JOURNAL OF VIROLOGY, Jan. 2005, p. 193–201 Vol. 79, No. 1
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.1.193–201.2005
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Novel Strategy for Generation and Titration of Recombinant Adeno-Associated Virus Vectors

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Received 1 June 2004/Accepted 10 August 2004

Recombinant adeno-associated virus (rAAV) vectors have many advantages for gene therapeutic applications compared with other vector systems. Several methods that use plasmids or helper viruses have been reported for the generation of rAAV vectors. Unfortunately, the preparation of large-scale rAAV stocks is labor-intensive. Moreover, the biological titration of rAAV is still difficult, which may limit its preclinical and clinical applications. For this study, we developed a novel strategy to generate and biologically titrate rAAV vectors. A recombinant pseudorabies virus (PrV) with defects in its gD, gE, and thymidine kinase genes was engineered to express the AAV *rep* and *cap* genes, yielding PS virus, which served as a packaging and helper virus for the generation of rAAV vectors. PS virus was useful not only for generating high-titer rAAV vectors by cotransfection with an rAAV vector plasmid, but also for amplifying rAAV stocks. Notably, the biological titration of rAAV vectors was also feasible when cells were coinfected with rAAV and PS virus. Based on this strategy, we produced an rAAV that expresses prothymosin α (ProT). Expression of the ProT protein in vitro and in vivo mediated by rAAV/ProT gene transfer was detected by immunohistochemistry and a bioassay. Taken together, our results demonstrate that the PrV vector-based system is useful for generating rAAV vectors carrying various transgenes.

Adeno-associated virus (AAV) is a member of the dependent parvovirus family that requires coinfection with a helper virus, such as adenovirus or herpesvirus, to undergo a productive infection in cultured cells (18). In addition to transducing both mitotic and postmitotic tissues, AAV binds to a cellular receptor, enters the cell, migrates to the nucleus, and delivers a single-stranded DNA genome to establish a latent state for long-term gene expression in the absence of coinfection with a helper virus. Because recombinant AAV (rAAV) vectors have deletions of the genes for all of the viral proteins, they offer advantages over some other viral vector systems, with long-term and high-level gene expression without a cellular immune response or toxicity even in immunocompetent hosts (4, 6). Several methods have been used to generate rAAV vectors (7). Originally, the construction of rAAV vectors involved the cotransfection of an AAV vector plasmid containing an expression cassette flanked by the AAV inverted terminal repeats (ITRs), which are the sole elements required for rescue, replication, packaging, and integration of AAV (23, 34), and an AAV helper plasmid encoding the Rep and Cap proteins into adenovirus-infected cells (30). Apart from the low frequency of transfecting both plasmids into the same cell, the separation of rAAV vectors from helper adenoviruses is required to minimize the risk of contamination with wild-type (wt) adenovirus. Recent improvements in rAAV packaging technology have made the production of high-titer rAAV more feasible. Helper virus-free methods for rAAV production have also been developed (9, 17, 39). These methods are based on the replacement of the helper virus with a helper plasmid carrying the adenovirus genome for helper functions, either in combination with the *rep* and *cap* genes or not, for rAAV production. These helper virus-free methods require successful transfection on a large scale, which is not easily achieved. To overcome this, several methods that use producer cell lines have been developed which still require adenovirus infection but bypass the necessity of transfection procedures. The improvement is the generation of Rep-inducible cell lines, with translational control of Rep production and an increase in Cap expression by driving *cap* transcription with a strong heterologous promoter (19). However, Rep-inducible cell lines do not produce rAAV more efficiently than conventional methods.

The efficient growth of AAV requires helper functions that are provided by a coinfecting adenovirus or herpesvirus. While adenovirus is an efficient helper virus for rAAV production, little consideration has been given to other helper viruses for AAV replication and packaging. Pseudorabies virus (PrV), a herpesvirus of swine, is also a fully competent helper virus for AAV replication (2). PrV contains several envelope glycoproteins that are important for interactions between virions and host cells. Glycoprotein D (gD) is essential for PrV entry into cells but is not required for the subsequent steps in virus replication (27), whereas glycoprotein E (gE) is dispensable for viral replication but is an essential protein for the transneuronal spread of PrV (26). Deletion of either the gD or gE gene reduces the virulence of PrV (25). Moreover, PrV-encoded thymidine kinase (TK) serves as another virulence factor to support viral DNA replication. PrV mutants that are defective in TK function reduce virulence, replication in peripheral target tissues, and migration to the central nervous system (14). For this study, we constructed a gD/gE/TK triply defective recombinant PrV, designated PS virus, to express the AAV

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Rep and Cap proteins and then employed it as a helper virus accompanied with packaging machinery for the production of rAAV vectors. With this system, rAAV vectors obtained from AAV plasmid vector transfection combined with PS virus infection to provide packaging and helper functions could be propagated to high titers in 293 cells that were coinfected with PS virus. Furthermore, rAAV vectors could be biologically titrated in this system. Therefore, this system facilitates the generation, amplification, and titration of rAAV vectors for gene therapy applications.

MATERIALS AND METHODS

Viruses, cells, and mice. PrV strain TNL is a virulent wt strain of PrV, while CW1 virus, derived from strain TNL, is a gD- and gE-negative and herpes simplex virus type 1 (HSV-1) TK-positive PrV strain (33). The TK expression cassette within the CW1 virus can be conveniently replaced with the AAV *rep* and *cap* genes by homologous recombination. Vero (African green monkey kidney cells), Vero-gD (PrV gD-overexpressing Vero cells) (33), 293 (human embryonic kidney cells), BHK (baby hamster kidney cells), and NIH 3T3 (mouse fibroblasts) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (or calf serum for NIH 3T3 cells), 2 mM L-glutamine, and 50 μg of gentamicin/ml. Female C3H/HeNCrj mice (6 to 8 weeks old) were obtained from the Laboratory Animal Center of the National Cheng Kung University Medical College. The principles of laboratory animal care were followed, and the animals were housed and fed in compliance with the operational guidelines of the Committee of the Animal Facility, National Cheng Kung University Medical College.

