HUMAN GENE THERAPY 10:1031–1039 (April 10, 1999)
Mary Ann Liebert, Inc.

Brief Report

Highly Purified Recombinant Adeno-Associated Virus Vectors Are Biologically Active and Free of Detectable Helper and Wild-Type Viruses

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ABSTRACT

Gene transfer vectors based on the replication-defective human parvovirus, adeno-associated virus type 2 (AAV-2), are viable candidates for *in vivo* and *ex vivo* human use. However, widespread testing of AAV vectors has been limited by difficulties in generating pure, high-titer vector stocks that are fully characterized. To address these issues, we have developed a single-step purification scheme using heparin affinity chromatography. Recovery from the crude lysate starting material exceeds 70%, and the end product rAAV vector is highly purified and appears to be free of adenovirus and cellular contaminates. Importantly, purified vectors retain predicted *in vivo* biologic activity. Concurrently, we have developed simple and rapid approaches for vector quantification using real-time PCR. These new methods, combined with the use of stable producer cell lines for rAAV production, make the commercial production of rAAV vectors for human use truly viable and pragmatic.

INTRODUCTION

RECOMBINANT ADENO-ASSOCIATED VIRUS (rAAV) vectors are being proposed as a gene transfer vehicles for an ever-widening array of human diseases. The interest in rAAV has been driven by the unexpected finding that these simple vectors can efficiently transduce a variety of postmitotic cells when administered directly to tissues and organs *in vivo* (Flotte *et al*., 1993; Kaplitt *et al*., 1994; Kessler *et al*., 1996; Xiao *et al*., 1996; Clark *et al*., 1997; Flannery *et al*., 1997; Koeberl *et al*., 1997; Snyder *et al*., 1997). Transduction and gene expression are long-lived, presumably because rAAV integrates into the host cell chromosome (Xiao *et al*., 1996; Clark *et al*., 1997; Fisher *et al*., 1997; Miao *et al*., 1998). Not surprisingly, these observations have moved the field of rAAV vector development ahead at a rapid pace.

The widespread use of rAAV vectors has been somewhat limited by difficulties associated with vector production and purification. Several viable approaches to vector production have been reported including stable producer cell lines (Clark *et al*., 1995; Tamayose *et al*., 1996; Gao *et al*., 1998; Liu *et al*., 1999) and transient cotransfection methods (Salvetti *et al*., 1998; Xiao *et al*., 1998). However, the downstream process of virion purification has remained cumbersome and the end products have not been well characterized. All experiments with rAAV vectors reported to date have been carried out with either unpurified vector (crude cell lysate), or vector purified over CsCl gradients. The unpurified preparations are obviously heavily contaminated with cellular and adenovirus proteins and nucleic acids. To date the effects of these contaminants on *in vitro* and *in vivo* transduction have not been fully addressed. CsCl gradients offer some variable degree of purification, but this is usually at the expense of total yield when compared with the starting cell lysate. Furthermore, the sheer volume of cell lysate required to produce useful amounts of rAAV vector cannot be reasonably purified using CsCl gradient centrifugation.

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To address these issues, we sought to develop a practical and efficient method for purification of rAAV vector particles. Our goal was to move directly from crude cell lysate to a single-step column chromatographic purification procedure. Herein, we describe a method for rAAV vector purification that is fast (<4 hr), efficient (>70% recovery), and reproducible. The resulting product retains biologic activity and is free of detectable cellular or adenoviral contaminants. In addition, we describe new methods for rapid and reproducible quantification of rAAV vectors using real-time polymerase chain reaction (PCR). These new methods, combined with the use of stable producer cell lines, make the commercial production of rAAV vectors for human use truly viable and pragmatic.

MATERIALS AND METHODS

Cell lines and viruses

HeLa cells were purchased from the American Type Culture Collection (Rockville, MD). rAAV producer HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, and G418 (active) (500 μg/ml). The construction of producer cell lines was detailed previously (Clark *et al.*, 1995).

Wild-type adenovirus type 5 was purified from infected HeLa cells by CsCl step and isopycnic gradient centrifugations according to standard protocols and titer determined by a 50% tissue culture infective dose ($TCID_{50}$) assay on 293 cells (Graham and Prevec, 1991). The presence or absence of replication-competent adenovirus in rAAV preparations was assayed by passing 1% of the purified rAAV stock onto 293 cells and scoring for adenovirus cytopathic effect (CPE) after 7 days. The presence of wild-type-like AAV was assessed by passing 1% of the rAAV stock onto normal HeLa cells in the presence of adenovirus (multiplicity of infection [MOI] of 20). At maximum adenovirus CPE (48 hr), a clarified cellular lysate was generated by three freeze–thaw cycles and residual adenovirus inactivated by heat treatment (56°C, 45 min). The entire clarified lysate was used to infect a second dish of HeLa cells in the presence of adenovirus (MOI of 20). Hirt DNA was isolated 48 hr later and Southern blot hybridization was performed using a *cap* gene probe. Control experiments demonstrated that the level of sensitivity in this double-passage experiment was 1 $TCID_{50}$ unit of wild-type AAV. The failure to detect monomeric and dimeric replicative intermediates after biologic amplification of our rAAV vector stocks was taken as evidence that contamination with wild-type-like AAV is $\leq$100 IU per rAAV preparation (typically 10^{12}–10^{13} total DNase-resistant particles [DNP]).

