Efficient Generation and Amplification of High-Capacity Adeno-Associated Virus/Adenovirus Hybrid Vectors

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Effective gene therapy is dependent on safe gene delivery vehicles that can achieve efficient transduction and sustained transgene expression. We are developing a hybrid viral vector system that combines in a single particle the large cloning capacity and efficient cell cycle-independent nuclear gene delivery of adenovirus (Ad) vectors with the long-term transgene expression and lack of viral genes of adeno-associated virus (AAV) vectors. The strategy being pursued relies on coupling the AAV DNA replication mechanism to the Ad encapsidation process through packaging of AAV-dependent replicative intermediates provided with Ad packaging elements into Ad capsids. The generation of these high-capacity AAV/Ad hybrid vectors takes place in Ad early region 1 (E1)-expressing cells and requires an Ad vector with E1 deleted to complement in trans both AAV helper functions and Ad structural proteins. The dependence on a replicating helper Ad vector leads to the contamination of AAV/Ad hybrid vector preparations with a large excess of helper Ad particles. This renders the further propagation and ultimate use of these gene delivery vehicles very difficult. Here, we show that Cre/loxP-mediated genetic selection against the packaging of helper Ad DNA can reduce helper Ad vector contamination by 99.98% without compromising hybrid vector rescue. This allowed amplification of high-capacity AAV/Ad hybrid vectors to high titers in a single round of propagation.

Viral gene delivery is currently the most efficient method to introduce foreign genetic information into target cells for the purpose of gene therapy. Considering the postmitotic status of most cells in the human body, it is important that viral vectors be able to transduce quiescent cells. In addition, the gene transfer vehicle should be minimally immunogenic and should provide sustained transgene expression. Adeno-associated virus (AAV)-based vectors have been shown to fulfill these requirements in animal models of human disease. Accordingly, they are being used in clinical trials for, among others, cystic fibrosis, limb girdle muscular dystrophy, and hemophilia B (1, 14, 19). The parental AAV is a small nonpathogenic parvovirus with an ~4.7-kb linear single-stranded DNA genome. AAV is a defective virus, since it requires the presence of a coinfecting helper virus (e.g., adenovirus) for productive infection. Adenovirus (Ad) helper activities are provided by E1a, E1b, E2a, V4 RNA, and E4 open reading frame (ORF) 6 expression. In the absence of helper virus, AAV DNA integrates with high prevalence into the AAV1 locus on the human chromosome 19. When a latently infected cell is superinfected with a helper virus, the AAV DNA is rescued from the host chromosomal DNA, replicates to high levels through double-stranded replicative intermediates, and at the end of the productive cycle, single-stranded genomes of both polarities are packaged into preformed empty capsids. The AAV genome consists of inverted terminal repeats (ITRs) flanking two genes: rep, encoding the nonstructural proteins Rep78, Rep68, Rep52, and Rep40, and cap, encoding the structural proteins VP1, VP2, and VP3. Due to the presence of palindromic sequences within the AAV ITRs, these elements can acquire characteristic T-shaped hairpin secondary structures. The 145-bp ITRs contain all cis-acting elements necessary for genome replication, packaging, and integration into host chromosomal DNA (reviewed in reference 3). Since the viral coding sequences are segregated from the cis-acting elements, AAV-based vectors can be produced devoid of viral genes. Typically, AAV vectors are generated by transfecting producer cells with a plasmid containing a recombinant AAV (rAAV) genome constituted exclusively of exogenous DNA flanked by AAV ITRs. In the presence of Ad helper functions and a plasmid expressing in trans the AAV rep and cap genes, the rAAV genome recapitulates the lytic phase of the wild-type virus by being rescued from the plasmid backbone, replicated, and packaged into AAV capsids (reviewed in reference 25). The fact that AAV vectors are based on a nonpathogenic defective virus contributes significantly to their attractiveness for human gene therapy. Moreover, rAAV can be made “gutless,” which prevents the expression of potentially immunogenic viral proteins and the elimination of transduced cells by the host immune system. Recent studies of the molecular fate of rAAV molecules in vivo indicate that the remarkably stable structures acquired by rAAV genomes also add to the long-term transgene expression achieved with these vectors. The persistent rAAV DNA forms are integrated genomes (21) as well as circular double-stranded high-molecular-
weight concatemers (5). In spite of steady improvements in rAAV production (15), vector purification (34), and capsid modification (11, 32), a fundamental problem associated with this highly promising vector system is its limited cloning capacity of approximately 4.7 kb (4). This precludes the delivery of large DNA molecules (e.g., the dystrophin ORF or the β-globin locus control region) in a single AAV particle. Other limitations of AAV-based vectors include their relatively slow nuclear import (2, 17), a high dependence on particular milieus for efficient intracellular trafficking (6, 23, 33), and, once in the nucleus, the requirement to convert the single-stranded DNA genomes into transcriptionally competent double-stranded templates (9, 10).

