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Quantification of High-Capacity Helper-Dependent Adenoviral Vector Genomes *In Vitro* and *In Vivo*, Using Quantitative TaqMan Real-Time Polymerase Chain Reaction

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Abstract

First-generation adenoviral (Ad) and high-capacity adenoviral (HC-Ad) vectors are efficient delivery vehicles for transferring therapeutic transgenes *in vivo* into tissues/organs. The initial successes reported with adenoviral vectors in preclinical trials have been limited by immune-related adverse side effects. This has been, in part, attributed to the use of poorly characterized preparations of adenoviral vectors and also to the untoward immune adverse side effects elicited when high doses of these vectors were used. HC-Ads have several advantages over Ads, including the lack of viral coding sequences, which after infection and uncoating, makes them invisible to the host's immune system. Another advantage is their large cloning capacity (up to ~35 kb). However, accurate characterization of HC-Ad vectors, and of contaminating replication-competent adenovirus (RCA) or helper virus, is necessary before these preparations can be used safely in clinical trials. Consequently, the development of accurate, simple, and reproducible methods to standardize and validate adenoviral preparations for the presence of contaminant genomes is required. By using a molecular method that allows accurate, reproducible, and simultaneous determination of HC-Ad, contaminating helper virus, and RCA genome copy numbers based on real-time quantitative PCR, we demonstrate accurate detection of these three genomic entities, within CsCl-purified vector stocks, total DNA isolated from cells transduced *in vitro*, and from brain tissue infected *in vivo*. This approach will allow accurate assessment of the levels and biodistribution of HC-Ad and improve the safety and efficacy of clinical trials.

OVERVIEW SUMMARY

Because high-capacity adenoviral (HC-Ad) vectors are deleted of all adenoviral protein-coding sequences, they cannot be titrated in assays that rely on vector replication. Furthermore, the contamination of HC-Ad preparations with helper virus and replication-competent adenovirus (RCA) needs careful evaluation, especially for future clinical preparations. Using a molecular method that allows accurate, reproducible, and simultaneous determination of HC-Ad, contaminating helper virus, and RCA genome copy numbers based on real-time quantitative PCR, we demonstrate accurate detection of these three genomic entities, within CsCl-purified vector stocks, total DNA isolated from cells transduced *in vitro*, and from brain tissue infected *in vivo*. This approach will allow accurate assessment of the levels and biodistribution of HC-

Ad and improve the required clinical standards for quality control to ensure the safety and efficacy of clinical trials.

INTRODUCTION

FIRST-GENERATION ADENOVIRAL (Ad) and high-capacity adenoviral (HC-Ad) vectors, with a relatively large capacity for encoding foreign DNA, can be produced at high titers and elicit efficient transduction of many cells and tissues *in vitro* (Hurtado-Lorenzo *et al.*, 2004; Bilbao *et al.*, 2005; Ohashi *et al.*, 2005; Ohbayashi *et al.*, 2005; Toietta *et al.*, 2005; Xiong *et al.*, 2006) and *in vivo* (Southgate *et al.*, 2000b; Windeatt *et al.*, 2000; Sarac *et al.*, 2002). Ad vectors are devoid of the essential E1a/1b genes (Cook *et al.*, 1986; Flint and Shenk, 1997; Castro *et al.*, 2003), and are thus routinely grown in cell lines that express these genes *in trans* to allow adenoviral replication and packaging. HC-Ad genomes retain only *cis*-acting adenoviral sequences necessary to replicate the viral vector genomes (inverted terminal repeats [ITRs]) and the packaging signal (ψ) sequence. The absence of wild-type adenoviral sequences from HC-Ad genomes results in lower immunogenicity *in vivo* and promotes safer, efficient gene transfer with long-lasting transgene expression (Morral *et al.*, 1998; Morsy *et al.*, 1998; Schiedner *et al.*, 1998). HC-Ads are grown with a helper virus that provides all essential adenoviral functions for replication *in trans*. The packaging sequence of the helper virus is flanked by either FRT (flippase [FLP] recombinase target) or *loxP* recombination sites, in 293-Cre or 293-FLPe cells. The helper viral genome undergoes recombination that deletes ψ and the helper virus genome is less efficiently packaged than the HC-Ad genome (Parks *et al.*, 1996; Umana *et al.*, 2001). With these current production methods, titers of contaminating helper virus are usually 100-fold less than that of HC-Ad genomes in final preparations (Ng *et al.*, 2001; Palmer and Ng, 2003, 2004).

Delivery of therapeutic transgenes by adenoviral vectors has been tested in clinical trials for numerous diseases, including cancer, metabolic disorders, and cystic fibrosis (Harvey *et al.*, 1999; Grines *et al.*, 2003; Hedman *et al.*, 2003; Raper *et al.*, 2003; Immonen *et al.*, 2004). However, gene therapy, as any therapeutic approach, can have serious side effects. In the case of a young male patient suffering from severe ornithine trans-carbamylase (OTD) deficiency, soon after administration of a first-generation adenoviral vector (6×10^{11} VP/kg) expressing OTD into the right hepatic artery, the patient developed a systemic immune response, most likely induced by delivery of a high dose of adenoviral vector (Raper *et al.*, 2003). This highlights the critical need to assess the quality of adenoviral vector preparations intended for clinical use, and a concerted effort was made immediately after this death to standardize physical and infectious titering of adenoviral vectors (Palmer and Ng, 2004).

HC-Ads have a higher cloning capacity; theoretically one can introduce up to 35 kb of foreign DNA. The toxicity of HC-Ad is much lower than that of Ad owing to the absence of an ongoing immune response against immunogenic viral epitopes expressed within infected cells because HC-Ads do not contain any wild-type adenoviral genes (Kochanek *et al.*, 2001). This results in much longer transgene expression *in vivo*, even when administered in the presence of a peripheral immune response to adenoviruses as would be encountered in patients preexposed to adenovirus who are undergoing clinical trials (Thomas *et al.*, 2001a,b, 2002). HC-Ads have been engineered to preclude the emergence of replication-competent adenovirus (RCA); however, RCA contamination still remains a theoretical possibility in these preparations and thus should be determined in addition to excessive helper virus contamination.

