Efficient Transgene Reconstitution with Hybrid Dual AAV Vectors Carrying the Minimized Bridging Sequences

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Abstract

A hybrid dual-vector system was developed recently as a universal platform to double the packaging capacity of recombinant adeno-associated virus (AAV). In this system, the expression cassette is split into two independent AAV vectors. A highly recombinogenic bridging DNA sequence is engineered in both vectors to mediate target gene-independent homologous recombination between the split vector genomes. In the prototype hybrid vectors, a 0.87-kb DNA fragment from the middle portion of the human placental alkaline phosphatase (AP) gene was used as the bridging sequence. Here we report the development of the minimized bridging sequences. Five independent bridging sequences (0.26 to 0.44 kb) were evaluated in MO59K cells and/or murine skeletal muscle in the context of the AP overlapping vectors and/or the β -galactosidase (LacZ) hybrid vectors. Robust reconstitution comparable to that of the original hybrid vectors was achieved from a 0.26-kb and a 0.27-kb bridging sequence. These newly developed bridging sequences greatly expand the utility of the hybrid dual AAV vector system for delivering larger therapeutic genes/expression cassettes.

Introduction

ADENO-ASSOCIATED VIRUS (AAV) is a single-stranded DNA virus. In a mature AAV virion, an ~ 4.7 -kb genome is encapsidated in an ~ 20 -nm icosahedral particle. The wild-type AAV genome is composed of a rep gene, a cap gene, and two flanking inverted terminal repeats (ITRs). A recombinant AAV vector is generated by replacing the wild-type AAV genes with a target gene expression cassette. During viral packaging, the viral genome is pumped into a preassembled empty capsid. The maximal size of the viral genome that can fill into a virion is dictated by the internal space in the empty particle (Dong *et al.*, 1996). The vector genome larger than 5 kb is rarely packaged (Grieger and Samulski, 2005; Dong *et al.*, 2010; Lai *et al.*, 2010b; Wu *et al.*, 2010). The small packaging capacity has significantly limited the application of AAV gene therapy to diseases that require a larger therapeutic expression cassette. To overcome this hurdle, a series of dual-vector strategies have been developed to double AAV packaging capacity (Ghosh and Duan, 2007; Lai *et al.*, 2010a). These include the cis-activation, trans-splicing, overlapping, and hybrid strategies. They are based on intergenome AAV ITR recombination or homologous recombination of the transgene sequence. The recently developed hybrid system integrates these recombination

pathways and offers the maximal flexibility and the highest efficiency (Ghosh *et al.*, 2008).

In the hybrid dual AAV vector system, one virion (the 5' vector) carries the head part of the expression cassette including the promoter, the 5'-end of the target gene, a splicing donor signal, and the bridging DNA sequence. The other virion (the 3' vector) carries the tail part of the expression cassette including the same bridging DNA sequence, a splicing acceptor signal, the 3'-end of the target gene, and the polyA signal. The reconstitution can be accomplished by ITR-mediated head-to-tail vector genome concatamerization and/or bridging DNA sequence-mediated homologous recombination. In the prototype hybrid vector system, a 0.87-kb DNA fragment from the middle portion of the human placental alkaline phosphatase (AP) gene was used as the bridging DNA sequence (Ghosh *et al.*, 2008). To further improve the hybrid dual-vector system, we split the original 0.87-kb bridging sequence into two one-half fragments and three one-third fragments. The reconstitution efficiency was tested in the AP overlapping vectors and the β -galactosidase (LacZ) hybrid vectors in mouse muscle. Two minimized bridging sequences (0.26 kb and 0.27 kb) were found to mediate robust reconstitution in cell culture *in vitro* and in murine muscle *in vivo*.

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Materials and Methods

Recombinant AAV vector production

The cis-plasmids used for AAV packaging are detailed in Table 1 (Duan *et al.*, 2000; Ghosh *et al.*, 2006, 2008). The plasmids that are designated as “this study” were generated using standard molecular cloning techniques. Details are

available upon request. The DNA fragments that were generated by PCR amplification were confirmed by restriction analysis and DNA sequencing. AP gene expression was driven by the Rous sarcoma virus promoter (RSV). LacZ gene expression was driven by the cytomegalovirus promoter (CMV). The AAV-6 packaging plasmids (pMT-Rep2 and pCMVCap6) were gifts from Dr. Dusty Miller at the Fred

