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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).

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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% CO_2–air atmosphere.

Plasmid construction. MT*rep*/CMV*cap* (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 *Hin*dIII/*Sna*BI 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-RS5 construct (Fig. 1A) was derived from plasmid pRS5 (12), which expresses *rep* gene sequences under control of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) promoter region. The *Sal*I-to-*Eco*RI fragment of the pBR322 backbone was substituted with a *Sal*I-to-*Eco*RI fragment from pGEM-1 to yield pGEM-RS5, capable of high-

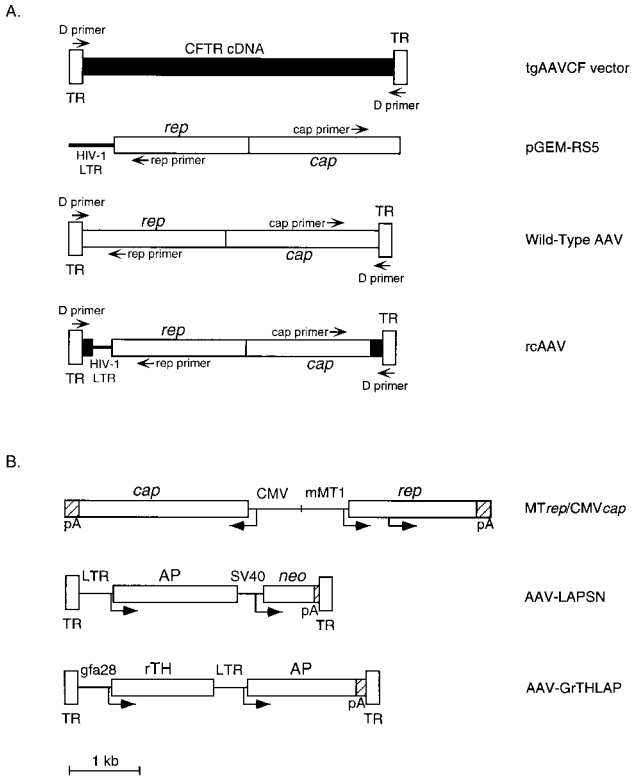

FIG. 1. Plasmid maps and PCR strategy for analyzing structures of AAV species. (A) The tgAAVCF vector, pGEM-RS5 packaging construct, wt AAV, and putative rcAAV chimera structures are shown schematically. Primers are shown as arrows; the D-region primer binds to the 5′ and 3′ TR regions in opposite orientations.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

copy-number maintenance in bacterial hosts. The AAV/Ad plasmid has been described elsewhere (33).

The AAV vector AAV-GrTHLAP (Fig. 1B) contains the AAV2 sequences from nt 1 to 193 and 4492 to 4680 which include the 145-bp TRs at each end (28, 35). Inserted between the AAV2 sequences is a glial fibrillary acidic protein promoter fragment (gfa28) (4) linked to the rat tyrosine hydroxylase cDNA (16) followed by the murine leukemia virus LTR linked to the human placental alkaline phosphatase (AP) coding sequence and, finally, the SV40 early polyadenylation site (29). The deduced sequence of the plasmid is available on request. The ptgAAVCF vector (Fig. 1A) consists of the 145-bp AAV2 TRs flanking the human cystic fibrosis transmembrane regulator (CFTR) cDNA (nt 133 to 4573) and a synthetic polyadenylation sequence based on that of the murine beta-globin gene at the 3′ end cloned in a pBR322-based plasmid backbone (1). The AAV vector AAV-LAPSN (Fig. 1B) contains the AP coding sequence and a neomycin phosphotransferase (*neo*) gene (30). Recombinant AAV vector plasmids were propagated in either *Escherichia coli* JC8111 (5) or *E. coli* SURE (Stratagene) to reduce the likelihood of deletions that can occur within the AAV TRs. AAV vector plasmid DNA was always generated from individual bacterial colonies, and the integrity of the TRs was confirmed by restriction enzyme analysis. In particular, *Ahd*I digestion appears to be diagnostic for TR deletions (data not shown). All plasmids were constructed by using standard techniques (31).

