adeno-associated virus (AAV) and lentivirus (HIV). It is not yet clear whether randomly integrating vectors such as recombinant AAV or lentivirus will be safe enough for use in a clinical setting. The concerns of wild-type contamination with HIV vectors does not need to be stressed. The episomal nature and mild symptoms of human Ad infection make them favorable candidates for gene therapy, and many clinical trials using Ads are already in progress.

Acknowledgments

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[48] Production and Purification of Recombinant Adeno-associated Virus

By WILLIAM W. HAUSWIRTH, ALFRED S. LEWIN, SERGEI ZOLOTUKHIN, and NICHOLAS MUZYCZKA

Introduction

Recombinant adeno-associated virus (rAAV), because of its simplicity, ability to infect a wide variety of dividing and nondividing cells, and lack of human pathogenicity, has proved to be a useful vector for efficient and long-term gene transfer *in vivo*. A variety of tissues have been successfully transduced, including retina,[1,2] lung,[3] muscle,[4–7] brain,[8] spinal

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0076-6879/00 $30.00

cord,[9] and liver.[10] Recombinant AAV vectors consist of a simple capsid with a single-stranded DNA genome containing short viral inverted terminal repeats (ITRs) but no viral coding sequences.[11,12] Most often rAAVs have been generated by cotransfection of adenovirus (Ad)-infected 293 cells with an rAAV vector plasmid and a wild-type AAV (wtAAV) helper plasmid.[13] Improvements in AAV helper design[14] as well as construction of noninfectious mini-Ad helper plasmids[15–17] have eliminated the need for adenovirus infection and improved the yield of rAAV per transfected cell. For normal retinal therapy experiments in small animal models, this general packaging protocol produces sufficient recombinant virus for injection typically into 50–100 eyes. However, for larger animals with human-sized eyes, scalable methods of rAAV production that do not rely on DNA transfection have also been developed.[18–21] These methods, generally involving the construction of producer cell lines and helper virus infection, are suitable for the higher volume production that may be needed for testing in larger eyes.

The traditional rAAV purification protocol involved the stepwise precipitation of rAAV with ammonium sulfate, followed by two or three rounds of CsCl density gradient centrifugation. Each gradient required fractionation and identification of the virus-containing regions by dot-blot hybridization or by polymerase chain reaction (PCR) analysis. Not only did this require up to 2 weeks to complete, it also often resulted in poor recovery and poor-quality virus. Such a lengthy production protocol com-

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bined with the growing demand for a wide variety of different rAAV stocks often taxed the capacities of vector production facilities. The need for a protocol that substantially reduces preparation time without sacrificing the quality and purity of the final product has been clear for some time. Such a protocol is described here. It is based on two improvements: (1) the observation that AAV binds to cell surface heparin sulfate proteoglycan,[22] and (2) a new bulk purification technique employing the nonionic gradient medium, iodixanol, which allows efficient binding of the virus to the affinity medium. This combination of techniques results in high recovery rates, improved viral infectivity, and rapid purification. The temporal order of steps for producing a pure rAAV includes (1) production of rAAV in crude lysates, (2) iodixanol density step gradient viral purification, (3) heparin affinity chromatography and virus concentration, and (4) characterization and quantification of the purified rAAV.

Production of Crude Cell Lysates Containing Recombinant Adeno-Associated Virus

The basic strategy for producing rAAV involves DNA transfection of a suitable human host cell in culture. Two plasmid DNAs are cotranfected, an AAV vector plasmid containing the AAV terminal repeat sequences (ITRs) and the promoter/gene of interest[1]; and a helper plasmid containing both the *rep* and *cap* AAV genes, required for packaging ITR flanked DNA, and the early genes of Ad, required for a productive AAV infection.[2] Human 293 cells have been and remain the primary host for rAAV production. Although alternatives exist, calcium phosphate-mediated cell transfection remains our standard technique for introducing plasmid DNAs into host cells. We employ the basic rAAV vector plasmid pTR-UF5[23] containing the humanized *gfp* gene under control of the cytomegalovirus (CMV) promoter as our usual starting vector. We also use the helper plasmid pDG, which contains both the AAV genes (*rep* and *cap*) and the adenovirus genes required for AAV propagation.[15] No replication-competent adenovirus has been detected with the use of this helper plasmid. To simplify the transfection protocol, the $CaPO_4$–DNA precipitate can be left in the medium for the entire incubation period of 48 hr without compromising cell viability. Transfection efficiency routinely reached 60% as judged by green fluorescent protein (GFP) fluorescence in host 293 cells. After harvesting the cells, virus is extracted by freezing and thawing the

[22] C. Summerford and R. J. Samulski, *J. Virol.* **72,** 1438 (1998).

[23] S. Zolotukhin, M. Potter, W. W. Hauswirth, J. Guy, and N. Muzyczka, *J. Virol.* **70,** 4646 (1996).

cells and the supernatant is then clarified by low-speed centrifugation. The use of sonication, microfluidizing, or detergent extraction (e.g., deoxycholate) does not appear to significantly increase the viral yields.

It is also possible to concentrate and purify rAAV from the cell culture medium instead of from material in the cell pellet. For such bulk purification from medium, virus from the cell supernatant can be precipitated with 50% (w/v) ammonium sulfate. However, this procedure is inefficient because, at the time of harvest (48 hr posttransfection), about 90% of the virus remains intracellular.

Host Cells. Low passage number (passage 29–40) 293 cells are propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). After passage 40 we observe a reproducible loss in recombinant viral titer. Therefore host cells are discarded after this passage number.

Vector Plasmid. The construction of pTR-UF5 has been described[8] and is available on request. Standard cloning procedures are used to delete the CMV promoter and/or the *gfp* gene in pTR-UF5 and insert the gene and regulatory sequences of interest into pTR-UF5. Because the packaging limit of the AAV capsid is about 4.9 kb, all DNA between (and including) ITRs must remain below this limit. Maximal space in the vector can be obtained by also deleting the herpesvirus thymidine kinase (TK) promoter and *neo* cassette in pTR-UF5, using a single *Sal*I digestion and reclosure.

