Short Technical Report

Development of Multiple Cloning Site *cis*-Vectors for Recombinant Adeno-Associated Virus Production

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ABSTRACT

Recombinant adeno-associated virus (rAAV) has become a very popular gene therapy vector in the past several years. A *cis*-plasmid is used to generate the rAAV stocks. In this plasmid, the entire expression cassette is incorporated between two AAV inverted terminal repeats. The construction of *cis*-plasmid has been problematic because of the high-frequency recombination of the viral inverted terminal repeats. Here we describe the design and construction of several multiple cloning site *cis*-plasmids that are driven by five different promoters, including the ubiquitous cytomegalovirus enhancer/chicken β -actin (CAG), cytomegalovirus (CMV), rous sarcoma virus (RSV), simian virus 40 (SV40), and a muscle-specific promoter (CK6). The application of these multiple cloning site *cis*-plasmids improves the cloning efficiency. As an example of the utilization of these multiple cloning site vectors, the prokaryotic β -galactosidase cDNA was cloned in the multiple cloning site *cis*-plasmids. High-level rAAV-mediated β -galactosidase expression was achieved in HeLa cells from CAG, CMV, RSV, and SV40 promoters, respectively, but not from the CK6 promoter. In vivo application in the adult mdx mouse (mouse

model for Duchenne muscular dystrophy) muscle revealed efficient transgene expression from CMV and CK6 promoters, followed by CAG and RSV promoters. The SV40 promoter was the least efficient.

INTRODUCTION

Adeno-associated virus (AAV) is a single-stranded *Parvoviridae* family DNA virus. Recombinant AAV (rAAV) has been used extensively as a gene delivery vehicle to transduce a wide range of cells in vitro and in vivo (1,10,20). Remarkable success has also been demonstrated recently in Phase I and II clinical trials in rAAV-based gene therapy studies (9,17,19).

The 4680-nucleotide-long wild-type AAV genome contains two major open reading frames that encode the viral replication proteins and the capsid proteins. Two 145-nucleotide-long viral inverted terminal repeats are incorporated at both ends of the viral genome. The inverted terminal repeat is one of the most essential elements in the AAV life cycle. It acts as the viral replication origin and is also responsible for the AAV packaging and integration. The only *cis*-acting viral element required in rAAV is the inverted terminal repeat. In rAAV, all the wild-type AAV open reading frames are replaced by the customer-favored transgene expression cassette. The rAAV viral stocks are pro-

duced with a *cis*-plasmid in which the transgene expression cassette is flanked by viral inverted terminal repeats. All the other factors that are required for rAAV replication and packaging are provided *in trans* by helper plasmids, viruses, and/or producer cell lines.

The palindromic sequence in the inverted terminal repeat forms a T-shaped hairpin structure. This unusual DNA structure harbors a very active recombination activity. Inverted terminal repeats form recombinant junctions with the cellular DNA at the AAV integration site in the host chromosome (12,22). Furthermore, intramolecular and intermolecular recombination between inverted terminal repeats lead to the formation of circular monomer and circular and linear concatamer AAV transduction intermediates (5,16). Homologous recombination between inverted terminal repeats also occurs in *E. coli* during the construction of the rAAV *cis*-plasmids. As a result of this recombination, inverted terminal repeat sequences and/or the transgene expression cassette are often rearranged or partially deleted during subcloning. A large number of clones have to be screened to detect the right one. This has been especially problematic when cloning sites are directly adjacent to the inverted terminal repeats (14,18). To increase the cloning efficiency and simplify the cloning procedure, we developed a series of multiple cloning site vectors based on the type-2

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Table 1. Cloning Capacity of the rAAV Multiple Cloning Site Vectors

