Gene Ther Mol Biol Vol 3, 67-74. August 1999.

Hepatocyte-specific gene expression by a recombinant adeno-associated virus vector carrying the apolipoprotein E enhancer and α_1-antitrypsin promoter

Research Article

Torayuki Okuyama[1,2], Motomichi Kosuga[1,2], Satori Takahashi[1], Kyoko Sasaki[1], and Masao Yamada[1]

Department of Genetics, National Children's Medical Research Center, Setagaya, Tokyo 154-8509 Japan, Department of Pediatrics, Keio University School of Medicine, Tokyo 160-8582

Correspondence: Torayuki Okuyama, M.D., Department of Genetics, National Children's Medical Research Center, 3-35-31 Taishido Setagaya-ku Tokyo 154-8509, Japan. Phone: +81-3-3414-8121 ext. 2752; Fax: +81-3-3414-3208; E-mail: tora@nch.go.jp

Key words: adeno-associated virus, liver-specific promoter, α_1-antitrypsin promoter, apolipoprotein E enhancer, gene therapy

Received: 30 October 1998; accepted: 10 November 1998

Summary

An adeno-associated virus vector was constructed to express exogenous genes to the liver. The original plasmid construct carried two expression units; a neomycin resistant gene and human α_1-antitrypsin cDNA under the control of hepatocyte specific transcription elements. Cells were transfected with the constructed plasmid DNA with another packaging plasmid, and recombinant adeno-associated viruses (rAAV) were then recovered after adenovirus infection. Alternatively, rAAV were recovered by transduction of DNAs of the packaging plasmid and adenovirus into pre-selected cells carrying constructed proviral DNA. When the transducing abilities were evaluated based on G418 resistant colony formation on HeLa cells, the latter method was found to give almost 10-fold more rAAV. We then isolated G418 resistant colonies and established several independent clones for the HeLa and Hepa1A cells infected with the rAAV. All of the eight clones derived from Hepa1A cells produced significant amounts of the human α_1-antitrypsin protein. In contrast, none of the five clones derived from HeLa cells produced a detectable level of α_1-antitrypsin. Our results suggest that liver-specific promoter and enhancer maintain the tissue specificity in the rAAV construct, and that the rAAV vector system would be useful in hepatocyte directed gene therapy.

I. Introduction

Liver-directed gene therapy could revolutionize treatments for many genetic disorders such as phenylketonuria, familial hypercholesterolemia and hemophilia (Ledley 1993). Adenoviral vectors efficiently transduce a gene into hepatocytes, easily achieve its expression at a therapeutic level for many diseases, but do not allow a long-term expression and repetitive administration because of the high immunogenicity (Jaffe et al. 1992; Okuyama et al. 1998). Retroviral vectors are also able to transduce an exogenous gene into hepatocytes, and a long term expression of the transduced gene has been identified in several experiments using rat or dog liver (Rettinger et al. 1994; Kay et al. 1992; Kay et al. 1993; Hafenrichter et al. 1994). However, the expression level is

generally too low for therapeutic treatments of patients because the transducing efficiency is extremely low.

We have previously demonstrated that a retroviral vector expressing an exogenous gene under the control of a human apolipoprotein E enhancer and α_1-antitrypsin promoter as well as an original retroviral LTR promoter dramatically increase the level of protein production after administration into the rat liver (Okuyama et al. 1996). The apolipoprtein E enhancer has been detected through studies on a gene cluster of apoE/C-I/C-II in human chromosome 19. Studies on transgenic mice disclosed that a 154 bp region located 15 kb downstream of the apolipoprotein E gene was responsible for the high level of expression in hepatocytes (Shachter et al. 1993; Simonet et al. 1993). A 420 bp segment of the 5' flanking region of the human α_1-antitrypsin gene contains distinct HNF-1 and HNF-2 binding sites, and both sites are responsible for strong and tissue-specific expression of α_1-antitrypsin (Li et al. 1988). Recently we found that rats administered the retroviral vector expressing human coagulation Factor X under the control of the 420 bp of α_1-antitrypsin promoter produced a therapeutic level of functional Factor X (Le et al. 1997). These observations suggested that with the retroviral vectors designed to express an exogenous gene under the control of promoter-enhancer complex of apolipoprotein E and α_1-antitrypsin, one is able to achieve significantly high levels of transgene expression. However, retroviral vectors can transduce foreign genes only into dividing cells, thus inducing the regeneration with partial hepatectomy which is essential for retroviral gene-transduction into hepatocytes. Although the mortality for a 70% hepatectomy is relatively low and the procedure could be justified for life threatening genetic deficiencies,alternative methods that circumvent partial hepatectomy are desirable.