We are developing a high-capacity AAV/Ad hybrid vector system that overcomes the limited packaging capacity of AAV particles and exploits the efficient cell cycle-independent Ad capsid-mediated nuclear gene delivery. This system is based on enlarged rAAV genomes provided with Ad packaging elements. In an earlier study, it was shown that in Ad early region 1 (E1)-complementing cells, these AAV/Ad chimeric genomes can be rescued, replicated, and packaged into Ad capsids in the presence of AAV rep gene products and an Ad vector with E1 deleted. The latter vector provided in trans AAV helper functions and Ad structural proteins (12). Although the feasibility of this approach has been demonstrated, the reliance on a replicating Ad vector led to the generation of high-capacity AAV/Ad hybrid vectors together with a very large excess of Ad particles with E1 deleted. The extreme contamination with helper Ad vectors with E1 deleted rendered the further propagation and ultimate use of AAV/Ad hybrid vectors very difficult. To overcome this problem, we tested whether Cre/loxP-mediated selection against the assembly of helper Ad particles, successfully employed in high-capacity Ad vector production (22), would allow us to rescue and efficiently propagate high-capacity AAV/Ad hybrid vectors. To this end, we generated PER.C6-based Cre recombinase-expressing cell lines and Ad vectors with E1 deleted containing packaging elements flanked by bacteriophage P1 loxP sequences (Ad.foxedΨ). Here we show that Ad.foxedΨ-infected Cre-expressing cells, Cre/loxP-mediated excision of the packaging elements rendered the helper Ad genomes packaging deficient while enabling them to replicate and express in trans AAV helper functions and Ad structural proteins necessary for the production of hybrid vector particles. Moreover, using this genetic selection against the packaging of helper Ad DNA, efficient amplification of high-capacity AAV/Ad hybrid vectors was achieved, circumventing the need for extensive serial propagations.

**MATERIALS AND METHODS**

**Cells.** For the production of viral vectors and for plaque assays, we used PER.C6 cells (7). These E1-transformed human embryonic retinoblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL) containing 10% fetal bovine serum (FBS) and 10 mM MgCl₂. Transduction experiments were performed in HeLa cells (American Type Culture Collection) cultured in DMEM supplemented with 5% FBS. Endpoint titrations of preparations of Ad vectors with E1 deleted were carried out in E1-complementing 911 cells (8) maintained in DMEM containing 10% FBS. All cells were cultured at 37°C in a humidified air–10% CO₂ atmosphere.