TABLE 1. THE CIS-PLASMIDS USED FOR AAV PRODUCTION

Abbreviation	Full name	Description	Reference
<i>Full-length vector</i>			
DD20	pcis.RSV.AP	A single intact full-length AP vector. This is the parental plasmid for all the overlapping vectors.	Duan <i>et al.</i> , 2000
<i>Overlapping vectors</i>			
AG3	pcisAPUpstream	The upstream vector of the original AP overlapping vector set. There is a 0.87-kb overlap between the upstream and downstream vectors.	Ghosh <i>et al.</i> , 2006
AG4	pcisAPDownstream	The downstream vector of the original AP overlapping vector set. There is a 0.87-kb overlap between the upstream and downstream vectors.	Ghosh <i>et al.</i> , 2006
AG36	pcis1/2Head.APUstream	The new upstream vector for the AP overlapping vectors that share a 0.44-kb overlap between the upstream and downstream vectors.	This study
AG37	pcis1/2Tail.APDownstream	The new downstream vector for the AP overlapping vectors that share a 0.43-kb overlap between the upstream and downstream vectors.	This study
AG71	pcis1/3Head.APUstream	The new upstream vector for the AP overlapping vectors that share a 0.27-kb overlap between the upstream and downstream vectors.	This study
AG72	pcis1/3Body.APUstream	The new upstream vector for the AP overlapping vectors that share a 0.34-kb overlap between the upstream and downstream vectors.	This study
AG73	pcis1/3Body.APDownstream	The new downstream vector for the AP overlapping vectors that share a 0.34-kb overlap between the upstream and downstream vectors.	This study
AG74	pcis1/3Tail.APDownstream	The new downstream vector for the AP overlapping vectors that share a 0.26-kb overlap between the upstream and downstream vectors.	This study
<i>Hybrid vectors</i>			
AG27	pcis.CMV.LacZ.Hybrid5'	The 5' vector of the prototype hybrid vectors that use the 0.87-kb AP fragment as the bridging sequence.	Ghosh <i>et al.</i> , 2008
AG28	pcis.CMV.LacZ.Hybrid3'	The 3' vector of the prototype hybrid vectors that use the 0.87-kb AP fragment as the bridging sequence.	Ghosh <i>et al.</i> , 2008
AG46	pcis1/2Head.LacZ.Hybrid5'	The 5' vector of the prototype hybrid vectors that use the 0.44-kb AP fragment as the bridging sequence.	This study
AG47	pcis1/2Head.LacZ.Hybrid3'	The 3' vector of the prototype hybrid vectors that use the 0.44-kb AP fragment as the bridging sequence.	This study
AG48	pcis1/2Tail.LacZ.Hybrid5'	The 5' vector of the prototype hybrid vectors that use the 0.43-kb AP fragment as the bridging sequence.	This study
AG49	pcis1/2Tail.LacZ.Hybrid3'	The 3' vector of the prototype hybrid vectors that use the 0.43-kb AP fragment as the bridging sequence.	This study
AG50	pcis1/3Head.LacZ.Hybrid5'	The 5' vector of the prototype hybrid vectors that use the 0.27-kb AP fragment as the bridging sequence.	This study
AG51	pcis1/3Head.LacZ.Hybrid3'	The 3' vector of the prototype hybrid vectors that use the 0.27-kb AP fragment as the bridging sequence.	This study
AG52	pcis1/3Body.LacZ.Hybrid5'	The 5' vector of the prototype hybrid vectors that use the 0.34-kb AP fragment as the bridging sequence.	This study
AG53	pcis1/3Body.LacZ.Hybrid3'	The 3' vector of the prototype hybrid vectors that use the 0.34-kb AP fragment as the bridging sequence.	This study
AG54	pcis1/3Tail.LacZ.Hybrid5'	The 5' vector of the prototype hybrid vectors that use the 0.26-kb AP fragment as the bridging sequence.	This study
AG55	pcis1/3Tail.LacZ.Hybrid3'	The 3' vector of the prototype hybrid vectors that use the 0.26-kb AP fragment as the bridging sequence.	This study

Hutchinson Cancer Research Center (Seattle, WA) (Allen *et al.*, 2000). Recombinant AAV-6 vectors were generated by a transient plasmid transfection protocol described before (Ghosh *et al.*, 2006). Viral stocks were purified through two rounds of isopycnic CsCl ultracentrifugation followed by extensive dialysis in HEPES buffer at 4°C. Viral titer and quality control were performed according to our previously published protocol (Bostick *et al.*, 2007; Ghosh *et al.*, 2007; Yue *et al.*, 2008).

