

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  $1 \times 10^5$  through  $1 \times 10^2$  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  $1 \times 10^5$  through  $1 \times 10^2$  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  $3 \times 10^8$  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) ( $1 \times 10^9$ ,  $3 \times 10^8$ , and  $1 \times 10^8$  copies) was loaded, which was derived from the GFP/Neo vector plasmid, pSR485a, 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

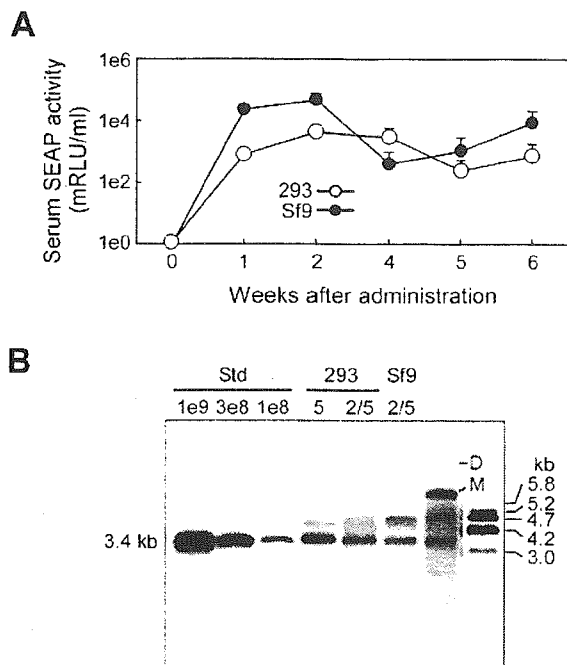


FIG. 8. (A) Serum SEAP activity following intramuscular injection of rAAV5-SEAP. Five mice each received a total of  $10^{11}$  vg of pseudotyped rAAV5-SEAP produced in 293 cells or Sf9 cells into tibialis anterior muscles. Blood was taken at the indicated weeks after injection. The serum SEAP activity 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  $10^5$ ) 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  $3 \times 10^5$  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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# Specific and Efficient Transduction of Cochlear Inner Hair Cells with Recombinant Adeno-associated Virus Type 3 Vector

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Recombinant adeno-associated virus (AAV) vectors are of interest for cochlear gene therapy because of their ability to mediate the efficient transfer and long-term stable expression of therapeutic genes in a wide variety of postmitotic tissues with minimal vector-related cytotoxicity. In the present study, seven AAV serotypes (AAV1–5, 7, 8) were used to construct vectors. The expression of EGFP by the chicken  $\beta$ -actin promoter associated with the cytomegalovirus immediate-early enhancer in cochlear cells showed that each of these serotypes successfully targets distinct cochlear cell types. In contrast to the other serotypes, the AAV3 vector specifically transduced cochlear inner hair cells with high efficiency *in vivo*, while the AAV1, 2, 5, 7, and 8 vectors also transduced these and other cell types, including spiral ganglion and spiral ligament cells. There was no loss of cochlear function with respect to evoked auditory brain-stem responses over the range of frequencies tested after the injection of AAV vectors. These findings are of value for further molecular studies of cochlear inner hair cells and for gene replacement strategies to correct recessive genetic hearing loss due to monogenic mutations in these cells.

**Key Words:** adeno-associated virus, serotype, gene transfer, cochlea, hair cells

## INTRODUCTION

The total number of hair cells in the cochlea is finite. They are not renewed and there is very little (if any) redundancy in this population. The irreversible loss of cochlear hair cells is presumed to be a fundamental cause of permanent sensorineural hearing loss. Gene transfer into hair cells presents numerous opportunities for protecting these cells. There is considerable interest in the development of viral vectors to deliver genes to the cochlea to counteract hearing impairment, and recent studies have focused on vectors based on adenovirus [1–3], herpes simplex virus [4–6], lentivirus [7], and adeno-associated virus (AAV) [8,9]. The patterns of vector-encoded transgene expression have been found to differ significantly among vectors. Cochlear hair cells can be efficiently transduced with adenovirus vectors [10–12].

However, these vectors were found to provoke a strong immune response that could damage recipient cells and compromise cochlear function [10,13,14]; they are also incapable of mediating prolonged transgene expression [15,16]. Although AAV vectors might overcome these problems, the transduction of hair cells by AAV2-derived vectors is controversial [8,10,17]. To our knowledge, other AAV serotypes have not yet been tested as cochlear gene transfer vectors *in vitro* or *in vivo*. AAV vectors are of interest in the context of gene therapy because they mediate efficient transfer and long-term stable expression of therapeutic genes in a wide variety of postmitotic tissues with minimal vector-related cytotoxicity.

In this study, we assessed the utility of seven AAV serotypes as vectors with the chicken  $\beta$ -actin promoter associated with a cytomegalovirus immediate-early

enhancer (CAG)-driven enhanced green fluorescent protein (EGFP) gene [18] in the murine cochlea. Vectors were introduced by microinjection through the round window membrane [19]. As a result, we determined that the specific and efficient gene transduction of inner hair cells could be achieved by using AAV type 3 vectors.

## RESULTS

### Expression Profile of EGFP in the Cochlea

Several cell types line the cochlear duct and support the hair cells (Fig. 1A). We carefully made a small opening in the tympanic bulla and injected vectors derived from the AAV1–4, 7, and 8 pseudotypes into the cochlea of two strains of mice (C57BL/6) and ICR) through the round window membrane (Fig. 1B). The mode of EGFP expression in various murine cochlear hair cells had a close similarity and was essentially equal for both strains. We determined the distribution of AAV vector-mediated EGFP expression throughout the cochlea for all serotypes tested (Table 1). A principal finding is that the inner hair cells in the organ of Corti showed clear evidence of EGFP expression with all of the AAV serotype-derived vectors except for the AAV4 vector (Fig. 2). This result indicates that most of the vectors (AAV1–5, 7, and 8) could efficiently transduce cochlear inner hair cells *in vivo* when slowly infused into the scala tympani. The AAV3-based vector was the most efficient and specific of the serotypes in transducing cochlear inner hair cells (Fig. 3). Transduction with  $5 \times 10^{10}$  genome copies (gc)/cochlea of the AAV3 vector resulted in robust transgene expres-

