Short Technical Report

Transposase-Mediated Construction of an Integrated Adeno-Associated Virus Type 5 Helper Plasmid

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ABSTRACT

Adeno-associated viruses (AAVs) are replication-defective parvoviruses that require helper virus functions for efficient productive replication. The AAVs are currently premier candidates as vectors for human gene therapy applications. In particular, much recent interest has been expressed concerning recombinant AAV serotype 5 (rAAV-5) vectors, as they appear to utilize cellular receptors distinct from those of the prototypical AAV serotype (AAV-2) and have been reported to have transduction properties *in vivo* that differ significantly from those of the prototype. One of the most popular current methods for the production of rAAVs involves co-transfection of human 293 cells with three plasmids: (i) an adenovirus (Ad)-derived helper plasmid containing Ad genes required for AAV replication, (ii) an AAV-derived plasmid encoding complementing AAV genes (i.e., the viral *rep* and *cap* genes), and (iii) a target plasmid containing a transgene of interest flanked by AAV inverted terminal repeats (ITRs) that confer packaging and replication capabilities upon the ITR-flanked heterologous DNA. Here we describe novel plasmid reagents designed for convenient and efficient production of rAAV-5. An integrated helper plasmid containing all Ad genes required for the efficient production of recombinant AAV, as well as the complementing AAV genes on the same plasmid

*backbone, was constructed via transposase-mediated insertion into an Ad helper plasmid of a transposable element containing the AAV-5 *rep* and *cap* genes linked to a selectable marker. This simple strategy can be used in the rapid and efficient construction of integrated helper plasmids derived from any reported AAV serotype for which a molecular clone exists.*

INTRODUCTION

Adeno-associated viruses (AAVs) possess biological properties that make them ideal candidates as vectors for somatic cell gene therapy, including (i) low immunogenicity, (ii) the ability to transduce post-mitotic tissues such as muscle and brain, (iii) the ability to mediate long-term expression of vector-encoded transgenes, and (iv) the potential for site-specific integration within human chromatin, thus ameliorating concerns of insertional mutagenesis (6–8,11,12,14).

The most widely used and best characterized of the primate AAVs is AAV serotype 2 (AAV-2). Recently, however, other serotypes of AAV have been cloned and molecularly characterized. These include AAV serotypes 1, 3, 4, 5, and 6 (2,4,5,13,15,18). At least two of these AAV serotypes (AAV-4 and AAV-5) appear to utilize cellular receptors distinct from those of AAV-2. AAV-5 has been shown to bind cell surface 2,3-linked sialic acid moieties, in contrast to AAV-2, which binds heparan sulfate proteoglycans (16,17). Although the receptor for AAV-4 has not been identified, it also appears to be distinct from

that of AAV-2 (4). The ability of the newly described AAV serotypes to bind different receptors suggests that these viruses may exhibit distinct cellular tropisms and that vectors derived from the respective serotypes may be utilized to target discrete tissues *in vivo*. Indeed, differences in tissue tropism have been reported for AAV serotypes 2, 4, and 5, particularly within the central nervous system and lung (6,19). In addition to its potential to target distinct tissues *in vivo*, recombinant AAV-5 (rAAV-5) has been reported to mediate transgene expression in several-fold excess to that of equivalent rAAV-2 vectors (3,7,11,19). This and other factors have led to much interest concerning the use of rAAV-5 for gene therapy studies. Although the reagents available for the production of rAAV-2 are varied and widely disseminated, reagents for the production of rAAV-5 are significantly fewer in number because of the recent characterization of this virus. Here we describe plasmid reagents useful in the production of rAAV-5, as well as a convenient strategy for the generation of integrated AAV helper plasmids via transposase-mediated insertion of complementing AAV *rep* and *cap* genes within an adenovirus (Ad) helper plasmid backbone.

Standard techniques for the construction of large recombinant DNA plasmids, such as direct ligation or homologous recombination in bacteria, suffer from several disadvantages. Direct ligation of large DNA fragments is an inefficient process; in fact, several attempts in our laboratory to directly clone AAV-5 *rep* and *cap* sequences into an Ad helper plasmid backbone

Table 1. rAAV-5 Titer

	Particles/mL ^a	Transducing Units/mL ^{a,b}	Total Particle Yield	Particles/Cell
Experiment 1	2.5×10^{12}	1.9×10^9	4.1×10^{12}	3.2×10^4
Experiment 2	3.5×10^{12}	3.1×10^9	4.4×10^{12}	3.4×10^4

^aPeak fraction
^bCOS-1 cells were seeded at 2×10^5 cells/well in 12-well cluster plates and transduced with serially diluted rAAV-5 the following day. GFP expression was monitored by fluorescence microscopy approximately 60 h after transduction. Each GFP-positive cell was defined as one transducing unit.