Containment. All stages of rAAV purification are carried out in a designated area physically separated from the rest of the facility. Virus-containing reagents are handled exclusively in biosafety cabinets. Only Quick-Seal tubes (Beckman, Palo Alto, CA) are used to purify recombinant virus.

Virus Production. To produce rAAV, a double cotransfection procedure at a 1 : 1 molar ratio is used to introduce the rAAV vector plasmid together with the helper plasmid pDG. Plasmid DNA for transfection is purified by a conventional alkaline lysis/CsCl gradient protocol. Care must be taken to avoid deleting ITR sequences during the growth of the vector plasmid in *Escherichia coli.* Because both flanking ITRs must be present in the vector plasmid DNA for rAAV packaging, it is important to confirm their presence prior to 293 cell transfection. We propagate vector plasmids in recombination-deficient Sure 2 cells (Stratagene, LaJolla, CA) for no more than 12 hr at 37°. To check the resultant vector plasmid DNA for intact ITRs, a small sample is digested with *Sma*I, which releases all DNA between the ITRs. ITR deletion will result in a loss of one of these flanking *Sma*I sites. Vector plasmid DNA containing less than about 10% deleted ITR (loss of a flanking *Sma*I site) will retain efficient packaging properties.

Protocol

1. Human 293 cells are split 1 : 2 the day prior to the experiment, so that, when transfected, the cells are about 75–80% confluent. Ten 15-cm plates are transfected as one batch.
2. To make the $CaPO_4$ precipitate, 180 μg of pDG is mixed with 180 μg of vector plasmid DNA in a total volume of 12.5 ml of 0.25 *M* $CaCl_2$.
3. Formation of the $CaPO_4$ precipitate is initiated at 37° by adding 12.5 ml of prewarmed 2× HBS (0.3 *M* NaCl, 10 m*M* KCl, 1.5 m*M* Na_2HPO_4, 10 m*M* dextrose, and 40 m*M* HEPES at pH 7.05) to the DNA–$CaCl_2$ solution.
4. The DNA is incubated for 1 min, then transferred into 200 ml of prewarmed DMEM–10% (v/v) FBS, stopping formation of the precipitate.
5. Medium is removed from the cells, 22 ml of the precipitated DNA–medium is immediately dispensed into each plate and cells are incubated at 37° for 48 hr. The $CaPO_4$ precipitate can remain on the cells during the whole incubation period without compromising cell viability.
6. Cells are harvested after 48 hr of incubation by centrifugation at 1140*g* for 10 min at room temperature, and the medium is discarded.
7. Cells are then lysed in 15 ml of 0.15 *M* NaCl, 50 m*M* Tris-HCl (pH 8.5) by three freeze–thaw cycles using alternating dry ice–ethanol and 37° baths.
8. Benzonase [Nycomed Pharma A/S (Roskilde, Denmark), pure grade] is then added to the mixture at 50 U/ml, final concentration, and the lysate incubated for 30 min at 37°.
9. The crude lysate is clarified by centrifugation at 3700*g* for 20 min at 4°. The virus-containing supernatant is considered the crude lysate that will require further purification by density gradient centrifugation.

Iodixanol Density Step Gradient Virus Purificaton

During initial attempts to design a more efficient purification method, it appeared that rAAV and cell proteins were forming aggregates in the crude cell lysate. When such complexes formed, the virus failed to display uniform biochemical properties, leading to poor viral recovery at all purification stages and making it difficult to develop a reproducible purification strategy. Furthermore, these apparently nonspecific interactions resulted in viral preparations contaminated with adventitious proteins, even after several rounds of CsCl gradient centrifugation. Therefore bulk purification of the crude lysate became the most crucial new step in rAAV purification.

To avoid aggregation, we investigated several alternative methods including the use of detergents and limited proteolytic digestion. Although

these methods provided some improvement during subsequent purification, they resulted in preparations that were more heat labile and/or sensitive to DNase. Ammonium sulfate precipitation, which has been used previously for concentrating virus, did not provide significant purification. Furthermore, residual ammonium sulfate in the virus pellet interfered with subsequent ion-exchange chromatography. Dialysis at this stage in the purification also led to aggregation and precipitation of virus and proteins resulting in poor rAAV recovery. A combination of $(NH_4)_2SO_4$ precipitation and hydrophobic phenyl-Sepharose chromatography also failed to produce a purified virus without substantial loss of viral infectivity. Cellufine sulfate chromatography had been proposed as a way to purify and concentrate rAAV from crude lysates.[24] In our hands, however, cellufine sulfate fails to reproducibly bind rAAV in the crude lysates made as described. To circumvent these problems, we developed a new strategy of purification based on density gradient centrifugation using iodixanol.

Iodixanol is an iodinated density gradient medium originally produced as an X-ray contrast compound for clinical use. Unlike CsCl and sucrose solutions commonly used in gradient fractionation of macromolecules, iodixanol solutions can be made isosmotic at all densities. This property suggests iodixanol could be a useful reagent for viral purification and analysis steps. The nonionic and inert nature of iodixanol allows electrophoretic analysis and viral infectivity assays to be carried out directly on gradient fractions. Because the viscosity of iodixanol solutions is also lower than that of sucrose of the same density, it is possible to use iodixanol fractions directly in subsequent chromatographic purification steps without dialysis or dilution.

Because the apparent density of macromolecules in iodixanol solutions is different from that in CsCl, the banding density of purified rAAV-UF5 was determined empirically by banding an aliquot of UF5 virus in a continuo s iodixanol gradient. The density of rAAV-UF5 was found to be 1.415 g/ml, equivalent to a 52% (w/v) solution of iodixanol. Opting to generate preformed step gradients in order to expedite this purification step, we adopted a 40% iodixanol (1.21 g/ml) step to accommodate rAAV/capsid protein complexes banding at slightly lower densities. This density is underlaid with a 60% step that acts as a cushion for any slightly denser rAAV particles. To locate the 40% iodixanol step and the 40%–60% density interface after centrifugation, we include phenol red dye (0.01 μg/ml) in the upper 25% and lower 60% density steps. The step gradient, consisting of 15, 25, 40, and 60% iodixanol, is run in a Beckman Ti70 rotor as described in the protocol below.

