growth factor (PDGF) receptors [27], may contribute to this phenomenon. Using other vector systems may result in different tissue specificity. Recently, transgene expression in the whole peritoneal cavity was observed by *ip* administration of polyethylenimine (PEI)/DNA complexes [28]. Further, in neonates, a long-term expression was observed in factor IX concentration, whereas in adult males a sharp decrease was observed at 12 weeks and later (Figure 3). When the peritoneum was analyzed, only the surface epithelium of the peritoneal tissue was transduced (Figure 4E), and it appeared to be responsible for continuously supplying the transgene product at a therapeutic level. These cells contain an extremely high copy number of transgenes even after a prolonged period of time (Figure 2C). The copy number of the vector genome within the peritoneum appears to be underestimated thus far because the whole peritoneal tissue was used for DNA extraction prior to Q-PCR. The presence of an extremely high copy number of vector genomes within the peritoneum is possibly related to the robust and persistent transgene expression in neonatal gene transfer. The mechanism for the persistence of high copy number and transgene expression is interesting and may offer important insights into the biology of the AAV vector.

Interestingly, a sex-related difference in transgene expression within the peritoneal tissues was observed after *ip* injection into adult mice regardless of the transgene. In a previous study, a sex-related difference in transgene expression was demonstrated in the liver, and an androgen-dependent pathway appeared to be involved [25,26]. We have also demonstrated an overwhelming sex-related difference in liver transduction efficiency in a mouse model [19]. Based on our knowledge, this is the first report that demonstrates a sex-related difference in transgene expression in tissues other than the liver. At present, it is not clear whether the same mechanism is involved in the peritoneal tissue. The difference may be a drawback when an attempt is made to transfer genes into females. However, our results indicate that this problem can be circumvented if neonates are targeted for gene therapy.

Neonatal gene transfer is also advantageous from an immunological point of view. Due to the immaturity of the neonatal immune system, tolerance to an 'immunogenic' transgene product can be induced. Recently, neonatal and fetal gene transfer experiments using adenoviral and retroviral vectors demonstrated the induction of tolerance to transgene products [14,15]. In our series of experiments, it is difficult to prove this point because all transgenes were expressed for a long period even in adults. Nonetheless, divergent levels of transgene expression between adults and neonates may reflect a difference in immunology, and needs to be analyzed in the future.

In conclusion, our findings support the efficacy of neonatal gene therapy and would help to design strategies for neonatal gene therapy using AAV vectors.

Acknowledgements

We thank Dr. Y. Hakamata (Animal Resource Project, Jichi Medical School) for providing technical assistance in the animal experiments. This work was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare, Japan; the 'High-Technology Research Center' Project for Private Universities: a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology, 2003–2007; and the 21st Century Centers of Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology.

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J Gene Med 2006; **8**: 990–997.
DOI: 10.1002/jgm

doi:10.1016/j.ymthe.2005.11.024

SHORT COMMUNICATION

Removal of Empty Capsids from Type 1 Adeno-Associated Virus Vector Stocks by Anion-Exchange Chromatography Potentiates Transgene Expression

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Available online 13 February 2006

Production of recombinant adeno-associated virus (rAAV) results in substantial quantities of empty capsids or virus-like particles (VLPs), virus protein shells without the vector genome. The contaminating VLPs would interfere with transduction by competing for cell-surface receptors and, when administered *in vivo*, contribute to antigen load, which may elicit a stronger immune response. Density-gradient ultracentrifugation provides a means to separate VLPs from rAAV particles, but is not feasible for large-scale preparations of vectors. Since the compositions of the VLP and vector differ by the single-stranded DNA genome, we hypothesized that the isoelectric point of the vector may differ from that of the VLP. In an attempt to separate type 1 rAAV particles from VLPs by ion-exchange chromatography, we tested a number of buffer systems and found that trimethylammonium sulfate, or $[(CH_3)_4N]_2SO_4$, effectively separated rAAV1 particles from VLPs. The rAAV1-GFP chromatographically separated from VLPs induced stronger GFP expression in HEK293 cells than rAAV1-GFP contaminated with VLPs. The transduction of mouse muscles with rAAV1-SEAP (secreted form of alkaline phosphatase) isolated from VLPs also showed higher serum SEAP levels than rAAV1-SEAP with VLPs. These results suggest that chromatographic separation of rAAV1 from empty capsids increased the efficacy of rAAV1.

Key Words: AAV vector, empty capsid, antichaotropic ion, chromatography

Recombinant adeno-associated virus (rAAV) is one of the promising gene transfer vectors and efficiently transduces neurons, hepatocytes, and skeletal muscle in rodent, dog, and nonhuman primate models [1]. AAV vectors produced with serotype 1 capsid protein transduce skeletal muscles particularly well compared to serotypes 2, 4, 5, and 6 [2]. Obtaining clinically meaningful levels of a therapeutic protein depends on several factors, including the amount of particles administered. A human clinical trial using rAAV2 expressing coagulation factor IX (up to 10^{13} particles/kg) in hemophilia B patients has been conducted, resulting in a partial, but transient, amelioration of symptoms. Thus, extrapolating from these earlier studies, more than 10^{15} particles of rAAV2 would be required for the complete correction of hemorrhagic tendency [3]. Using serotypes with higher biological activities may reduce the dose; even so, large animal studies comparing the efficacy of rAAV1 and other serotypes indicated that large particle numbers of rAAV1 would still be required for human application [2,4].

rAAV is usually produced by plasmid transfection of HEK293 cells with two or three plasmids: AAV helper plasmid encoding *rep* and *cap* genes devoid of inverted terminal repeat (ITR) sequences, AAV vector plasmid harboring the therapeutic gene between the ITRs, and a plasmid containing a minimal set of adenovirus helper genes, E2A, VARNA, and E4orf6. Since the structural and nonstructural genes, as well as the *cis*-acting elements of AAV type 1 and AAV type 2, are highly conserved, it is possible to package the gene of interest between the type 2 ITRs into the coexpressed type 1 capsid [5], which is composed of VP1, VP2, and VP3 polypeptides with a stoichiometry of 1:1:10. In transfected HEK293 cells, the expression of the three structural proteins forms virus-like particles (VLPs) or empty capsids independent of vector DNA replication and packaging. The maturation of particles occurs during vector DNA replication and

1525-0016/$30.00

doi:10.1016/j.ymthe.2005.11.024

particles with vector genomes appear [6]. However, the fraction of VLPs that acquire vector DNA remains a minor component of the total particles in the cell. The ratio of empty to filled particles can range from 10:1 to 4:1 [7,8]. Without a DNA payload, the presence of VLPs in the rAAV stocks would diminish the effect by competing for cell-mediated processes, such as receptor binding and uptake, as well as providing a source of antigen that may elicit a stronger immune response *in vivo* against AAV vectors [9]. It is therefore desirable to eliminate empty capsids from rAAV vector stocks. The only established method to isolate rAAV from empty capsids is density gradient ultracentrifugation using CsCl or other density materials, which relies on the difference in the buoyant density between DNA-filled and empty particles. However, ultracentrifugation is not readily adaptable to the large-scale preparation of rAAV, especially for clinical grade material. In addition to density gradient centrifugation, other physicochemical differences may lead to exploitable processes for separating VLPs from vector particles. Although affinity column chromatography does not distinguish between vector and VLP, the process is scalable and also provides a higher level of purification than CsCl ultracentrifugation [7,10,11]. In addition, chromatography can preserve more infectious rAAV particles [10].

All members of the *Parvoviridae* are structurally similar and have linear, single-stranded DNA genomes. It is possible that the presence of encapsidated DNA alters the isoelectric point (p*I*) of the AAV particles. We postulated that if the p*I* of rAAV differs from that of the empty capsid, then rAAV separation from empty capsid is possible using high-resolution chromatography.

For starting materials, we used empty and filled rAAV1 particles independently obtained by CsCl density ultracentrifugation and subsequent chromatography, as described in the Fig. 1 legend. We characterized each type of particle by density, DNA content, protein composition, and biological activity (data not shown). We confirmed the purity of rAAV or empty particles by silver staining of the samples resolved on an SDS–PAGE gel (Fig. 1A). We examined the elution profile of rAAV1 containing the green fluorescent protein (GFP) gene and type 1 empty capsid on a high-resolution anion-exchange column, Mini Q 4.6/50 PE (Amersham Biosciences, Piscataway, NJ, USA). Full rAAV particles, equivalent to 5×10^{10} vector genomes (vg), or an equivalent quantity of empty particles, were bound to the column in a low-salt buffer of 20 mM Tris–HCl (pH 8.4), 20 mM NaCl, and 4% glycerol; they were eluted with a linear 20–300 mM NaCl gradient at pH 8.4. Although there is overlap at the base of the peaks, Fig. 1A shows that the empty particles (broken line) eluted at a lower salt concentration than rAAV (solid line). While the resolution of the empty and filled particle fractions was not optimal, the ability to elute the two types of particles selectively was a very encouraging result. To increase the resolution of the eluted particle peaks, we surveyed an extensive range of elution buffers and found that the use of so-called antichaotropic ions, such as NH_4^+, $(CH_3)_4N^+$, PO_4^{3-}, and SO_4^{2-}, was capable of resolving rAAV from empty capsids better than using NaCl gradients. Fig. 1B shows a representative chromatogram of the mixture of rAAV1-GFP particles and VLPs eluted with a linear 20–300 mM Na^+ or $(CH_3)_4N^+$ gradient. Among the buffers we tested, $[(CH_3)_4N]_2SO_4$ or trimethylammonium sulfate most effectively separated the rAAV particles (F) from empty capsids (E). We also examined a weaker anion, $(C_2H_5)_4N^+$, for the separation of rAAV, which more efficiently separated the rAAV particles from empty capsids. However, the solution containing $(C_2H_5)_4N^+$ was viscous and disrupted the rAAV particles. NH_4^+ also isolated rAAV1 from empty particles. The ammonium ion, however, is volatile at high pH and the ammonium solution is not stable over time.

The pH of the buffers is also important for chromatography. The elution of rAAV and empty particles at different pH is shown in Fig. 1C. We loaded the mixture of purified rAAV and empty particles onto the column and eluted them with a linear 20–300 mM $(CH_3)_4N^+$ gradient at pH 7.5, 8.0, 8.5, or 9.0. The separation of the two peaks was better at pH 8.5 or 9.0 than at lower pH. Since the empty and filled AAV particles are unstable at a higher pH [12], we used buffers at pH 8.5 in the subsequent experiments.

Our final goal was to develop a chromatographic method for the purification of a large quantity of type 1 rAAV particles free of empty particles. We next tested the separation of approximately 10^{13} vg of rAAV1-GFP from empty particles. We produced rAAV particles and released them from HEK293 cells, as described in the legend to Fig. 1. After low-speed centrifugation, we again centrifuged the cleared cellular lysate for 10 min at 30,000*g* at 4°C and filtered the supernatant through 0.45- and 0.2-μm membrane filters. We diluted the lysate four times with a dilution buffer of 20 mM Tris–HCl (pH 8.4), 2 mM $MgCl_2$, and 4% glycerol and loaded it onto a 10-mm × 60-cm Tricorn column (Amersham Biosciences) packed with POROS HQ 50-μm medium (Applied Biosystems, Foster City, CA, USA). rAAV1 was eluted with a linear 50–400 mM NaCl gradient (250 ml). We collected the fractions containing rAAV1 and diluted them threefold with the dilution buffer and loaded them onto the second anion-exchange column (5 mm × 10 cm) packed with POROS HQ 10-μm matrix (Applied Biosystems). The rAAV1 was again eluted with a linear 50–400 mM NaCl gradient (25 ml). We further purified the rAAV1 by gel filtration column chromatography, as described in the Fig. 1 legend. We mixed the fractions containing rAAV together and diluted them with 4 volumes of the dilution buffer and loaded them onto a high-resolution column (5 mm × 20 cm) filled with POROS HQ 10-μm equilibrated with 25 mM *N*-methyldiethanolamine (pH 8.5) and 10 mM $[(CH_3)_4N]_2SO_4$. Bound viral particles were eluted

with a 10–125 mM $[(CH_3)_4N]_2SO_4$ gradient over 38 ml at a flow rate of 0.5 ml/min. A representative chromatogram (Fig. 2A) shows that the two peaks were observed as expected. The peak that appeared earlier or later corresponded to empty capsids or rAAV particles. The analysis of each fraction by Western blotting with an anti-type 5 VP antibody, which was cross-reactive with type 1 VP protein (middle), revealed that the first, larger peak contained much more AAV VP protein (fractions 19 through 22). The second, smaller peak also contained VP protein, although the amount was smaller (fractions 23 through 26). Quantification using real-time PCR indicated that the majority of rAAV vector genome was in fractions 23 through 26 (bottom), corresponding to the second peak fractions. Electron microscopy of a sample from the pooled peak fractions confirmed that the earlier peak corresponded to empty capsids and the later one corresponded to rAAV particles (insets in Fig. 2A). Since a single run was not sufficient to separate completely the empty from the full capsids, we repeated the high-resolution chromatography step. After the first separation, more than 90% of contaminating empty capsids was removed. The second run was able to eliminate empty particles further and we obtained a rAAV stock with less than 5% empty particles. Table 1 summarizes the recovery of rAAV1 particles after high-resolution column chromatography for the removal of empty particles. After two rounds of chromatography, we were able to recover approximately 50% of rAAV1-GFP.