Viruses. Adenovirus type 5 was grown in 293 cells and purified by CsCl step gradients followed by isopycnic centrifugation in CsCl. Adenovirus stocks were titered on 293 cells by 50% tissue culture infective dose ($TCID_{50}$) endpoint assay with detection by fluorescein isothiocyanate-conjugated antihexon antibody (Biodesign). AAV serotype 2 was grown in 293 cells and purified by two rounds of isopycnic gradient CsCl purification (23). AAV2 stocks were dialyzed against phosphate-buffered saline and heat treated at 56°C for 30 min to remove any residual contaminating adenovirus. AAV2 stocks were titered on A549 cells by $TCID_{50}$ endpoint assay using rabbit anti-*rep* antiserum as previously described (36).

AAV vector production. The tgAAVCF vector was prepared as previously described (12), with minor modifications. Semiconfluent 293 cells were infected with adenovirus type 5 at a multiplicity of infection (MOI) of 5, followed by transfection with 5 μg each of pGEM-RS5 and ptgAAVCF per 10^6 cells, using LipofectAmine (Gibco). Cells were harvested 3 days following transfection, followed by cell disruption in a Branson cup horn sonicator. tgAAVCF vector was purified by an initial isopycnic centrifugation in CsCl, treatment with DNase, and a second separation by isopycnic centrifugation in CsCl. Purified vector was dialyzed into Ringer's balanced salt solution containing 5% glycerol and heat inactivated at 56°C for 5 to 10 min to remove any residual infectious adenovirus. Purified stocks were aliquoted and stored at −70°C.

AAV-LAPSN and AAV-GrTHLAP vector stocks were prepared as previously described (2), with slight modifications. Human 293 cells were seeded at 5×10^6 cells per 10-cm-diameter dish and infected with adenovirus (MOI = 5) approximately 1 h before transfection. Calcium phosphate precipitates were formed with 10 μg of AAV packaging plasmid and 10 μg of vector plasmid per ml and added to the adenovirus-infected 293 cells (1 ml per 10-cm-diameter plate). The cultures were harvested when maximum cytopathic effect was observed, usually 48 to 72 h after adenovirus infection. The cells were scraped into the overlying medium (10 ml/dish) and lysed by three freeze-thaw cycles in a dry ice-ethanol bath. The lysates were then pelleted by centrifugation through 40% (wt/vol) sucrose in phosphate-buffered saline. The viral pellet was resuspended in Ringer's solution with CsCl and centrifuged, and fractions (500 μl) were collected. The peak fraction was determined by Southern hybridization for the vector genomes. The fractions were dialyzed against Ringer's solution across a 50,000-molecular-weight-cutoff membrane to remove the CsCl and incubated at 56°C for 1 h to inactivate any residual adenovirus.

Titer determination and transduction. The titers of the vectors containing AP were determined by counting AP-positive (AP^+) foci of cells after transduction of the immortalized epithelial cell line IB3 (19). AAV-LAPSN-infected 293 cells were isolated by incubating 1.5×10^5 AP^+ focus-forming units (FFU) with 5×10^6 cells for 24 h. The cells were then plated at low density and selected in 1 mg of active G418 (Gibco) per ml, and individual colonies were expanded for Southern analysis using an *Eag*I/*Sph*I fragment containing the *neo* gene as a probe.

Vector particle numbers were determined by Southern blot analysis following DNase treatment of vector stocks (20, 30). A comparison of this signal to the transduction titer determined by AP staining was used to generate the particle-to-infectivity ratio for the vector stocks.

Sequential amplification assay for rcAAV detection. Human 293 cells were seeded at either 2.5×10^5 cells per T-25 flask or 5×10^6 cells per 10-cm-diameter dish. The next day, the cultures were infected with adenovirus (MOI = 5) and infected with vector or wt AAV. After 72 h, either the cells were harvested and episomal DNA was isolated by the method of Hirt (21) or the cells were scraped into the medium, subjected to three cycles of freeze-thaw in a dry ice-ethanol bath, and centrifuged to pellet the cell debris. The clarified crude lysate was incubated at 56°C for 1 h to inactivate the adenovirus, and 500 μl was added to a fresh plate of 293 cells with or without fresh adenovirus (MOI = 5) for a second round of amplification. After an additional 72 h, the cells were scraped into the medium and pelleted, and total genomic DNA was prepared by standard techniques (31). The DNA was subjected to Southern analysis and hybridized with either a ^{32}P-labeled *Bgl*II fragment from pAV2 comprising the entire AAV2 genome or a *Hin*dIII/*Sna*BI fragment of pAV2 containing the *cap* sequence by using standard techniques (31).