Our initial attempts to purify rAAV with discontinuous iodixanol gradi-

[24] K. Tamayose, Y. Hirai, and T. Shimada, *Hum. Gene Ther.* **7**, 507 (1996).

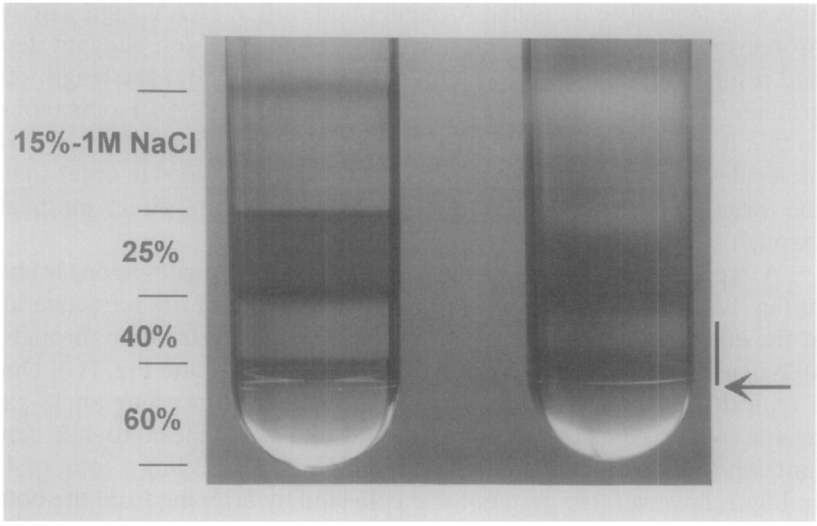
ents gave inconsistent results. As mentioned earlier, rAAV aggregates with proteins in the cell lysate. This apparently changes virion buoyant density and results in distribution of rAAV particles throughout the length of the gradient. To solve this problem, we modified the top (15%) iodixanol step so that it contained 1 *M* NaCl to disrupt ionic interactions between macromolecules. High salt was not added to the remaining steps in order to band the virus under isosmotic conditions, hence allowing direct purification through subsequent chromatographic stages.

A typical gradient, before and after the 1-hr centrifugation, is shown in Fig. 1A, left and right tubes, respectively. A plot of the refractive index at the end of the run is shown in Fig. 1B. rAAV is distributed through the 40% density step (Fig. 1A, shown by the vertical bar, and Fig. 1C). During a typical production run, the virus is recovered by inserting an 18-gauge needle connected to a 5-ml syringe about 2 mm below the 60-to-40% density junction (indicated by the arrow in Fig. 1A) and collecting a total of 4 ml. In Fig. 1, however, the gradient was collected by dripping from the bottom and collecting 1-ml fractions for detailed analysis. The bulk of the rAAV virus bands within the 40% density step (Fig. 1C, fractions 5–8). An opaque band that migrates at the 40-to-25% iodixanol junction consists mostly of cellular proteins (Fig. 1C, fractions 9 and 10) and contains less then 5% of the total rAAV. A small amount of rAAV also bands at the 40-to-60% junction (Fig. 1B and C, fraction 5). Approximately 75–80% of the rAAV in the crude lysate is recovered in the iodixanol fraction.

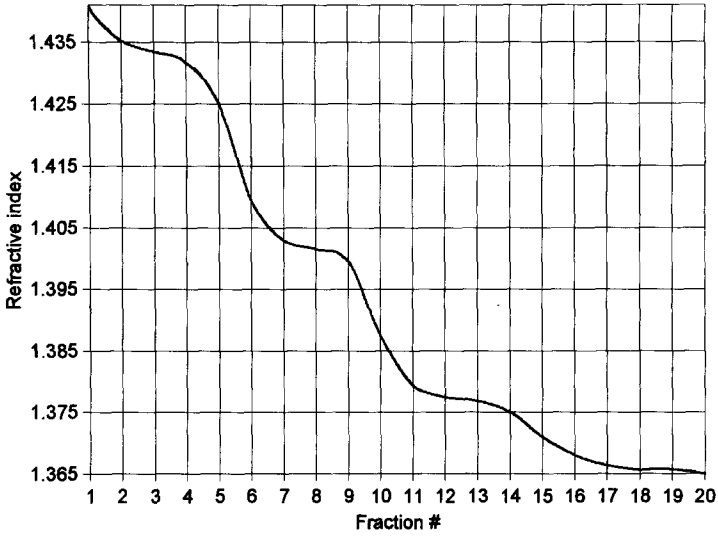
The nucleic acid–protein ratio in rAAV-UF5 is different from wtAAV because the size of the DNA packaged is 3.4 kb, or approximately 70% of the wild-type genome size. We have used the same protocol with no modifications to purify more than 50 different rAAV vectors, in which the size of the packaged genome ranged from 2.3 to 4.9 kb. Regardless of the size, there is no substantial difference in the banding pattern of rAAV on the iodixanol step gradient. Therefore, there is no need to modify the concentrations of iodixanol in the gradient steps to accommodate rAAV genomes in this size range.