Cell lysate preparation and column chromatography

rAAV producer cells were seeded in 175-cm^2 flasks in DMEM containing 5% FBS. The next day, cells were infected with adenovirus (MOI of 50) in fresh DMEM containing 2% FBS. At maximum adenoviral cytopathic effect (48 hr), cells were collected and pelleted at 2000 $\times$ *g* for 15 min at 4°C. The cell pellet was resuspended in 20 m*M* Tris (pH 8.0)–150 m*M* NaCl at a final cell density of 5×10^6 cells/ml. Deoxycholate (DOC) was added to a final concentration of 0.5% and incubated for 30 min at 37°C. Lysate viscosity was reduced by the concurrent addition of Benzonase (50 units/ml; American International Chemical, Natick, MA). Cellular debris was then removed by centrifugation at 3000 $\times$ *g* for 15 min at 4°C. The clarified lysate was heated to 56°C for 45 min to inactivate adenovirus and then frozen at −20°C until processing. On thawing, any flocculent precipitate was removed by centrifugation (2000 $\times$ *g*), and the supernatant was filtered through a 2-μm pore size disk filter.

A Biocad Sprint high-performance liquid chromatography (HPLC) system (PerSeptive Biosystems, Framingham, MA) was used in conjunction with a POROS HE1/M heparin column (20-μm bead size) to generate all chromatographic separation data shown. The high-pressure column (1.7-ml bed volume) was equilibrated with 17 ml of 20 m*M* Tris (pH 8)–100 m*M* NaCl. Clarified cell lysates (500–1000 ml) were applied at a flow rate of 3–5 ml/min at ambient temperature using the internal HPLC pumps. The system back pressure was continually monitored and never exceeded 600 psi. After sample loading, the column was washed with equilibration buffer (typically 35 ml) until the A_{280} returned to baseline ($\geq$0.005). Bound material was eluted by application of a linear NaCl gradient (0.1–1 *M*) at a flow rate of 3 ml/min, and 1-ml gradient fractions were collected. After column purification, small aliquots (20 μl) of peak protein-containin g fractions were analyzed by sodium dodecyl sulfate-polyacrylam ide gel electrophoresis (SDS-PAGE) and SYPRO-Orange (Molecular Probes, Eugene, OR) staining to visualize the eluted proteins. Virus-containin g fractions (typically two or three) were pooled, dialyzed against multiple changes of 20 m*M* Tris (pH 8.0)–1 m*M* $MgCl_2$–150 m*M* NaCl, and stored in aliquots at −80°C in 5% glycerol. The total protein content in the purified stocks was determined using the NanoOrange protein quantitation kit according to the manufacturer instructions (Molecular Probes).

Quantification of rAAV particles

DNase-resistant particle values were determined for highly purified rAAV viral stocks by two methods. The first method was a standard DNase digestion/dot-blot protocol performed as previously described (Clark *et al.*, 1995). The second method used the Perkin Elmer (PE)-Applied Biosystems (Foster City, CA) Prism 7700 sequence detector system and is detailed in the caption to Fig. 3.

The Prism 7700 primer and fluorescent probe sets used for rAAV quantification and final PCR working concentrations were as follows: SIVgp130/160: primers—5′ cgctatttccaagaagcagtacaa 3′ (300 n*M*) and 5′ cccacgcgcttgcaa 3′ (300 n*M*); probe—5′ FAM (6-carboxy-fluorescein) ctctcgcgcaagtttccaccacg TAMRA (6-carboxy-tetramethyl-rhodamine) 3′ (150 n*M*).

RESULTS

Characterization of rAAV binding to chromatography media

Previous work by others had shown that rAAV could be concentrated by low-pressure cation-exchange chromatography (Tamayose *et al.*, 1996), and this suggested to us that it should

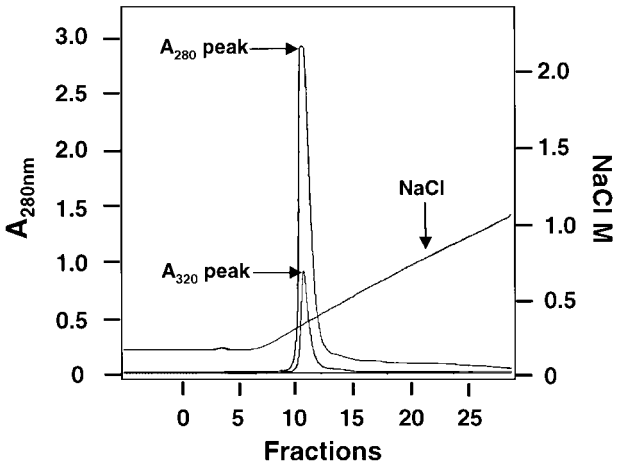

FIG. 1. Purification of rAAV using heparin as the affinity ligand. A POROS HE/M column (1.7-ml bed volume) was equilibrated in buffer (20 m*M* Tris, pH 8; 100 m*M* NaCl) and then a clarified cell lysate (600 ml from 3×10^9 cells) containing rAAV/SIVgp130 was applied at a flow rate of 5 ml/min at ambient temperature. The column was washed with equilibration buffer until the A_{280} returned to baseline. Bound material was eluted by application of a linear NaCl gradient (0.1–1.0 *M*) and 1-ml fractions were collected. Shown are the tracings for A_{280}, A_{320}, and NaCl molarity.

be possible to purify particles using this or a similar approach. In pilot experiments, we empirically chose to evaluate two readily available solid-phase chromatographic media: heparin and strong anion exchanger (quaternized polyamine-Mono Q). The first set of experiments were performed using a low-pressure heparin column (HiTrap; Pharmacia, Piscataway, NJ) and showed unequivocally that rAAV bound to the column in low salt and could easily be eluted with a salt gradient. Under these conditions, vector-containing fractions were contaminated with multiple protein species, but the purification was superior to CsCl gradient fractionation using the same starting material (not shown). Similar (but less compelling) data were obtained with Mono Q.

