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Factors Influencing Recombinant Adeno-Associated Virus Production

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ABSTRACT

Recombinant adeno-associated virus (rAAV) is produced by transfecting cells with two constructs: the rAAV vector plasmid and the rep-cap plasmid. After subsequent adenoviral infection, needed for rAAV replication and assembly, the virus is purified from total cell lysates through CsCl gradients. Because this is a long and complex procedure, the precise titration of rAAV stocks, as well as the measure of the level of contamination with adenovirus and rep-positive AAV, are essential to evaluate the transduction efficiency of these vectors *in vitro* and *in vivo*. Our vector core is in charge of producing rAAV for outside investigators as part of a national network promoted by the Association Française contre les Myopathies/Généthon. We report here the characterization of 18 large-scale rAAV stocks produced during the past year. Three major improvements were introduced and combined in the rAAV production procedure: (i) the titration and characterization of rAAV stocks using a stable rep-cap HeLa cell line in a modified Replication Center Assay (RCA); (ii) the use of different rep-cap constructs to provide AAV regulatory and structural proteins; (iii) the use of an adenoviral plasmid to provide helper functions needed for rAAV replication and assembly. Our results indicate that: (i) rAAV yields ranged between 10^{11} to 5×10^{12} total particles; (ii) the physical particle to infectious particle (measured by RCA) ratios were consistently below 50 when using a rep-cap plasmid harboring an ITR-deleted AAV genome; the physical particle to transducing particle ratios ranged between 400 and 600; (iii) the use of an adenoviral plasmid instead of an infectious virion did not affect the particles or the infectious particles yields nor the above ratio. Most of large-scale rAAV stocks (7/9) produced using this plasmid were free of detectable infectious adenovirus as determined by RCA; (iv) all the rAAV stocks were contaminated with rep-positive AAV as detected by RCA. In summary, this study describes a general method to titrate rAAV, independently of the transgene and its expression, and to measure the level of contamination with adenovirus and rep-positive AAV. Furthermore, we report a new production procedure using adenoviral plasmids instead of virions and resulting in rAAV stocks with undetectable adenovirus contamination.

OVERVIEW SUMMARY

Production of recombinant adeno-associated virus (rAAV) relies upon a complex and relatively inefficient purification procedure. Thus, a precise titration of the rAAV stock, as well as its characterization to measure contaminating adenovirus and rep-positive AAV particles, are essential to evaluate the efficiency of these vectors *in vitro* and *in vivo*. We describe here the development of a modified RCA using a stable rep-cap HeLa cell line. Eighteen large-scale rAAV stocks, produced by our vector core during the past year, were titered and characterized using this modified RCA. This assay was used to monitor the consequences of some major modifications introduced in the rAAV production procedure and particularly the use of an adenoviral plasmid to produce rAAV stocks free of infectious adenoviral particles.

INTRODUCTION

WILD-TYPE ADENO-ASSOCIATED VIRUS (wtAAV) is a naturally defective parvovirus that requires co-infection with a helper virus, such as adenovirus or herpes virus, to establish

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a productive infection. The virus is not associated with any human disease and has been shown to have a broad host range of infection *in vitro*. AAV has a relatively simple genome organization composed of two major genes coding for the regulatory (rep) and structural (cap) proteins. Three viral promoters located at map unit 5 (p5), 19 (p19), and 40 (p40) control the synthesis of mRNA coding for the four Rep and the three Cap proteins. The viral genome is flanked by 145 bases inverted terminal repeats (ITRs) which contain palindromic sequences necessary *in cis* for replication of the viral genome (Leonard and Berns, 1994).

Recombinant AAV viruses (rAAV) are derived by deleting the rep and cap genes, which are replaced by the transgene and the transcriptional control elements needed for its expression. The only viral sequences retained *in cis* are the viral ITRs (Muzyczka, 1992). The ability of rAAV to transduce tissues efficiently in mice such as the muscle, the retina, or the liver (Kessler *et al.*, 1996; Xiao *et al.*, 1996; Zolotukhin *et al.*, 1996; Fisher *et al.*, 1997; Flannery *et al.*, 1997; Herzog *et al.*, 1997; Koeberl *et al.*, 1997; Snyder *et al.*, 1997) and to lead to a prolonged gene expression with little to no pathology makes this virus unique among the family of viral vectors. However, widespread use of rAAV is hampered by the relatively cumbersome and inefficient procedure needed to produce it at high titers and in sufficient amount for *in vivo* experiments. The standard procedure relies upon the transfection of 293 cells with two plasmids: a plasmid providing *in trans* the rep and cap functions and the rAAV vector plasmid itself. After subsequent infection with an adenovirus, rAAV particles are assembled in the nuclei of the cells concomitantly with adenoviral particles. rAAV stocks are obtained after purification from total cell lysates through CsCl gradients (Snyder *et al.*, 1996). Because of this relatively long and complex procedure, a precise titration and characterization of the rAAV stock is essential to evaluate the transduction efficiency *in vitro* and *in vivo* of rAAV stocks.

Our laboratory is part of a national vector cores network funded by the Association Française contre les Myopathies/Généthon, which provides viral vectors for outside investigators. We report here the characterization of 18 large-scale rAAV stocks produced by our vector core during the past year. Three critical modifications were introduced and combined in the rAAV production procedure: (i) the titration and the characterization of rAAV stocks using a stable rep-cap HeLa cell line; (ii) the use of different rep-cap expression plasmids; (iii) the use of an adenoviral plasmid to provide helper functions needed for rAAV replication and assembly.

MATERIALS AND METHODS

Cell lines and viruses

293 and HeLa cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; SIGMA) supplemented with 10% heat-inactivated fetal calf serum (FCS; SIGMA) and 1% (vol/vol) penicillin/streptomycin (GIBCO BRL, 5,000 U/ml). Adenoviruses used are: wild-type adenovirus type 5 (wtAd5) (ATCC VR-5), Ad.dl324 (a gift from Transgène, France), and the double thermosensitive Ad.dts (Moullier P., unpublished data) which cumulates the ts125 mutation in the E2a region and the ts149 mutation located in the E2b DNA polymerase (Ginsberg *et al.*, 1977). All of these adenoviruses were produced and titered on 293 cells using the standard procedures (Graham and Prevec, 1991). Cells and adenoviruses were tested for the absence of wtAAV by PCR as indicated below.

DNA constructs

Rep-Cap Plasmids: The pspRC plasmid (Fig. 1A) contains the ITR-deleted AAV genome (positions 190–4,484 bp). It was excised as an *Xba* I fragment from the psub201 plasmid (Samulski *et al.*, 1989) and inserted into the *Xba* I site of pSP72 plasmid (Promega).

The pspRCC plasmid (Fig. 1A) contains the rep gene (190–2,278 bp of wtAAV) followed by the bovine growth hormone (bGH) gene poly(A) signal and by the cytomegalovirus (CMV) promoter leading the expression of the cap ORF