To determine the resolving capacity of the iodixanol gradient, an experiment was performed in which viral lysates obtained from 5, 10, or 15 plates (15-cm diameter) were loaded onto separate gradients. This was equivalent to 1.6, 3.1, or 4.7×10^8 human 293 cells, respectively. After centrifugation, rAAV was recovered from each gradient as described above, and aliquots from each gradient subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2). The three viral capsid proteins VP1, VP2, and VP3 constituted the major species seen at all concentrations, suggesting that good separation of rAAV was achieved even at the highest concentration of lysate used. However, a significant increase in background contaminating protein was seen when the pooled lysate from 15 plates was

A


15%-1M NaCl
25%
40%
60%

B


Refractive index
1.435
1.425
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1.405
1.395
1.385
1.375
1.365
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fraction #

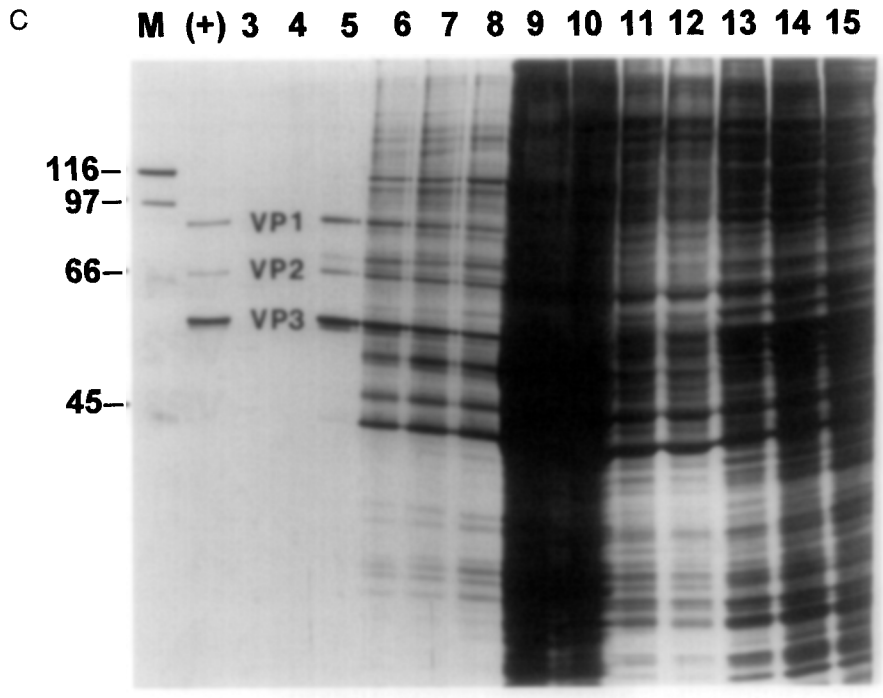


FIG. 1. Iodixanol step gradient for the purification of rAAV. (A) Preformed gradients shown before (left tube) and after (right tube) a 1-hr spin. The red or yellow tinge in the 60 and 25% steps indicates inclusion of phenol red in these steps. The positions of the density steps are shown on the left and the distribution of rAAV through the gradient is shown by the vertical bar. Typically, virus was collected by a needle puncture at the position on the side of the tube as indicated by the arrow. (B) A plot of the refractive index of 1-ml fractions collected from the bottom of the tube on the left in (A). (C) Silver-stained SDS–protein gel analysis of iodixanol fractions. rAAV was collected by dripping 1.0-ml fractions from the bottom of the gradient shown on the right in (A). Equivalent amounts of each fraction were then loaded onto a 12% (w/v) SDS–acrylamide gel and electrophoresed for 5 hr at 200 V. The numbers on the top of the gel correspond to the fraction numbers; only fractions 3–15 are shown. VP1, VP2, and VP3 indicate the position of viral capsid proteins. Lane (+) contains purified rAAV virus as a positive control. Lane M, protein standards whose molecular masses are shown on the left in kilodaltons.

used. Therefore, we routinely load rAAV lysate from only 10 plates per gradient. As a practical matter, this means that a 1-hr iodixanol gradient run in a Ti70 rotor would accommodate 3.1×10^9 cells from which approximately 10^{14} virus particles or about 10^{12} infectious units could be purified.

Protocol

1. A discontinuous step gradient is formed by underlayering and displacing the less dense cell lysate with iodixanol, 5,5′-[(2-hydroxy-1-3-propan-

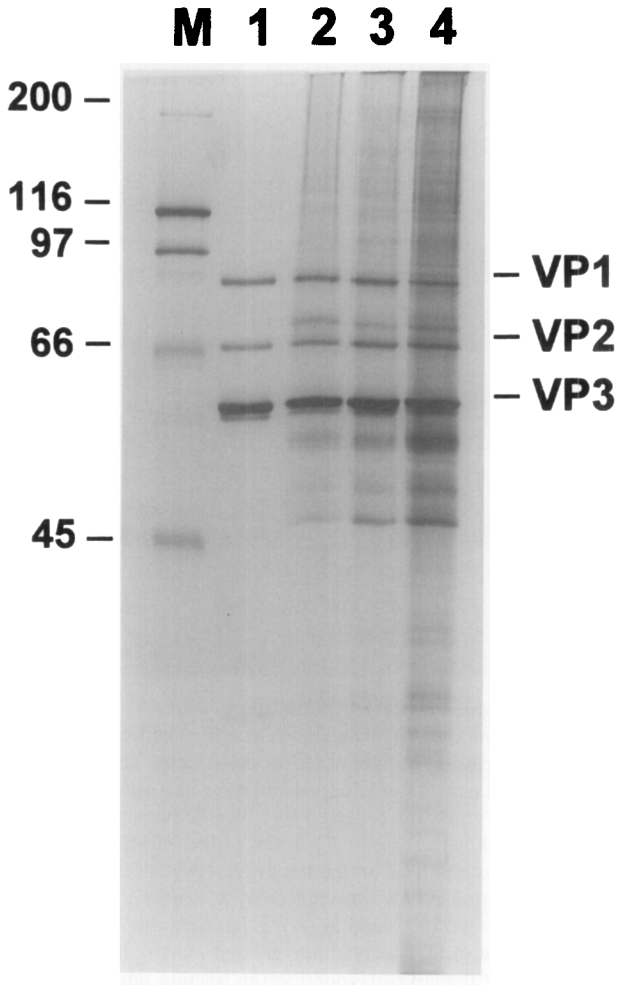


FIG. 2. Silver-stained SDS–protein gel analysis of the resolving capacity of an iodixanol step gradient. The virus lysate from 5, 10, or 15 dishes (lanes 2, 3, and 4, respectively) was purified on a single iodixanol gradient of the type shown in Fig. 1A. After the virus was collected, equivalent amounts of rAAV were loaded from each gradient onto an SDS–acrylamide gel as described in Fig. 1C. VP1, VP2, and VP3 indicate the positions of the viral capsid proteins. Lane 1 contains purified virus as a positive control. Lane M, standard proteins whose molecular weights are indicated on the left. See text for additional details.

