to 10- and 100-fold, respectively, relative to the wild-type E1. Interruption of the *E1B55K* and *protein IX* genes had a modest effect on vector production. Interestingly, expression of anti-apoptotic E1B19K cellular homologues such as Bcl-2 or Bcl-x_L fully complemented E1B19K mutants for AAV vector production. These findings may be valuable for the future development of packaging cell lines for AAV vector production.

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Adeno-associated virus (AAV)-based vector systems are particularly attractive vehicles for clinical applications requiring long-term *in vivo* gene expression from post-mitotic tissues. AAV vectors have been shown to promote stable expression of a wide variety of transgenes in numerous tissues, including skeletal and cardiac muscle, liver, the central nervous system and retina (Rabinowitz & Samulski, 1998). Overt evidence of inflammation is either minimal or non-existent in target tissues immediately following AAV vector administration. Furthermore, cytotoxic T-lymphocyte responses are not normally elicited to transgene products delivered by AAV vectors, even when such proteins are foreign to the host (Jooss *et al.*, 1998). AAV vectors are considered to be relatively safe because the parental virus is non-pathogenic and unable to replicate in the absence of a co-infecting helper virus. Additionally, current production methods have reduced the regeneration of replication competent wild-type AAV during vector production to undetectable levels (Allen *et al.*, 1997). Finally, the robust protein capsid of AAV makes AAV vectors particularly amenable to existing production methods for protein pharmaceuticals (Gao *et al.*, 2000) and confers upon them desirable drug stability characteristics.

AAV2, the parent virus from which the vector system is derived, is replication defective and requires co-infection of helper viruses to propagate. Adenovirus (Atchinson *et al.*, 1965) and herpes virus (Buller *et al.*, 1981) act as complete helpers and vaccinia virus (Schlehofer *et al.*, 1986) acts as a partial helper. The set of adenoviral (type 2 or 5) genes that facilitate AAV2 propagation has been defined and consists of E1A, E1B55K, the VA RNAs, E2A and E4orf6 (Samulski & Shenk, 1988). E1A acts as a cue to begin virus replication by up-regulating transcription from the *rep* gene promoters, P5 and P19 (Tratschin *et al.*, 1984) and by activating the early adenovirus promoters. E1A is also required to drive the host cell into the S-phase of the cell cycle for viral DNA replication because the AAV encoded proteins are not capable of this function. An adverse effect of E1A is that it stabilizes p53, which leads to apoptosis (Lowe *et al.*, 1993). To prevent this, the E1B55K and the E4orf6 proteins form a complex with p53 and cause it to be degraded through ubiquitin-mediated proteolysis (Querido *et al.*, 1997; Steegenga *et al.*, 1998). Later in infection, E1B55K and E4orf6 form a heterodimer that causes the preferential export of AAV and adenoviral late mRNAs from the nucleus while inhibiting the transit of adenoviral early and cellular mRNAs (Pilder *et al.*, 1986). The 72 kDa DNA-binding protein encoded by E2A has functions in viral DNA replication, viral mRNA processing and export, and AAV promoter regulation (Carter *et al.*, 1992; Ward *et al.*, 1998; Chang & Shenk, 1990). It causes an increase in the intracellular levels of the

single- and double-stranded forms of the AAV genome, the spliced forms of the rep proteins, and dramatically increases capsid protein production. Lastly, the VA RNAs inhibit the interferon-inducible eIF-2 protein kinase, thereby circumventing this cellular anti-viral mechanism from blocking viral protein translation (West *et al.*, 1987).

With respect to E2A, E4orf6 and the VA RNAs, the helper gene requirement for AAV vector and virus production is identical. We and others, have shown that plasmids encoding these genes, when cotransfected into 293 cells along with plasmids encoding rep/cap and a vector, mediate higher levels of vector production than that produced by adenovirus infection (Xiao *et al.*, 1998; Matsushita *et al.*, 1998). This so-called 'triple plasmid' transfection method forms the basis of the current scale-up vector production effort at Avigen and has a respectable mean production efficiency of 1×10^{13} vector genomes produced per 850 cm^2 roller bottle. A report was published describing a method for producing AAV vector in 293 cells using only E4orf6 as the helper gene (Allen *et al.*, 2000). This method requires the use of a heterologous promoter to drive the capsid gene and is about 10-fold less productive than methods using a plasmid encoding all three adenoviral helper genes (unpublished data).

The genes of the E1 region have not been analysed for their contribution to AAV vector production. In this study, we have investigated the role of the *E1A* and *E1B* genes in AAV vector production by using a series of E1 mutant plasmids and cell lines that lack adenoviral genes. E1A was required for efficient vector production. In contrast to the helper requirements for AAV production, our data indicated that *E1B19K* gene greatly augmented vector production, however, *E1B55K* gene did not.

The contributions of each of the component genes from the E1 region to AAV helper function was assessed by creating a set of plasmids with mutations in the *E1A*, *E1B19K*, *E1B55K* or *protein IX* genes and then testing them for their ability to support transfection-based AAV vector production. At least one truncation or one deletion mutation was made for each gene (Fig. 1).