PCR and sequencing. The following primers were used for PCR amplification for analysis of rcAAV: D region, 5′-CTCCATCACTAGGGGTTCC-3′ (AAV2 nt 126 to 144); rep, 5′-GGCAGATGCCCGTCAAGGT-3′ (AAV2 nt 379 to 361); and cap, 5′-CAGAGATGTGTACCTTCAG-3′ (AAV2 nt 4026 to 4044). The PCR amplification strategy and locations of primer pairs are shown in Fig. 1A. Episomal DNA was subjected to 35 cycles of PCR amplification using the D-region–rep or D-region–cap primer set. Products were separated on a 1% 3:1 agarose-NuSieve gel and detected by ethidium bromide staining. PCR products were isolated from the gel and cloned into the pCRII vector (Invitrogen) for sequence analysis using an ABI 370A sequencer (Applied Biosystems).

RESULTS

Detection of rcAAV 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 4484 flanked by adenovirus type 5 TRs (33). However, we found that vector stocks produced with AAV/Ad tested positive for rcAAV 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 ^{32}P-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 4×10^8 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 was used in conjunction with an amplimer from the 5′ end of *rep*. In a similar fashion, the 3′ end of the recombinant was analyzed by using an amplimer from the 3′ end of *cap* in conjunction with the D-region amplimer. PCR of Hirt supernatants from wt AAV amplified in 293 cells by using the 5′ amplimer set produced the expected product of 253 bp (Fig. 3A). However, Hirt supernatants from the 293-amplified tgAAVCF showed multiple discrete products, ranging from 200 to 600 bp in length, none of which comigrated with the wt AAV product. PCR with the 3′ amplimer set produced the expected 529-bp product when wt AAV was used (Fig. 3B). In contrast, the tgAAVCF vector preparation produced two discrete products of approximately 550 and 800 bp that did not comigrate with the wt product. PCR amplification of plasmids pGEM-RS5 and ptgAAVCF in the same reaction did not yield detectable products with either the 5′ or 3′ primer set (data not shown). These data indicated that novel rcAAVs had been generated during production of the tgAAVCF vector preparation.

To further characterize the rcAAV molecules present in the Hirt supernatants derived from the tgAAVCF preparation,

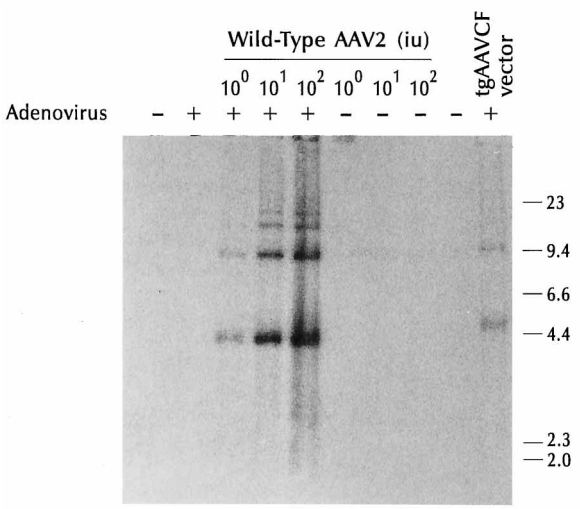

FIG. 2. Detection of a replicating AAV species in the tgAAVCF vector. Test samples were added to 293 cells with or without adenovirus, the cells were cultured for 72 h, and episomal DNA samples were analyzed for AAV2 hybridizing species. Sizes are given in kilobase pairs at the right.