ediyl)bis(acetylamino)]bis[*N,N'*-bis(2,3 dihydroxypropy)-2,4,6-triiodo-1,3-benzenecarboxamide], prepared using a 60% (w/v) sterile solution of Opti-Prep (Nycomed). Specifically, 15 ml of the clarified lysate is transferred into Quick-Seal Ultra-Clear 25 × 89 mm centrifuge tubes (Beckman), using a syringe equipped with a 1.27 × 89 mm spinal needle. Care is taken to avoid bubbles that could interfere with subsequent filling and sealing of the tube. A variable-speed peristaltic pump (model EP-1; Bio-Rad, Hercules, CA) is used to underlay in order: 9 ml of 15% (w/v) iodixanol and 1 *M* NaCl in PBS–MK buffer [1× phosphate-buffered saline (PBS), 1 m*M* $MgCl_2$, and 2.5 m*M* KCl]; 6 ml of 25% iodixanol in PBS–MK buffer containing phenol red [2.5 μl of a 0.5% (w/v) stock solution per milliliter of the iodixanol solution]; 5 ml of 40% iodixanol in PBS–MK buffer; and finally, 5 ml of 60% iodixanol in PBS–MK buffer containing phenol red (0.01 μg/ml).

2. The centrifuge tubes are then sealed and centrifuged in a type 70 Ti rotor (Beckman) at 350,000*g* for 1 hr at 18°.

3. Using the phenol red in the 25 and 60% iodixanol steps to identify density, 4 ml of the clear 40% step is aspirated after puncturing the tube on the side with a syringe equipped with an 18-gauge needle with the bevel facing upward.

Recombinant AAV in the 40% iodixanol fraction is further purified by the method described below.

Heparin Affinity Chromatography

Heparinized chromatographic supports have been successfully used for the purification of many heparin-binding macromolecules, including viruses such as CMV.[25] Heparin is the glycosaminoglycan moiety covalently bound to the protein core of proteoglycans found in mast cells. It is closely related to heparan sulfate (HS), the glycosaminoglycan (GAG) chain of the HS proteoglycan. The latter has been shown to be a cell surface receptor that mediates AAV cellular binding.[22] Covalent binding of heparin molecules to the matrix through its reducing end mimicks the orientation of the naturally occurring GAGs.[26] To take advantage of the structural similarities between heparin and HS, we tested heparin affinity chromatography as a final purification step after iodixanol gradients.

Heparin is a heterogeneous carbohydrate molecule composed of long unbranched polysaccharides modified by sulfation and acetylation. The

[25] J. Neyts, R. Snoeck, J. Balzarini, J. D. Esko, A. Van Schepdael, and E. De Clercq, *Virology* **189,** 48 (1992).

[26] V. D. Nadcarni, A. Pervin, and R. J. Linahrdt, *Anal. Biochem.* **222,** 59 (1994).

degree of sulfation strongly correlates with its virus-binding capacity.[27] Therefore, we anticipated that heparinized matrices from different vendors might display different affinity toward rAAV. We therefore tested a number of heparin ligand-containing media, including three column chromatography media manufactured by Sigma (St. Louis, MO), heparin–agarose type I, heparin–agarose type II-S, heparin–agarose type III-S, as well as Affi-Gel heparin gel (Bio-Rad). Affi-Gel heparin gel and heparin–agarose type III-S columns bound less than 50% of the applied virus and, therefore, were not considered further. Either heparin–agarose type I or heparin–agarose type II-S prepacked 2.5-ml columns were efficient in both retaining and subsequently releasing rAAV. The type II-S column, however, is less selective, binding many cellular proteins along with the virus. In contrast, heparin–agarose type I columns were the best among those tested in terms of binding specificity and virus recovery, and is currently the virus-binding medium of choice.

Silver-stained SDS–acrylamide gel electrophoresis of rAAV-UF5 fractions at different stages of purification is shown in Fig. 3. Virus in iodixanol gradient fractions prepared from cells transfected with pTR-UF5/pDG (lane 2, Fig. 3) was directly applied to a heparin–agarose type I column (lane 4, Fig. 3) and eluted with 1 *M* NaCl as described below. The 1 *M* NaCl fraction contained 34.6% of the input rAAV (Table I), and was more than 95% pure, as judged by silver-stained SDS gel analysis. The heparin–agarose affinity fractionation yielded consistently higher purity virus than the traditional protocol that used ammonium sulfate precipitation and two rounds of CsCl gradient centrifugation (Fig. 3, lane 7). Iodixanol/heparin–agarose preparations also typically had the best particle-to-infectivity ratios.

Protocol

1. A prepacked 2.5-ml heparin–agarose type I column (Sigma) is equilibrated with 20 ml of PBS–MK (see above) under gravity. Alternatively, the columns can be placed inside 15-ml screw-cap conical tubes (Sarstedt) and spun at 200 rpm for 5 min in a type J6-HC centrifuge (Beckman). After each spin the flow-through is discarded and fresh buffer is added, and the washing repeated three more times.
2. The rAAV iodixanol fraction is then applied to the equilibrated column under gravity, and the column is washed with 10 ml of PBS–MK buffer either under gravity or by gentle batch spinning as described above.
3. Recombinant AAV is eluted with the same buffer containing 1 *M* NaCl under gravity or gentle centrifugation (100 rpm). After applying 10

[27] B. C. Herold, S. I. Gerber, T. Polonsky, B. J. Belval, P. Shaklee, and K. Holme, *Virology* **206,** 1108 (1995).