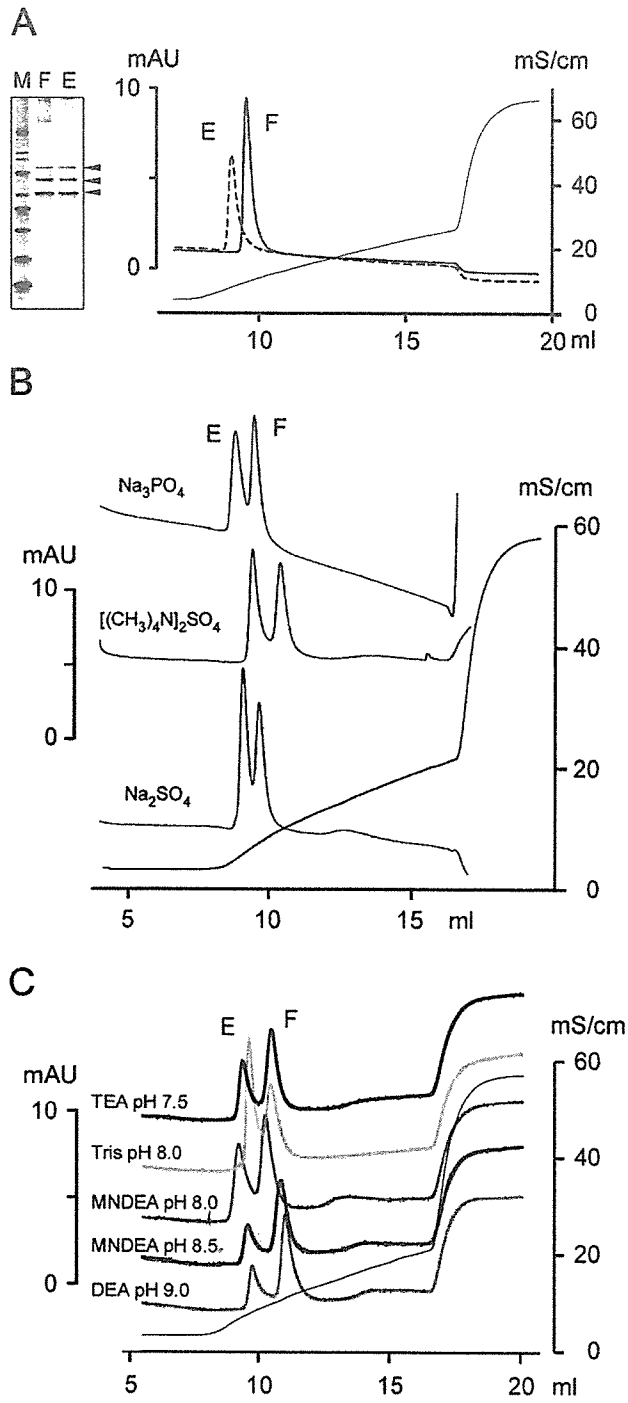

FIG. 1. (A) Elution profile of DNA-filled or rAAV1 and empty particles on a high-resolution anion-exchange column. For the production of rAAV1-GFP, HEK293 cells at 80% confluency (approximately 10^5 cells/cm^2) in a 225-cm^2 flask were cotransfected with 26.7 μg of an AAV vector plasmid harboring a humanized GFP gene (Stratagene, Palo Alto, CA, USA) under the control of the cytomegalovirus immediate early gene promoter (CMV) between the type 2 ITRs, 26.7 μg of an AAV1 helper plasmid carrying type 2 *rep* and type 1 *cap* genes [5], and 26.7 μg of an adenovirus helper plasmid using the calcium precipitation method. Two days after transfection, the cells were pelleted by centrifugation and lysed in 2 ml (per 225-cm^2 flask) of lysis buffer (20 mM Tris–HCl (pH 8.4), 150 mM NaCl, 2 mM $MgCl_2$, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (Merck, Darmstadt, Germany), 60 U/ml benzonase (Merck)) and incubated at 37°C for 30 min. After low-speed centrifugation, solid CsCl was added to the lysate to produce a buoyant density of 1.36 g/cm^2 and the samples were centrifuged for 24 h at 36,000 rpm at 21°C in an SW40Ti rotor (Beckman Coulter, Fullerton, CA, USA). rAAV1-containing fractions were collected and spun once again. rAAV1-GFP was then loaded on a gel-filtration column (HiPrep 16/60 Sephacryl S-300 HR; Amersham Biosciences) preequilibrated with 50 mM Hepes (pH 7.4), 0.3 M NaCl, 2 mM $MgCl_2$ to eliminate further cellular contaminants. Type 1 empty capsids were also generated in 293 cells transfected with a type 1 AAV helper plasmid alone and purified as for rAAV particles except for the CsCl density of 1.30 g/cm^2. Their purity was confirmed by silver staining of the SDS–PAGE gel using the SilverQuest silver staining kit (Invitrogen, Carlsbad, CA, USA). Arrows indicate VP1, VP2, and VP3 polypeptides. Approximately 5×10^{10} vg of rAAV1-GFP (F) or an equivalent quantity of type 1 empty particles (E) was loaded onto a Mini Q 4.6/50 PE column (Amersham Biosciences) controlled by an ÄKTA FPLC system (Amersham Biosciences). The bound particles were eluted over 10 ml with a linear 20 to 300 mM NaCl gradient at pH 8.4. The profile is represented as the absorbance at 280 nm (mAU). Buffer conductance (mS/cm) is indicated by the thin line. M, molecular weight standards. (B) Chromatogram of the mixture of AAV1 and empty particles in antichaotropic buffers with a 20 to 300 mM Na^+ or $[(CH_3)_4N]_2^+$ gradient. The earlier elution from the column represents empty particles (E) and DNA-filled or rAAV1 (F) eluted at a higher salt concentration. (C) The effect of pH on the elution of rAAV1 and empty particles. A buffer of 25 mM triethanolamine (TEA) at pH 7.5, Trizma (Tris) at pH 8.0, *N*-methyldiethanolamine (NMDEA) at pH 8.0 or 8.5, or diethanolamine (DEA) at pH 9.0 with a 10 to 150 mM $[(CH_3)_4N]_2SO_4$ gradient was used for elution.

doi:10.1016/j.ymthe.2005.11.024

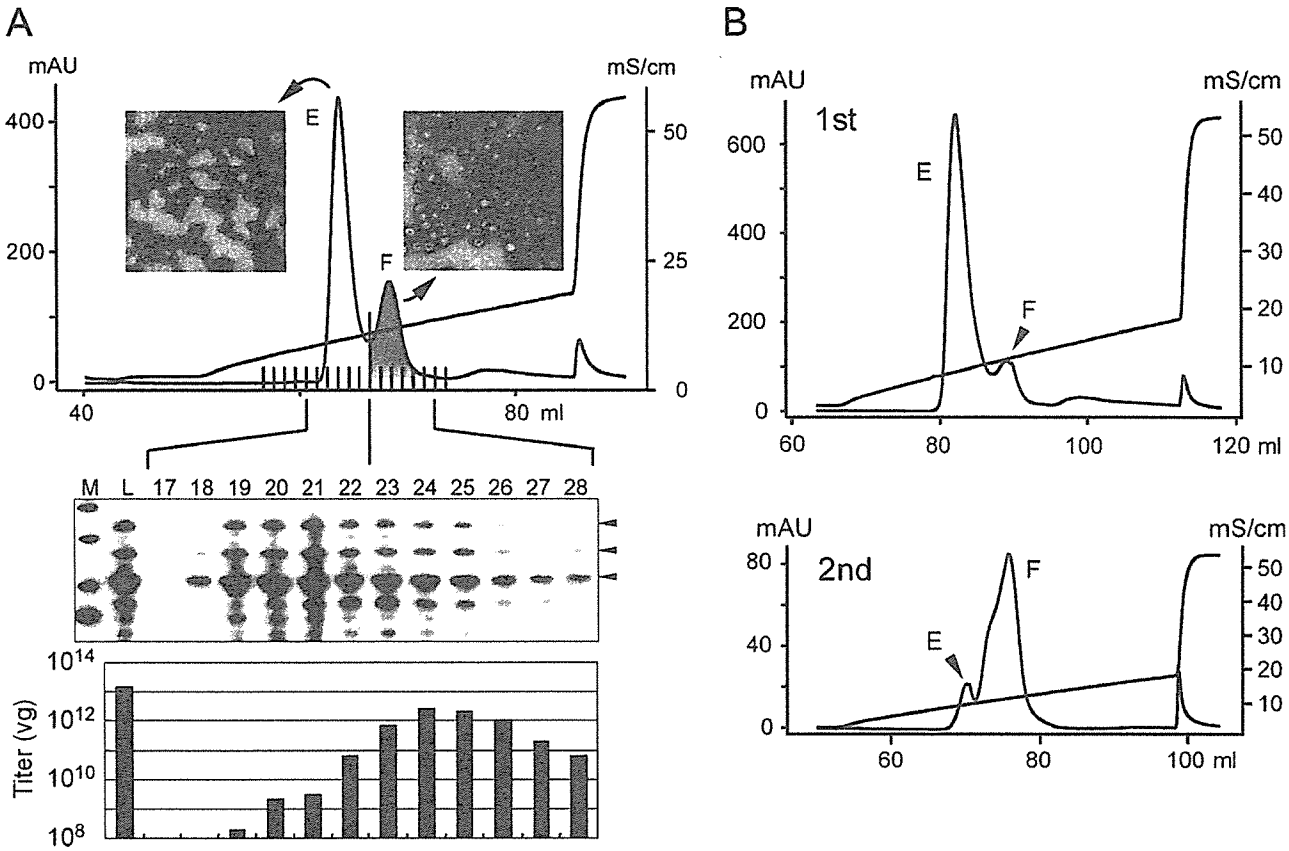

FIG. 2. (A) A representative chromatogram of a rAAV1-GFP preparation. Approximately 10^{13} vg of vector particles were generated and purified as described in the legend to Fig. 1 and finally loaded onto a 5-mm × 20-cm Tricorn column (Amersham Biosciences) packed with POROS HQ 10-μm matrix (Applied Biosystems) equilibrated with 25 mM *N*-methyldiethanolamine (pH 8.5) and 10 mM $[(CH_3)_4N]_2SO_4$. Bound viral particles were eluted with a 10–125 mM $[(CH_3)_4N]_2SO_4$ gradient over 38 ml at a flow rate of 0.5 ml/min. F and E indicate filled and empty particles, respectively. Electron microscopy of negatively stained samples from each peak is shown as an inset. After 1-ml fractionation samples were analyzed on a 4–12% NuPAGE gel (Invitrogen), the separated proteins were transferred to a Durapore membrane (Millipore, Bedford, MA, USA) and incubated with a rabbit polyclonal anti-type 5 VP antibody. After incubation with a secondary anti-rabbit immunoglobulin G labeled with horseradish peroxidase (Pierce, Milwaukee, WI, USA), chemiluminescent signals were detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce) (middle). The fraction number is indicated above each lane. VP1, VP2, and VP3 capsid proteins are indicated by arrows. A sample from each fraction was also analyzed by real-time PCR to quantify the GFP vector DNA using a primer set specific to the CMV promoter, as previously described [15]. M, molecular weight standard; L, loaded sample. (B) An example of separation of rAAV1-GFP from empty particles by two runs of the high-resolution column chromatography. The first run was able to eliminate more than 90% of the contaminating empty capsids (E) from rAAV1-GFP (F). Reloading of the eluate from the first run further removed the contaminating empty particles.

We assessed the biological activity of the rAAV1-GFP isolated by column chromatography. We infected HEK293 cells with rAAV1-GFP samples, before or after chromatographic removal of empty particles, at the particle per cell numbers indicated (Fig. 3A). Seven days after transduction, we examined the cells under a fluorescence microscope. To quantify the GFP fluorescence, we also analyzed the cells by flow cytometry as described [13]. The analysis gave the percentage positive cells and the average GFP fluorescence, which refers to the average fluorescence intensity in the subpopulation of GFP-positive cells. The fluorescence volume represents a summation of GFP fluorescence within the subpopulation of GFP-positive cells, which was calculated to be equal to the fraction of GFP-positive cells in the sample population times the mean fluorescence intensity. When HEK293 cells were infected with either rAAV1-GFP at more than 10^4 vg per cell, both vectors transduced almost all the infected cells. However, the volume of GFP

TABLE 1: Recovery of rAAV1-GFP after removal of empty capsids

Preparations	Load	After 1st run (%)	After 2nd run (%)
#1	1.2×10^{13}	7.6×10^{12} (63.3)	5.0×10^{12} (41.7)
#2	3.3×10^{13}	2.4×10^{13} (72.7)	1.7×10^{13} (51.5)
#3	1.3×10^{13}	8.6×10^{12} (66.2)	6.8×10^{12} (52.3)

Number of rAAV1 particles was determined by real-time PCR. The percent recovery was calculated by dividing the number of rAAV1 particles loaded onto the first high resolution column by the number of rAAV particles recovered after chromatography.