Adeno-associated virus is a replication-defective parvovirus that is being developed as a vector for human gene therapy (Laughlin et al. 1986). One advantage of AAV as a vector is that it can transduce genes into postmitotic cells like cells of the Central Nervous System (Kaplitt et al. 1994), lung epithelial cells (Flotte et al. 1993), or muscle fiber cells (Fisher et al. 1996). Since most hepatocytes *in vivo* are also in the growth arrested state, AAV vectors is expected to be suitable for *in vivo* hepatocyte-directed gene therapy. However, little is known about the transduction and expression efficiency of rAAV in hepatocytes *in vitro* as well as *in vivo* (Flotte et al. 1995; Fisher et al. 1997; Snyder et al. 1997). In this study, we generated a recombinant adeno-associated virus containing hepatocyte-specific expression unit, and evaluated its transduction efficiency, tissue specificity, and level of expression in gene-transduced cells of hepatocyte origin.

II. Results

A. Generation of rAAV vector containing a liver-specific promoter and enhancer

The structure of the rAAV vector TRNAEAT containing liver-specific transcriptional elements is shown in **Fig. 1**. The vector contains the human α_1-antitrypsin (hAAT) cDNA as a reporter gene downstream of the 420bp 5' flanking sequence of its own gene. The enhancer region located in the apoE/C-I/C-II gene locus is necessary for high level expression of the apoE gene in livers of transgenic mice (Schacter et al. 1993). In an attempt to further enhance expression of the hAAT promoter from an rAAV vector, four copies of ApoE enhancer were placed upstream of the hAAT promoter (Simonet et al. 1993). In addition to these 2.8-kb hepatocyte-specific expression units of hAAT, an 1.5 kb expression cassette of neomycin resistant gene under the control of mouse phosphoglycerate kinase promoter (Soriano et al. 1991; Adra et al. 1987) was also introduced into the AAV vector plasmid pTR (+).

B. Comparison of the efficiency of rAAV generation

We produced rAAV vectors via two different methods. Method I was a conventional co-transfection method described before (Zolotukhin et al. 1996). Briefly, HeLa cells were co-transfected with the vector plasmid pTRNAEAT and packaging plasmid pIM45 (Peel et al. 1997) at a molar ratio of 1:2 using the calcium-phosphate method, followed by the infection of human adenovirus 5 at MOI.2. For Method II, we established a stable HeLa cell line "HeLa-TRNAEAT" carrying proviral sequences of AAV-TRNAEAT by transfecting HeLa cells with pTRNAET following the selection in G418. HeLa-TRNAEAT cells were transfected with pIM45 and infected with Ad5. Then, wild type HeLa cells were infected with the rAAV generated by these two methods and selected with G418 to determine the Neo-resistant titers. The rAAV titer of the viral stock from Method I was $0.8\pm0.4x10^4$ cfu/ml, while those from Method II was $1.0\pm0.2x10^5$ cfu/ml (**Fig. 2**). We were able to generate rAAV stocks with 10 fold higher titer using Method II. Moreover, since more consistent and reproducible results were obtainable with the latter method, we used Method II for generating rAAV for further analysis.

C. Testing the rAAV vectors for infectivity to human and rodent hepatoma cell lines

We determined the Neo^R titers of HepG2 and Hepa1A cells to evaluate the infectivity of the rAAV to cell lines of hepatocyte origin. The Neo^R titers for HepG2 and Hepa-