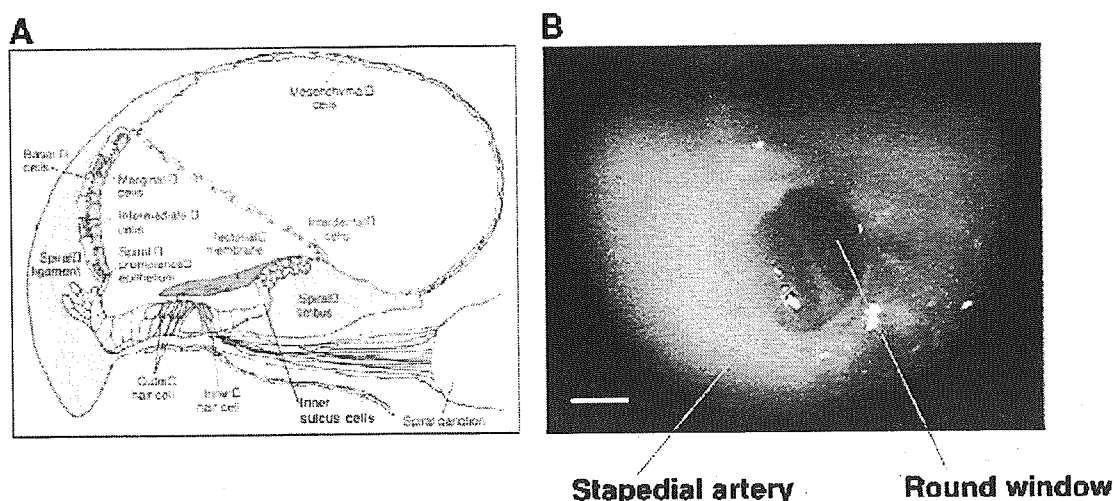
sion in the inner hair cells. The spiral ganglion cells showed significantly higher levels of fluorescence per unit area with the AAV5-based vector (Fig. 2n), and the spiral ligament cells were transduced prominently with the AAV1 and AAV7 vectors (Figs. 2d and 2r). Histological sections of cochleae injected with the AAV4 vector identified EGFP-positive cells predominantly in connective tissue within the mesothelial cells beneath the organ of Corti and in mesenchymal cells lining the perilymphatic fluid spaces (Figs. 2j and 2l). Furthermore, we detected intense expression with the AAV5- and AAV8-based vectors in the inner sulcus cells and in Claudius' cells (Figs. 2p and 2x). We did not detect notable levels of gene expression in the outer hair cells, supporting pillar cells, or stria vascularis cells for any serotype.

### Long-term Expression of EGFP

We examined cochlear expression of the EGFP transgene in animals sacrificed at 1–12 weeks. Expression persisted in cochlear tissues for up to 3 months after infusion, while the extent of expression peaked at 2 weeks.

### Transgene Activity

We determined the percentage of inner hair cells transduced with the AAV3 vector. The mid- to high-frequency regions of the cochlea were efficiently transduced, as shown in Fig. 3. Almost all of the inner hair cells in the basal and middle cochlear regions were transduced with the AAV3 vector (Fig. 4). Transgene expression was not detected in the hair cells of the apical turn of the cochlea. The predominant expression in the middle and basal cochlear turns is reasonable, as the virus



**FIG. 1.** (A) Schematic diagram of a cross section of the cochlea, demonstrating the scala vestibuli, scala tympani, and scala media or cochlear duct. The organ of Corti rests on the basilar membrane, with the hair cell cilia embedded in the gelatinous tectorial membrane. The outer margin of the cochlear duct contains the stria vascularis. Reproduced, by permission of the publisher, from [44]. (B) Direct visualization of the round window membrane in the right ear. The upper side of the picture is the back of the mouse and the right side is the head of the animal. The stapedial artery, a branch of the internal carotid artery, transverses an open bony semicanal within the round window niche. Bar denotes 500  $\mu$ m, 15 $\times$  original magnification.

TABLE 1: Expression of transgene in the mouse cochlea with vectors derived from the AAV1-4, 7, and 8 pseudotypes

Vector	Inner hair cells	Outer hair cells	Spiral ganglion	Stria vascularis	Spiral ligament	Spiral limbus	Reissner's membrane	Inner and outer pillar cells	Inner sulcus cells	Deiter's cells	Claudius' cells	Hensen's cells	Mesenchymal cells
AAV1	+++	-	++	-	++	++	++	-	+	-	-	-	++
AAV2	++	-	+	-	+	+	-	-	-	-	-	-	-
AAV3	++++	-	-	-	-	-	-	-	-	-	-	-	-
AAV4	-	-	-	-	-	-	-	-	-	-	-	-	+
AAV5	+++	-	+++	-	+	++	+	-	++	-	+	-	++
AAV7	+++	-	+	-	+++	++	-	-	+	-	+	-	++
AAV8	++++	-	-	-	+	+	-	-	++	-	+	-	+

The level of expression was graded by fluorescence intensity on a four-level scale (+, ++, +++, +++) depending on the pixel/unit area count. + means the strongest intensity of EGFP expression, + means the weakest intensity of EGFP expression, while - means no fluorescence.

was slowly infused into the scala tympani adjacent to the most basal turn of the cochlea. The percentage of transduced inner hair cells from the basal (high frequencies) to the apical (low frequencies) cochlear regions is shown in Fig. 4.