For vector construction the plasmid pE1, which encodes the *E1A*, *E1B19K*, *E1B55K* and *protein IX* genes, was created from Ad2 DNA (Invitrogen). Briefly, the *Afl*III fragment (nt positions 142–5927) of Ad2 was cloned into the *Afl*III site of pBR322 (New England Biolabs) to generate pE1. pE1A-825stop was constructed by the insertion of an adapter (CCGGACTAATTAACTAGT), which includes a stop codon and an *Spe*I site, into the *Bsp*EI site of pE1. Similarly, pE1B19K-1912stop, pE1B55K-2243stop,

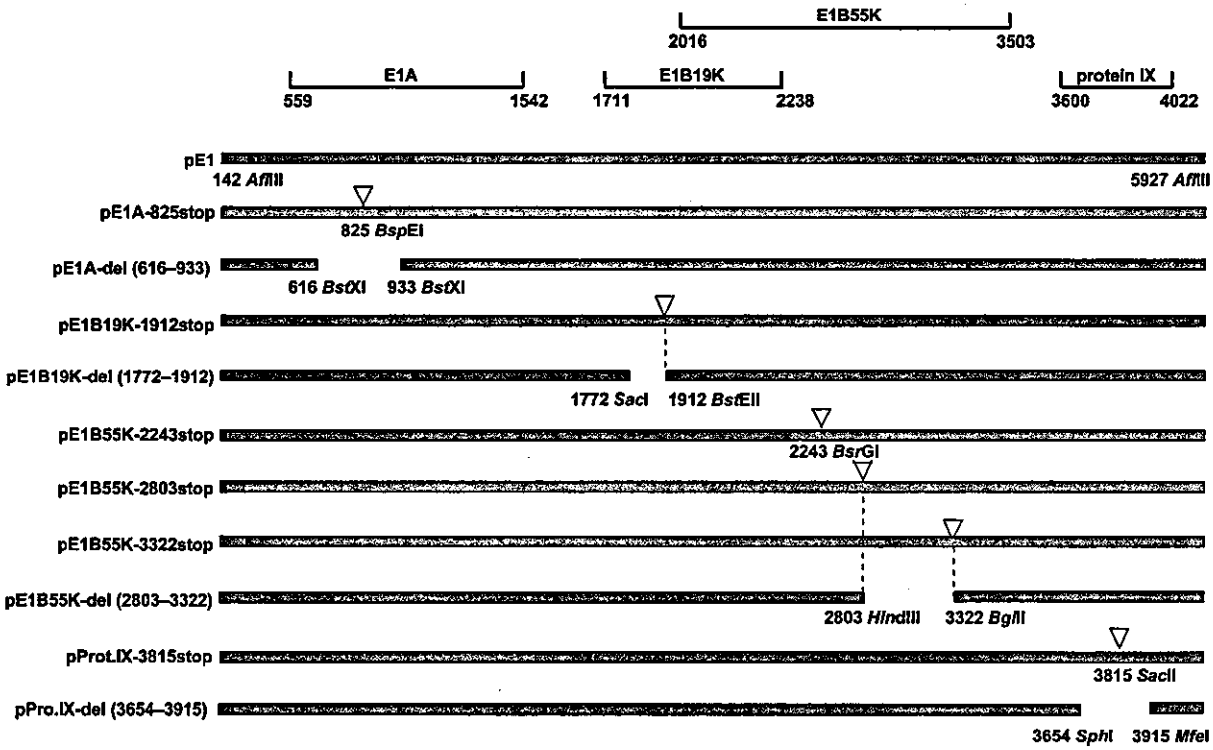

Fig. 1. Schematic representation of plasmids harbouring adenoviral E1 mutants used in this study. A 5·8 kb DNA fragment of adenovirus type 2 was cloned into the *Afl*II site of pBR322. pE1 encodes the entire E1 region, and the E1 mutant plasmids shown here were derived from it. The vertical flags mark the positions of inserted stop codons. The gaps in pE1A, pE1B19K, pE1B55K or pProt.IX constructs represent deletions.

pE1B55K-2803stop and pE1B55K-3322stop were made by the insertion of oligonucleotides into the *Bst*EII, *Bsr*GI, *Hin*dIII and *Bgl*II sites of pE1, respectively. pE1A-del (616–933) has a deletion of a 318 bp segment (positions 616–933 in Ad2). pE1B19K-del (1772–1912) and pE1B55K-del (2803–3322) have the same deletions as *dl337* (Pilder *et al.*, 1984) and *dl338* (Pilder *et al.*, 1986), respectively, used by Samulski & Shenk (1988) to examine E1 helper function for AAV2 production. Briefly, pE1B19K-del (1772–1912) lacks sequences between positions 1772 and 1912, and pE1B55K-del (2803–3322) lacks sequences between positions 2803 and 3322. pProt.IX-3815stop was constructed by the insertion of oligonucleotides into a *Sac*II site. pProt.IX-del (3654–3915) lacks a 262 bp segment (between positions 3654 and 3915 of Ad2).