Several assays are currently available for determining the titers of first-generation Ad and HC-Ad vectors. These employ a diverse range of biological, molecular, and physical methods for the determination of total viral particles, therapeutic virus titer, and contamination with either RCA or helper virus in a viral preparation (Southgate *et al.*, 2000a; Ma *et al.*, 2001; Ng *et al.*,

2002b; Reddy *et al.*, 2002; Schiedner *et al.*, 2002; Palmer and Ng, 2004; Brunetti-Pierri *et al.*, 2005). Here we describe the development and characterization of a single quantitative polymerase chain reaction (qPCR) assay that can be used to assess the number of vector genomes, contaminating helper genomes, and replication-competent Ad genomes present within HC-Ad and Ad vector preparations at the same time from the same sample. This single method can be applied to purified viral aliquots, viral DNA isolated from infected cells *in vitro*, and ultimately from infected organs *in vivo*. U.S. Food and Drug Administration (FDA) guidelines require the use of therapeutic adenoviral vectors with a ratio of total viral particles to therapeutic viral particles of no more than 30:1. This single method ought to streamline adherence to new FDA guidelines and improve the ability to characterize the purity of therapeutic adenoviral vector preparations to be used for the safe, efficient delivery of genes in preclinical models and in clinical trials.

MATERIALS AND METHODS

Generation of adenoviral vectors

HC-Ad rescue, amplification, and scaling up—The FLPe/FRT helper-dependent system consists of a helper virus (FL helper), that is, a modified first-generation, E1-deleted vector, with FRT sites flanking the packaging signal (ψ) (Umana *et al.*, 2001) (Fig. 1B); a transcriptional cassette that expresses luciferase; and a 293 cell line derivative expressing FLP recombinase (293-FLPe) (Maione *et al.*, 2001; Umana *et al.*, 2001). To rescue the corresponding HC-Ad vector, the HC-Ad genomic DNA was released from the pSTK mCMV- β gal-TK plasmid by overnight digestion with *PmeI*. HC-Ad plasmid DNA (5 μ g) was transfected into 293-FLPe cells as previously described (Southgate, 2000a). After six amplification passages done in 10-cm dishes (2×10^6 cells), and scaling up done in 32 dishes (150 mm, 1×10^7 cells), the viral vector was purified by two CsCl step gradients as described (Southgate *et al.*, 2000a). The HC-Ad band was collected as described above and was dialyzed (Spectra/Por dialysis membrane; Spectrum Laboratories, Rancho Dominguez, CA) (Ng *et al.*, 2002b). The virus suspension was taken out of the dialysis cassette, and 10 μ l aliquots were stored at -80°C . HC-Ad preparations used to produce the data presented in Tables 1 and 2 were scaled up with the Cre-*loxP* system as previously described in detail (Palmer and Ng, 2003, 2004).

Purification and characterization of first-generation Ads—Two E1-deleted first-generation adenoviral vectors (Ad-hCMV- β gal and Ad-mCMV- β gal), were used in this study (Shering *et al.*, 1997; Gerdes *et al.*, 2000; Thomas *et al.*, 2000, 2001b). The scaling up, purification, and titration of first-generation Ads have been described previously in detail (Southgate *et al.*, 2000a). Both Ad preparations were titrated on the basis of infectious viral particles (infectious units, IU), β -galactosidase activity (blue-forming units, BFU), and total viral particles (absorbance at a wavelength of 260 nm, OD₂₆₀). Each Ad preparation was evaluated for the presence of bacterial lipopolysaccharide (LPS) and replication-competent adenovirus (RCA), as described previously (Dion *et al.*, 1996; Southgate *et al.*, 2000a). Ad-hCMV- β gal and Ad-mCMV- β gal tested negative for LPS and RCA contamination.

Generation of standard plasmid for qPCR determinations

Four independent PCR products were generated by amplification with the following primers and target sequences. To amplify a portion of the L3 region of adenovirus pJM17, a plasmid that carries the genomic sequence of adenovirus with a deletion in the E1 gene region (Shering *et al.*, 1997; Gerdes *et al.*, 2000) was used as a PCR template along with L3-specific primers (L3 forward, 5'-AGAAGCTTAGCATCCGTTACTC-GAGTTGG-3'; and L3 reverse, 5'-ATAAGCTTGCATGTTG-GTATGCAGGATGG-3'); a portion of the adenovirus E1a region was amplified with genomic DNA extracted from 293 E1-expressing cells as a PCR template

along with E1a-specific primers (E1a forward, 5'-ATGAATTCCGATCTTACCTGCC-CACGAGG-3'; and E1a reverse, 5'-ATGAATTCCCAAACC-CACCACTCTATCACC-3'); a sequence specific to cosmid-346 used in the gutless backbone was generated (Umana *et al.*, 2001) with plasmid DNA from pSTK120 as a template along with cosmid-specific primers (Umana *et al.*, 2001) (primers: Cosmid-346 forward, 5'-ATGGATCCAACTTGGCTCGCT-CCTCC-3'; and Cosmid-346 reverse, 5'-ATGGATCCACT-GTGGGCTATGTAGTGGTGGG-3'); a region corresponding to a region of the luciferase (Luc) gene located in the helper virus was amplified with plasmid DNA from pGL3Promoter vector (GenBank U47298) as a template and luciferase-specific primers (primers: Luciferase forward, 5'-TATCTAGAGCA-GAAGCTATGAAACGATATGG-3'; and Luciferase reverse, 5'-TATCTAGACCTGGTAATCCGTTTTAGAATCC-3'). The cycling conditions for all four specific amplifications were 95°C for 4 min, followed by 30 cycles of 95°C for 1 min, 58.5°C for 30 sec, and 72°C for 1 min, with a final elongation at 72°C for 7 min. Each of the PCR amplification products was gel purified and ligated into pGemTEasy plasmid (Promega, Madison, WI). The PCR products were excised with the corresponding restriction enzymes, gel purified, and cloned into pBluescript II SK(−) (Stratagene, La Jolla, CA), as described below (Fig. 1A). The PCR product containing L3 sequences was excised from pGemTEasy with *HindIII*; the E1a PCR product was excised from pGemTEasy with *EcoRI*; HC-Ad-specific sequence Cosmid-346 was excised from pGemTEasy with *BamHI*; and the luciferase PCR product was excised from pGemTEasy with *XbaI*. All the PCR fragments were ligated sequentially into pBluescript II SK(−) in order to generate the standard plasmid (pSt) (total size of pSt, 4004 bp) (Fig. 1A). Plasmid DNA for pSt was purified with a Qiagen maxiprep kit (QIAGEN-tip 500; Qiagen, Chatsworth, CA) and quantified by OD₂₆₀ (DU 640; Beckman Coulter, Fullerton, CA). The reading obtained was 0.2795 optical density units, corresponding to 1.39 µg/µl and to 1.66×10^{12} total copies of the plasmid.