PCR products from both the 5′ and 3′ amplification reactions were cloned into the pCRII cloning vector (InVitrogen) and sequenced. Four clones were isolated from the 5′ reaction, and two clones were isolated from the 3′ reaction. All of the clones showed linkage between the TR D region derived from the tgAAVCF vector and either *rep* or *cap* sequences found on the pGEM-RS5 packaging plasmid. The sequences 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 MT*rep*/CMV*cap* (Fig.

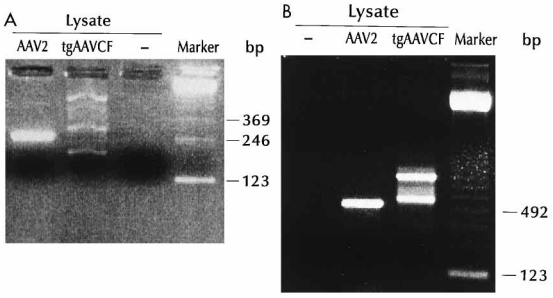

FIG. 3. PCR analysis of rcAAV present in the tgAAVCF preparation with 5′ amplimers (A) or 3′ amplimers (B).

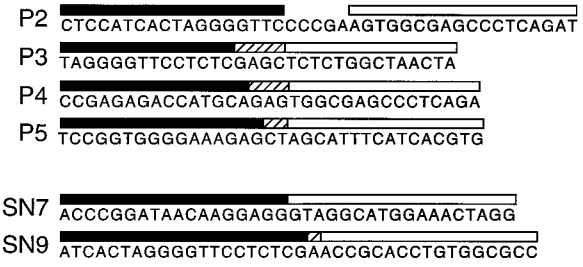

FIG. 4. Fine structure analysis of crossover sequences. P2 to P5 are TR D-region–rep-amplified species; SN7 and SN9 are TR D-region–cap-amplified species. ■, vector plasmid; □, packaging plasmid; ▨, shared sequence; blank, new sequence.

1B). This plasmid uses heterologous promoters to transcribe mRNA containing the *rep* coding sequence, similar to pRS5, as well as the *cap* ORFs. The transcription units were inverted, relative to each other, to eliminate the possibility of generating rcAAV by a simple deletion of the intervening sequence and recombination with the vector to add AAV TRs. We compared the production of AAV-LAPSN using MT*rep*/CMV*cap* to that using AAV/Ad in parallel. Both packaging plasmids were able to provide the necessary AAV *rep* and *cap* functions required for production of vector. Vector yield was evaluated by measuring AP^+ FFU on IB3 cells. Use of the AAV/Ad plasmid resulted in an AP vector titer of $\sim 5 \times 10^5$ FFU per 5×10^6 cells transfected; use of MT*rep*/CMV*cap* resulted in approximately 3×10^5 FFU per 5×10^6 cells. In crude lysates before CsCl purification, we have obtained up to 10 AP^+ FFU per cell with either packaging plasmid system (data not shown). These results indicate that the MT*rep*/CMV*cap* construct can be used to produce AAV vectors with yields comparable to those seen with AAV/Ad.

The AAV-LAPSN stocks produced by using MT*rep*/CMV*cap* and AAV/Ad were analyzed for the presence of rcAAV. Equal numbers of infectious virions (2.5×10^5 AP^+ FFU) were analyzed by the sequential amplification assay. The rcAAV was easily detected in AAV-LAPSN produced by using AAV/Ad but was not detectable even after two rounds of amplification when the MT*rep*/CMV*cap* construct was used to generate the vector (Fig. 6). These data show that in contrast to AAV/Ad, the vector produced with MT*rep*/CMV*cap* was not contaminated with rcAAV.