**DNA constructions.** Recombinant DNA techniques were performed using established methods (24) or according to the instructions supplied with specific reagents. Escherichia coli strain DH5α (Gibco BRL) was used for all DNA transformations. To make construct pCMV.TA, the plasmid pHUD15-1 (13) was linearized with EcoRI, treated with the large fragment of E. coli DNA polymerase I (Klenow; Gibco BRL), and digested with HindIII. To 1.0-kb fragment containing the tetacycline resistance transcriptional activator (tTA) coding sequence was then cloned into EcoRV- and BamHI-digested pDNA3.1/Hygro− (Invitrogen). Plasmid pRSV.Cre.IRESpuro, used to generate the PER.I.TA.Cre cell lines, contains a bicistronic expression cassette composed of the Rous sarcoma virus (RSV) promoter, the bacteriophage P1 cre gene, and the eukaryophilic cytomegalovirus immediate-early (IECMV) promoter of pRSVpuro2 was replaced with an RSV promoter. To this end, pRSVpuro2 was digested with NcoI and EcoRV, and the 4.5-kb digestion product containing the vector backbone was purified from gel and dephosphorylated using thermosensitive alkaline phosphatase (Gibco BRL). Next, this DNA molecule was ligated to the filled-in 0.4-kb MluI-HindIII fragment from pRSVpuro (GenBank accession no. M77786) containing the RSV promoter. This gave rise to plasmid pRSV.IRESpuro. Finally, the 1.1-kb XhoI-MluI fragment from plasmid pBS185 (Gibco BRL) with the cre gene was filled in and inserted into Nhel-linearized and Klenow-blunted pRSV.IRESpuro.

To generate pHV.EGFP, the ORF coding for the aestuorea victoria enhanced green fluorescent protein (EGFP) was excised from plasmid pEGFP.N1 (Clontech) with XhoI and cloned into the unique SpeI site of the eukaryotic expression plasmid pEFO (30), resulting in construct pEFO.EGFP. After SaII and AarI digestion of pEFO.EGFP, the 4.4-kb insert containing the EGFP expression cassette was isolated and blunt ended with T4 DNA polymerase (Gibco BRL). Next, this DNA fragment was inserted into the EcoRV site of pHZeoAdTRP, resulting in the construct pZeoAdTRP.EFO.EGFP. pZeoAdTRP was created by inserting into the BamHI site of cloning vector pZeoSV2A+ (Invitrogen) the 0.5-kb BglII fragment from pAdTRP.R (12) comprising the first 454 bp of the Ad serotype 5 (Ad5) genome. Subsequently, the EGFP expression cassette and contiguous Ad sequence were excised from pZeoAdTRP.EFO.EGFP with Msel. This insert was ligated to the 6.7-kb filled-in ClaI-BglII fragment from pPAAAdTRP.R.5 (12) consisting of the AAV ITRs and vector backbone. This plasmid cloning step created the hybrid vector shuttle construct pPAAAd/TRP.5 (23) containing the 0.5-kb AdTRP.R insert and the 6.7-kb digestion product of pPAAAdTRP.R.5 (12) containing the 3.3-kb AdTRP.R insert, which was digested with StuI and ligated to the SpeI site of pAAV. Next, this DNA fragment was inserted into the EcoRV site of pZeoAdTRP, resulting in construct pZeoAdTRP.EFO.EGFP. pHV.IRESpuro2 was obtained by inserting a 9.1-kb AdE1 flanking fragment from intron 44 of the human dystrophin gene (GenBank accession no. M66524) into the unique SpeI site of pAAV. AdCMV. The hybrid vector shuttle construct pHV.IcaZ was generated by insertion of a 9.6-kb SpeI flanking fragment from intron 44 of the human dystrophin gene into the SpeI site of pAAVAd TRP.R (12). pHV.IcaZ was obtained by exactly the same procedure as described for pHV.IacZ except that the Ad terminal repeat (TR)-specific primer initially used to amplify the ade1-exacting elements had the sequence 5′-CCAA GTTAGAC1TCTTATTTGGATAGAC1GCAAATAGTAT3′. The reverse primer was pPS5.PRep contains the AAV rep ORF under the control of its endogenous promoters, whereas pCMVE2a has the Ad5 E2a gene driven by the IE-CMV promoter from cloning vector pcDNA3 (Invitrogen). The E2a gene encodes the Ad DNA-binding protein (DBP).