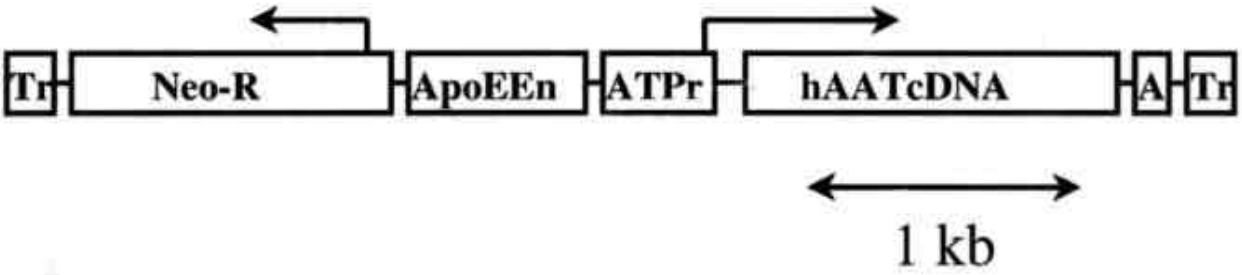

Figure 1. Schematic presentation of recombinant adeno-associated virus "TRNAEAT". Two expression cassettes were introduced in an opposite direction to each other between two terminal inverted repeat sequences of AAV. Expression of neomysin resistant gene is under the control of phophorylgycerate kinase promoter, and expression of human α_1-antitrypsin cDNA is expected to be under the control of human α_1-antitrypsin promoter and apolipoprotein E enhancer. Tr, inverted terminal repeat sequence of adeno-associated virus; Neo-R, expression cassette for neomycin resistant gene; ApoEEn, 4 copies of apolipoprotein E enhancer; hAATcDNA, cDNA for human α_1-antitrypsin; A, polyA signal of SV40

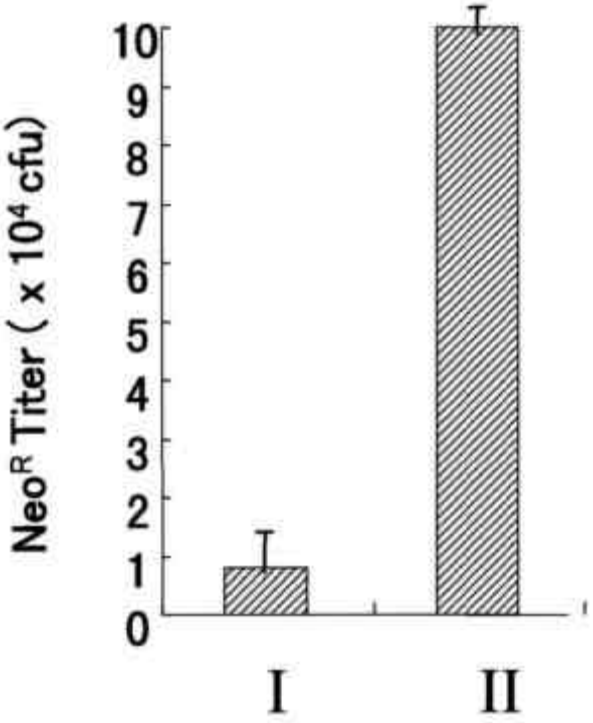

Figure 2. Neo^R titers of rAAV "TRNAET" in HeLa cells. The viral stocks of rAAV "TRNAEAT" were prepared by two different methods, Method I and Method II (see details in text), and Neo^R titers of each viral stock were determined in HeLa cells. Data is presented as the average ± SE.

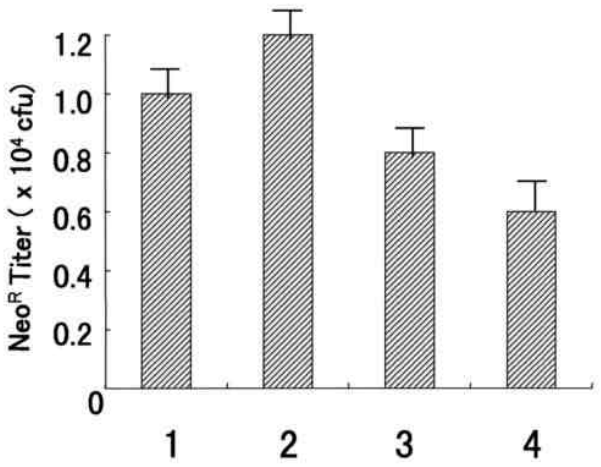

Figure 3. Infectivity of rAAV in different cell lines. Neo^R titers of the rAAV prepared with Method II were determined with 4 different cell lines, HeLa (Lane 1), NIH3T3 (Lane 2), HepG2 (Lane 3), and Hepa1A (Lane 4). Data is presented as the average ± SE.

1A cells were 0.8±0.2x10^5 cfu/ml and 0.6±0.1x10^5 cfu/ml, respectively. The same viral solution was used to calculate the titer in HeLa cells and NIH3T3 cells (**Fig. 3**). The relative infectivity of the rAAV in HepG2 and Hepa1A cells compared to those of HeLa cells were 0.75 and 0.67, respectively. These observations suggested that it is possible to transduce exogenous genes into cell lines of hepatocyte origin with similar efficiency as into HeLa or NIH3T3 cells using the rAAV gene transfer system.