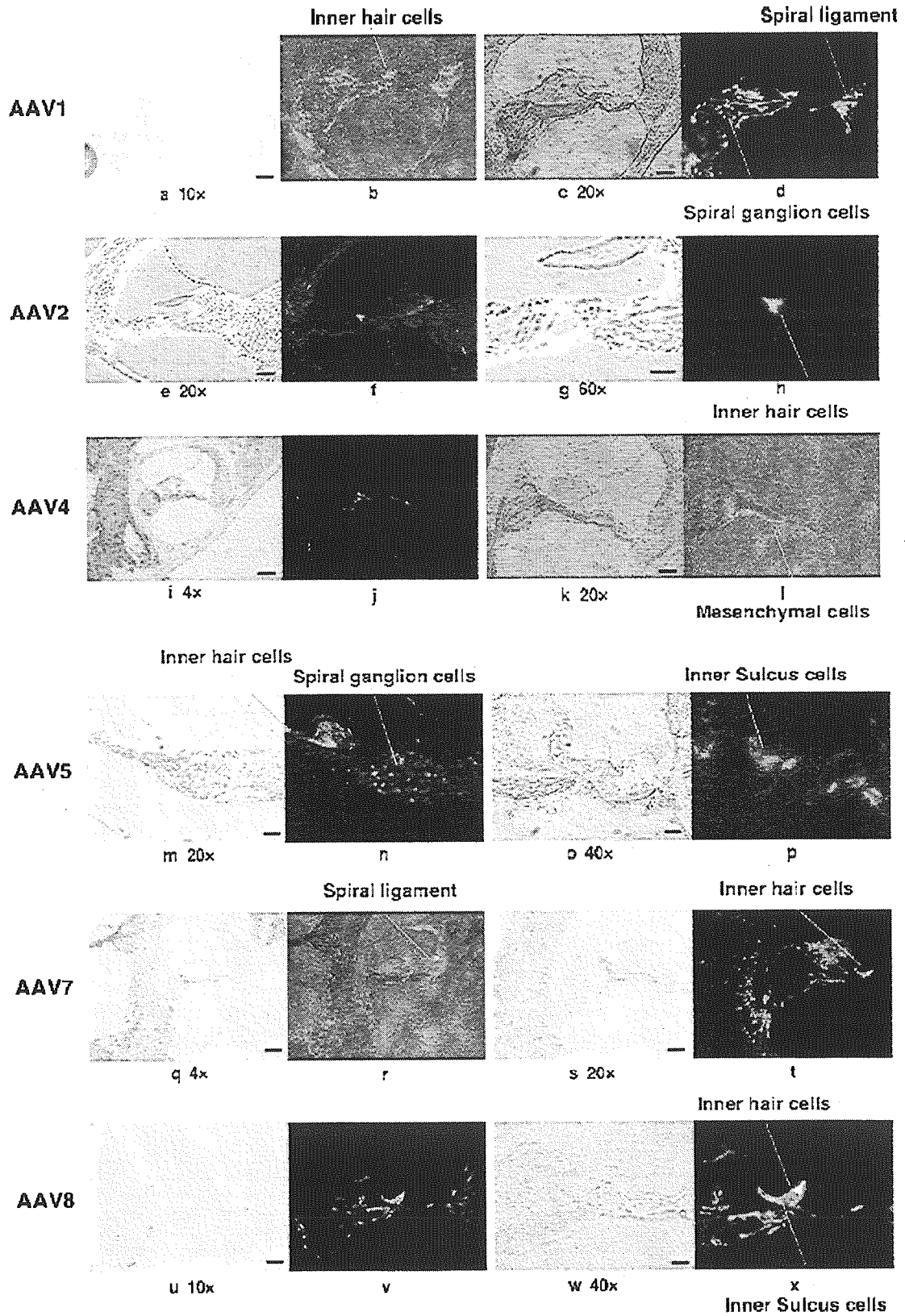
### Cytotoxicity

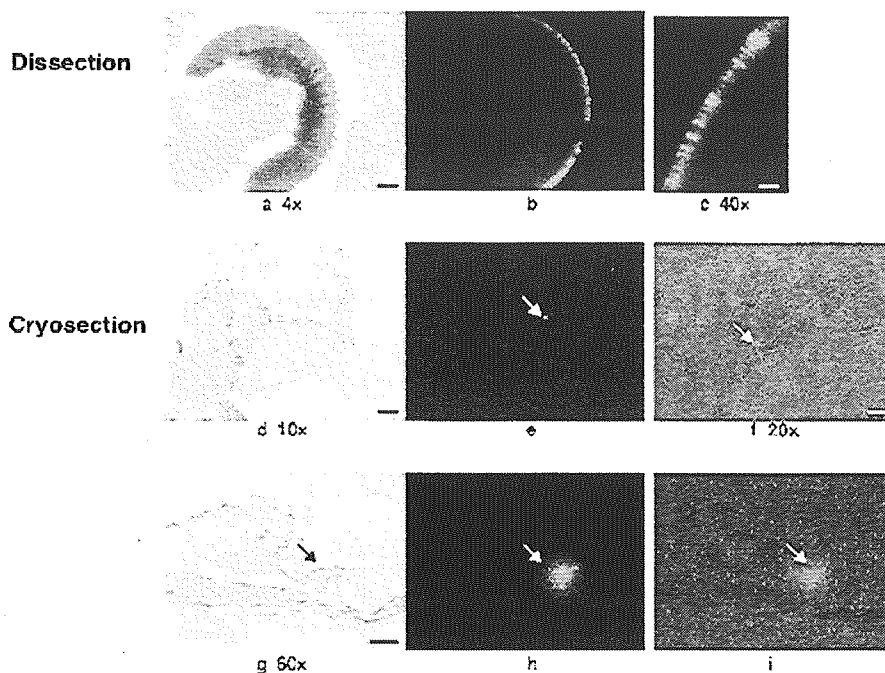
We detected no deleterious effects on the viability of transduced cells. We compared evoked auditory brainstem response (ABR) threshold levels before and after injection, using a two-way repeated measure of the analysis of variance. There was no significant loss in ABR and hence no change in cochlear function for up to 10 days following vector infusion (Figs. 5A and 5B). In addition, the cellular and tissue architecture of experimental cochleae remained intact. There was no evidence of endolymphatic hydrops after AAV vector injection in any of the animals. We observed no significant destruction of the inner or outer hair cells (Fig. 5C).

### DISCUSSION

In the present study, we assessed the utility of vectors derived from seven AAV serotypes for gene delivery into the cochlea. Our results showed that the AAV3 vector was the most efficient and specific in transducing cochlear inner hair cells, although these cells could also be transduced with AAV1, 2, 5, 7, and 8 vectors. The transduction efficiency of the spiral ganglion by the AAV5 vector was particularly high, followed by that of the AAV1, AAV2, and AAV7 vectors. The efficient and specific transduction of inner hair cells with the AAV3 vector suggests that it recognizes a unique host range with a distinct cellular receptor. Transduction efficiency is dependent on initial viral binding (a property of the viral capsid), entry, and various postentry processes such as intracellular trafficking and second-strand synthesis [20-22]. The genome size of AAV vectors has also been demonstrated to affect transduction efficiency [23]. Comparisons of the serotypes have indicated that heterogeneity in the capsid-encoding regions and a differential ability to transduce cells may be associated with different receptor and co-receptor requirements for cell entry [24]. However, the receptors and co-receptors of AAV3 have not yet been clearly identified.