Generation of recombinant PrV. The plasmid pDgD/rep-cap was derived from pDelta-gD (33) by replacement of the HSV TK expression module with an AAV *rep-cap* module. The fragment containing the *rep* and *cap* genes along with their native p5, p19, and p40 promoters was excised from pXX2 (39) by XbaI digestion followed by a fill-in reaction with Klenow polymerase and was then ligated into pDelta-gD that had been digested with EcoRI and HindIII followed by a fill-in reaction with Klenow polymerase. The targeting deletion vector pDelta-gD harbored 5′- and 3′-flanking regions to provide homology for homologous recombination with the CW1 virus at its unique EcoRI and HindIII sites. Using a previously described strategy of homologous recombination (33), we transfected Vero-gD cells with the pDgD/rep-cap plasmid and infected them 24 h later with the CW1 virus at 1.75×10^7 50% tissue culture infective doses ($TCID_{50}$). After an overnight incubation, the culture medium was supplemented with acyclovir (64 μM) to isolate TK-negative PrV. The resulting plaque-purified gD/gE/TK triply defective mutant carrying the AAV *rep* and *cap* genes, designated PS virus, was propagated for 10 passages in the presence of acyclovir.

Production of rAAV vectors. pSub201 (31) is an AAV-based plasmid that contains 96% of the internal AAV-2 coding sequence located between the two ITRs to accommodate replacement with a promoter and a gene of interest to generate rAAV plasmids. The β-actin promoter-driven AAV vector plasmid pSub201/β was derived from pSub201 by replacement of the XbaI-restricted *rep-cap* region with the PvuII-to-HpaI fragment encompassing the rat β-actin promoter sequence of pJ6Ω (38). The pSub201/β vector plasmid can be used to insert a transgene flanked by the ITRs. The pSub201/EGFP plasmid was used to produce a trackable enhanced green fluorescent protein (EGFP)-rAAV to assess our AAV production system. It was constructed by subcloning an EGFP cDNA obtained from pRUFneo/EGFP restricted with BamHI and BglII into the BamHI and BglII sites of pSub201/β. Similarly, a prothymosin α (ProT) cDNA was obtained from EcoRI- and NotI-restricted pRUF/ProT (32) and cloned into the EcoRI and NotI sites of pSub201/β, resulting in pSub201/ProT. rAAV vectors were produced by use of either the PS virus packaging-helper system or an AAV helper-free system (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. 293 cells were transfected with pSub201/EGFP or pSub201/ProT by the calcium phosphate precipitation method and then plated in 35-mm-diameter culture dishes followed by infection with various doses of PS virus. rAAV vectors were harvested 72 h after infection, and their titers were determined.

Titration of rAAV/EGFP by a fluorescent cell assay and the $TCID_{50}$ method. 293 cells were infected with rAAV/EGFP, the infected cells were trypsinized, and cells expressing EGFP were counted by use of a hemocytometer and fluorescence microscopy 48 h later. The titers of rAAV/EGFP are expressed as EGFP transduction units (TU) per well. Because a cytopathic effect (CPE) was observed only for cells that were coinfected with rAAV and PS virus, and not for those infected with either virus alone, we were able to determine rAAV titers by the $TCID_{50}$ method. BHK cells (2×10^5/well) were cultured in 24-well plates for 24 h, infected with different doses of rAAV/EGFP for 24 h, and subsequently infected with 10^6 $TCID_{50}$ of PS virus. The cells were observed daily for CPE and stained with a 10% formalin–0.05% crystal violet solution. According to the onset of CPE, the biological titers of rAAV vectors were determined by the $TCID_{50}$ method.

Detection of replication-competent PrV by the CPE method. rAAV stocks were tested for contaminating replication-competent PrV on Vero cells by the CPE method. The virulent PrV strain TNL, which induces severe CPE in BHK cells, was used as a positive control for the assay (33).

In vitro transduction of rAAV vectors and characterization of transgene expression. Seventy-two hours after infection with rAAV/ProT, NIH 3T3 cells were fixed in 3.7% formaldehyde, treated with cold acetone, and quenched in 3.7% H_2O_2 for the detection of ProT expression by immunohistochemical staining with a mouse monoclonal antibody against human ProT (38). After sequential incubations with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin G and aminoethyl carbazole as a substrate chromogen, the slides were counterstained with hematoxylin. To assess cell proliferation induced by ProT, we infected NIH 3T3 cells cultured in 96-well plates with rAAV/ProT at different doses in quadruplicate and incubated them for 4 days. PP23, a ProT-overexpressing NIH 3T3 clone (38), served as a positive control. [^{3}H]thymidine (0.5 μl/well) was added to each well 18 h before harvest. The cells were harvested, and the [^{3}H]thymidine incorporated into the DNA was counted by use of a Matrix 9600 direct β counter (Packard, Meriden, Conn.).

In vivo delivery of rAAV vectors to muscle tissues. To monitor transgene expression in skeletal muscle, we inoculated mice intramuscularly in the posterior thigh with rAAV/ProT (31.8 $TCID_{50}$) suspended in 0.5 ml of phosphate-buffered saline (PBS) containing charcoal or with the vehicle. After 10 days, the mice were sacrificed and the muscle at the site of injection was examined for ProT expression by immunohistochemistry as previously described (33).