Biological and physical titration of HC-Ad or Ad preparations and contaminants

Titration of HC-Ad vector and contaminating helper virus by biological methods

—Biological titrations of HC-Ad vectors expressing β-galactosidase were performed on 293 cells and expressed as blue-forming units (BFU) per milliliter. We used 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of transduced 293 cells as described in detail previously (Umana *et al.*, 2001; Lowenstein, 2002). FL helper virus was titrated by quantifying the infectious units (IU) per milliliter, using the end-point cytopathic event (CPE) method in 96-well plates (Umana *et al.*, 2001; Lowenstein, 2002). Titers expressed as infectious units per milliliter were calculated by dividing the dilution factor by the highest viral vector dilution tested that exhibited CPE. Adenovirus Reference Material (ARM), that is, wild-type adenovirus serotype 5, was obtained from the American Type Culture Collection (VR-1516; ATCC, Manassas, VA). The biological titer that we obtained for the ARM was 4.92×10^{11} IU/ml (ATCC value was 7×10^{10} IU/ml).

Physical titration of adenoviral vectors by spectrophotometry—On the basis of the well-characterized absorption of pure adenovirus at 260 nm (Maizel *et al.*, 1968), an OD₂₆₀ of 1.00 corresponds to a viral particle concentration of 1.1×10^{12} VP/ml based on a 36-kb size of the wild-type particle genome. Thus, total viral particles for HC-Ad and Ad vectors were calculated as follows: $\text{VP/ml} = (\text{OD}_{260} \times \text{dilution factor} \times 36 \text{ kb}) / (\text{size of the vector [kb]} \times 9.09 \times 10^{-13})$; where 9.09×10^{-13} is the extinction coefficient for wild-type adenovirus.

Preparation of viral vector genomic DNA

Purification of adenoviral genomic DNA from CsCl-purified viral preparations—

Both HC-Ad vector and contaminating FL helper virus genome copy numbers within CsCl-purified virus preparations were measured by multiplex real-time qPCR. To purify adenoviral DNA, three independent adenoviral preparations, each 20 µl, were incubated with 400 µl of

ProtK-SDS solution (proteinase K [0.5 mg/ml], 10 mM Tris-HCl [pH 7.5], 0.5% sodium dodecyl sulfate [SDS], 10 mM EDTA [pH 8]) for 3 hr at 37°C. DNA was then precipitated by addition of 1/10 vol of 3 M sodium acetate (pH 5.2) and a 2.5 vol of 95% ethanol, rinsed with 70% ethanol, dried, and resuspended in 25 µl of water.

Purification of adenoviral genomic DNA from cells infected with HC-Ad-mCMV-βgal or Ad-mCMV-βgal—Flasks (25 cm²) of 90% confluent 293 cells (2.00×10^6) were infected with HC-Ad-mCMV-βgal at a multiplicity of infection (MOI) of 100 BFU/cell in a volume of 1 ml; the cells were washed with phosphate-buffered saline (PBS) after 1 hr of adsorption to remove unabsorbed virions (Palmer and Ng, 2004). Four hours postinfection, the cells were detached from the flask and washed with PBS and intracellular DNA was extracted as previously described (Ma *et al.*, 2001). The cell pellet was resuspended in 200 µl of 100 mM Tris-HCl, pH 8, and cells were broken by three cycles of freezing and thawing and treated with 10 µg of DNase I in the presence of 2 µl of 2 M MgCl₂, followed by a 30-min incubation at 37°C. DNase I activity was inactivated by adding 8 µl of 0.5 M EDTA followed by a 10-min incubation at 37°C, and heat inactivation. Subsequent proteinase K digestion, DNA precipitation, and resuspension were performed as described above for viral DNA purification. Cosmid, L3, and E1a quantifications were performed by qPCR.

Purification of adenoviral genomic DNA from rat brain tissue transduced with HC-Ad-mCMV-βgal-WPRE or Ad-mCMV-βgal—Adult female C57BL/6 mice (body weight, 18–25 g) were used for *in vivo* HC-Ad- or Ad-mediated gene delivery. Four mice were injected with vector HC-Ad-mCMV-βgal or Ad-mCMV-βgal via the striatum (coordinates from bregma: anterior, 0.5 mm; lateral, 2.2 mm; ventral, 3.0 mm), using a 10-µl Hamilton syringe (Smith-Arica *et al.*, 2000). For each animal, a total volume of 1 µl of vector diluted in 0.9% (w/v) saline was injected in the striatum over a 3-min period. Subsequent to vector injection, the needle was left in place for a further 2 min before careful needle retraction. Control mice received 1-µl saline injections. Two days postinjection, both untreated and treated animals were killed and brains were perfused with approximately 100 ml of oxygenated Tyrode's solution (0.14 M NaCl, 1.8 mM CaCl₂, 2.7 mM KCl, 0.32 mM NaH₂PO₄, 5.6 mM glucose, and 11.6 mM NaHCO₃) by means of transcardial perfusion. Once taken out of the scalp, brains were stored at –80°C until processed for DNA purification.

Brain tissue was removed from animals ($n = 4$) after perfusion with Tyrode's solution, at 2 days postinjection. A starting amount of 25 mg of brain tissue was dissected from the area surrounding the injection site and homogenized. DNA was isolated from the homogenate, using a DNeasy tissue kit (Qiagen). DNA was eluted in a volume of 100 µl and concentration was determined with a spectrophotometer. A total of 5 µl of DNA was used for quantification by real-time PCR specific to the cosmid, L3, or E1 region present in the different vector genomes as described above. Data were expressed as the number of viral copies of each region present in the total DNA isolated from the brain of each animal. Results were expressed as means $\pm$ SEM.