Encouraged by these observations, we turned our attention to the development of methods that would be useful for larger scale purification. For this purpose, we chose to use high-flow "perfusion" HPLC resins specifically engineered for semi-preparative biological applications (POROS; PerSeptive Biosystems). In pilot experiments similar to those described above, we again demonstrated that rAAV particles reversibly bound to heparin (POROS HE/M) and to another resin, POROS HQ (a quaternized polyethyleneimine). After additional trial runs in which elution parameters were optimized, we arrived at a set of conditions for the heparin resin that allowed for single-step purification of rAAV; those conditions are described below in an example of a typical purification run.

Previously frozen, clarified cell lysate derived from rAAV producer cells (rAAV/SIVgp 130) was applied to a POROS heparin column. The column was washed extensively and then bound material was eluted with a linear NaCl gradient. The elution profile (shown in Fig. 1) revealed a single protein peak (280 nm) that corresponded to a particulate peak (320 nm) that eluted at approximately 400 m*M* NaCl. Gradient fractions were collected and analyzed as described below. Control adenovirus-infected HeLa cell lysates failed to yield any detectable protein peaks at ~400 m*M* NaCl.

SDS-PAGE and immunoanalysis of gradient fractions

To determine the composition of the gradient fractions, we analyzed each fraction by SDS-PAGE and visualized the proteins using a sensitive fluorescent stain (SYPRO-Orange). Not surprisingly, the fractions with the highest $A_{280/320}$ readings also contained the most abundant protein by SDS-PAGE (Fig. 2A). The three polypeptide bands in fractions 10–13 appeared to correspond (by mobility) to VP1, VP2, and VP3 of AAV. To confirm that the observed polypeptides were AAV-2 structural proteins, we performed Western blot analysis using a mouse monoclonal antibody to the AAV capsid (Fig. 2B).

Although fluorescence staining of the SDS-PAGE gel indicated that these fractions were largely free of contaminating proteins, they were also examined for the presence of adenoviral structural proteins. Fractions were again analyzed by Western blot, using a rabbit anti-adenovirus polyclonal antiserum (Fig. 2C). We failed to detect any contaminating adenoviral proteins in peak rAAV-containing fractions (10–13). In fractions eluted by higher salt, two immunoreactive adenovirus species were detected. On the basis of apparent molecular mass, these proteins were tentatively identified as adenovirus polypeptides II (hexon) and IIIa (hexon-associated protein). Fortuitously, the rabbit anti-adenovirus polyclonal antiserum also reacted with bovine serum albumin, thereby demonstrating the absence of albumin in the AAV fractions. Thus, we concluded that fractions 11–13 were essentially composed of AAV proteins.

Finally, we assayed each fraction for rAAV genome-containing particles (Fig. 2D). The assay was designed to detect DNase-resistant particles (DRPs) and is described in the following section. Not surprisingly, the peak of rAAV DRP activity correlated with the peak of AAV capsid proteins.

Quantitative PCR measurement of rAAV DRPs and infectivity titer

With the development of a single-step chromatographic method for generating highly purified stocks of rAAV, we needed a rapid yet simple assay for viral particles and infectivity that could keep pace with production runs. To that end, we developed PCR-based methods to assay for DRPs and infectivity. The "DRP PCR Taqman" assay is essentially a modified dot-blot protocol whereby purified rAAV is serially diluted and sequentially digested with DNase I and proteinase K. A portion of the treated sample is then subjected to quantitative PCR analysis using the PE-Applied Biosystems Prism 7700 sequence detector system (Holland *et al.*, 1991; Gibson *et al.*, 1996). A typical amplification profile of HPLC-purified rAAV is shown in Fig. 3. A dilution (10^{-4}) of rAAV/SIVgp130 was sequentially digested with DNase I and proteinase K and then subjected to quantitative PCR analysis. The plasmid standard amplification curve corresponding to each initial template copy number standard is shown, along with the diluted rAAV/SIVgp130 sample. The rAAV/SIVgp130 genome copy number was determined by extrapolation (using system software) from the internal plasmid DNA standard curve (which consistently yields a coefficient of linearity of $\geq$0.995). The