were unsuccessful. Also, the paucity of unique restriction sites within most large DNA fragments limits the versatility with which these molecules may be recombined. Although homologous recombination in bacteria overcomes many of the pitfalls of traditional direct ligation techniques, this method re-

quires regions of significant homology between the donor and recipient DNAs, as well as suitable strains of *E. coli*. Here we describe an efficient transposase-mediated recombination strategy for the construction and selection of an integrated AAV-5 helper plasmid. This strategy uses commonly available

Figure 1. Strategy for the transposase-mediated construction of an integrated AAV helper plasmid. A synthetic transposon bearing the AAV-5 *rep* and *cap* genes linked to a bacterial kanamycin resistance gene and flanked by high-efficiency transposase recognition sequences (known as "mosaic ends", ME) was PCR-amplified from a plasmid clone and randomly inserted into an Ad-derived helper plasmid via an in vitro reaction using purified transposase. Integrants were isolated by electroporation into recipient bacteria, followed by selection on solid growth medium containing kanamycin. Individual colonies were transferred to kanamycin-containing liquid medium in a 96-well array and replica plated into liquid medium containing ampicillin. Filled circles represent bacterial growth. Bacteria containing a recombinant plasmid in which the synthetic transposon has integrated into the ampicillin resistance gene of the Ad-derived helper plasmid are identified by a kanamycin-resistant, ampicillin-sensitive phenotype.

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laboratory strains of *E. coli* and is independent of DNA restriction sites within the donor and recipient molecules. The strategy described here can be used to construct an integrated AAV helper plasmid derived from any serotype for which a molecular clone exists.

MATERIALS AND METHODS

Plasmid Construction

The adenovirus serotype 5 (Ad5)-derived helper plasmid, pSR449B, was constructed as follows. A 3.6-kb *PmeI-MfeI* fragment containing the Ad5 VA gene was excised from plasmid pAdEasy™-1 (Quantum Biotechnologies, Montreal, Canada) and cloned between the compatible *EcoRI-SmaI* sites of pBlueScript® II SK (Stratagene, La

Jolla, CA, USA), thus generating the intermediate construct pSR448. Next, an 11.7-kb *BamHI* fragment containing the E4 and E2a transcription units of Ad5 was isolated from pAdEasy-1 and cloned into the unique *BamHI* site of pSR448, yielding the Ad helper plasmid pSR449B.

To generate an integrated AAV helper plasmid containing all Ad genes necessary for productive AAV replication on the same plasmid backbone as the complementing AAV-5 *rep* and *cap* genes, a three-step approach was used. First, a synthetic transposon consisting of a kanamycin-resistance gene and multiple cloning site flanked by high-efficiency Tn5 transposase recognition sequences (EZ::TN™ <KAN-2> Transposon; Epicentre Technologies, Madison, WI, USA) was PCR-amplified using the primer 5'-GGCAGCTGTC-

TCTTATACACATC-3' (*PvuII* site underlined), which is specific for the Tn5 recognition sequences at each end of the transposon. The PCR product was digested with *PvuII* and cloned between the *PvuII* sites of pMOD™-2<MCS> (Epicentre Technologies), yielding plasmid pSR481. Next, the AAV-5 *rep* and *cap* genes, along with a flanking simian virus 40 (SV40) *ori*/enhancer region, were PCR-amplified from plasmid p5RepCapB (5) using the forward and reverse primers 5'-GCCCTAGAGCGCGTATGAGTTCTCGC-G-3' and 5'-AATTAACCCTCACTAAAGGG-3', respectively. The resulting amplified DNA was digested with *XbaI* (underlined in forward primer) and *HindIII* (internal to the PCR product) and cloned into the *XbaI-HindIII* sites of pSR481 (to generate plasmid pSR483), thus linking AAV-5 *rep* and *cap* sequences to the kanamycin-resistance gene of the transposable element cloned within pSR481. The *kan^r-rep-cap* transposon was PCR-amplified from pSR483 using plasmid backbone-specific primers flanking the multigene cassette. The resulting PCR product was reacted with EZ::TN Transposase (Epicentre Technologies) in the presence of an equimolar amount of the target plasmid (pSR449B), according to the manufacturer's suggested protocol. The transposase reaction was terminated by the addition of SDS (0.1% final concentration), and a portion of the reaction was used to transform electrocompetent *E. coli* strain DH10B™ (Invitrogen, Carlsbad, CA, USA). Electrotransformed bacteria were spread onto LB-agar plates containing 50 µg/mL kanamycin. After overnight incubation at 37°C, kanamycin-resistant colonies were individually transferred to the wells of a 96-well flat-bottom tissue culture dish containing 200 µL/well LB medium supplemented with 50 µg/mL kanamycin. Following overnight incubation, the 96-well plate was replica plated into a second 96-well plate containing LB medium with 50 µg/mL ampicillin and incubated for approximately 16 h. Kanamycin-resistant, ampicillin-sensitive clones were expanded and screened by restriction digest for insertion of the *kan^r-rep-cap* cassette.

