Identification and Elimination of Replication-Competent Adeno-Associated Virus (AAV) That Can Arise by Nonhomologous Recombination during AAV Vector Production

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Adeno-associated virus (AAV) vector preparations are often contaminated with variable amounts of replication-competent AAV (rcAAV), which may influence the behavior of these vectors both in cultured cells and in animals. A packaging plasmid/vector plasmid system containing no significant homology and lacking the wild-type AAV p5 promoter was constructed to eliminate the production of wild-type AAV by recombination. Still, rcAAV was detected in vector produced by cotransfection of these plasmids at large scale. Sequence analysis revealed that nonhomologous recombination was responsible for the generation of these novel rcAAVs. A new AAV packaging plasmid carrying separate rep and cap expression cassettes in opposite transcriptional orientations was constructed. AAV vector preparations produced by using this packaging construct did not contain rcAAV.

Adeno-associated virus (AAV) vectors are attractive for gene therapy because they are based on a nonpathogenic human parvovirus whose replication is dependent on a helper virus, typically adenovirus (28). The genome is composed of a linear, single-stranded DNA molecule (4,680 bases) including a 145-base terminal repeat (TR) at each end (35). Either the plus or minus strand can be encapsidated by AAV structural proteins to generate infectious virions (32). Two open reading frames (ORFs) encoding the nonstructural and structural viral proteins are present on alternatively spliced transcripts originating at three distinct promoters (7). The replication (rep) gene encodes at least four protein products (26), two expressed from the p5 promoter (Rep 78 and Rep 68) and two expressed from the p19 promoter (Rep 52 and Rep 40) on alternatively spliced transcripts (8). The structural capsid proteins (VP1, VP2, and VP3) are encoded by the right half of the AAV genome (cap) on alternatively spliced transcripts originating at the p40 promoter (37).

Typically, recombinant AAV vector virions are produced by transient cotransfection of the vector plasmid with a plasmid containing the AAV packaging functions. The most widely used packaging system, AAV/Ad (33) (described in Results), uses complementary plasmids which may contain no shared sequences, depending on the vector used. This system has been reported to produce vector that contains no wild-type (wt) AAV. However, a recent report (12) has demonstrated the presence of replication-competent AAV (rcAAV) in vector preparations produced using AAV/Ad. This is not surprising, given that recombination between two partial AAV plasmids sharing only a single restriction site can reconstitute wt AAV (34).

To obviate this problem, an alternate packaging system that replaces the AAV p5 promoter with a heterologous promoter was developed (12). This system is inherently incapable of generating wt AAV, as there are 119 bases of wt AAV sequence that are not present in either packaging or vector constructs. Small-scale vector preparations made by using this system did not contain rcAAV (12), but we find that large-scale preparations are infrequently contaminated with rcAAV by using a sensitive amplification assay. Sequence analysis revealed that the rcAAV resulted from nonhomologous recombination between AAV vector and packaging DNAs.

To overcome this problem, the AAV rep and cap genes were split into two separate transcription units, each under the control of heterologous transcriptional regulatory sequences. Both transcription units were placed on a single plasmid, but in opposing transcriptional orientations to reduce the likelihood of recombination resulting in the generation of rcAAV. Large-scale AAV vector preparations produced with this packaging vector were free of contaminating rcAAV.

MATERIALS AND METHODS

Cell culture. 293, A549, and IB3 cells have been described elsewhere (15, 25, 44). All cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in a 5% CO2-air atmosphere.

Plasmid construction. MDRp/CMVcap (Fig. 1B) was generated from a plasmid containing AAV serotype 2 (pAV2) (24). A fragment containing the entire rep coding sequence (from nucleotides [nt] 311 to 2253 [35]) was inserted into a mouse metallothionein I expression vector consisting of a promoter sequence from nt −592 to +69 (3) and a polyadenylation signal from nt +925 to +1242 (14). The cap open reading frame was isolated on a HindIII/SmaI fragment (nt 1983 to 4493) (35) and cloned downstream of the human cytomegalovirus major immediate-early (IE) gene promoter (nt −672 to +71) followed by the simian virus 40 (SV40) early polyadenylation signal (6, 29). The transcription units were then subcloned into a pKS Bluescript vector (Stratagene) in opposite transcriptional orientations (Fig. 1B). The deduced sequence of the plasmid is available on request. The pGEM-SSS construct (Fig. 1A) was derived from plasmid pBSK12 (12), which expresses rep gene sequences under control of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) promoter region. The SalI-to-EcoRI fragment of the pBR322 backbone was substituted with a SalI-to-EcoRI fragment from pGEM-1 to yield pGEM-SSS, capable of high-