A second AAV vector can be produced in the absence of detectable rcAAV by using MT*rep*/CMV*cap*. We tested the MT*rep*/CMV*cap* system for production of rcAAV-free AAV vector stocks by using a second AAV vector, AAV-GrTHLAP (Fig. 1B). AAV-GrTHLAP produced by using MT*rep*/CMV*cap* yielded 6×10^5 AP^+ FFU per 5×10^6 cells transfected. AAV-GrTHLAP produced by using AAV/Ad had a yield of 2×10^6 AP^+ FFU per 5×10^6 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 (2.5×10^6 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 2.5×10^6 AP^+ FFU, the AAV-GrTHLAP vector produced with MT*rep*/CMV*cap* 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 MT*rep*/CMV*cap* 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 dilute plate and expanded for further analysis. Total cellular DNA from six representative clones was prepared, digested with either *Hin*dIII or *Eco*RI, and analyzed by Southern hybridization with a *neo* probe (Fig. 8). *Hin*dIII and *Eco*RI cut 5′ of the *neo* gene in AAV-LAPSN, and the distances from the *Hin*dIII and *Eco*RI 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 *Eco*RI digest of clone 5

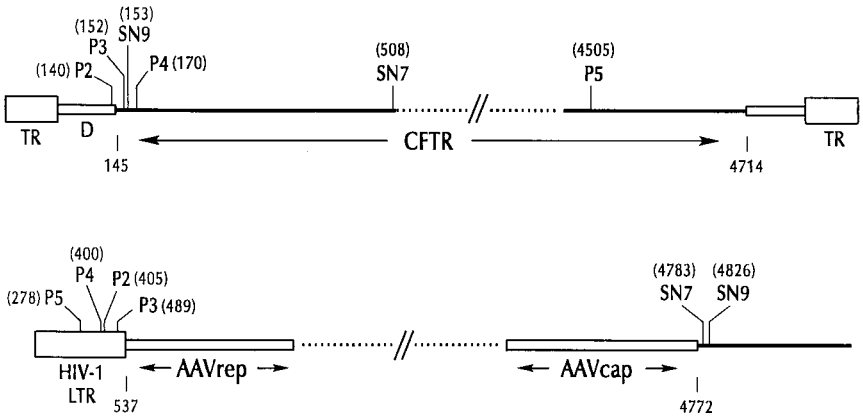

FIG. 5. Analysis of crossover points of cloned rcAAVs. P2 to P5 are TR D-region–rep-amplified species; SN7 and SN9 are TR D-region–cap-amplified species. Numbers refer to nucleotide numbers of the recombination junction. Top, tgAAVCF vector; bottom, pGEM-RS5 vector (not to scale).

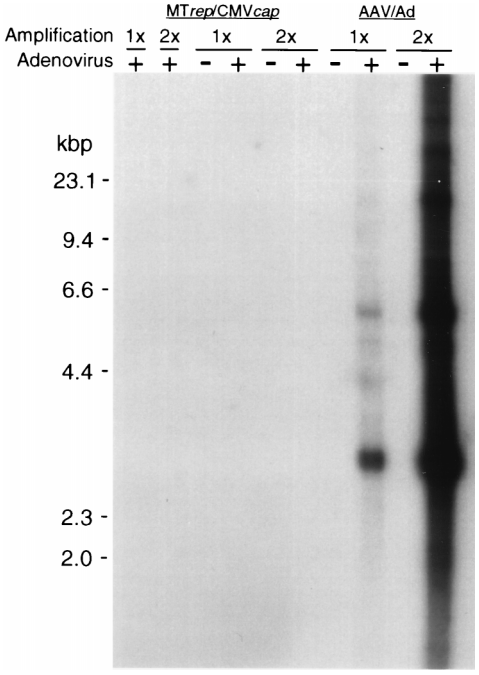

FIG. 6. Detection of rcAAV in AAV-LAPSN virions produced with AAV/Ad but not with MT*rep*/CMV*cap*. Shown is Southern analysis of total genomic DNA harvested from 293 cells after one (1×) or two (2×) rounds of infection with adenovirus alone (MOI = 5) or AAV-LAPSN (2.5×10^5 AP^+ FFU) with or without adenovirus (MOI = 5). The samples were digested with *Hin*dIII and probed with the AAV *cap* gene fragment. *Hin*dIII 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 2.8-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).