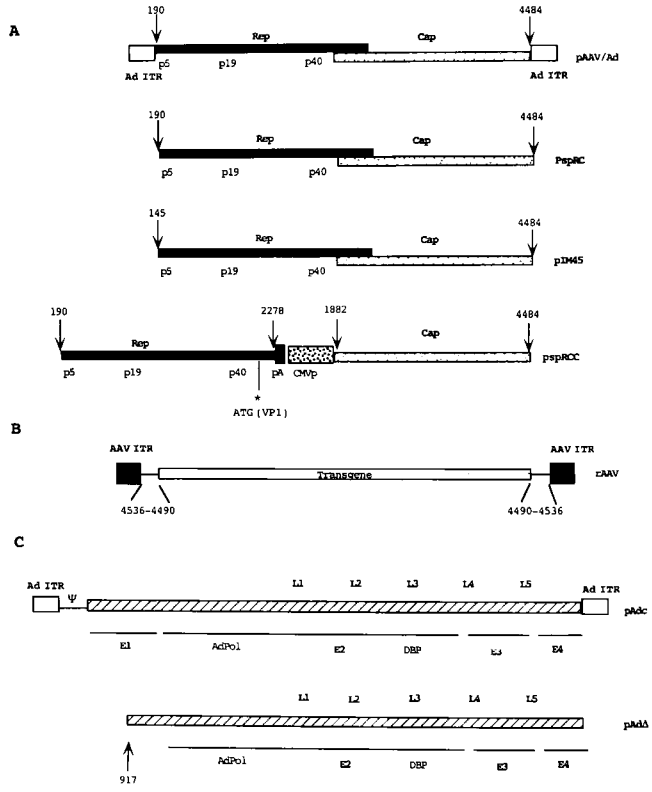

FIG. 1. Constructs used for rAAV production. A. Rep-cap constructs. Constructs pAAV/Ad and pIM45 are described in Samulski *et al.* (1989) and Pereira *et al.* (1997), respectively. The pspRC and the RepCMVCap constructs are described in Materials and Methods. Numbers on the top correspond to position on the wild-type AAV genome. CMVp, CMV promoter; pA, poly(A) signal from the bovine growth hormone gene. B. rAAV plasmids derived from the psub201 plasmid (Samulski *et al.*, 1989). Numbers refer to wild-type AAV sequences maintained in this plasmid and flanking the viral ITRs. C. Adenoviral plasmids: pAdc has the entire wild-type Ad5 genome. Plasmid pAdΔ has a deletion of the 5′ and 3′ ITRs, the ψ and E1 regions until position 917 of wtAd5 (Champion-Arnaud *et al.*, manuscript in preparation).

(1,882–4,484 bp of wtAAV). This plasmid was derived from pspRC by partially deleting the Cap ORF with *Xho* I, which cuts at position 2,232 of wtAAV (upstream of the stop signal for Rep 68 and Rep40) and further downstream in the plasmid backbone. The 324-bp poly(A) signal from the bGH gene was then inserted downstream of the rep open reading frame (ORF) to give the pspRep/pA plasmid. This construct codes for Rep 78 and Rep 52 proteins and contains the p40 promoter of AAV. To complete the Rep ORF, a 90-bp polymerase chain reaction (PCR) fragment including region 2,193–2,278 bp of wtAAV was obtained using the following primers: 5′-atgatttaaatcaggtttggctgccg-3′ (positions 2,187–2,212 of wtAAV) and 5′-gctctagatgagcttccaccactgtc-3′ (positions 2,278–2,251 of wtAAV). This PCR fragment, which includes a mutated VP1 start site (underlined in the primer sequence), was inserted between the *Swa* I and *Xba* I sites of the pspRep/pA plasmid to give plasmid pspRep/pA ΔVP1. To obtain plasmid pspRCC, a cassette composed of the cap ORF (1,882–4,484 bp of wtAAV) placed under the control of the CMV promoter (873 bp) was inserted downstream the poly A signal of pspRep/pA ΔVP1 at the unique *Pvu* II site.

rAAV Vector Plasmids: These were derived from psub201 (Samulski *et al.*, 1989) by deleting the rep-cap *Xba* I or *Sna* BI region and replacing it with different expression cassettes (Fig. 1B).

Adenoviral Plasmids: Two adenoviral plasmids were generated (Fig. 1C): (i) plasmid pAdc contains the complete adenoviral genome cloned into the SuperCos plasmid (Stratagene); (ii) the pAdΔ plasmid contains an adenoviral genome with both ITRs, the packaging signal (ψ), and the E1 region deleted, also cloned into the SuperCos plasmid (Champion-Arnaud *et al.*, manuscript in preparation).

rAAV production

rAAV was produced using the procedure detailed in Fig. 2: on day 1, 25 15-cm plates of 293 cells (at ~80% of confluence) were co-transfected by the calcium phosphate method with the rep-cap and the vector plasmids (12.5 μg each). Six hours later, cells were washed with DMEM and infected with adenovirus with a multiplicity of infection (moi) of 10 in DMEM 5% FCS (Fig. 2A). Under these conditions, a cytopathic effect (CPE) was visible approximately 3 days later. When using the Ad.dts adenovirus, the cells were incubated at 32°C, which is the permissive temperature for adenoviral growth. Alternatively, when rAAV was produced using an adenoviral plasmid (Fig. 2B), each dish was transfected on day 1 with three plasmids: the rep-cap, the vector (12.5 μg each) and the pAdc or pAdΔ plasmids (25 μg). Six hours later, cells were washed and incubated in DMEM 5% FCS. No cytopathic effect was evident under these conditions and cells were usually harvested 3 days later unless otherwise indicated.

To purify rAAV particles, cellular pellets (each corresponding to six 15-cm plates) were resuspended in 20 ml of 10 m*M* HEPES pH 7.6, 150 m*M* NaCl buffer and lysed by three cycles of freeze/thawing (dry ice with ethanol/37°C water bath). The cell lysate was then centrifuged at 3,000 rpm for 15 min to remove cellular debris and further clarified by centrifuging at 10,000 × *g* (Beckman rotor JA17) for 10 min at 4°C. The supernatant was then precipitated by addition of the same volume of cold saturated $(NH_4)_2SO_4$ pH 7.0 and incubation for 20 min on ice. After centrifugation at 12,000 × *g* for 20 min at 4°C (Beckman rotor JA17), the supernatant was removed and the pellet was resuspended in 3 ml of phosphate-buffered saline (PBS) pH 7.0, which was then loaded on top of a CsCl step gradient composed of 3 ml of 1.5 grams/ml and 3 ml of 1.35 CsCl in PBS (Beckman Optiseal centrifuge tubes). The gradients were centrifuged for 6 hr (minimum time required to reach equilibrium) to overnight at 67,000 rpm at 15°C (Beckman rotor 90Ti). After centrifugation, a band is visible in the middle of the tube, whichever adenoviral helper system was used (virus or plasmid). Ten fractions (20 drops each) were recovered from the bottom of the tube using the Beckman Fraction Recovery System and analyzed by dot blot to identify those containing rAAV genomes (see below). Usually, rAAV particles are concentrated within six fractions located below the band. The rAAV-containing fractions were then pooled and dialyzed for 24 hr against three changes of PBS supplemented with 0.9 m*M* $CaCl_2$ and 0.5 m*M* $MgCl_2$. The viral suspension was then aliquoted and stored at −80°C without adding any stabilizer. The final titer of the rAAV preparation was determined using a frozen aliquot of virus following the methods described below.

Titration of rAAV stocks

Two different methods are used to measure the rAAV titer: (i) the dot blot analysis to measure the number of particles/ml based on the quantification of viral DNA; (ii) a modified Replication Center Assay (RCA) to measure the number of infectious particles/ml, as well as the level of contaminating adenovirus and rep-positive AAV.

Dot Blot Analysis: 1- and 10-μl of the viral stock are incubated with 20 U of DNase I (Boehringer Manheim) in 200 μl of DMEM for 1 hr at 37°C. Two hundred microliters of 2× Proteinase K solution (20 m*M* Tris-HCl pH 8.0, 20 m*M* EDTA pH 8.0, 1% SDS) are then added and the samples incubated further for 1 hr at 37°C. Viral nucleic acid are then purified by a phenol/chloroform extraction, precipitated after addition of NaOAc/ethanol, and incubated for 20 min at −80°C. After 30 min of centrifugation at 15,000 × *g*, the nucleic acid pellet was washed in 75% ethanol and resuspended in 400 μl of 0.4 *M* NaOH, 10 m*M* EDTA. After heating at 100°C for 5 min, the DNA is loaded on a Zetaprobe membrane (Biorad) using a dot blot apparatus. As a standard for the determination of the amount of viral DNA, several dilutions of rAAV vector plasmid used to produce the virus were loaded on the same membrane. After blotting, the membrane was prehybridized for 30 min at 60°C. A denatured fluorescein-labeled probe (Amersham, *Gene Images* random prime labeling module) corresponding to the cDNA included in the rAAV vector was then added and incubated overnight at 60°C. The following day, the membrane was processed according to the manufacturer's protocol (Amersham, *Gene Images* CDP-Star detection module) and exposed to autoradiography film.