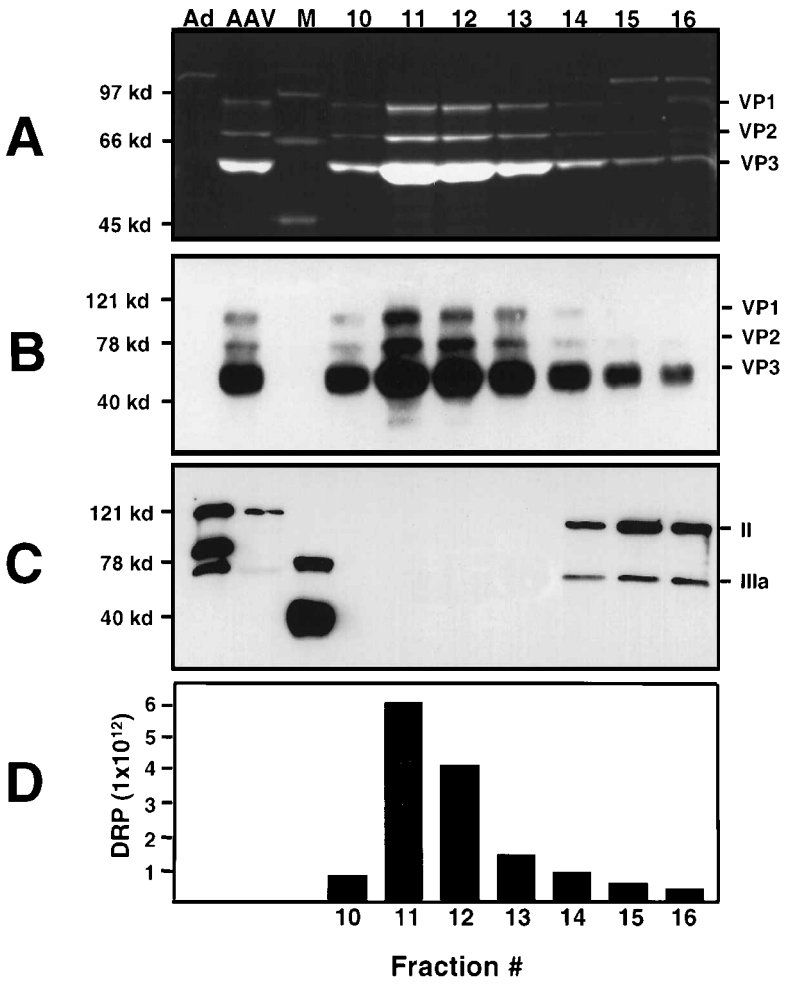

FIG. 2. SDS-PAGE analysis of HPLC-purified gradient fractions. A portion of individual gradient fractions (20 μl) from the chromatographic separation shown in Fig. 1 was resolved on a standard 10% SDS-polyacrylamide gel. (**A**) Protein bands were visualized using the SYPRO-Orange protein staining reagent. According to the manufacturer (Molecular Probes), the sensitivity for this stain is 1–10 ng per band. The image was captured using UV scanning illumination (520 nm long-pass filter with a 20-sec integration time; Bio-Rad Fluor-S MultiImager). Lane 1 (Ad) contains 10^8 IU of CsCl-banded adenovirus type 5. Lane 2 (AAV) contains partially purified AAV-2 as a control. Lane 3 (M) shows protein size markers. Lanes 4–10 are individual gradient fractions 10–16, respectively. (**B**) Western blots were performed using standard methods with slight modifications (Mendelson *et al.*, 1986; Vincent *et al.,* 1997). Here, a gel identical to that in (**A**) was probed with and anti-AAV Cap monoclonal primary antibody (clone B1, 1:10 dilution; American Research Products). The secondary antibody was anti-mouse IgG conjugated to horseradish peroxidase (1:10,000 dilution; Sigma). The blot was developed using an enhanced chemiluminescence peroxidase substrate (ECL; Amersham Life Sciences) followed by brief exposure (60 sec) to X-ray film. According to the manufacturer, the sensitivity of this blotting method is ~1 ng per band. Each fraction was shown to contain AAV capsid proteins, with the peak in fractions 11–13. No AAV proteins were found in lane 1 containing the adenovirus control. (**C**) A duplicate gel was probed for the presence of contaminating adenovirus proteins. The primary antibody was a polyclonal rabbit anti-adenovirus antiserum (1:5000 dilution; kind gift from J. Rose, NIAID). The secondary antibody was anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution; Sigma). The blot was developed as described in (**B**). Three dominant proteins were detected in lane 1 that probably corresponded to polypeptide II (120 kDa), polypeptide IIIa (63 kDa), and a third unknown protein (78 kDa) that was probably bovine serum albumin. The polyclonal sera also cross-reacted with the marker proteins in lane 3 (bovine serum albumin at 78 kDa and carbonic anhydrase at 39.5 kDa). The AAV preparation in lane 2 was clearly contaminated with adenovirus proteins, in contrast to fractions 10–13, which were free of detectable adenovirus (and bovine serum albumin). Later fractions (14–16) had small amounts of AAV and adenovirus proteins. (**D**) Individual fractions (10–16) were assayed for total DRP by quantitative PCR as described in the caption to Fig. 3. Earlier fractions (5–9) contained <1% of the total DRPs recovered (data not shown). The absolute number of rAAV DRPs in a fraction correlated with the amount of rAAV capsid protein.