Primers and probes for qPCR

Specific pairs of primers and TaqMan probes were designed with the Primer Express software program, obtained from Applied Biosystems (Foster City, CA). Primers and probe sequences were as follows: (1) HC-Ad Forward, 5'-CAGCTTTCAGATGGAGACAGGAA-3'; HC-Ad Reverse, 5'-CCCCGCCCTTCTCCTGACT-3'; HC-Ad Probe, 6-FAM-5'-CTCCTCCCAATTGCCTATGCTGCAAC-3'-TAMRA (detecting cosmid-346 bp 14615–14894 sequence present in the pSTK120 gutless backbone plasmid); (2) Luciferase-Helper virus Forward, 5'-AACGTGAATTGCTCAACAGTATGG-3'; Luciferase-Helper virus Reverse, 5'-TTGCAACCCCTTTT-GGAAA-3'; Luciferase-Helper virus Probe, 6-VIC-5'-

CATTT-CGCAGCCTACCGTGGTGTTC-3'-TAMRA (detecting Luciferase gene present in FL helper virus); (3) L3-Adenovirus Forward, 5'-GAGTTGGCACCCCTATTCTGA-3'; L3-Adenovirus Reverse, 5'-ATGCCACATCCGTTGACTTG-3'; L3-Adenovirus Probe, 6-VIC-5'-CCACCCGTGTGTACCTGGTG-GACA-3'-TAMRA (detecting Ad5 gene L3-1 bp 14315–14549 sequence, present in all helper first-generation viruses); and (4) E1-Adenovirus Forward, 5'-GGGTGAGGAGTTTGTGTGA-GATTATG-3'; E1-Adenovirus Reverse, 5'-TCCTCCGGT-GATAATGACAAGA-3'; E1-Adenovirus probe, TET-5'-AG-CACCCCGGGCACGGTTG-3'-TAMRA (detecting Ad5 gene E1a, present in replication-competent adenovirus particles) (Fig. 1B–E).

The pure spectral peaks of the dyes used in this paper, FAM, TET, and VIC, are at 520, 540, and 550 nm. Most dyes have emission spectral tails into the red, a fact that can interfere with the spectra of dyes with emission maxima at longer wavelengths. However, this is overcome with the software that is used to run the ABI PRISM 7900HT sequence detection system. The software analyzes the composite spectrum of the wavelength of each dye and compensates for the presence of overlapping fluorescence spectra, using a process called “multicomponenting.” Using this approach, we determined that two fluorescent probes can be run together without any significant interference between the emission spectra of the dyes, and this is seen when such results are compared with those obtained with each fluorescent probe used by itself (results not shown). Nevertheless, we experienced inconsistent and variable results when we attempted to run all three fluorescent dyes together in the same sample (results not shown). Therefore, we chose to run all samples according to a duplex strategy (cosmid-FAM with luciferase-VIC, or L3-VIC with E1a-TET). Results performed by two different operators were comparable, and gave values within the boundaries of statistical experimental error. Further, qPCR was performed on three dilutions of each sample (10^{-3} , 10^{-4} , and 10^{-5}), and assayed in triplicate, to verify assay repeatability.

Generation of a standard curve by qPCR

To generate the standard curve, serial dilutions of standard plasmid (pSt) DNA were prepared fresh each time the qPCR was performed, with concentrations ranging from 10^7 to 10^2 molecules/5 μ l. The equivalency between molecular weight of the standard plasmid and the number of copies was calculated by considering the pSt molecular weight ($MW_{pSt} = 4004$ bp) and the equivalency between base pairs (bp) and daltons (Da) (1 bp = 635 Da). $Mass_{pSt}$ (Da) = 4004 bp/molecule $\times$ 635 Da/bp = 2.54×10^6 Da/molecule. Using the conversion factor 1.65×10^{-18} μ g/Da to obtain the equivalency of mass (in micrograms) per molecule of pSt, we multiplied 2.54×10^6 Da/molecule $\times$ 1.65×10^{-18} μ g/Da = 4.195×10^{-12} μ g/molecule. We measured pSt DNA concentration on the basis of absorbance at 260 nm and used the standard equivalency between optical density units measured at 260 nm (OD_{260}) and mass, that is, 1 OD = 50 μ g/ml, to calculate the mass of each standard point in the qPCR standard curve.

Quantification of HC-Ad, FL helper, and Ad by qPCR

Vector genomic DNAs of Ad and HC-Ad vectors were evaluated in three different dilutions per assay: 10^{-3} to 10^{-5} . In a total volume of 50 μ l per reaction, 5 μ l of the corresponding template DNA was added as described below. On the basis of the desired target sequence (HC-Ad, helper virus, or first-generation Ad) the corresponding sets of primers plus probe were used (Fig. 1B–E). To generate the qPCR standard curve, 10-fold dilutions of the standard plasmid, ranging from 10^2 to 10^7 copy numbers, were set up in triplicate for use as template. For the samples corresponding to Ad-derived vectors, the above-mentioned dilutions were made and added to the master mix once dispensed into the wells. All the reagents except the template were added into a master solution, which was then loaded into the 96-well plate (45 μ l/well), using an automatic micropipette (Research pro; Eppendorf, Hamburg, Germany). Special care was taken in order to avoid bubble formation. After 5 μ l of the template was added

to each well, the plate was sealed (optical cover, ABI PRISM 7900HT sequence detection system) and spun down for 5 min at 4°C. The reaction was performed under the following conditions: at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min (ABI PRISM 7900HT).

Genome copy numbers of the HC-Ad vector and contaminating FL-helper virus within vector stocks were calculated by comparing threshold cycle (C_t) values of each sample with the standard curve, using ABI PRISM 7900HT software. To determine final genome copy numbers of HC-Ad vector and contaminating helper virus, the total number of DNA molecules was corrected for the dilution factor and the volume of DNA used in the qPCR, using the following formula: number of copies = quantity $\times$ dilution factor $\times$ 1000 μ l/5 μ l. The quantity is an extrapolation made by the software, using the standard curve values; the dilution factor is the inverse of the dilution of template detected; and the ratio 1000 μ l/5 μ l is the dilution factor to calculate the copy number per milliliter, considering that a volume of 5 μ l of template was added to the reaction.

Real-time qPCR to measure RCA contamination

DNA was purified from 10 μ l aliquots for both HC-Ad-mCMV- β gal and ARM, as described above. Absorbance at 260 nm was measured in order to determine DNA concentration; 10^7 genome copies per 5 μ l were used in the assay. Seven different reaction mixtures were evaluated by qPCR, all of them containing 10^7 HC-Ad-mCMV- β gal genome copies mixed with 10^7 to 10^1 ARM total genome copies per reaction tube. Controls containing 10^7 HC-Ad-mCMV- β gal genome copies or 10^7 ARM genome copies per reaction were also quantified.

Biological assay for RCA detection in an HC-Ad-mCMV- β gal vector preparation

HC-Ad-mCMV- β gal vector was evaluated for RCA contamination by the supernatant rescue assay, using HeLa cells as described previously (Dion *et al.*, 1996; Southgate *et al.*, 2000a), by inoculating 10^9 VP of HC-Ad-mCMV- β gal vector with 10, 100, or 1000 of ARM viral particles; or with 10^9 VP of HC-Ad-mCMV- β gal used as a control.