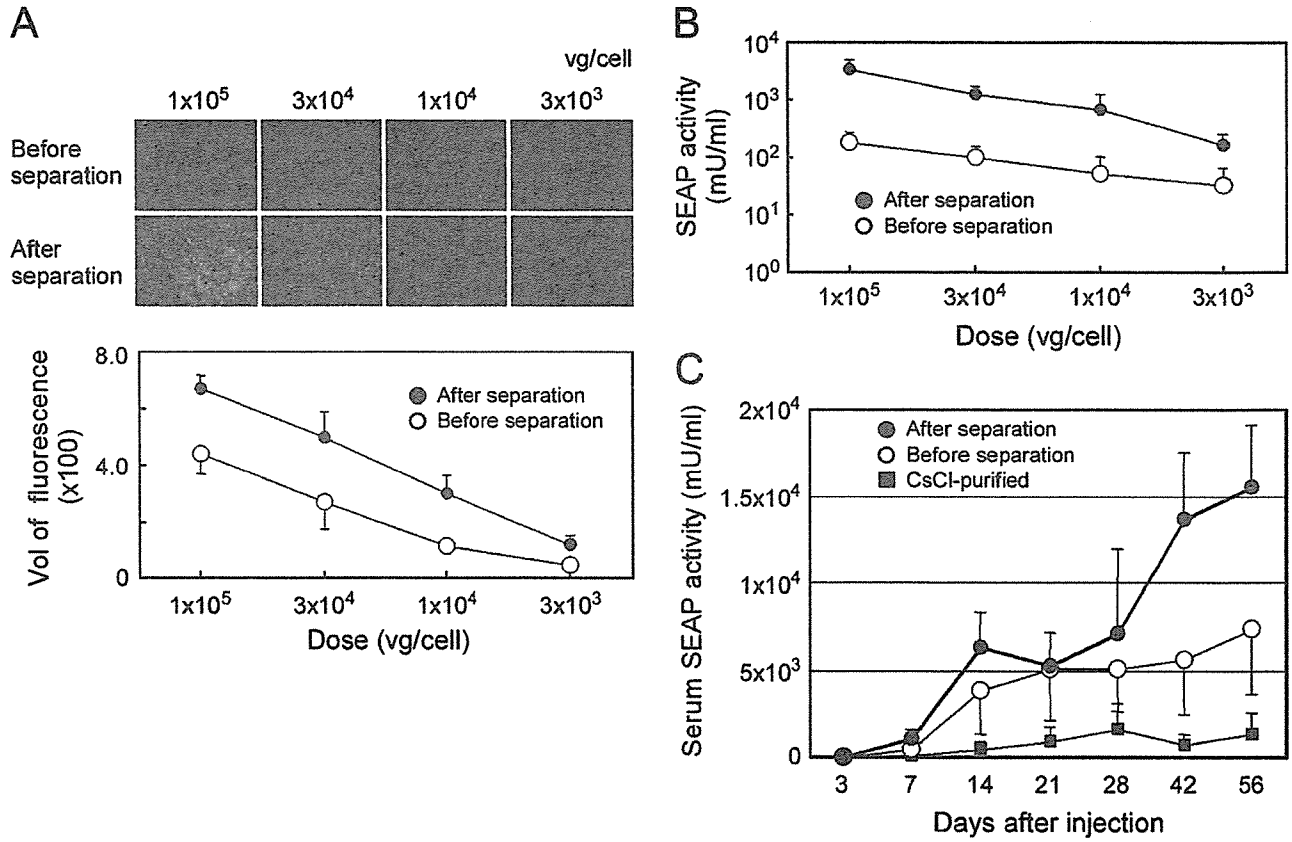

FIG. 3. (A) Transduction of HEK293 cells with rAAV1-GFP chromatographically separated from empty particles. 293 cells were infected with rAAV1-GFP before or after column chromatography intended to separate empty particles at the doses indicated. The GFP-expressing cells were analyzed by flow cytometry. The volume of GFP fluorescence was obtained by calculating (the fraction of GFP-positive cells) × (the average GFP fluorescence). Data represent means and standard deviation of experiments performed in triplicate. (B) The SEAP activity of the culture supernatant after infection of HEK293 cells with rAAV1-SEAP contaminated with or without empty particles. HEK293 cells were infected with type 1 SEAP vector before or after chromatographic removal of empty capsids at doses ranging from 1×10^5 through 3×10^3 vg per cell in triplicate. Results are expressed as means ± SD. (C) The serum SEAP levels after injection of rAAV1-SEAP into mouse muscles. A total of 10^{10} vg of rAAV1-SEAP particles before or after high-resolution chromatography or rAAV1-SEAP purified by CsCl ultracentrifugation was injected into mouse tibialis anterior muscles in triplicate and blood was taken from 3 through 56 days after injection.

fluorescence obtained by rAAV1-GFP separated from empty capsids was larger than that by rAAV contaminated with empty particles. We also infected HEK293 cells with rAAV1 expressing the human secreted alkaline phosphatase (SEAP). We excised the SEAP gene from pSEAP2-Basic (Clontech, Mountain View, CA, USA) with *Nru*I and *Sal*I and blunt-ended the resulting 1.8-kb fragment and inserted it between the type 2 ITRs. We used the resulting plasmid for transfection of HEK293 cells and purified rAAV1-SEAP as described above. We measured the SEAP activity in the culture supernatants 1 week after infection by using the SEAP Report Gene Assay (Roche Diagnostics, GmbH, Penzberh, Germany) according to the manufacturer's instructions. The rAAV1-SEAP separated from empty particles induced higher SEAP levels than rAAV1-SEAP contaminated with empty capsids at the doses tested (Fig. 3B). These results suggested that contaminating empty capsids interfered with the transduction of HEK293 cells by rAAV1.

To investigate the efficacy of rAAV1 *in vivo*, we injected rAAV1-SEAP (10^{10} vg) into mouse tibialis anterior muscles in triplicate. We used rAAV1-SEAP before chromatographic separation of empty capsids and CsCl-banded rAAV1-SEAP as controls. Fig. 3C shows the time course of the serum SEAP levels after the injection of SEAP vectors. rAAV1-SEAP purified by anion-exchange chromatography induced the highest levels of serum SEAP activity. The rAAV1-SEAP purified by column chromatography, but contaminated with empty particles, expressed lower levels of SEAP. CsCl-banded SEAP vector showed the lowest level, although the difference in the SEAP activity among the three groups was not statistically significant due to the small number of animals employed. The serum SEAP level at 56 days postinjection with the rAAV1 vector from which empty capsids were removed by chromatography was 10 times higher than that with the rAAV-SEAP from which empty capsids were excluded by CsCl ultracentrifugation, which may be due

doi:10.1016/j.ymthe.2005.11.024

to the impurity and/or the damage of CsCl-purified rAAV1 [14]. These results again indicated that the removal of empty particles from rAAV1 stocks by chromatography potentiated the SEAP expression in the muscles.

In summary, we report here a method for the selective removal of empty capsids from type 1 AAV vector. The chromatographic separation obtained pure rAAV1 stocks contaminated with less than 5% empty capsids. This method can remove empty capsids without the loss of the efficacy of rAAV1 and is easily scalable to a large volume. It will be useful for the purification of large quantities of rAAV1 for large-animal or human applications.

ACKNOWLEDGMENTS

We thank Robert Kotin (Laboratory of Biochemical Genetics, NIH) for his critical review of the manuscript. This work was supported in part by grants from the Ministry of Health, Welfare, and Labor of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Technology of Japan and the High-Tech Research Center Project for private universities matching fund subsidy from the Ministry of Education, Science, Sports, and Technology of Japan.

RECEIVED FOR PUBLICATION SEPTEMBER 25, 2005; REVISED NOVEMBER 25, 2005; ACCEPTED NOVEMBER 28, 2005.

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JOURNAL OF VIROLOGY, Feb. 2006, p. 1874–1885 Vol. 80, No. 4
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.4.1874–1885.2006

Scalable Generation of High-Titer Recombinant Adeno-Associated Virus Type 5 in Insect Cells

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Received 14 June 2005/Accepted 27 November 2005

We established a method for production of recombinant adeno-associated virus type 5 (rAAV5) in insect cells by use of baculovirus expression vectors. One baculovirus harbors a transgene between the inverted terminal repeat sequences of type 5, and the second expresses Rep78 and Rep52. Interestingly, the replacement of type 5 Rep52 with type 1 Rep52 generated four times more rAAV5 particles. We replaced the N-terminal portion of type 5 VP1 with the equivalent portion of type 2 to generate infectious AAV5 particles. The rAAV5 with the modified VP1 required α2-3 sialic acid for transduction, as revealed by a competition experiment with an analog of α2-3 sialic acid. rAAV5-GFP/Neo with a 4.4-kb vector genome produced in HEK293 cells or Sf9 cells transduced COS cells with similar efficiencies. Surprisingly, Sf9-produced humanized *Renilla* green fluorescent protein (hGFP) vector with a 2.4-kb vector genome induced stronger GFP expression than the 293-produced one. Transduction of murine skeletal muscles with Sf9-generated rAAV5 with a 3.4-kb vector genome carrying a human secreted alkaline phosphatase (SEAP) expression cassette induced levels of SEAP more than 30 times higher than those for 293-produced vector 1 week after injection. Analysis of virion DNA revealed that in addition to a 2.4- or 3.4-kb single-stranded vector genome, Sf9-rAAV5 had more-abundant forms of approximately 4.7 kb, which appeared to correspond to the monomer duplex form of hGFP vector or truncated monomer duplex SEAP vector DNA. These results indicated that rAAV5 can be generated in insect cells, although the difference in incorporated virion DNA may induce different expression patterns of the transgene.

Recombinant adeno-associated virus (rAAV) is being developed as a gene transfer vector. rAAV based on serotype 2 (rAAV2) successfully transduces nondividing cells, including muscle, liver, and brain cells (29). Conventional rAAV production requires packaging of rAAV DNA into type 2 capsids by transient transfection of HEK293 cells with two or three plasmids: an AAV helper plasmid encoding *rep* and *cap* genes devoid of inverted terminal repeat (ITR) sequences, a vector plasmid harboring the therapeutic gene between ITRs, and an adenovirus helper plasmid expressing E2A, virus-associated (VA) RNA, and E4orf6. Transient cotransfection is the major limitation for scale-up of rAAV production. Since rAAV can be purified using column chromatography, which can result in highly purified rAAV while eliminating other contaminating viruses, some efforts were made to develop rAAV production systems by using recombinant mammalian viruses such as adenovirus (10) or herpes virus (4) which do not rely on the plasmid transfection and therefore may be amenable to scale-up production.

Recombinant baculoviruses based on the *Autographa californica* nuclear polyhedrosis virus are widely employed for production of heterologous proteins in cultured insect cells. The highly active, late *A. californica* nuclear polyhedrosis virus promoters, such as polyhedrin and p10 promoters, regulate the expression of heterologous proteins, resulting in large amounts of foreign proteins. Insect cells may be grown in suspension cultures in volumes ranging from shake flasks of sizes from, e.g., 50 to 400 ml, up to commercial-size bioreactors, e.g., 1,000 liters and larger. Recently, we described a highly scalable and efficient method for packaging rAAV2 in insect cells by use of baculovirus expression vectors (31). The ease of scale-up production is perhaps the most attractive feature of this production system. Infection of insect cells in suspension culture with recombinant baculoviruses eliminates the transfection process. Standard downstream processing to recover rAAV, such as tangential flow filtration and column chromatography, is readily applied.

In addition to vectors derived from serotype 2, other serotypes, utilizing different cell surface receptors, constitute a vector set from which an appropriate vector can be selected for a specific application. AAV5 is the most divergent dependovirus characterized (2), and type 5 AAV vectors have desirable properties that differ from other serotype vectors. AAV5 utilizes different receptors from other serotypes (14, 30), and rAAV5 has demonstrated different tropism from AAV2 (5), thus making it worthwhile to establish a method to produce rAAV5 in insect cells.

AAV is a member of the family *Parvoviridae*. The genome of AAV is a linear, single-stranded DNA of 4.7 kb in length. The ITRs flank the unique coding sequences for the nonstructural replication initiator proteins, Rep, and the structural capsid proteins, VP. The ITRs serve as origins of DNA replication and may also function as the packaging signal. Type 2 Rep78 is generated by the p5 promoter, while Rep68 is translated from spliced mRNA from the p5 promoter. The small Rep polypep-

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tides Rep52 and Rep40 are expressed by the p19 promoter with nonspliced or spliced mRNA. The p40 promoter regulates expression of capsid proteins VP1, VP2, and VP3. Alternate usage of two splice sites and translation of VP2 at a non-AUG codon results in a stoichiometry of 1:1:10 of VP1, VP2, and VP3. Both p5 proteins Rep78 and Rep68 are AAV origin binding proteins, and the presence of either is required for AAV DNA replication and processing replicative intermediates of the virus DNA (13). Also, either Rep52 or Rep40 is necessary for packaging the single-stranded, linear virion genome into preformed empty capsids (17). The transcriptional map of type 5 AAV differs from that of type 2; the p7 promoter or p19 promoter transcribes mRNA for Rep78 or Rep52. Type 5 AAV does not encode the spliced form of Rep polypeptides Rep68 and Rep40 (25). Structural protein VP1 is a minor constituent in the AAV capsid. But the VP1-unique portion of approximately 140 amino acid residues is highly conserved among different serotypes and has a phospholipase A_2 motif. The YXGGX and HDXXY motifs (where X is any amino acid residue) in phospholipase A_2 indicate the catalytic site and Ca^{2+} binding loop, respectively (see Fig. 3A). Enzymes classified into the secretory phospholipase A_2 family hydrolyze the ester bond at the 2-acyl ester position of glycerophospholipids in the presence of Ca^{2+} and are involved in many aspects of cellular pathways, such as lipid membrane metabolism and signal transduction pathways (1, 21). The VP1-unique portion of parvovirus is required for transfer of the virus from late endosomes to the nucleus (36). A mutant virus lacking the VP1-unique portion or the activity of phospholipase is not processed properly, and thus no virus or vector genes are expressed.

In the present study, we describe a rAAV5 production system based on recombinant baculovirus and insect cells. In order to achieve high production levels of rAAV5 particles, we replaced a portion of the VP1 polypeptide with the corresponding portion of type 2. The VP1 substitution did not alter the tropism of rAAV5, which behaved indistinguishably from rAAV5 with wild-type VP1. In an attempt to improve the yields of rAAV5 particles, we used type 1 Rep52 instead of type 5, which resulted in the production of more than 5×10^4 vector genomes (vg) per insect cell.

