active gassing [20]. The physical titer of the viral stock was determined by dot-blot hybridization with plasmid standards.

HDAC inhibitors. The HDAC inhibitor FR901228 (obtained from Fujisawa Pharmaceutical Co., Ltd.) is a depsipeptide fermentation product from *Chromobacterium violaceum* [21]. FR901228 strongly inhibits the proliferation of tumor cells by arresting cell cycle transition and is now being tested in clinical trials [22]. FR901464 (obtained from Fujisawa Pharmaceutical Co., Ltd.) and TSA (Sigma–Aldrich Corp., St. Louis, MO, USA) are also prepared as HDAC inhibitors [21].

Cells and culture. The malignant human glioma cell line U251MG, the malignant rat glioma cell line 9L, the laryngeal epidermoid carcinoma cell line HEp-2, and the human maxillary sinus cancer cell line NKO-1 were used in this study. Cells were cultured in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2. Human embryonic kidney 293 cells were cultured with D-MEM:F12 (1:1 mixture) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2. Luciferase assay was performed on the luminometer (Fluoroskan Ascent FL, Thermo Labsystems, Beverly, MA, USA) using the Bright-Glo Reagent kit (Promega, Madison, WI, USA).

FACS analysis. Approximately 5×10^4 cells were analyzed on the FACScan (Becton–Dickinson, San Jose, CA, USA) with CellQuest software (Becton–Dickinson). Cells were incubated with a PE-labeled monoclonal antibody (13C2) specific for human integrin v chain (CD51; Cymbus Biotechnology Ltd., Chandlers Ford, UK) for 30 min on ice. The 7-aminoactinomycin-D (Via-Probe; Pharmingen, San Diego, CA, USA)-negative cell fraction, which contains the viable cells, was used to detect EGFP- and/or PE-positive cells.

Western blot analysis. Detection of histone acetylation by FR901228 in U251MG cells was performed as described [7]. Western blot analysis of the cells incubated in the presence or absence of FR901228 for 24 h was performed using either a rabbit polyclonal antibody against histone H3 or one against acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:2000 in 5% milk. The probed membrane was incubated with an anti-rabbit immunoglobulin horseradish peroxidase-linked antibody and developed by ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Determination of transgene copy number. Tumor cells were infected with 1×10^4 genome copies/cell of rAAV in the presence of FR901228. The high-molecular-weight DNA was extracted from the cells (DNA Extraction Kit; Qiagen, Inc., Hilden, Germany) 0, 2, 4, 12, and 24 h later. The copy numbers were determined by quantitative PCR analysis of 100 ng of the DNA by using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) as described in the supplementary information.

mRNA analysis of coreceptors for the AAV. U251MG cells were incubated with recombinant AAV either alone (1×10^4 genome copies/cell) or together with FR901228 (0.3 or 3 ng/ml) for 24 h. mRNA was isolated from the cell culture using an RNeasy mini kit (Qiagen) and reverse-transcribed into a single-stranded cDNA using the SuperScript Preamplification System (Invitrogen, Carlsbad, CA, USA). FGF-R1 or PDGF-R mRNA was quantitated by real-time PCR as described in the supplementary information.

PCR analysis of immunoprecipitated DNA. Chromatin immunoprecipitation was performed following the Upstate Biotechnology ChIP kit protocol. U251MG cells were transduced with AAV vector at 1×10^4 genome copies/cell, pCMV-EGFP, or pAAV2EGFP in the presence or absence of the 1 ng/ml FR901228. Twenty-four hours after the transduction, chromatin proteins of interest were cross-linked to DNA. After preclearing, isotype-antibody control or anti-acetylated histone H3 or anti-histone H3 antibody (Upstate Biotechnology) was added to the sonicated chromatin solution and incubated overnight at 4°C with agitation. Resulting immune complexes were collected by the salmon sperm DNA–protein A agarose slurry. The eluted samples were treated with proteinase K and purified by phenol/chloroform extraction. Precipitated DNAs were analyzed for the vector-derived promoter by quantitative PCR with an ABI Prism 7700 sequence detection system as described in the supplementary information.

In vivo analysis of enhanced transgene expression. U251MG cells were treated with PBS ($n = 3$) or transduced with a recombinant AAV2 expressing luciferase (AAV2Luc) at 1×10^4 genome copies/cell for 1 h ($n = 5$), and then 3×10^6 of the transduced cells in 100 μl PBS containing 25% (v/v) basement membrane matrix (Matrigel; BD Biosciences, Franklin Lakes, NJ, USA) were inoculated subcutaneously into male BALB/c *nu/nu* mice (Clea Japan, Tokyo, Japan) along with intraperitoneal injection of FR901228 at 1 mg/kg or the same volume of vehicle. Twenty-four hours after the administration of FR901228, optical bioluminescence imaging was performed using the CCD camera (Xenogen Corp., Alameda, CA, USA). After intraperitoneal injection of reporter substrate D-luciferin (375 mg/kg body wt), mice were imaged for scans.

To analyze the effect of FR901228 on the enhanced tumor elimination *in vivo*, 9L tumor cells were transduced with an AAV5TK at 1×10^4 genome copies/cell for 1 h, and then 3×10^6 of the transduced cells in 100 μl PBS containing 25% (v/v) Matrigel were inoculated subcutaneously into BALB/c mice. The tumor-bearing animals received an intraperitoneal injection of FR901228 at 3 mg/kg (group 1, $n = 6$; group 3, $n = 10$) or PBS (group 2, $n = 6$). The animals were also exposed to ganciclovir at 100 mg/kg per day (groups 2 and 3) or PBS (group 1) for 14 consecutive days by intraperitoneal placement of the miniosmotic pumps (Alzet, Palo Alto, CA, USA) according to the manufacturer's instructions. Tumor growth was monitored two to three times a week by measuring two perpendicular tumor diameters using calipers and the volumes were calculated as $a \times b^2 \times 0.5$, where a is the length and b is the width of the tumor in millimeters. Animals with tumors larger than 2 cm in diameter were euthanized.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymthe.2005.11.010.