Modified RCA: This protocol is similar to the one previously described (Yakobson *et al.*, 1987; McLaughlin *et al.*, 1988), but instead of using wtAAV we used a stable HeLa cell clone expressing *rep-cap* (HeLaRC32) (Salvetti *et al.*, manuscript in preparation). Briefly, HeLaRC32 and control HeLa cells were

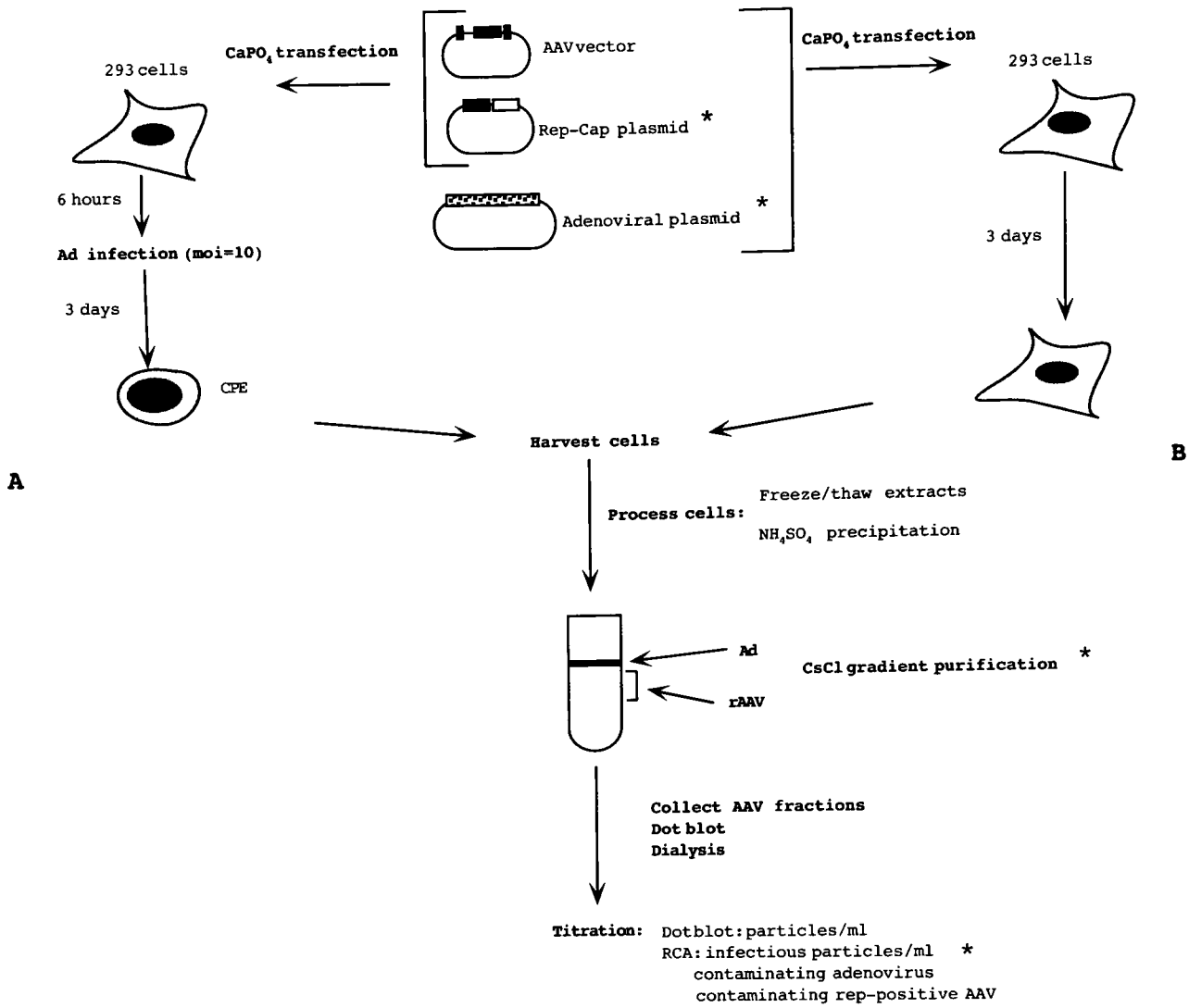

FIG. 2. rAAV production protocol. rAAV was produced using two different protocols. A. 293 cells were transfected with the rAAV vector and the rep-cap plasmid (1:1 ratio) and infected 6 hr later with adenovirus. When a cytopathic effect (CPE) was evident, cells were harvested and processed as described in Materials and Methods. B. 293 cells were transfected with the rAAV vector, the rep-cap plasmid and the adenoviral plasmid (1:1:2 ratio), washed after 6 hr, collected 3 days later and processed. After centrifugation, rAAV-containing fractions were pooled, dialyzed, and titered by dot blot and RCA as described in Materials and Methods. Asterisks indicate the steps that were modified from the original protocol (Snyder *et al.*, 1996).

seeded the previous day in a 48-well plate (8×10^4 cells/well). The following day, cells are infected with 2 μl of pure or diluted rAAV with or without wild-type adenovirus (moi of 50) in 200 μl of DMEM and 5% FCS. Twenty four hours later, cells were trypsinized, washed in PBS, and filtered through a Zetaprobe membrane (Biorad). Filters were soaked in 0.5 *M* NaOH, 1.5 *M* NaCl for 5 mn and then neutralized in a 1 *M* Tris HCl pH 7.0, 2× SSC solution. Filters were hybridized overnight to a fluorescein-labeled probe corresponding to the cDNA included in the rAAV vector and then processed as previously described.

LacZ forming unit (LFU) assay

To measure the transducing activity of rAAV harboring the β-galactosidase (β-Gal) gene (*LacZ*), HeLa cells, seeded the day before at 2×10^5 cells per well (24-well plate), were infected with pure or diluted rAAV in DMEM and 5% FCS in the presence or in the absence of wtAd5 (moi of 50). Twenty-four hours later, cells were washed in PBS, fixed with 0.5% glutaraldehyde for 5 min at room temperature, and then stained with 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) for 6 hr at 37°C.

Southern blots on low-molecular-weight DNA

For extracting low-molecular-weight DNA, cells were trypsinized and lysed in a solution of 10 m*M* Tris HCl pH 8.0, 10 m*M* EDTA, 1% SDS for 30 min at 37°C. After addition of Proteinase K at 500 μg/ml final (Boehringer Manheim), the lysate was incubated for 2 hr at 37°C. To precipitate high-molecular-weight DNA, 5 *M* NaCl was added to the cell lysate (fi-

nal concentration of 1.1 *M*) and incubated overnight at 4°C. High-molecular-weight DNA was pelleted by centrifugation at 10,000 rpm for 20 min at 4°C and the supernatant was extracted twice with chloroform at room temperature. The nucleic acid was then precipitated with ethanol and resuspended in 10 m*M* Tris pH 8.0, 1 m*M* EDTA containing 200 μg/ml of RNase (Boehringer Manheim). After incubation for 15 min at room temperature, the DNA is extracted twice with chloroform and ethanol/NaOAc precipitated. The final pellet was resuspended in 10 m*M* Tris pH 8.0, 1 m*M* EDTA and stored at −20°C. For analysis, the DNA was digested with *Dpn* I (which cleaves only input methylated plasmid DNA), run on a 1% agarose gel, and transferred under alkaline conditions on a Hybond N^+ membrane (Amersham). The membrane is hybridized to a fluorescein-labeled probe and processed as described above.

Detection of rep sequences by PCR

Cells and adenoviral stocks were routinely assayed for the presence of rep-positive AAV by PCR. The PCR primers were: Rep1 (5′-TATTTAAGCCCGAGTGAGCA-3′), which corresponds to positions 255–275 of wild-type AAV in the p5 promoter; Rep3 (5′-AAAGTTCTCATTGGTCCAGT-3′), which corresponds to positions 1,417–1,397 of wild-type AAV in the Rep52/40 ORF. PCR was carried using *Taq* polymerase (GIBCO-BRL) for 25 cycles (30 sec at 94°C, 30 sec at 55°C, 33 sec at 72°C) in a Perkin-Elmer thermocycler (Gene Amp PCR System 9600).

