THE JOURNAL OF GENE MEDICINE
J Gene Med 2000; **2**: 444–454.

RESEARCH ARTICLE

Scaleable chromatographic purification process for recombinant adeno-associated virus (rAAV)

Catherine R. O'Riordan*
Amy L. Lachapelle
Karen A. Vincent
Samuel C. Wadsworth

Genzyme Corporation, 31 New York Avenue, Framingham, MA 01701-9322, USA

*Correspondence to: Catherine R. O'Riordan, Genzyme Corporation, 31 New York Avenue, Framingham, MA 01701-9322, USA.
E-mail: coriordan@genzyme.com

Received: 9 May 2000
Revised: 3 July 2000
Accepted: 4 July 2000
Published online: 6 July 2000

Abstract

Background Adeno-associated virus (AAV) is a human parvovirus currently being developed as a vector for gene therapy applications. Traditionally AAV has been purified from cell lysates using CsCl gradients; this approach however is not likely to be useful in large-scale manufacturing. Moreover gradient-purified AAV vectors tend to be contaminated with significant levels of cellular and adenoviral proteins and nucleic acid. To address the issue of purification we have developed a process scale method for the rapid and efficient purification of recombinant AAV (rAAV) from crude cellular lysates.

Methods The preferred method for the purification of rAAVβgal includes treatment of virally infected cell lysates with both trypsin and nuclease followed by ion exchange chromatography using ceramic hydroxyapatite and DEAE-Sepharose in combination with cellufine sulphate affinity chromatography.

Results Purification of rAAV particles from crude cellular lysates co-infected with adenovirus was achieved using column chromatography exclusively. Column-purified rAAV was shown to be greater than 90% pure, free of any detectable contaminating adenovirus, biologically active, and capable of directing efficient gene transfer to the lungs of both cotton rats and mice.

Conclusions This study demonstrates the feasibility of using column chromatography alone for the isolation of highly purified rAAV vector. The methods described here are advancements in procedures to purify rAAV and are adaptable for commercial production of clinical-grade rAAV vector.

Keywords adeno-associated virus; vector purification; gene therapy

Introduction

Recombinant adeno-associated virus (rAAV) vectors are being proposed as a gene transfer vehicle for an increasing number of human diseases. Over an extended period of time these vectors have been shown to transduce a variety of cells when administered directly to tissues and organs *in vivo* [1–12]. Long-term expression (1.5 years) after direct rAAV injection into immunocompetent mouse muscle and brain has been reported [4,13], while rAAV vectors have been tested in a clinical trial for gene therapy of cystic fibrosis without any signs of toxicity or immune complications [14]. One of the attractive features of rAAV vectors is that transduction and gene expression are persistent, presumably because rAAV integrates into the host cell chromosome [4–6,15]. In addition this vector system appears to elicit limited immune responses because of limited ability to transduce dendritic cells [16].

As the utility of rAAV as a gene therapy vector has been demonstrated, it is necessary to develop methods to improve both its production and purification. In an attempt to increase vector production, many studies have focused on optimization of the original three-component AAV packaging system described by Samulski *et al*. [17]. These studies focused on finding a beneficial balance between *rep* and *cap gene* expression from the packaging plasmid as a means of obtaining optimal rAAV production [18–21]. An additional modification of the original packaging system was introduced where the essential adenovirus genes were provided on a helper plasmid [22], while more recently a two-component packaging system has been described [21] which results in high-titer rAAV vector stocks without the production of detectable wild-type AAV or adenovirus. Another novel approach to overcoming the problem of producing high-titer rAAV was described by Gao *et al*. [23]. This approach involves use of a cell line, B50, stably transfected with a rep/cap-containing plasmid. B50 is infected with an adenovirus defective in E2b, followed by a hybrid virus where the AAV vector is cloned in the E1 region of a replication-defective adenovirus. This results in a 100-fold amplification and rescue of the AAV genome, leading to a high yield of recombinant AAV that is free of replication-competent AAV.

Many approaches to improve purification of rAAV vectors have also been reported. Considerable effort has been invested in developing protocols to purify rAAV by CsCl centrifugation [24,25] which has resulted in cleaner rAAV stocks, however CsCl gradients offer a variable degree of purification with poor vector yields. Furthermore, the volume of cell lysate required to produce useful amounts of rAAV vector cannot easily be processed using gradient centrifugation. More recently, improved methods of purification of rAAV have been reported which have used a biocompatible density gradient medium, iodixanol, either alone [26], or in combination with heparin or sulphate ion exchange chromatography [27]. Although these methods describe significant improvements over CsCl gradient purification, they still require gradient centrifugation which is not a feasible approach for large-scale commercial production. Clark *et al*. [28] describe a purification process which involves use of a single heparin sulphate chromatography step. A potential problem with use of this one affinity chromatography step is that many cellular proteins can associate with heparin making it necessary to design further purification methods to remove contaminating heparin-binding proteins.

In this study we describe methods to reproducibly purify rAAV. The procedure is rapid, yielding purified rAAV vector which is greater than 90% pure and free of detectable contaminating adenovirus. A significant advantage of this method is that it is scaleable for commercial purposes, being designed to process large volumes of cell lysate. In addition, column-purified rAAV is shown to maintain biological activity, directing efficient gene transfer to the lungs of both cotton rats and mice *in vivo*.

Materials and methods

Cell lines and viruses

The 293 cell line, an Ad type 5-transformed human embryonic kidney cell line [29], was propagated in Dulbecco's modified Eagle's medium-high glucose (DME), 20 mM glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml at 37°C and 5% CO_2. Ad type 5 mutant ts 149 (Ad5ts149) [30] used as a helper virus in these studies has reduced ability to replicate viral DNA at the non-permissive temperature (39°C) due to a temperature-sensitive mutation in the DNA polymerase encoded by Ad early region 2 [31]. Ad5ts149 was grown in 293 cells at the permissive temperature (33°C) and purified by CsCl gradient centrifugation.

Plasmid

Vector sequences are encoded by a plasmid, pNTC3CMV*β*gal, which was constructed by removing the AAV sequences between bp 275 (EcoRV) and 4498 (SnaBI) of the plasmid pNTC3M1 [32] and replacing them with a CMV-*β*gal-SV40polyA cassette isolated from the plasmid pCMV*β* (Clontech). To package rAAV-*β*gal, Rep and Cap proteins are provided in *trans* by the helper plasmid, p5rep-Δ-CMVcap in which the *rep* genes are expressed from the endogenous AAV promoters (p5, p19) but transcription of the *cap* gene is directed by the CMV promoter [20].