The helper activities of the various E1 plasmids were assayed by cotransfecting them with a plasmid encoding both an AAV CMV*lacZ* vector and rep/cap (pW4389LacZ), and a plasmid encoding the adenovirus-2 VA RNA, E2A and E4 regions (Pladeno5), into KB or HeLa cells, and then quantifying *lacZ* vector production as described previously (Matsushita *et al.*, 1998). AAV vector was harvested 40 or 72 h after transfection and stocks were prepared by the freeze-thaw method. AAV vector production was quantified by titration of the vector stocks in 293 cells in the presence of adenovirus, followed by X-Gal staining and manual counting by light microscopy. For each experiment, all constructs were tested using triplicate production cultures, and all experiments were conducted at least three times, independently.

Elimination of the entire E1 region resulted in 2 (HeLa cells) to 3 log (KB cells) reduction in vector production relative to production in the presence of pE1, a plasmid encoding the entire E1 region ($P<0{\cdot}01$ by Student's *t*-test) (Fig. 2a, b). Disruption of the *E1A* genes, whether by truncation or deletion, caused 1 (HeLa cells) to 1·5 log (KB cells) reduction in vector production ($P<0{\cdot}01$). Truncations or deletions in the *E1B19K* gene also resulted in substantial reduction in vector production, 1 log in HeLa cells and greater than 2 logs in KB cells ($P<0{\cdot}01$). The lesser severity of the E1B19K mutant in HeLa cells, relative to KB cells, may be due to the relatively high level of Bcl-2 expression in HeLa cells (Liang *et al.*, 1995), or the human papilloma virus E6/E7 genes they harbour. The E6/E7 genes have been shown to facilitate some of the processes in AAV replication (Walz *et al.*, 1997). In most cases, disruption of the *E1B55K* and *protein IX* genes had a modest effect on vector production in either HeLa or KB cells. Two constructs, pE1B55K-2243stop and pProt.IX-3815stop showed fivefold reduction in vector yield in KB cells but little reduction in HeLa cells.

Our results differ substantially from those of Samulski & Shenk (1988) who examined the effect of E1B adenovirus mutants on AAV2 production, DNA replication, and mRNA and protein expression. This group found that an E1B19K adenovirus-2 mutant (*dl337*) mediated efficient AAV production from HeLa cells transfected with a plasmid encoding an AAV wild-type provirus (pSM620) but that

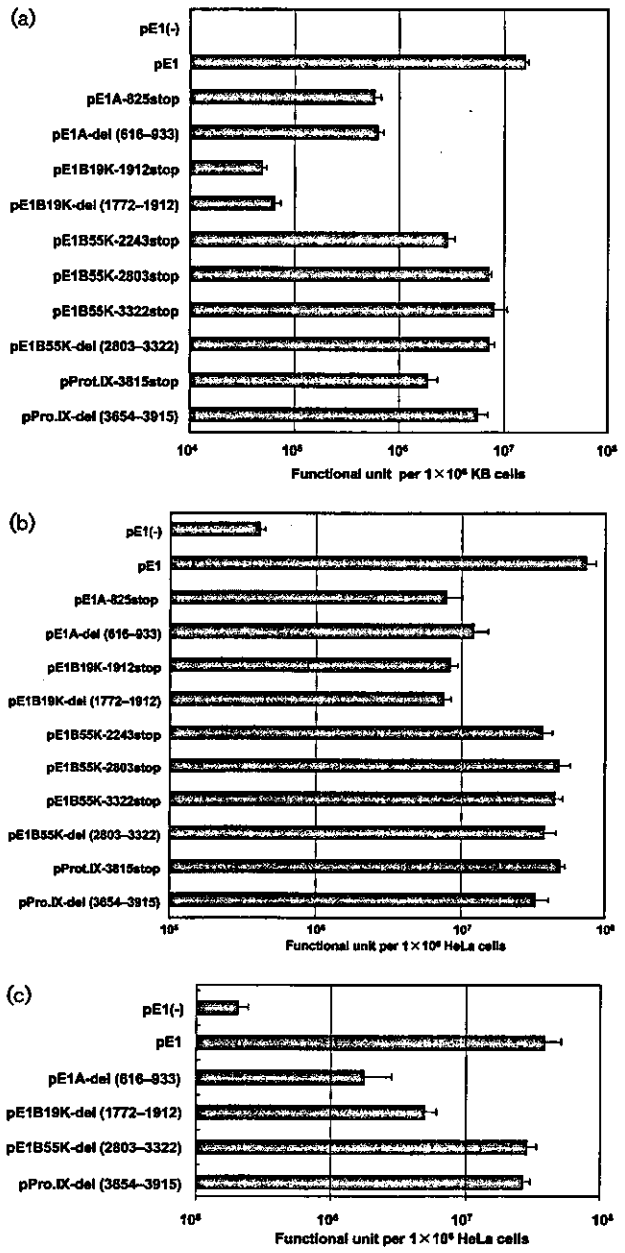

Fig. 2. Comparison of E1 mutant plasmids with respect to AAV helper function in KB (a) and HeLa cells (b) at 72 h after transfection, or in HeLa cells (c) at 40 h after the transfection. AAV *lacZ* vector was produced by the transfection of HeLa or KB cells with pW4389lacZ (encodes rep/cap and an AAV *lacZ* vector) and pladeno 5 (encodes the E2A, E4 and VA RNA regions), in the presence and absence of the indicated E1 plasmids. AAV vector production was assessed by titration of *lacZ* vector in 293 cells. pE1 (-) is identical to pBR322 without the expression cassette. Each bar represents the mean value obtained from triplicate cultures, and the error bar represents the standard deviation.