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

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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′-gatccATGGAGCTGGTCGGGTGGCTGGTGGA-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

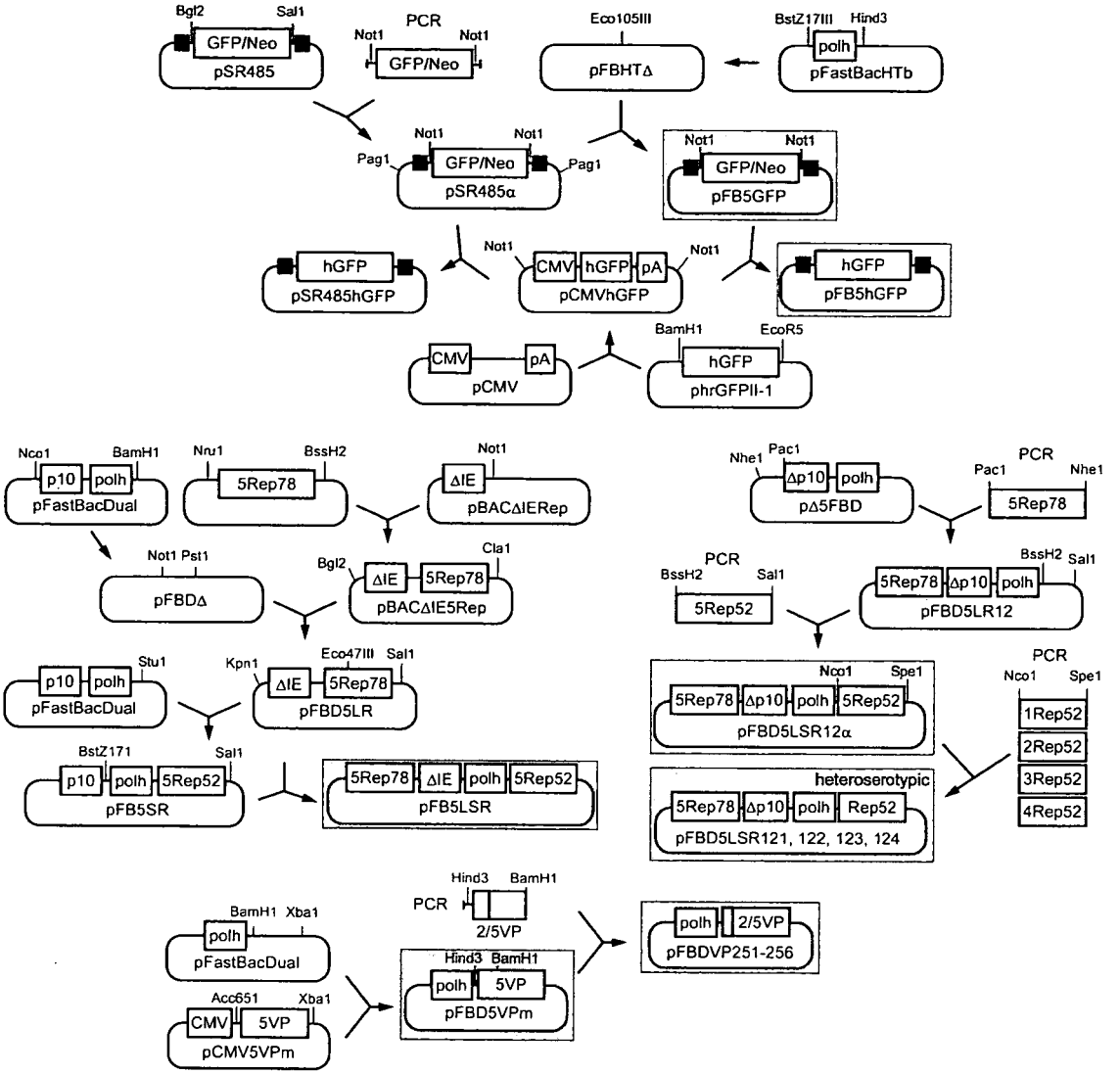

FIG. 1. Flow chart of plasmid construction. See Materials and Methods for details. Plasmids on gray backgrounds were used for generation of recombinant baculovirus vectors. Black boxes, type 5 ITR sequence; p10, p10 promoter; polh, polyhedrin promoter; pA, simian virus 40 polyadenylation sequence.

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]-l-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-

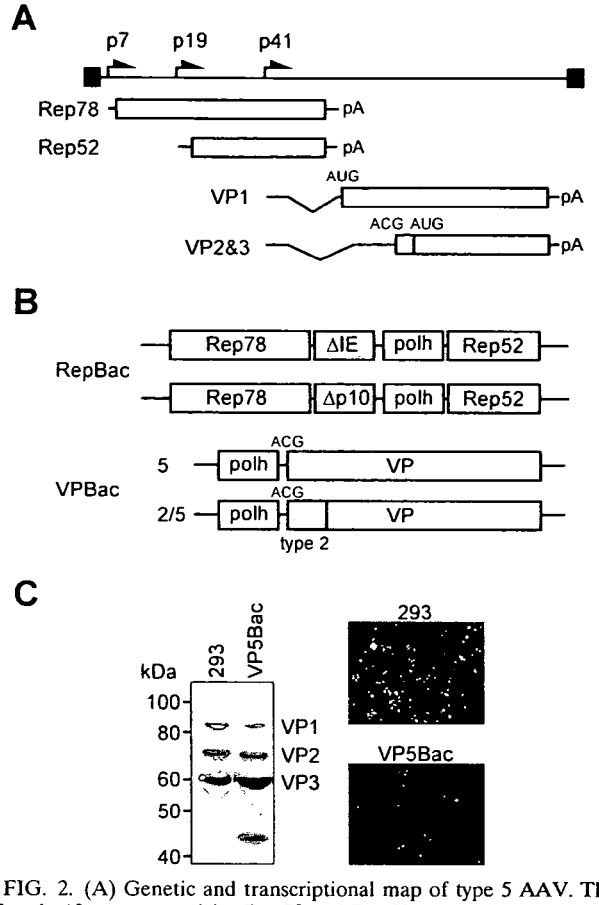

FIG. 2. (A) Genetic and transcriptional map of type 5 AAV. The p7 and p19 promoters drive Rep78 and Rep52, respectively. The p41 promoter transcribes two mRNAs. One expresses VP1, and the other is for VP2 and VP3. The initiation codon for VP2 is ACG, which is poorly utilized for translation, leading 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.