In vitro studies

MO59K cells were purchased from the American Type Culture Collection (Manassas, VA). These cells were derived from human glioma cells (Allalunis-Turner *et al.*, 1993). MO59K cells were grown in F12/Dulbecco's modified Eagle's medium (1:1). The culture was supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were propagated in a 5% CO₂ atmosphere at 37°C. Prior to dual-vector coinfection experiments, single-vector infection was performed in 70% confluent MO59K cells at a multiplicity of infection (MOI) of 500 vg particles/cell (for the overlapping vectors) or 10,000 vg particles/cell (for the hybrid vectors). Transgene expression was not detected from individual vectors carrying the fragmented expression cassettes. To evaluate the transduction efficiency of the various AP overlapping vectors, 70% confluent MO59K cells were coinfecting with both the upstream and downstream vectors at an MOI of 500 vg particles/vector/cell. To evaluate the transduction efficiency of the various LacZ hybrid vectors, 70% confluent MO59K cells were coinfecting with both the 5' and the 3' vectors at an MOI of 10,000 vg particles/vector/cell. Transduction efficiency was determined at 48 hr by cytochemical staining (for both AP overlapping vectors and LacZ hybrid vectors) and β-galactosidase assay (for LacZ hybrid vector coinfection), as we described before (Ghosh *et al.*, 2008).

In vivo evaluation of the LacZ hybrid vectors in murine muscle

All animal experiments were approved by the Animal Care and Use Committee at the University of Missouri and were in accordance with NIH guidelines. Seven-week-old male C57Bl/10 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in specific-pathogen-free animal care facilities and kept under a 12-hr light (25 lux)/12-hr dark cycle with free access to food and water.

Recombinant AAV-6 LacZ hybrid vectors were delivered to the tibialis anterior (TA) muscle as we described before (Ghosh *et al.*, 2006, 2008). Each coinfection consisted of 1×10^{10} vg particles/vector/muscle. LacZ expression was analyzed 6 weeks later by histochemical staining and β-galactosidase assay (Applied Biosystems, Bedford, MA) using published protocols (Ghosh *et al.*, 2006, 2008).

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed with the SPSS software (SPSS, Chicago, IL). Statistical significance was determined by one-way ANOVA followed by Bonferroni *post hoc* analysis. Difference was considered significant when $p < 0.05$.

Results

Splitting the 0.87-kb prototype bridging sequence in half differentially affects the transduction efficiency of the overlapping and hybrid AAV vectors

To minimize the size of the bridging sequence, we first split the 0.87-kb prototype bridging sequence into two fragments and tested their recombination efficiency in the context of the AP overlapping vectors. The 5' half (0.44 kb)

FIG. 1. *In vitro* analysis of the AAV-6 AP overlapping vectors and the AAV-6 LacZ hybrid vectors that are based on the 0.43- and 0.44-kb bridging sequences. **(A)** Left panel: Schematic outline of the overlapping regions in the original and two new AP overlapping vectors. In the original AP overlapping vectors, the upstream and downstream vectors shared a 0.87-kb fragment (the middle part of the AP gene). In the 1/2 head and 1/2 tail overlapping vectors, the upstream and downstream vectors shared a 0.44-kb (the head half of the 0.87-kb fragment) and a 0.43-kb (the tail half of the 0.87-kb fragment), respectively. Right panel: Quantification of AP-positive cells after co-infection (500 vg particles/vector/cell). $n = 5$ per group. **(B)** Left panel: Schematic outline of the LacZ hybrid vectors that used the 0.87-kb (original LacZ hybrid vectors), 0.44-kb (1/2 head LacZ hybrid vectors), and 0.43-kb (1/2 tail LacZ hybrid vectors) regions of the AP gene as the bridging sequences. Right panel: Quantification of β-galactosidase activities in coinfecting cells (10,000 vg particles/vector/cell). *Significantly higher than other groups. $n = 3$ for the original LacZ hybrid vectors, $n = 6$ per group for the 1/2 head and 1/2 tail LacZ hybrid vectors.

was termed the 1/2 head fragment, and the 3' half (0.43 kb) was termed the 1/2 tail fragment. Two sets of the overlapping AP vectors were generated. In one set, the upstream and the downstream vectors shared the 0.44-kb 1/2 head fragment (Fig. 1A, Table 1). This set was called the 1/2 head overlapping vectors. The other set shared the 0.43-kb 1/2 tail fragment and was called the 1/2 tail overlapping vectors (Fig. 1A, Table 1). Despite an ~50% reduction of the size of the overlapping region, the newly generated overlapping vectors yielded a transduction efficiency similar to that of the original overlapping vectors in MO59K cells (Fig. 1A).