E1B55K (*dl338*) and E4orf6 (*dl355*) adenovirus mutants did not. AAV virion production was measured at a 40 h time point. The E1B55K and E4orf6 defects were caused by a delay in AAV mRNA accumulation that resulted in delays in viral DNA replication, capsid expression and ultimately virus production. AAV mRNA, DNA and capsid protein concentrations in cultures infected with E1B55K and E4orf6 mutants eventually reached levels seen in cultures infected by wild-type adenovirus but at longer time points, 72–96 h for adenovirus mutants compared with 24–40 h for wild-type adenovirus.

An important difference between our study and that of Samulski & Shenk (1988) was the timing of AAV/AAV vector harvest, 40 h in our study versus 72 h in theirs. Therefore, we examined a subset of the E1 region plasmids in transfection experiments using the same 40 h time point for vector harvest (Fig. 2c). The results were essentially similar to those at the 72 h time point and still differed from those produced by the adenovirus mutants. This observed difference in helper gene requirement may be attributable to technical factors associated with using virus infection or DNA transfection. A possible explanation for the conclusions reached by Samulski & Shenk (1988) might be the differences in the growth rates of the adenovirus mutants tested. The E1B55k mutant, *dl338*, was reported to grow inefficiently (100-fold reduced relative to wild-type) in HeLa cells (Pilder *et al.*, 1986) while the E1B19K mutant, *dl337*, was reported to be less defective (about 10-fold reduced relative to wild-type) (Pilder *et al.*, 1984). The lag in AAV mRNA, DNA and virus production seen with the E1B55K mutant may be simply because of a slow growing helper virus, resulting in low copy numbers of all of the adenovirus helper genes, and may not be directly due to the lack of the mutated gene. The observation that E1B19K is apparently not required for adenovirus mediated AAV production is harder to explain. It is tempting to speculate that the transfection-based production system benefits from additional anti-apoptitic activity provided by E1B19K. If this is true, this requirement does not appear to be cell-type or transfection-reagent specific (calcium phosphate and polycation-based transfection reagents both show an E1B19K effect, data not shown), and may have something to do with the adenoviral helper gene dose or kinetics of expression. Other differences between the two methods of identifying AAV helper function include: transfection method, the packaging of AAV virus versus a vector, and the use of replicating helper (AAV) versus non-replicating plasmid helpers. Full resolution of these issues will require further experimentation.

The adenovirus *E1B19K* gene, and its cellular homologues Bcl-2 and Bcl-x_L, encode anti-apoptotic proteins that function by inhibiting proapoptotic Bcl-2 homologues, such as Bax and Bak, by forming inactive heterodimers with them. To determine whether other anti-apoptotic members of the Bcl-2 family could augment AAV vector production, plasmid vectors expressing the *E1B19K*, *Bcl-2* or *Bcl-x_L* gene