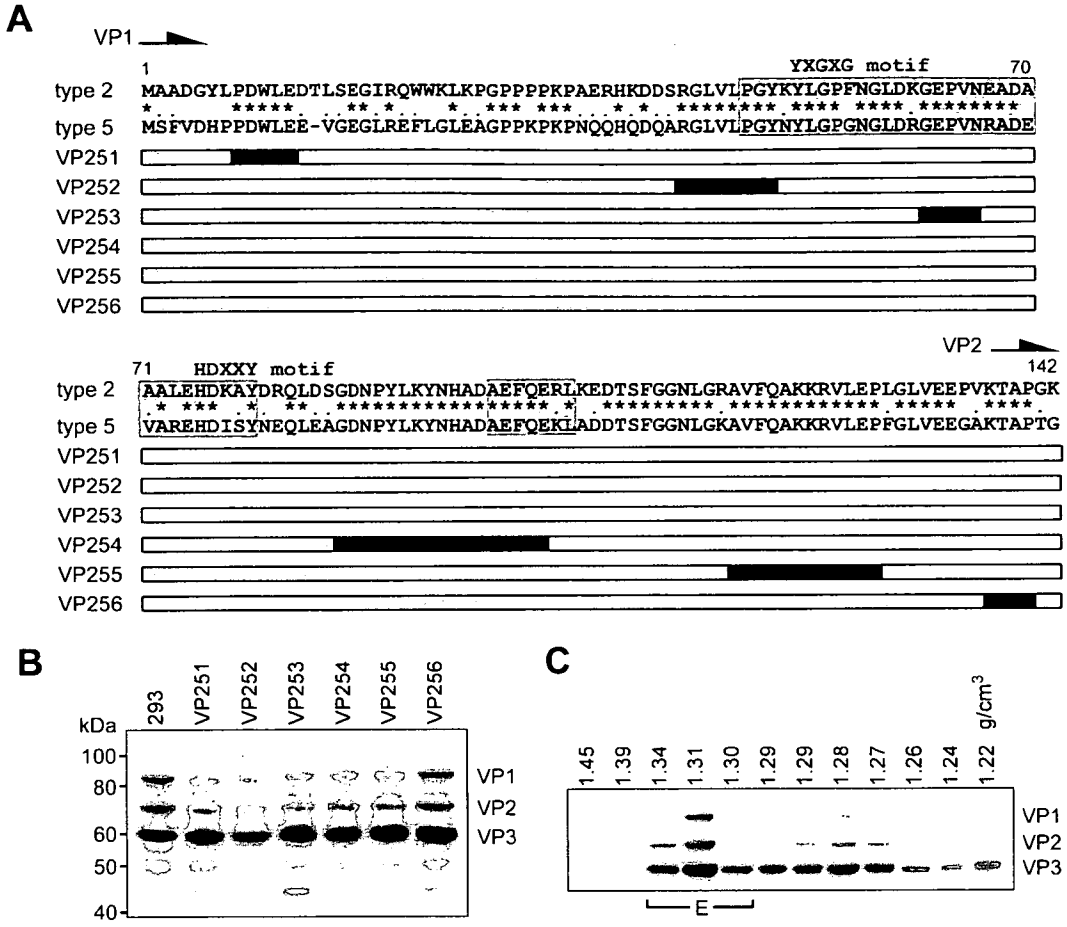

FIG. 3. (A) Chimeric VP genes constructed. The portions derived from type 2 are indicated in gray, while those from type 5 are in white. The common portions are indicated in black. The phospholipase A_2 motifs are shaded. The YXGGX and HDXXY motifs (where X is any amino acid residue), indicating the 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-

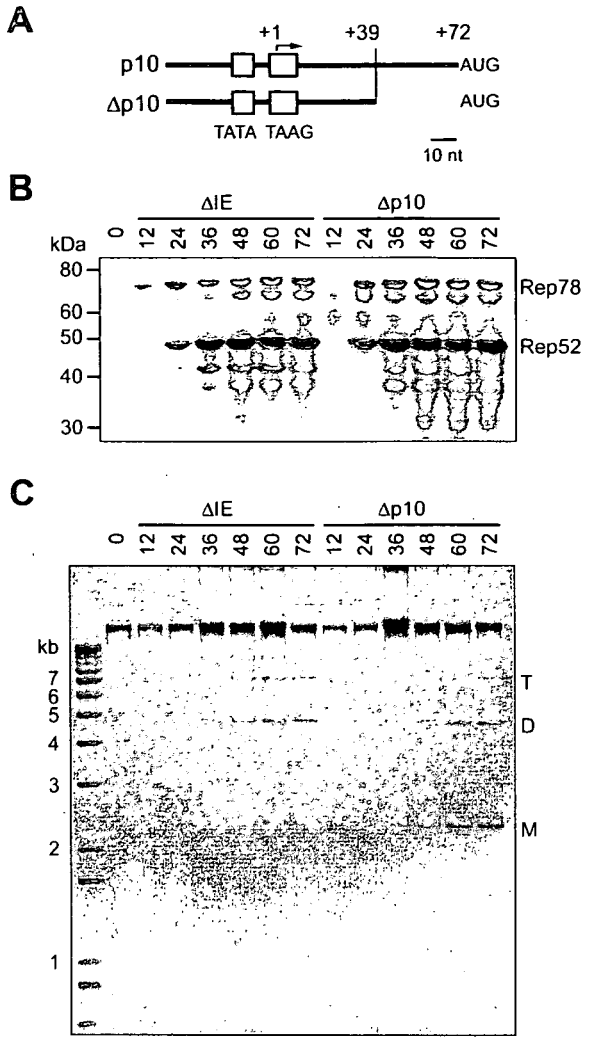

FIG. 4. (A) Map of the Δp10 promoter used for Rep78 expression. The sequence between positions +39 and +72 is deleted in the Δp10 promoter, where the T of the TAAG sequence or the transcription start site (marked with a bent arrow) is defined as +1 and the A of the p10 protein AUG codon is defined as +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