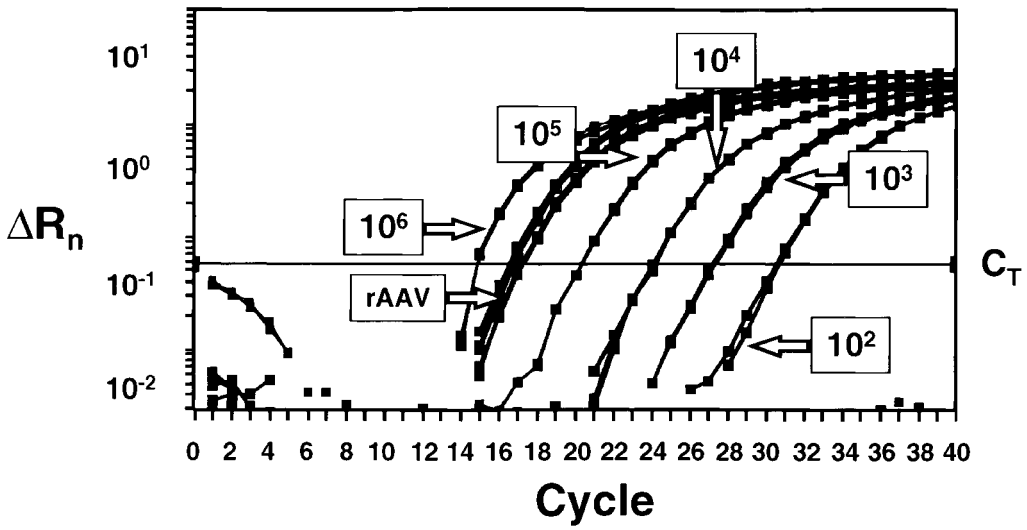

FIG. 3. Real-time PCR for rAAV DRP quantification. A PE-Applied Biosystems Prism 7700 sequence detector was used for quantitative PCR. A 10^{-4} dilution of the viral stock was prepared in 1× PCR buffer (50 m*M* KCl, 10 m*M* Tris-HCl [pH 8.3], 5 m*M* $MgCl_2$, and 0.01% [w/v] gelatin). The samples were digested with DNase I (350 units) for 30 min at 37°C, while minus DNase I samples were maintained at 4°C. After DNase I digestion, 10 μg of proteinase K was added to all samples and incubated at 50°C for 1 hr. This was followed by a 20-min incubation at 95°C to inactivate the proteinase K. Digested sample (2.5 μl) was added to 22.5 μl of a standard Taqman master mix (PE-Applied Biosystems). Concurrently, a DNA plasmid standard curve was set up in duplicate using 10^2–10^6 AAV genome equivalents. Because the AAV genome is single stranded, the plasmid standard curve values (double stranded) were doubled to account for the difference. To derive calculated DRP values, the initial template value was multiplied by the dilution factor (10^4), and then by 400 to derive a titer per milliliter (2.5 μl × 400 = 1 ml). All plasmid DNA concentrations were determined by fluorometer/Hoescht dye readings. A semilog plot of ΔR_n versus PCR cycle number is shown. The control plasmid (pAAV/CMV/SIVgp160) copy number (boxed values shown as 10^x) amplification curves are labeled according to the initial template copy number analyzed (10^2–10^6). The amplification profile of a 10^{-4} dilution of rAAV/SIVgp130 is also shown (boxed as rAAV). Fluorescence emission data were plotted as a function of the increase in reproter fluorescence (ΔR_n) versus copy number. On the basis of these data, a copy number standard curve was then calculated by plotting the threshold cycle (C_T) versus input copy number (coefficient of linearity, 0.997). The unknown rAAV/SIVgp130 sample possessed an average initial starting genome copy number of 7.4×10^5 by extrapolation from the plasmid standard curve. No template control samples (NTC; run in triplicate) were analyzed and yielded an average C_T value of 39.7. Amplification and detection were achieved using the SIVgp130/160 primer/probe set (see Materials and Methods).

genome copy number for this rAAV/SIVgp130 sample was calculated to be 7.4×10^5 genome copies per reaction, which corresponded to a titer of 3×10^{12} DRP/ml. Further validation of this general assay was carried out by analyzing multiple preparations of different vectors and comparing the values to standard dot-blot methods (Clark *et al.*, 1995); agreement was always within a factor of 2.

A second assay, developed for infectious rAAV titration, also used a quantitative PCR approach. We previously demonstrated the utility of a stable *rep–cap*-expressing HeLa cell line (C12) for replication-based rAAV titration (Clark *et al.*, 1995, 1996). We further modified that approach to rapidly derive infectious end-point rAAV titers using the PE 7700 (Fig. 4). Pooled fractions (11–13) from Fig. 2 were serially diluted and used to infect C12 cells in the presence (+Ad) or absence of adenovirus (–Ad). The average threshold cycle (ΔC_T) for all positive samples (10^{-9}–10^{-11} dilutions) was ≥12. This particular viral preparation had a positive end point at the 10^{-11} dilution, at which four of four replicates scored positive for viral genome replication; all replicates were negative at the 10^{-12} dilution. Thus, the end-point titer was 3.16×10^{11} IU/ml (1.0-ml initial sample infection volume).