RESULTS

Description of the rAAV production method

rAAV was initially produced following the protocol described by Snyder *et al.* (1996). Briefly, 25 15-cm plates of 293 cells are transfected with two plasmids: one harboring the rep and cap genes and the other the rAAV vector (Fig. 2A). Six hours after transfection, cells are infected with adenovirus (wild type, or Ad.dts). When a cytopathic effect appeared, cells were harvested and lysed and extracts were purified through two cesium chloride gradients. A technical change was introduced in the purification step on the CsCl gradient reducing the centrifugation time down to 6 hr to reach equilibrium. This modification did not affect the rAAV yields but instead increased the final volume of virus. Beside this technical improvement, three major modifications were introduced in the rAAV production procedure (Fig. 2): the first one concerns the titration method employed to measure the number of infectious particles produced; the second one is related to the use of different rep-cap expression constructs; finally, the third modification concerns the use of an adenoviral plasmid instead of an adenoviral particle to provide helper functions needed for rAAV replication and assembly.

A summary of the 18 large-scale rAAV stocks produced is presented in Table 1. A detailed analysis of these results in relation with the major modifications introduced in the standard protocol is presented below.

TABLE 1. CHARACTERIZATION OF THE 18 LARGE-SCALE rAAV STOCKS

Vector name	*Size (b)*	*rep-cap plasmid*	*Virus or plasmid*	*rAAV Titer*			*Contaminations*[c]		*Vol. (ml)*
				p./ml[a]	*i.p./ml*[b]	*ratio*	*Ad. i.p./ml*	*rep+ AAV i.p./ml*	
AAVCMVLacZ	4873	pAAV/Ad	wtAd5	1.2×10^{12}	1.0×10^{9}	1.2×10^{3}	5×10^{4}	4.5×10^{7}	1.6
AAVCMVGDNF	3000	pAAV/Ad	wtAd5	2.5×10^{11}	1.5×10^{7}	1.7×10^{4}	7.5×10^{4}	5.5×10^{5}	1.7
AAVCMVGDNF	3000	pAAV/Ad	wtAd5	8.0×10^{11}	2.0×10^{8}	4.0×10^{3}	1.0×10^{5}	4.0×10^{7}	2.8
AAVCMVLacZ	4873	pspRC	wtAd5	1.6×10^{12}	9.2×10^{10}	17.3	7×10^{3}	1.0×10^{5}	1.1
AAVPGKβGLU	3854	pspRC	Ad.dts	3.0×10^{11}	9.5×10^{9}	31.5	3.1×10^{4}	1.0×10^{5}	3.0
AAVPGKhALD	3565	pspRC	Ad.dts	1.4×10^{11}	1.8×10^{10}	7.8	1.3×10^{4}	1.0×10^{4}	3.1
AAVCMVApoE4	2609	pspRC	wtAd5	4.7×10^{10}	1.1×10^{10}	4.2	1.1×10^{4}	1.5×10^{4}	3.6
AAVCMVEpo/rtTa	5017	pspRC	wtAd5	2.5×10^{11}	2.1×10^{10}	11.9	2.3×10^{4}	1.3×10^{6}	4.1
AAVPGKnlsLacZ	4712	pspRC	wtAd5	2.5×10^{11}	9.0×10^{10}	2.8	2.2×10^{4}	5.0×10^{5}	4.2
AAVCMVnlsLacZ	4641	pspRC	pAdc	5.1×10^{11}	1.6×10^{10}	31.8[3]	$<5 \times 10^{2}$	4.5×10^{5}	7.6
AAVLTRApoE	3700	pspRC	pAdc	3.0×10^{11}	3.7×10^{10}	8.1	2.5×10^{3}	5.5×10^{5}	7.6
AAVLTREpo	3310	pspRC	pAdc	3.7×10^{11}	4.2×10^{10}	8.8	$<5 \times 10^{2}$	4.0×10^{5}	7.2
AAVCMVnlsLacZ*	4641	pspRC	pAdc	3.0×10^{11}	2.5×10^{10}	12.0	$<5 \times 10^{2}$	7.0×10^{4}	13.4
AAVCMVβGLU	4717	pspRC	pAdc	3.8×10^{10}	1.5×10^{10}	2.5	$<5 \times 10^{2}$	4.5×10^{4}	6.8
AAVCMVEpo	2238	pspRC	pAdc	1.2×10^{11}	5.4×10^{10}	2.2	$<5 \times 10^{2}$	5.0×10^{4}	6.9
AAVCMVvEGF	2080	pspRC	pAdc	1.8×10^{11}	7.4×10^{10}	2.4	$<5 \times 10^{2}$	2.5×10^{5}	7.5
AAVCMVnlsLacZ*	4641	pspRC	pAdc	7.8×10^{10}	5.5×10^{10}	1.4	$<5 \times 10^{2}$	1.5×10^{5}	14.0
AAVCMVβGLU*	4717	PSPRC	pAdc	3.4×10^{11}	6.6×10^{10}	5.1	2.5×10^{3}	1.3×10^{5}	11.4

Each vector was produced from 25 15-cm plates of 293 cells except for stocks marked with an asterisk which were produced from 50 15-cm plates of cells. The vector name indicates the promoter and the transgene inserted between AAV ITRs. The second column indicates the rAAv size (ITR to ITR), the third, the rep-cap construct used for the production, and the fourth whether adenovirus (wtAd5 or Ad.adts) or an adenoviral plasmid (pAdc) was used. The last column indicates the final volume of virus after CsCl gradient purification and dialysis. rAAV stocks listed below the darker line in the middle of the table were purified following the centrifugation conditions described in Materials and Methods. Those listed above were purified following the protocol described by Snyder *et al.* (1996). p./ml; particles/ml; i.p./ml: infectious particles/ml.

[a]rAAV titer measured by dot blot.

[b]rAAV titer measured by RCA.

[c]Contaminations with adenovirus and rep-positive AAV particles were also measured by RCA.

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Use of a stable rep-cap HeLa cell clone for titration and characterization of rAAV stocks

Quantification of viral DNA by dot blot is generally used to measure the amount of rAAV particles. This assay, however, does not provide any information about the number of infectious rAAV particles that relies either on the detection of the rAAV transducing activity or in the measure of infectious particles in a Replication Center Assay (RCA). This latter method is particularly interesting because it does not depend upon the expression of the transgene but only upon the ability of rAAV to infect a target cell (generally 293 cells) and to replicate its genome in the presence of adenovirus and Rep proteins. In the originally described RCA (Yakobson *et al.*, 1987; McLaughlin *et al.*, 1988), Rep proteins are provided by adding wtAAV, which requires a restricted area to prevent any contamination. Our rationale was to circumvent the use of wtAAV in this assay by developing a stable cell clone expressing Rep proteins. A stable HeLa cell clone with two integrated copies of an ITR-deleted AAV genome (HeLaRC32 cells) was thus generated (Salvetti *et al.*, manuscript in preparation). In the modified RCA, HeLaRC32 or control HeLa cells are infected with different dilutions of rAAV in the presence or in the absence of wild-type adenovirus and individually analyzed for the presence of replicating rAAV using a transgene probe. A typical result obtained using this assay is presented in Fig. 3 where the number of spots obtained with the heLaRC32 cells infected with rAAV and adenovirus can be translated as the number of infectious rAAV particles, and the one obtained with the HeLaRC32 cells infected with rAAV, but in the absence of adenovirus, as the number of contaminating infectious adenoviral particles. As expected, no signal was detected when HeLaRC32 cells were infected with adenovirus alone. Finally, when control HeLa cells were infected with rAAV and adenovirus, some signal was always detected, suggesting that rAAV was amplified in these cells (Fig. 3). A plausible interpretation of this result is that rAAV stocks are contaminated with rep-positive AAV particles. This last result was thus used to measure the number of rep-positive AAV particles in all the large-scale rAAV stocks. The development of this modified RCA allowed us to characterize fully the effect of each modification introduced in the rAAV production protocol as described below.