Large-scale transfection

Prior to transfection of 293 cells for large-scale growth of rAAV*β*gal, the cells were seeded in roller bottles such that they would reach 60–80% confluence on the day of transfection (final density was approximately 10^8 cells/bottle). Transfection was carried out in OptiMem medium using lipid #53 : DOPE [20]. Cells were infected with Ad5ts149 at a MOI of 20 at the time of transfection and incubated at 39°C for 48 h prior to harvest.

Chromatographic procedures

Column resins were tested for their separation characteristics using a Pharmacia FPLC.

Extraction of rAAV*β*gal from 293 cells

The cells were harvested and collected by centrifugation at 1000 *g* in a Sorvall RC-3B centrifuge. Cell pellets were frozen at −80°C for further use or were resuspended in 10 mM sodium phosphate, 10 mM NaCl, 10% glycerol, 2 mM $MgCl_2$, pH 6.4. Following resuspension the cells were treated with benzonase® (American International

Chemical, Inc., Natick, MA, USA) for 1 h at room temperature followed by trypsin treatment 0.05% for 1 h in the presence of 1% Tween-80. The resulting lysate was filtered through a 0.8 μm Millex HV filter unit before chromatography. Routinely 10–15 roller bottles were harvested equivalent to 1–5 e 9 cells.

BioRad ceramic hydroxyapatite (80 μm)

Cell lysates from 293 cells infected with adeno-associated virus (in the presence of Ad5ts149) were chromatographed on a hydroxyapatite resin (40 ml) which was pre-equilibrated with 10 mM sodium phosphate pH 6.4, containing 10 mM NaCl, 0.1% Tween-80, 10% glycerol and 2 mM $MgCl_2$. rAAVβgal was applied to the resin in the same buffer. Bound proteins were eluted from the resin using a linear salt gradient (120 ml) of 10–400 mM sodium phosphate at pH 6.4. Fractions collected from the resin were assayed for rAAVβgal proteins by immunoblotting using an antibody against the three capsid proteins of rAAVβgal, VP1, VP2 and VP3 (Catalog 03-65158, American Research Products, Belmont, MA, USA). Fractions eluted from the resin were also assayed for adenoviral-contaminating proteins by immunoblotting using an anti-adenoviral antibody.

DEAE chromatography

A DEAE Macroprep resin (BioRad) (5 ml) was equilibrated with 10 mM sodium phosphate buffer containing 50 mM NaCl, 10% glycerol, pH 7.5. rAAVβgal-containing fractions eluted from the hydroxyapatite column were pooled and dialyzed into the same buffer used for equilibration of the DEAE resin. A linear gradient (50 mM–1 M NaCl in 10 mM sodium phosphate, pH 7.5, 10% glycerol, 0.05% Tween-80) was applied to the resin at a flow rate of 5 ml/min, the volume of the gradient was 50 ml. Bound proteins were eluted from the resin and collected in 2.5-ml fractions. Each fraction was assayed for rAAVβgal proteins and contaminating adenoviral proteins (Coomassie blue staining and immunoblotting). In addition all fractions were assayed for rAAVβgal and Ad5ts149 infectivity by titer analysis. Fractions which were positive for both rAAVβgal protein and infectivity were pooled and chromatographed further using a Cellufine sulphate resin.

Cellufine® sulphate resin (Amicon)

Cellufine sulphate resin (3 ml column volume) was equilibrated with phosphate buffer saline (PBS) containing 10% glycerol. Fractions eluted from the DEAE resin containing both rAAVβgal proteins and DNA were pooled and applied to the resin at a flow rate of 4 ml/min. The resin was washed with 250 mM NaCl and a linear salt gradient (0.25–1 M NaCl in PBS/10% glycerol) was applied. The volume of the gradient was 10 ml. The eluted fraction and the flow through were analyzed for both rAAVβgal and Ad5ts149 proteins (immunoblotting) and infectivity (titer analysis). The final fraction was also assayed for rAAVβgal DNA by slot blot analysis.

SDS-PAGE analysis

One-dimensional SDS-PAGE was performed using 10–20% gradient (Daiichi) gels. Samples were boiled for 5 min in SDS-PAGE reducing buffer 125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% (wt/vol) SDS, 0.05% bromophenol blue, 0.5% β-mercaptoethanol before loading on gel. Proteins in the gel were detected using Coomassie blue or silver stain. For immunoblotting, PVDF membranes (Novex) were pre-wetted with methanol and soaked in 10 mM CAPS, pH 11.0 containing 10% methanol. Gels were equilibrated in this transfer buffer for 10 min and then blotted at 30 V for 1 h in a Novex Blot Module. After transfer membranes were blocked with 1% dried milk in TBS (20 mM Tris-HCl, pH 7.5 containing 150 mM NaCl) for 1 h. After blocking, the membranes were probed with anti-VP1, VP2, VP3 antibody or anti-adenoviral antibody in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 and 0.05% Tween 20 (TBST) containing 0.1% BSA for 1 h. The membranes were incubated with horseradish peroxidase labeled anti-mouse or anti-goat IgG for 45 min and the immunoreactive bands visualized by chemiluminescence using the BM Chemiluminescent Western Blotting Detection System (Boehringer Mannheim).

Measurement of infectious particles

The rAAVβgal titer was determined by an end-point dilution assay on 293 cells. In brief, 293 cells were plated into a 96-well micro-titer plate, 100 μl of 5 e 5 cells/ml for each well. In a separate plate a 200-μl aliquot of virus sample diluted 1 : 100 was added to the first column and was serially diluted two-fold across the plate. A 100 μl-aliquot of each well was transferred to its identical position in the 293 seeded plate and allowed to incubate at 37°C in a humidified air 5% CO_2 incubator for 2 days. The media was removed by aspiration and the cells were stained with X-gal (5 bromo-4-chloro-3-indol-β-D-galactopyranoside) for 20–24 h. Titers were calculated with a computer program based on Karber's method [33]. The titer of contaminating Ad5ts149 was determined using a similar end-point dilution assay except staining was for hexon using fluorescence isothiocyanate (FITC)-conjugated anti-hexon antibody [34].

Slot blot analysis of column fractions for detection of rAAV DNA

Column fractions were assayed for rAAVβgal DNA by slot blot analysis as described by Vincent *et al.* [20].