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FIG. 1. Plasmid maps and PCR strategy for analyzing structures of AAV species. (A) The tgAAVCF vector, pGEM-RS5 packaging construct, wt AAV, and putative rAAV chimera structures are shown schematically. Primers are shown as arrows; the D-region primer binds to the 5′ and 3′ TR regions in opposite orientations. (B) Maps of the AAV packaging plasmid MTrep/CMVcap and the AAV-LAPSN and AAV-GrTHLAP vector constructs. Arrows indicate transcriptional start sites, and open boxes indicate coding regions. Abbreviations: pA, polyadenylation signal; cap, AAV capsid ORF; CMV, human cytomegalo-virus major IE gene promoter; MT, mouse metallothionein I gene promoter; rep, AAV replication ORF; TR, AAV terminal repeat; gfa28, glial fibrillary acidic protein promoter; rTH, rat tyrosine hydroxylase cDNA; LTR, retroviral long terminal repeat; AP, human plasmatic alkaline phosphatase coding sequence; neo, neomycin phosphotransferase.

RESULTS

Detection of rAAV in vector preparations produced by using AAV packaging constructs with linked rep and cap genes. Production of AAV vectors is routinely accomplished using AAV/Ad to provide rep and cap functions. This plasmid contains AAV2 nucleotides 191 to 4483 flanked by adenovirus type 5 TRs (33). However, we found that vector stocks produced with AAV/Ad tested positive for rAAV by an infectious center assay. Briefly, 293 cells were infected with serial
dilutions of vector stock in the presence or absence of adenovirus for ~28 h, were suspended by trypsinization, and were aspirated onto Hybond N membranes (Amersham International, Amersham, England) for hybridization to 32P-labeled DNA fragments containing the AAV2 rep and cap genes (20). Adenovirus-dependent amplification of the rep and cap sequences in single cells was compared to the vector titer to determine the level of rcAAV contamination. Vector prepared with AAV/Ad contained rcAAV at an average of ~1% of the vector titer but ranged from as little as 0.0001% to as high as 5%, depending on the preparation tested.

An alternative packaging plasmid to AAV/Ad, pRS5, lacks TR sequences and contains a substitution of the AAV p5 promoter by a portion of the HIV-1 LTR (12). This plasmid cannot recombine with vector plasmid to produce wt AAV, as both vector and packaging plasmids lack the AAV p5 promoter. pRS5 was found to produce approximately 10-fold-higher titers of an AAV-neo vector compared to AAV/Ad, and rcAAV was not detected in small-scale preparations (12).

Large-scale production of an AAV vector containing the human CFTR gene (Fig. 1A) was accomplished by using the AAV packaging plasmid pGEM-RS5 (Fig. 1A). The tgAAVCF virions were produced by cotransfection of 4 × 10^6 of the 293 cells with plasmids ptgAAVCF and pGEM-RS5 in the presence of adenovirus. Aliquots of vector virions (2 × 10^7 DNase-resistant vector genomes) were screened by the amplification assay for the presence of rcAAV. A single round of amplification showed an assay sensitivity of 1 IU (as determined by TCID_{50} endpoint assay) of wt AAV, and no signal was seen with adenovirus alone. Even though generation of wt AAV by recombination between vector and packaging plasmids was impossible, some large-scale preparations of the tgAAVCF vector showed an AAV2-hybridizing signal (Fig. 2). A representative Southern analysis revealed bands of ~4.5 and 9 kb, approximately the same size as the monomer and dimer replicative forms of wt AAV (Fig. 2). These data show that the tgAAVCF vector preparation was contaminated by rcAAV.