MATERIALS AND METHODS

Plasmid construction. A flow chart of plasmid construction is shown in Fig. 1. pSR485 is an AAV5 vector plasmid harboring green fluorescent protein (GFP) and neomycin (Neo) genes between the ITRs (27). NotI sites were introduced outside the GFP/Neo expression cassette by PCR amplification using primers 5′-GATCGTCGACGCGGCCGCTCTCAGTACAATCTGCTCTGATGCC and 5′-AGTCGTCGACGCGGCCGCCTGCAGGCATGCAAGCTTGTGAAAAA AATGC. The NotI sites (underlined) were introduced. The resulting 4-kb DNA fragment was inserted into the BglII-SalI (blunt) sites of pSR485 (pSR485α). pFB5GFP was constructed by insertion of the 4.8-kb PagI fragment from pSR485 into the Eco105III site of pFBHTΔ, which was derived from pFBHTb (Invitrogen, Carlsbad, CA) after removal of the polyhedrin promoter with BstZ17I and HindIII digestion. A humanized *Renilla* GFP (hGFP) gene was excised from phrGFPII-1 (Stratagene, La Jolla, CA) by treatment with BamHI and EcoRV and subcloned into an expression plasmid regulated by the cytomegalovirus (CMV) immediate-early promoter (pCMV). The resulting plasmid, pCMVh GFP, was treated with NotI to cut out the entire hGFP expression cassette, which was inserted into the corresponding site of pSR485α or pFB5GFP (pSR485hGFP or pFB5hGFP, respectively). A human secreted alkaline phosphatase (SEAP) gene was excised from pSEAP2-Basic (Clontech, Mountain View, CA) with NruI and SalI, and the resulting 1.8-kb fragment was blunt-ended and inserted into pCMV. The entire SEAP cassette was then excised with NotI and inserted into the corresponding site of pAAVGFP or pFBGFPR (31) between the type 2 ITRs (pAAVSEAP or pFBSEAP, respectively). The type 5 p5 Rep open reading frame (ORF) equivalent to type 2 Rep78 was PCR amplified from pAAV5-2 (2) by using primers 5′-GAAGAAGCGCGCGTATGAGTTCT CGCGAGACTTC and 5′-CGATTTACTGTTCTTTATTGGCATCGTCAA AATC and inserted into a cloning vector. The Rep ORF was cut out by NruI and BssHII, blunt-ended, and subcloned into the NotI site (blunt) of pBACΔIERep (31), which was then treated with BglII and ClaI and blunt-ended, and the resulting 2.1-kb fragment was inserted into the NotI-PstI (blunt) sites of pFBDΔ (pFBD5LR). pFBDΔ is a derivative of FastBac Dual (Invitrogen) generated by the removal of the polyhedrin and p10 promoters with NcoI and BamHI treatment. The small Rep ORF was cut out from pFBD5LR by partial digestion with Eco47III and SalI, and the resulting 1.3-kb fragment was blunt-ended and inserted into the StuI site of pFastBac Dual (pFBD5SR). pFBD5SR was then digested with BstZ17I and SalI and treated with T4 DNA polymerase, and the resulting 1.4-kb fragment was inserted into the KpnI site (blunt) of pFBD5LR (pFBD5LSR). To generate the truncated p10 promoter, complementary 5′-phosphorylated oligonucleotides 5′-TAAAATCGCGAC and 5′-CATGGTCGC GATTTTAAT were annealed to each other and inserted into the PacI-NcoI sites of pFastBac Dual (pΔ5FBD). The type 5 Rep78 gene was PCR amplified with primers 5′-GCGCTTAATTAAAATCGCTAGTATGGCTACCTTCTATGA AGTCATT-3′ and 5′-GATCGCTAGCTTACTGTTCTTTATTGGCATCGT CA-3′ and subsequently digested with PacI and NheI and inserted into the PacI-NheI sites of pΔ5FBD (pFBD5LR12) (the Rep78 ORF is capitalized)). The type 5 Rep52 gene amplified using primers 5′-GATCGCGCGCCATGGCGCTCG TCAACTGGCTCGTGGAG-3′ and 5′-GATCGTCGACTTACTGTTCTTTAT TGGCATCGTCA-3′ was digested with BssHII and SalI and inserted into the corresponding sites of pFBD5LR12 (pFBD5LSR12α). To replace type 5 Rep52 on pFBD5LSR12α with type 1, 2, 3, or 4 Rep52, PCR was conducted with sense primer 5′-gatcccATGGAGCTGGTCGGGTGGCTGGTGGA-3′ and antisense primer 5′-gatcactagtTTATTGCTCAGAAACACAGTCATCCA-3′ (for type 1 or 3) or 5′-gatcactagtTTATTGTTCCATGTCACAGTCATCCA-3′ (for type 4) from AAV1 (purchased from American Type Culture Collection), an AAV2 helper plasmid pHLP19 (20), p3-2 (22), or p4-2 (3) (NcoI and SpeI sites are underlined). The resulting 1.2-kb DNA was digested with NcoI and SpeI and inserted into the corresponding sites of pFBD5LSR12α (pFBD5LSR121, pFBD5LSR122, pFBD5LSR123, and pFBD5LSR124). The resulting recombinant baculoviruses expressing type 5 Rep78 and type 1, 2, 3, 4, or 5 Rep52 are designated Rep5/1, 5/2, 5/3, 5/4, and 5/5, respectively. The type 5 VP ORF was obtained by PCR amplification from pAAV5-2 by using primers 5′-gtcaagcttcct gttaagACGTCTTTTGTTGATCACCCTCCAGATTGGT-3′ and 5′-cgaatctagaTT AAAGGGGTCGGGTAAGGTATCG-3′. The sequence corresponding to the VP ORF is capitalized, and the initiation codon was mutated to ACG to reduce its translational efficiency. The 2.2-kb PCR product was cloned into pCMV (pCMV5VPm). The plasmid was digested with Acc65I and treated with T4 DNA polymerase and subsequently with XbaI to excise the VP ORF, which was then inserted into the BamHI (blunt)-XbaI sites of pFastBac Dual (pFBD5VPm). Plasmid expressing a chimeric VP was constructed by the use of an overlapping-PCR method as follows. VP251 was generated by PCR from pAAV5-2 using primers #30 and #31 (Table 1). The resulting PCR product was treated with BamHI and HindIII and cloned into the corresponding sites of pFBD5VPm. For VP252 construction, the type 2 VP portion was PCR amplified with primers #32 and #34 from pHLP19. The type 5 VP was amplified with primers #33 and #31. After gel purification, the two PCR products were combined and subjected to the second round of PCR using primers #31 and #32. Chimeric VP253, -254, -255, and -256 were produced in the same way except for primers for the first round of PCR. For VP253, primers #32 and #36 were used to amplify the type 2 VP1 portion and #31 and #35 to amplify the type 5 VP portion (see Fig. 3A). A PCR-generated chimeric VP1 gene was digested with HindIII and BamHI and inserted into the HindIII-BamHI sites of pFBD5VPm.

Cell culture. HEK293 cells were maintained in Dulbecco's modified Eagle's medium–F-12 (1:1, vol/vol; Invitrogen) supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO). *Spodoptera frugiperda* Sf9 cells (Invitrogen) were grown at 27°C in shake flask cultures containing Sf-900 II SFM (Invitrogen) supplemented with 10% fetal calf serum.

Western blotting and silver staining. Cells were lysed in 1× sodium dodecyl sulfate sample buffer and resolved on a 4 to 12% NuPAGE Bis-Tris gel (Invitrogen). After electrophoresis, separated proteins were transferred to a Durapore membrane filter (Millipore, Bedford, MA) and incubated with a primary antibody, either an anti-Rep monoclonal antibody (303.9; Research Diagnostics, Flanders, NJ) at a dilution of 1:200 or a polyclonal anti-type 5 VP antibody raised against a portion of type 5 VP3 polypeptide at a dilution of 1:50,000. The blots

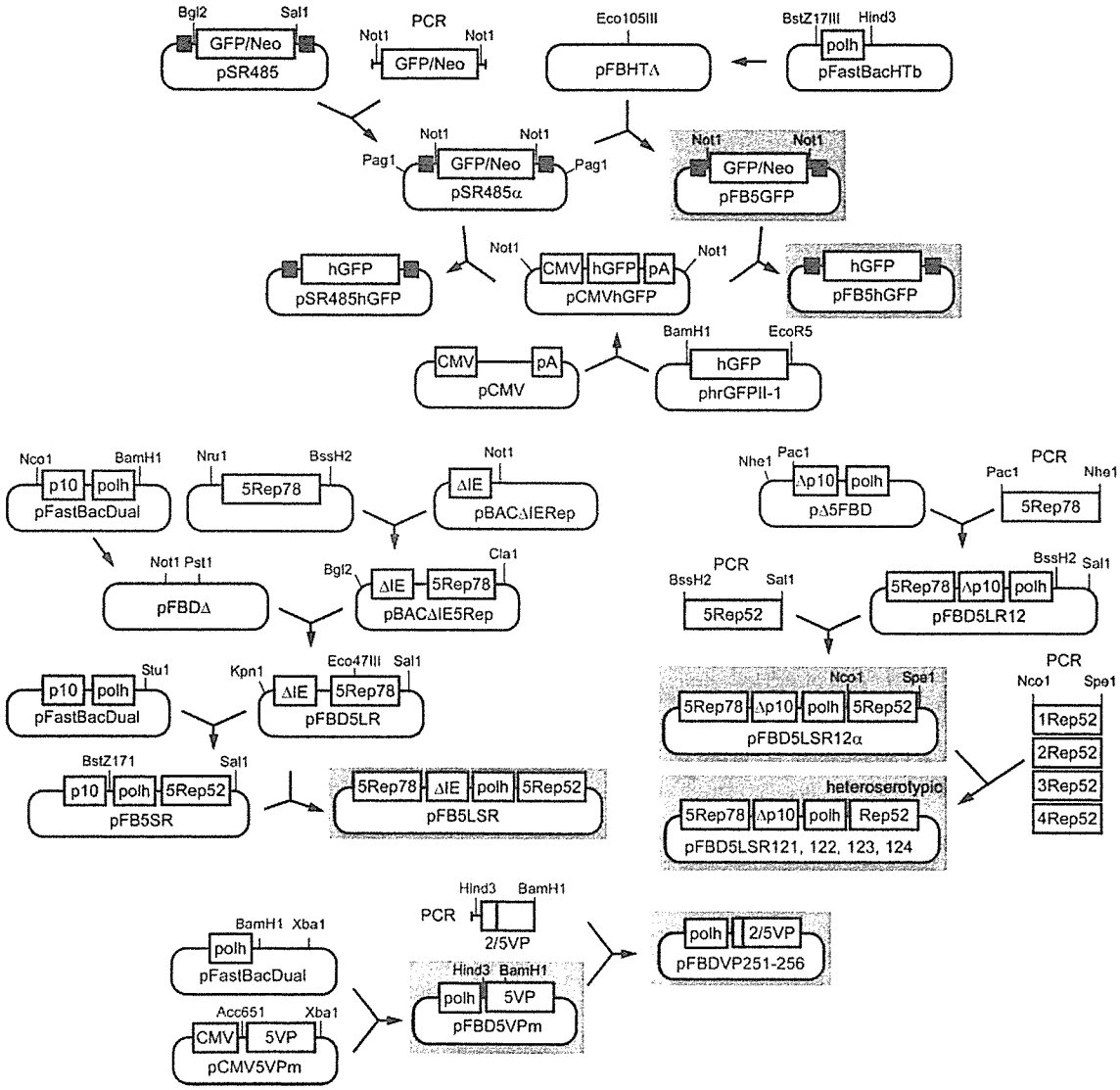

FIG. 1. Flow chart of plasmid construction. See Materials and Methods for details. Plasmids on gray backgrounds were used for generation of recombinant baculovirus vectors. Black boxes, type 5 ITR sequence; p10, p10 promoter; polh, polyhedrin promoter; pA, simian virus 40 polyadenylation sequence.

were then incubated with a secondary anti-mouse or anti-rabbit immunoglobulin G labeled with horseradish peroxidase at a dilution of 1:7,500 (Pierce, Milwaukee, WI). Membranes were incubated in Tris-buffered saline with Tween 20 (TBS-T) (10 mM Tris-HCl [pH 7.6], 0.15 M NaCl, 0.05% Tween 20, 5% nonfat dry milk). Antibodies were added to TBS-T for 1 h. After incubation, membranes were washed three times for 10 min each in TBS-T. All steps were performed at ambient temperature. The development of chemiluminescence catalyzed by horseradish peroxidase was performed according to the manufacturer's instructions (SuperSignal West Pico chemiluminescent substrate; Pierce), and the signals were detected with an X-ray film. Silver staining was performed using a SilverQuest silver staining kit (Invitrogen) according to the manufacturer's instructions.

Analysis of replicated rAAV DNA in Sf9 cells. Sf9 cells (2×10^5 cells per well) in 12-well plates were infected with GFP with or without Rep baculoviruses at a multiplicity of infection (MOI) of 3 and incubated at 27°C for 3 days. After incubation, extrachromosomal DNA was isolated by the method of Hirt (12) and a volume corresponding to 2×10^4 cells was resolved on a 0.8% agarose gel in Tris-borate buffer. Ethidium-stained gel was visualized under UV.

Production of rAAV5 in HEK293 cells. To produce rAAV5-GFP in mammalian cells, HEK293 cells at 80% confluence (approximately 10^5 cells per cm^2) in a 225-cm^2 flask were cotransfected with 27 μg of an AAV vector plasmid and 53 μg pSR487 by the calcium phosphate coprecipitation method. pSR487 harbors type 5 *rep* and *cap* genes and adenovirus E2A, E4orf6, and VA genes (27). Two days after transfection, rAAV5 was purified as described below. For production of pseudotyped type 5 rAAV-SEAP, HEK293 cells were cotransfected with pAAVSEAP; a Rep plasmid expressing type 2 Rep78, Rep68, Rep52, and Rep40; a VP plasmid expressing VP254; and an adenovirus helper plasmid.

Production and purification of rAAV5 in Sf9 cells. Typically, 4×10^8 Sf9 cells (2×10^6 cells per ml) were infected with a Rep baculovirus (RepBac), a VP baculovirus (VPBac), and a GFP baculovirus (GFPBac) with an MOI of 1 per baculovirus construct. To generate pseudotyped 2/5 rAAV-SEAP, Sf9 cells were infected with a RepBac expressing type 2 Rep78 and Rep52, VP254Bac, and SEAPBac. Pseudotype virus refers to the ITRs of one serotype packaged into a capsid derived from a different AAV serotype. For example, rAAV2/5 consists of AAV2 ITRs packaged into an AAV5 capsid. Three days after infection, the cells were pelleted by centrifugation and lysed in a lysis buffer of 20 mM Tris-HCl (pH 8.4), 50 mM NaCl, 2 mM $MgCl_2$, 0.4% deoxycholic acid, 0.5% 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (Merck, Darmstadt, Germany), and 60 U per ml of Benzonase (Merck) and incubated at 37°C for 30 min. The concentration of NaCl in the cell lysate was adjusted to 150 mM and incubated for an additional 30 min. Solid CsCl was added to obtain a final density of 1.36 g/cm^3. After centrifugation at 36,000 rpm for 24 h at 21°C using an SW40 Ti rotor (Beckman, Fullerton, CA), fractions containing rAAV5 were recovered and subjected to a second round of CsCl ultracentrifugation. For some experi-

TABLE 1. Oligonucleotides used for construction of chimeric VP genes

Primer	Sequence[a]
#30	5′-gtcaaagcttcctgttaagAcGGCTGCCGAcGGTTATCTaCCcGA TTGGTTGGAAGAAGTTGGTGAAGGT-3′
#31	5′-GCTGGGATCCGCTGGGTCCAGCTTCGGCGT-3′
#32	5′-gtcaaagcttcctgttaagAcGGCTGCCGAcGGTTATCTaCCcGA TTGGTTGGAGGAC-3′
#33	5′-ACAGCAGGGGTCTTGTGCTGCCTGGTTATAACTA-3′
#34	5′-TAGTTATAACCAGGCAGCACAAGACCCCTGCTGT-3′
#35	5′-GACTCGACAAGGGAGAGCCTGTCAACAGGGCAGA-3′
#36	5′-TCTGCCCTGTTGACAGGCTCTCCCTTGTCGAGTC-3′
#37	5′-GAGACAACCCGTACCTCAAGTACAACCACGCGGA-3′
#38	5′-TCCGCGTGGTTGTACTTGAGGTACGGGTTGTCTC-3′
#39	5′-GAGCAGTCTTCCAGGCGAAGAAAAGGGTTCTCGA-3′
#40	5′-TCGAGAACCCTTTTCTTCGCCTGGAAGACTGCTC-3′
#41	5′-AGGAACCTGTTAAGACGGCCCCTACCGGAAAGCG-3′
#42	5′-CGCTTTCCGGTAGGGGCCGTCTTAACAGGTTCCT-3′

[a] The HindIII or BamHI sites are underlined. The initiation codon for the VP1 gene was mutated to ACG. The possible splicing donor site was destroyed by introducing silent mutations. The VP ORFs are capitalized, and mutated nucleotides are indicated by lowercase letters.

ments, rAAV5 was further purified by anion-exchange column chromatography. CsCl-banded rAAV5 fractions were dialyzed against a buffer of 20 mM Tris-HCl (pH 8.4), 20 mM NaCl, 2 mM $MgCl_2$, and 4% glycerol and loaded onto a HiTrap Q Sepharose XL column (1-ml bed volume; Amersham Biosciences, Piscataway, NJ). Bound rAAV5 was eluted with a 20 to 500 mM linear NaCl gradient. Fractions containing rAAV5 were dialyzed against a buffer of 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, and 5% sorbitol and stored at −80°C until use. The titer of rAAV was determined by real-time PCR with CMV-specific primers 5′-TATGGAGTTCCGCGTTACATAACTTACGGT-3′ and 5′-GACTAATACGTAGATGTACTGCCAAGTAGG-3′ on an HT7000 genetic analyzer (Applied Biosystems, Foster City, CA). Dilutions of pSR485 were employed as a copy number standard.