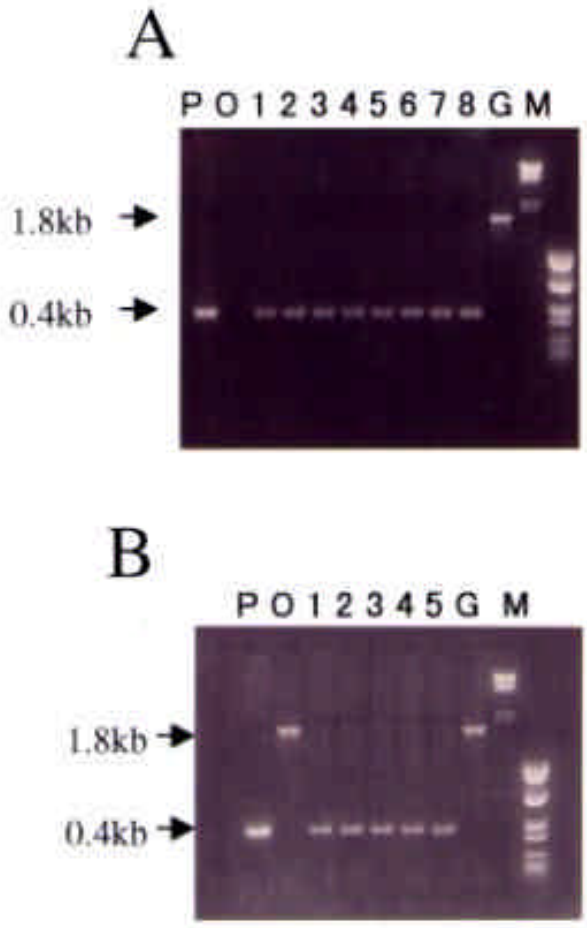

Figure 4. Detection of proviral genome sequence in Hepa1A and HeLa cells infected with rAAV "TRNAET". A 400 bp DNA region of human α_1-antitrypsin was amplified using purified genomic DNA of rAAV-infected cells as templates. (**A**) PCR results for the eight Hepa 1A clones. Template DNA samples of PCR reactions were as below, pTRNAET (Lane P), wild type Hepa1A (Lane 0), rAAV-infected Hepa1A clones No.1-No.8 (Lane 1-Lane 8), and wild type HepG2 (Lane G). Lane M stands for DNA size markers, *Hind*I II digested Lambda DNA (left) and *Sau*3AI digested PUC 19 (right). The 400 bp amplified DNA fragments were identified in Lanes 1 to 8, indicating that proviral sequences of the rAAV were introduced into all of the eight clones. This signal was not identified in Lane 0 and Lane G, but a 1.8 kb signal was identified in Lane G instead. This corresponds to the DNA amplified from human genome for α_1-antitrypsin. (**B**) PCR results for five independent HeLa cell clones. Template DNA samples of PCR reactions were wild type HeLa cells (Lane 0), and rAAV-infected HeLa clones No.1-No.5 (Lane 1-Lane5). Lane P, Lane G, and Lane M were same as for **Figure 4A**. PCR of the five HeLa clones and wild type HeLa cells resulted 1.8 kb DNA fragments amplified from human gene for α_1-antitrypsin, but only AAV-infected HeLa clones showed 0.4 kb fragments corresponding to the rAAV proviral sequence.

Table 1. Comparison of levels of human α_1-antitrypsin (hAAT) expression in Hepa1A cells infected with rAAV "TRNAEAT" and retroviral vector "ApoE(-)haat-LTR". (Data is presented as the average (±SE)

Vectors for hAAT gene transduction	**hAAT expression (ng/ million cells / 24 h)**
rAAV-TRNAEAT	103.8±23.5
retroviral vector "ApoE(-)hAAT-LTR"	71.0±6.6

D. Cell-type specific expression of cDNA for human α_1-antitrypsin in Hepa1A cells transduced with the rAAV "TRNAEAT"

Hepa1A cells and HeLa cells infected with the rAAV were selected in G418 for 14 days, and eight Hepa1A and five HeLa clones were isolated. To verify that the cDNA region of hAAT was introduced into the cells, the 400 bp partial hAAT cDNA was amplified using purified genomic DNA as template. The expected DNA fragments were amplified in all HeLa and HepG2 clones, indicating that the expression unit of hAAT was integrated into the chromosomal DNA of the cells with the rAAV vector (**Fig. 4A** and **4B**). In HeLa and HepG2 cells, we identified faint 1.8-kb amplified DNA fragments, corresponding to the endogenous human hAAT gene. The hAAT protein secreted into the media of each clone was assayed by ELISA using human-specific antibody for α_1-antitrypsin. All eight clones of Hepa1A cells secreted hAAT into the media. The average amount of hAAT protein secreted from the rAAV-infected Hepa1A cells was 103.8 ng /10^6cells /24 hours (**Fig. 5A**). This represented 31% of hAAT secreted from one million cells of wild type HepG2, and was similar to the level of hAAT secreted from Hepa1A cells infected with retroviral vector "apoE(-) haat-LTR" carrying an identical liver-specific expression cassette for hAAT (**Table 1**). On the other hand, none of the HeLa clones obtained by the infection of rAAV-TRNAEAT secreted detectable levels of hAAT protein in cultured media, although expression units for hAAT were administered into the host cell chromosome (**Fig. 5B**). These results suggested that rAAV-TRNAEAT was able to express exogenous genes exclusively in cells of hepatocyte origin.