**Characterization of rcAAV.** A PCR strategy was designed to distinguish between possible wt AAV contamination of the tgAAVCF vector and a recombination event resulting in the generation of a novel rcAAV (Fig. 1A). Molecules replicated in the amplification assay were assumed to have TR sequences present at each end and to contain rep and cap sequences found in the packaging plasmid. To analyze the 5' end of this putative recombinant, an amplimer corresponding to the TR D region derived from the packaging plasmid. The locations of the junctions and the regions of overlap between plasmids ptgAAVCF and pGEM-RS5 are shown in Fig. 4. The recombinant events were essentially nonhomologous, as the junction regions had only 0 to 4 bases of overlap. The locations of the junctions relative to the entire plasmid sequences are depicted in Fig. 5. The analysis revealed that the recombination breakpoint within the tgAAVCF vector was not conserved, that both the 5' and 3' TRs were capable of participation, and that the 5' TR could be found on either end of the rcAAV molecule (Fig. 5). The identified pGEM-RS5 breakpoints were all upstream of the start codon for the rep proteins at the 5' end and were downstream of the termination site for the cap proteins at the 3' end. This observation indicates that the ORFs were required to produce replication-competent recombinants, as expected.

**Production of AAV vector free of contaminating rcAAV.** In a further attempt to produce AAV vectors free of rcAAV, the rep and cap genes were split into two separate inverted transcription units on a single plasmid called MTrep/CMVcap (Fig.
resulted in an AP vector titer of \( \frac{1}{3} \times 10^5 \) FFU on IB3 cells. Use of the AAV/Ad plasmid resulted in approximately \( 3 \times 10^5 \) FFU per 5 \( \times 10^6 \) cells transfected; use of MT/CMVcap resulted in approximately \( 3 \times 10^5 \) FFU per 5 \( \times 10^6 \) cells. In crude lysates before CsCl purification, we have obtained up to 10 AP species.

A second AAV vector can be produced in the absence of detectable rcAAV by using MTrep/CMVcap. We tested the MTrep/CMVcap system for production of rcAAV-free AAV vector stocks by using a second AAV vector, AAV-GrTHLAP (Fig. 1B). AAV-GrTHLAP produced by using MTrep/CMVcap yielded \( 6 \times 10^5 \) AP FFU per 5 \( \times 10^6 \) cells transfected. AAV-GrTHLAP produced by using AAV/Ad had a yield of \( 2 \times 10^6 \) AP FFU per 5 \( \times 10^6 \) cells transfected. Both preparations showed a vector genome-containing particle-to-infectivity ratio of \(~200\) particles per AP FFU (data not shown). The AAV-GrTHLAP virions generated with each of the packaging plasmids were then tested for the presence of rcAAV. Vector \( (2.5 \times 10^5 \) AP FFU) was used to infect 293 cells in the presence of adenovirus and subjected to the sequential amplification assay. A standard curve of wt AAV plus adenovirus was also included to quantify the amount of rcAAV present. Southern analysis revealed that as little as 1 IU of wt AAV could be detected in this assay, and detection of wt AAV was absolutely dependent on the presence of adenovirus during infection (Fig. 7). While rcAAV was detected in the AAV-GrTHLAP vector produced with AAV/Ad at a concentration of 1 to 10 IU per \( 2.5 \times 10^5 \) AP FFU, the AAV-GrTHLAP vector produced with MTrep/CMVcap was completely free of rcAAV in this assay (Fig. 7).

**Stable vector transduction in the absence of rcAAV.** The rep proteins are involved in the site-specific integration of wt AAV in human chromosome 19 (13). Contaminating rcAAV in vector stocks could supply rep functions in trans, altering the biologic behavior of AAV vectors. Therefore, we confirmed that vector stocks free of contaminating rcAAV were able to mediate stable gene transduction. AAV-LAPSN contains the neo gene in addition to the alkaline phosphatase gene. 293 cells were transduced with the AAV-LAPSN stock produced by using MTrep/CMVcap at an MOI of 0.03 AP FFU/cell. After a 24-h exposure to virus, the cells were plated at low density and selected in G418. Single colonies were isolated from a 24-h exposure to virus, the cells were plated at low density and selected in G418. Single colonies were isolated from a dilute plate and expanded for further analysis. Total cellular DNA from six representative clones was prepared, digested with either HindIII or EcoRI, and analyzed by Southern hybridization with a neo probe (Fig. 8). HindIII and EcoRI cut 5' of the neo gene in AAV-LAPSN, and the distances from the HindIII and EcoRI sites to the 3’ TR of AAV-LAPSN are 1,083 and 3,363 bp, respectively. All of the samples displayed unique bands consistent with the integration of a full-length AAV-LAPSN genome except for the EcoRI digest of clone 5.