Statistical analyses

Comparisons between vector titers expressed as viral particles, blue-forming units, and genomes (cosmid or L3 sequences), were done by Bonferroni's multiple comparison test as a post-ANOVA analysis; significant differences are indicated ($p < 0.001$) (see Fig. 3A–D). Comparisons between titers obtained as infectious units and as contaminating helper virus genomes (luciferase sequences) were analyzed by a nonparametric t test; significant differences are indicated ($p < 0.05$) (Fig. 3A and B). Comparison of median genome copy number with the input value was done by Wilcoxon signed-rank test; differences were considered significant when $p < 0.05$ (Tables 1 and 2).

RESULTS

Development of qPCR assay to detect total copy numbers of cosmid (HC-Ad specific), luciferase (helper virus specific), L3 (first-generation Ad specific), and E1a (RCA specific) DNA sequences

We constructed a standard plasmid (pSt) (Fig. 1A) encoding (1) a 235-bp fragment from the L3 Ad gene (present in all first-generation vectors and in the FL-helper virus genome), (2) a 239-bp fragment of the E1a Ad gene (present only in the replication-competent Ad genome and in 293 cells), (3) a 280-bp fragment of Cosmid-346 (a sequence present only in HC-Ad vector genomes), and (4) a 289-bp fragment of luciferase cDNA (a sequence present only within the FL-helper virus). Plasmid pSt was developed to produce accurate standard curves

from known starting concentrations of target DNA to assess the number of adenoviral vector genomes present in purified adenoviral preparations, in viral DNA extracted from infected cells, or tissue samples. Primer and probe sets were designed to amplify regions from cosmid (Fig. 1B), Luc (Fig. 1C), L3 (Fig. 1C–E), and E1a (Fig. 1E). The cosmid probe was conjugated with FAM and E1a was conjugated with 6-TET, whereas both Luc and L3 were conjugated with VIC fluorophores. Luc and L3 cannot be measured in the same sample, because of the use of the same fluorophore for both probes. Emission spectra of FAM, VIC, and 6-TET do not overlap with one another, and this allows us to detect simultaneously cosmid, E1a, and either Luc or L3 in the same assay. Simultaneous detection of E1a, cosmid, and Luc or L3 was not possible because of artifacts observed in the quantification values when compared with the single detections run as controls.

In vitro quantification of HC-Ad, helper virus, Ad, and RCA genomes from adenoviral vector preparations, using qPCR

We observed consistent, reproducible qPCR cycle values for the detection of cosmid (Fig. 2A), Luc (Fig. 2C), L3 (Fig. 2E), and E1a genome copies (Fig. 2G), using pSt. A linear relationship was observed between the number of cycles and the $\log_{10}$ dilution when 102–107 pSt copies were assayed. The correlation coefficient, r^2 , was >0.99 for all standard curves. We next used these standard curves to calculate the total number of HC-Ad or contaminating helper virus genomes present in three serial dilutions of purified HC-Ad-mCMV- β gal-TK DNA (10^{-3} , 10^{-4} , and 10^{-5}). The threshold values for detecting cosmid, Luc, L3, and E1a were set at 10 standard deviations above the baseline value (previously fixed between cycles 3 and 15, using ABI PRISM sequence detection system software) (Fig. 2B, D, F, and H).

qPCR compares favorably with existing biological and physical methods for titrating HC-Ad and Ad vectors

Titration of CsCl-purified preparations of HC-Ad-mCMV- β gal and HC-Ad-mCMV- β gal-TK were compared in biological and physical titration assays (i.e., X-Gal histochemistry [BFU] and optical density [VP] assays; Southgate *et al.*, 2000a), with the qPCR assay described herein. Helper virus contamination was assessed as infectious units. For both preparations, viral particle numbers were significantly higher than in qPCR and biological activity determinations, which were comparable (Fig. 3A and B). The level of helper virus contamination in the HC-Ad-mCMV- β gal and HC-Ad-mCMV- β gal-TK preparations was calculated by qPCR to be 1.3×10^9 helper and 4.0×10^8 genome copies/ml, respectively. These values were significantly higher than the biological titration results for the contaminating helper virus present in HC-Ad-mCMV- β gal, that is, 2.7×10^8 IU/ml ($p < 0.05$) (Fig. 3A), or in HC-Ad-mCMV- β gal-TK, that is, 2.7×10^7 IU/ml ($p < 0.05$) (Fig. 3B), consistent with previously published reports (Kreppel *et al.*, 2002; Reddy *et al.*, 2002). RCA, evaluated by E1a detection according to the qPCR method, was negative for both HC-Ad viral preparations, in agreement with the biological assay results (data not shown).

It was important to validate the qPCR system in Ads, to determine whether values obtained by qPCR are similar to values obtained by biological assays, which measure infectious viral particles. This is because HC-Ad vectors cannot be titered by methods based on the determination of infectious units. We used two CsCl-purified first-generation viral vector preparations expressing β -galactosidase under the control of either the hCMV promoter, Ad-hCMV- β gal (Parks *et al.*, 1996); or the mCMV promoter, Ad-mCMV- β gal (Gerdes *et al.*, 2000). The molecular titer of Ad-hCMV- β gal detected by qPCR using the L3 primer and probe set was 1.4×10^{11} genome copies/ml, which was not significantly different from the biological titer obtained as blue-forming units, 6.8×10^{10} BFU/ml ($p > 0.05$) or the biological titer obtained by end-point dilution of infectious particles ($p > 0.05$) (Fig. 3C). The molecular titer of Ad-mCMV- β gal detected by qPCR using the L3 primer and probe set was 1.1×10^{12} genome

copies/ml, which was not significantly different from the biological titer obtained as blue-forming units, 3.4×10^{10} BFU/ml ($p > 0.05$) or the infectious unit titer obtained by end-point dilution ($p > 0.05$) (Fig. 3D). The total number of viral particles detected for Ad-hCMV- β gal was 6.1×10^{12} VP/ml, significantly higher than the titers observed as blue-forming units ($p < 0.001$) or infectious units ($p < 0.001$) and by qPCR ($p < 0.001$) (Fig. 3C). Similarly, the total number of viral particles detected for Ad-mCMV- β gal was 3.8×10^{12} VP/ml, significantly higher than the titers observed as blue-forming units ($p < 0.001$) or infectious units ($p < 0.001$) and by qPCR ($p < 0.01$) (Fig. 3D). RCA, evaluated by E1a detection according to the qPCR method, was negative for both adenoviral preparations, in agreement with the biological assay results (data not shown).