Evaluation of different rep-cap expressing plasmids for rAAV production

The pAAV/Ad plasmid, described in the standard rAAV production method (Samulski *et al.*, 1989), harbors the AAV rep-cap sequences flanked by the adenovirus ITRs (Fig. 1A). After producing three rAAV stocks with the pAAV/Ad plasmid, the following rAAV stocks were produced using the pspRC construct, which harbors only the rep-cap sequences in a psp72 backbone (Fig. 1A). The use of the pspRC plasmid for large-scale rAAV production did not affect the total number of physical particles recovered, which ranged between 10^{11} and 5×10^{12} total particles, but instead increased the infectious particles yields, as measured by RCA (Table 1 and Fig. 4). Indeed, particles to infectious particles ratios ranged between 10^3 and 10^4 when the pAAV/Ad was the trans-complementing plasmid, whereas, rAAV stocks produced with the pspRC plasmid con-

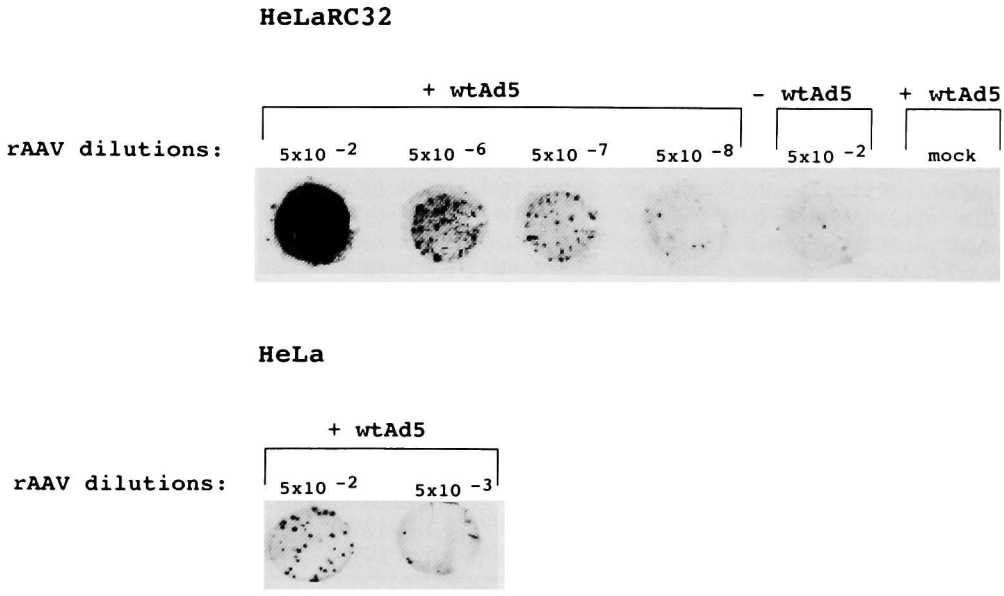

FIG. 3. Characterization of rAAV by a modified RCA. HeLaRC32 cells, harboring two integrated copies of an ITR-deleted AAV genome (Salvetti *et al.*, manuscript in preparation) or control HeLa cells were infected with different dilutions of the rAAV stocks and either infected or not with wtAd5 (see Materials and Methods for details). Twenty-four hours later, cells were trypsinized, filtered through a membrane, lysed, and the filters were hybridized overnight with a transgene probe. The number of infectious rAAV particles is determined by counting the number of spots on HeLa32RC cells in the presence of adenovirus. The same assay performed in the absence of adenovirus gives the level of contamination with infectious adenoviral particles. No signal is detected when HeLaRC32 cells are infected with adenovirus alone. Finally, the result on control HeLa cells in the presence of adenovirus gives the level of contamination with rep-positive AAV.

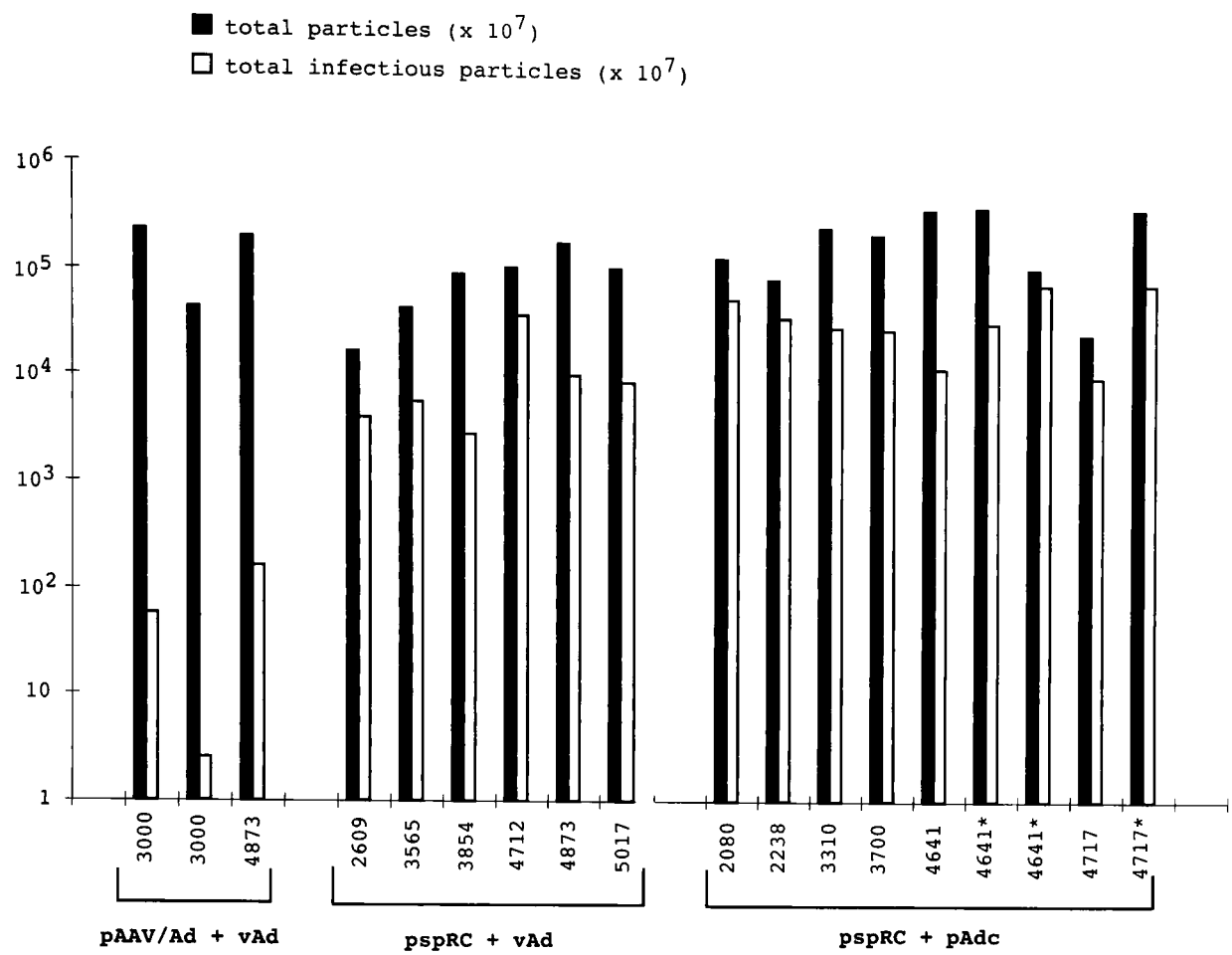

FIG. 4. Comparison of rAAV yields produced with different rep-cap plasmids and either adenovirus or an adenoviral plasmid. rAAV stocks described in Table 1 were titered by dot blot and RCA to measure, respectively, the number of particles and infectious particles. The *x* axis indicates the size of each rAAV and the production protocol followed: *pAAV/Ad + vAd*, transfection of the rAAV vector with the pAAV/Ad plasmid followed by infection with adenovirus; *pspRC + vAd*, transfection of the rAAV vector with the pspRC plasmid followed by infection with adenovirus; *pspRC + pAdc*, transfection of the rAAV vector, the pspRC construct, and the adenoviral plasmid pAdc harboring the entire adenoviral genome. All of the rAAV stocks were prepared from 25 15-cm plates of 293 except for stocks marked with an asterisk that were prepared from 50 15-cm plates of cells.

sistently showed a ratio below 50 and this independently of the adenoviral helper functions provided (adenoviral virions or adenoviral plasmid).