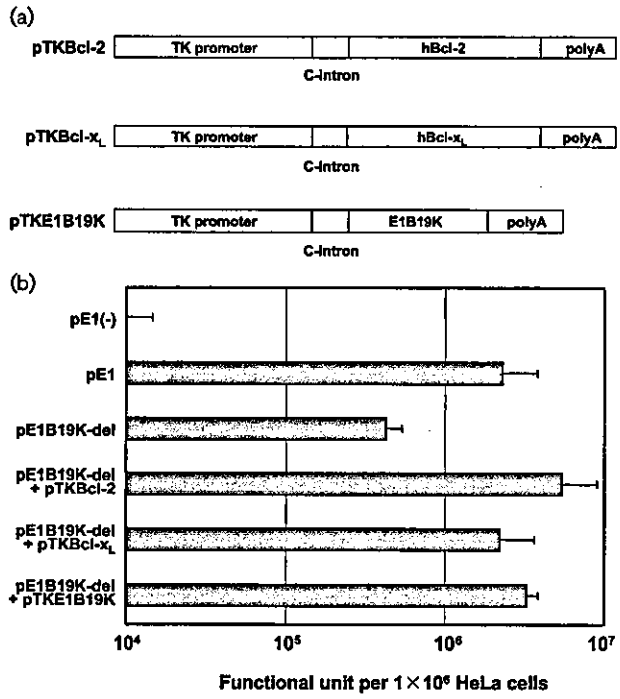

Fig. 3. (a) Schematic representation of Bcl-2, Bcl-x_L and E1B19K expression plasmids. TK promoter, HSV-*tk* promoter; C-intron, chimeric CMV/β-globin intron; polyA, SV40 late polyadenylation signal; hBcl-2, human Bcl-2 cDNA; hBcl-x_L, human Bcl-x_L cDNA; and E1B19K, adenovirus type 2 early region 1B 19 kDa protein gene. (b) Bcl-2 family members complement the vector production defect of an E1B19K mutant in HeLa cells. AAV *lacZ* vector was produced by the transfection of HeLa cells with pW4389lacZ (encodes rep/cap and an AAV *lacZ* vector), pladeno 5 (encodes the E2A, E4 and VA RNA regions), and pE1B19K-del (1772–1912), in the presence and absence of the indicated plasmids expressing Bcl-2 family genes, including E1B19K. AAV vector production was assessed by titration of *lacZ* vector in 293 cells. Each bar represents the mean value of triplicate cultures and the error bar represents the standard deviation.

products were tested for their ability to complement the vector production defect of the E1B19K deletion mutant, pE1B19K-del (1772–1912) (Fig. 3a). pTKPRMCS was assembled by the removal of a *Renilla* luciferase (R*luc*) reporter gene from pRL-TK (Promega) (between the *Nhe*I and *Xba*I sites) and insertion of a multiple cloning site (between the *Kpn*I and *Xba*I sites) from pBluescript II (Stratagene). pTK-Bcl-2 and pTK-Bcl-x_L were created by the insertion of human Bcl-2 and Bcl-x_L cDNA sequences, respectively, into pTKPRMCS. pTK-E1B19K was constructed by the insertion of the E1B19K fragment into pTKPRMCS. As shown in Fig. 3(b), plasmids expressing E1B19K, Bcl-2 or Bcl-x_L restored vector production of the E1B19K deletion mutant to levels equivalent to that produced by the wild-type pE1 plasmid. The use of the medium strength HSV-*tk* promoter to drive the expression

of the Bcl-2 homologues was essential for helper function. CMV-driven constructs produced low vector yields in a dominant fashion and caused a substantial increase in apoptosis (data not shown).

The fact that E1B19K mutants can be complemented by similarly anti-apoptotic cellular homologues such as Bcl-2 or Bcl-x_L suggests a common mechanism, the inhibition of Bak/Bax-mediated apoptosis. Interestingly, no increase in DNA ladder formation is seen in HeLa cells when transfected with E1B19K mutant plasmids relative to wild-type plasmids (data not shown). Consequently, the mechanism of vector production augmentation is not clear.

Current transfection-based AAV vector production methods are sufficient to commercially support gene therapy applications with large doses and small patient populations (e.g. haemophilia, other genetic diseases) or applications with small doses and large patient populations (e.g. Parkinson's disease). Applications with large doses and large patient populations (e.g. heart failure) will be a challenge for transfection-based production methods that scale linearly. Consequently, the construction of a producer cell line that is both helper virus-free, and suspension culture-adaptable, is of great interest. This is a formidable task since many of the viral helper proteins are toxic to the cell either alone (e.g. E2A) or in combination with other helper functions (e.g. E4orf6 and E1B55K, E1A and *rep*). The task is further complicated by genes such as E1B19K that must be expressed in a rather precise manner. Packaging cell lines containing inducible E1 genes, along with the E2a, VA and E4 regions, and an integrated AAV vector have been produced but were found to suffer from relatively low vector yield and substantial production instability (Qiao *et al.*, 2002). Both of these problems were likely due to, or exacerbated by, helper gene toxicity. Our data indicates that one source of toxicity, the inhibition of host mRNA nuclear export mediated by the E4orf6/E1B55K heterodimer, could be eliminated by not including the E1B55K gene in packaging cell lines.

Defining the minimum set of helper genes necessary for efficient vector production is the first step in creating suitable packaging cell lines for AAV vectors. Using our transfection-based assay, we define that set to be E1A, E1B19K, the VA RNAs, E2A and E4orf6 genes.

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References

Allen, J. M., Debelak, D. J., Reynolds, T. C. & Miller, A. D. (1997). Identification and elimination of replication-competent adeno-associated virus (AAV) that can arise by nonhomologous recombination during AAV vector production. *J Virol* **71**, 6816–6822.

Allen, J. M., Halbert, C. L. & Miller, A. D. (2000). Improved adeno-associated virus vector production with transfection of a single helper adenovirus gene, E4orf6. *Mol Ther* **1**, 88–95.

Atchinson, R. W., Casto, B. C. & Hammon, W. M. (1965). Adenovirus-associated defective virus particles. *Science* **149**, 754–756.

Buller, R. M., Janik, J. E., Sebring, E. D. & Rose, J. A. (1981). Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. *J Virol* **40**, 241–247.

Carter, B. J., Antoni, B. A. & Klessig, D. F. (1992). Adenovirus containing a deletion of the early region 2A gene allows growth of adeno-associated virus with decreased efficiency. *Virology* **191**, 473–476.

Chang, L. S. & Shenk, T. (1990). The adenovirus DNA-binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters. *J Virol* **64**, 2103–2109.