In the current study, we found that cochlear inner hair cells could be transduced with six AAV serotypes, although Lalwini *et al.* [8] reported that outer hair cells could be transduced with a low titer ( $1 \times 10^6$  viral particles/ml) of AAV2 *in vivo*. After injecting the AAV2 vector, we found that the spiral ganglion neurons, the inner hair cells, and the cells in the spiral ligament were all transduced. This transduction pattern differs from that reported in previous studies [8,10,17], and this discrepancy might be due to the different delivery methods and dissimilar promoters. Although the CAG promoter directs





**FIG. 3.** Cochlear transduction with AAV3-CAG-EGFP. Dissected cochleae and cryosections show transgene expression in inner hair cells. (a) A light photomicrograph of the basal turn of the cochlea is shown, illustrating its laminar structure. (b) A fluorescence photomicrograph of this dissection. (c) A higher magnification view of the dissection shown in (b), illustrating a row of inner hair cells in the organ of Corti expressing EGFP. (d–i) Representative photomicrographs from three magnifications of a radial cochlear cryosection. (d) Light photomicrography of an intact cochlear duct. Fluorescence photomicrography of this duct is shown in (e). (h and i) A higher magnification of (e), illustrating EGFP expression within inner hair cells. Cryosections show transgene expression in the inner hair cells (arrows). Scale bars: 4 ·, 250  $\mu$ m; 10 ·, 100  $\mu$ m; 20 ·, 50  $\mu$ m; 40 ·, 25  $\mu$ m; 60 ·, 25  $\mu$ m.

higher expression than do the cytomegalovirus (CMV) and EF-1 $\alpha$  promoters [25], each promoter drives reporter gene expression in different cell types [26,27].

Cell-specific or -selective infectivity of the viral vectors suggests the presence of various factors to introduce the distinct expression patterns of the transgenes. Spiral ganglion neurons and glial cells can be transduced with a lentivirus–GFP construct *in vitro* but not *in vivo* [7]. The differential transducibility under *in vivo* and *in vitro* conditions reflects a high degree of structural isolation of the spiral ganglion and other cell types—such as the cells on the periphery of the endolymph—from the perilymph into which the viral vector was introduced. The strict separation of the endolymph from the perilymph is maintained by tight junctions that line the boundary between these fluid chambers. The size of the viral particle may contribute to the observed variability in transgene expression promoted by different vectors. The diameters of adenovirus and retrovirus (including lentivirus) particles are approximately 75 nm and greater than 100 nm, respectively, while the diameters of AAV vectors are typically 11–22 nm [28,29]. Thus, the larger size of lentiviruses and adenoviruses may limit their subsequent

dissemination from the perilymph into the endolymph. The variable patterns of adenovirus- and lentivirus-mediated gene expression seen with different methods of inoculation may be due to the inoculation route, the volume and number of viral particles, differences in viral preparation, or differences in the method of transgene detection. The introduction of adenovirus vectors by cochleostomy or with an osmotic pump via the round window leads to a more efficient transduction of cochlear hair cells [30–32]. The apical domain (apical membrane and stereocilia) of cells in the sensory epithelium (hair cells and supporting cells) is bathed in endolymph, while the basal–lateral domain is immersed in perilymph. Access of the viral vectors to the endolymphatic space by cochleostomy may facilitate the transduction of hair cells and supporting cells. However, although the cochleostomy procedure has been tested, inoculation into the membranous labyrinth could not be confirmed [32]. In the present study, AAV vectors were found to infect cochlear hair cells easily *in vivo*, via round window injection.

Gene transfer into the cochlea through the round window membrane is ideal, because this procedure

**FIG. 2.** Transduction of the cochleae by AAV1-, AAV2-, AAV4-, AAV5-, AAV7-, and AAV8-based vectors. (a, c, e, g, i, k, m, o, q, s, u, and w) Light photomicrographs of cochlear cryosections. (b, d, l, h, j, l, n, p, r, t, v, and x) Fluorescence photomicrographs (green fluorescence from transgene). The spiral ligament cells were transduced prominently with the AAV1 and AAV7 vectors (d and r). Transgene expression in inner hair cells was detected with AAV1-, AAV2-, AAV5-, AAV7-, and AAV8-based vectors (b, h, n, t, and x). AAV4-based vector faintly transduced mesenchymal cells (j and l). The spiral ganglion cells showed significant levels of fluorescence with the AAV5-based vector (n). Intense fluorescence was detected with the AAV5- and AAV8-based vectors in the inner sulcus cells (p and x). Scale bars: 10 ·, 100  $\mu$ m; 20 ·, 50  $\mu$ m; 40 ·, 25  $\mu$ m; 60 ·, 25  $\mu$ m.



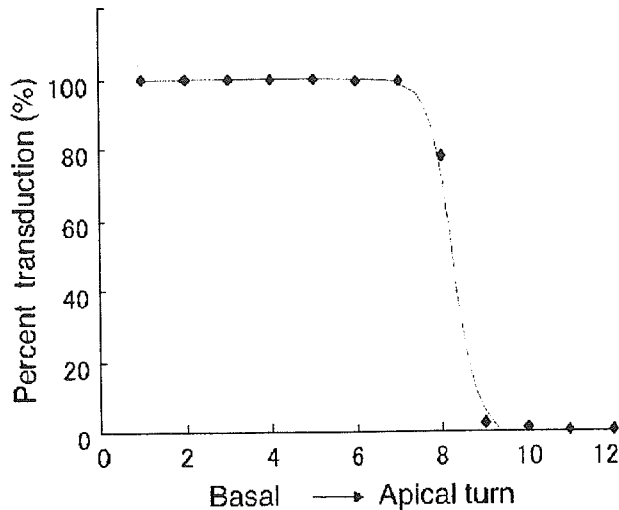


FIG. 4. EGFP expression profile of inner hair cells transduced with AAV3, as shown for a cross section subdivided into 12 segments ranging from the basal (high frequencies) to the apical (low frequencies) cochlear regions.