RESULTS

Generation of recombinant packaging-helper PrV. A fragment containing the AAV *rep* and *cap* genes isolated from pXX2 (39) was used to replace the HSV TK module in pDelta-gD, providing homology regions for homologous recombination with PrV, resulting in pDgD/rep-cap. gD-expressing Vero-gD cells were transfected with pDgD/rep-cap followed by infection with the CW1 virus on the following day (33). The rAAV vector, designated PS virus, carrying the *rep* and *cap* genes from AAV was then selected by acyclovir treatment. A schematic representation of the strategy for constructing PS virus and the rAAV vector is shown in Fig. 1. The plaque-purified PS virus was further confirmed by detection of the AAV *rep* gene by PCR analysis and restriction digestion (data not shown). The PS virus, which is a gD/gE/TK triply defective PrV carrying the AAV *rep* and *cap* genes under the control of their native p5, p19, and p40 promoters, was then tested for the ability to provide packaging and helper functions for rAAV vector production.

Optimization of rAAV generation by packaging-helper PS virus. To test the feasibility and efficiency of rAAV vector production with PS virus as a packaging and helper virus, we needed to examine two parameters that may affect the rAAV yield, namely, the transfection efficiency of the AAV vector plasmid and the amount of PS virus used for infection. In order to investigate whether the rAAV yield could be improved by increasing the transfection efficiency of the pSub201/EGFP plasmid, we infected 293 cells that had been transfected with pSub201/EGFP at various transfection efficiencies with 3.72×10^6 $TCID_{50}$ of PS virus. As shown in Fig. 2A, the yield of rAAV vectors positively correlated with the transfection efficiency of the rAAV vector plasmid, as determined by EGFP expression

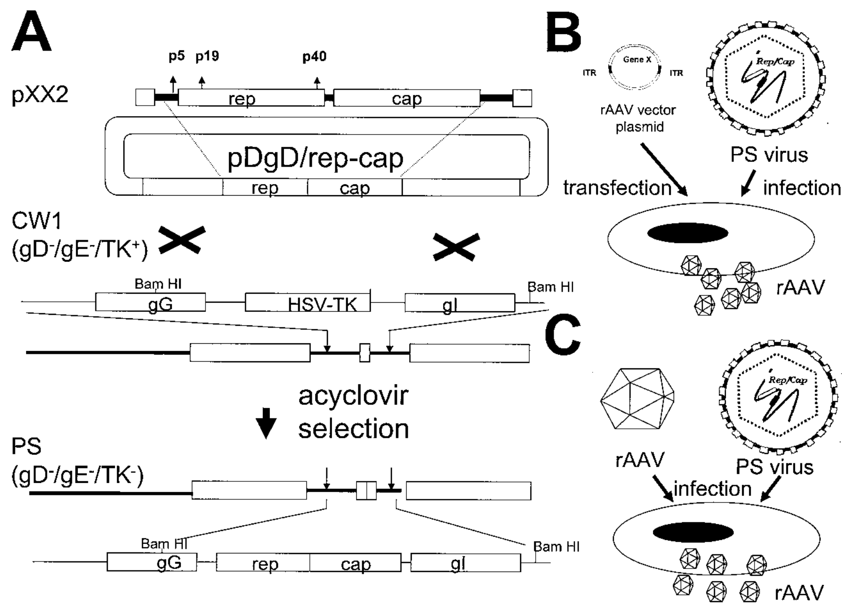

FIG. 1. Schematic presentation of the strategy for generating PS virus, a gD/gE/TK-defective PrV carrying the AAV *rep* and *cap* genes, and an rAAV vector. (A) The AAV gene cassette containing the *rep* and *cap* genes as well as their native p5, p19, and p40 promoters was excised from the AAV packaging plasmid pXX2 and cloned into the targeting deletion vector pDelta-gD to generate pDgD/rep-cap. The CW1 virus is a gD/gE-negative and HSV TK-positive recombinant PrV strain, with the HSV TK gene replacing the gD gene in the PrV genome. Vero-gD cells were transfected with the pDgD/rep-cap plasmid followed by infection with the CW1 virus. The resulting PS virus was generated by homologous recombination in the presence of acyclovir selection. (B) Gene "X," a transgene of interest, was inserted into pSub201/β, a β-actin promoter-driven rAAV vector plasmid flanked by the ITRs of AAV. 293 cells were transfected with the rAAV vector plasmid followed by infection with PS virus, resulting in the generation of rAAV vectors. (C) rAAV vectors generated as shown in panel B were used to directly infect 293 cells concurrent with PS virus infection, resulting in the amplification of rAAV vectors.

in transfected 293 cells under fluorescence microscopy. Another factor that influenced rAAV production was the amount of PS virus used for infection. When the transfection efficiency of rAAV/EGFP in 293 cells achieved 70%, larger amounts of PS virus used for infection resulted in higher rAAV yields, as measured in EGFP TU (Fig. 2B). Taken together, the transduction efficiency of the rAAV vector plasmid and the amount of packaging-helper PS virus significantly influenced the resulting titers of rAAV stocks obtained with our vector production system.

Biological titration of rAAV by coinfection with PS virus. Because AAV is a naturally defective parvovirus, it causes no CPE in cultured cells unless it is in the presence of a helper virus, such as adenovirus or herpesvirus. However, wt adenovirus serving as a helper virus generally induces CPE, which makes titration of rAAV by the $TCID_{50}$ method impossible. In the system described here, since PS virus has defective gD, gE, and TK genes, it is very attenuated and causes no CPE in non-gD-complementing cells (33). We therefore determined the titers of rAAV vectors by the $TCID_{50}$ method with BHK cells that were coinfected with PS virus. Figure 3 shows that rAAV induced CPE in BHK cells in a dose-dependent manner when it was coinfected with PS virus, whereas rAAV or PS virus alone did not induce CPE. Furthermore, we also compared the correlation between TU and $TCID_{50}$ when rAAV/EGFP was applied. Ten $TCID_{50}$ of rAAV/EGFP generated 397 TU on 293 cells.