III. Discussion

In this report we have shown that a recombinant adeno-associated virus vector is able to transfer an exogenous gene into human or rodent cells of hepatocyte origin with a similar efficiency as in HeLa cells or in NIH3T3 cells, and

that it is possible to obtain hepatocyte specific transgene expression using a liver-specific promoter and enhancer.

We previously generated a retroviral vector expressing α_1-antitrypsin cDNA under the control of the enhancer-promoter complex of apolipoprotein E and α_1-antitrypsin. This retroviral vector, apoE(-)haat-LTR, showed markedly increased expression of α_1-antitrypsin in rat liver *in vivo* (Okuyama et al. 1996). The rAAV vector TRNAEAT contains the same liver-specific expression cassette, and the levels of transgene expression in Hepa1A cells infected with rAAV-TRNAEAT were similar to those in the same cells infected with the retroviral vector apoE(-)haat-LTR. These *in vitro* results suggest that a high level of expression could be expected *in vivo* using rAAV-TRNAEAT, if similar gene transduction efficiency with retoroviral vectors is obtainable using the rAAV gene transfer system.

In order to evaluate the level of expression in rat liver *in vivo*, however, it is necessary to prepare high titer viral stocks. It is difficult to generate high titer AAV viral particles in large scale with the conventional co-transfection method (Peel et al. 1997). To circumvent this problem, we established a HeLa cell line encoding the proviral genome sequence of the rAAV-TRNAEAT. Using this cell line, it was possible to obtain more than 10 fold higher titer viral stocks easily, and this result was highly reproducible (**Fig. 2**). Flotte et al. (1995) tried a similar approach using 293 cells, and were successful in generating rAAV with a 5 fold higher titer compared with the conventional co-transfection method.

One of the potential advantages of rAAV for hepatic gene therapy is that it is possible to transduce genes into non-dividing cells (Podsakoff et al. 1994). Recently Snyder et. al. (1997) reported persistent transgene expression in mouse after a simple intraportal infusion of the rAAV expressing human Factor IX under control of MuLV LTR promoter/enhancer. This result suggests that the rAAV gene transfer system is promising for *in vivo* liver-directed gene therapy. However, one major disadvantage of *in vivo* hepatic gene transfer is that it is difficult to restrict gene transduction to hepatocytes, because there are many non-parenchymal cells, such as Kuppfer cells and sinusoidal endothelial cells, in the liver in addition to hepatocytes. Here we demonstrated that cell-type specific transgene expression was achievable by rAAV carrying liver-specific promoter enhancer sequences. The vector system described here has the potential advantage of eliminating the risk of miss-targeting, a problem encountered when rAAV vectors are used as an *in vivo* gene delivering vehicle.