In vivo measurement of β-galactosidase expression

The activity of rAAVβgal particles was also assessed by measuring the level of gene transfer (β-galactosidase) into the lungs of mice and cotton rats. BALB/c mice were instilled intranasally with 4 e 4 IU of rAAVβgal either alone or in the presence of 2 e 8 IU wtAd 5 (three animals per treatment group). For comparison animals were also administered Ad2/βgal 2 (adenovirus 2 vector containing a nuclear-localized β-galactosidase gene driven by the CMV promoter) at 1 e 7 IU. At 3 days post-administration, animals were sacrificed and the right caudal lobe of the lung was analyzed for β-galactosidase activity by the AMPGD assay while the left lobe was fixed and stained with X-gal. Adult cotton rats were instilled intranasally with rAAVβgal virus (non-nuclear localized β-galactosidase, 6.4 e 4 IU per cotton rat) both alone and in the presence of Ad5ts149 (5 e 7 IU). Animals were sacrificed 2 days post-administration (three animals per treatment group). Prior to X-gal staining the left lobes of the lungs were fixed by instillation in 2% formaldehyde and 0.2% glutaraldehyde. The right lobes were taken for biochemical analysis (AMPGD assay) for β-galactosidase activity.

Results

Characterization of recombinant AAV binding to chromatography media: ceramic hydroxyapatite chromatography

Following lysis of rAAVβgal-infected cells in the presence of trypsin, the lysate was treated with the nuclease benzonase® which enzymatically degrades host cell, non-encapsidated or incomplete rAAVβgal nucleic acids [35]. Trypsin treatment of cell lysates greatly reduced the viscosity of the lysate causing less rAAVβgal aggregation while allowing greater separation of rAAVβgal from adventitious proteins during column chromatography.

Cell lysates from 293 cells producing rAAVβgal (in the presence of Ad5ts149) were chromatographed on a BioRad ceramic hydroxyapatite resin (CHA) pre-equilibrated with 10 mM sodium phosphate, pH 6.4 containing 10 mM NaCl and 10% glycerol. Both rAAVβgal and Ad5ts149 bound to the CHA resin and were eluted using an increasing salt gradient (10–400 mM sodium phosphate, pH 6.4). Figure 1 shows a typical elution profile. rAAVβgal was resolved into a sharp peak and eluted from the resin at 125 mM sodium phosphate. Fractions across the peak were pooled and assayed for rAAVβgal proteins using an antibody against the three capsid proteins, VP1, VP2 and VP3. Recovery of rAAVβgal infectious particles was determined by titer analysis. The rAAVβgal peak contained only 5% of the total protein initially present in the whole cell lysate while 100 % of the rAAVβgal activity was recovered (Table 1). Table 2 shows the average recovery of protein and rAAV activity for five purification runs; typically 11% of total protein is recovered following CHA chromatography with a 88% recovery of rAAV activity. SDS-PAGE analysis of the pooled fraction (Figure 4A, lane 2) showed that there were still contaminating proteins co-eluting with the rAAVβgal which included Ad5ts149 proteins as determined by immunoblotting (data not shown). The majority of Ad5ts149 proteins and infectious Ad5ts149 particles eluted from the CHA resin over a broad range of concentration of sodium phosphate. Although CHA chromatography was not effective at resolving rAAVβgal

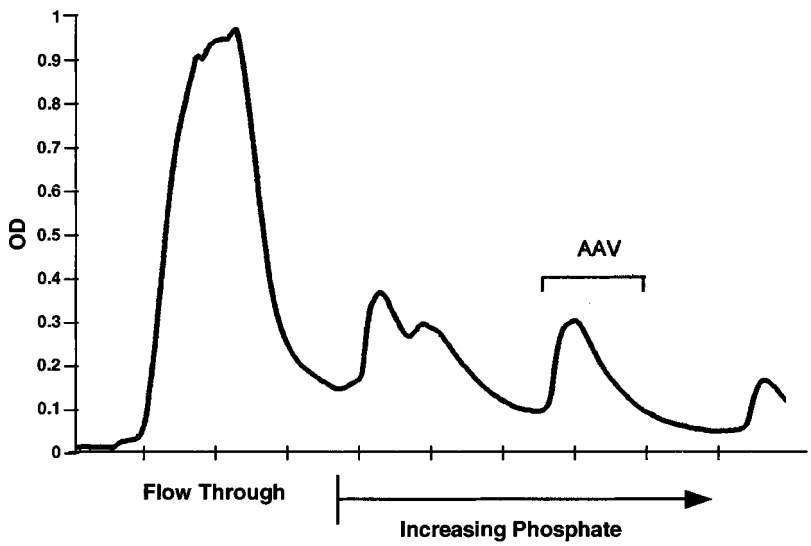

Figure 1. Hydroxyapatite chromatography of 293 cell lysate containing rAAVβgal. Representative profile of 280 nm absorbance of fractions eluted from the hydroxyapatite resin using a sodium phosphate gradient is shown. A gradient of sodium phosphate (10–400 mM, pH 6.4) was applied to the resin and the eluted fractions were tested for rAAVβgal proteins by Western blot analysis and infectivity by titer analysis. By these criteria rAAVβgal eluted from the resin at 125 mM sodium phosphate and is indicated on the elution profile. Fractions across the rAAVβgal peak were pooled and further purified by DEAE-chromatography

Table 1. Summary of chromatographically purified rAAVβgal ($n=2$)

Sample	HA load	HA eluate	DEAE eluate	CS eluate
Total protein (mg)	236	13	5	0.6
Volume (ml)	110	55	10	2
Total rAAVβgal (IU)	3.92 e 9	7.24 e 9	2.47 e 9	1.11 e 9
Total Ad5ts149 (IU)	3.75 e 8	3 e 7	6 e 6	4.8 e 4
Percentage Ad5ts149 activity removed	NA	90%	80%	>99%

Total >99.99% of contaminating Ad5ts149 activity removed. The average Ad5ts149 activity remaining after a three-column purification run is 4.8e4 ($\pm$4e4), $n=2$. Ad5ts149 activity represents 0.03% $\pm$0.015% of the total rAAVβgal activity.

Table 2. Column performance (average of five runs)

Sample	Percentage protein remaining[a]	Percentage protein cumulative[b]	Percentage rAAV activity[a]	Percentage rAAV activity cumulative[b]
HA load	100	100	100	100
HA eluate	11	11	88	88
DEAE eluate	30	3	60	52.8
CS eluate	8	0.4	48	30

[a]Individual column performance.
[b]Cumulative performance.

particles from Ad5ts149 particles, greater than 90% of the Ad5ts149 infectivity is removed with this one chromatographic step (Table 1).