Competition experiment with a type 2 or type 5 AAV receptor analog. COS cells were plated in a 12-well plate at 30% confluence 24 h prior to infection. rAAV2-GFP or rAAV5-GFP was incubated in 0 or 20 μg per ml of heparin (Sigma-Aldrich), an analog of heparan sulfate proteoglycan (HSPG), for 2 h at room temperature. The cells were infected with adenovirus (3 PFU per cell) at 37°C for 2 h. The cells were washed with medium and then infected with rAAV2-GFP at 10^4 vg per cell or rAAV5-GFP at 10^5 vg per cell. At 24 h postinfection, the cells were visually examined under a fluorescent microscope and the percentages of positive cells were determined by flow cytometric analysis of 10^5 infected cells. Experiments were performed in triplicate. Competition experiments with α2-3 sialic acid were performed as described previously (14). COS cells were plated at 30% confluence 1 day before infection in a 12-well plate. The cells were infected with adenovirus (3 PFU per cell) and incubated at 37°C for 2 h. The adenovirus-containing medium was removed, and the cells were washed with medium. The cells were then infected with rAAV2-GFP (10^4 vg per cell) or rAAV5-GFP (10^5 vg per cell) for 1.5 h in 0 or 0.5 mM 3′-*N*-acetylneuraminyl-*N*-acetyllactosamine (3′-SLN) (Sigma-Aldrich), an analog of α2-3 sialic acid. The cells were washed twice with medium and further incubated for 1 day. The cells were then examined for GFP fluorescence, and the number of positive cells was measured by flow cytometry.

Treatment of cells with neuraminidase. COS cells were infected with adenovirus at 3 PFU per cell for 1 h at 37°C. The cells were treated with 0.08 U per ml of neuraminidase (*Vibrio cholerae*, type III; Sigma-Aldrich) for 1 h and infected with rAAV2-GFP at 10^4 vg per cell or rAAV5-GFP at 10^5 vg per cell for 2 h. The infected cells were then washed twice with medium and incubated for 1 additional day. The GFP-positive cells were counted by flow cytometry. Experiments were done in triplicate.

Muscle injection of rAAV5 in mice. A total of 10^{11} vg of pseudotyped rAAV5-SEAP produced in either 293 cells or Sf9 cells were injected into murine tibialis anterior muscles and blood was taken at the indicated weeks after injection. The serum SEAP activity was measured by a SEAP report gene assay (Roche Diagnostics, GmbH, Penzberg, Germany). The mouse study was approved by a review board at Jichi Medical School.

RESULTS

Construction of recombinant VP and Rep baculoviruses. Production of rAAV2 in insect cells uses three baculovirus vectors providing the following: (i) genes for three AAV structural proteins that form the virus capsid (VP1, VP2, and VP3), (ii) two of the AAV nonstructural proteins for replication and encapsidation (Rep78 and Rep52), and (iii) AAV vector DNA consisting of the gene of interest flanked by the AAV origins of replication (ITRs). In the presence of the AAV nonstructural proteins, the AAV vector DNA is "rescued" from the baculovirus genome and replicates as AAV via the ITRs (31).

Similarly to AAV type 2, the type 5 capsid proteins VP1, VP2, and VP3 are synthesized from two spliced mRNAs arising from the p41 promoter (Fig. 2A) (25). One mRNA is translated into VP1, while another transcript encodes VP2 and VP3. The initiation codon for VP2 is ACG, which is poorly utilized, resulting in the ribosome scanning through to the VP3 initiation codon AUG. The alternate usage of two acceptor sites and the poor utilization of the ACG initiation codon for VP2 are responsible for the 1:1:10 stoichiometry of VP1, VP2, and VP3. As shown in our previous report, the type 2 VP gene with an AAV intron does not express all of the VP polypeptides in insect cells (31). Mutating the VP1 AUG initiation codon to ACG resulted in production of VP1, VP2, and VP3 with a stoichiometry of approximately 1:1:10 from a single transcript without alternate splicing (31). Based on our initial success with AAV2, we constructed a similar type 5 VP baculovirus (VP5Bac) that harbored a type 5 VP gene where the initiation codon for VP1 was changed to ACG (Fig. 2B). Although this VP5Bac was able to produce type 5 capsids into which type 5 AAV vector DNA was incorporated, VP1 was poorly expressed compared to that synthesized in 293 cells (Fig. 2C). The resulting rAAV5-GFP particles poorly transduced COS cells. The calculated ratio of vector genomes to transducing units for the Sf9 cell-produced rAAV5-GFP was 10 times higher than the ratio for the 293 cell-produced counterpart. The VP1 polypeptides have phospholipase A_2 activity and are critical for efficient transfer of the viral genome from late endosomes to the nucleus (36). The efficiency with which a scanning eukaryotic ribosome recognizes an AUG codon for translational initiation is dependent on the local sequence context of the codon. The sequence ACCAUGG is optimal for initiation (18). Residue G at +4 seems particularly important for translation from a non-AUG codon where the A of the AUG codon is defined as +1 (11). In type 2 VP1, the nucleotide at +4 is G while the corresponding nucleotide at +4 in type 5 is U. To increase the efficiency of translation from an ACG codon for type 5 VP1 in insect cells, we tested some VP1 mutants that introduced a G residue at +4. However, these mutants also failed to produce infectious type 5 AAV particles (data not shown). The VP1-unique portion is conserved well among different serotypes compared to the VP3 portion that constitutes the majority of the viral capsids and is responsible for receptor binding specificity. The type 5 VP1-unique portion is approximately 70% identical to the equivalent portion of type 2 (Fig. 3A), while the type 5 VP3 portion is 60% homologous to the equivalent portion of type 2 (2). Since we successfully produced rAAV2 that was as infectious as the 293 cell-produced one, we tested a series of chimeric capsids between types 2 and 5 in which a part of the type 5 VP1-unique portion was replaced by the corresponding portion of type 2 VP1. Figure 3A shows the chimeric VP1 genes constructed. Figure 3B shows the Western analysis of type 5 VP poly-

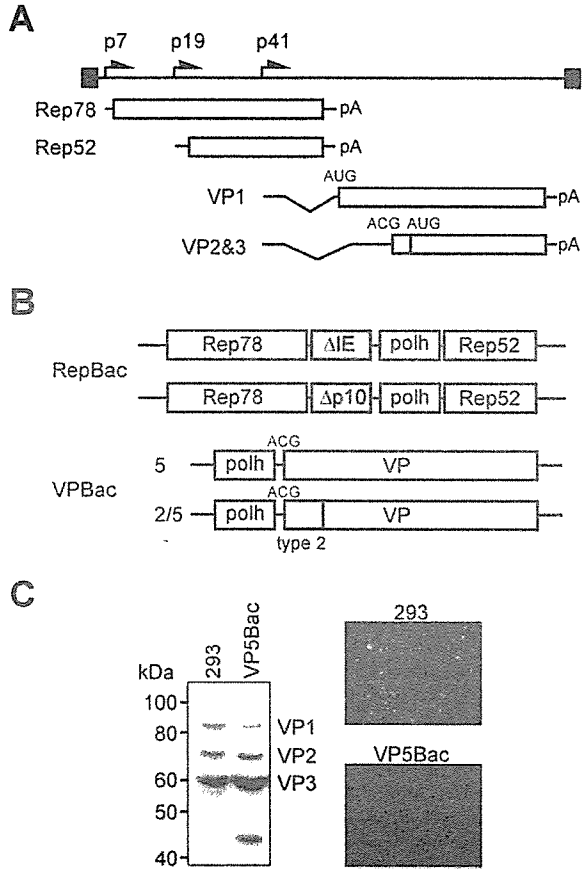

FIG. 2. (A) Genetic and transcriptional map of type 5 AAV. The p7 and p19 promoters drive Rep78 and Rep52, respectively. The p41 promoter transcribes two mRNAs. One expresses VP1, and the other is for VP2 and VP3. The initiation codon for VP2 is ACG, which is poorly utilized for translation, leading to production of a smaller amount of VP2 polypeptides than VP3 polypeptides. The ITRs are indicated by black boxes. pA, polyadenylation signal sequence. (B) Recombinant baculoviruses (rBac) constructed. An rBac for Rep utilizes a truncated promoter for the immediate-early 1 gene of *Orgyia pseudotsugata* nuclear polyhedrosis virus (ΔIE) for type 5 Rep78, and another RepBac expresses Rep78 under the control of a truncated p10 promoter (Δp10). See Fig. 4A for details. Either RepBac uses the polyhedrin promoter (polh) for Rep52. For expression of type 5 capsid proteins, a recombinant baculovirus that harbored a VP gene on which the initiation codon for VP1 is mutated to ACG was constructed (VP5Bac). Another series of VPBacs that had the type 5 VP1 gene partially replaced by the corresponding portion of type 2 VP1 at the N terminus was generated. (C) Western analysis of Sf9 cells infected with VP5Bac. The initiation codon for VP1 was mutated to an ACG codon, which enabled synthesis of VP1, -2, and -3 from a single VP mRNA. The amount of VP1 synthesized was extremely small compared to that in 293 cells. rAAV5-GFP generated with VP5Bac was used for the infection of COS cells at 10^5 vg per cell. The number of GFP-positive cells was 10% of the number of positive cells obtained with rAAV5-GFP produced in 293 cells.

peptides produced with VP251Bac through VP256Bac. Each VPBac produced chimeric VP1 at levels comparable to those of VP2. Formation of empty capsids was confirmed by CsCl density gradient analysis of Sf9 cell lysate infected with VP254Bac, as shown in Fig. 3C. The peak of VP polypeptides came to the fraction of 1.31 g/cm^3, a buoyant density of empty capsids. The GFP gene between the type 5 ITRs could be packaged into each type of chimeric capsid, and all of the chimeric rAAV5-GFPs except VP251 could transduce COS cells with efficiency similar to that of 293 cell-produced rAAV5-GFP (data not shown). The yields of rAAV5-GFP produced with VP253Bac or VP254Bac were approximately 1.2 times higher than others, although the difference was not statistically significant. We thus used VP254Bac to produce rAAV5 for the next experiments.

The initial Rep baculovirus for type 2 rAAV production drove type 2 Rep72 expression with a truncated promoter for the immediate-early 1 gene of *Orgyia pseudotsugata* nuclear polyhedrosis virus (ΔIE) and type 2 Rep52 under the control of the polyhedrin promoter (31) (Fig. 2B). The AAV5 genome encodes nonstructural proteins Rep78 and Rep52 (Fig. 2A). Similarly, we constructed a Rep baculovirus that expressed type 5 Rep78 and Rep52 under the control of the ΔIE promoter and the polyhedrin promoter, respectively. The titers of the type 2 or type 5 Rep baculoviruses, however, were lower than those of other recombinant baculovirus vectors (e.g., VPBac, GFPBac). The immediate-early 1 gene promoter becomes active at the early stage of baculovirus infection, and we thought that early expression of Rep78 in insect cells might negatively affect the yields of recombinant baculoviruses. The very late p10 promoter, which is widely used for recombinant protein production, is active at the latest stage of baculovirus infection. Thus, to delay and suppress the expression of Rep78, we tested a series of truncated p10 promoters. First, we screened the truncated p10 promoters for production of type 2 rAAV and selected one that generated high-titer rAAV2. Figure 4A shows the map of the p10 promoter and the truncated p10 promoter we constructed. The upstream TAAG sequence does not affect the activity of the p10 promoter (32). The sequence between the TAAG sequence and the p10 protein initiation codon at +72 (where the transcription start site is defined as +1) is called the burst sequence and is required for the "burst" of expression of the p10 protein at the very late stage of baculovirus infection. The *vlf-1* transactivator interacts with the burst sequence and strongly stimulates the transcription from the p10 promoter (35). To construct a weak p10 promoter (Δp10), we removed the burst sequence between positions +39 and +72 from the original p10 promoter. The Δp10 promoter was best for the production of rAAV2 among a series of truncated p10 promoters we examined. The titers of recombinant baculoviruses with the Δp10 promoter were comparable to those of other recombinant baculoviruses. The Δp10 promoter was transferred to express type 5 Rep78 (Fig. 2B). Figure 4B compares the time courses of type 5 Rep expression by ΔIE and Δp10 promoters over 72 h after infection, indicating that the Δp10 promoter-driven Rep78 expression was detected at 24 h after infection while the ΔIE promoter expressed Rep78 as early as 12 h after infection. To examine whether this modest difference in the levels of Rep78 affected replication of the AAV vector DNA, we isolated the low-molecular-weight DNA from the Sf9 cells infected with hGFP baculovirus and a Rep baculovirus (Fig. 4C). A ladder of replicative forms (RF) of rAAV5 DNA began to appear at 36 h postinfection in either case. The expected size of rAAV5-hGFP or monomer RF is 2.4 kb and the sizes of dimer and trimer RF are 4.8 and 7.2 kb, which is consistent with the result of the agarose gel electrophoresis.