![Diagram](https://example.com/diagram.png)

**Fig. 5.** Analysis of crossover points of cloned rcAAVs. P2 to P5 are TR D-region–rep-amplified species; SN7 and SN9 are TR D-region–cap-amplified species. Numbers refer to nucleotide numbers of the recombination junction. Top, tgAAVCF vector; bottom, pGEM-RS5 vector (not to scale).
The data presented here and in another recent report on August 14, 2017 by guest

DISCUSSION

AAV vector production has a history of vector contamination with wt virus (see reference 28 for a comprehensive review). Transfection analyses of plasmids containing intact AAV and a variety of deletion mutants have yielded important insights into recombinational events that serve to produce phenotypically wt virus. For example, analysis of cloned AAV mutant genomes has demonstrated that the rcAAV-free AAV-LAPSN stocks generated with MTrep/CMVcap are capable of integration into the genomes of transduced cells.

FIG. 6. Detection of rcAAV in AAV-LAPSN virions produced with AAV/Ad but not with MTrrep/CMVcap. Shown is Southern analysis of total genomic DNA harvested from 293 cells after one (1×) or two (2×) rounds of infection with adenovirus alone (MOI = 5) or AAV-LAPSN (2.5 × 10^3 AP+ FFU) with or without adenovirus (MOI = 5). The samples were digested with HindIII and probed with the AAV cap gene fragment. HindIII cuts once within wt AAV2, just 3’ of the P40 transcription start site, and splits the AAV genome into two fragments migrating at ~1.8 and ~2.8 kbp (35), of which only the 2.6-kbp fragment containing the AAV cap sequence is detected by the cap probe. The second major band at ~5.6 kbp is likely due to the detection of tail-to-tail dimers of the rcAAV replicative forms as predicted by models for AAV replication (28).

FIG. 7. Detection of rcAAV in AAV-GrTHLAP virions produced with AAV/Ad but not with MTrrep/CMVcap. Shown is a Southern blot of total genomic DNA harvested from 293 cells after two rounds of infection with AAV-GrTHLAP (2.5 × 10^3 AP+ FFU) with or without adenovirus (MOI = 5). The samples were digested with HindIII and probed with an AAV cap gene fragment. A standard curve of wt AAV infection is shown to the right.

(12) show that generation of rcAAV clearly does occur in the AAV/Ad system.

The pGEM-RS5 system was evaluated for large-scale production of vector free of rcAAV. Generation of genotypically wt AAV by recombination between the vector and packaging plasmid is not possible, as the p5 promoter region from wt AAV is deleted from both the vector and the packaging plasmid. Surprisingly, phenotypically wt AAV was detected in some vector preparations by using the pGEM-RS5 system. Evaluation of these rcAAV species shows genomes that have vector-derived TR sequences at either end, with variable lengths of CFTR vector sequences incorporated. The pGEM-RS5-derived portions included variable amounts of the HIV-1 LTR promoter region followed by rep and cap sequences. It should be noted that the amplification assay and PCR analysis impose the following constraints on detection of replicating material. First, the rcAAV genome must have TR sequences at both ends to allow for amplification in the presence of adenovirus. Second, the rep gene must be present and must be downstream from a functional promoter element. Third, in order for efficient genome encapsidation and purification of the rcAAV to occur, the rcAAV molecule must be similar in size to wt AAV. These constraints select against recombinant genomes that could not be efficiently produced or amplified.

The mechanism for generation of the rcAAV identified in the present study must involve nonhomologous recombination, as there are no areas of overlap between the vector and packaging plasmid DNA. Sequence analysis of two rcAAV clones, P2 and SN7, revealed that the site of recombination occurred in a region containing no sequence identity between the parental sequences (Fig. 4). Four additional clones analyzed showed a maximum of 4-bp overlap at each junction.