Amplification of rAAV via coinfection with PS virus. We next tested the feasibility of amplifying rAAV vectors through coinfection with PS virus. Not surprisingly, PS virus could also serve as a packaging and helper virus in cells that were directly infected with the rAAV vector, resulting in the amplification of rAAV stocks. When rAAV and PS virus coinfected 293 cells, an amplification of the rAAV vector was observed (Fig. 4). Infections with PS virus (0.9×10^6 to 5.4×10^6 $TCID_{50}$) along with 10^3 TU of rAAV vectors led to 200- to 500-fold amplifications of input rAAV/EGFP in 6×10^6 293 cells. As shown in Table 1, serial passages of rAAV vectors with PS virus resulted in vector amplification. With our packaging-helper PS virus system, rAAV/EGFP vectors could be amplified 300- to 380-fold in each passage. The total amplification of rAAV vectors was $>10^7$-fold after three cycles of passages. However, with the three-plasmid helper-free system, no amplification of the vectors occurred when 293 cells were directly infected with rAAV vectors.

Production of rAAV vectors that are free of replication-competent PrV. To test whether rAAV stocks made by using PS virus as the packaging and helper virus were free of replication-competent PrV, we infected Vero cells with rAAV stocks and monitored them for CPE by crystal violet staining. No CPE was detected on Vero cells 5 days after incubation with rAAV stocks at any dilution tested, whereas CPE was evident in those infected with the virulent PrV strain TNL at dilutions of 10^{-1} to 10^{-5} (Fig. 5A). Upon photomicrographic examination, Vero cells exhibited severe CPE following infection with PrV strain TNL, whereas cells infected with rAAV stocks appeared as healthy as mock-infected cells (Fig. 5B),

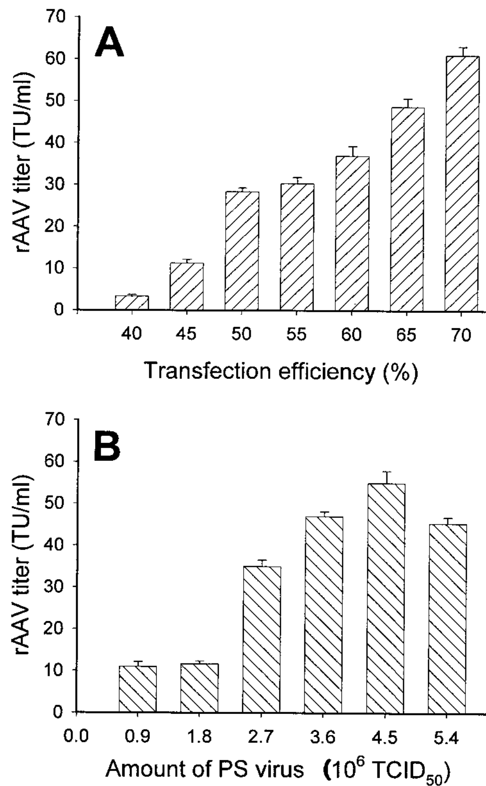

FIG. 2. Influences of transfection efficiency and helper virus titer on rAAV yields. (A) 293 cells that had been transfected with pSub201/EGFP at different transfection efficiencies were plated in 35-mm-diameter culture dishes and infected with PS virus (3.72×10^6 $TCID_{50}$). (B) pSub201/EGFP-transfected cells with a 70% transfection efficiency were infected with different amounts of PS virus, ranging from 0.9×10^6 to 5.4×10^6 $TCID_{50}$. After 72 h, the cells were harvested, and rAAV vectors were determined by a fluorescent cell assay and expressed in EGFP TU.

suggesting that rAAV stocks produced by the PS virus packaging-helper system were free of replication-competent PrV.

In vitro and in vivo transduction of rAAV vector carrying the ProT gene. We next produced an rAAV/ProT vector expressing ProT by using the PS virus system and tested its ability to

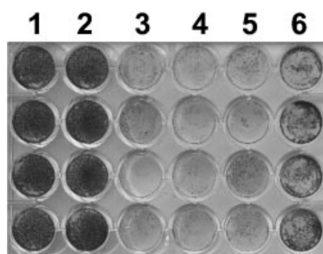

FIG. 3. Determination of rAAV titers by the $TCID_{50}$ method via coinfection with PS virus. BHK cells (2×10^5/well) were cultured in 24-well plates for 24 h, infected with serial twofold dilutions of rAAV/EGFP for 24 h, and subsequently infected with 10^6 $TCID_{50}$ of PS virus. The cells were also infected with either PS virus or rAAV vector alone, which served as negative controls. The cells were observed daily for CPE and were stained with crystal violet 6 days later. Lane 1, PS virus only; lane 2, rAAV vector only; lanes 3 to 6, serial twofold dilutions of rAAV vectors, starting from lane 3, in the presence of PS virus.