The LacZ hybrid vectors were then generated using either the 0.44-kb 1/2 head fragment or the 0.43-kb 1/2 tail fragment as the bridging sequences (Fig. 1B, Table 1). Surprisingly, the transduction efficiency was significantly reduced in these hybrid vectors. It only reached about half of that of the original LacZ hybrid vectors in MO59K cells (Fig. 1B).

Identification of two <0.3-kb, highly recombinogenic regions in the 0.87-kb prototype bridging sequence

We also split the 0.87-kb prototype bridging sequence into three fragments. They represented the 5'-end (1/3 head, 0.27

kb), the middle (1/3 body, 0.34 kb), and the 3'-end (1/3 tail, 0.26 kb) of the prototype bridging sequence (Figs. 2 and 3, Table 1). Transduction efficiency of the overlapping vectors based on the one-third split was evaluated in MO59K cells (Fig. 2). Whereas the 1/3 head and 1/3 tail AP overlapping vectors yielded expression comparable to that of the original AP overlapping vectors, the overlapping vectors that shared the 0.34-kb 1/3 body fragment resulted in poor expression (Fig. 2B).

Three sets of the LacZ hybrid vectors were engineered using the 0.27-kb 1/3 head, 0.34-kb 1/3 body, and 0.26-kb 1/3 tail fragments (Fig. 3A, Table 1). Coinfection was performed in MO59K cells. Minimal expression was observed

FIG. 2. Evaluation of the AAV-6 AP overlapping vectors based on the 0.26-, 0.27-, and 0.34-kb overlapping sequences in MO59K cells. (A) Schematic outline of the overlapping regions in the original and new overlapping vectors. (B) Quantification of AP-positive cells after coinfection (500 vg particles/vector/cell). *Significantly lower than other groups. $n = 5$ per group.

FIG. 3. Evaluation of the AAV-6 LacZ hybrid vectors based on the 0.26-, 0.27-, and 0.34-kb bridging sequences in MO59K cells. (A) Schematic outline of the LacZ hybrid vectors that used the 0.87-kb (original LacZ hybrid vectors), 0.26-kb (1/3 head LacZ hybrid vectors), 0.34-kb (1/3 body LacZ hybrid vectors), and 0.26-kb (1/3 tail LacZ hybrid vectors) regions of the AP gene as the bridging sequences. (B) Quantification of β -galactosidase activities in coinfecting cells (10,000 vg particles/vector/cell). *Significantly lower than other groups. $n = 3$ for the original LacZ hybrid vectors, $n = 6$ per group for the other LacZ hybrid vectors.

from the set that used the 0.34-kb 1/3 body fragment as the bridging sequence. However, the transgene expression levels of the other two sets reached that of the original hybrid vectors (Fig. 3B).

The 0.27-kb and the 0.26-kb bridging sequences mediate robust hybrid vector reconstitution in mouse muscle in vivo

To validate the utility of the newly generated LacZ hybrid vectors, we compared their transduction efficiency with that of the original LacZ hybrid vectors in the TA muscle in C57Bl/10 mice. Coinfection was performed with 1×10^{10} vg particles/vector/muscle. At 6 weeks after coinfection, we examined LacZ expression by histochemical staining in cryomuscle sections and β -galactosidase assay in whole-muscle lysates (Fig. 4). Strong LacZ staining was observed in muscles that were coinfecting with the original hybrid vectors

(Fig. 4A) (Ghosh *et al.*, 2008). The hybrid vectors that built on the 0.27-kb 1/3 head and the 0.26-kb 1/3 tail fragments also resulted in intense staining. Consistent with the results of MO59K cells, we only detected faint LacZ staining from the hybrid vectors carrying the 0.34-kb bridging sequence. Quantification of LacZ-positive cells and β -galactosidase activities confirmed the observation (Fig. 4B).