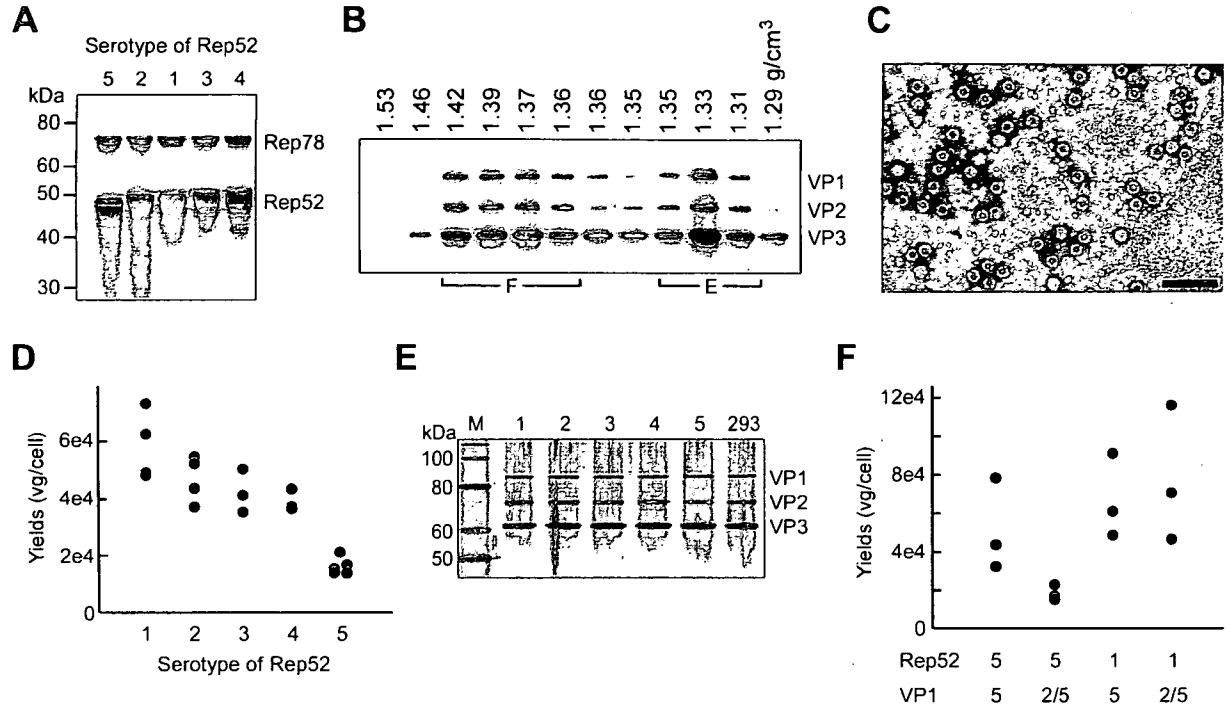

FIG. 5. (A) Western analysis of RepBacs expressing type 5 Rep78 and type 1, 2, 3, 4, or 5 Rep52 with an anti-Rep antibody. (B) Analysis of Sf9 cells coinfected with Rep, VP254, and hGFP baculoviruses by CsCl density gradient ultracentrifugation. Three days after infection, the cells were lysed and subjected 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

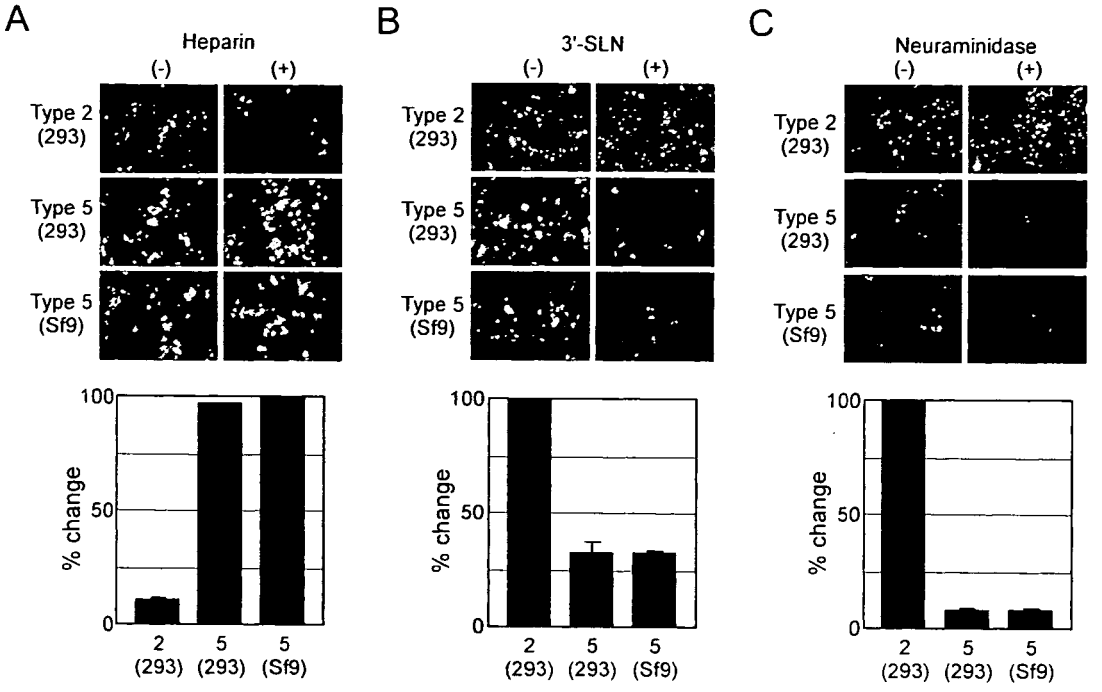

FIG. 6. (A) Heparin, an analog of HSPG, does not inhibit transduction of COS cells infected with rAAV5-GFP/Neo produced in insect cells. Cells were infected with adenovirus at 3 PFU per cell and incubated at 37°C for 2 h. After being washed with medium, the cells were infected with rAAV2-GFP/Neo produced 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

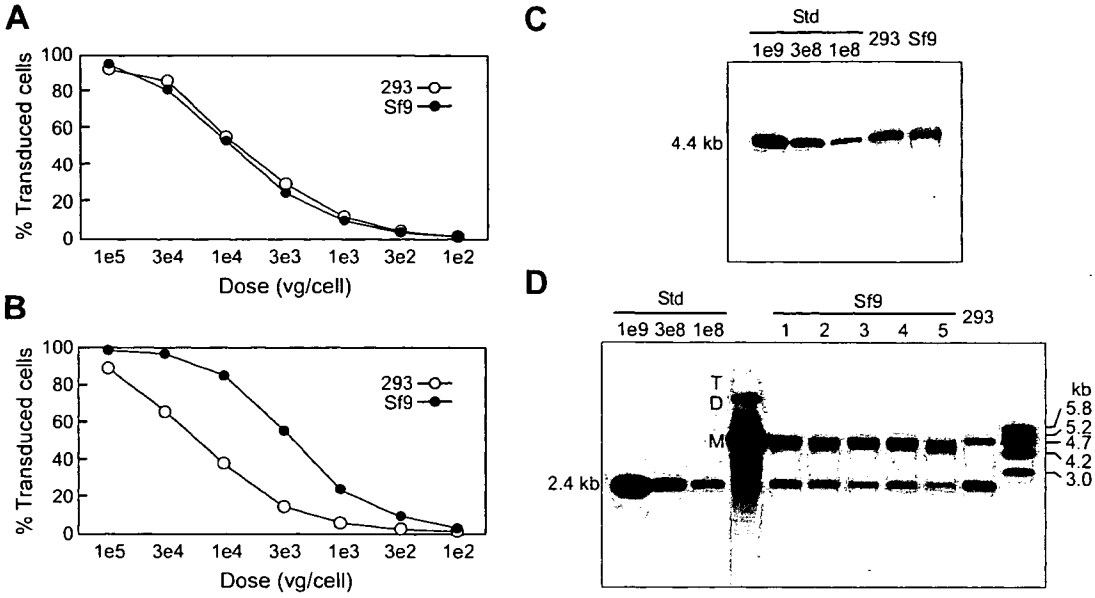

FIG. 7. Comparison of infectivities of rAAV5 produced in 293 cells and rAAV5 produced in Sf9 cells. (A) COS cells were infected with rAAV5-GFP/Neo produced in 293 cells or Sf9 cells at the doses indicated, ranging from 1×10^5 through 1×10^2 vg per cell. One day after infection, the cells were examined 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