DEAE Ion exchange chromatography: methods to separate Ad5ts149 from rAAVβgal

Separation of rAAVβgal from Ad5ts149 and contaminating proteins was achieved by DEAE ion exchange chromatography. The pooled rAAVβgal fractions from the hydroxyapatite resin were dialyzed into 10 mM sodium phosphate, pH 7.5 containing 50 mM NaCl, 10% glycerol and 0.05% Tween-80 and applied to a DEAE resin which was pre-equilibrated with the same buffer. Both rAAVβgal and Ad5ts149 bound to the resin and were resolved into two separate peaks following elution with a salt gradient (50 mM–1 M NaCl). rAAVβgal eluted at 200 mM salt (Figure 2) while the Ad5ts149 remained more tightly bound to the resin eluting later at a higher salt concentration (Figure 2). Thus rAAVβgal and Ad5ts149 can be effectively separated using DEAE ion exchange chromatography. The recovery of rAAVβgal infectious particles over the DEAE column was 30% (Table 1) with only 2% of the protein present in the initial whole cell lysate remaining. On average the cumulative recovery of protein following DEAE chromatography was 3% with a 52.8% recovery of rAAV infectious particles (Table 2). SDS-PAGE analysis of the rAAVβgal-containing fraction from the DEAE resin (Figure 4A, lane 3) indicated that there were still some residual contaminating proteins co-purifying with the rAAVβgal. Western blot analysis of this rAAVβgal fraction using an anti-adenoviral antibody showed no detectable contaminating Ad5ts149 proteins, however titer analysis indicated that there was a low level of contaminating Ad5ts149 infectious particles. This remaining Ad5ts149 activity was less than 20% of the total activity loaded onto the resin (Table 1). Thus DEAE-Sepharose was an effective resin at removing both cellular and Ad5ts149 proteins. To reduce the level of Ad5ts149 infectious particles further, affinity chromatography using a cellufine sulphate resin was used.

Cellufine sulphate chromatography

Cellufine sulphate is a cellulose matrix with sulfonate groups esterified at the number 6 carbon of the repeating glucose subunits. Binding of proteins to this resin is

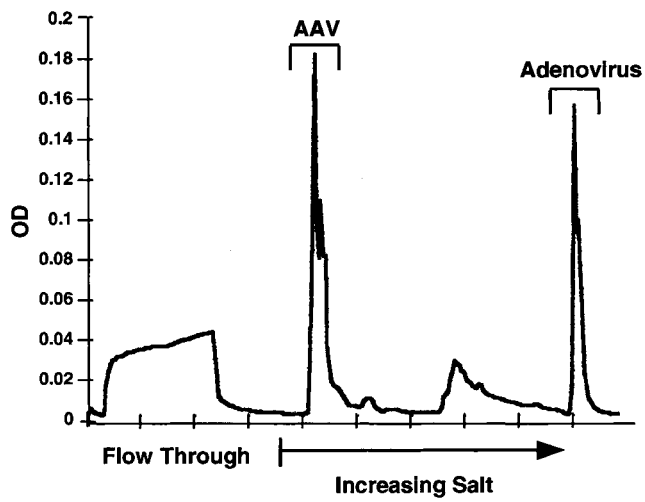

Figure 2. Ion exchange chromatography of rAAVβgal using DEAE. The pooled rAAVβgal fractions eluted from the hydroxyapatite resin were dialyzed into 10 mM sodium phosphate, pH 7.5 containing 50 mM NaCl, 10% glycerol and 0.05% Tween-80 and applied to a DEAE resin equilibrated with the same buffer. A representative profile of 280 nm absorbance of fractions eluted from the DEAE resin using an increasing salt gradient (50 mM–1 M NaCl) is shown. rAAVβgal eluted from the resin at 200 mM salt and is indicated on the profile. Fractions across the rAAVβgal peak were pooled and further purified using a cellufine sulphate resin

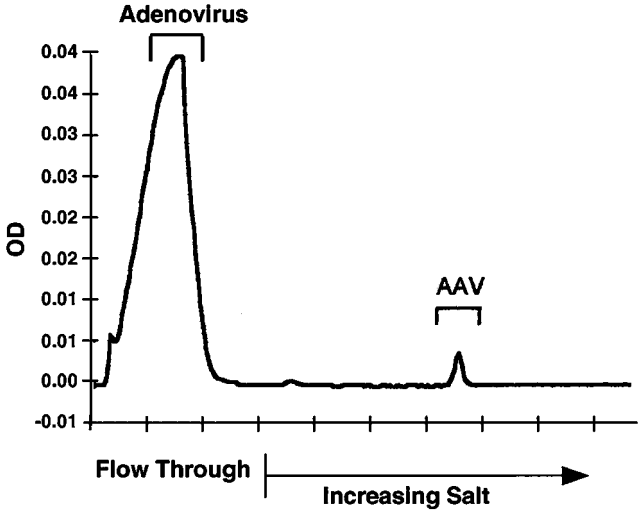

Figure 3. Cellufine sulphate chromatography of rAAVβgal. The pooled rAAVβgal fractions eluted from the DEAE resin were applied to a cellufine sulphate resin. A representative profile of 280 nm absorbance of fractions eluted from the cellufine sulphate resin using an increasing salt gradient (0.25–1 M NaCl in PBS) is shown. rAAVβgal eluted from the resin at approximately 475 mM NaCl

thought to occur through the polysaccharide moieties thereof. We predicted that rAAVβgal should bind to the cellulose sulphate matrix as the native receptor for AAV has been shown to contain sialic acid. Treatment of cells with neuraminidase prevents AAV attachment, suggesting that the sialic acid residue is important for AAV binding [36]. More recently a membrane-associated heparan sulphate proteoglycan was identified as a receptor for AAV [37], while several groups have demonstrated the use of heparin sulphate as an affinity matrix for the purification of rAAV particles [27,28]. Under the buffer conditions used, rAAVβgal bound to the cellufine sulphate resin and was eluted at 475 mM salt (Figure 3), while Ad5ts149 was recovered in the flow through. Table 1 shows the results for recovery of rAAVβgal and Ad5ts149 infectious particles following chromatography on the cellufine sulphate resin. Approximately 45% of the rAAVβgal activity was recovered in the cellufine sulphate eluate while less than 1% of the applied Ad5ts149 activity was recovered. Figure 4A, lane 5 shows the SDS-PAGE analysis of the final fraction of purified rAAVβgal. In summary, cellufine sulphate reduced the level of contaminating Ad5ts149 by greater than 99% (Table 1) in addition to reducing other cellular protein contaminants. Thus the Ad5ts149 titer was decreased from 1.2 e 9 IU in the crude lysate to 4.8 e 4 IU ($\pm$4e4) after a three-column purification (compare with rAAVβgal titer of 1.11 e 9 of infectious particles). Another important consequence of the cellufine sulphate chromatography was concentration of the rAAVβgal for further use in *in vitro* and *in vivo* studies.