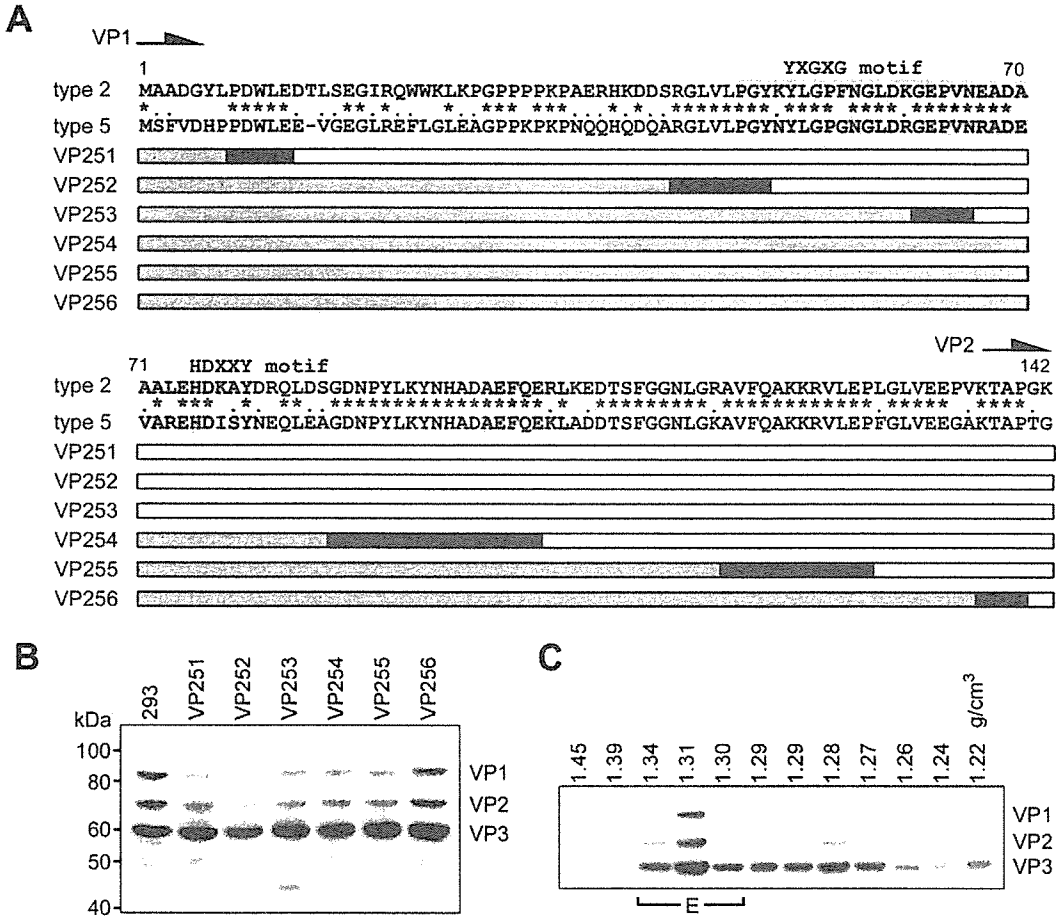

FIG. 3. (A) Chimeric VP genes constructed. The portions derived from type 2 are indicated in gray, while those from type 5 are in white. The common portions are indicated in black. The phospholipase A_2 motifs are shaded. The YXGGX and HDXXY motifs (where X is any amino acid residue), indicating the catalytic site and Ca^{2+} binding loop, respectively, are also shown. The amino acid residues common between type 2 and type 5 are indicated by asterisks. Amino acid residues classified into the same group are indicated by dots. (B) Expression of chimeric VP polypeptides in Sf9 cells. One microgram of cell lysate was resolved onto a 4 to 12% NuPAGE Bis-Tris gel in MOPS (morpholinepropanesulfonic acid) buffer (Invitrogen). Separated proteins were transferred to a Durapore membrane (Millipore), and VP proteins were detected with a rabbit polyclonal antibody raised against the type 5 VP3 portion. The lane labeled 293 shows lysate from HEK 293 cells transfected with pSR487, a hybrid plasmid harboring type 5 AAV *rep* and *cap* genes and adenovirus E2A, VA RNA, and E4orf6 genes (27). Lanes labeled VP251 through VP256 indicate lysates from Sf9 cells infected with recombinant baculovirus expressing chimeric VP. (C) Chimeric VP between types 2 and 5 is able to form empty particles. Sf9 cells (1×10^7 cells) infected with a VP2/5Bac, VP253Bac, were lysed as described in Materials and Methods. Solid CsCl was added to make a buoyant density of 1.30 g/cm^3. After ultracentrifugation for 24 h at 36,000 rpm at 21°C using an SW40 Ti rotor (Beckman), 1-ml fractionations were collected. A portion of each fraction was resolved onto a 4 to 12% NuPAGE gel in MOPS buffer, transferred to a Durapore membrane, and detected with a rabbit anti-type 5 VP polyclonal antibody. The buoyant density of each fraction is indicated above each lane. Fractions that contain empty capsids are indicated by E.

Heteroserotypic small Rep can package rAAV5 DNA into type 5 capsids. The insect cell-based production system for rAAV2 or rAAV1 can generate more than 4×10^4 particles of rAAV per Sf9 cell. However, the yields of rAAV5 produced with either ΔIE or Δp10 RepBac were approximately 1×10^4 to 2×10^4 vg per Sf9 cell. Rep52, or small Rep protein, has been implicated in encapsidation of the AAV genome (17). To establish a high-titer production system, we investigated the use of other serotypes of Rep52 for rAAV5 production. We replaced the type 5 Rep52 with serotype 1, 2, 3, or 4 Rep52 on the Δp10 RepBac. Figure 5A shows the results of Western blotting of Sf9 cells infected with Rep baculoviruses expressing type 5 Rep78 under the control of the Δp10 promoter and serotype 1, 2, 3, 4, or 5 Rep52 driven by the polyhedrin promoter. To generate rAAV5, Sf9 cells were coinfected with hGFPBac, VP254Bac, and a RepBac with the indicated serotype Rep52 at an MOI of 1. Sf9 cells infected with hGFPBac and VP254Bac along with RepBac producing type 1 Rep52 were processed by CsCl density centrifugation, and fractions were analyzed for capsid antigen by Western blotting (Fig. 5B). Two peaks of VP proteins were detected; the higher-buoyant-density peak, from 1.42 to 1.36 g/cm^3, presumably consists of a vector genome containing rAAV5 particles. Another peak, at 1.33 g/cm^3, represents empty capsids, indicating that type 1 Rep52 packaged serotype 5 rAAV DNA into type 5 capsids. When a RepBac that expressed only type 5 Rep78 was used, no rAAV5 particles were produced, confirming that heteroserotypic small Rep indeed packaged type 5 rAAV DNA into type 5 capsids. The cell lysate was loaded directly onto an anion-exchange column, and purified particles were investigated un-

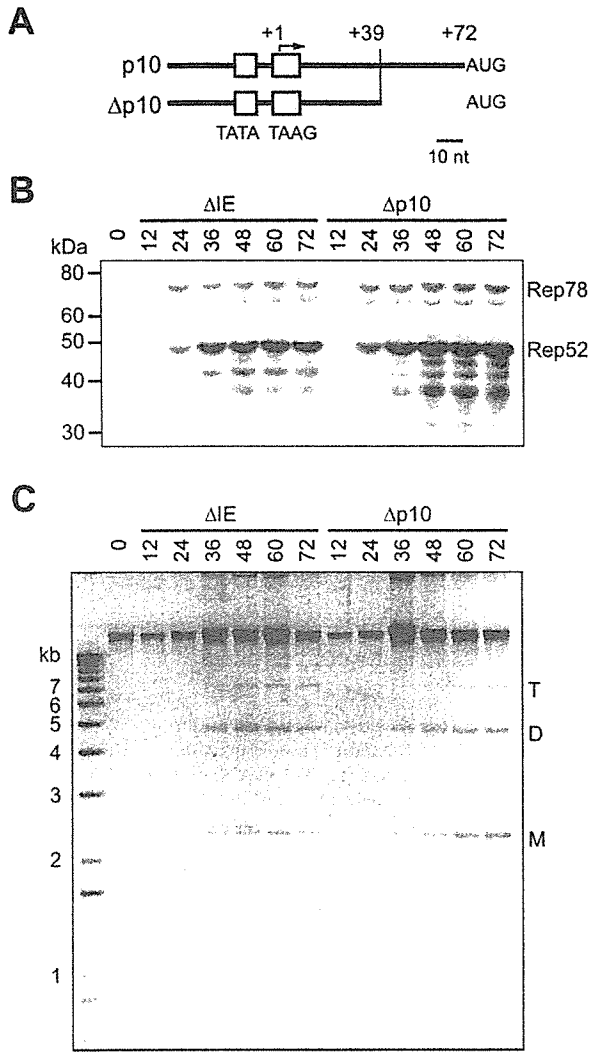

FIG. 4. (A) Map of the Δp10 promoter used for Rep78 expression. The sequence between positions +39 and +72 is deleted in the Δp10 promoter, where the T of the TAAG sequence or the transcription start site (marked with a bent arrow) is defined as +1 and the A of the p10 protein AUG codon is defined as +72. The original AUG codon for the p10 protein was mutated to ACT with pFastBac Dual (Invitrogen). The positions of the TATA box and the TAAG sequence are indicated. (B) Time course of Rep78 expression by ΔIE or Δp10 promoter. Sf9 cells were infected with a Rep baculovirus, and the cells were harvested at the times indicated (in hours) for Western analysis with a monoclonal anti-Rep antibody. (C) Replication of hGFP vector DNA in insect cells. Sf9 cells were coinfected with a Rep baculovirus and an hGFP baculovirus at 1 PFU per cell and incubated for the times indicated (in hours). Low-molecular-weight DNA was isolated, and DNA equivalent to 10^5 cells was resolved onto a 1% agarose gel. T, trimer replicative form; D, dimer; M, monomer.

der electron microscopy, showing typical rAAV particles of a diameter of 20 nm in addition to empty capsids (Fig. 5C). According to the staining pattern, approximately 30% of capsids contained vector genomes. In another experiment, rAAV5-hGFP was purified with two rounds of CsCl ultracentrifugation and the titers of rAAV5-hGFP were determined by real-time PCR using a pair of CMV-specific primers. Figure 5D summarizes the yields of rAAV5-hGFP with the use of different serotypes of small Rep. The titer of rAAV5-GFP produced with type 1, 2, 3, or 4 small Rep was 56,000 ± 3,200 (n = 4), 41,000 ± 18,900 (n = 4), 42,000 ± 7,300 (n = 3), or 39,000 ± 3,500 (n = 3) particles per Sf9 cell, respectively, while that of rAAV5-GFP produced using AAV5 Rep52 was 13,500 ± 3,200 (n = 5). The rAAV5-hGFP particles produced with the indicated serotype Rep52 were further purified by anion-exchange column chromatography, and a total of 3×10^9 vg of either rAAV5-hGFP were then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and examined by silver staining along with 293 cell-produced rAAV5-hGFP (Fig. 5E). Densitometric analysis indicated that the intensities of the VP3 bands were almost equal to one another.

Type 5 vector DNA was packaged into type 5 capsids consisting of chimeric VP1 between types 2 and 5 in the baculovirus system. To examine the possible effect of the chimeric VP1 on packaging of type 5 vector DNA with heteroserotypic Rep52, we tested the production of rAAV5-hGFP by using either Rep5/1Bac or Rep5/5Bac and VP5Bac or VP254Bac. Interestingly, the yields of rAAV5 produced with type 5 Rep52 and type 2/5 chimeric capsids were constantly lower than yields produced with other combinations (Fig. 5F). Type 1 Rep52 was capable of packaging type 5 vector DNA into type 5 capsids and type 2/5 chimeric capsids with similar levels of efficiency. Although the result was not conclusive, the presence of a type 2 VP1-unique portion might interfere with type 5 Rep52 packaging rAAV5 DNA into type 5 capsids in insect cells.