Recovery of starting material after column purification

Using the assays described above, we were able to calculate the yield of particles recovered in the column fractions with respect to the starting material (crude lysate). In the example run detailed above, the recovery was 84% (DRPs). We have now analyzed nine separate purified preparations from four different producer cell lines and have observed an average recovery of 73%. The amount of vector remaining in the column flowthrough was on average less than 10% of the total applied particles (as estimated by DNase-resistant dot-blot hybridization or DRP Taqman analysis). Other important observations include the following: (1) the average DNA-containing particle-to-infectivity (i.e., DRP/IU) ratio for these preparations was, on average, 50. Interestingly, the largest vector construct (rAAV/β-gal—104.7% of wild-type genome length), had a higher DRP/IU ratio of 105 when compared with the three smaller vector constructs (DRP/IU ratio of 22). Regardless, these overall values are in agreement with previously reported rAAV particle-to-infectio us unit ratios (Salvetti *et al.*, 1998); (2) viral producer cells yielded ≥10^3–10^4 DRPs/cell that were free of detectable wild-type-like AAV particles (≤1 wild-type-

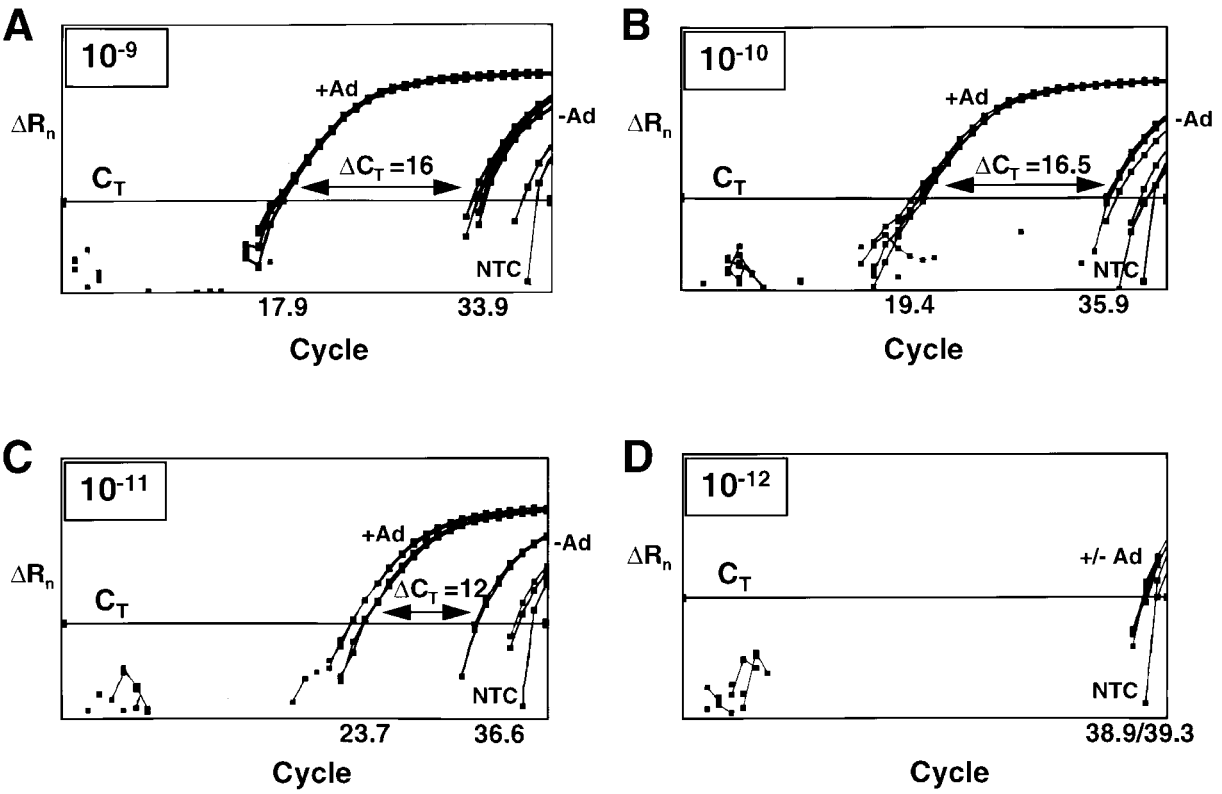

FIG. 4. Real-time PCR for rAAV infectious unit titration. We have previously detailed the use of a stable *rep–cap*-expressing cell line (C12) for the infectious titration of rAAV on the basis of the ability of rAAV to replicate in this cell line following coinfection with adenovirus (Clark *et al.*, 1995, 1996). This approach is now extended by using real-time PCR. Serial 10-fold dilutions of a purified viral stock were prepared in DMEM supplemented with 2% FBS and used to infect C12 cells (replicates of four) in the presence of adenovirus type 5 (MOI of 20). The cells were lysed 24 hr later and total genomic DNA isolated from each individual well using Qiaprep genomic anionic exchange columns (Qiagen). A fifth control sample, also prepared from each dilution, was not coinfected with adenovirus (minus adenovirus input control). Approximately 500 ng of the total genomic DNA (20% of the sample) was subjected to a standard Taqman PCRs (25-μl total volume) for 40 cycles. For each sample, two duplicate PCR were performed and the average calculated. Samples were scored positive for rAAV replication on the basis of a $\geq$4 cycle differential in the average C_T values between the plus adenovirus samples versus the minus adenovirus input control sample. Shown in (**A**)–(**D**) are the semilog plots of ΔR_n versus PCR cycle number as a function of the original rAAV stock dilution that was used to infect target C12 cells: (**A**) 10^{-9}; (**B**) 10^{-10}; (**C**) 10^{-11}; (**D**) 10^{-12}. At each dilution, the amplification profiles for DNA samples isolated in the presence (+Ad) or absence (–Ad) of coinfecting adenovirus are shown. The average threshold cycle (C_T) for the +Ad and –Ad (input control) samples is given below the *x* axis in each panel. The three no temple control (NTC) reactions for this plate are shown at bottom right in (**A**)–(**D**) and possessed an average C_T of 38.6. The difference in the input control samples (–Ad) and the replicating samples (+Ad) is given by the ΔC_T value. Cell and adenovirus alone control infections were always performed in parallel and yielded C_T values similar to the NTC control values. Amplification and detection were achieved using the SIVgp130/160 primer/probe set (see Materials and Methods).

like AAV/IU/10^{9-10} rAAV IU); and (3) the specific activity (DRPs per microgram of total protein) of each viral stock was calculated, and the average for the nine preparations was 2.7×10^{10} DRP/μg of total protein.