Discussion

A dual-vector system significantly broadens AAV gene therapy applications. Several dual-vector approaches have been developed by manipulating the vector genome. Among these, the trans-splicing and the overlapping vectors have been shown to lead to expression comparable to that of a single AAV for certain genes, such as the 6-kb mini-dystrophin gene (via the trans-splicing approach) and the AP gene (via the overlapping approach) (Halbert *et al.*, 2002; Lai

FIG. 4. *In vivo* performance of the AAV-6 LacZ hybrid vectors based on the minimized bridging sequences. (A) Representative LacZ histochemical staining of the TA muscle coinfecting with different sets of the LacZ hybrid vectors (1×10^{10} vg particles/vector/muscle). Scale bar applies to all images. (B) Quantification of the transduction efficiency in muscle section (percentage of LacZ-positive cells; left panel) and muscle lysate (β -galactosidase activity; right panel). *Significantly lower than other groups. $n = 4$ per group. Color images available online at www.liebertonline.com/hum.

et al., 2005; Ghosh *et al.*, 2006, 2007). However, the success of these strategies is highly dependent on the molecular property of the target gene. To overcome this hurdle, we recently developed a hybrid dual-vector system (Ghosh *et al.*, 2008).

The hybrid dual vectors are capable of reconstituting the split vector genome fragments in a transgene-independent manner. This is achieved via a highly recombinogenic bridging sequence. In the prototype hybrid vectors, the bridging sequence is a 0.87-kb fragment from the middle region of the AP gene. In a proof-of-principle study, the hybrid vectors based on this 0.87-kb bridging sequence showed superior transduction efficiency over the traditional trans-splicing and overlapping vectors (Ghosh *et al.*, 2008). Nevertheless, a wide application of the hybrid vector system has been limited. First, the size of the bridging sequence remains too big for certain transgenes. Considering the 5-kb packaging limit of a single AAV virion, dual vector is expected to double the packaging capacity to 10 kb. The inclusion of two 0.87-kb bridging sequences (one for each vector) essentially reduces the available space for the expression cassette to ~8.2 kb. Second, for transgenes that are larger than 10 kb (such as the full-length dystrophin coding sequence), one may have to use a tri-vector strategy for delivery. In this case, two different bridging sequences will be needed to mediate ordered reconstitution among three vectors.

To address these issues, we further dissected the 0.87-kb prototype bridging sequence. We hypothesized that the high recombinogenic property of this 0.87-kb sequence could be narrowed down to a smaller region. We split the 0.87-kb sequence to either two (0.43 and 0.44 kb) or three (0.26, 0.27, and 0.34 kb) smaller fragments. We first tested whether these fragments could mediate efficient homologous recombination in the context of the AP overlapping vectors in cultured cells. Except for the 0.34-kb fragment, the other four fragments appeared quite competent, and they all yielded AP expression comparable to that of the original AP overlapping vectors (Figs. 1 and 2). We then engineered these fragments into the LacZ hybrid vectors. As expected, the set based on the 0.34-kb fragment resulted in minimal expression (Fig. 3B). Interestingly, the sets based on the 0.43-kb and 0.44-kb fragments showed reduced expression compared with that of the prototype LacZ hybrid vectors (Fig. 1B). Nevertheless, robust reconstitution was achieved in the sets based on the 0.26-kb and 0.27-kb fragments (Fig. 3B). To validate the *in vitro* results, we further examined the transduction efficiency of the three LacZ hybrid vectors (two best and one poorest) in mouse muscle. Consistent with the cell line data, the hybrid vectors built on the 0.26-kb and 0.27-kb bridging sequences were at least as efficient as that of the original LacZ hybrid vectors (Fig. 4). As a matter of fact, the LacZ staining appeared slightly stronger and β -galactosidase activity appeared higher, although it did not reach statistical significance.

The reduced reconstitution of the hybrid vectors based on the 0.44-kb and 0.43-kb bridging sequences, as well as the poor reconstitution of the vectors (overlapping and hybrid) carrying the 0.34-kb 1/3 body fragment, is intriguing. Initial analysis did not yield any notable features (such as the GC content) or sequence motifs that may explain the inhibitory effect. Future studies are needed to determine the underlying mechanism(s).

In summary, we have identified two minimized yet highly recombinogenic bridging sequences. We also demonstrated that these bridging sequences are suitable for *in vivo* application in the hybrid dual AAV vectors. These newly developed bridging sequences greatly expand the utility of the hybrid dual AAV vector system for delivering larger therapeutic genes/expression cassettes.

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Author Disclosure Statement

No competing financial interests exist.

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