Other rep-cap constructs were further tested on small-scale rAAV productions performed on two 15-cm plates of 293 cells. The pspRC plasmid was compared to two other constructs: (i) plasmid pIM45 (Pereira *et al.*, 1997), which harbors approximately the same rep-cap region extended on the 5′ end of 45 bp from wtAAV (Fig. 1A); (ii) plasmid pspRCC, which harbors the rep ORF under the control of the native AAV promoters followed by the cap ORF under the transcriptional control of the CMV promoter (Fig. 1A). This last construct was used to test whether expression of the cap gene under the control of a strong heterologous promoter can increase the viral titer as recently reported (Vincent *et al.*, 1997). rAAV produced on small-scale experiments was purified on a CsCl gradient and characterized by dot blot, RCA, and by a LFU on HeLa cells. As indicated in Table 2, approximately the same number of particles was measured by dot blot. Similarly, the number of infectious or transducing particles was not significantly affected by the rep-cap plasmid used. The reason for the difference between our result and that reported by Vincent *et al.* (1997) is presently not understood, although they used adenoviral virions as helper whereas we used an adenoviral plasmid (see below).

Use of an adenoviral plasmid for rAAV production

Recombinant AAV stocks were initially produced using either wtAd5 or Ad.dts adenovirus. None of these virions actually made any difference in terms of rAAV production (Table 1). Subsequently, we tested whether adenoviral virions could be replaced by plasmid pAdc harboring the complete adenoviral genome from wtAd5 (Fig. 1C). The production protocol, thus, consisted of an initial transfection of three plasmids (Fig. 2B): the vector, the rep-cap, and the adenoviral plasmid (1:1:2 ratio). No cytopathic effect was observed even five days after transfection. Consequently, to purify rAAV, cells were usually

TABLE 2. EVALUATION OF DIFFERENT REP-CAP EXPRESSION PLASMIDS FOR rAAV PRODUCTION

	Part/ml[a]	*Inf. part./ml*[b]	*LFU/ml*[c]
pspRC	1.7×10^{10}	1.2×10^{9}	2.0×10^{6}
pIM45	8.5×10^{9}	3.2×10^{8}	6.6×10^{5}
RepCMVCap	3.4×10^{10}	5.8×10^{8}	2.0×10^{6}

rAAV was produced from two 15-cm plates of 293 cells transfected with the AAVCMVnlsLacZ vector, the adenoviral plasmid pAdc, and the indicated rep-cap construct. Cells were collected 3 days after transfection and cell extracts purified as described in Materials and Methods. The final volume of virus was of 7 ml for each stock.

[a]Stock titered by dot blot.

[b]Stock titered by RCA.

[c]Stock titered by LFU assay performed on HeLa cells.

scraped from the plates 3 days after transfection and processed as previously described.

Titration of rAAV stocks, produced using this new procedure, indicated that replacement of adenoviral virions by the pAdc plasmid did not affect the particles yields, which ranged between 10^{11} and 5×10^{12} total particles (Table 1 and Fig. 4). Similarly, the infectious particles yields remained unchanged (Fig. 4). However, some variability in the production yields using the pAdc plasmid was observed and we suspect that this result may be related to the inconsistent quality of the pAdc plasmid and/or the same variability in transfection efficiencies.

We next compared the pAdc plasmid with plasmid pAdΔ, which harbors an adenoviral genome lacking the two ITRs, the packaging signal, and the E1 region (Fig. 1C). Two 15-cm plates of 293 cells were transfected with the vector, the pspRC plasmid, and either the pAdc or the pAdΔ constructs. To determine the optimal harvesting time for rAAV production, the cells were collected at 72, 96, and 120 hr post-transfection. Cellular extracts were purified on a CsCl gradient and the results of the dot blot and of the RCA are presented in Table 3. The same rAAV titer ranging between 10^{10} and 5×10^{10} particles/ml was obtained with both plasmids. Incubation of the cells for more than 3 days did not significantly affect the particles/ml titer, however, a slight reduction in the infectious particles yields was observed.

Both the pAdc and the pAdΔ adenoviral plasmids, generated a slight band observed at equilibrium in the CsCl gradients at a position similar to mature adenoviral particles. We were not able, however, to detect plaques after incubation of 293 cells with an aliquot of the band obtained with the pAdc plasmid.

More importantly, no adenoviral contamination was observed in most of the large-scale rAAV stocks (7/9) produced using the pAdc plasmid as detected by RCA (Table 1). Also, the low level of adenoviral contamination ($2.5\ 10^{3}$ i.p./ml) observed in two rAAV stocks has not yet been confirmed by other methods.

Detection of rep-positive AAV in the rAAV stocks

The rAAV stocks were tested in a modified RCA for the presence of contaminating rep-positive AAV. Infection of control HeLa cells with different dilutions of rAAV and adenovirus resulted in the detection of some level of replicating rAAV (detected with a transgene probe) in all the rAAV stocks (Fig. 3). This suggested that some Rep activity had been transferred to these cells. This level of contamination ranged between 10^{4} and 5×10^{7} infectious rep-positive AAV particles/ml and this independently of the use of adenovirus or of an adenoviral plasmid (Table 1). To substantiate this result, 293 cells were infected with rAAVCMVnlsLacZ, produced either with adenovirus or the pAdc plasmid, in the absence or in the presence of Ad.dl324, and low-molecular-weight DNA was analyzed on a Southern blot using a LacZ probe. As shown in Fig. 5, the typical AAV replicative forms were detected when cells were co-infected with adenovirus, whereas only input single-stranded DNA was seen in cells infected with rAAVCMVnls LacZ alone. This result suggested that particles containing rep sequences and/or Rep proteins are present in the rAAV stocks. To check for the presence of rep sequences, DNA was extracted from 10 μl of a rAAV stock (approximately 10^{9} particles) and analyzed by PCR using primers located in the p5 promoter and the rep gene (see Materials and Methods). As expected, an amplification product was detected in all the rAAV stocks (data not shown). Altogether, these data suggest that some rep DNA is packaged during rAAV assembly in 293 cells.

Assay of rAAV transducing activity in vitro *and* in vivo

Because nearly all rAAV stocks described in Table 1 were produced for external investigators, most of them were not directly assayed in the laboratory and experiments are still underway.

To relate the RCA and the dot blot titers to the *in vitro* trans-

TABLE 3. COMPARISON OF THE TWO ADENOVIRAL PLASMIDS pADC AND pADΔ FOR rAAV PRODUCTION

	72 hr		*96 hr*		*120 hr*	
	part/ml[a]	*inf. part./ml*[b]	*part/ml*[a]	*inf. part./ml*[b]	*part/ml*[a]	*inf. part./ml*[b]
pAdc	3.5×10^{10}	1.4×10^{9}	2.5×10^{10}	6.0×10^{8}	2.1×10^{10}	3.5×10^{7}
pAdΔ	3.5×10^{10}	2.2×10^{9}	2.0×10^{10}	1.2×10^{9}	2.5×10^{10}	1.0×10^{8}

rAAV was produced from two 15-cm plates of 293 cells transfected with the pspRC construct, the AAVCMVnlsLacZ vector, the indicated adenoviral plasmid. Cells were collected 72, 96, and 120 h after transfection and cell extracts purified as described in Materials and Methods. Each stock was titered by:

[a]Dot blot.

[b]RCA.