requires simple surgery without cochlear trauma [19]. Another critical factor in assessing the utility of a gene transfer vector is safety. Factors determining safety include the toxicity of the gene transfer agent itself, the provocation of immune responses, the generation of replication-competent virus, and the risk of creating genetically modified cells by insertional mutagenesis. The cells and tissues within the AAV-EGFP-perfused cochleae were free from inflammation and were generally intact. No pathological changes were observed in the organ of Corti, stria vascularis, or spiral ganglion cells. The long-term expression of EGFP within the cochlear tissues is consistent with data obtained from other animal models and different organ systems [9,33]. Since EGFP is known to introduce cellular toxicity, vectors expressing physiologically therapeutic proteins would achieve longer transduction periods than EGFP. Gene transfer into the inner hair cells presents numerous opportunities for auditory neuroscience. Potential applications include the localization of proteins by expression of tagged constructs, the generation of dominant-negative or antisense knockouts of endogenous proteins, the rescue of mutant phenotypes to identify disease genes, and perhaps even the treatment of auditory disorders. Advances in the molecular basis of auditory diseases have allowed the identification of a number of genetic disorders such as presbycusis, acoustic trauma, and ototoxicity. The development of gene therapy now allows us to evaluate the effects of transferring therapeutic genes into the inner ear by several different strategies. The expression of marker genes in the inner ear tissue has been demonstrated. Further studies will improve our understanding of cochlear function as well as provide

for the development of novel therapies for a wide variety of inner ear diseases. Intracochlear gene transfer using AAV vectors has been established as a viable experimental proposition. Future study will include the transfer of functioning genes *in vivo* and the development of alternative vectors. While clinical application may be some way off, it is vital that gene delivery techniques are optimized in anticipation of future need.

In conclusion, the data presented in this paper demonstrate successful gene transfer into several types of cochlear cells *in vivo* with AAV-based vectors. Interestingly, the AAV3 vector promoted inner hair cell-specific transduction. These findings are of value for further molecular studies of the cochlear inner hair cells and for gene replacement strategies to correct hereditary hearing loss due to specific monogenic mutations affecting cochlear inner hair cells.

## MATERIALS AND METHODS

**Construction and preparation of proviral plasmids.** The AAV vector proviral plasmid pAAV2-LacZ harbors an *Escherichia coli*  $\beta$ -galactosidase expression cassette with the CMV promoter, the first intron of the human growth hormone gene, and the SV40 early polyadenylation sequence, which are flanked by inverted terminal repeats (ITRs) [34]. The LacZ expression cassette of pAAV2-LacZ was ligated to *NotI*-excised pAAV5-RNL [35] to form the proviral plasmid pAAV5-LacZ. The pAAV2-CAG-EGFP-WPRE construct consists of the EGFP gene under the control of the CAG promoter (the chicken  $\beta$ -actin promoter associated with the cytomegalovirus immediate-early enhancer) and WPRE (woodchuck hepatitis virus posttranscriptional regulatory element) flanked by ITRs. The WPRE cassette augments the stability of transgene mRNA [36] and increases EGFP expression levels, thereby ensuring long-term transgene expression. A *Bam*HI-*Xba*I fragment containing the EGFP cDNA excised from pEGFP-1 and a *Hind*III fragment containing the WPRE sequence excised from pBS II SK'WPRE-B11 (a gift from Dr. J. Donello) was ligated to *Xho*I linkers and cloned into an *Xho*I site of pCAGGS (a gift from Dr. J.-I. Miyazaki) to create pCAG-EGFP-WPRE. The EGFP expression cassette from pCAG-EGFP-WPRE was ligated to the *NotI*-excised pAAV2-LacZ and pAAV5-RNL [35] to form the proviral plasmids pAAV2-CAG-EGFP-WPRE and pAAV5-CAG-EGFP-WPRE, respectively. The AAV-helper plasmid harbors Rep and Cap. The adenovirus helper plasmid pAdeno5 (identical to pVAE2AE4-5) encodes the entire E2A and E4 regions and the VA RNA I and II genes [37]. Plasmids were purified with the Qiagen plasmid purification kits (Qiagen K.K., Tokyo, Japan).

**Recombinant AAV vector production.** Vectors derived from the AAV1-4, 7, and 8 pseudotypes were produced with the AAV packaging plasmid pAAV1RepCap (for AAV1) [38], pHLF19 (for AAV2), pAAV3RepCap (for AAV3) [39], pAAV4RepCap (for AAV4) [40], pAAV7RepCap (for AAV7) [41], or pAAV8RepCap (for AAV8) [41] and the AAV proviral plasmid pAAV2-LacZ or pAAV2-CAG-EGFP-WPRE. The plasmids pAAV5RepCap [35] and pAAV5-LacZ, or pAAV5-CAG-EGFP-WPRE, were used to produce vector with the AAV5 pseudotype [42]. Seven AAV serotype vectors were produced as previously described by the three-plasmid transfection adenovirus-free protocol [37]. Briefly, three days before transfection, 293 cells were plated onto a 10-tray Cell Factory (Nalge Nunc International, Rochester, NY, USA;  $6 \times 10^7$  cells/10-tray). The cells were cotransfected with 650  $\mu$ g each of the proviral plasmid, the AAV vector packaging plasmid, and the adenovirus helper plasmid pAdeno5 [34] by the calcium phosphate coprecipitation method. The medium was changed following incubation for 6-8 h at 37°C. Recombinant AAV was harvested 72 h after transfection by three freeze/thaw cycles. The crude viral lysate was purified twice on a cesium chloride two-tier centrifugation