To construct the helper Ad shuttle plasmid pAd.foxedΨ with E1 deleted, the 5′ end of the Ad.Cscluc genome (22) was amplified by PCR using elongase enzyme mix (Gibco BRL) in combination with the upstream primer 5′-ACCTT AATTTACATCATCATAATATACCTTTA′ containing a PacI site and the downstream primer 5′-GCA TGCAGGCCGTGGTACAAATAGT3′. The mixture was heated at 94°C for 30 s followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 90 s. The reaction was completed by a 10-min incubation at 68°C. The resulting DNA fragment contained the left Ad TR, the Ad packaging elements AI through AIV, and a truncated A repeat V (27). These packaging elements are flanked by a direct repeat of loxP sites from bacteriophage P1. Subsequently, the PCR product was inserted into the plasmid pCR2.1 using the TA cloning system (Invitrogen), giving rise to construct pCR.AdTR.foxedΨ. The nucleotide sequence of the cloned PCR fragment was determined by the dideoxy chain termination method using an ABI PRISM 3700 DNA analyzer and the ABI PRISM BigDye Terminator version 3.1 cycle-sequencing system (Applied Biosystems). To knock out the PacI recognition sequence located between the loxP sites, pCR.AdTR.foxedΨ was partially digested with PacI. Following purification of the linearized pCR.AdTR.foxedΨ, the DNA was blunt ended with T4 DNA polymerase and recircularized using T4 DNA ligase (Gibco BRL). These maneuvers generated the construct pCR.AdTR.foxedΨ.PsiI, containing a 0.5-kb PacI fragment and the Ad packaging elements flanked by loxP sites. pAdMire was derived from the cloning vector pBR322 and...
contains the Ad5 sequence from nucleotides 1 through 454, a multiple cloning site, and the Ad5 sequence from nucleotides 3551 to 6096. The PacI site upstream of the Ad TR in pAd.floxAp was used to linearize the shuttle plasmid before transfection.

**Generation, purification, and titration of helper Ad vectors with E1 deleted.** The helper Ad vector Ad.floxAp/E1 was deleted was generated through homologous recombination in PER.C6 cells transfected with 6 μg of PacI-digested pWEAd/A/H1-IIrTR.pac.RbB5 (20) and 2 μg of PacI-treated pAd.floxAp. The linearized constructs were diluted in DMEM to a total volume of 100 μl, gently mixed with 100 μl of a 1:1 suspension of lipofectamine (Gibco BRL) in DMEM, and incubated for 30 min at room temperature. Subsequently, the DNA-liposome complexes were supplemented with 2.3 ml of DMEM and added to a confluent monolayer of PER.C6 cells washed with serum-free medium. After incubation for 2 h at 37°C, 2.5 ml of DMEM containing 20% FBS and 20 mM MgCl2 was added to the cell culture. At 24 h posttransfection, the culture medium was replaced by 5 ml of DMEM containing 10% FBS and 10 mM MgCl2, and the cells were incubated for another 24 h at 37°C. Next, the cells were trypsinized and transferred to an 80-cm2 culture flask (Nunc). Six to 8 days after transfection, the cells were harvested and lysed by repeated freezing and thawing. The cellular debris was pelleted by centrifugation, and the supernatant was stored at −20°C. Subconfluent monolayers of PER.C6 cells were then inoculated with a 1:1 mixture of the clarified cell lysate to produce an initial vector stock. Next, a plaque assay on PER.C6 cells was performed, and vectors derived from single isolated plaques were expanded for large-scale production.

For the preparation of large stocks of Ad vectors with E1 deleted, subconfluent monolayers of PER.C6 cells in 16 175-cm2 culture flasks (Nunc) were infected with plaque-purified virions. At the stage of pronounced cytopathic effect (CPE), the cell cultures were harvested by centrifugation, lysed by freezing and thawing, and resuspended in 10 mM sodium phosphate (pH 7.2 to 7.4) containing 7% glycerol. This material was then incubated for 30 min at 37°C in the presence of 0.5% (wt/vol) deoxycholic acid. Next, MgCl2 and DNase I (Roche) were added to final concentrations of 30 mM and 7 μg/ml, respectively. After homogenization, an incubation period of 20 min at 37°C followed. Subsequently, cell debris was removed by low-speed centrifugation, and the recovered supernatants were loaded onto a block gradient consisting of 1.4- and 1.2-g/ml CsCl solutions containing 5% (vol/vol) glycerol and buffered with 30 mM Tris-HCl (pH 8.0). After centrifugation at 21,000 rpm for 2 h at 10°C in an SW28 rotor (Beckman), the vector was concentrated at the interface between the two CsCl solutions and was retrieved by puncturing the centrifugation tube. The recovered particles were transferred to a quick-seal tube (Beckman) containing a Tris-buffered 1.33-g/ml CsCl solution and spun overnight at 55,000 rpm in a VTI 65.1 rotor (Beckman) at 10°C. After sedimentation, the vector particles were collected, and the CsCl was removed by extensive dialysis against phosphate-buffered saline with 5% (wt/vol) sucrose. The purified Ad vector batches were divided into aliquots and stored at −80°C.