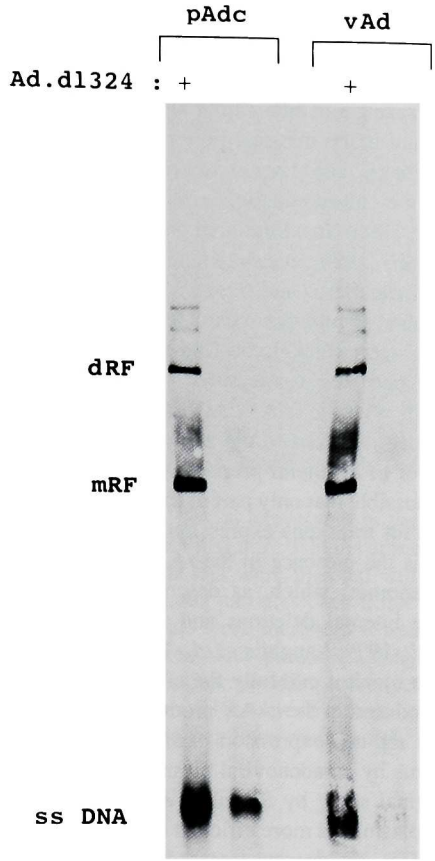

FIG. 5. Analysis of the replication of rAAVCMVnlsLacz in 293 cells. 293 cells were infected with rAAVCMVnlsLacZ (moi of 100 as defined by RCA), produced either with adenovirus (vAd) or the adenoviral plasmid (pAdc), in the presence or in the absence of Ad.dl324 (moi of 10). Three days later, cells were harvested and lysed, and low-molecular-weight DNA was extracted, run on a gel, transferred to a membrane, and hybridized to a LacZ probe as described in Materials and Methods. ss DNA, Single-stranded DNA; mRF, monomer double-stranded DNA; dRF, dimer double-stranded DNA.

ducing activity of rAAV, we used a vector encoding the nls-LacZ gene under the control of the CMV promoter. Two large-scale stocks of rAAVCMVnlsLacZ, produced either with adenovirus (vAd) or with the pAdc adenoviral plasmid, were assayed *in vitro* in an infectious LFU on HeLa cells (Table 4). Typically, a ratio ranging from 10 to 50 was observed between the infectious particles and the LFU measured on HeLa cells in the presence of adenovirus. This ratio was further increased 10-fold if the LFU assay was performed in the absence of adenovirus. The particles to LFUs ratio ranges for both rAAV stocks from 4×10^2 to 6×10^2 These data indicate that, at least *in vitro*, rAAV produced with either adenovirus or an adenoviral plasmid is functional. Furthermore, *in vitro* assays also indicate that the rAAVCMVnlsLacZ virus produced with the pAdc plasmid is, as generally described for rAAV, resistant to heat treatment (30 min at 56°C), to repeated freeze and thaw (at least twice), and is also stable at least 3 days at 4°C (data not shown).

A different sensitivity between these two assays, RCA and LFU, can explain the discrepancy between the number of infectious and transducing particles (Couffinhal *et al.*, 1997). However, two other hypotheses can also be evoked: (i) not all the rAAV genomes able to replicate and thus detected in the RCA can lead to the production of the LacZ protein 24 hr after infection; (ii) the Rep proteins produced in the HeLaRC32 cells, used for the RCA but absent in the control HeLa cells used for the LFU assay, account for this difference. To test this last hypothesis, rAAVCMVnlsLacZ was used to infect either HeLa or HeLaRC32 cells in the presence or absence of adenovirus, followed by an X-Gal staining 24 hr later. LFU titers remained essentially unchanged using the HeLaRC32 cells as compared with control cells. This result indicates that even in the presence of Rep proteins, the rAAV titer as measured by the LFU assay is not equivalent to the number of infectious particles measured by the RCA.

To test the *in vivo* transducing activity of rAAVCMVnlsLacZ (produced with the pAdc plasmid), 2.5×10^8 infectious particles (measured by RCA) were injected in the rat tibialis anterior muscle (3 animals were injected). Animals were sacrificed 1 month post injection and X-Gal staining revealed the presence of transduced fibers in most of the tissue sections (Fig. 6). Transduction efficiency was evaluated to range between 10–20% of the fibers.

Other *in vivo* data were obtained after injection in the mouse

TABLE 4. MEASURE OF INFECTIOUS AND TRANSDUCING rAAVCMVnlsLacZ PARTICLES PRODUCED USING EITHER AD.d1324 (vAd) OR AN ADENOVIRAL PLASMID (pAdc)

	part/ml[a]	*inf. part./ml*[b]	*LFU/ml*[c] *(+wtAd5)*	*LFU/ml*[c] *(−wtAd5)*	*Part/ inf. part*	*Inf. part/ LFU*
vAd	1.7×10^{11}	6.6×10^9	4.1×10^8	ND	25	16
pAdc	3.0×10^{11}	2.5×10^{10}	5.0×10^8	1.4×10^7	12	50

Rep-cap functions were provided by the pspRC construct. In the case of the stock obtained with vAd, the virus was produced from 20 15-cm plates of 293 cells and the final volume was of 6.8 ml. In the case of the stock obtained with pAdc, the virus was produced from 50 15-cm plates of 293 cells and the final volume was of 13.4 ml. Recombinant AAV was titered by:

[a]Dot blot.

[b]RCA.

[c]LFU assay on Hela cells in the presence or in the absence of wtAd 5.

ND, not done.

muscle of rAAV encoding the murine erythropoietin (mEpo) under the control or not of a doxycycline-inducible promoter (Bohl *et al.*, 1998). Two different vectors were used in this study: (i) the rAAVCMVEpo/rtTa (produced with adenovirus), which harbors the mEpo cDNA under the control of the tetO-CMV promoter, as well as the reverse transactivator (rtTA) under the control of the Moloney long terminal repeat (LTR). This vector was injected into the tibialis anterior muscle of mice (2.1×10^9 or 4.2×10^9 infectious particles/muscle), and transgene expression was monitored by measuring erythropoietin (Epo) secretion and increase of the hematocrit. The data obtained up to 6 months after injection indicate that Epo secretion can be switched on and off depending on the presence or absence of doxycycline in the drinking water. (ii) Epo secretion was also achieved after injection in the mouse muscle of rAAVCMVEpo (produced with the pAdc adenoviral plasmid) in which the mEpo cDNA is placed under the control of the Moloney LTR. In summary, these *in vivo* data indicate that rAAV produced with adenovirus or the adenoviral plasmid, pAdc, is functional.

DISCUSSION

Because of some unique properties among viral vectors, the use of rAAV for gene transfer is becoming widespread. However, efficient production of these vectors still relies upon methods that are cumbersome and have to be performed at a large scale to obtain a sufficient amount of virus for *in vivo* experiments. Furthermore, the need for helper adenovirus for rAAV assembly leads to the concomitant production of adenoviral particles, which are difficult to separate from rAAV particles. In this study, we report the improvements achieved for the production and the characterization of rAAV stocks.

We believe that a major technical improvement is the centrifugation step needed to purify rAAV. By changing the CsCl gradient conditions, we considerably shortened the protocol because equilibrium in the gradient can be reached by centrifuging for 6 hr instead of 48 hr. However, development of new methods such as chromatography or affinity columns should also greatly improve the purification procedure in a near future (Tamayose *et al.*, 1996).

The second improvement described in this study is the characterization of rAAV stocks. In many studies, titers, and consequently moi, are given as genome particles per milliliter (measured by dot blot). This parameter does not provide information on the rAAV infectivity and on the level of contamination with adenovirus and rep-positive AAV. The lack of such data when performing *in vitro* and *in vivo* experiments, using total cellular extracts or purified virus, makes any comparative evaluation of rAAV-mediated gene transfer quite difficult. We developed a general titration method based on the use of an HeLa rep-cap stable cell line. This method can be applied to any viral stock produced whichever transgene is present. It allows the measurement of infectious rAAV particles as well as of the level of contamination with infectious adenovirus and rep-positive AAV (Fig. 3). A similar stable HeLa rep-cap cell clone has been previously described and used to develop a titration method for infectious rAAV (Clark *et al.*, 1995, 1996). However, in that titration assay, replicative forms were analyzed on a Southern blot, 60 hr after infection of the HeLa rep-cap cells (that is late in the AAV growth cycle) with different dilutions of rAAV and adenovirus. In our assay, cells are individually analyzed (Fig. 3) for the presence of replicating rAAV DNA 24 hr after infection, which is the minimal time for allowing replication of viral DNA, but is short enough to prevent the virus from being released in the culture medium, spreading to other cells in the well (Carter, 1990). In addition, in the titration assay developed by Clark *et al.* (1996), measurements of the level of contamination with adenovirus and rep-positive AAV were lacking.