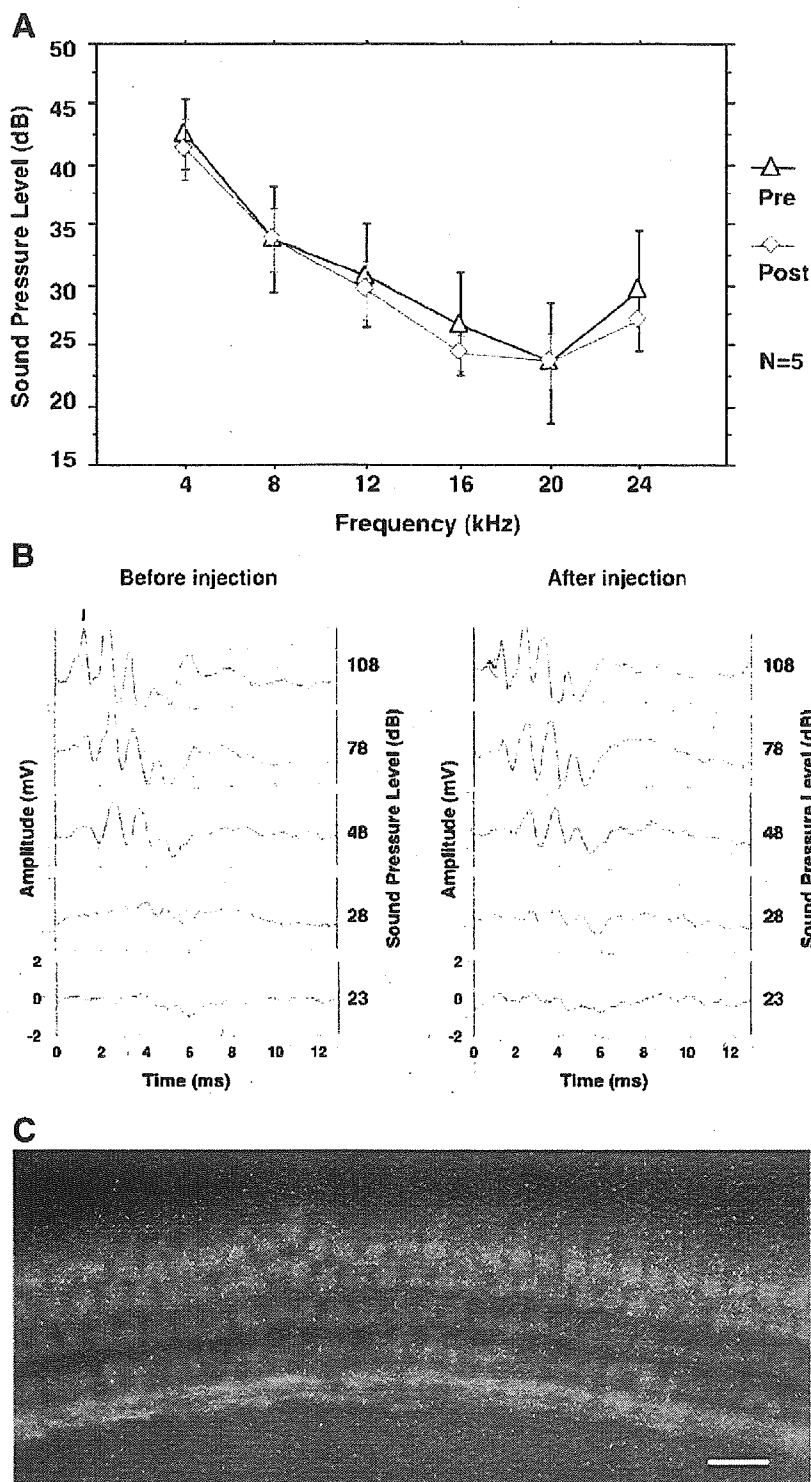


FIG. 5. (A) ABR threshold (mean  $\pm$  SD) at each frequency tested preoperatively (pre) versus postoperatively (post). (B) Example of ABR waveforms in C57BL/6j at various stimuli (16 kHz; 108 dB, 78 dB, 48 dB, 28 dB, and 23 dB). ABR were tested in the transduced ear prior to viral injection and 10 days after injection. Wave I was measured to analyze the activity of the cochlea. (C) F-actin staining showing that no outer hair cells were lost from inoculated cochleae. Original magnification 40 $\times$ ; scale bar, 25  $\mu$ m.

gradient as described previously [24]. The viral stock was treated with DNase and titrated by quantitative real-time PCR with plasmid standards [43].

*Surgical procedures and cochlear perfusions.* All animal studies were performed in accordance with the guidelines issued by the committee on animal research of Jichi Medical School and approved by its ethics



committee. Sixty female C57BL/6J mice (4 weeks of age; CLEA Japan, Tokyo, Japan) and 40 male ICR mice (2 months of age; Japan SLC, Shizuoka, Japan) were utilized. The mice were initially anesthetized with ketamine (50 mg/kg) and the analgesic xylazine (5 mg/kg). A postauricular approach was used to expose the tympanic bony bulla. A small opening (2 mm) in the tympanic bulla was carefully made to allow access to the round window membrane. In the tested groups, 5  $\mu$ l AAV vector solution ( $5 \times 10^{10}$  gc) was microinjected into the cochlea through the round window over 10 min with a glass micropipette (40  $\mu$ m in diameter) fitted on a Univentor 801 syringe pump (Serial No. 170182, High Precision Instruments, Univentor Ltd., Malta) [19]. A small plug of muscle was used to seal the cochlea and the surgical wound was closed in layers and dressed with antibiotic ointment. Five mice of each strain received control cochlear perfusions with artificial perilymph (145 mM NaCl, 2.7 mM KCl, 2 mM MgSO<sub>4</sub>, 1.2 mM CaCl<sub>2</sub>, 5 mM Hepes) alone. Each AAV-EGFP serotype was injected into five mice of each strain. Another 20 C57BL/6J mice were injected with the AAV3 vector to study long-term expression.

**Cochlear function assessment using ABR.** To assess the physiological status of experimental ears, auditory thresholds were determined with multiple frequency and intensity tone bursts by performing ABR audiometry with Tucker-Davis Technologies and Scope v3.6.9 software (Power Lab/200; ADInstruments, Castle Hill, Australia). Tone pipes were introduced into the operated ears of the anesthetized mice, and evoked potentials were recorded using needle electrodes inserted through the skin. ABR were elicited and measured 256 times at 4, 8, 12, 16, 20, and 24 kHz frequencies with tone bursts in systematic 5-dB steps. The rise/fall times for the tone bursts were 0.1 ms/rise/ms flat (cosine gate). Free-field system was used as a calibration procedure. Wave I was measured to analyze the activity from the cochlea. The lowest stimulus level that yielded a detectable ABR waveform was defined as the threshold. ABR were tested in the infused ear prior to surgery and 10 days postsurgery. Data were statistically analyzed using repeated-measures analysis of variance followed by paired Student's *t* test performed with StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). Values of *P* < 0.05 were considered significant.