Comparison of qPCR and supernatant rescue bioassay for detection of RCA within Ad and HC-Ad vector preparations

An international standard for adenovirus has been developed, called Adenoviral Reference Material (ARM). ARM can be used to compare and standardize tests performed in different laboratories. We wished to determine the accuracy and reproducibility of our qPCR approach, using ARM as a standard. We performed a biological assay, that is, a supernatant rescue assay (Dion *et al.*, 1996), wherein 10, 100, or 1000 ARM viral particles were added to 10^9 HC-Ad-mCMV- β gal VP. These spiked samples were then used to infect HeLa cells, as outlined in Materials and Methods. Whereas 10 ARM viral particles did not show any CPE in spiked vector preparations, evidence of CPE was observed when using HC-Ad-mCMV- β gal spiked with either 100 or 1000 ARM VP, indicating that in our hands the biological RCA assay has a limit of detection of 10–100 RCA (Fig. 4A). The sensitivity of the biological RCA assay as performed in our laboratory was lower than the sensitivity reported previously (Dion *et al.*, 1996). This could be due to a number of reasons, including the nature of the wild-type viruses used in the assays. We next determined the levels of RCA in the vector preparation by qPCR using the E1a primer and probe set. The qPCR assay was performed with 10^1 to 10^7 ARM genome copies added to 10^7 HC-Ad-mCMV- β gal genome copies. Our results show that detection is specific and that quantification presents linear behavior (linear regression, $R^2 = 0.9997$) between the dilutions spiked with 10^2 and 10^7 E1a genome copies. Using the qPCR assay, 66% of the samples were RCA positive when 10^7 genome copies of HC-Ad-mCMV- β gal were spiked with 10^1 genome copies of ARM (we detected two positive samples out of three; that is, 66% of the samples were RCA positive) (Fig. 4B and C).

Determination of adenoviral genomes from viral DNA extracted from cells infected in vitro

Next, we wished to determine whether qPCR could be used to calculate the ratio of total adenoviral vector genomes to infectious adenoviral genomes in either Ad or HC-Ad preparations. 293 cells were infected with 2×10^8 BFU (MOI of 2) of either HC-Ad-mCMV- β gal-WPRE or Ad-mCMV- β gal for 4 hr, which is a time point allowing for DNA nuclear entry but precedes the onset of viral DNA replication (Ng *et al.*, 2002a). At this time, intracellular adenoviral genomes were harvested and purified, and qPCR was performed. Viral genome copies 4 hr after infection of 293 cells were not significantly different from the viral input preparation ($p > 0.05$) (Table 1). As a control, we infected 293 cells with 2×10^8 IU of ARM (2.4×10^8 genome copies, independently determined by qPCR). We obtained an average yield of 1.8×10^8 genome copies of ARM from 293 cells 4 hr later and this was not significantly different from the total number of input genomes ($p > 0.05$), suggesting that infection of 293 cells for 4 hr was sufficient to allow entry into the cells of the majority of adenoviral genomes.

Determination of adenoviral genomes from viral DNA extracted after infection of brain stratum in vivo

A useful application of qPCR would be enable the detection and quantification of the total number of adenoviral genomes present in tissue samples after infection *in vivo*. Mice were injected with 1×10^7 BFU of either HC-Ad-mCMV- β gal-WPRE or Ad-mCMV- β gal, delivered into the striatum. Genome quantification from DNA isolated from striatal tissue 2 days after injection was performed by qPCR. After injection of 6.1×10^7 total genome copies of HC-Ad-mCMV- β gal-WPRE, we detected an average of 6.3×10^6 cosmid copies. Similarly, after injection of 4.8×10^7 genome copies of Ad-mCMV- β gal, we detected an average of 9.2×10^6 total L3 genome copies (Table 2). Although the number of input genomic copies was higher than the total copies detected 2 days after infection, no significant difference was detected between biological titers injected (blue-forming units) and qPCR values from total DNA isolated from brain tissue 2 days after infection ($p > 0.05$).

DISCUSSION

Doses of adenoviral vectors that could be safely used in pre-clinical models, such as rodents and primates, have been determined experimentally. It has been extensively demonstrated that innate and adaptive immune responses are key mediators of detrimental side effects to high doses of vector (Thomas *et al.*, 2000, 2001b; Bouchard *et al.*, 2003; Fitzgerald *et al.*, 2003; Raper *et al.*, 2003; Peden *et al.*, 2004; Sabatino *et al.*, 2005). In this paper, we describe the development and characterization of an accurate molecular method to determine the concentration and purity of adenoviral vectors in vector aliquots, *in vitro* and *in vivo*.

Biological methods such as the determination of infectious units in plaque assays have routinely been used to titrate first-generation adenoviral vectors. Because HC-Ad vectors cannot be titered by plaque assays or any type of biological titration method requiring vector replication, alternative methods are required. The most popular methods for titrating HC-Ad have either assessed transgene expression, or used molecular and physical methods, that is, dot blots, Southern blots, qPCR, or spectrometry. Levels of contaminating helper virus have been assessed by biological methods (infectious units) or molecular methods (qPCR, dot blot, or other DNA-based techniques) (Ma *et al.*, 2001; Ng *et al.*, 2002b; Reddy *et al.*, 2002; Schiedner *et al.*, 2002; Palmer and Ng, 2004; Brunetti-Pierri *et al.*, 2005). Titrations relying on transgene expression depend on the strength of the promoter used and the availability of sensitive detection methods for each particular transgene. Because the quantitative assessment of transgene expression will vary between vectors, it cannot provide a standardized method to titer all HC-Ad vectors and Ads. Importantly, titration values based on transgene expression will also depend on promoter strength (Bowers *et al.*, 2000), the transgene itself (Sastry *et al.*, 2002), the orientation of the expression cassette within the vector genome (Shering *et al.*, 1997; Sandig *et al.*, 2000), and the sensitivity of assays used to quantify levels of expression, that is, biological assays, ELISA, or radioimmunoassays. The cell type used to titer the vectors can also affect levels of transgene expression, especially if the promoter driving transgene expression is cell-type specific (Gerdes *et al.*, 2000). Ideally, the method to assess vector titers should be independent of levels of transgene expression. To overcome these limitations, qPCR methods have been developed to directly measure the number of genomes of HC-Ad, helper virus, or RCA (Umana *et al.*, 2001; Reddy *et al.*, 2002; Palmer and Ng, 2003; He *et al.*, 2005).