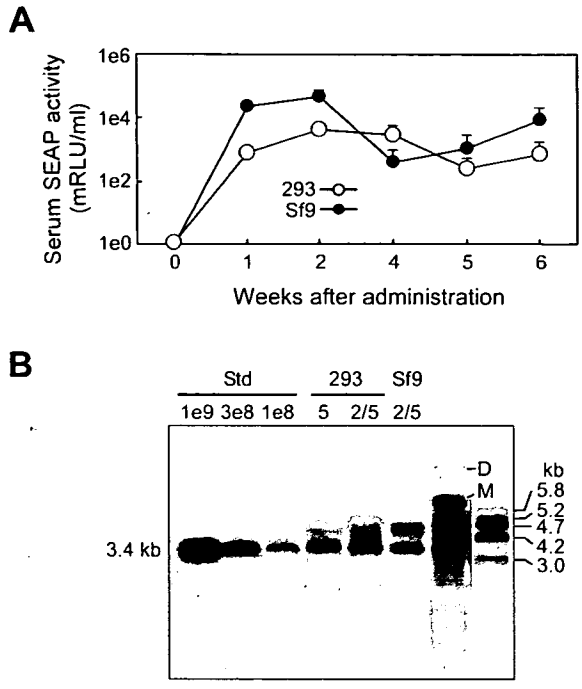

FIG. 8. (A) Serum SEAP activity following intramuscular injection of rAAV5-SEAP. Five mice each received a total of 10^{11} vg of pseudotyped rAAV5-SEAP produced in 293 cells or Sf9 cells into tibialis anterior muscles. Blood was taken at the indicated weeks after injection. The serum SEAP activity was 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.

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Technical Report

Large-Scale Production of Recombinant Viruses by Use of a Large Culture Vessel with Active Gassing

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ABSTRACT

Adenovirus and adeno-associated virus (AAV) vectors are increasingly used for gene transduction experiments. However, to produce a sufficient amount of these vectors for *in vivo* experiments requires large-capacity tissue culture facilities, which may not be practical in limited laboratory space. We describe here a large-scale method to produce adenovirus and AAV vectors with an active gassing system that uses large culture vessels to process labor- and cost-effective infection or transfection in a closed system. Development of this system was based on the infection or transfection of 293 cells on a large scale, using a large culture vessel with a surface area of 6320 cm^2. A minipump was connected to the gas inlet of the large vessel, which was placed inside the incubator, so that the incubator atmosphere was circulated through the vessel. When active gassing was employed, the productivity of the adenovirus and AAV vectors significantly increased. This vector production system was achieved by improved CO_2 and air exchange and maintenance of pH in the culture medium. Viral production with active gassing is particularly promising, as it can be used with existing incubators and the large culture vessel can readily be converted for use with the active gassing system.

OVERVIEW SUMMARY

Large-scale production of recombinant viruses, using a large culture vessel with active gassing, is superior to protocols using standard tissue culture plates or flasks because of the higher capacity for cell growth. Although a previous protocol for recombinant virus production in a large culture vessel had the problem of insufficient transduction efficiency resulting from inadequate gas exchange, a method to use active gassing successfully improved productivity of recombinant viruses. Development of a vector production system on a large scale, using commercially available large culture vessels, allows us to process labor- and cost-effective manipulation in a closed system.

INTRODUCTION

ADENOVIRUS AND ADENO-ASSOCIATED VIRUS (AAV) VECTORS are highly efficient for transduction in many gene therapy studies (Okada *et al.*, 2002b, 2004; Ito *et al.*, 2003; Nomoto *et al.*, 2003; Yamaguchi *et al.*, 2003; Mochizuki *et al.*, 2004; Yoshioka *et al.*, 2004; Liu *et al.*, 2005). However, current production methods rely on the manipulation of many individual flasks and are not generally considered appropriate for scaling-up of production because it would be a time-consuming and labor-intensive process. Therefore, alternative tissue culture vessels with higher capacity for cell growth, such as a 10-tray Cell Factory (CF10; Nalge Nunc International, Rochester, NY) with a surface area of 6320 cm^2, could be suitable for scaling-up of

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vector production (Okada *et al.*, 2002a). This device is easy to handle and can be used for efficient cell culture on a large scale in a closed system requiring only an air filter (Berger *et al.*, 2002; Tuyaerts *et al.*, 2002). Nevertheless, a previous protocol for recombinant virus production in the CF10 had the problem of insufficient scaling-up of vector production (Liu *et al.*, 2003). In that protocol, inadequate gas exchange between the culture vessel and the incubator might have been the cause of the inefficient yield.

We consequently adapted an active gassing system to generate large numbers of recombinant viruses in the CF10. The purpose of this active gassing is to control and maintain CO_2 tension and pH in the growth medium by passing a gas mixture through the CF10. For many types of cells, pH is an important parameter for controlling cell growth. This can be achieved by gassing with CO_2 in atmospheric air in the incubator. Enhanced gas exchange in a large culture vessel should improve both viral infectivity and plasmid transfection efficiency. In combination with the previously described method of using the CF10 (Okada *et al.*, 2002a), we have now created a simple and highly efficient system of producing vector stock on a large scale. Presented here is a labor- and cost-effective method for large-scale production of adenovirus and AAV vectors with an active gassing system that uses a large culture vessel to achieve transfection or infection in a closed system.

MATERIALS AND METHODS

Cell culture with active gassing

Propagation of vectors was based on the infection or transfection of human embryonic kidney-derived 293B cells (Yamaguchi *et al.*, 2003) by using either a flask with a surface area of 225 cm^2 (Falcon, T-225; BD Biosciences Discovery Labware, Bedford, MA) or the CF10, as described previously (Okada *et al.*, 2002a). Cells were cultured in Dulbecco's modified Eagle's medium and nutrient mixture F12 (DMEM–F12: Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator. First, cells were plated at 2.3×10^6 cells per T-225 or at 6.5×10^7 cells per CF10 to achieve a monolayer at 20 to 40% confluency when cells initially attach to the surface of the flask. The volume of medium used per flask was 40 ml per T-225 or 1120 ml per CF10. Subsequently, cells were grown to a confluency of 70–90% over the next 48 to 72 hr for adenovirus infection or plasmid transfection. An aquarium pump (NISSO, Tokyo, Japan) was used to circulate air through the CF10 with 5% CO_2 and humidity control by an incubator. The CF10 was mounted with a bacterial air filter (bacterial air vents; Pall Gelman Sciences, Ann Arbor, MI) to connect the aquarium pump. The pump was connected to the gas inlet of the CF10 and the CF10 was placed inside the incubator, so that the incubator atmosphere was circulated through the CF10. The flow through the CF10 was maintained at 500 ml/min. Culture medium was sampled periodically, and the CO_2 concentrations and pH were estimated with a blood gas analyzer (Nova PHOX; Diamond Diagnostics, Holliston, MA). Glucose levels of the culture medium were also estimated with a glucose meter (Glutest Sensor, Glutest Ace GT-1640; Sanwa Kagaku Kenkyusho, Nagoya, Japan).