**Histology.** Cochlear transgene expression patterns were determined for all AAV serotypes by visualizing EGFP expression. The animals were sacrificed 10 days after injection, and intracardiac perfusion was performed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The cochleae were harvested and the stapes footplates were removed. For AAV3-mediated transduction, the animals (five mice for each time point) were sacrificed 1, 2, 4, 8, or 12 weeks after inoculation. Postfixation was carried out in 4% PFA for 4 h at 4 °C, and decalcification was performed in 10% EDTA for 12 days at room temperature. The cochlear half-turns were microdissected and processed and the other half-turns were prepared by cryosection (10  $\mu$ m) to detect EGFP expression by using an Olympus IX70 (Olympus Corp., Tokyo, Japan) fluorescence microscope with a standard fluorescein isothiocyanate filter set and Studio Lite software (Olympus Corp.). Cells that exhibited fluorescence were considered positive for transgene expression. The level of expression was graded by fluorescence intensity on a four-point scale (+, ++, +++, +++) depending on the pixel/unit area count. Hair cell counts were carried out with dissected cochlea.

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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<sup>2</sup>. 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<sub>2</sub> 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<sup>2</sup>, 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; Tuyraerts *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<sub>2</sub> 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<sub>2</sub> 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<sup>2</sup> (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<sub>2</sub> incubator. First, cells were plated at  $2.3 \times 10^6$  cells per T-225 or at  $6.5 \times 10^7$  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<sub>2</sub> 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<sub>2</sub> 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 mM CaCl<sub>2</sub>. This solution was gently added to an equal volume of 2× HEPES-buffered saline (HBS: 290 mM NaCl, 50 mM HEPES buffer, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 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 pre-warmed fresh DMEM-F12 containing 2% FBS. Cell suspensions were collected 72 hr after transfection and centrifuged at  $300 \times g$  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 mM Tris-HCl [pH 8.0], 150 mM 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 ( $1 \times 10^{11}$  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<sub>2</sub>, 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<sub>2</sub> 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<sub>2</sub> 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<sup>2</sup>, a CF10 with a surface area of 6320 cm<sup>2</sup>, 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.

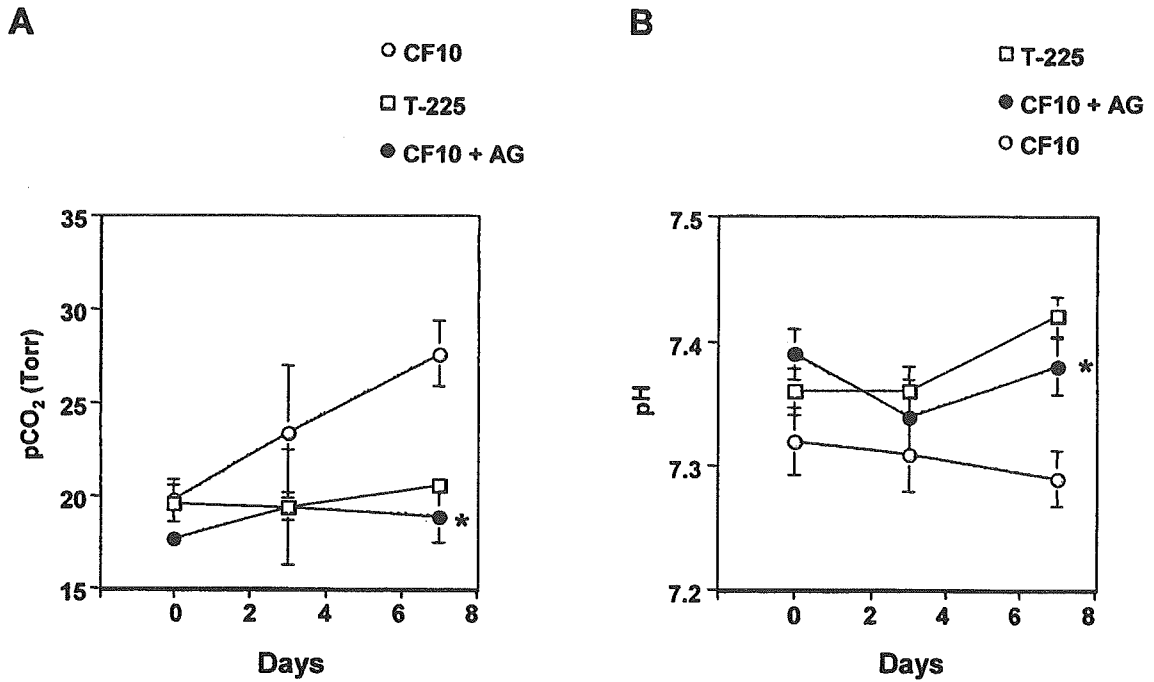
#### Efficient AAV vector production in a large culture vessel with active gassing

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<sub>2</sub> 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-

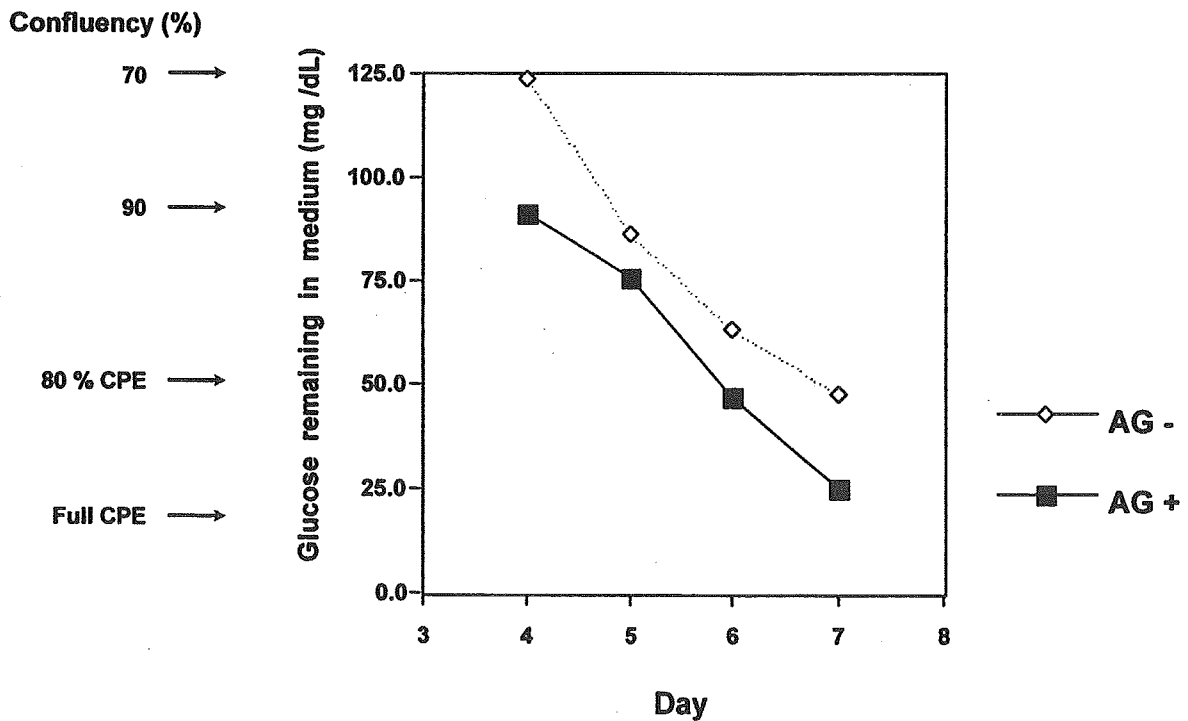
TABLE 1. INCREASED CELL GROWTH AND VECTOR YIELD WITH ACTIVE CO<sub>2</sub> AND AIR EXCHANGE<sup>a</sup>