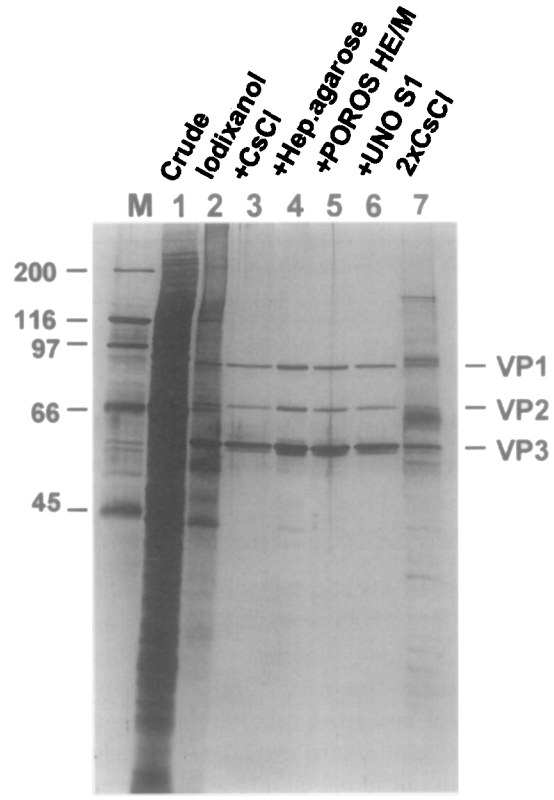


FIG. 3. Silver-stained SDS–acrylamide gel electrophoresis of rAAV-UF5 at various stages of purification. VP1, VP2, and VP3 indicate the position of the three AAV viral capsid proteins. Lanes 1–6 are various fractions obtained from virus preparations efirst employing an iodixanol gradient and then the indicated step from crude viral lysate as described. Lanes 5 and 6 are results from POROS HE/M and UNO S1 columns, respectively, and are not discussed specificially in text. Lane 7 contains a separate rAAV-UF5 preparation that was purified by the conventional method of ammonium sulfate precipitation followed by two CsCl gradients. A comparison of viral titers from each purification scheme is shown in Table I.

ml of the elution buffer, the first 2 ml of the eluant is discarded, and the virus collected in the subsequent 3.5 ml of elution buffer.

Concentration of Recombinant Adeno-Associated Virus

The heparin sulfate column-purified rAAV is then concentrated and desalted by centrifugation through BIOMAX 100 K filters (Millipore, Bedford, MA) according to the manufacturer instructions. The 1 *M* salt in the

TABLE I
COMPARISON OF IODIXANOL/HEPARIN AGAROSE AND $(NH)_4SO_4$/CsCl PURIFICATION[a]

Purification	Particles by QC-PCR ($\times 10^{-11}$)	Infectious units by ICA ($\times 10^{-9}$)	Particle-to-infectivity ratio
NH_4SO_4/2× CsCl	0.2	0.012	1667
Iodixanol/ heparin–agarose	1.0	1.5	67

[a] Crude rAAV stock was made by cotransfection of 293 cells with pDG and pTR-UF5 and purified by either ammonium sulfate and two cesium chloride gradients or by iodixanol/heparin agarose fractionation. Note that the quoted physical and biological titers are assayed before viral concentration, a step that further increases titers at least 10-fold.

initial buffer is effectively exchanged by three cycles of concentration, dilution with lactated Ringer solution, and recentrifugation.

Characterization of Purified Recombinant Adeno-Associated Virus

An important index of virus quality is the ratio of physical particles to infectious particles in a given preparation. To characterize rAAV, we routinely titer both physical and infectious rAAV particles. We have found that a simple quantitative competitive (QC)-PCR assay (Fig. 4A) for physical particle titers is easier and more reliable than the conventional dot-blot assay. As the competitive standard a pTR-UF5 plasmid DNA containing a small internal deletion in the *neo* gene has been created. Infectious titers are determined by an infectious center assay (ICA). To avoid adventitious contamination of rAAV stocks with wtAAV, the use of wtAAV as a helper in the ICA has been eliminated. This was made possible by the use of the C12 cell line[21] containing integrated wtAAV *rep* and *cap* genes, for both the infectious center assay and the fluorescent cell assay. Adenovirus serotype 5 (Ad5), used to coinfect C12 along with rAAV for the ICA assay, is titered using the same C12 cell line in a serial dilution assay of cytopathic effect (CPE). The amount of Ad5 producing well-developed CPE at 48 hr postinfection on C12 cells is used to provide rAAV helper function. The physical particle titer and infectious titers typically differ by about a factor of 100 or less in an acceptable preparation (Table I).

Protocol

Quantitative-Competitive Polymerase Chain Reaction Assay for Recombinant Adeno-Associated Virus Physical Particles

1. The purified, concentrated viral stock is first treated with DNase I to digest any contaminating unpackaged DNA. Ten microliters of a purified

virus stock is incubated with 10 U of DNase I (Boehringer Mannheim, Indianapolis, IN) in a 100-μl reaction mixture, containing 50 m*M* Tris-HCl (pH 7.5), 10 m*M* $MgCl_2$ for 1 hr at 37°.

2. At the end of the reaction, 10 μl of 10× proteinase K buffer [10 m*M* Tris-HCl (pH 8.0), 10 m*M* EDTA, 1% (w/v) SDS final concentration] is added, followed by the addition of 1 μl of proteinase K (18.6 mg/ml; Boehringer Mannheim). The mixture is then incubated at 37° for 1 hr.

3. Viral DNA is purified by two phenol–chloroform extractions, followed by chloroform extraction and ethanol precipitation using 10 μg of glycogen as a carrier. The DNA pellet is then dissolved in 100 μl of water.