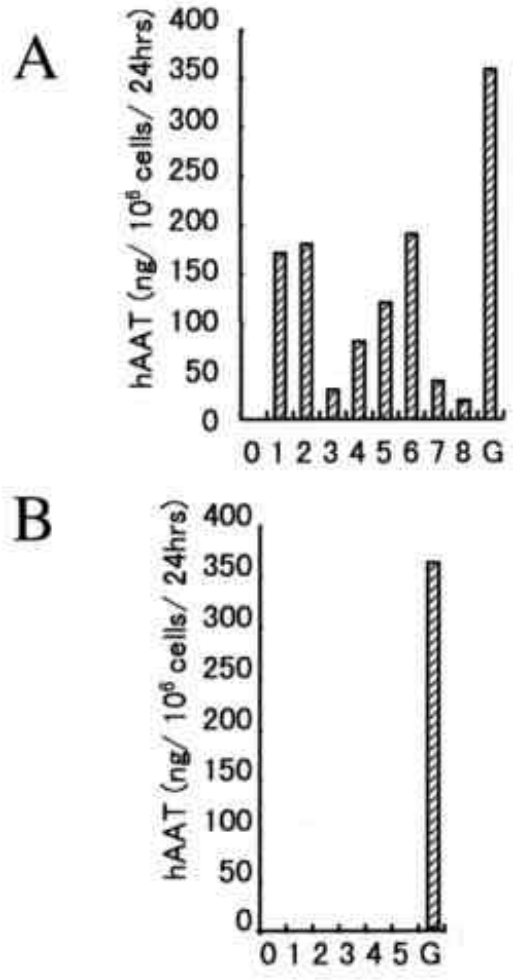

Figure 5. Quantification of human α_1-antitrypsin secreted from rAAV-infected Hepa 1A and HeLa cells. The amount of human α_1-antitrypsin protein secreted into the media was determined by ELISA. (A) The results of ELISA assay in wild type Hepa1A cells (Lane 0), in rAAV-infected Hepa1A clones No.1-No.8 (Lane1-Lane8), and in wild type HepG2 cells (Lane G). (B) The results of ELISA assay in wild type HeLa cells, rAAV-infected HeLa clones No.1-No.5 (Lane 1-Lane 5), and wild type HepG2 (Lane G). Data is presented as the average of three independent assays.

IV. Materals and Methods

A. Plasmid construction

The plasmid pIM45, encoding rep and cap genes of AAV, and pTR(+) for constructing vector plasmid of rAAV were generous gifts from Dr. Nick Muzyckzucka of the University of Florida. The structure of retroviral vector plasmid pAp(-)hAAT-LTR was described elsewhere (Okuyama et al. 1996). The plasmid pAp(-)hAAT-LTR was linealized at the unique *Bgl*II site, and then partially digested with *Bam*HI. The 2.2 kb *Bgl*II-*Bam*HI DNA fragment containing 4 copies of the 154 bp

apolipoprotein E enhancer region, 400 bp of human α_1-antitrypsin promoter sequence, and 1.2kb cDNA for human α_1-antitrypsin was gel-isolated and ligated with *Bgl*II-digested pTR(+) generating the plasmid pTRAET.

pTR(+) is a plasmid for constructing rAAV, using E.coli strain JC8111 (Deiss et al. 1990) as host cells for transformation. The plasmid pTRAET was again digested and linealized with *Bgl*II, blunt-ended with the Klenow fragment of E.coli DNA polymerase, and ligated with the 1.6 kb expression cassette of the neomycin resistant gene under the control of mouse phosphorylglycerol kinase promoter, isolated from another plasmid, pPGKNeo (Soriano et al. 1991). The plasmid pTRNAEAT was generated based on this cloning process.

B. Production of rAAV vector "TRNAEAT"

HeLa cells were maintained with DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Sanko Junyaku Co. Ltd., Tokyo Japan). The rAAV TRNAEAT was generated by two different methods. Method I involved conventional co-transfection. HeLa cells were transfected with pTRNAEAT and pIM45 at a molar ratio of 1:2 using calcium phosphate precipitation method described before (Chen and Okayama 1987). 24 hours after the transfection, the cells were infected with wild type adenovirus Ad5 with MOI.2 for 2 hours. Three days after the transfection, the cells were harvested, lysed by freezing and thawing 5 times, and incubated at 60°C for one hour to inactivate co-existing adenoviruses. In Method II, we established the stable HeLa cell clone first. HeLa cells were transfected with the plasmid pTRNAEAT and selected with 600μg/ml G418 (GIBCO BRL) for 14 days, and established the HeLa cell clone HeLa-NAEAT carrying the proviral genome sequences in its chromosomes. To produce rAAV, HeLa-NAEAT was transfected with pIM45, and the transfected cells were treated in the same way as for Method I.

C. Determination of neo^R titers of the rAAV "TRNAEAT"

HeLa and HepG2 cells were maintained with DMEM supplemented with 10% fetal bovine serum, NIH3T3 cells were maintained with DMEM with 10% calf serum (Sanko Junyaku Co. Ltd.), and Hepa1A cells, cells from a mouse hepatoma cell line, (Darlington 1987) were maintained with 75% MEM (GIBCO BRL), 25% Waymouth (GIBCO BRL), 10% fetal bovine serum. Serial dilutions of the viral stocks were made with DMEM, and certain amounts were added into the media of the plates culturing HeLa cells for 4 hours. Then cells were washed with PBS twice, and fed with fresh media for two more days. Two days after the infection, 600μg/ml of G418 (GIBCO BRL) was added to the media, and the culture was continued until distinct colonies were identified. The titer of each viral stock solution was calculated by counting the numbers of the G418 resistant colonies on the plates. The neo^R titers of the rAAV to NIH3T3 cells, Hepa1A cells, and HepG2 cells were determined in the same way except for the concentrations of G418 used for the selection: 400 μg/ml in NIH3T3 cells, 600 μg/ml in Hepa1A cells, and 800 μg/ml in HepG2 cells, respectively.