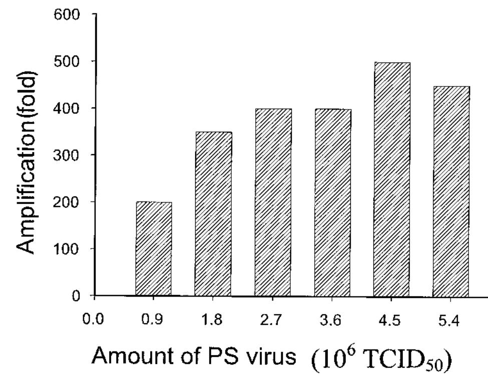

FIG. 4. Determination of the optimal amount of PS virus required for rAAV amplification. 293 cells were infected with rAAV/EGFP (10^3 TU) for 24 h, followed by infection with different amounts of PS virus, ranging from 0.9×10^6 to 5.4×10^6 $TCID_{50}$. After 72 h, the cells were harvested, and rAAV titers were determined by a fluorescent cell assay and expressed in EGFP TU. The amount of amplification was calculated by dividing the titer of the output virus by that of the input virus.

mediate gene transfer. ProT, a putative thymic hormone, has been shown to exert antitumor effects on murine bladder cancer when delivered by retroviral vectors (32). Expression of the ProT protein was detected in rAAV/ProT-infected NIH 3T3 cells by immunohistochemistry at 72 h postinfection (Fig. 6A), but it was not detected in mock-infected cells (Fig. 6B). Furthermore, the expression of ProT in skeletal muscle in vivo was examined 10 days after intramuscular injections of rAAV/ProT or PBS into mice. ProT was stained red by immunohistochemical staining, while black spots were charcoal particles that were coadministered to facilitate the location of the injection site. Expression of the ProT protein was detectable in muscle tissues from mice who were injected with rAAV/ProT (Fig. 6C), whereas no apparent red staining was found in muscle tissues from PBS-treated mice (Fig. 6D). Since ProT promotes cell proliferation (38), we tested whether the proliferation of NIH 3T3 cells could be enhanced by rAAV/ProT treatment. Figure 7 shows that rAAV/ProT enhanced the proliferation of NIH 3T3 cells in a dose-dependent manner. Taken together, these results indicate that the transgene carried by the rAAV vector generated by our system can be expressed in vitro and in vivo. More importantly, the protein encoded by the transgene retained its biological activity.

DISCUSSION

The use of rAAV vectors for gene transfer has been successful in achieving long-term high-level gene expression in vivo. rAAV vectors have become increasingly popular as vectors for gene therapy and functional genomic studies. However, their use is hampered by cumbersome packaging protocols for vector production. This difficulty has led to development in numerous aspects of rAAV vectors in order to increase their production. Most strategies have involved the use of adenovirus to provide the helper functions for rAAV production. An rAAV vector is typically produced by transfecting cells with an rAAV vector plasmid and a packaging plasmid that expresses the AAV *rep* and *cap* genes, followed by infection with adenovirus to provide helper functions for effi-

TABLE 1. Serial passages of rAAV/EGFP via coinfection with PS virus resulted in rAAV vector amplification[a]

Passage no.	Input vector (TU)	Output vector (TU)		Amplification (fold)		Total amplification	
		PS virus system	Helper-free system	PS virus system	Helper-free system	PS virus system	Helper-free system
0		65	<3				
1	10^3	3.0×10^5	1.7×10^3	300	1.7	300	1.7
2	10^4	3.7×10^6	ND	370	ND	1.1×10^5	ND
3	2×10^4	7.8×10^6	ND	380	ND	4.2×10^7	ND

[a] For passage number 0, 10 μg of pSUB201/EGFP was transfected into 293 cells (3×10^6) by use of PS virus as a packaging-helper virus or by use of a three-plasmid helper-free system. For passages 1 to 3, 293 cells were infected with rAAV/EGFP (input vector), followed by infection with PS virus (4.65×10^7 $TCID_{50}$) 24 h later. After 72 h, the cells were harvested, and titers of output rAAV vectors were determined by a fluorescent cell assay and expressed in EGFP TU. ND, not done.

cient AAV replication and assembly (30). Another approach to rAAV vector production is based on the use of stable *rep*- and *cap*-expressing cell lines. Infection of the cell lines with an adenovirus is required for the activation of the *rep* and *cap* genes, whereas the AAV vector is delivered by either transfection or infection with an adenovirus-AAV hybrid vector (22, 35). Some stable cell lines containing both the *rep* and *cap* genes and the AAV genome only require infection with adenovirus for the production of AAV (8). With these approaches, the need for the helper adenovirus for rAAV vector production leads to the concomitant production of infectious adenovirus particles, which are difficult to separate from rAAV stocks. The removal of adenovirus has relied on physical methods, such as $CsCl_2$ gradient centrifugation, column chromatography, and heat, to inactivate any residual adenovirus particles that may still be present. The presence of adenovirus or adenoviral proteins is a potential source of contamination that may induce unwanted immune responses in vivo. With these concerns, new vector production systems have been developed that are free of replicating adenovirus. The minimal adenoviral