Name	Construct	Optimal Insert ^a	Maximal Insert ^b	scAAV Insert ^c	Multiple Cloning Sites (5'→3')
pDD1	pcis.RSVmcs	3228	3548	888	<i>KpnI-NheI-HindIII-NheI-NotI-XhoI-SfiI-BamHI</i>
pDD2	pcis.CMVmcs	3161	3481	821	<i>NheI-XhoI-EcoRI-MluI-KpnI-XbaI-SalI-AccI-EagI-NotI</i>
pDD3	pcis.CMVmcs-intron	2964	3284	624	<i>NheI-XhoI-EcoRI-MluI-KpnI-XbaI-SalI-AccI-EagI-NotI</i>
pDD4	pcis.SV40mcs	3535	3855	1195	<i>NheI-XhoI-EcoRI-MluI-KpnI-XbaI-SalI-AccI-EagI-NotI</i>
pDD5	pcis.SV40mcs-intron	3338	3658	998	<i>NheI-XhoI-EcoRI-MluI-KpnI-XbaI-SalI-AccI-EagI-NotI</i>
pDD344	pcis.CK6mcs	3421	3741	1081	<i>XhoI-EcoRI-MluI-KpnI-SalI-NotI</i>
pDD345	pcis.CAGmcs	3157	3477	817	<i>SalI-XhoI-EcoRI-MluI-KpnI-XbaI-SalI</i>

scAAV, self-complementary AAV.

^aOptimal Insert: The expected size of the inserted transgene (bp) based on the rAAV size of 4680 nucleotides.

^bMaximal Insert: The expected size of the inserted transgene (bp) based on the rAAV size of 5000 nucleotides.

^cscAAV Insert: The expected size of the inserted transgene (bp) based on the rAAV size of 2340 nucleotides.

AAV inverted terminal repeat. We incorporated the most commonly used viral promoters [such as cytomegalovirus (CMV), rous sarcoma virus (RSV), and simian virus 40 promoter (SV40)] and the synthetic promoters [such as muscle-specific CK6 and the ubiquitous cytomegalovirus enhancer/chicken β -actin (CAG) promoter]. A poly(A) signal was also included at the 3'-end of the multiple cloning sites to facilitate transgene expression. A simple, one-step insertion of the target cDNA into the multiple cloning sites will yield the *cis*-plasmid for rAAV production.

MATERIALS AND METHODS

Construction of Multiple Cloning Site *cis*-Plasmids

The type-2 AAV inverted terminal repeat from psub201 is used in all the multiple cloning site vectors (13). To generate the pcis.RSVmcs (pDD1), the 1084-bp RSV expression cassette (RSV-mcs-pA) was released from pREP4 (Invitrogen, Carlsbad, CA, USA) by *SalI* digestion and blunted into the *XbaI* sites in psub201. The pcis.CMVmcs-intron (pDD3) was generated by the blunt-end ligation of the 1348-bp *BamHI/BglII* fragment from pCI (Promega, Madison, WI, USA) to the *XbaI* sites in psub201. Similarly, a 983-bp *BamHI/BglII* fragment from pSI (Pro-

mega) was used to generate pcis.SV40mcs-intron (pDD5). The synthetic intron in both pDD3 and pDD5 was removed by discarding a 197-bp *AflIII* fragment. The resulting plasmids are designated as pDD2 (pcis.CMVmcs) and pDD4 (pcis.SV40mcs). The pcis.CAGmcs (pDD345) was generated by replacing the SV40 promoter (*SnaBI/XhoI*) in pDD4 with the 855-bp *XbaI* (blunted)/*XhoI* CAG promoter fragment. The muscle-specific CK6 promoter was a gift from Dr. Jeff Chamberlain at the University of Washington (8). The pcis.CK6mcs (pDD344) was generated by swapping the SV40 promoter (*SnaBI/NheI*) in pDD4 with the 613-bp *NotI* (blunted)/*SpeI* CK6 promoter fragment. The intactness of the

inverted terminal repeat sequence in all the clones is screened by three restriction enzymes including *BssHII* (cuts at the A arm in the inverted terminal repeat), *MscI* (cuts at the junction of A and D arms), and *SmaI* (cuts at the C arm). The correct clones were further confirmed by sequencing. All the restriction sites in each multiple cloning site have also been confirmed by enzymatic digestion.