The infectious titers of stocks of Ad vectors with E1 deleted were determined by end-point dilution in 96-well plates containing 5 x 10^5 HeLa cells per well. Two weeks after infection with serially diluted Ad vector preparations, wells with CPE were marked with the aid of an Olympus CK2-TR optical microscope. The control vector Ad.ΔE3 was deleted and containing a nonfloxed packaging signal was generated, purified, and titrated as described for Ad.floxAp.

**Generation of PER.tTA and PER.tTA.Cre cell lines.** The parental cell line PER.tTA was obtained by stable transfection of PER.C6 cells with pCMV.ΔC. Hygromycin B phosphotransferase was used as a selectable marker, and for selection, culture medium containing 50 μg of hygromycin B (Gibco BRL)/ml was used. To generate PER.tTA.Cre cell lines, PER.tTA cells were transfected with pRSV.Cre.IRESpu, and individual clones were selected and expanded in the presence of puromycin (Clontech) at a final concentration of 0.4 μg/ml. The use of PER.tTA instead of PER.C6 as the parental cell line might allow future optimization of vector production through regulated gene expression. The Cre-negative control cell line PER.tTA.Puro5 was generated by stable transfection of PER.tTA cells with pRSV.IRESpu.

**Cre activity assays.** Six million E1-complementing cells (i.e., PER.C6, PER.tTA.puro5, and PER.tTA.Cre cell clones 100, 43, 76, and 36) were seeded in 25-cm2 culture flasks (Nunc). The next day, Ad.floxAp/E1 was added to the cells at a multiplicity of infection (MOI) of 5 infectious units (IU)/cell. After a 24-h incubation period, the inoculum was removed and DMEM supplemented with 10% FBS and 10 mM MgCl2 was added to the infected cells. Approximately 72 h posttransfection, the DNA was extracted according to the modified Hirt protocol previously described in detail (12). After agarose gel electrophoresis, the DNA was transferred by capillarity to a nylon membrane (Hybond N; Amersham Pharmacia Biotech) and incubated with an [α-32P]dCTP-labeled DNA fragment containing nucleotides 1 through 191 of the Ad5 genome. This probe was labeled using the RITs RadPrime DNA labeling system (Gibco BRL).

In parallel, 6 x 10^6 PER.tTA.Cre76 or PER.tTA.Cre43 cells seeded in triplicate in 25-cm2 culture flasks were infected for 24 h with either Ad.floxAp or Ad.ΔE3 at an MOI of 5 IU/cell. After the development of complete CPE, the cells were harvested and lysed by repeated freezing and thawing, and the cellular debris was removed by centrifugation at 208 x g for 10 min. The recovered supernatants were clarified by filtration through 0.45-μm-pore-size filters (Millipore). The yields of both Ad vectors with E1 deleted were determined by comparison on 911cell lines.

**Rescue, amplification, and titration of high-capacity AA/VAd hybrid vectors.** Six million PER.tTA.Cre76 cells were seeded in 100-mm-diameter culture dishes (Greiner). After overnight incubation, the cell monolayers were transfected with a mixture of 5 μg of AA/VAd hybrid vector shuttle DNA, 5 μg of pCMV.E2a, and 5 μg of pks.P5.Rep. The absence of pks.P5.Rep in control samples was compensated for by adding 5 μg of pUC19 carrier DNA (Gibco BRL). Before incubation with the transfection medium, the producer cells were harvested and lysed by three cycles of freezing and thawing. The cellular debris was pelleted by centrifugation at 208 x g for 10 min, and the recovered supernatants were clarified by filtration through 0.45-μm-pore-size filters.