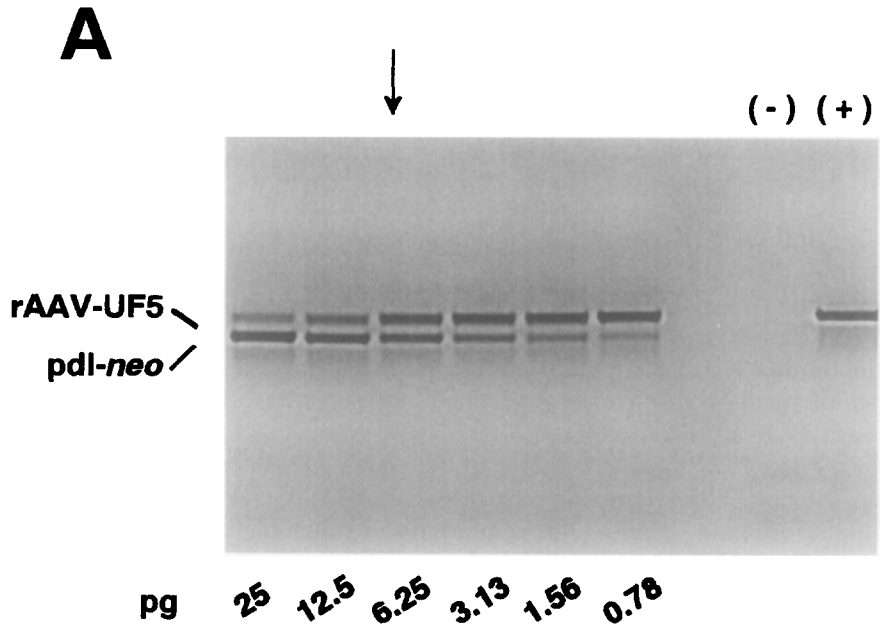
4. PCR mixtures contain 1 μl of the diluted viral DNA and twofold serial dilutions of the internal standard plasmid DNA. The most reliable range of standard DNA was found to be between 1 and 100 pg. Aliquots of each PCR are then analyzed by 2% (w/v) agarose gel electrophoresis until the two PCR products, one from the rAAV DNA template and one from the competitor template, are resolved (Fig. 4A). An image of the ethidium bromide-stained gel is then digitized using an ImageStore 7500 system (UVP, Upland, CA) and the densities of the target and competitor bands in each lane measured using the ZERO-Dscan Image Analysis System, version 1.0 (Scanalytics, Billerica, MA). The ratios are plotted as a function of the standard DNA concentration. At ratio of 1, the mass of viral DNA equals the mass of competitor DNA. This value is then used to determine the DNA concentration of the virus stock, thus allowing an estimate of the physical viral titer.

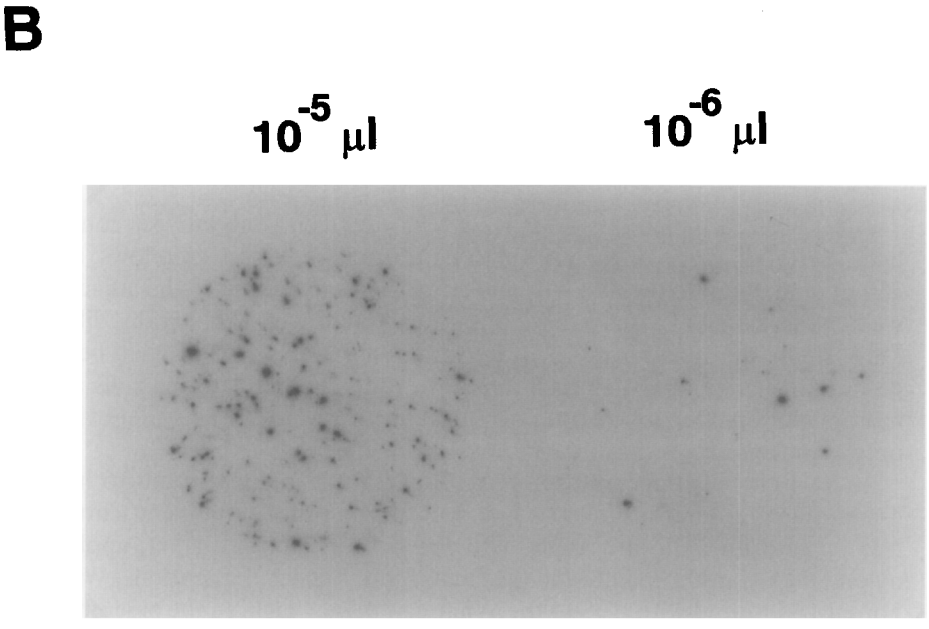
Infectious Center Assay

A modification of a previously published protocol[12] is used to measure the ability of the recombinant virus to infect C12 cells, unpackage, and replicate its DNA.

1. C12 cells are plated in DMEM in a 96-well dish at about 75% confluence. Serial dilutions of the rAAV to be titered are set up as follows: add 250 μl of medium to the first well and add 225 μl of medium to the adjacent wells. Add 2.5 μl of virus to be titered to the first well and serially dilute (10× steps) by transferring 25 μl per dilution to the adjacent well, being certain to change tips after each dilution. Add adenovirus to the cells at a multiplicity of infection (MOI) of 20. Leave a few wells as "adenovirus only" controls.

2. At 40 hr postinfection, set up a 12-port filter manifold built to hold 4-mm-diameter filters as follows: first, wet two pieces of Whatman (Clifton, NJ) paper with PBS and apply it to the entire manifold. Next, apply a nylon DNA transfer membrane (MagnaGraph; Micron Separations, Westboro, MA) wetted with PBS to the Whatman paper. Tighten the assembled


A
(-) (+)
rAAV-UF5
pdl-neo
pg 25 12.5 6.25 3.13 1.56 0.78


B
10-5 µl
10-6 µl

manifold and fill each well of the manifold with 5 ml of PBS. (Alternatively, individual glass-fritted disk 4-mm-diameter filter holders fitted into a vacuum manifold can be used.)

3. Detach cells from the 96-well dish by pipeting vigorously eight times and apply each infected cell sample to one well of the manifold. Wash the well with 200 μl of PBS and apply this also to the appropriate well. After transferring all of the cells to the filter manifold, allow 5 min to pass before gentle vacuum (about 1 cm of water) is applied.