Gao, G., Qu, G., Burnham, M. S. & 7 other authors (2000). Purification of recombinant adeno-associated virus vectors by column chromatography and its performance *in vivo*. *Hum Gene Ther* **11**, 2079–2091.

Jooss, K., Yang, Y., Fisher, K. J. & Wilson, J. M. (1998). Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol* **72**, 4212–4223.

Liang, X. H., Mungal, S., Ayscue, A., Meissner, J. D., Wodnicki, P., Hockenbery, D., Lockett, S. & Herman, B. (1995). Bcl-2 proto-oncogene expression in cervical carcinoma cell lines containing inactive p53. *J Cell Biochem* **57**, 509–521.

Lowe, S. W., Ruley, H. E., Jacks, T. & Housman, D. E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**, 957–967.

Matsushita, T., Elliger, S., Elliger, C., Podsakoff, G., Villarreal, L., Kurtzman, G. J., Iwaki, Y. & Colosi, P. (1998). Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* **5**, 938–945.

Pilder, S., Logan, J. & Shenk, T. (1984). Deletion of the gene encoding the adenovirus 5 early region 1b 21,000-molecular-weight polypeptide leads to degradation of viral and host cell DNA. *J Virol* **52**, 664–671.

Pilder, S., Moore, M., Logan, J. & Shenk, T. (1986). The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol Cell Biol* **6**, 470–476.

Qiao, C., Li, J., Skold, A., Zhang, X. & Xiao, X. (2002). Feasibility of generating adeno-associated virus packaging cell lines containing inducible adenovirus genes. *J Virol* **76**, 1904–1913.

Querido, E., Marcellus, R. C., Lai, A., Charbonneau, R., Teodoro, J. G., Ketner, G. & Branton, P. E. (1997). Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. *J Virol* **71**, 3788–3798.

Rabinowitz, J. E. & Samulski, J. (1998). Adeno-associated virus expression systems for gene transfer. *Curr Opin Biotechnol* **9**, 470–475.

Samulski, R. J. & Shenk, T. (1988). Adenovirus E1B 55-Mr polypeptide facilitates timely cytoplasmic accumulation of adeno-associated virus mRNAs. *J Virol* **62**, 206–210.

Schlehofer, J. R., Ehrbar, M. & zur Hausen, H. (1986). Vaccinia virus, herpes simplex virus, and carcinogens induce DNA amplification in a human cell line and support replication of a helpervirus dependent parvovirus. *Virology* **152**, 110–117.

Steegenga, W. T., Riteco, N., Jochemsen, A. G., Fallaux, F. J. & Bos, J. L. (1998). The large E1B protein together with the E4orf6 protein

target p53 for active degradation in adenovirus infected cells. *Oncogene* **16**, 349–357.

Tratschin, J. D., West, M. H., Sandbank, T. & Carter, B. J. (1984). A human parvovirus, adeno-associated virus, as a eucaryotic vector: transient expression and encapsidation of the procaryotic gene for chloramphenicol acetyltransferase. *Mol Cell Biol* **4**, 2072–2081.

Walz, C., Deprez, A., Dupressoir, T., Durst, M., Rabreau, M. & Schlehofer, J. R. (1997). Interaction of human papillomavirus type 16 and adeno-associated virus type 2 co-infecting human cervical epithelium. *J Gen Virol* **78**, 1441–1452.

Ward, P., Dean, F. B., O'Donnell, M. E. & Berns, K. I. (1998). Role of the adenovirus DNA-binding protein in *in vitro* adeno-associated virus DNA replication. *J Virol* **72**, 420–427.

West, M. H., Trempe, J. P., Tratschin, J. D. & Carter, B. J. (1987). Gene expression in adeno-associated virus vectors: the effects of chimeric mRNA structure, helper virus, and adenovirus VA1 RNA. *Virology* **160**, 38–47.

Xiao, X., Li, J. & Samulski, R. J. (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**, 2224–2232.

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Roles of Bim in Apoptosis of Normal and Bcr-Abl-Expressing Hematopoietic Progenitors

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Bcr-Abl kinase is known to reverse apoptosis of cytokine-dependent cells due to cytokine deprivation, although it has been controversial whether chronic myeloid leukemia (CML) progenitors have the potential to survive under conditions in which there are limited amounts of cytokines. Here we demonstrate that early hematopoietic progenitors (Sca-1$^+$ c-Kit$^+$ Lin$^-$) isolated from normal mice rapidly undergo apoptosis in the absence of cytokines. In these cells, the expression of Bim, a proapoptotic relative of Bcl-2 which plays a key role in the cytokine-mediated survival system, is induced. In contrast, those cells isolated from our previously established CML model mice resist apoptosis in cytokine-free medium without the induction of Bim expression, and these effects are reversed by the Abl-specific kinase inhibitor imatinib mesylate. In addition, the expression levels of Bim are uniformly low in cell lines established from patients in the blast crisis phase of CML, and imatinib induced Bim in these cells. Moreover, small interfering RNA that reduces the expression level of Bim effectively rescues CML cells from apoptosis caused by imatinib. These findings suggest that Bim plays an important role in the apoptosis of early hematopoietic progenitors and that Bcr-Abl supports cell survival in part through downregulation of this cell death activator.