For the propagation of high-capacity AA/VAd hybrid vectors, 6 x 10^6 PER.tTA.Cre76 cells were transfected with 7.5 μg of pks.P5.Rep and 7.5 μg of pCMV.E2a. Twenty-four hours later, the transfection medium was replaced with 10 ml of DMEM containing 10% FBS, 10 mM MgCl2, and Ad.floxAp/E1 at an MOI of 2.5 IU/cell. After incubation for 5 h at 37°C, the inoculum was aspirated and 10 ml of DMEM supplemented with 10% FBS plus 10 mM MgCl2 was added to the cell culture. At the stage of complete CPE, the producer cells were harvested and lysed by three cycles of freezing and thawing. The debris was pelleted by centrifugation at 208 x g for 10 min, and the recovered supernatants were clarified by filtration through 0.45-μm-pore-size filters.

Mycoplasma HeLa cells were used to determine background signal intensities. The infectious titers of stocks of Ad vectors with E1 deleted were determined by flow cytometric analyses as follows. HeLa cells were seeded at 10^5/well in a 24-well plate (Greiner). The next day, the confluent HeLa cell layers received 500 μl of fivefold serially diluted Ad vector stocks. Forty-eight hours later, the cells were analyzed for GFP expression using a FACSort flow cytometer (Becton Dickinson) equipped with a 488-nm-wavelength argon laser. Routinely, 10,000 events were acquired and stored in list mode files. Analyses were performed with CellQuest software (Becton Dickinson Immunocytometry Systems), and titers were calculated from the linear range of the dose-response curves. Mock-infected HeLa cells were used to determine background signal intensities.

**Extraction of AA/VAd hybrid vector DNA and AAV ITR-specific PCR.** Aliquots of hybrid vector preparations were treated with DNase I at a final concentration of 0.4 μg/ml at 30 min for 37°C in the presence of 10 mM MgCl2. Next, the nuclease was inactivated by addition of EDTA (pH 8.0), sodium dodecyl sulfate, and proteinase K (Roche) at final concentrations of 10 mM, 0.5%, and 0.1 μg/ml, respectively. The samples were then incubated at 50°C for 1 h and extracted twice with a mixture of Tris-buffered phenol, chloroform, and isomyl alcohol (24:24:1) and once with chloroform. The DNase I-resistant templates were recovered by centrifugation after ethanol and salt precipitation, resuspended in demineralized water, and subjected to touchdown PCR. The primers targeting the 5’ termini of AAV/Ad hybrid vector genomes had the sequences 5’-CAGTCTTGGCCACATC CCGTCG-3’ and 5’-TATATTCCTCACATGAGGGCAGGAGC-3’, whereas the 3’ extremity of AAV/Ad.EGFP DNA was targeted by primers with the sequences 5’-ACCCGACCATAGTAAAGGGCAGC-3’ and 5’-TTAAGGACTCATCGAGG C-3’. These PCR mixtures contained 2% (vol/vol) glycerol and 2.5 μl of SuperTag polymerase (HT Biotechnology) were subjected to the following cycling conditions: a denaturation step at 94°C for 3 min was followed by 20 cycles of 94°C for 30 s, an annealing period of 90 s with the temperature decreasing 0.5°C every cycle from 69 to 59°C, and a 1-min extension step at 68°C. Twenty-three additional cycles were carried out at the final touchdown PCR