We developed a qPCR assay that is highly reproducible, allows a high throughput of samples, and can accurately quantify genome copy numbers over a large linear range (10^2 – 10^7 copies of standard plasmid per qPCR). Measurements can be done on genomic DNA purified from pure viral preparations, from infected cells, or from infected tissues *in vivo*. We found a consistent difference between qPCR values and optical density of purified viral preparations

whereby optical density was higher than the number of genomes present. This could be due to the loss of viral DNA during precipitation for qPCR. However, titration against ARM, the international standard Adenovirus Reference Material used to standardize biological and molecular assays, confirmed that our method of extracting viral DNA accurately quantified the number of viral genomes present, indicating that optical density values tend to overestimate the number of vector genomes present in a sample. It has been reported in the literature that the OD₂₆₀ values of HC-Ad preparations can vary as a result of (1) number of cells used for production, (2) the cell line, (3) the type and amount of protein expressed by the vector, and (4) the method of purification (Kreppel *et al.*, 2002). This is because residual cellular components such as proteins contaminating the viral preparation can significantly contribute to the total absorbance at 260 nm and the presence of these residual cellular components varies from preparation to preparation.

Here we show that qPCR can be used to quantitate both first-generation and high-capacity adenoviral vectors under a variety of conditions: purified vector stocks, *in vitro* after infection of cellular monolayers, and also quantification of vector genomes in the brain after infection *in vivo*. Our data indicate that qPCR can be used to accurately ascertain the presence and number of HC-Ad viral genomes as well as the presence of helper viral contamination or RCA within tissue samples. We found that the number of genomes injected into the brain of animals is not significantly different from the number of genomes that we detect in DNA purified from the brain tissue 2 days after infection. This was also observed when cells were infected *in vitro*. Therefore, qPCR allows an additional assessment of the quality of the preparation, namely, the percentage of total adenoviral genomes that express the transgene *in vivo*.

We also used qPCR to assess the levels of RCA within Ad vector preparations. The supernatant rescue assay as described by Dion *et al.* (1996) is able to detect an 85% incidence of CPE in the secondary plates at a frequency of 10 RCA added to 10⁹ Ad vector PFU; and 36% incidence of CPE in the secondary plates at a frequency of 1 RCA added to 10⁹ Ad vector PFU. Using our qPCR assay we can detect a 66% incidence of E1a-positive samples when 10 ARM genome copies were added to the HC-Ad vector sample (Fig. 4C). Thus, our data suggest that the qPCR has a sensitivity that is somewhat lower than that of the biological RCA assay as reported by Dion *et al.* (1996). Differences between these assays performed in separate laboratories are not unexpected.

In summary, careful and systematic validation of adenoviral preparations is an essential prerequisite for the development of safe and effective therapies in human patients. We have developed a fast, reliable, and sensitive qPCR method that allows accurate quantification of adenoviral vector genomes within first-generation and high-capacity vector preparations. We show that this method allows us to determine simultaneously contaminating genomes present in adenoviral preparations, such as RCA and helper virus, and this will improve our ability to screen the quality of vector preparations. Furthermore, we demonstrate that qPCR can be used to accurately quantify adenoviral genomes in tissue samples, which will allow accurate assessment of both the biodistribution and half-life kinetics of adenovirus-based therapeutic vectors in preclinical animal models and in clinical trials using DNA extracted from tissue biopsies.

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FIG. 1.

Schematic diagram indicating the genomic elements of high-capacity (HC-Ad) vectors, FL helper adenovirus, and wild-type adenovirus. (A) Schematic diagram of the standard plasmid constructed on the basis of pBluescript backbone. PCR products from Ad5 gene L3 (235 bp), Ad5 gene E1a (239 bp), cosmid-346 (280 bp), and luciferase (289 bp) were ligated into the multiple cloning site at *HindIII*, *EcoRI*, *BamHI*, and *XbaI* restriction sites, respectively. (B) HC-Ad vector genome containing the packaging signal, stuffer sequences, transcription cassette, and ITR genomic sequences. Cosmid-346 primers (black arrows) recognize a region, present only in HC-Ad vectors, that is detected by a FAM-conjugated probe (light blue box). (C) FRT-helper adenovirus genome containing the packaging signal (ψ) flanked by FRT sites,

stuffer sequences, luciferase cassette, and ITR genomic sequences. Luciferase primers (black arrows) recognize a region of DNA present only in helper virus that is detected with a VIC-conjugated probe (purple box). (**C** and **D**) Ad5 gene L3 primers (black arrows) recognize a region of DNA present on both helper viruses and wild-type adenovirus and is detected with a VIC-conjugated probe (pink box). (**E**) Wild-type Ad5 genome containing the packaging signal, E1a region, L3 region, and ITR genomic sequences (highlighted). Ad5 gene E1a primers (black arrows) recognize a region of DNA present only on wild-type adenovirus 5 that is detected with a TET-conjugated probe (dark blue box).

FIG. 2.

Standard calibration curves for HC-Ad and Ad vectors and quantification by real-time PCR. Shown are standard calibration curves of threshold cycle values (C_t) versus copy numbers ($\log_{10}$), using serial dilutions of pSt for HC-Ad vectors (A), FL helper virus (C), Ad vectors (E), and RCA (G). The coefficient of correlation (r^2) and slope (A, C, E, and G) are indicated. The standard curve (solid dots) was generated with serial dilutions of standard plasmid DNA containing 10^2 to 10^7 copies. Three dilutions of HC-Ad-mCMV- β gal-TK vectors (10^{-3} , 10^{-4} , and 10^{-5}) were amplified with qPCR primer and probe sets for cosmid (B) or luciferase (D). Three dilutions of Ad-hCMV- β gal vector (10^{-3} , 10^{-4} , and 10^{-5}) were amplified with qPCR primer and probe sets for L3 (F) and E1a (H). All reactions were monitored by changes

in fluorescence (ΔR_n) at each qPCR cycle. The values for each dilution in the standard plot and in the amplification plots are the result of three independent repetitions.

FIG. 3.