D. Isolation of Hepa1A and HeLa cell clones infected with rAAV "TRNAEAT"

Hepa 1A cells and HeLa cells were infected with rAAV "TRNAEAT" for 4 hours at MOI. 0.1, and two days after the infection, 600 μg/ml of G418 was added to the media. About two weeks after the infection, several colonies were picked up, and further propagated. Finally we established eight Hepa1A clones, and five HeLa clones. Purified genomic DNA samples from these cells were used as templates of PCR reactions for detecting the 400bp DNA region of human α_1-antitrypsin cDNA. Forward and reverse primer sequences were 5'-CACTCAGAAGCCTTCACTGTCA-3', and 5'-ACCCAGCT GGACAGCTTCTT-3'. Thirty cycles of PCRs were performed at 1 minute of 95°C, 1 minute of 57°C, and 2 minutes of 72°C. Since the forward and reverse primers were synthesized based on the sequence of exon1 and 2 of human α_1-antitrypsin gene, this PCR reaction was expected to generate a 1.8kb DNA fragment covering the whole of intron 1 and the part of exon 1 and 2 of the human genomic DNA (Long et al. 1984).

E. Quantification of human α_1-antitrypsin produced from rAAV-infected HeLa and Hepa1A cells

24 hour-cultured media were used for ELISA assay to quantify the amounts of human α_1-antitrypsin secreted from the cells. The assay was performed in 96-well microtitration plates. Goat anti-human α_1-antitrypsin antibody, and peroxidase-conjugated goat anti-human α_1-antitrypsin antibody were purchased from Cappel (Durham, NC). After 2-hour incubation at room temperature with goat anti-human α_1-antitrypsin antibody (2 mg/well), non-specific binding was blocked by overnight incubation with 200 ml of 3% BSA and 0.02% sodium azide in PBS at 4°C. After rinsing with washing buffer, 200ml of the cultured media or control samples (purified human α_1-antitrypsin, Sigma, St. Louis MO) were added. The standard curve was made from 0 to 100 ng/ml. The microtitration plates were incubated for 3 hours at room temperature and washed four times with PBS. Then 200 ml of peroxidase-conjugated goat anti-human α_1-antitrypsin antibody (15mg/ml) was added to each well. After incubation for 2 hours at room temperature, the wells were rinsed five times and 200ml of substrate solution containing 10 mg o-phenylendiamine hydrochloride (Sigma), 10ml 30% H_2O_2, and 25ml citrate-phosphate buffer pH5 was added. The reaction was stopped by the addition of 50ml of 3 M H_2SO_4.

Acknowledgements

We thank Dr. Nick Muzyczka for recombinant adeno-associated virus construction, and Ms. K. Saito for editorial assistance. This work was supported in part by grants for pediatric research and gene therapy research from the Ministry of Health and Welfare of Japan.

References

Adra CN, Boer PH, McBurney MW (**1987**) Cloning and expression of the mouse pgk-1 gene and the nucleotide sequence of its promoter. **Gene** 60, 65-74

Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA Mol Cell Biol 7, 2745-2752

Darlington GJ (**1987**) Liver cell lines. Methods in **Enzymology** 151, 19-39

Deiss V, Tratschin JD, Weitz M, Siegl G (**1990**) Cloning of the human parvovirus B19 genome and structural analysis of its palindromic termini. **Virology** 175, 247-254

Fisher KJ, Gao GP, Weitzman MD, DeMatteo R, Burda JF, Wilson JM (**1996**) Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. **J Virol** 70, 520-532

Fisher KJ, Jooss K, Alston J, Yang Y, Haecker SE, High K, Pathak R, Raper SE, Wilson JM (**1997**) Recombinant adeno-associated virus for muscle directed gene therapy. **Nat Med** 3, 306-312

Flotte TR, Afione SA, Conrad C, McGrath SA, Solow R, Oka H, Zeitlin PL, Guggino WB, Carter BJ (**1993**) Stable in vivo expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. **Proc Natl Acad Sci U S A** 90, 10613-10617

Flotte TR, Barraza-Ortiz X, Solow R, Afione SA, Carter BJ, Guggino WB (**1995**). An improved system for packaging recombinant adeno-associated virus vectors capable of in vivo transduction. **Gene Ther** 2, 29-37