Construction of β -Galactosidase Expressing *cis*-Plasmids

The pcisCAGLacZ (pDD385) was generated by cloning the 3.4-kb β -galactosidase cDNA into the *KpnI/XbaI* site in pDD345. The pcisCK6LacZ (pDD384) was generated by cloning the 3.4-kb β -galactosidase cDNA into the *KpnI/SalI* site in pDD344. The pcisCMVLacZ (pDD382) was generated by cloning the 3.4-kb β -galactosidase cDNA into the *NheI/XhoI* site in pDD344. The pcisRSVLacZ (pDD17) has been previously described (7). The pcisSV40LacZ (pDD22) was generated by cloning the 3.4-kb β -galactosidase cDNA into the *NotI* site in pDD4.

Generation of rAAV Stock and Infection of HeLa Cells and Murine Skeletal Muscle

The rAAV stocks were generated according to a previously described pro-

Figure 1. Schematic representation of the multiple cloning site *cis*-plasmid structure. Five commonly used promoters were used to drive transgene expression in these multiple cloning site *cis*-plasmids. The diagram is not drawn to scale.

tocol (7). HeLa cell infection was performed at a MOI of 100 viral particles/cell in serum-free DMEM for 2 h. At the end of infection, the serum level was brought to 10% by supplementing with FBS. Cell lysate was harvested 36 h after infection, and the β -galactosidase activity was measured as described earlier (6). All the animal experiments were carried out in accordance with the National Institutes of Health guidelines and the Institution guidelines of the University of Missouri. The six-month-old mdx mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). A total of 2.5×10^{10} viral particle from each indicated rAAV stock was microinjected into the tibialis anterior muscle as previously described (6). The β -galactosidase expression was examined one month after infection by the histochemical LacZ staining and the quantitative enzyme activity assay as previously described (6).

RESULTS

Establishment of Multiple Cloning Sites *cis*-Plasmids for rAAV Production

Eight different serotypes of the rAAV vectors have been described so far. The difference in the capsids offered significant advantages in the rAAV mediated gene transfer for different tissues (3,23). Interestingly, the *cis*-

plasmid harboring type-2 AAV inverted terminal repeat can be efficiently cross-packaged by viral capsids from all the other serotypes. This pseudotyping manipulation will likely enhance the tissue-type-specific applications of the multiple cloning site vectors. To generate the multiple cloning site vectors that suit different applications, we have chosen the well-characterized type-2 AAV inverted terminal repeat as our multiple cloning site *cis*-plasmid back-

Figure 2. The transduction profiles of five β -galactosidase-expressing rAAV viruses generated with multiple cloning site *cis*-plasmids. The relative levels of β -galactosidase activity are shown from rAAV-infected HeLa cells (mean $\pm$ SEM; $n = 3$, 36 h after infection) and the tibialis anterior muscles of the mdx mice (mean $\pm$ SEM; $n = 4$, one month after infection).

Figure 3. The β -galactosidase expression in the mdx mouse skeletal muscle from rAAVs driven by CAG, CK6, CMV, RSV, and SV40 promoters, respectively. A 1-mm-thick central belly muscle piece is stained for β -galactosidase at room temperature for 15 h before embedding. The representative photomicrographs (5- μ m paraffin section) from four independent samples are shown for each condition (top panel, 5 $\times$ objective lens magnification; bottom panel, 20 $\times$ objective lens magnification). The centrally localized myofiber nucleus seen at the high magnification (20 $\times$) is a characteristic feature of the mdx mouse muscle. The muscle sections are counterstained with hematoxylin-eosin. The scale bar in the top panel represents 200 μ m. The scale bar in the bottom panel represents 50 μ m.

bone (13). Figure 1 depicts the common structure of the multiple cloning site *cis*-plasmids. All the multiple cloning site sequences were derived from the commonly used eukaryotic expression vectors.