In the chronic phase, chronic myeloid leukemia (CML) is characterized by massive proliferation of granulocytes in the peripheral blood and their progenitors in the bone marrow. Abnormal hematopoietic stem cells harboring the *Bcr-Abl* chimeric gene still differentiate into mature granulocytes with apparently normal function but gradually come to occupy the hematopoietic space. They subvert the system controlling their homeostasis in the body and thus accumulate in large numbers. Because cytokines are considered to play critical roles in this homeostasis, dysregulation of cytokine-mediated cell death, cell survival, or cell division by Bcr-Abl may be responsible for leukemogenesis. Indeed, among multiple systems regulating diverse cell functions, including cell proliferation, differentiation, and apoptosis, which are dysregulated by Bcr-Abl, the reversal of apoptosis caused by cytokine deprivation is one of the most consistently observed effects (reviewed in references 15 and 23). This finding has been repeatedly demonstrated by use of different experimental systems that include murine interleukin-3 (IL-3)-dependent Baf-3 and 32D cells (8, 9, 11, 22, 28, 32, 37, 39).

We and others have investigated this cytokine-dependent cell survival system in hematopoietic progenitors by using IL-3-dependent cells and demonstrated that two distinct signaling pathways support cell survival. One pathway emanates from the membrane-proximal region of the common receptor chain (βc chain) shared by IL-3 and granulocyte-macrophage colony-stimulating factor, which activates JAK-STAT pathways and transcriptionally upregulates Bcl-x_L expression (14, 45, 46). The other pathway functions via the distal portion of the βc chain and activates Ras pathways (26, 27, 30). Because experiments using Baf-3 cells expressing truncated forms of the βc chain revealed that signals from its proximal portion support cell survival only transiently, signals from its distal region, especially the activation of Ras pathways, were considered to be indispensable for long-term cell survival supported by cytokines (26; also reviewed in reference 35).

Recent progress has revealed that cell death decisions are implemented through an evolutionarily conserved mechanism (or general apoptosis program) in which members of the Bcl-2 superfamily play the central roles (reviewed in references 1 and 7). The anti- or proapoptotic family members regulate the translocation of cytochrome *c* from mitochondria to the cytosol, an event that ultimately activates the caspase cascade, while members of the BH3-only subfamily of cell death activators inhibit the function of the antiapoptotic Bcl-2 family members by binding to them. In mammals, more than three factors have been identified to be members of each subfamily. Redundancy in each category of the Bcl-2 superfamily has been explained, at least partially, by the tissue- and/or stimulus-specific response of each family member. We therefore concentrated on identifying the major Bcl-2 superfamily mem-

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TABLE 1. Primers used in this study for real-time quantitative RT-PCR

Gene product	Product size (bp)	Forward primer	Reverse primer
A1	168	GGGAAGATGGCTGAGTCTGAGCTCATG	TGACTTCAGATTCTTTTCAACTTC
Bad	233	CCACCAACAGCTATCATGGAGGCGC	GCTCTTTGGGCGAGGAAGTCCCTTG
Bax	162	AATATGGAGCTGCAGAGGATGATTG	GCACTTTAGTGCACAGGGCCTTGAG
Bcl-2	261	GTGGTGGAGGAACTCTTCAGGGATG	GGTCTTCAGAGACAGCCAGGAGAAATC
Bcl-x_L	293	GTAGTGAATGAACTCTTTCGGGATGG	ACCAGCCACAGTCATGCCCGTCAGG
BimEL	324	AGTGGGTATTTCTCTTTTGACACAG	TCAATGCCTTCTCCATACCAGACG
Bim(si)	119	AATGTCTGACTCTGACTCTCGGAC	TCTCCGCAGGCTGCAATTGTCTAC
Mcl-1	259	GTAATGGTCCATGTTTTCAAAGATG	AAGCCAGCAGCACATTTCTGATGCC
DP5/Hrk	189	AGACCCAGCCCGGACCGAGCAA	AATAGCACTGGGGTGGCTCT
28S rRNA	324	ACGCAGGTGTCCTAAGGCGAGCTC	CACGACGGTCTAAACCCAGCTCAC

ber that is regulated by signals from the distal portion of the βc chain, especially via Ras pathways. We and others have found that mRNA and protein expression levels of Bim, a member of the BH3-only death activator subfamily, are downregulated by IL-3 through either the Ras/Raf/mitogen-activated protein kinase (MAPK) or the Ras/phosphatidylinositol 3-kinase (PI3-K) pathway in Baf-3 cells (13, 44). Bim was isolated independently by two groups that exploited its ability to bind Bcl-2 or Mcl1 (20, 36). Alternative splicing gives rise to three variants, BimEL, BimL, and BimS, each of which contains the BH3 domain and functions as a death inducer. It was shown that Bim was induced in Baf-3 cells by IL-3 deprivation but not by other apoptotic triggers, such as DNA damage or Fas, and that enforced expression (but not overexpression) of each form of Bim induced apoptosis in Baf-3 cells even in the presence of IL-3 (44). In addition to Bim induction in hematopoietic cells, the induction of Bim by deprivation of nerve growth factor (NGF) in primary cultures of rat sympathetic neurons, as well as in neuronally differentiated rat pheochromocytoma PC-12 cells, has been reported (5, 40, 49). These findings suggest that the level of Bim expression is a major determinant of cell fate regulated by cytokines.