Comparing qPCR and established assays for accuracy and sensitivity when measuring HC-Ad vector preparations. Total HC-Ad viral genomes, helper virus contamination, and replication-competent adenovirus (RCA) were titrated in two HC-Ad preparations: HC-Ad-mCMV-βgal (A) and HC-Ad-mCMV-βgal-TK (B), using molecular, biological, or physical methods. Blue-forming units (BFU) were used to titer vector genomes expressing β-galactosidase; infectious units (IU) were used to titer contaminating helper virus in 293 cells. Molecular titrations using qPCR were used to measure HC-Ad genomes (cosmid), contaminating helper virus (L3 or luciferase), and contaminating RCA (E1a). Total viral particles were measured by absorbance at 260 nm. Titters are indicated in the corresponding units per milliliter. (C and D) Ad vector preparations. Two first-generation adenoviral vectors, Ad-hCMV-βgal (C) and Ad-mCMV-βgal (D), were titrated by molecular, biological, or physical methods as described in Materials and Methods. Blue-forming units (BFU), viral particles (VP), and infectious units (IU) were used to titer Ad-hCMV-βgal or Ad-mCMV-βgal first generation vectors. Molecular titrations

using qPCR were used to measure Ad genomes (L3) and contaminating RCA (E1a). Total viral particles were measured by absorbance at 260 nm. Titers are indicated in the corresponding units per milliliter. Results are expressed as means $\pm$ SEM ($n = 3$). RCA detection for the four vectors described was negative.

FIG. 4.

Detection of RCA, using qPCR or biological assays. **(A)** The sensitivity of the biological assay for RCA detection was assessed by adding different amounts of ARM (10, 100, and 1000 VP) to 10^9 VP of HC-Ad-mCMV- β gal vector and evaluating CPE on HeLa cells. **(B)** We analyzed the relationship between theoretical ARM copies added versus the total number of copies detected by qPCR ($n = 3$). We found that there was a linear relation (linear regression, $R^2 = 0.9997$). Standard deviations of three samples analyzed are indicated. **(C)** Sensitivity of the qPCR assay for RCA detection was assessed by adding different amounts of ARM genome copies (ranging from 10 to 10^7 genome copies) to 10^9 HC-Ad-mCMV- β gal genome copies. E1a and cosmid copies were quantified by qPCR. Results are expressed as means $\pm$ SEM ($n = 3$).

Table 1

qPCR Quantification of Intracellular DNA after *In Vitro* Infection^a

Vector	Input		qPCR		
	Infectious (BFU or IU)	Genomic (copies)	Cosmid	L3	E1
HC-Ad-mCMV-βgal-WPRE	2.00 × 10 ⁸	1.22 × 10 ⁹	9.19 (±0.80) × 10 ⁷	7.58 (±0.24) × 10 ⁵	Neg
1			10.5 (±0.16) × 10 ⁷	8.13 (±0.12) × 10 ⁵	Neg
2			9.31 (±0.45) × 10 ⁷	4.16 (±0.25) × 10 ⁵	Neg
Ad-mCMV-βgal	2.00 × 10 ⁸	9.63 × 10 ⁸	Neg	2.61 (±0.05) × 10 ⁸	Neg
1			Neg	1.50 (±0.09) × 10 ⁸	Neg
2			Neg	1.18 (±0.73) × 10 ⁸	Neg
3			Neg	1.82 (±0.58) × 10 ⁸	1.69 (±0.24) × 10 ⁸
ARM	2.00 × 10 ⁸	2.39 × 10 ⁸			

Abbreviations: ARM, Adenovirus Reference Material; BFU, blue-forming units; IU, infectious units; qPCR, quantitative PCR; Neg, negative.

^a293 cells (2.00 × 10⁶) were infected with either HC-Ad-mCMV-βgal-WPRE or Ad-mCMV-βgal (100 BFU/cell) or with ARM (100 IU/cell). After 4 hr of incubation at 37°C, the cells were harvested and processed for intracellular DNA. Cellular lysates were treated subsequently with DNase I and proteinase K as described in Materials and Methods; DNA was precipitated and re-suspended in 25 μl of DEPC-treated water. Three independent repetitions of the infection done with the same high-capacity or first-generation vector expressing β-galactosidase are shown. ARM was used as control. Standard deviations correspond to three repetitions for qPCR quantifications. Intracellular DNA from mock-infected cells quantified for cosmid, L3, and E1a was negative. No differences were found between input and qPCR genome quantification from intracellular DNA. Results are expressed as means ± SEM (n = 3). Titers for the HC-Ad-mCMV-βgal-WPRE vector preparation used the experiments described here and in Table 2 were as follows: (1) cosmid (HC-Ad) = 1.49 × 10¹² genomes/ml; (2) L3 (helper virus) = 1.06 × 10¹⁰ genomes/ml; (3) biological activity of HC-Ad = 2.44 × 10¹¹ BFU/ml; (4) infectious activity of helper virus = 1.0 × 10⁶ IU/ml; (5) physical titer = 5.74 × 10¹² VP/ml. The titers for the Ad-mCMV-βgal vector preparation are given in Fig. 3D.

Table 2

Vector Genome Quantification in Brain Tissue DNA after Injection^a

Vector	Input		qPCR	
	Infectious (BFU)	Genomic (copies)	Cosmid	E1
HC-Ad-mCMV-βgal-WPRE	1.00 × 10 ⁷	6.11 × 10 ⁷	7.04 (±0.26) × 10 ⁶ 8.16 (±0.30) × 10 ⁶ 4.42 (±0.30) × 10 ⁶ 5.57 (±0.43) × 10 ⁶	6.58 (±0.13) × 10 ⁴ 7.06 (±0.36) × 10 ⁴ 5.84 (±0.17) × 10 ⁴ 13.99 (±0.22) × 10 ⁴
Ad-mCMV-βgal	1.00 × 10 ⁷	4.82 × 10 ⁷	Neg Neg Neg Neg	7.47 (±0.89) × 10 ⁶ 7.25 (±0.45) × 10 ⁶ 11.3 (±0.54) × 10 ⁶ 10.79 (±0.89) × 10 ⁶
Saline	—	—	Neg	Neg

^aFour mice were injected in the brain with 1.00 × 10⁷ BFU of either HC-Ad-mCMV-βgal-WPRE or Ad-mCMV-βgal. Two days post-injection, mice were perfused and brains were removed, and treated for DNA purification as described in Materials and Methods; DNA was eluted in 100 μl of AE buffer. The standard error from the mean corresponds to three repetitions for qPCR quantifications. No specific detection for cosmid, L3, and E1a from saline-injected animals was obtained. Total genome copies injected are indicated as input value, and total genome copies obtained by qPCR for cosmid, L3, and E1a are indicated with the value calculated, considering the total volume of DNA extracted per brain. No differences were found between input and qPCR genome quantification from injected brain tissue. Results are expressed as means ± SEM (n = 3). Titers for the HC-Ad-mCMV-βgal-WPRE vector preparation are given in detail in the footnote to Table 1. Titers for the Ad-mCMV-βgal vector preparation are given in Fig. 3D.