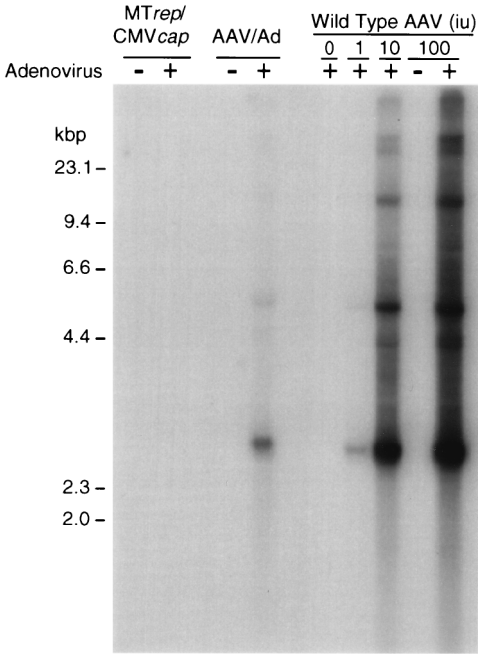

FIG. 7. Detection of rcAAV in AAV-GrTHLAP virions produced with AAV/Ad but not with MT*rep*/CMV*cap*. Shown is a Southern blot of total genomic DNA harvested from 293 cells after two rounds of infection with AAV-GrTHLAP (2.5×10^6 AP^+ FFU) with or without adenovirus (MOI = 5). The samples were digested with *Hin*dIII and probed with an AAV *cap* gene fragment. A standard curve of wt AAV infection is shown to the right.

(Fig. 8). The pattern of hybridization indicates that the lines contained a single or low number of integrated AAV-LAPSN genomes. This analysis confirmed that the rcAAV-free AAV-LAPSN stocks generated with MT*rep*/CMV*cap* are capable of integration into the genomes of transduced cells.

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 cotransfection of TR^+ rep^- cap^- genomes with TR^- rep^+ cap^+ genomes results in the production of phenotypically wt AAV (34). This process is quite efficient with regions of genome overlap of several hundred base pairs but occurs even with overlap of only 6 bp at each end. More recently, the AAV/Ad packaging system, consisting of vector and helper plasmids without significant overlap but containing abutting homology, has been described (33). Initial reports indicated that the AAV/Ad system did not produce phenotypically wt AAV at detectable frequency (33). Recently, however, the necessity for increasing vector production for gene therapy purposes resulted in the development of sensitive amplification assays like the one described in this report. The data presented here and in another recent report (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 mamma-

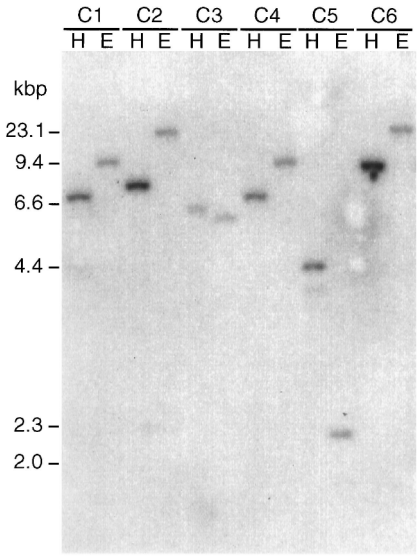

FIG. 8. Analysis of integrated AAV-LAPSN vector genomes in clonal G418-resistant 293 lines. Total cellular DNA was isolated from six independent AAV-LAPSN-transduced lines (C1 to C6) and digested with either *Hin*dIII (H) or *Eco*RI (E). After transfer, the blot was probed with a DNA fragment containing the *neo* gene.

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 MT*rep*/CMV*cap* construct allowed production of vector stocks free of rcAAV. Two vectors (AAV-LAPSN and AAV-GrTH-LAP) were evaluated, and up to 2.5×10^6 AP^+ 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 MT*rep*/CMV*cap* 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 MT*rep*/CMV*cap* 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 virus LTR 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 be not a safety issue but rather a complicating factor in experiments designed to investigate AAV biology.