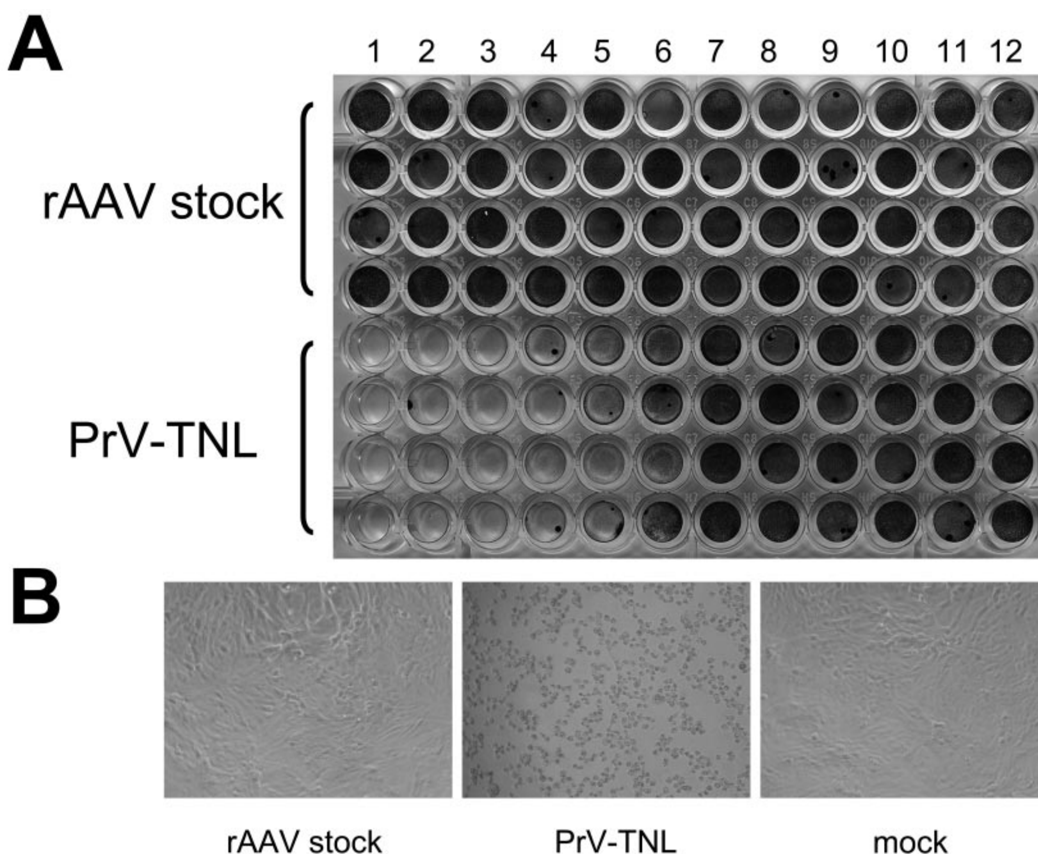

FIG. 5. Analysis of presence of replication-competent PrV in rAAV stocks by the CPE method. (A) Confluent Vero cells cultured in 96-well plates were infected with serial 10-fold dilutions, ranging from 10^{-1} to 10^{-11}, of an rAAV stock generated by the PS packaging-helper system. Meanwhile, Vero cells were infected with PrV strain TNL or mock infected to serve as positive and negative controls, respectively, in the CPE assay. At 5 days postinfection, the cells were stained with crystal violet. Lanes 1 to 11, serial 10-fold dilutions (10^{-1} to 10^{-11}) of rAAV or PrV TNL stock, starting from lane 1; lane 12, mock infected. (B) CPE was detected in Vero cells infected with PrV strain TNL, but not in those infected with the rAAV vector, by photomicrographic examination at 5 days postinfection. Original magnification, ×200.