The major difference in these multiple cloning site vectors is the source of the promoter used to drive transgene expression. The size of the promoter also determined the cloning capacity of each individual multiple cloning site *cis*-plasmid (Table 1). The optimal cloning capacity is based on the physical length of the wild-type AAV genome. Because functional rAAV particles can be efficiently packaged up to approximately 110% of the wild-type AAV genome size (4), we have also listed the maximal cloning capacity for each multiple cloning site *cis*-plasmid. The self-complementary AAV represents a novel AAV packaging concept (11). According to this theory, when the size of the rAAV genome approaches half of the wild-type AAV size, a pair of plus- and minus-strand proviral genomes is co-packaged into a single viral particle. More efficient gene transfer is achieved from the self-complementary AAV as a result of bypassing second-strand synthesis (11). This information is extremely useful for small-size transgene. In Table 1, we have also provided the optimal cDNA insert size for generating self-complementary AAV from each multiple cloning site *cis*-plasmids.

In Vitro and In Vivo Expression of the β -Galactosidase from rAAV

To test the function of the multiple cloning site vectors described earlier, a series of β -galactosidase rAAV *cis*-plasmids were constructed by one-step subcloning. Type-2 rAAV stocks were generated. In vitro infection of the HeLa cells demonstrated compatible high-level β -galactosidase expression from rAAV carrying CAG, CMV, RSV, and SV40 promoters, respectively. As expected, only background level of enzyme activity was detected from rAAV carrying muscle-specific CK6 promoter in the HeLa cells (Figure 2). However, when these viruses were used to infect the skeletal muscles of the six-month-old mdx mice ($n = 4$ for each group),

the highest transduction levels were observed in viruses carrying CK6 and CMV promoters one month after infection. The level of β -galactosidase expression was slightly lower from the CAG and RSV promoters. The SV40 promoter gave the lowest level of expression (Figures 2 and 3).

DISCUSSION

The construction and application of the multiple cloning site vector are the most traditionally adapted and widely used approaches in biomedical research. Here we have characterized several multiple cloning site *cis*-plasmids for rAAV production. The unique restriction sites encoded in these vectors have offered a wide range of selection for straightforward subcloning of the cDNA of interest. Furthermore, all the molecular manipulations are carried out at a distance from the viral inverted terminal repeat to reduce the inverted terminal repeat-mediated recombination and the subsequent cloning error (D. Duan, unpublished observation).

One of the most common methods to optimize the gene transfer vehicle for a particular tissue and/or application is to screen for the best transcription regulatory elements, especially the promoter. In this regard, the CAG promoter has been found to yield the highest level of liver transduction in an AAV vector, following portal vein injection (21). On the other hand, studies in the primary sensory neuronal culture and the cystic fibrosis patient airway epithelial had identified the CMV promoter as the strongest promoter in the setting of adenoviral vectors (15,24). The availability of the multiple *cis*-plasmid multiple cloning site vectors described here provides a working foundation for the development of optimized rAAV vectors for specialized applications.

An important compound factor in optimizing promoters for the gene transfer vector is the tissue specificity. A recent study in the γ -sarcoglycan knockout mouse model of muscular dystrophy suggested that a tissue-specific promoter might be essential for the high-level, persistent AAV transduction in tissues with a high inflammatory background (2). In that model system, a

significant drop in the level of therapeutic γ -sarcoglycan expression was found one month after infection, when it was expressed from the CMV promoter. The pathological changes in the mdx mouse skeletal muscle are also characterized by substantial inflammation. Surprisingly, despite the fact that prokaryotic β -galactosidase is very immunogenic, similar levels of efficient transgene expression were achieved from rAAV carrying either muscle-specific CK6 promoter or ubiquitous CMV promoter. It is possible that the pathological process and/or specific pieces of skeletal muscle used in the study (2) (tibialis anterior muscle in our study vs. quadriceps in the other study) may have contributed to the different observations. A more systemic comparison will eventually help to determine the most appropriate promoter(s) for rAAV-mediated gene therapy in the inflammatory dystrophic muscle.

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