Insect cell-produced rAAV5 infects cells via an α2-3 sialic acid receptor. AAV2 capsids utilize HSPG as a primary coreceptor to infect target cells (30), whereas AAV5 capsids require α2-3 sialic acid for efficient uptake (14). rAAV5 capsids generated in Sf9 cells are composed of VP1 partially replaced with type 2 VP1. The domains involved in receptor binding are within the VP3 portion (16), and the type 2 VP1-unique portion does not appear to be involved in attachment to target cells (19). To determine whether rAAV5 chimeric capsid particles infect cells via sialic acid and not via HSPG, we performed competition experiments with receptor analogs. The results of the heparin competition study show that rAAV2-GFP failed to transduce COS cells in the presence of heparin, an analog of heparan sulfate, as expected (Fig. 6A, top panels). By contrast, rAAV5-GFP produced in 293 cells (Fig. 6A, middle panels) or insect cells (Fig. 6A, bottom panels) was able to express GFP in COS cells irrespective of the presence of heparin, suggesting that Sf9 cell-produced rAAV5-GFP did not utilize HSPG as a primary coreceptor. The number of GFP-expressing cells was counted by flow cytometry, and the percent change in transduction compared to transduction in the absence of heparin was calculated, which clearly corroborated the observation with fluorescent microscopy. We next examined whether insect cell-produced rAAV5-GFP infects cells via α2-3 sialic acid. As shown in Fig. 6B, COS cells were infected with rAAV5 generated in 293 cells (middle panels) or Sf9 cells (bottom panels) in the presence or absence of an analog of α2-3 sialic acid, 3′-SLN. The analog inhibited GFP expression in COS cells by both 293 cell- and Sf9 cell-produced rAAV5-GFP, suggesting that rAAV5-GFP produced in insect cells infected cells via α2-3 sialic acid as did 293 cell-produced rAAV5. To confirm that rAAV5-GFP derived from insect cells utilized sialic acid as a cell attachment receptor, we infected cells denuded of sialic acid by neuraminidase treatment. The

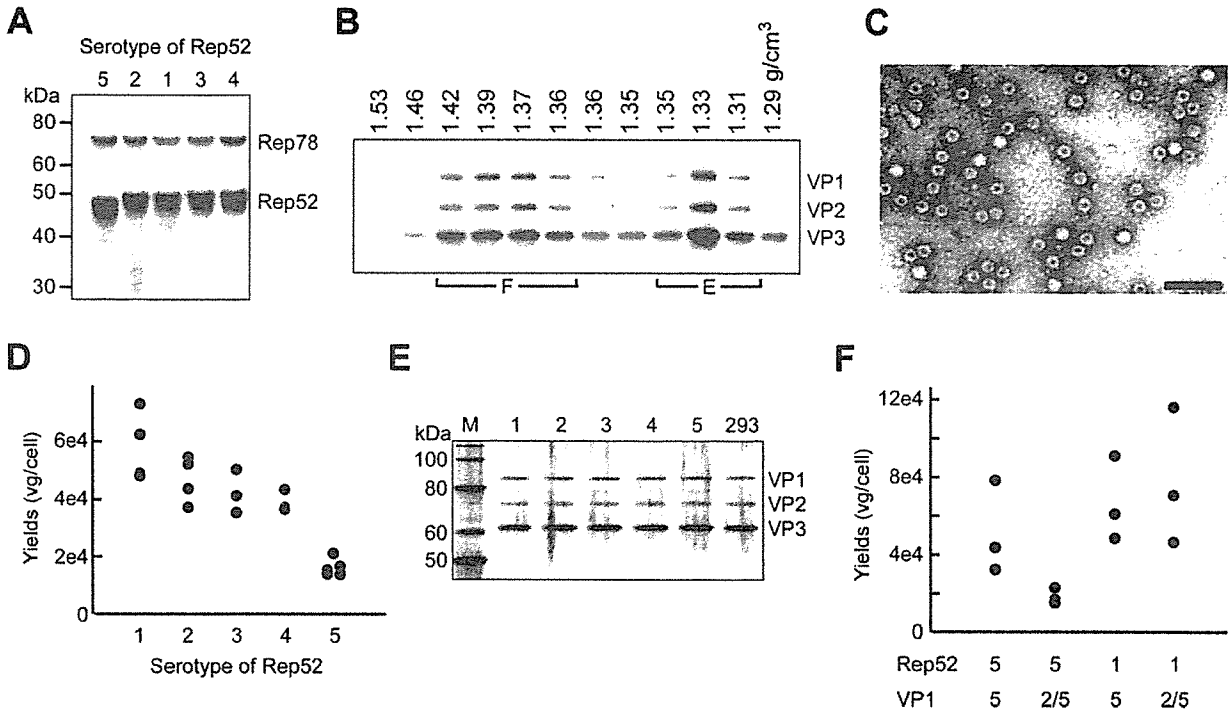

FIG. 5. (A) Western analysis of RepBacs expressing type 5 Rep78 and type 1, 2, 3, 4, or 5 Rep52 with an anti-Rep antibody. (B) Analysis of Sf9 cells coinfected with Rep, VP254, and hGFP baculoviruses by CsCl density gradient ultracentrifugation. Three days after infection, the cells were lysed and subjected to ultracentrifugation. F, filled, or containing rAAV particles; E, empty capsids. (C) Negative staining of rAAV5-hGFP particles purified with ion-exchange column chromatography alone. Particles were stained with 2% uranyl acetate. Magnification, ×100,000. Bar, 100 nm. (D) Generation of rAAV5-hGFP produced with different serotypes of Rep52. The yield of rAAV5-GFP produced with type 1, 2, 3, 4, or 5 small Rep was 56,000 ± 3,200 (n = 4), 41,000 ± 18,900 (n = 4), 42,000 ± 7,300 (n = 3), 39,000 ± 3,500 (n = 3), or 13,500 ± 3,200 (n = 5) particles per Sf9 cell, respectively. (E) Analysis of rAAV5-hGFP produced in insect cells or 293 cells by silver staining. rAAV5-hGFP (3×10^9 particles) produced with serotype 1, 2, 3, 4, or 5 and that produced in 293 cells were resolved onto a 4 to 12% NuPAGE Bis-Tris gel (Invitrogen). Lane M, molecular size markers. (F) Comparison of the yields of rAAV5-GFP produced with type 1 or type 5 Rep52 and VP5Bac or VP2/5Bac. Sf9 cells were coinfected with hGFPBac, Rep5/1Bac or Rep5/5Bac, and VP5Bac or VP254Bac at an MOI of 1 in each of three independent experiments. The rAAV5-hGFP produced was purified by two rounds of CsCl density gradient ultracentrifugation, and the genomic titer was determined by real-time PCR.

result shows that prior incubation with neuraminidase significantly inhibited the transduction of COS cells mediated by rAAV5-GFP produced in 293 cells and Sf9 cells (Fig. 6C).

Comparison of transduction efficiencies with rAAV5 in cultured cells. We next compared the efficacy of rAAV5-GFP/Neo produced in Sf9 cells to that for a mammalian-cell-produced counterpart. COS cells were infected with either Sf9-produced or 293-produced rAAV5-GFP/Neo at 1×10^5 through 1×10^2 vg per cell for 1 day, and the number of GFP-positive cells was counted by flow cytometry. As shown in Fig. 7A, both Sf9-produced and 293-produced rAAV5-GFP/Neo showed similar dose-response curves. In addition, the vector genome-to-transducing unit ratio was calculated based on the number of GFP-positive cells at 3×10^3 vg per cells. Three independently produced samples were examined, and the vector genome-to-transducing unit ratio for Sf9-produced rAAV5-GFP was $3.9 \times 10^4 \pm 1.6 \times 10^4$ (mean ± standard deviation), while the ratio for 293-produced rAAV was $3.6 \times 10^4 \pm 1.2 \times 10^4$. These results indicated that insect cell-generated rAAV5-GFP/Neo had a similar ability to transduce COS cells. Although the capsids produced in Sf9 cells contain type 2/5 chimeric VP1 and those produced in HEK293 cells were composed of original type 5 VP1, rAAV5-GFP/Neo derived from Sf9 cells and that derived from HEK293 cells did not show any significant difference in GFP expression in COS cells, suggesting that the difference in the VP1-unique portion did not impact the expression of the transgene or affect the intracellular processing of type 5 capsids in COS cells. We also compared transduction efficiencies of rAAV5-hGFP generated in Sf9 cells and rAAV5-hGFP generated in HEK293 cells. Surprisingly, the dose-response curve obtained by Sf9-produced rAAV5-hGFP shifted to the right and the number of GFP-positive cells at the dose of 3×10^3 vg per cell was five times larger than that for 293-produced rAAV5-hGFP (Fig. 7B). Since the substitution of the type 5 VP1-unique portion with the equivalent portion of type 2 did not impact the GFP expression in COS cells (Fig. 7A), we explored the rAAV genomes packaged into vector capsids. Virion DNA was isolated and analyzed on an alkaline gel. After electrophoresis, the DNA was transferred to a nylon membrane and hybridized with a ^{32}P-labeled CMV-specific probe. The GFP/Neo DNA packaged into AAV5 capsids is essentially the same in size and amount as expected (Fig. 7C). We next analyzed virion DNA isolated from rAAV5-hGFP produced with the indicated serotype Rep52 in insect cells, as well as 293-produced rAAV5-hGFP (Fig. 7D). The encapsidated hGFP DNA is present as

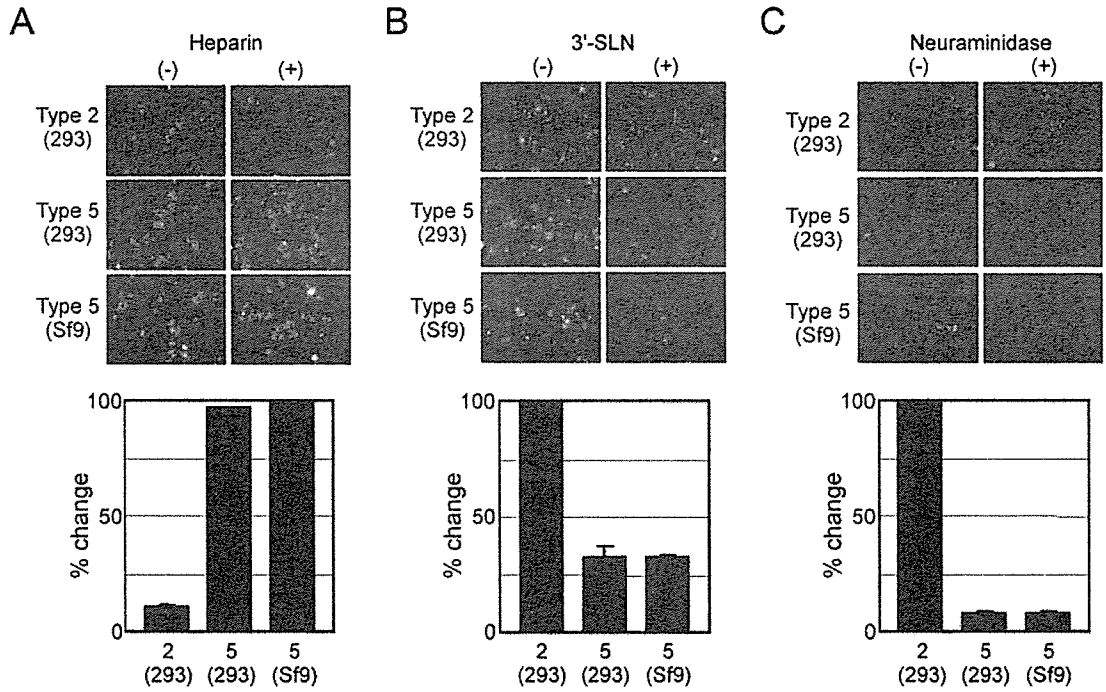

FIG. 6. (A) Heparin, an analog of HSPG, does not inhibit transduction of COS cells infected with rAAV5-GFP/Neo produced in insect cells. Cells were infected with adenovirus at 3 PFU per cell and incubated at 37°C for 2 h. After being washed with medium, the cells were infected with rAAV2-GFP/Neo produced in 293 cells at 10^4 vg per cell or rAAV5-GFP/Neo generated in 293 cells or Sf9 cells at 10^5 vg per cell in the presence or absence of 20 μg per ml of heparin in triplicate. One day after infection, the cells were observed under a fluorescent microscope. The number of GFP-positive cells was also counted by flow cytometry. Data are presented as percent change in transduction compared to transduction in the absence of heparin. (B) An analog of α2-3 sialic acid inhibits both 293 cell- and Sf9 cell-produced rAAV5-GFP/Neo. COS cells were infected with an adenovirus at 3 PFU per cell and incubated at 37°C for 2 h. After being washed with medium, the cells were infected with rAAV2-GFP/Neo at 10^4 vg per cell or rAAV5-GFP at 10^5 vg per cell for 1.5 h in the presence of 0 or 0.5 mM 3′-SLN (Sigma-Aldrich). The cells were washed twice with medium and further incubated for 1 day. The number of GFP-expressing cells was measured by flow cytometry (n = 3). Data are presented as percent change in transduction compared to transduction in the absence of the analog. (C) Neuraminidase treatment of COS cells inhibits transduction with rAAV5-GFP generated in 293 cells or Sf9 cells. COS cells were infected in triplicate with adenovirus at 3 PFU per cell for 1 h at 37°C, treated with 0.08 U per ml of neuraminidase (*Vibrio cholerae*, type III; Sigma-Aldrich) for 1 h, and infected with 10^5 vg per cell of rAAV5-GFP produced in 293 cells or Sf9 cells for 2 h. COS cells were similarly treated and also infected with 10^4 vg per cell of rAAV2-GFP/Neo. The infected cells were then washed twice with medium and incubated for an additional day. After incubation, the cells were observed under fluorescent microscopy and the number of GFP-positive cells was counted by flow cytometry.

two DNA species. The higher-mobility virion DNA corresponds with 2.4-kb hGFP vector DNA or a single-stranded monomer, which is confirmed by comigration with a 2.4-kb vector DNA obtained by treatment with a restriction enzyme of the hGFP vector plasmid, pSR485hGFP. The lower-mobility DNA is the same in size as the monomer RF or duplex form of hGFP DNA (Fig. 7D) isolated from Sf9 cells coinfected with RepBac and hGFPBac (Fig. 4C). The intensity of the larger virion DNA, which was quantified with an imaging analyzer, was roughly double that of shorter DNA for each rAAV5 produced in Sf9 cells. If the larger virion DNA is a monomer duplex form and thus has two CMV promoter sequences hybridizing to a CMV probe, then we estimated that the quantity of the double-stranded monomeric form was equal to that of the single-stranded monomer. The ratio of the amount of the monomer duplex form to the amount of the single-stranded monomer form in the rAAV5-hGFP virion produced in 293 cells is 1 to 3.5. AAV particles have been shown to package two copies of vector genomes that are less than 50% of the 4.8-kb AAV genome, and the packaged vector DNA appeared to be monomeric single-stranded and double-stranded RF (6). For gene expression, the single-stranded vector genome has to be converted to a double-stranded form by either second-strand synthesis (8, 9) or annealing of complementary strands (23). The monomeric duplex vector DNA, on the other hand, can function directly as a template for mRNA synthesis. Thus, the more potent gene expression mediated by rAAV5-hGFP generated in Sf9 cells is probably due to the presence of the encapsidated monomer duplex form.