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REFERENCES

1. **Afione, S. A., C. K. Conrad, W. G. Kearns, S. Chunduru, R. Adams, T. C. Reynolds, W. B. Guggino, G. R. Cutting, B. J. Carter, and T. R. Flotte.** 1996. In vivo model of adeno-associated virus vector persistence and rescue. J. Virol. **70:**3235–3241.
2. **Alexander, I. E., D. W. Russell, A. M. Spence, and A. D. Miller.** 1996. Effects of gamma irradiation on the transduction of dividing and nondividing cells in brain and muscle of rats by adeno-associated virus vectors. Hum. Gene Ther. **7:**841–850.
3. **Bacolla, A., and F. Y.-H. Wu.** 1991. Mung bean nuclease cleavage pattern at a polypurine.polypyrimidine sequence upstream from the mouse metallothionein-I gene. Nucleic Acids Res. **19:**1639–1647.
4. **Besnard, F., M. Brenner, Y. Nakatani, R. Chao, H. J. Purohit, and E. Freese.** 1991. Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. J. Biol. Chem. **266:**18877–18883.

5. **Boissy, R., and C. R. Astell.** 1985. An *Escherichia coli recBCsbcBrecF* host permits the deletion-resistant propagation of plasmid clones containing the 5′-terminal palindrome of minute virus of mice. Gene **35:**179–185.
6. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41:**521–530.
7. **Carter, B. J.** 1992. Adeno-associated virus vectors. Curr. Opin. Biotechnol. **3:**533–539.
8. **Chejanovsky, N., and B. J. Carter.** 1989. Mutagenesis of an AUG codon in the adeno-associated virus *rep* gene: effects of viral DNA replication. Virology **173:**120–128.
9. **Dong, J., P. Fan, and R. A. Frizzell.** 1996. Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. Hum. Gene Ther. **7:**2101–2112.
10. **Dorsett, D. L., I. Keshet, and E. Winocour.** 1983. Quantitation of a simian virus 40 nonhomologous recombination pathway. J. Virol. **48:**218–228.
11. **Fisher, K. J., G.-P. Gao, M. D. Weitzman, R. DeMatteo, J. F. Burda, and J. M. Wilson.** 1996. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. J. Virol. **70:**520–532.
12. **Flotte, T. R., X. Barraza-Ortiz, R. Solow, S. A. Afione, B. J. Carter, and W. B. Guggino.** 1995. An improved system for packaging recombinant adeno-associated virus vectors capable of *in vivo* transduction. Gene Ther. **2:**29–37.
13. **Giraud, C., E. Winocour, and K. I. Berns.** 1994. Site-specific integration by adeno-associated virus is directed by a cellular DNA sequence. Proc. Natl. Acad. Sci. USA **91:**10039–10043.
14. **Glanville, N., D. M. Durnam, and R. D. Palmiter.** 1981. Structure of mouse metallothionein-I gene and its mRNA. Nature **292:**267–269.
15. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of human cell line transformed by DNA from human adenovirus type 5. J. Virol. **36:**59–74.
16. **Grima, B., A. Lamouroux, F. Blanot, N. F. Biguet, and J. Mallet.** 1985. Complete coding sequence of rat tyrosine hydroxylase mRNA. Proc. Natl. Acad. Sci. USA **82:**617–621.
17. **Grossman, Z., E. Winocour, and K. I. Berns.** 1984. Recombination between simian virus 40 and adeno-associated virus: virion coinfection compared to DNA cotransfection. Virology **134:**125–137.
18. **Grossman, Z., K. I. Berns, and E. Winocour.** 1985. Structure of simian virus 40-adeno-associated virus recombinant genomes. J. Virol. **56:**457–465.
19. **Halbert, C. L., I. E. Alexander, G. M. Wolgamot, and A. D. Miller.** 1995. Adeno-associated virus vectors transduce primary cells much less efficiently than immortalized cells. J. Virol. **69:**1473–1479.
20. **Halbert, C. L., T. A. Standaert, M. L. Aitken, I. E. Alexander, D. W. Russell, and A. D. Miller.** 1997. Transduction by adeno-associated virus vectors in the rabbit airway: efficiency, persistence, and readministration. J. Virol. **71:**5932–5941.
21. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