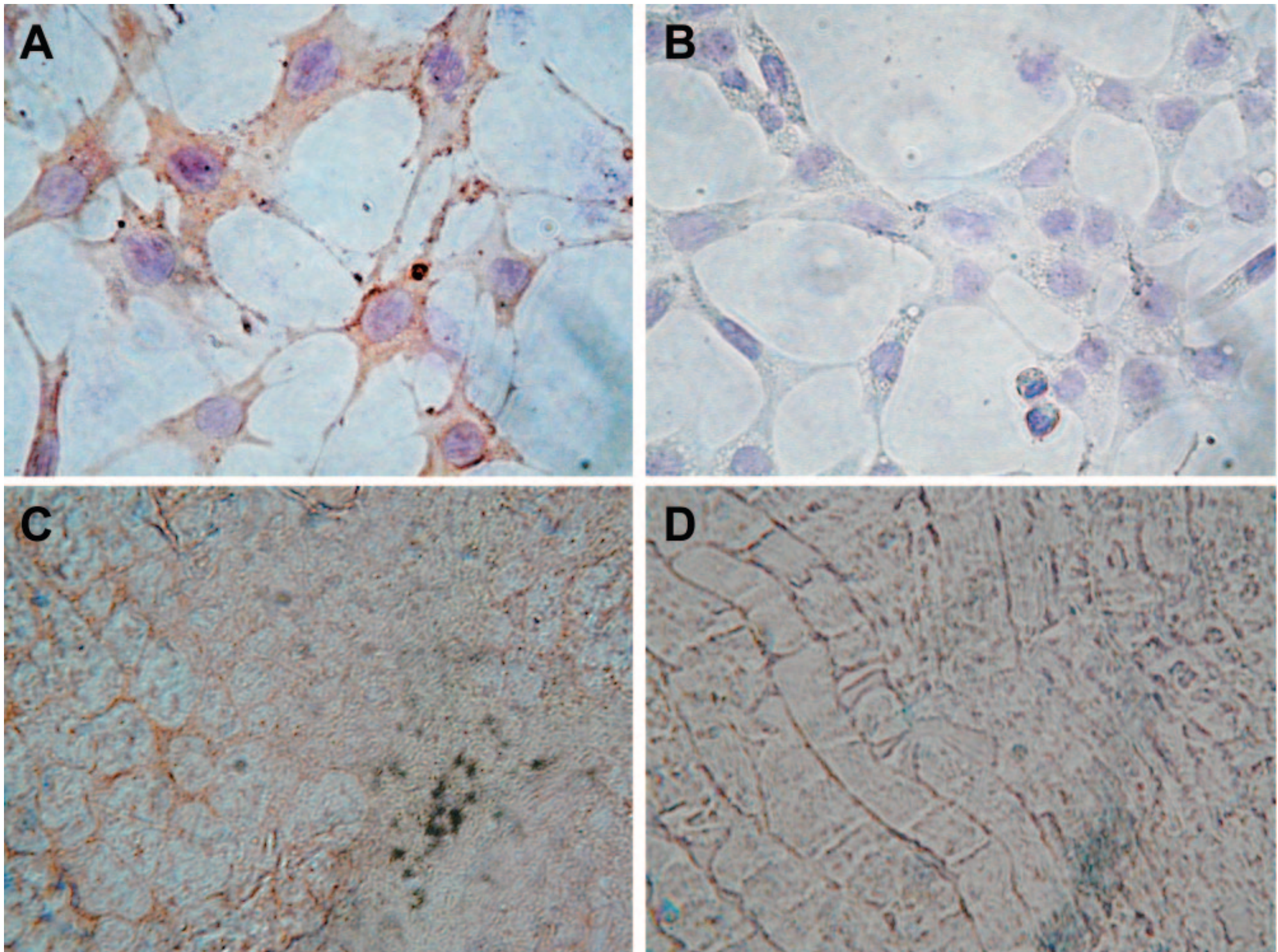

FIG. 6. ProT expression in cells infected with rAAV/ProT in vitro and in vivo, as determined by immunohistochemistry. The expression of ProT was observed in rAAV/ProT-infected (A) but not mock-infected (B) NIH 3T3 cells. Original magnification, ×400. Expression of the ProT protein was detected in rAAV/ProT- and charcoal particle-injected skeletal muscles from C3H/HeN mice (C), but not in their vehicle-control counterparts (D). Original magnification, ×200. Note that ProT stained red and that charcoal particles, which were coadministered to facilitate the location of the injection sites, were present as black spots in panels C and D.

components that are necessary for the helper function have been cotransfected as a noninfectious helper plasmid into 293 cells that provide the adenovirus E1A and E1B gene products, thereby eliminating the chance of adenovirus contamination (39). Furthermore, a single helper plasmid that incorporates adenovirus helper genes and the *rep* and *cap* genes also provides an efficient packaging system without contaminating adenovirus when transfected into 293 cells (9, 17). Therefore, these helper virus-free production systems using plasmid DNAs to provide essential adenovirus functions have the notable advantage of the absence of replicating adenovirus.

HSV is able to support the replication and packaging of AAV (5). HSV-1 amplicons expressing the AAV Rep and Cap proteins along with HSV-1 helper functions have been shown to support the replication and packaging of rAAV vectors (12). The titer of an rAAV vector produced with an HSV-1 amplicon made by using an HSV-1 strain lacking ICP27, a protein that is required for HSV-1 replication, as a helper virus is equal to that achieved by supplying *rep* and *cap* by transfection followed by adenovirus superinfection. In contrast to the case when adenovirus serves as a helper virus, the replication of these HSV-1 amplicon stocks is not markedly inhibited by the expression of the Rep protein. An HSV-1 strain lacking the gene for the essential glycoprotein H (gH) has also been used to support the replication of HSV amplicons containing both an rAAV vector genome and a helper genome encoding *rep* and *cap* (40). Furthermore, an ICP27-defective HSV-1 strain expressing the Rep and Cap proteins has been constructed to serve as a single infectious helper virus to support rAAV replication and packaging (11). This recombinant HSV-1 vector has been found to be 100 times more efficient at producing rAAV than the HSV-1 amplicon system (12). Recently, Booth et al. (3) constructed HSV/AAV hybrid vectors in which all of the components necessary for rAAV production are contained within the HSV helper genome rather than also requiring a stably transfected cell line or transient transfection, thus allowing rAAV vector production from a single infection step. They demonstrated that rAAV vectors can be produced from both nonreplicating (with ICP27 deleted) and replicating HSV/AAV hybrid vectors.

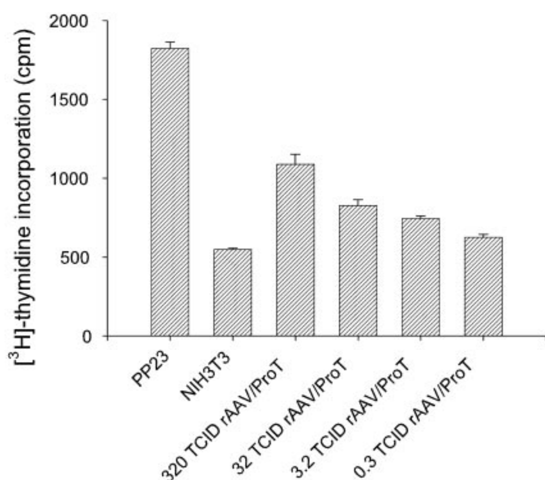

FIG. 7. Determination of the biological activity of ProT in cells infected with rAAV/ProT by a cell proliferation assay. NIH 3T3 cells were infected with different doses of rAAV/ProT for 4 days. The cells were pulsed with [^{3}H]thymidine for 18 h before being harvested, and cell proliferation was determined by measuring [^{3}H]thymidine incorporation. Data are presented as the means of six determinations ± standard errors. PP23, a ProT-overexpressing NIH 3T3 clone, served as a positive control.