Figure 4A shows an enrichment of rAAVβgal purified by column chromatography while Figure 4B shows an immunoblot of the same fractions. rAAVβgal was estimated to be greater than 90% pure by densitometry of a Coomassie stained gel (Figure 4C), while silver stain analysis of the purified rAAVβgal showed no remaining contaminating proteins (Figure 4D). The overall yield of rAAVβgal infectious units using column chromatography was approximately 30% (Tables 1 and 2). This is a significant improvement over the yield of virus achieved using ultracentrifugation/CsCl purification which routinely is 10–12% (data not shown). The particle : IU ratio of the rAAVβgal purified by column chromatography was on average about 75 suggesting that column purification of rAAVβgal did not enrich for empty particles. Thus rAAVβgal can be purified to high yield and purity by column chromatography. Further characterization of the column-purified rAAVβgal was achieved using *in vivo* experiments with cotton rats and mice.

Co-administration of rAAVβgal and Ad5ts149 to the cotton rat and mouse lung

Since we could show that rAAVβgal purified by column chromatography remained infectious for cells in culture, the next step was to study the infectivity of column-purified rAAVβgal virus *in vivo*. Adult cotton rats and BALB/c mice were instilled intranasally with rAAVβgal virus (non-nuclear localized β-galactosidase) either alone or in the presence of Ad5ts149 or wtAd5. Co-infection with a replication-competent adenovirus was carried out to study the adenovirus-mediated enhancement of rAAV transduction previously observed *in vitro* and *in vivo* [38,39]. Figures 5 and 6 show the levels of β-galactosidase activity (RLU mg/protein) that were measured in the lungs of these animals. Co-infection of rAAVβgal with Ad5ts149 enhanced the rAAV-mediated gene transfer to the lungs of cotton rats; this is reflected in the increased levels of β-galactosidase in lung homogenates from these animals (Figure 5). Similarly in mice, co-administration of wtAd5 with rAAVβgal to the lung enhanced AAV-mediated gene transfer (Figure 6). The amount of gene expression achieved with 4 e 4 IU rAAVβgal is similar to that observed with 1 e 7 IU Ad2/βgal 2 alone (Figure 6). In order to determine which cells were targeted in the lung, X-gal staining was performed on cotton rat lungs that had been instilled with rAAVβgal and Ad5ts149. The lungs were fixed by inflation in 2% formaldehyde and 0.2% glutaraldehyde prior to staining. Figure 7A shows an outer view of X-gal stained cotton rat lungs that were instilled with rAAVβgal with Ad5ts149, while Figure 7B shows a dissected lung from the same animal revealing X-gal staining of lung airways. To demonstrate that rAAV vectors can accomplish gene transfer to the appropriate cells in the lung, a dissected lung from the cotton rat that received both rAAVβgal and Ad5ts149 was embedded in plastic, subjected to histological sectioning and counter-stained with nuclear fast red. Figure 7C shows positive staining cells in the airway epithelium suggesting that rAAVβgal can infect this cell type.