4. Allow the nylon membrane to air dry for 5 min on Whatman 3MM paper. Denature the DNA on the filter for 5 min in 0.5 *M* NaOH, 1.5 *M* NaCl and then blot on Whatman paper. Neutralize the membrane for 5 min in 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.8, and blot on Whatman paper. Rinse in 4× SSC (1× SSC is 0.15 *M* Nacl plus 0.015 *M* sodium citrate) for 30 sec and air dry for 10 min. Microwave the nylon membrane on a high setting for 4 min to fix the DNA. *Caution:* Be sure to have about 300 ml of water in a separate beaker in the microwave or the membrane may catch on fire.

5. Prehybridize the membrane in HM [7% (w/v) SDS, 0.25 *M* $NaHPO_4$ (pH 7.2), 1 m*M* EDTA (pH 8.0)] at 65° for at least 2 hr before adding the probe. The probe is a ^{32}P-labeled riboprobe against any portion of the DNA in the rAAV being titered. Add the probe in 100 ml of HM to the membrane in a glass hybridization cylinder and hybridize at 65° for 12 hr. Wash the membrane twice for 1 hr each in HM at 60°, dry, and expose the filter to standard X-ray film.

An example of filter autoradiographs is shown in Fig. 4B. To calculate the infectious titer of the rAAV preparation, count the number of positive cells on the filters (optimally 20–200 positive dots per filter) and correct for the dilution of the stock used on that filter.

FIG. 4. Titering of rAAV stocks by the QC-PCR assay (A) or infectious center assay (B). (A) Agarose gel electrophoresis of QC-PCR products (negative image). The top band is the product obtained from rAAV-UF5 viral DNA; the bottom band (pdl-*neo*) is the PCR product obtained from the standard competitor plasmid, which contains a small deletion in the *neo* gene sequence that is amplified. The numbers below each lane show the amount of input competitor DNA in each reaction. The viral DNA sample was diluted 1000-fold to achieve a template concentration that results in linear amplification of the input DNA. Symbols (−) and (+) indicate negative (no template) and positive (vector DNA without competitor) controls in the PCR. The arrow indicates the concentration at which the target template and the standard template were equivalent. (B) Autoradiographic image of nylon filters containing products of an infectious center assay, in which C12 cells had been infected with rAAV-UF5 and Ad5 and hybridized with ^{32}P-labeled GFP probe. Numbers at the top show the respective dilutions of the rAAV used to infect the C12 cells. The assay was typically linear in the range of 10–200 spots.

Discussion

The history of rAAV production is not without controversy. While some investigators report efficient rAAV-mediated transduction, others have found a strong dependence of transduction on helper virus adenovirus contaminants,[28] wtAAV contaminants,[12,29] or the growth state of the cells being transduced.[30] Crude rAAV preparations have also been considered a source of protein for artifactual transduction.[31] Some of the variability associated with rAAV transduction *in vivo* is undoubtedly due to intrinsic properties of the target cells. Some cells, for example, are relatively deficient in the high-affinity heparin proteoglycan receptor[22] and others may be incapable of efficiently synthesizing the transcriptionally active form of the rAAV genome.[28,32] However, a significant aspect of rAAV cell transduction variation undoubtedly relates to the methods used to purify rAAV and resultant contaminants present in the final preparation. In general, there has been a correlation between the success of rAAV vectors *in vivo* and the generation of high-titer, helper virus-free rAAV. Under optimal conditions, as few as 10–40 infectious particles of rAAV have been found to be sufficient to transduce one cell *in vivo*.[2,8,9]

The use of an iodixanol gradient rather than CsCl gradients as the initial rAAV purification from a crude lysate deserves examination because CsCl density centrifugation remains the standard in many laboratories. We encounter at least three problems during purification of rAAV using conventional CsCl centrifugation methods. First, rAAV is often nonspecifically bound to cellular protein and helper adenovirus. Such associations lead to an aggregation of rAAV, reducing yields and purity. Often the final rAAV stock contains cell remnants or serum proteins that may compromise subsequent interpretation of *in vivo* data by triggering immune responses. Second, the conventional purification method often produces viral preparations with particle-to-infectivity ratios greater than 1000, whereas ratios approaching 20:1 should be achievable[12] (Table I). Third, conventional purification takes up to 2 weeks to complete and often results in a substantially lower overall yield. In contrast, iodixanol has proved to be an excellent

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bulk purification method that accomplishes three things: first, rAAV from the crude lysate is purified at least 100-fold. Even if helper adenovirus helper is present, this contamination is reduced by a factor of 100. Second, rAAV is purified and concentrated in a nonionic and relatively nonviscous medium that can be loaded onto virtually any kind of chromatographic matrix. Finally, for reasons that remain unclear, iodixanol prevents rAAV aggregation and the associated loss of virus accompanying most other bulk purification and column chromatography methods. Typically, 70–80% of the starting infectious units are recoverable after iodixanol gradient fractionation and, unlike other purification methods, this step is reproducible. In combination with final purification on heparin resins, iodixanol gradients therefore allow a fast, simple, and reproducible rAAV purification protocol, one that is amenable to scale-up and yields stocks of high titer and purity.

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[49] Ribozymes in Treatment of Inherited Retinal Disease

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Introduction

The medical applications of ribozymes were recognized soon after RNA catalysis was discovered in the early 1980s.[1] RNA enzymes, or ribozymes, promote a variety of reactions involving RNA and DNA molecules including site-specific cleavage, ligation, polymerization, and phosphoryl exchange.[2] Naturally occurring ribozymes fall into three broad classes: (1) RNase P, (2) self-splicing introns, and (3) self-cleaving viral agents. RNase P is required for tRNA processing. Self-splicing introns include the group I and II introns of bacteria, mitochondria, and chloroplasts. Self-cleaving agents include hepatitis delta virus and components of plant viroids that

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