In vivo *transduction using HPLC-purified rAAV*

To confirm that HPLC-purified rAAV vectors retained *in vivo* biologic activity, four adult BALB/c mice were injected with rAAV/β-gal into the hind limb quadriceps. Four weeks after injection, the quadriceps muscle was harvested, cryosectioned, and stained for β-galactosidase activity. A significant fraction of the muscle from all four animals was transduced with the purified vector (a representative section is shown in Fig. 5). There was no detectable inflammatory response to the itnroduced vector. These data suggest that contaminating proteins, either cellular or adenoviral, are not required for efficient *in vivo* transduction of mouse muscle by rAAV vectors.

DISCUSSION

As rAAV vectors gain popularity among those interested in human gene transfer, it becomes increasingly important for vector technology to keep pace. Even though substantial progress has been made in understanding the biology of rAAV, little attention has been paid to important practical issues such as vector production, purification, and characterization of the end

product. The findings reported in this article will allow for the routine production of highly purified rAAV vectors suitable for animal and human investigation. The methods we describe are relatively rapid, inexpensive, and amenable to high-volume throughput. Recovery of the starting material is high and the vector maintains predicted *in vivo* biologic activity.

Heparin affinity chromatography has been used to purify a variety of biologic molecules, including viruses (Navarro del Canizo *et al*., 1996; Katayama *et al*., 1998), and was thus a logical starting point for our investigation. Our determination that heparin-based affinity chromatography works quite well for rAAV-2 isolation is further supported by the observation that heparin sulfate proteoglycan can serve as a cellular receptor or coreceptor for AAV-2 (Summerford *et al*., 1998). We have not assayed other AAV serotypes for heparin affinity, but predict (on the basis of an amino acid identity of ~87%) that AAV-3-based vectors might also demonstrate an affinity for heparin.

The end product of this simple procedure is essentially free of detectable contaminates, including adenovirus polypeptides. Other investigators have pointed out that rAAV vector production systems using plasmid DNA to provide essential adenovirus functions have a notable advantage, namely, the lack of replicating adenovirus (Salvetti *et al*., 1998; Xiao *et al*., 1998). Hence, the potential for adenovirus contaminates is eliminated. Our purification protocol allows for the use of replicating adenovirus (at high MOIs) without the concern that the final product will contain adenovirus-derived proteins. Importantly, we have documented that a highly purified rAAV vector retains the ability to transduce muscle *in vivo*, even in the absence of contaminating molecules that might have served as facilitators of transduction (Alexander *et al*., 1994, 1997; Halbert *et al*., 1995; Russell *et al*., 1995; Clark *et al*., 1996; Ferrari *et al*., 1996; Fisher *et al*., 1996). In more recent experiments (not shown), we have gone on to show efficient rAAV-mediated transduction of mouse liver, CNS, and muscle with four different highly purified rAAV vector constructs.

Our vector preparations appeared to be highly purified on the basis of three criteria. First, in peak protein gradient fractions, we typically analyzed ~5 μg of total protein per lane by SDS-PAGE (Fig. 2A) and failed to observe any contaminating polypeptides. On the basis of the sensitivity of our fluorescent stain (1–10 ng per band), our preparations therefore contain $\leq$0.2% of any specific contaminating cellular or adenovirus protein. Second, the Western blot protocol we used to detect adenoviral protein contamination possessed a sensitivity of detection of ~1 ng per band. Thus, our highly purified rAAV stocks apparently contained $\leq$0.02% of any one specific adenovirus protein. Finally, we have derived an estimate of stock purity on the basis of viral specific activity. For nine individual production runs, we calculated a mean value of 2.7×10^{10} DRP/μg of total protein in the final product. Assuming a protein molecular mass of an intact AAV-2 particle to be 4×10^6 Da (Laughlin *et al*., 1979), 1 μg of AAV-2 virions would be equal to 1.5×10^{11} particles. Therefore, on average, our rAAV stocks contained approximately 20% of the maximal number of theoretical particles. Our particle estimates measure only DNase-resistant particles, and thus, empty particles (non-DNA-containing) would not be counted. Bearing this in mind, the average calculated ratio of empty to DNA-containing particles in our preparations was 5.6:1. Because cell culture-derived AAV is known to contain significant amounts of empty capsids (Carter *et al*., 1979; Laughlin *et al*., 1979), these calculations are entirely consistent with a highly purified final product consisting primarily (if not entirely) of rAAV. We also observed an average DNase-resistant particle-to-infectious unit ratio of 50 for the nine preparations. Therefore, the average total parti-