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

<sup>a</sup>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<sup>2</sup> in the presence or absence of active gassing (AG) were compared with that in 28 flasks with a surface area of 225 cm<sup>2</sup> each.

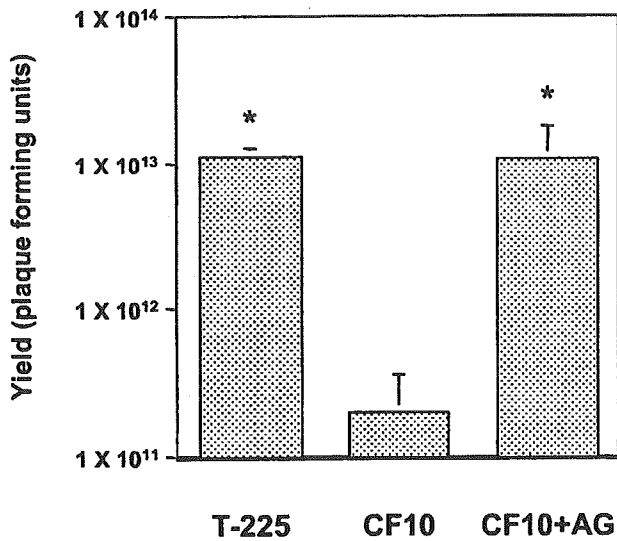


**FIG. 1.** Improved CO<sub>2</sub> and air exchange and maintenance of pH in conditioned medium after recombinant adenovirus infection. Subsequent to adenovirus infection in a normal flask with a surface area of 225 cm<sup>2</sup> (T-225) or a large culture vessel (a 10-tray Cell Factory [CF10] with a surface area of 6320 cm<sup>2</sup>) in the presence or absence of active gassing (AG), CO<sub>2</sub> 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.



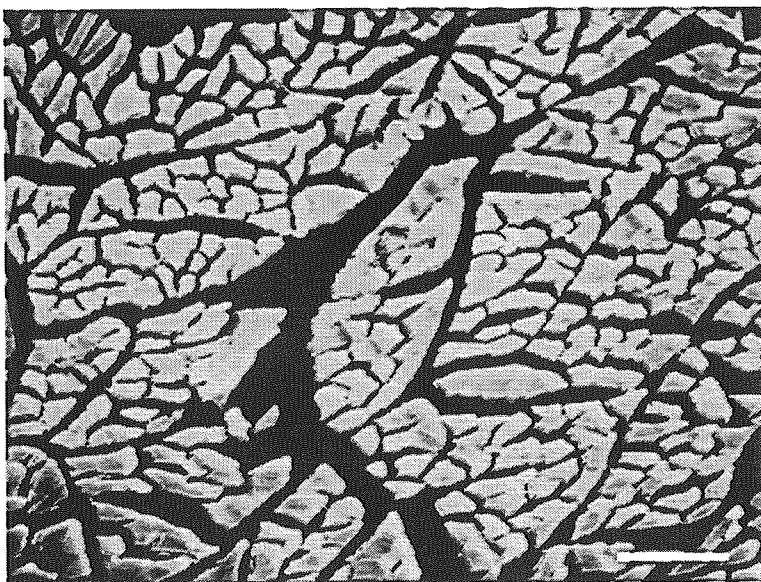


**FIG. 3.** Improved production of adenovirus vector. Adenovirus vector was propagated in 28 T-225 flasks ( $n = 4$ ), a CF10 ( $n = 3$ ), or a CF10 in the presence of active gassing (CF10 + AG,  $n = 3$ ). Adenovirus vector expressing an EGFP reporter gene was generated in two independent experiments. The average number of plaque-forming units (PFU) was assessed by TCID<sub>50</sub>. \* $p < 0.05$  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  $2.0 \times 10^{13}$  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) ( $1 \times 10^{11}$



**FIG. 4.** Transduction of muscles with AAV vectors *in vivo*. Five-week-old male Sprague-Dawley rats were injected with AAV1-EGFP ( $1 \times 10^{11}$  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  $\mu\text{m}$ .

**TABLE 2.** ENHANCED GAS EXCHANGE AND MAINTENANCE OF pH IN CONDITIONED MEDIUM AFTER PLASMID TRANSFECTION<sup>a</sup>

	$p\text{CO}_2$ (Torr)	pH
CF10	$25.6 \pm 1.1$	$7.23 \pm 0.03$
CF10 + AG	$14.2 \pm 0.1$	$7.40 \pm 0.01$

<sup>a</sup>Three days after plasmid transfection by using CF10 in the presence or absence of active gassing (AG), CO<sub>2</sub> concentrations and pH in the conditioned medium were estimated. Means  $\pm$  standard deviations are shown ( $n = 4$ ).

**TABLE 3.** IMPROVED YIELDS OF RECOMBINANT AAV TYPE 1 BY ACTIVE GAS EXCHANGE<sup>a</sup>

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

<sup>a</sup>Titers of AAV1-EGFP were determined as genome copies by dot-blot analysis of DNase-treated stocks. AG, active gassing. Means  $\pm$  standard deviations are shown ( $n = 4$ ).

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).

## DISCUSSION

Successful vector production in a large culture vessel was achieved by improvement of CO<sub>2</sub> 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-

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  $1.0 \times 10^{13}$  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<sub>2</sub> 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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## 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<sup>2</sup>. 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<sub>2</sub> 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<sup>2</sup>, could be suitable for scaling-up of

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