The number of infectious particles measured by our modified RCA is approximately 50-fold higher than the number of transducing particles as measured for example by the LacZ forming units with the rAAVCMVnlsLacZ (Table 4). Whether this difference is linked to the assay used to detect transgene expression or to a general property of rAAV remains still unclear. It is possible that only part of the pool of replicating DNA is available for transgene expression, at least *in vitro*. Another possibility is the presence in the rAAV stock of defective interfering genomes, which, as described for wild-type AAV, would have internal deletions and still retain the viral ITRs (Carter *et al.*, 1979; Laughlin *et al.*, 1979). This modified RCA was used to monitor carefully the effect of two major modifications introduced in the rAAV production protocol: (i) the use of different rep-cap expression plasmids; (ii) the replacement of adenovirus by an adenoviral plasmid.

The original study by Samulski *et al.* (1989) describes the pAAV/Ad plasmid as more efficient than a plasmid without the adenoviral ITR. Yields were assessed by looking at rAAV transducing activity (conferring neomycin resistance) using a non-purified cell lysate. Our results obtained with different rAAV vectors produced on a large scale and purified through a cesium gradient clearly do not confirm this initial observation. Not only was the total particles yield not affected, but also the number of infectious particles was increased when the two adenoviral ITRs were removed from the rep-cap construct. Indeed, in all of the rAAV stocks produced with the pspRC plasmid, the particles to infectious particles ratios were always less than 50, while they ranged between 10^3 and 10^4 in the rAAV stocks produced with the pAAV/Ad plasmid (Table 1). It should be noted, however, that only three large-scale rAAV stocks produced with the pAAV/Ad plasmid were analyzed by RCA. New large-scale experiments performed in parallel using either the pspRC or the pAAV/Ad plasmid are underway to further confirm this result.

Rep and Cap proteins levels are an important parameter to consider for rAAV production. In our production protocol, Rep proteins are always expressed under the control of the native AAV p5 and p19 promoters (pspRC construct). This configuration was chosen to preserve, as much as possible, the natural cascade of trans-activations and/or repressions occuring during AAV life cycle in the presence of adenovirus (Pereira *et al.*, 1997). A recent study indicates, however, that decreasing the level of Rep 78/68 enhances rAAV yields. Indeed, Li *et al.* (1997) reported that the use of a rep-cap construct harboring a mutation of the ATG site of Rep 78/68 to ACG results in an eight-fold increase in the production of rAAV infectious particles. Conversely, overexpression of CAP proteins by using an heterologous promoter such as CMV has also been shown to increase the rAAV yield (Vincent *et al.*, 1997). The replace-

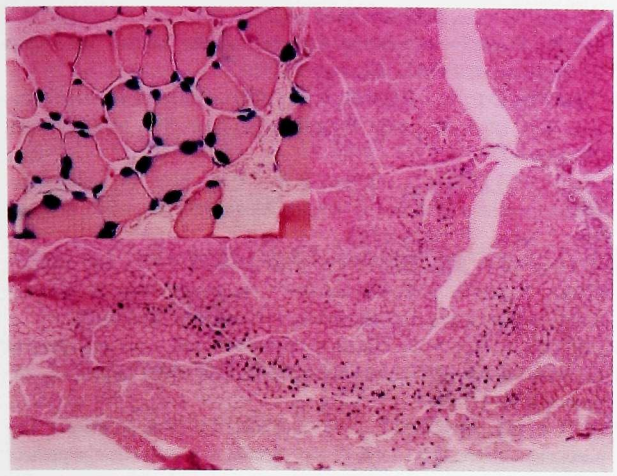

FIG. 6. Nuclear targeted β-Gal expression in the rat muscle after injection of rAAVCMVnlsLacZ. Tibialis anterior muscles of three 9-week-old Wistar rats were injected with 2.5×10^8 rAAVCMVnlsLacZ infectious particles each (three sites of injection per muscle). Animals were sacrificed 4 weeks after injection. Muscles were fixed with paraformaldehyde, stained overnight at 37°C with X-Gal, paraffin embedded, and sectioned into 4-μm sections which were counterstained with Kernechtrot solution. Magnifications: main panel, 100×; inset panel, 600×.

ment of the p40 promoter, leading Cap expression, for the CMV promoter did not result, in our hands, in a higher rAAV yield as measured by the number of physical and infectious particles (Table 2). It should be noted, however, that the moi of adenovirus and particularly the ratio between replicating adenovirus and rep-cap DNA copies is probably a key factor in determining Rep and Cap expression levels and consequently efficient rAAV production. Thus, the use of an adenoviral plasmid, instead of adenovirus, might account for the difference between our results and those published by Vincent *et al.* (1997). Evaluation of other constructs include strong heterologous promoters leading Cap expression as well as modification of the ATG site of Rep to ACG is presently underway.

The second modification introduced in the rAAV production protocol was to replace adenovirus with an adenoviral plasmid. Two constructs were tested: one harboring the entire adenoviral genome and the second harboring deletions of the ITRs, the ψ and E1 regions (Fig. 1C). Despite the large size of these plasmids (over 40 kb), rAAV production was not decreased after transfection into 293 cells. rAAV obtained under these conditions displayed the same physical properties than rAAV produced with adenovirus (infectious particles to LFU ratio and heat stability). Recently, other investigators have also described the rAAV production using adenoviral plasmids that harbor either an E1-deleted adenoviral genome or only the minimal adenoviral functions needed for rAAV production (J. Kleinschmidt, personal communication) (Ferrari *et al.*, 1997). In our hands, most of rAAV stock obtained using the pAdc plasmid were free of detectable adenoviral contamination as determined by RCA. Two rAAV preparations, however, displayed some level of contamination by infectious adenovirus as measured by RCA (2.5×10^3 infectious particles/ml). We are currently confirming this contamination.

In vivo experiments using rAAV produced with the pAdc plasmid indicate that these viruses are competent for transducing muscle cells in mice and rats. It is possible, however, that this relatively pure rAAV displays a different kinetic and transduction efficiency *in vivo* as compared to rAAV produced with adenovirus.

Contamination of the rAAV stocks with rep-positive AAV is a challenging issue. Indeed, some studies (Allen *et al.*, 1997; Halbert *et al.*, 1997) have reported that rAAV preparations are contaminated with such particles revealed either by RCA or by dot blot using in both cases a rep-cap probe. The extent of contamination published ranges from 0.0001 to 10% of the rAAV stocks. In the study of Allen *et al.* (1997), the contaminating rep-positive AAV has been characterized following sequential amplification in adenovirus-infected cells. All rep-positive AAV genomes sequenced have at least a portion of the AAV ITRs and a nonhomologous recombination leading to the insertion of rep-cap sequences close to AAV ITRs was proposed to explain the emergence of such contaminant (Allen *et al.*, 1997).

Our RCA assay indicates that amplification of the vector can occur in HeLa cells in the presence of adenovirus. Furthermore, replicative forms were also detected in low-molecular-weight DNA extracted from rAAV and adenovirus-infected 293 cells (Fig. 5). Both of these observations suggest the presence of rep-positive particles in rAAV stocks. The extent of this contamination represents, on average, 0.001% of infectious rAAV particles (also measured by RCA). However, because synthesis of AAV double-stranded DNA forms (mRF and dRF) is due only to the activity of cellular polymerases, one could argue that rAAV amplification detected in the RCA results from Rep-independent (mRF and dRF) and Rep-dependent (ssDNA) hybridization signals. On the other hand, the presence of contaminating rep-positive AAV was confirmed by a PCR assay designed to detect rep sequences. Altogether, these observations strongly suggest the presence of rep-positive AAV particles, even though the extent of this contamination might be overestimated by the RCA.

Finally, some studies have suggested that Rep proteins might be associated with AAV particles (wild type or recombinant) either on the outside of the particle, by a covalent linkage to AAV DNA (Prasad and Trempe, 1995; Prasad *et al.*, 1997), or in the inside (Kube *et al.*, 1997). Our observations do not exclude this possibility and it is possible that replication of rAAV, detected in infected cells in the presence of adenovirus, also results from the transfer of Rep proteins to the cells. Extensive characterization of rAAV preparations will help defining the relative proportions of particles containing rep DNA and/or Rep proteins.

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