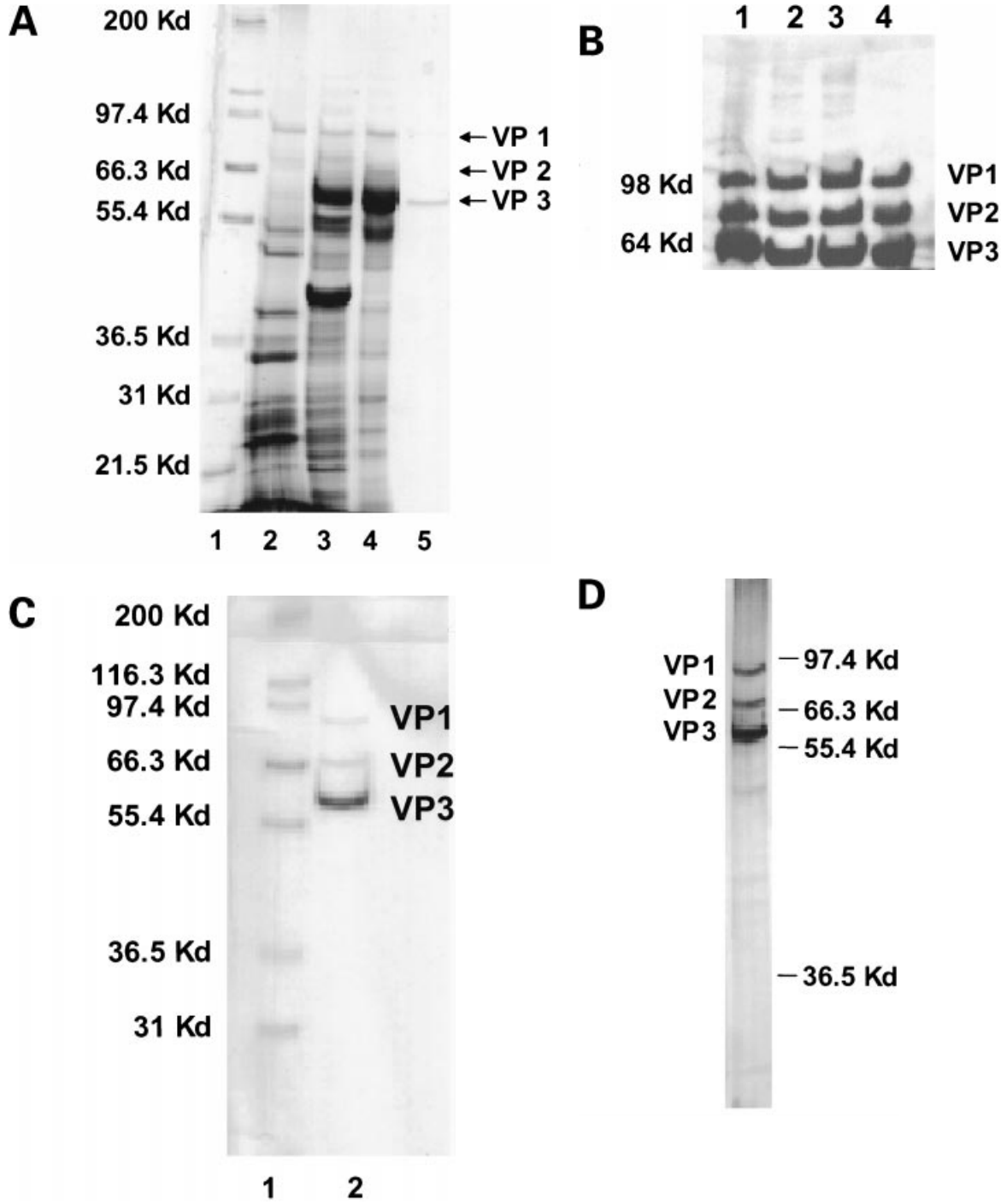

Figure 4. Analysis of purified rAAV*β*gal. (A) Aliquots containing 0.5% (volume) of the pooled rAAV*β*gal-containing fraction from each resin were subjected to SDS-PAGE (10–20% acrylamide) followed by Coomassie blue staining. Lane 1: Molecular weight standards; lane 2: hydroxyapatite load (293 cell lysate containing rAAV*β*gal); lane 3: rAAV*β*gal-containing fractions eluted from the hydroxyapatite resin (also DEAE resin load); lane 4: rAAV*β*gal-containing fractions eluted from the DEAE resin (also cellufine sulphate load); lane 5: rAAV*β*gal-containing fractions eluted from the cellufine sulphate resin. (B) Immunoblot of rAAV*β*-gal-containing fractions (shown in 4A); Lane 1: hydroxyapatite load (293 cell lysate containing rAAV*β*gal); lane 2: rAAV*β*gal-containing fractions eluted from the hydroxyapatite resin (also DEAE resin load); lane 3: rAAV*β*gal-containing fractions eluted from the DEAE resin (also cellufine sulphate load); lane 4: rAAV*β*gal-containing fraction eluted from the cellufine sulphate resin. (C) Approximately 100 µg rAAV*β*gal purified by hydroxyapatite, DEAE and cellufine sulphate chromatography was concentrated and electrophoresed on a SDS-PAGE gel (10–20%) and stained using Coomassie blue. Densitometric analysis of this gel determined that the rAAV*β*gal was greater than 90% pure. (D) Silver stain analysis of rAAV*β*gal (1 µg) purified by hydroxyapatite, DEAE and cellufine sulfate chromatography

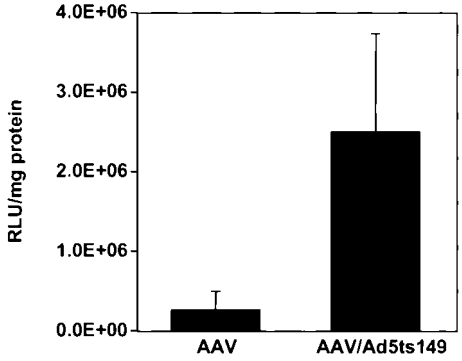

Figure 5. Co-administration of rAAV*β*gal and Ad5ts149 to the cotton rat lung. Adult cotton rats were instilled intranasally with rAAV*β*gal virus (non-nuclear localized *β*-galactosidase, 6.4 e 4 IU per cotton rat) both alone and in the presence of 5 e 7 IU Ad5ts149 (contains a temperature-sensitive mutation in the DNA polymerase encoded by adenovirus early region 2). Animals were sacrificed 2 days post-administration and the right lobes of the lungs were removed for biochemical analysis (AMPGD assay for *β*-galactosidase activity) while the left lung was fixed and stained for X-gal (see Figure 7). The level of *β*-galactosidase activity measured in the lungs of these animals is expressed as RLU (relative light units) per milligram of lung tissue. Values are the mean ± SD

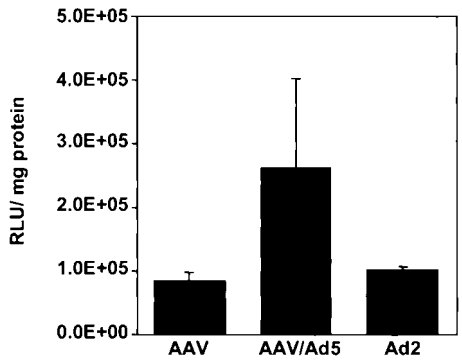

Figure 6. Co-administration of rAAV*β*gal and wtAd5 to the mouse lung. Balb/c mice were instilled intranasally with 4 e 4 IU rAAV*β*gal either alone or in the presence of 2 e 8 IU wtAd5. For comparison, animals were also administered Ad2/*β*gal2 (adenovirus 2 vector containing a nuclear localized *β*-galactosidase gene driven by the CMV promoter) at a dose of 1 e 7 IU. Three days post-administration the animals were sacrificed and the right caudal lobe of the lung was analyzed for *β*-galactosidase activity by the AMPGD assay. The level of *β*-galactosidase activity measured in the lungs of these animals is expressed as RLU (relative light units) per milligram of lung tissue. Values are the mean ± SD

Discussion

Evaluation of rAAV-based vectors for gene therapy requires supplies of purified high titer virus for escalated dose and repeated dose animal studies. Here we have described procedures for the purification of rAAV using column chromatography, which is a prerequisite for the production of large quantities of this vector. In addition we show that rAAV purified by column chromatography can infect the lung cells of both cotton rats and mice *in vivo*, thus demonstrating its potential use as a vector for gene therapies of the lung.

The preferred process for the purification of rAAV*β*gal includes treatment of the virally infected cell lysate with both trypsin and nuclease followed by ion exchange chromatography using ceramic hydroxyapatite and DEAE-Sepharose. A final purification step was achieved using a cellufine sulphate resin. Before chromatography, treatment of the cell lysate with benzonase was necessary to remove unwanted DNA. Further treatment of the lysate with trypsin improved both the purity and yield of rAAV*β*gal during column purification and also greatly facilitated the clarification of cell lysates by filtration. This we attribute to decreased interaction of rAAV with adventitious proteins and less aggregation of rAAV particles.

Ceramic hydroxyapatite chromatography was the most effective method for initial purification of rAAV*β*gal. The advantages of the CHA resin include its high protein binding characteristics and its scaleability; thus the first step in chromatography of rAAV*β*gal allows the processing of large quantities of virally infected cell lysates. Although CHA chromatography was not effective at separating Ad5ts149 from rAAV*β*gal, it resulted in inactivation of Ad5ts149 particles with a concomitant reduction (>90%) in Ad5ts149 titer (Table 1). Inactivation of Ad5ts149 particles following CHA chromatography may be a consequence of the architecture of this resin. Fractionation of the adenoviral capsid (70–80 nm diameter) into 80 nm-diameter pores in the CHA resin likely occurs, possibly resulting in damage to the fiber protein with subsequent loss of infectivity.

The procedure described here for the preparation and initial purification of virally infected cell lysates offers several advantages over existing procedures [27,28]. Clark *et al*. [28] lysed vector producer cells by exposing them to deoxycholate detergent. The clarified lysate was heated to inactivate the helper adenovirus used in their production of rAAV. This combination of detergent lysis and heat treatment is likely to be responsible for denaturation and removal of potential contaminants. In contrast, Zolotukhin *et al*. [27] used a protocol whereby cell lysate was prepared by successive freeze/thaw cycles followed by gradient purification using iodixanol. Although both procedures offer considerable advancements over previous protocols and provide practicable approaches for the purification of research grade vector, it is not clear whether they could easily be scaled for commercial production of rAAV vectors.