A general-purpose AAV-5 vector plasmid bearing a Rous sarcoma virus

Figure 2. Novel reagents for the production of AAV-5. (A) Plasmid pSR449B contains the Ad5 VA, E2a, and E4 transcription units in a high copy number plasmid backbone. When transfected into an E1a-expressing cell line, such as human 293 cells, the cloned Ad genes support productive AAV replication. (B) Plasmid pSR487 is an integrated AAV helper plasmid derived from pSR449B and encodes the AAV-5 *rep* and *cap* genes on the same plasmid backbone as the Ad helper functions. (C) Plasmid pAAV5-7D05 contains an RSV promoter immediately upstream of a multiple cloning site and flanked by AAV-5 inverted terminal repeat sequences (ITRs). The ITRs are the sole *cis*-acting sequences required for the excision, replication, and packaging of AAV-5 DNA. (D) Plasmid pSR485 is derived from pAAV5-7D05 and contains an AAV-5 ITR-flanked GFP gene under the control of a human CMV immediate-early promoter and an SV40 promoter-driven neomycin resistance gene.

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(RSV) promoter immediately upstream of a multiple cloning site was constructed in a two-step process. First, the AAV-5 ITR element of pAAV5LacZ (5) was isolated as a 275-bp *KpnI*-*BglIII* fragment and cloned between the *BamHI*-*KpnI* sites of pSP72 (Promega, Madison, WI, USA) to generate pAAV5-ITR. Next, the cloned AAV-5 ITR element along with additional polylinker sequences was excised from pAAV5-ITR as a 280-bp *BglIII*-*XhoI* fragment and ligated to the 3.25-kb *BamHI*-*XhoI* fragment of pAAV5LacZ to generate the final product, pAAV5-7D05. An AAV-5 vector encoding the GFP and aminoglycoside phosphotransferase (*neo^r*) genes was cloned by PCR amplification of the cytomegalovirus (CMV) promoter-driven GFP gene and SV40 promoter-driven *neo* gene from plasmid pQBI-25 (Quantum Biotechnologies) with primers 5'-GGGAGAT-CTCCCGATCCCCTATGGTCGAC-3'

and 5'-GCCAAGCTTGTGAAAAAATGCTTTATTTG-3'. The amplified PCR product was digested with *BglIII* and *HindIII* (underlined within their respective primers) and ligated between the *BglIII*-*HindIII* sites of pAAV5-7D05, thus replacing the RSV promoter with the GFP/*neo* expression cassette.

Cell Culture and Vector Production

COS-1 cells (SV40-transformed African green monkey kidney cells) and 293T cells (Ad5-transformed human embryonic kidney cells expressing SV40 large T antigen) were maintained in DMEM containing 25 mM glucose, 100 µg/mL streptomycin, 100 U/mL penicillin, and 10% FBS. For vector production, 293T cells were seeded at a density of 7×10^6 cells/15-cm plate the day before transfection. Two experimental preparations of 10 15-cm plates each were produced. Transfection was by cal-

cium-phosphate precipitation using 40 µg integrated helper plasmid (pSR487) and 15 µg AAV-5 GFP construct (pSR485) per 15-cm plate. Approximately 48 h after transfection, the cells were harvested by gentle scraping, rinsed with PBS, and suspended in TD buffer (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.7 mM K_2HPO_4 , and 2 mM $MgCl_2$). Cells were lysed by three cycles of freezing and thawing followed by centrifugation to remove cellular debris. The cell lysate was adjusted to 0.5% sodium deoxycholate and 300 U/mL Benzonase (a recombinant endonuclease; Sigma, St. Louis, MO, USA), and then incubated for 1 h at 37°C. DNase-resistant AAV-5 particles were purified by isopycnic banding in a CsCl density gradient. The purified vector was extensively dialyzed against PBS-2 mM $MgCl_2$ and titered by real-time PCR analysis using the CMV promoter-specific primers 5'-TGGTGATGCGGTTT-TGGCAG-3' and 5'-AATGGGGCGGAGTTGTTACGAC-3'. Vector aliquots were stored at -70°C.