In addition to its role as a key intracellular factor for cytokine-mediated cell survival, Bim was demonstrated to be an essential regulator of the total number of white blood cells by analysis of Bim-deficient mice (6). Bim-deficient mice have increased numbers of mature monocytes, granulocytes, and lymphocytes but not erythrocytes in the peripheral blood, with overgrowth of hematopoietic precursors in the bone marrow. This prompted us to investigate the roles of Bim as a possible downstream target of Bcr-Abl by using our previously established transgenic (tg) mice in addition to the conventional experimental systems for CML, such as cell lines established from patients in the blast crisis (BC) phase and cytokine-dependent cells expressing Bcr-Abl. In these tg mice, Bcr-Abl is expressed under the control of the *tec* tyrosine kinase promoter that is active in immature myeloid progenitors (16, 17, 33). Virtually all of these mice develop CML-like disease, namely, proliferation of mature myeloid precursors and megakaryocytes in the bone marrow with increased granulocytes and platelets in the peripheral blood and progressive anemia, within 8 months of birth. They generally die of the disease within 15 months (18). Moreover, when they are intercrossed with p53 haplo-deficient mice, they develop T-cell leukemia and lack functional p53 (19), indicating that this model mimics human CML in both the chronic and BC phases. Here we show that Bim plays an important role in the apoptosis of early hematopoietic progenitors and that Bcr-Abl supports cell survival in part through the downregulation of this cell death activator.

MATERIALS AND METHODS

Mice. $p210^{bcr/abl}$ tg (*BCR-ABL*$^{tg/-}$) mice were previously described (18). Because the founder mice were generated by using ova derived from (C57BL × DBA)F_2 (BDF_2) mice and the tg progeny were generated by intercrossing the tg mice with BDF_1 mice, the genetic background of the *BCR-ABL*$^{tg/-}$ mice was a mixture of C57BL/6 and DBA. We used the normal littermates of these mice (*BCR-ABL*$^{-/-}$) as controls in this study.

Primary culture and isolation of cytokine-dependent hematopoietic progenitors. Mice that were 8 to 12 weeks of age were sacrificed, and bone marrow cells were harvested by a standard procedure. Cells were cultured for 5 days in serum-free medium (SF-O2; Sanko Junyaku, Tokyo, Japan) containing 10 ng of thrombopoietin (TPO) per ml and 50 ng of stem cell factor (SCF) per ml. After Ficoll gradient centrifugation to separate dead cells and mature granulocytes, cells expressing lineage-specific markers (CD4, CD8, CD11b, CD41, or Gr-1) were eliminated by using magnetic beads conjugated with specific antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). More than 90% of lineage marker-negative (Lin^-) cells obtained by this procedure were positive for c-Kit. These cells were further divided into Sca-1-enriched (Sca-1^+ c-Kit^+ Lin^-) and Sca-1-depleted (Sca-1^- c-Kit^+ Lin^-) fractions by using magnetic beads conjugated with Sca-1 antibody. Viable cell counts were determined by trypan blue dye exclusion in triplicate assays. Morphology was determined by using cytospin preparations stained with May-Giemsa solution.

TUNEL analysis. Cells in the Sca-1-enriched fraction were cultured in cytokine-free medium for different periods. Cells were harvested and fixed with 4% paraformaldehyde for 20 min, and a terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) assay was performed with an apoptosis detection kit according to the manufacturer's directions (Promega, Madison, Wis.). Cells were then stained with 1 μg of propidium iodide per ml. Cytospin preparations were made, and the incorporation of dUTP was analyzed with a laser cytoscan (Olympus, Tokyo, Japan).

Real-time quantitative RT-PCR. Total cellular RNA was isolated with an Isogen kit according to the manufacturer's instructions (Wako Pure Chemicals, Osaka, Japan). RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif.). Real-time PCR was carried out with an ABI 7700 instrument and SYBR green PCR master mix (Applied Biosystems, Weiterstadt, Germany), which allows real-time monitoring of the increase in PCR product concentration after every cycle based on the fluorescence of the double-stranded-DNA-specific dye SYBR green. The number of cycles required to produce a product detectable above background levels was measured for each sample and used to calculate differences (*n*-fold) in starting mRNA levels for each sample. Because we had observed that levels of β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA, which are generally used for monitoring equal loading of RNA, were rapidly downregulated in the course of apoptosis by cytokine deprivation in murine IL-3-dependent cell lines (data not shown), we used 28S rRNA as an internal control. The gene primers, selected to cross introns, are listed in Table 1. The real-time reverse transcription (RT)-PCR products were resolved on a 2% agarose gel containing ethidium bromide to confirm that only single bands of the predicted size were visible.