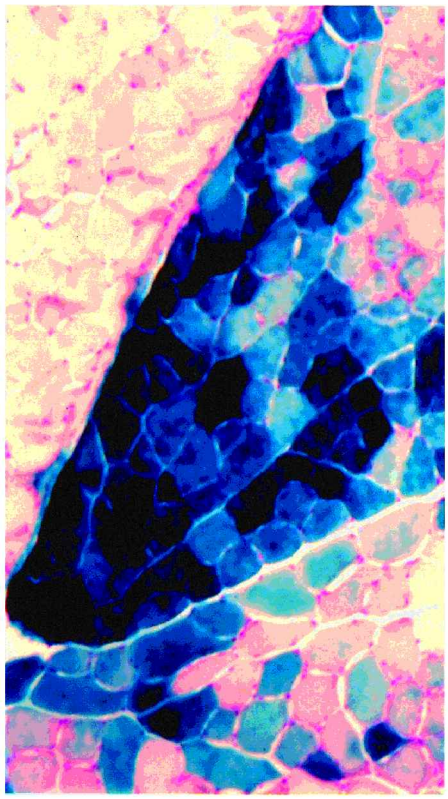

FIG. 5. β-Galactosidase activity in mouse quadriceps muscle after injection of highly purified rAAV/β-gal. All animal experiments were approved by the Children's Hospital Institutional Animal Care and Use Committee. Adult male BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were injected with 50 μl of rAAV/β-gal containing 10^{11} DRPs along the longitudinal axis of the quadriceps muscle, using a 28-guage needle. The animals were sacrificed 28 days later and the entire quadriceps muscle group was removed and bisected along the transverse plane. One-half of the excised muscle was placed in a cryoprotective medium (O.C.T., Tissue Tek, Miles) and rapidly frozen in liquid nitrogen. The remaining muscle was fixed in 10% formalin and paraffin embedded. Histologic evaluation and assessment of β-galactosidase activity were performed on each animal. Cryosections (6 μm) were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 100 m*M* sodium phosphate buffer (pH 7.3) for 5 min at 4°C. Fixed sections were stained with X-Gal substrate solution [1-mg/ml concentration of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in 100 m*M* sodium phosphate containing 1.3 m*M* $MgCl_2$, 3 m*M* $K_3Fe(CN)_6$, and 3 m*M* $K_4Fe(CN)_6$ at pH 7.3] for 16 hr at room temperature and counterstained with nuclear fast red. Paraffin sections (4 μm) of muscle from each animal were stained with hematoxylin and eosin and examined for evidence of an inflammatory response (not shown). A representative cross-section of muscle stained for β-galactosidase activity is shown. Original magnification: $\times$ 40.

cle (non-DNA plus DNA-containing)-to-infectious unit ratio for these stocks was estimated to be 370:1. If one excludes the largest rAAV vector genome (rAAV/β-gal) from consideration, then a more realistic estimate of the total DNase-resistant particle-to-infectious unit ratio is 60–120:1.

Concurrent with the development of our purification methods, we sought a further evolution of methods for rAAV vector quantification. The adaptation of real-time PCR for vector quantification greatly speeds up particle titer determination, and is applicable regardless of the vector promoter or transgene. For infectivity titrations, real-time PCR allows the end user to observe actual amplification kinetics. Thus, one is able to document low levels of rAAV DNA replication, which consequently yield large ΔC_T values when compared with input control samples. Hence, low levels of input virus can be readily distinguished from true rAAV genome replication, a feature lacking in standard end-cycle PCR amplification protocols.

We have not detected the presence of infectious wild-type-like AAV particles in our preparations. Our assay for infectious wild-type-like AAV involves double-blind passage of 1% of a given rAAV preparation in the presence of adenovirus; the sensitivity of this assay is 1 $TCID_{50}$ of wild-type AAV. Thus, for a typical preparation of rAAV containing 3×10^{13} DRPs, we conclude that there is $\leq$1 infectious wild-type-like AAV particle in 3×10^{11} rAAV DRPs. Importantly, studies have documented the presence of wild-type-like AAV in rAAV vector stocks generated by transient cotransfection methods (Allen *et al.*, 1997; Wang *et al.*, 1998). The formation of wild-type-like AAV species was shown to occur as a result of multiple illegitimate recombination events, presumably occurring between AAV helper and rAAV vector plasmids. Considering that our rAAV vectors are produced from stably transduced cell lines, the initial intranuclear copy number is typically 5–20 integrated copies of the helper and vector sequences (our unpublished observations, 1999). This stands in stark contrast to exogenously transfected cells, which are known to contain significantly higher levels of the introduced plasmid DNA ($\geq 10^2$–10^3 copies/cell). Consequently, the potential for illegitimate recombination events would be expected to be somewhat greater in these transient transfection systems (Allen *et al.*, 1997; Wang *et al.*, 1998).

Production of highly purified, adenovirus-free, wild-type AAV-free rAAV preparations is now feasible at a scale useful for human trials. Our simple single-column procedure is adaptable for research or commercial-scale production, and it is hoped will further the widespread testing and consideration of rAAV vectors for human gene transfer applications.

ACKNOWLEDGMENTS

The authors thank Dr. David Brigstock for assistance with the initial heparin chromatography experiments and Anna Mendoza, Sara Bailey, Maera Flynn, and Ruju Chen for expert technical assistance. This work was supported by The Children's Hospital Viral Vector Core Laboratory and by grants from the National Institutes of Health, Children's Hospital Research Foundation, and Alkermes, Inc.

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Received for publication October 6, 1998; accepted after revision January 29, 1999.