Other than adenovirus and HSV-1, few studies have explored the possibility of using other helper viruses for rAAV replication and packaging. PrV, which belongs to the same subfamily as HSV, is also a fully competent helper virus for AAV replication (2). PrV has a broad host range and productively infects birds and most mammals, except for horses and higher primates, including humans, who are resistant to infection. Although PrV does not infect humans naturally, there are still concerns about using an animal virus as a helper virus to produce rAAV vectors. We therefore sought to employ a replication-defective PrV strain to construct a single helper virus to provide replication and packaging functions for rAAV vector production. Since the TK activity of herpesviruses has been associated with virulence, inactivation of the TK gene in the PrV genome attenuates the virus (14). With regard to safety concerns for gene therapy, recombinant viral vectors should not spread out to be transmitted to other uninoculated individuals. For this study, we developed a novel system for the generation of rAAV vectors by using a recombinant PrV strain carrying the AAV *rep* and *cap* genes to provide helper and packaging functions. PS virus, a gD/gE/TK triply defective PrV encoding *rep* and *cap*, is able to supply all *trans* factors and helper functions that are required for rAAV packaging. Using PrV, which is nonpathogenic for primates, as a helper virus for rAAV production may be safer than using HSV, a human pathogen. Furthermore, the replication of PS virus is not required for efficient replication and packaging of the rAAV vector. The use of noncomplementing cells, such as 293 cells, permitted the production of the rAAV vector without generating additional infectious PS virus. The helper PS virus would therefore be effectively eliminated from the rAAV vectors produced. Our data demonstrate that no detectable replication-competent PrV was generated during the production of rAAV stocks.

The large-scale production of rAAV vectors is required for preclinical and clinical studies. The application of recombinant PrV to introduce *rep* and *cap* as well as helper virus functions into cells to produce rAAV vectors has many advantages. As shown in Table 1, the use of PS virus to provide packaging and helper functions led to a significantly higher efficiency of rAAV production than that seen with a commercially available plasmid-based system. PS virus is flexible and can be utilized to produce rAAV vectors from either rAAV vector plasmid-transfected or rAAV-infected cells. Although the transfection efficiency of the calcium phosphate precipitation method varies with cell types, it usually reaches 10^{-3} to 10^{-5} (10). If a three-plasmid system were used for rAAV generation, theoretically the transfection efficiency would be estimated to be 10^{-9} to 10^{-15} for the successful transfection of three plasmids into the same cell. Since in our system only one plasmid is required for transfection into cells in conjunction with infection with the gD/gE/TK-defective PrV carrying the *rep* and *cap* genes, the transfection efficiency would be expected to be higher than that of a two- or three-plasmid system, thus resulting in a more efficient production of rAAV vectors. More importantly, rAAV vectors generated by transient transfection of an rAAV vector plasmid concurrent with PS virus infection can be further propagated in 293 cells that are coinfected with PS virus, which apparently eliminates the need for transient transfection every time when producing rAAV vectors. After three cycles of serial passages, the total amplification of rAAV vectors was $>10^7$-fold. Therefore, our PS packaging and helper system based on gD/gE/TK-defective recombinant PrV may be useful for high-titer rAAV vector production.

For large-scale use of a vector in clinical trials, the development and availability of high-throughput production, an efficient concentration, and purification of the vector as well as reliable titration methods for viral particles are essential prerequisites. The establishment of scalable systems for AAV vector production has been explored with various systems, including insect cells. Urabe et al. (36) demonstrated that an rAAV vector produced in insect cells was indistinguishable from one produced in mammalian cells in its physical and biological properties. This robust system provides a simple, cost-effective method for AAV vector production on a large scale. While progress has been achieved in the production and purification of rAAV particles, there is still room for improvement in quantitative procedures. So far, most assays are based on the capability of the virus to transduce cultured cells. After transduction, either the intracellular replication of the recombinant virus genomes or the expression of an easily detectable transgene is measured (1, 20, 29). Examples of these assays are fluorescence cell assays based on fluorescence-activated cell sorting analysis (24), fluorescence microscopy (37), and serial dilution replication assays (13, 29). Although all of these methods are of proven value for the quantitation of viral titers, they can be influenced by parameters such as the helper virus used (15), the choice of promoter and cell type used for infection (29), and the concentration of magnesium (21), leading to significant differences in the resulting rAAV titers. Other assays are independent of the infectivity of the virus. They depend on lysis of the core unit and release of the single-stranded DNA. Serial dilutions of the dot-blotted DNA can be compared to a plasmid probe, and the number of particles can then

be calculated (16). More recently, a real-time PCR method that is independent of marker gene expression was presented for the quantitation of rAAV particles. The primers are targeted for the detection of marker genes, therapeutic genes, or the promoter driving the transgenes (13, 28). For clinical applications of rAAV, the reporter genes must be removed from the vector plasmid and can therefore not be used for titration. One of the possible reasons for more clinical trials using adenovirus than those using other viruses is the feasibility of performing biological titration with recombinant adenoviral vectors. Although virus titers determined by biological titration are lower than those obtained by chemical titration, biological titers should be more reliable for estimating the effects of viral vectors in vitro and in vivo. Using the PS helper virus reported here, we demonstrated the biological titration of rAAV vectors by the $TCID_{50}$ method, which may benefit further applications of rAAV vectors in preclinical and clinical studies.

In conclusion, this is the first report to exploit a gD/gE/TK-defective PrV carrying the *rep* and *cap* genes as a single helper virus that is capable of supporting replication and packaging of an rAAV vector. The PS packaging and helper system for the production of rAAV vectors described in the present study may provide the following advantages. This system is much simpler than plasmid-based protocols because a high successful transfection rate is not required. Furthermore, rAAV vectors can be serially packaged and amplified in 293 cells after coinfection with PS virus. Notably, the biological titration of rAAV vectors is feasible with this system. Combined with recently developed purification procedures for AAV (7), PS virus is an ideal packaging and helper virus for the production of high-titer rAAV vectors, which should permit the widespread use of rAAV vectors for clinical applications.

ACKNOWLEDGMENTS

We are indebted to J. Samulski (University of North Carolina, Chapel Hill) for providing the pSub201 and pXX2 plasmids.

This work was supported by grants from the National Science Council, Taiwan (NSC90-2318-B-006-003-M51 and NSC 91-2320-B-006-085).

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