Construction and propagation of adenoviral vectors

A recombinant adenoviral vector, Ad-EGFP, was constructed using an adenoviral DNA–protein complex without a transgene insert (AVC2.null) (Okada *et al.*, 1998); it carried the cytomegalovirus (CMV) promoter, cloning sites, a simian virus 40 (SV40) intron, and the SV40 polyadenylation signal. To generate Ad-EGFP encoding enhanced green fluorescent protein (EGFP), a *SpeI–ClaI* fragment containing the *EGFP* cDNA excised from pEGFP-1 (BD Biosciences Clontech, Palo Alto, CA) was inserted into the *XbaI* and *NspV* sites in the DNA–protein complex, AVC2.null, using the direct *in vitro* ligation technique (Okada *et al.*, 1998). The ligated DNA–protein complex was introduced into 293 cells by the calcium phosphate transfection method. Viral plaques on 293 cells were isolated, amplified, and titrated by standard techniques. To amplify the vector in 293 cells, half the medium in the tissue culture flasks was exchanged with fresh DMEM–F12 containing 10% FBS 1 hr before infection. Cells were infected with the virus at 10 multiplicities of infection (MOI) per cell. Cells were incubated to reach full cytopathic effect, and crude viral lysate was purified by two rounds of CsCl two-tier centrifugation. The average number of plaque-forming units (PFU) was assessed on the basis of the 50% tissue culture infective dose. The number of vector particles was estimated by dot-blot hybridization of DNase I-treated stocks with plasmid standards.

Construction and propagation of AAV vectors

AAV1-EGFP, a recombinant AAV type 1 expressing the *EGFP* gene under the control of the CAG promoter (modified chicken β-actin promoter) with the CMV-IE enhancer, was generated by the following procedure. A *BamHI–XbaI* fragment containing EGFP cDNA excised from pEGFP-1 and a *HindIII* fragment containing the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence excised from pBluescript II SK(+)WPRE-B11 (a gift from T. Hope, University of Illinois at Chicago, Chicago, IL) was cloned into an *XhoI* site of pCAGGS (a gift from J.-i. Miyazaki, Osaka University Graduate School of Medicine, Japan) to create pCAG-EGFP-WPRE, using an *XhoI* linker. The EGFP expression cassette in pCAG-EGFP-WPRE was ligated to *NotI*-excised pAAV-LacZ to form the proviral vector plasmid pAAV2-CAG-EGFP-WPRE. AAV viral stocks were prepared according to a previously described protocol (Okada *et al.*, 2002a) with minor modifications. Half the medium in tissue culture flasks was exchanged with fresh DMEM–F12 containing 10% FBS 1 hr before plasmid transfection. Subsequently, cells were cotransfected with 23 μg (per T-225) or 650 μg (per CF10) of each of the following plasmids: a proviral vector plasmid, AAV-1 chimeric helper plasmid p1RepCap (Mochizuki *et al.*, 2004), and adenoviral helper plasmid pAdeno, by a calcium phosphate coprecipitation method. Each of the vector and helper plasmids was added to 4 ml (per T-225) or 112 ml (per CF10) of 300 m*M* $CaCl_2$. This solution was gently added to an equal volume of 2× HEPES-buffered saline (HBS: 290 m*M* NaCl, 50 m*M* HEPES buffer, 1.5 m*M* Na_2HPO_4, pH 7.0) and immediately mixed by gentle inversion three times to form a uniform solution. This solution was immediately mixed with fresh DMEM–F12 containing 10% FBS outside the flasks to produce a homogeneous plasmid solution mixture. Subsequently, medium in the

culture flasks was entirely replaced with this plasmid solution mixture. At the end of incubation for 6 hr, the plasmid solution mixture in the culture flasks was replaced with prewarmed fresh DMEM–F12 containing 2% FBS. Cell suspensions were collected 72 hr after transfection and centrifuged at 300 × *g* for 10 min. Each cell pellet was resuspended in 2 ml (per T-225) or 56 ml (per CF10) of Tris-buffered saline (TBS: 100 m*M* Tris-HCl [pH 8.0], 150 m*M* NaCl). Recombinant AAV was harvested by three cycles of freeze–thawing of each resuspended pellet. Crude viral lysate was then purified twice by passage through a CsCl two-tier centrifugation gradient, as described previously (Okada *et al.*, 2002b). The viral stock was titrated by dot-blot hybridization of DNase I-treated stocks with plasmid standards. To confirm transgene expression with the propagated vector *in vivo*, 5-week-old male Sprague-Dawley rats were injected via the anterior tibial muscle with AAV1-EGFP (1×10^{11} genome copies per rat). Fifteen weeks after injection, the rats were sacrificed and expression was confirmed by fluorescence microscopy.

Statistical analysis

Statistical significance was determined on the basis of an unpaired, two-tailed p value and Student t test, and a p value less than 0.05 was considered significant.

RESULTS

Improved gas exchange and maintenance of pH in medium after recombinant adenovirus infection

Propagation of vectors was based on infection or transfection of 293 cells on a large scale. A minipump was connected to the gas inlet of the CF10 and placed inside the incubator, so that the atmosphere in the incubator, containing 5% CO_2, was circulated through the CF10. The gas flow for circulation through the CF10 was maintained at 500 ml/min. An appropriate gas flow rate was important to give a uniform distribution of the gas in the individual trays of the CF10. A flow less than 200 ml/min gave uneven distribution of the gas, and significantly influenced cell growth. Gas flow that was too high also disturbed the uniformity of cell density. Appropriate cell density and uniform distribution of cells are critical to achieve successful gene transduction. Application of active gassing significantly increased cell growth in the CF10 (Table 1). CO_2 concentrations in the media stayed at their initial levels when using either a T-225 or CF10 with active gassing (Fig. 1A). In contrast, the CO_2 concentration inside the CF10 increased subsequent to adenovirus infection in the absence of active gassing. The pH of culture medium in the CF10 with active gassing was close to that in the T-225 and significantly higher than that in the CF10 without active gassing (Fig. 1B).

Monitoring of cell numbers and time point for harvest

The glucose level was monitored as an index for tracing cell growth and cytopathic effect in the CF10 to avoid the necessity for a specialized microscope to monitor cells in the large culture vessel. The glucose level decreased with increasing cell confluency and progression of cytopathic effect (CPE) (Fig. 2). When 80% CPE was reached, the glucose level was reduced to about 50 mg/ml. When glucose levels were less than 25%, the cells showed full CPE and this was regarded as the appropriate time for harvest.

Improved adenovirus vector production in a large culture vessel with active gassing

We estimated the adenovirus vector yield propagated by using 28 T-225 flasks with a surface area of 225 cm^2, a CF10 with a surface area of 6320 cm^2, or a CF10 in the presence of active gassing. When active gassing was used with the CF10, the productivity of the adenovirus vectors was dramatically increased, by 53.4 times compared with that in the CF10 without active gassing (Fig. 3). The vector yield per producer cell in the CF10 was also significantly improved in the presence of active gassing (Table 1). The PFU-to-particle ratios for vectors produced in the T-225, CF10, and CF10 with active gassing were 1:7, 1:15, and 1:10, respectively.