An additional ion exchange resin was necessary for resolution of rAAV*β*gal particles from Ad5ts149 particles; this was achieved by chromatography on DEAE-Sepharose. Overall the characteristics of DEAE chromatography were very consistent and reproducible; the recovery of rAAV*β*gal infectious particles was typically in the range 30–60% (Tables 1 and 2). The loss of virus with this resin could be attributed to selective pooling of the column fractions, however there was significant purification of rAAV*β*gal with removal of both irrelevant

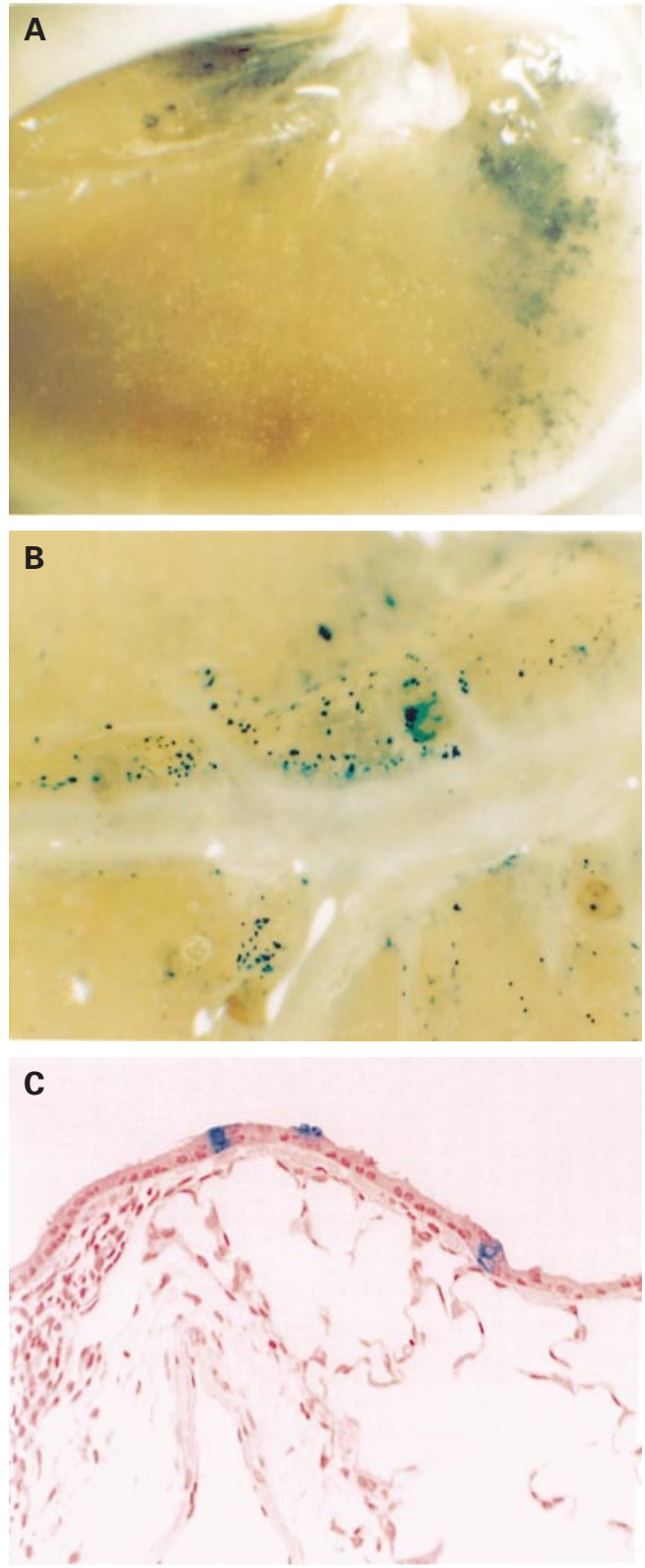

Figure 7. Localization of gene expression in the lungs of cotton rats. The left lungs of the cotton rats instilled intranasally with rAAV*β*gal in the presence of Ad5ts149 were fixed by inflation in 2% formaldehyde and 0.2% glutaraldehyde prior to X-gal staining. (A) An outer view of a lung from an animal that received both rAAV*β*gal and Ad5ts149. (B) A dissected lung from an animal that received both rAAV*β*gal and Ad5ts149. (C) The lungs in (A) and (B) were embedded in plastic, subjected to histological sectioning and counterstained with nuclear fast red. The photograph shows positive-staining cells in the airway epithelium

cell proteins and Ad5ts149 proteins. Final purification and concentration of rAAV*β*gal was achieved using a cellufine sulphate resin, an affinity matrix with binding properties mediated by sulphated polysaccharide groups. It is likely that the recently described heparin affinity resin [27,28] could be substituted for this cellufine sulphate

step. Heparin is a heterogeneous carbohydrate molecule composed of long unbranched polysaccharides modified by sulfations and acetylations and should therefore have similar binding characteristics to cellufine sulphate. The use of such an affinity matrix, which uses a molecule that is essentially analogous to the natural receptor of the virus [37], would likely achieve even more selective purification of rAAV particles.

The use of chromatography for the purification of recombinant adeno-associated viruses for use in gene therapies provides an effective alternative to CsCl density gradient ultracentrifugation. There are several advantages related to this methodology, but most importantly it consistently yielded rAAV that had a particle : IU ratio of less than 100. This is a significant improvement over purification methods involving CsCl which can yield rAAV virus preparations with particle : IU ratios up to 1000 [28]. Moreover, rAAVβgal which was highly purified by column chromatography was shown to infect lung cells in both the cotton rat and mouse suggesting that the methods described here produce biologically active rAAV particles capable of directing efficient gene transfer. An important aspect of the combination of the purification steps identified in this study is that each can be adapted for high-volume, clinical-grade vector production which produces rAAV that is greater than 90% pure (average duration of the purification procedure is 1.5 days). In addition the procedure described here can be used to purify rAAV vector in production methods where adenovirus is required. Other recently reported methods for column purification of rAAV [26,27] include a gradient centrifugation step, which is not easily adapted to the large-scale production of vector. Similarly, Clark *et al*. [28] describe a purification method using a single affinity chromatography step yielding rAAV, which would be unlikely to meet the necessary requirements for purity of a clinical-grade vector.