RESULTS AND DISCUSSION

The efficient manipulation and/or cloning of the large DNA fragments necessary for the production of most recombinant DNA viruses can be problematic, often requiring alternatives to standard cloning techniques. One such alternative used in the construction of adenovirus vectors and more recently in the construction of an integrated AAV-2 helper plasmid is homologous recombination in bacteria (9,10). This approach requires significant regions of homology between the donor and recipient DNA molecules, as well as the use of a *recA⁺* strain of *E. coli* (typically strain BJ5183). Another approach to generate large, plasmid-based recombinant molecules is transposase-mediated recombination. An advantage to this approach is that it does not require DNA homology for recombination and is relatively independent of the size of the donor or recipient DNA molecules. Also, it can be performed in vitro in a brief enzyme-catalyzed reaction using purified transposase. However, a potential disadvantage to certain applications of transposition is the random nature of the insertion event.

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In our desire to generate an integrated AAV helper plasmid for the production of recombinant AAV-5, we sought to utilize the ease and efficiency of in vitro transposition, while at the same time requiring targeted insertion of the transposable element. To this end, we have devised a selection strategy that allows random insertion of a gene (or genes) of interest into a target vector, followed by bacterial transformation and antibiotic-mediated selection of plasmid integrants containing insertions within a defined region of the target plasmid (Figure 1). In this strategy, an artificial transposable element containing the AAV-5 *rep* and *cap* genes linked to a bacterial kanamycin-resistance cassette and flanked by Tn5 transposase recognition sequences was integrated into an Ad helper plasmid, pSR449B (Figure 2A), via a transposase-mediated in vitro reaction. Since the Ad genes within pSR449B are essential for AAV

production, and therefore could not be disrupted, we identified recombinant plasmid molecules in which the insertion event occurred within the Ad helper plasmid-encoded ampicillin resistance gene (*Amp^r*). Bacterial cells were electroporated with a portion of the traspo-

sition reaction and then selected on LB-agar plates containing kanamycin to recover target plasmids that underwent an insertion event. Kanamycin-resistant colonies were then transferred to a 96-well array containing LB medium supplemented with kanamycin (LB-kana-

Figure 3. AAV-5-mediated GFP expression in transduced COS-1 cells. COS-1 cells were transduced with recombinant AAV-5 produced by co-transfection of 293T cells with the integrated AAV helper plasmid pSR487 and the AAV vector plasmid pSR485. (A) COS-1 cells transduced with approximately 100 transducing units/cell. (B) Control cells that did not receive viral vector.

mycin) and subsequently replica plated into LB-ampicillin medium. Bacteria containing target plasmids in which the transposition event disrupts the Amp^r gene present a kanamycin-resistant, ampicillin-sensitive phenotype. Individual kanamycin-resistant, ampicillin-sensitive clones were amplified and analyzed by restriction endonuclease digestion of isolated plasmid DNA to determine the approximate location of the insertion event. One clone was chosen for further analysis, and the exact integration site within this clone was determined by DNA sequencing. This selection strategy facilitated the rapid and efficient construction of a novel AAV-5 helper plasmid, pSR487 (Figure 2B), in which the Ad helper functions and complementing AAV-5 genes are on the same plasmid backbone.

To assess the ability of the integrated AAV-5 helper plasmid to direct the rescue, replication, and packaging of an AAV-5 ITR-flanked transgene, we first generated a novel AAV-5 GFP vector by replacing the RSV promoter element of plasmid pAAV5-7D05 (Figure 2C) with a GFP/neomycin-resistance cassette, thus yielding pSR485 (Figure 2D). Plasmid pSR485 was co-transfected with pSR487 into recipient human 293T cells. Transfected cell lysates were treated with DNase to remove contaminating vector plasmid DNA, and nuclease-resistant rAAV virions were purified by CsCl density gradient centrifugation. DNase-resistant vector stocks were characterized by real-time PCR analysis of vector genome content, as well as by the ability to transduce a GFP transgene into recipient COS-1 cells (Figure 3). Co-transfection of 10 15-cm dishes containing approximately 1.3×10^7 293T cells/dish at the time of transfection yielded approximately 4×10^{12} total DNase-resistant vector particles in parallel experiments (Table 1). This corresponds to the generation of greater than 10^4 vector particles/cell. The titer of the peak CsCl density gradient fraction exceeded 2×10^{12} particles/mL in each vector preparation. The transducing unit titer was determined by inoculating COS-1 cells with serial dilutions of the rAAV-5 GFP vector and enumerating GFP-positive cells. A transducing unit titer of $2\text{--}3 \times 10^9$ transducing units/mL was observed.

This number is in agreement with the approximately 1000:1 particle-to-transducing unit ratio commonly reported for AAV vectors titrated in the absence of helper virus functions (1,5).

In conclusion, we have used a transposase-mediated recombination strategy to generate rapidly and efficiently an integrated AAV-5 helper plasmid. This plasmid facilitates the production of high-titer recombinant AAV-5 particles in a simple two-plasmid transfection protocol. In addition, we present rAAV-5 plasmid reagents useful for the construction of rAAV-5 vectors bearing other reporter and/or therapeutic transgenes.

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