Efficient AAV vector production in a large culture vessel with active gassing

Enhanced gas exchange in a large culture vessel should also improve vector production through plasmid transfection. AAV vectors were produced in a large vessel by a three-plasmid transfection adenovirus-free protocol (Okada *et al.*, 2002b). Three days after plasmid transfection, the CO_2 concentrations in medium from the CF10 in the presence of active gassing were significantly less than those without active gassing (Table 2). The pH of the culture medium in the CF10 with active gassing was also improved. The CF-10 with active gassing was compatible with the three-plasmid transfection protocol for recombinant AAV production. When we used active gassing, the vec-

TABLE 1. INCREASED CELL GROWTH AND VECTOR YIELD WITH ACTIVE CO_2 AND AIR EXCHANGE[a]

Flask	*Number of cells harvested*	*Vector yield per cell (PFU/cell)*
225-cm^2 flask	$(1.4 \pm 0.2) \times 10^9$ (per 28 flasks)	7.9×10^3
CF10	$(4.9 \pm 1.6) \times 10^8$	4.1×10^2
CF10 + AG	$(1.3 \pm 0.3) \times 10^9$	8.2×10^3

[a]At the time of cell harvest after adenovirus infection, cell growth and vector yield per cell in a CF10 with a surface area of 6320 cm^2 in the presence or absence of active gassing (AG) were compared with that in 28 flasks with a surface area of 225 cm^2 each.

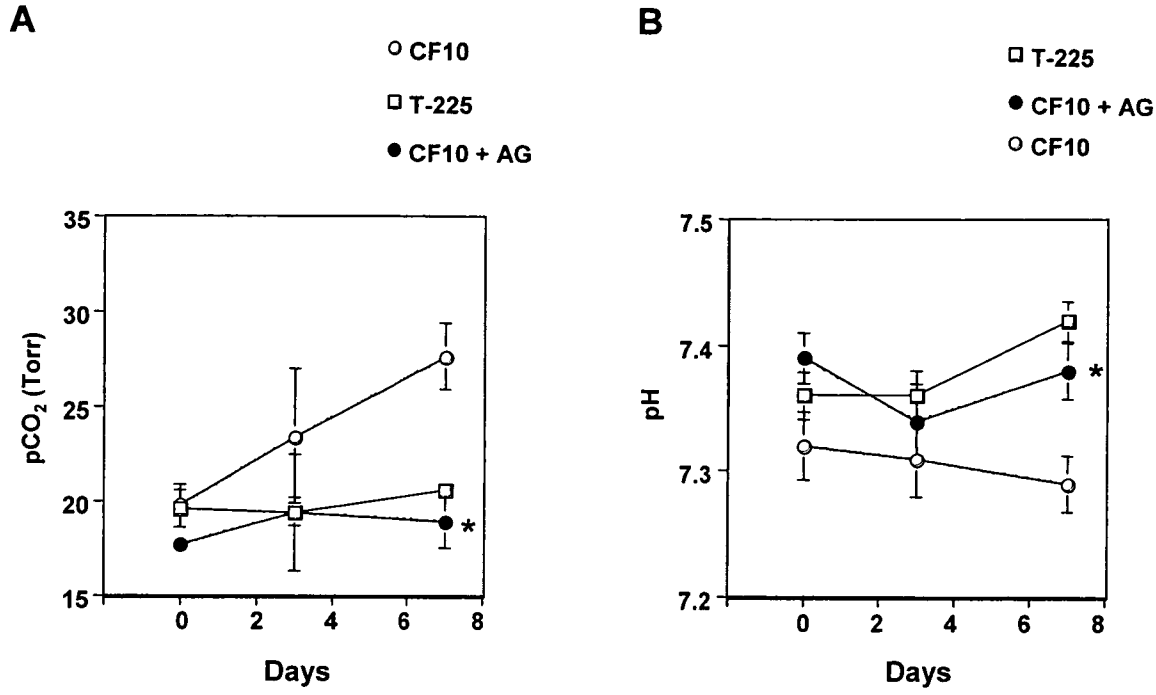

FIG. 1. Improved CO_2 and air exchange and maintenance of pH in conditioned medium after recombinant adenovirus infection. Subsequent to adenovirus infection in a normal flask with a surface area of 225 cm^2 (T-225) or a large culture vessel (a 10-tray Cell Factory [CF10] with a surface area of 6320 cm^2) in the presence or absence of active gassing (AG), CO_2 concentrations (**A**) and pH (**B**) in conditioned medium were determined ($n = 4$). Asterisk indicates $p < 0.05$ in comparison with a CF10 without AG.

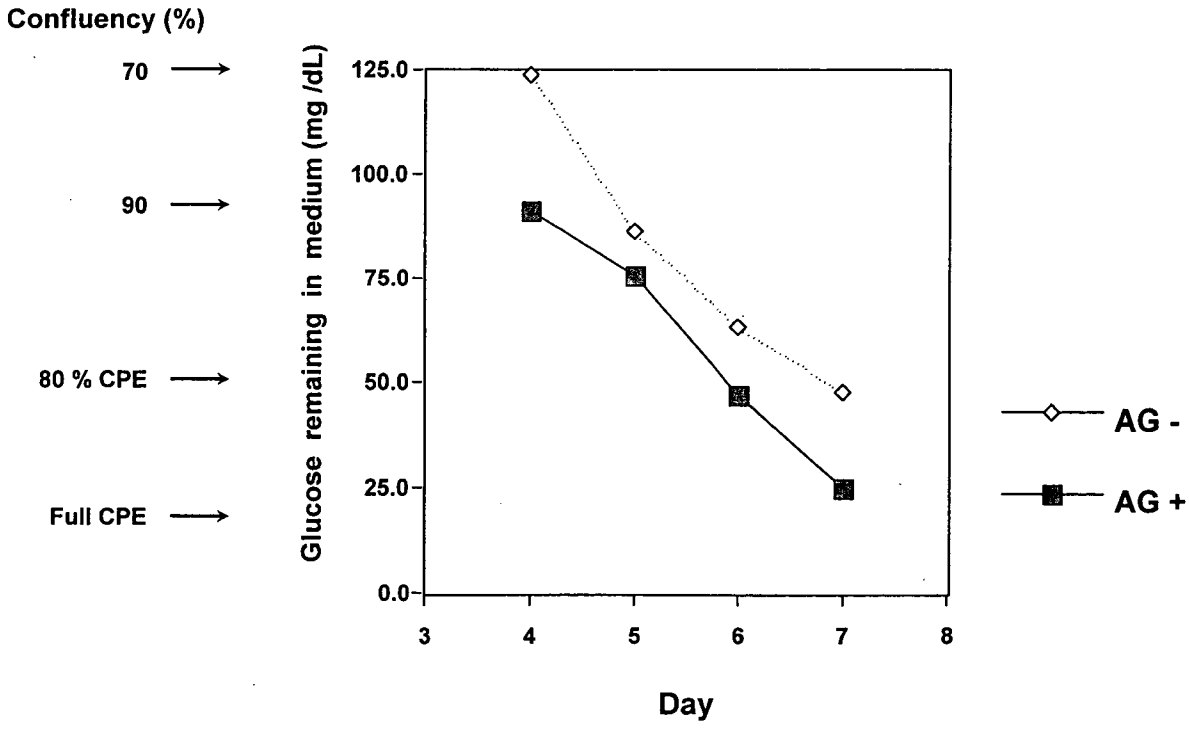

FIG. 2. Glucose reading to monitor cell growth. Glucose reading of culture medium was used as an index to monitor cell growth and cytopathic effect (CPE) in the CF10 to avoid the need for a specialized microscope. Cells were infected with recombinant adenovirus in the presence or absence of active gassing (AG) when 90% confluent.