Recombination following plasmid transfection into mammal...
lian cells has been well documented (10, 27, 39, 40). In the specific case of AAV, recombinants between wt AAV and SV40 have been investigated by use of an infectious center assay (17). Both viral coinfection and plasmid cotransfection gave rise to SV40/AAV recombinants capable of replication and encapsidation in Cos cells, at a relatively high frequency of 1 in 1,000 cells. The presumed mechanism is nonhomologous recombination, as there are no extensive sequence homologies between the two viral genomes. Molecular analysis of the SV40/AAV recombinants demonstrated conservation of both the SV40 ori region and the size of the total hybrid genome; however, breakpoints for the recombinational events were not conserved (18).

The separation of rep and cap genes achieved with the MTrep/CMVcap construct allowed production of vector stocks free of rcAAV. Two vectors (AAV-LAPSN and AAV-GrTH-LAP) were evaluated, and up to 2.5 FFU of the vectors were shown to be free of rcAAV, using the sequential amplification assay (Fig. 6 and 7). Despite the use of heterologous promoters, yields comparable to those generated with AAV/Ad were obtained. The vector titers appear lower than values reported in the literature due to differences in titer determination. Many recent reports include vector titers determined in the presence of adenovirus. Adenovirus coinfection can increase the apparent titer of AAV vectors up to 1,000-fold (11, 38). In addition, the AP titer reported in this study represents gene transfer as well as expression, as opposed to assays designed to measure the ability of vector genomes to replicate in the presence of adenovirus and AAV rep protein.

An rcAAV-free vector produced by using MTrep/CMVcap was used to stably transduce cells to G418 resistance. Southern analysis of AAV-LAPSN-transduced clones showed a restriction pattern consistent with the integration of vector genomes in each clone (Fig. 8). This observation confirms that AAV vectors can stably integrate and persist in transduced cells in the absence of contaminating rcAAV.

The design of the MTrep/CMVcap packaging plasmid employs several features designed to reduce the likelihood of rcAAV formation. It lacks all of the replication and encapsidation signals for AAV. It contains heterologous promoters substituted for the AAV p5 and p40 promoters. The rep and cap genes were placed in opposite transcriptional orientations relative to each other. A simple two-step recombination event leading to addition of flanking TRs would result in a genome exceeding the packaging limit for AAV (9). These modifications would require a minimum of three recombinations to produce an infectious rcAAV. A similar packaging plasmid containing the Rous sarcoma virusLTR and the cytomegalovirus IE promoter substituted for the AAV p5 and p40 promoters, respectively, but maintaining an AAV-like orientation of the rep and cap transcription units was recently described (38). The authors report having generated a small-scale, crude vector preparation of a β-galactosidase vector (TRlacZ) free of contaminating wt AAV. The frequency of recombination events leading to the generation of rcAAV in small-scale, single-plate preparations can be low, resulting in the generation of rcAAV-free stocks even with the AAV/Ad and pGEM-RS5 packaging plasmids. It remains to be determined whether large-scale, purified vector preparations made by using this system will remain rcAAV free, as no data were reported for large-scale preparations that were produced in this study (38).

The consequences of rcAAV contamination of vector stocks may influence our understanding of the biology of AAV, AAV vectors, and procedures for gene therapy. Coinfection of a cell with both rcAAV and a therapeutic AAV vector may affect the conversion of single- to double-stranded genomes in the presence of adenovirus (11). Studies indicate that transduction of mouse hepatocytes with AAV vectors is enhanced by the presence of wt AAV (22). In a separate study, wt AAV interfered with AAV vector transduction of rabbit lung epithelial cells (20). Coinfection of vector and rcAAV may also lead to mobilization of the vector upon adenovirus infection (28). The expression of AAV open reading frames in infected cells could result in an immune response against the transduced cells. Transduction with adenovirus vectors has been associated with the development of cytolytic T-cell responses to adenovirus proteins expressed in target cells (41) and the production of neutralizing antibodies capable of blocking gene transfer (42, 43). In contrast to retroviruses, adenovirus, and herpesvirus, however, AAV is not a pathogen; the presence of rcAAV in vector stocks may not be a safety issue but rather a complicating factor in experiments designed to investigate AAV biology.

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