22. **Koeberl, D. D., I. E. Alexander, C. L. Halbert, D. W. Russell, and A. D. Miller.** 1997. Persistent expression of human clotting factor IX from mouse liver after intravenous injection of adeno-associated virus vectors. Proc. Natl. Acad. Sci. USA **94:**1426–1431.
23. **Laughlin, C. A., M. W. Myers, D. L. Risin, and B. J. Carter.** 1979. Defective-interfering particles of the human parvovirus adeno-associated virus. Virology **94:**162–174.
24. **Laughlin, C. A., J.-D. Traschin, H. Coon, and B. J. Carter.** 1983. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. Gene **23:**65–73.
25. **Lieber, M., B. Smith, A. Szakal, W. Nelson-Rees, and G. Todaro.** 1976. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int. J. Cancer **17:**62–70.
26. **Mendelson, E., J. P. Trempe, and B. J. Carter.** 1986. Identification of the *trans*-acting Rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. J. Virol. **60:**823–832.
27. **Miller, C. K., and H. M. Temin.** 1983. High-efficiency ligation and recombination of DNA fragments by vertebrate cells. Science **220:**606–609.
28. **Muzyczka, N.** 1992. Use of adeno-associated virus as a general transduction vector for mammalian cells. Curr. Top. Microbiol. Immunol. **158:**97–129.
29. **Reddy, V. B., B. Thimmappaya, R. Dhar, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman.** 1978. The genome of simian virus 40. Science **200:**494–502.
30. **Russell, D. W., A. D. Miller, and I. E. Alexander.** 1994. Adeno-associated virus vectors preferentially transduce cells in S phase. Proc. Natl. Acad. Sci. USA **91:**8915–8919.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
32. **Samulski, R. J., Long-Sheng Chang, and T. Shenk.** 1987. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication. J. Virol. **61:**3096–3101.
33. **Samulski, R. J., Long-Sheng Chang, and T. Shenk.** 1989. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J. Virol. **63:**3822–3828.
34. **Senapathy, P., and B. J. Carter.** 1984. Molecular cloning of adeno-associated virus variant genomes and generation of infectious virus by recombination in mammalian cells. J. Biol. Chem. **259:**4661–4666.
35. **Srivastava, A., E. W. Lusby, and K. I. Berns.** 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. J. Virol. **45:**555–564.
36. **Trempe, J. P., E. Mendelson, and B. J. Carter.** 1987. Characterization of adeno-associated virus rep proteins in human cells by antibodies raised against rep expressed in Escherichia coli. Virology **161:**18–28.
37. **Trempe, J. P., and B. J. Carter.** 1988. Alternate mRNA splicing is required for synthesis of adeno-associated virus VP1 capsid protein. J. Virol. **62:**3356–3363.
38. **Vincent, K. A., S. T. Piraino, and S. C. Wadsworth.** 1997. Analysis of recombinant adeno-associated virus packaging and requirements for *rep* and *cap* gene products. J. Virol. **71:**1897–1905.
39. **Winocour, E., and I. Keshet.** 1980. Indiscriminate recombination in simian virus 40-infected monkey cells. Proc. Natl. Acad. Sci. USA **77:**4861–4865.
40. **Winocour, E., V. Lavie, and I. Keshet.** 1983. Structure of simian virus 40-ϕX174 recombinant genomes isolated from single cells. J. Virol. **48:**229–238.
41. **Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gonczol, and J. M. Wilson.** 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc. Natl. Acad. Sci. USA **91:**4407–4411.
42. **Yang, Y., G. Trinchieri, and J. M. Wilson.** 1995. Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung. Nat. Med. **1:**890–893.
43. **Yei, S., N. Mittereder, K. Tang, C. O'Sullivan, and B. C. Trapnell.** 1994. Adenovirus-mediated gene transfer for cystic fibrosis: quantitative evaluation of repeated in vivo vector administration to the lung. Gene Ther. **1:**192–200.
44. **Zeitlin, P. L., L. Lu, J. Rhim, G. Cutting, G. Stetten, K. A. Kieffer, R. Craig, and W. B. Guggino.** 1991. A cystic fibrosis bronchial epithelial cell line: immortalization by adeno-12-SV40 infection. Am. J. Respir. Cell Mol. Biol. **4:**313–319.