In conclusion, advancements in procedures both to produce and purify rAAV vectors makes the production of biologically active rAAV stocks for gene transfer studies in both preclinical and clinical studies a more achievable goal.

Acknowledgements

We thank the Gene Transfer Analysis group at Genzyme for their participation in the β-galactosidase and histological assays, and Terry Choquette for her participation in assay development.

References

1. Flotte TR, Afione SA, Conrad C, *et al*. Stable *in vivo* expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc Natl Acad Sci U S A* 1993; **90**: 10613–10617.
2. Kaplitt MG, Leone P, Samulski RJ, *et al*. Long-term gene expression and phenotypic correction using an adeno-associated virus vector in the mammalian brain. *Nat Genet* 1994; **8**: 148–154.
3. Kessler PD, Podsakoff GM, Chen X, *et al*. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc Natl Acad Sci U S A* 1996; **93**: 14082–14087.
4. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* 1996; **70**: 8098–8108.
5. Clark KR, Sferra TJ, Johnson PR. Recombinant adeno-associated virus vectors mediate long-term transgene expression in muscle. *Hum Gen Ther* 1997; **8**: 659–669.
6. Fisher KJ, Jooss K, Alston J, *et al*. Recombinant adeno-associated virus for muscle directed gene therapy. *Nat Med* 1997; **3**: 306–312.
7. Flannery JG, Zolotukhin S, Vaquero MI, *et al*. Efficient photoreceptor targeted gene expression *in vivo* by recombinant adeno-associated virus. *Proc Natl Acad Sci U S A* 1997; **94**: 6916–6921.
8. Koeberl DD, Alexander IE, Halbert CL, *et al*. Persistent expression of human clotting facter IX from mouse liver after intravenous injection of adeno-associated virus vectors. *Proc Natl Acad Sci U S A* 1997; **94**: 1426–1431.
9. Snyder RO, Miao CH, Patjin GA, *et al*. Persistent and therapeutic concentration of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat Genet* 1997; **16**: 270–276.
10. Greelish JP, Su LT, Lankford EB, *et al*. Stable restoration of the sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector. *Nat Med* 1999; **5**: 439–443.
11. Dudus L, Anand V, Acland GM, *et al*. Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. *Vision Res* 1999; **39**: 2545–2553.
12. Bennett J, Maguire AM, Cideciyan AV, *et al*. Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina. *Proc Natl Acad Sci U S A* 1999; **96**: 9920–9925.
13. McCown TJ, Xiao X, Li J, *et al*. Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res* 1996; **713**: 99–107.
14. Flotte T, Carter B, Conrad C, *et al*. A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. *Hum Gene Ther* 1996; **7**: 1145–1159.
15. Miao CH, Snyder RO, Schowalter DB, *et al*. The kinetics of rAAV integration in the liver. *Nat Genet* 1998; **19**: 13–15.
16. Jooss K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol* 1998; **72**: 4212–4223.
17. Samulski RJ, Chang LS, Shenk T. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J Virol* 1989; **61**: 3096–3101.
18. Flotte TR, Barraza-Ortiz X, Solow R, *et al*. An improved system for packaging recombinant adeno-associated virus vectors capable of *in vivo* transduction. *Gene Ther* 1995; **1**: 29–37.
19. Li J, Samulski RJ, Xiao X. Role of highly regulated *rep* gene expression in adeno-associated virus vector production. *J Virol* 1997; **71**: 5236–5243.
20. Vincent KA, Piraino ST, Wadsworth SC. Analysis of recombinant adeno-associated virus packaging and requirements for *rep* and *cap* gene products. *J Virol* 1997; **71**: 1897–1905.
21. Grimm DK, Kern A, Rittner K, Kleinschimdt JA. Novel tools for production and purification of recombinant AAV vectors. *Hum Gene Ther* 1998; **9**: 2745–2760.
22. Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 1998; **72**: 2224–2232.
23. Gao GP, Qu G, Faust LZ, *et al*. High-titer adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus. *Hum Gen Ther* 1998; **9**: 2353–2362.
24. Bartlett JS, Xiao X, Samulski RJ. Adeno-associated virus vectors for gene transfer. In *Protocols for Gene Transfer in Neuroscience: Towards Gene Therapy of Neurological Disorders*, Loewestein PR, Enquist LW (eds). Wiley: New York, 1996; 115–130.
25. Snyder RO, Xiao X, Samulski RJ. Production of recombinant adeno-associated viral vectors. In *Current Protocols in Human Genetics*, Dracopoli N (ed.). Wiley: New York, 1996; 12.1.1–12.1.20.
26. Hermens W, Brake OT, Duzhuizen PA, *et al*. Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation

of vector stocks for gene transfer in the nervous system. *Hum Gene Ther* 1999; **10**: 1885–1891.
27. Zolotukhin S, Byrne BJ, Mason E, *et al*. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* 1999; **6**: 973–985.
28. Clark RK, Liu X, McGrath JP, Johnson PR. Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Hum Gene Ther* 1999; **10**: 1031–1039.
29. Graham FL, Smiley J, Russel WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 1977; **36**: 59–74.
30. Einsinger MJ, Ginsberg, HS. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. *J Virol* 1972; **10**: 328–339.
31. Stillman BW, Tamanoi F, Mathews MB. Purification of an adenovirus-coded DNA polymerase that is required for initiation of DNA replication. *Cell* 1982; **31**: 613–623.
32. Chejanovsky N, Carter BJ. Replication of a human parvovirus nonsense mutant in mammalian cells containing an inducible amber suppressor. *Virology* 1989; **171**: 239–247.
33. Lynn DE. A BASIC computer program for analyzing endpoint assays. *BioTechniques* 1992; **12**: 880–881.
34. Rich DP, Couture LA, Cardoza LM, *et al*. Development and analysis of recombinant adenoviruses for gene therapy of cystic fibrosis. *Hum Gene Ther* 1993; **4**: 461–476.
35. Huyghe BG, Liu X, Sutjipto S, *et al*. Purification of a type 5 recombinant adenovirus encoding human p53 by column chromatography. *Hum Gene Ther* 1995; **11**: 1403–1416.
36. Cotmore SF, Tattersall P. The autonomously replicating parvoviruses of vertebrates. *Adv Virus Res* 1987; **33**: 91–97.
37. Summerford C, Samulski RJ. Membrane-associated heparan sulphate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 1998; **72**: 1438–1445.
38. Fisher KJ, Gao GP, Weitzman MD, *et al*. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J Virol* 1996; **70**: 520–532.
39. Ferrari FK, Samulski T, Shenk T, Samulski RJ. Second-strand synthesis is a rate limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol* 1996; **70**: 3227–3234.