Hafenrichter DG, Wu X, Rettinger SD, Kennedy SC, Flye MW, Ponder KP (**1994**) Quantitative evaluation of liver-specific promoters from retroviral vectors after in vivo transduction of hepatocytes. **Blood** 84, 3394-3404

Jaffe HA, Danel C, Longenecker G, Metzger M, Setoguchi Y, Rosenfeld MA, Gant TW, Thorgeirsson SS, Stratford-Perricaudet LD, Perricaudet M, Pavirani A, Lecocq JP, Crystal RG (**1992**) Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. **Nat Genet** 1, 372-378

Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ (**1994**) Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. **Nat Genet** 8, 148-154

Kay MA, Li Q, Liu TJ, Leland F, Toman C, Finegold M, Woo SL (**1992**) Hepatic gene therapy, persistent expression of human alpha 1-antitrypsin in mice after direct gene delivery in vivo. **Hum Gene Ther** 3, 641-647

Kay MA, Rothenberg S, Landen CN, Bellinger DA, Leland F, Toman C, Finegold M, Thompson AR, Read MS, Brinkhous KM, Woo SLC (**1993**) In vivo gene therapy of hemophilia B, sustained partial correction in factor IX-deficient dogs. **Science** 262, 117-119

Laughlin CA, Cardellichio CB, Coon HC (**1986**). Latent infection of KB cells with adeno-associated virus type 2. **J Virol** 60, 515-524

Le M, Okuyama T, Cai SR, Kennedy SC, Bowling WM, Flye MW, Ponder KP (**1997**) Therapeutic levels of functional human factor X in rats after retroviral-mediated hepatic gene therapy. **Blood** 89, 1254-1259

Ledley FD (**1993**) Hepatic gene therapy, present and future. **Hepatology** 18, 1263-1273

Li Y, Shen RF, Tsai SY, Woo SL (**1988**) Multiple hepatic trans-acting factors are required for in vitro transcription of the human alpha-1-antitrypsin gene. **Mol Cell Biol** 8, 4362-4369

Long GL, Chandra T, Woo SL, Davie EW, Kurachi K (**1984**). Complete sequence of the cDNA for human alpha 1-antitrypsin and the gene for the S variant. **Biochemistry** 23, 4828-4837

Okuyama T, Huber RM, Bowling W, Pearline R, Kennedy SC, Flye MW, Ponder KP (**1996**) Liver-directed gene therapy, a retroviral vector with a complete LTR and the ApoE enhancer-alpha 1-antitrypsin promoter dramatically increases expression of human alpha 1-antitrypsin in vivo. **Hum Gene Ther** 7, 637-645

Okuyama T, Li X-K, Funeshima N, Fujino M, Sasaki K, Kita Y, Kosuga M, Takahashi M, Saito H, Suzuki S, Yamada M. (**1998**) Fas-mediated apoptosis is involved in the elimination of gene-transduced hepatocytes with E1/E3-deleted adenoviral vectors. **J Gastroenterol Hepatol** 13, 5113-5118.

Peel AL, Zolotukhin S, Schrimsher GW, Muzyczka N, Reier PJ (**1997**). Efficient transduction of green fluorescent protein in spinal cord neurons using adeno-associated virus vectors containing cell type-specific promoters. **Gene Ther** 4, 16-24

Podsakoff G, Wong KK Jr, Chatterjee S (**1994**). Efficient gene transfer into nondividing cells by adeno-associated virus-based vectors. **J Virol** 68, 5656-5666

Rettinger SD, Kennedy SC, Wu X, Saylors RL, Hafenrichter DG, Flye MW, Ponder KP (**1994**) Liver-directed gene therapy, quantitative evaluation of promoter elements by using in vivo retroviral transduction. **Proc Natl Acad Sci USA** 91, 1460-1464

Shachter NS, Zhu Y, Walsh A, Breslow JL, Smith JD (**1993**) Localization of a liver-specific enhancer in the apolipoprotein E/C-I/C-II gene locus. **J Lipid Res** 34, 1699-1707

Simonet WS, Bucay N, Lauer SJ, Taylor JM (**1993**). A far-downstream hepatocyte-specific control region directs expression of the linked human apolipoprotein E and C-I genes in transgenic mice. **J Biol Chem** 268, 8221-8229

Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, Nagy D, Gown AM, Winther B, Meuse L, Cohen LK, Thompson AR, Kay MA (**1997**). Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. **Nat Genet** 16, 270-276

Soriano P, Montgomery C, Geske R, Bradley A (**1991**). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. **Cell** 64, 693-702

Zolotukhin S, Potter M, Hauswirth WW, Guy J, Muzyczka N (**1996**) A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. **J Virol** 70, 4646-4654