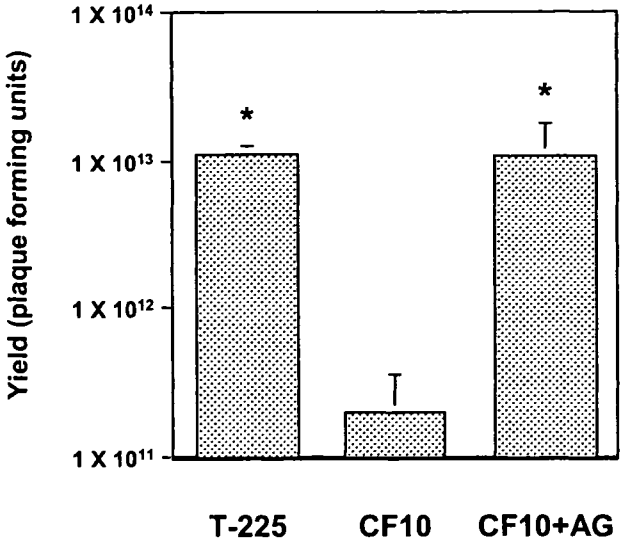

FIG. 3. Improved production of adenovirus vector. Adenovirus vector was propagated in 28 T-225 flasks ($n = 4$), a CF10 ($n = 3$), or a CF10 in the presence of active gassing (CF10 + AG, $n = 3$). Adenovirus vector expressing an EGFP reporter gene was generated in two independent experiments. The average number of plaque-forming units (PFU) was assessed by $TCID_{50}$. $^*p < 0.05$ in comparison with a CF10 without AG.

tor yield per cell was increased significantly, by 3.5 times (Table 3). Although vector yield was dependent on the transgene and construct, production of vector particles at up to 2.0×10^{13} genome copies per CF-10 was achieved.

Transduction of muscles with AAV vectors produced in a large culture vessel with active gassing

Five-week-old male Sprague-Dawley rats were injected with AAV1-enhanced green fluorescence protein (EGFP) (1×10^{11} genome copies per rat) via the anterior tibial muscle. Fifteen weeks after injection, the rats were sacrificed to confirm expression by fluorescence microscopy. The injected sites showed efficient expression of EGFP (Fig. 4).

TABLE 2. ENHANCED GAS EXCHANGE AND MAINTENANCE OF pH IN CONDITIONED MEDIUM AFTER PLASMID TRANSFECTION[a]

	pCO_2 (Torr)	pH
CF10	25.6 ± 1.1	7.23 ± 0.03
CF10 + AG	14.2 ± 0.1	7.40 ± 0.01

[a]Three days after plasmid transfection by using CF10 in the presence or absence of active gassing (AG), CO_2 concentrations and pH in the conditioned medium were estimated. Means ± standard deviations are shown ($n = 4$).

TABLE 3. IMPROVED YIELDS OF RECOMBINANT AAV TYPE 1 BY ACTIVE GAS EXCHANGE[a]

	Yield per vessel	Yield per cell
CF10	$(2.2 \pm 0.5) \times 10^{12}$	$(3.1 \pm 0.6) \times 10^{3}$
CF10 ± AG	$(1.0 \pm 0.7) \times 10^{13}$	$(1.1 \pm 0.7) \times 10^{4}$

[a]Titers of AAV1-EGFP were determined as genome copies by dot-blot analysis of DNase-treated stocks. AG, active gassing. Means ± standard deviations are shown ($n = 4$).

DISCUSSION

Successful vector production in a large culture vessel was achieved by improvement of CO_2 and air exchange along with maintenance of pH in the medium. Adenovirus production was enhanced by more than 50 times with the active gassing sys-

FIG. 4. Transduction of muscles with AAV vectors *in vivo*. Five-week-old male Sprague-Dawley rats were injected with AAV1-EGFP (1×10^{11} genome copies per body) via the anterior tibial muscle. Fifteen weeks after injection, the rats were killed to confirm expression by fluorescence microscopy. Scale bar: 100 μm.

tem. CF-10 with active gassing was also compatible with the three-plasmid transfection protocol for recombinant AAV production. When we used active gassing, the productivity of the AAV vectors was significantly increased.

In a direct comparison with vectors generated in ordinary culture flasks, viruses from the CF10 with active gassing were equivalent regarding function and bioactivity. The use of a CF10 with active gassing thus resulted in the production of vectors equivalent to those obtained in conventional culture dishes, but with a dramatically reduced workload. An average yield of approximately 1.0×10^{13} PFU requires as many as 28 T-225 flasks, according to our previous protocol. Alternatively, only one CF10 with active gassing was enough to achieve the same amount of virus. Furthermore, the PFU-to-particle ratio was also increased with the use of active gassing, suggesting improved bioactivity of the viruses. We used this system to amplify various adenovirus vectors. Although vector yield was dependent on the transgene and construct, a proportional increase in yield relative to surface area was achieved (data not shown).

The system was also compatible with plasmid transfection for recombinant AAV production. Active gassing combined with a large culture vessel significantly increased the productivity of the AAV vectors. The effect of enhanced gas exchange on the productivity of AAV vectors was less than the effect on the productivity of adenovirus vectors. Because lactate production accompanied by adenovirus replication is much greater than that with AAV, the protection of cells against pH drop by maintaining the CO_2 tension might be a plausible explanation for the preferential effect on adenovirus production. Transient transfection in a large culture vessel also provides a simple and flexible method of producing lentivirus-based vectors (Karolewski *et al.*, 2003). Therefore, our protocol would also be applicable to the efficient production of lentivirus- or retrovirus-based vectors.

Because this system fits into existing incubators and current vessels can readily be converted to the active gassing system, the viral production protocol using a CF-10 coupled with active gassing has practical utility for growing recombinant virus stocks in a limited laboratory space. This system has proven successful in our repeated manipulations and appears particularly promising. We have used the CF10 with the active gassing system in more than 400 vector preparations in the course of our more recent gene therapy experiments. The system allows us to perform a considerable number of *in vivo* experiments and to validate our studies.

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