Comparison of efficacies of rAAV5 in vivo. To compare the efficacies of rAAV5 produced in 293 cells and rAAV5 produced in Sf9 cells, we constructed a type 5 vector that expressed human SEAP. rAAV5 particles produced in Sf9 cells consisted of chimeric VP1 between type 2 and type 5. To eliminate the possible difference in intracellular processing of rAAV5 particles due to replacement of the type 5 VP1-unique portion with the equivalent one of type 2, we compared the in vivo activities of rAAV5 particles containing type 2/5 VP1 polypeptides produced in insect and mammalian cells. Five mice each intramuscularly received a total of 10^{11} vg of rAAV5-SEAP generated in either 293 cells or Sf9 cells, and serum SEAP levels were monitored. As shown in Fig. 8A, the expression profile of the Sf9-produced type 5 SEAP vector differed from that of the 293-produced one. The rAAV5-SEAP

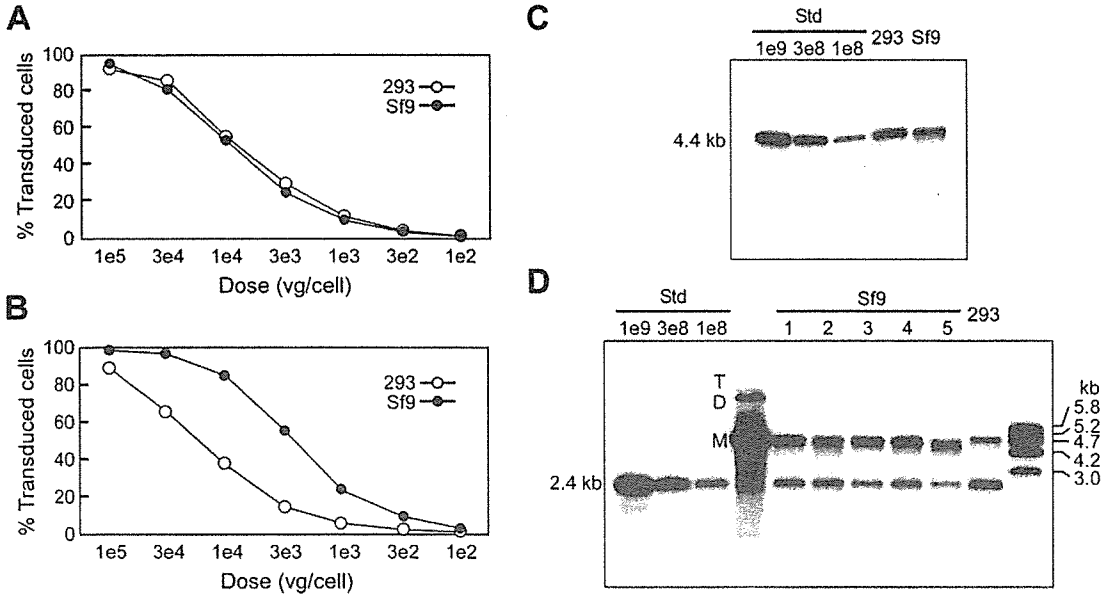

FIG. 7. Comparison of infectivities of rAAV5 produced in 293 cells and rAAV5 produced in Sf9 cells. (A) COS cells were infected with rAAV5-GFP/Neo produced in 293 cells or Sf9 cells at the doses indicated, ranging from 1×10^5 through 1×10^2 vg per cell. One day after infection, the cells were examined for GFP fluorescence by flow cytometry in triplicate. (B) COS cells were infected with rAAV5-hGFP produced in HEK293 cells or Sf9 cells at a dose of 1×10^5 through 1×10^2 vg per cell for 1 day. The number of GFP-positive cells was counted cytometrically. (C) Analysis of GFP/Neo vector virion DNA on an alkaline gel. Virion DNA was isolated from rAAV5-GFP/Neo produced in HEK293 cells or Sf9 cells by treatment with proteinase K, and samples equivalent to 3×10^8 vg were resolved onto a 0.8% alkaline gel. The DNA was then transferred to a nylon membrane and hybridized to a ^{32}P-labeled CMV-specific probe. A 4.4-kb-size copy number standard (Std) (1×10^9, 3×10^8, and 1×10^8 copies) was loaded, which was derived from the GFP/Neo vector plasmid, pSR485α, with a restriction enzyme that cut out the vector portion. (D) Alkaline agarose gel electrophoresis of virion DNA isolated from rAAV5-hGFP. Vector DNA isolated from rAAV5-hGFP particles produced with type 1, 2, 3, 4, or 5 Rep52 was analyzed along with 293-produced rAAV5-hGFP. Low-molecular-weight DNA isolated from insect cells infected with RepBac and hGFPBac (Fig. 4C) serves as a reference for monomer (M), dimer (D), and trimer (T) replicative forms.

generated in HEK293 cells showed a gradual increase in serum SEAP activity over 1 month after injection, which is a typical expression pattern by rAAV-mediated transduction. The Sf9-produced rAAV5-SEAP induced levels of SEAP activity at 1 or 2 weeks after injection that were more than 30 or 10 times higher, respectively, than those of the 293-produced rAAV5-SEAP, and the serum SEAP activity by Sf9 produced rAAV5-SEAP decreased at 4 weeks after injection. There was no significant difference between the two groups after 4 weeks following administration. We also analyzed the SEAP vector DNA on an alkaline gel (Fig. 8B). The expected size of rAAV5-SEAP vector genomes is 3.4 kb. The majority of 293-produced rAAV5-SEAP DNA is single-stranded monomer in both type 5 capsids and type 2/5 chimeric capsids. In addition to the 3.4-kb single-stranded vector genome, DNA extracted from Sf9 cell-produced rAAV5 particles contained an additional DNA of approximately 4.7 kb. One model for AAV packaging proposes that when the size of vector DNA is larger than the size of the wild-type AAV, 4.7 kb, the vector DNA is cleaved to 100% of the AAV genome during packaging into virion (6). The 4.7-kb virion DNA may be a cleaved product of duplex multimers synthesized in Sf9 cells.

DISCUSSION

Recent advances in understanding of biology of AAV and in production of rAAV have facilitated the use of rAAV as a gene transfer vector. A human clinical trial with rAAV2 expressing a coagulation factor IX has shown that intramuscular delivery of more than 10^{15} rAAV2 particles would be required for amelioration of hemophilia B (15). Currently, the widely employed method for production of rAAV is transfection of packaging cells, such as HEK293 cells, with plasmids carrying AAV and adenovirus genes. Plasmid transfection is more easily adaptable to packaging different serotype AAV vectors than establishing a packaging cell line. However, the transfection process is the limiting step in rAAV production, which requires adherent HEK293 cells on a two-dimensional surface for efficient production of rAAV.

The production of other AAV serotype-derived vectors has been described previously (26) and follows the strategy developed for rAAV2 (20). Some modifications have been reported, such as lipofection of 293 cells in suspension culture in serum-free media, which makes the handling of the cells and the purification step easier (28). However, the use of a lipid reagent for transfection may be neither cost-effective nor scalable. A recombinant herpes simplex virus harboring type 5 *rep* and *cap* genes was created to eliminate the transfection process (33), although the yields of rAAV5 were low. The baculovirus/insect cell-based rAAV5 production system presented here does not require plasmid transfection and is scalable. By extrapolation from culture volume, we expect to obtain more than 10^{14} particles of rAAV5-GFP from a 1-liter culture. This is consistent with yields of rAAV1 or rAAV2 produced in Sf9 cell cultures (20a, 31).

To produce infectious rAAV5 particles in insect cells, we

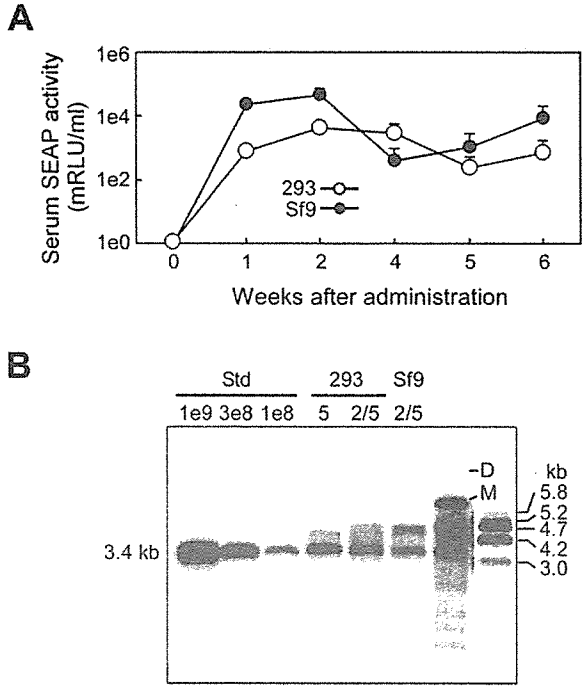

FIG. 8. (A) Serum SEAP activity following intramuscular injection of rAAV5-SEAP. Five mice each received a total of 10^{11} vg of pseudotyped rAAV5-SEAP produced in 293 cells or Sf9 cells into tibialis anterior muscles. Blood was taken at the indicated weeks after injection. The serum SEAP activity was measured by a SEAP report gene assay (Roche Diagnostics, GmbH, Penzberg, Germany). RLU, relative light units. (B) Molecular analysis of SEAP vector DNA. Vector genomes were isolated from type 5 or type 2/5 SEAP vector particles produced in HEK293 cells or from Sf9-produced rAAV5-SEAP. Extrachromosomal low-molecular-weight DNA isolated from Sf9 cells infected with RepBac and SEAPBac was also analyzed. Copies (10^9 through 10^8) of copy number standard (Std) vector DNA derived from SEAP vector plasmid, pAAVSEAP, were also loaded. M, monomer replicative form of SEAP vector genomes; D, dimer replicative form.

inserted an N-terminal portion of type 2 VP1 into the corresponding site of type 5 VP1. The N termini of VP1 polypeptides contain the phospholipase A_2 motif and are essential to viral infectivity (36). Electron microscopy indicated that the VP1-unique portion is hidden within type 2 capsids and appears on the surface of the capsids during the infectious pathway in cells (19). The VP1-unique portion is well conserved among different AAVs. Comparison of the portion among serotypes 1 through 4 and 6 revealed that one serotype is more than 80% identical to another. The type 5 VP1-unique portion is 70 to 75% identical to that of other serotypes, while the sequence alignment of VP2 or VP3 of AAV1 through AAV6 showed that type 5 is approximately 55% identical to other serotypes. The initial trial mutation of the start codon for type 5 VP1 gene to ACG failed to produce infectious rAAV5 particles due to low synthesis of VP1 polypeptide (Fig. 2C). However, the successful generation of rAAV2 particles in insect cells and the notion that the VP1-specific region is well conserved among different serotypes led us to construct a chimeric type 5 VP1 polypeptide whose N-terminal portion was partially replaced by the equivalent portion of type 2. The transduction of COS cells and mouse muscles with rAAV5 produced in insect cells clearly indicated that the chimeric VP1, VP254, could confer infectivity to it (Fig. 7 and 8).

The strategy of producing AAV "pseudotyped" vectors, typically consisting of AAV2 ITR and non-AAV2 capsids, such as AAV4 and AAV5, has been reported previously (2, 3, 26, 34). We first tested similar pseudopackaging of rAAV DNA type 2 ITRs into type 5 capsids with type 2 RepBac in insect cells. However, the yields of vector particles produced were four times lower than those reached by packaging type 5 DNA into type 5 capsids (data not shown). We also examined the production of rAAV5 by packaging type 2 AAV DNA with type 2 Rep78 and type 5 Rep52 into type 5 capsids, which also resulted in low yields of rAAV5 (data not shown). The production of type 5 vector in 293 cells by transfection with a type 5 vector plasmid and a type 5 *rep cap* plasmid usually yields more than 10^4 particles per HEK293 cell, and the production of pseudotyped type 5 vectors by using a type 2 AAV vector plasmid and type 2 *rep* and type 5 *cap* plasmid recovers 3×10^3 particles per cell (unpublished observation), an observation consistent with the production in Sf9 cells.

Using Sf9 cells, we found that Rep52 proteins of other serotypes were capable of packaging DNA with type 5 ITRs into type 5 capsids more efficiently than type 5 Rep52. Type 2 small Rep protein has been shown to package the AAV2 genome into preformed capsid with its helicase activity in collaboration with large Rep protein (7, 17). The small Rep protein associates with Rep78/68 (24) and probably specifically interacts with large Rep protein during encapsidation (7). The basis for the improved AAV packaging with non-type 5 Rep52 remains to be elucidated. To exclude the possibility that cellular proteins and/or baculovirus proteins played a major role in packaging type 5 DNA, we used a RepBac that expressed only type 5 Rep78 for production of type 5 rAAV. No rAAV5 particles were recovered from the recombinant baculovirus-infected Sf9 cells (data not shown), suggesting that the small Rep protein is absolutely required for generating rAAV5 in insect cells. As shown in Fig. 5F, the fact that the partial replacement of the VP1-unique portion with the corresponding portion of type 2, the strategy we took to generate infectious type 5 particles in insect cells, inhibited type 5 Rep52-mediated introduction of type 5 ITR genomes into type 5 capsids may only indicate the role of the type 2 VP1-unique portion as a physical barrier during packaging of rAAV genomes into capsids. We believe that under a special circumstance, such as in invertebrate cells, heteroserotypic Rep52 is superior to type 5 Rep52 in packaging rAAV DNA with type 5 ITR into type 5 capsids. It is interesting to examine whether other serotypes of Rep52 can package type 5 rAAV DNA into type 5 capsid in mammalian cells. We are currently investigating the packaging of type 5 genome into type 5 capsids with different serotypes of Rep52 in HEK293 cells.

The majority of the vector genome of rAAV5 produced in HEK293 cells in the present study is in single-stranded monomeric form, irrespective of the size of the vector genome (Fig. 7C and D and 8B). However, when the size of vector DNA is shorter than the size of the wild-type AAV genome, insect cells tend to package longer, 4.7-kb DNA into type 5 capsids. The 4.7-kb longer virion DNA in Sf9-produced rAAV5 appears to be a cleavage product of multimers of replicated vector genomes. If the size of a multimer is within the packaging limit,

it is efficiently introduced into AAV capsids. If a multimer is larger than 4.8 kb in size, a partially truncated multimer is packaged into AAV capsids in insect cells (6). Sequencing of 4.7-kb DNA packaged into virions will be a key to disclosing the difference between packaging of vector DNA into capsids in HEK293 cells and insect cells. The difference in packaged virion DNA between rAAV5 produced in human cells and in insect cells provides important information on designing vector DNA for production of rAAV5 in insect cells.

In summary, we developed a new method for production of rAAV5 in insect cells, which offers a better alternative to the existing production methods of rAAV5, although the vector genomes packaged into capsids differ in size from rAAV5 produced in HEK293 cells. The robust generation in suspension culture will facilitate the use of type 5 rAAV not only for basic studies but also for clinical studies.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Health, Welfare, and Labor of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Technology of Japan, and High-Tech Research Center Project for private universities (matching-fund subsidy from the Ministry of Education, Science, Sports, and Technology of Japan). This research was also supported in part by the Intramural Research Program of the NHLBI, NIH.

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