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RESULTS

Characterization of PER.tTA.Cre cell lines. Bacteriophage P1 34-bp loxP sequences are the targets for dimers of the site-specific 38-kDa bacteriophage P1 Cre recombinase. When two loxP sites are in a directly repeated configuration, Cre catalyzes the excision of the intervening DNA. Therefore, this site-specific recombination reaction enables the conditional deletion of DNA sequences located between loxP sites (floxed), depending on the presence or absence of Cre protein. We have generated PER.C6-based Cre-expressing cell lines to test whether assembly of helper Ad vectors could be prevented through Cre/loxP-mediated removal of their packaging signals without compromising high-capacity AAV/Ad hybrid vector yields. For this purpose, we constructed the plasmid pRSV.Cre.IRESpuro, which directs expression of a bicistronic mRNA encoding both Cre recombinase and puromycin N-acetyltransferase. Twenty-five single-cell clones were obtained by stable transfection of pRSV.Cre.IRESpuro into PER.tTA cells. These cell lines displayed various degrees of Cre activity. Three clones did not show detectable levels of functional Cre protein, whereas the PER.tTA.Cre clones 100, 43, 76, and 36 had the highest recombination activities (see below). The levels of functional Cre recombinase were evaluated by determining the extent to which the packaging elements of Ad.floxedΨ genomes could be excised. To this end, two assays were set up. One assay was based on restriction enzyme fragment length analyses of de novo-synthesized Ad.floxedΨ DNA extracted from PER.tTA.Cre cells (Fig. 1A). Control samples consisted of Ad.floxedΨ DNA extracted from PER.C6 cells (7) and from PER.tTA cells stably transfected with the cre-negative pRSV.IRESpuro construct. pRSV.IRESpuro has precisely the same genetic constitution as pRSV.Cre.IRESpuro except for the absence of the cre ORF. Southern blot analyses of SphI-digested Ad.floxedΨ DNA extracted from four PER.tTA.Cre clones (i.e., 100, 43, 76, and 36) showed very high site-specific recombinase activity, since 491-bp fragments, expected from template DNA molecules that went through Cre/loxP-mediated excision, were the strikingly prominent species (Fig. 1B). SphI-digested Ad.floxedΨ DNA extracted from either Cre-negative cell line yielded exclusively 751-bp fragments corresponding to unrecombined templates (Fig. 1B). In addition, the signal intensities indicate that the Ad.floxedΨ genomes replicated to the same extent regardless of the presence or absence of Cre. This is important, because the major late promoter that drives expression of most Ad structural proteins is dependent on viral DNA replication for full activation (18). Another assay consisted of quantifying infectious vector particles produced by Cre-positive cells infected with Ad.floxedΨ or a control with a nonfloxed packaging signal Ad vector (Ad.Ψ). Data obtained from clones PER.tTA.Cre76 and PER.tTA.Cre43 showed that Cre/loxP-mediated excision of the Ad packaging elements resulted in an average reduction of helper Ad vector titer of 99.4 and 99.9%, respectively (Fig. 2). Taken together, these data indicate that production of Ad.floxedΨ was efficiently blocked at the DNA-packaging level as a consequence of the very robust expression of functional Cre protein in the selected PER.tTA.Cre cell clones.

High-capacity AAV/Ad hybrid vectors can be generated in E1-transformed Cre-expressing cells using helper Ad.floxedΨ vectors. Although the high-capacity AAV/Ad hybrid vector principle has been demonstrated, the dependence on a replicating Ad vector with E1 deleted led to the production of hybrid vector particles (3.1 × 10^6 LTU/ml) together with a large excess of helper Ad (3.2 × 10^9 lacZ transducing units [LTU]/ml) (12). Considering that, especially at high MOI, the E1 deletion can be partially complemented by E1-like cellular activities (29), first-generation Ad vectors can be toxic to cells due to leaky viral gene expression and limited viral replication. As a consequence, the utility of preparations containing small amounts of AAV/Ad hybrid vector and high titers of Ad vector...
with E1 deleted is very limited. Hence, we decided to test the capacity of the Cre/loxP system to prevent the assembly of helper Ad vectors with E1 deleted while still enabling the rescue of AAV/Ad chimeric genomes into Ad capsids. To this end, the pHV.EGFP shuttle construct containing an AAV/Ad chimeric genome consisting of AAV ITRs flanking the left Ad TR plus contiguous packaging signal, an EGFP expression cassette, and stuffer DNA (Fig. 3A) was transfected into PER.tTA.Cre76 cells together with an AAV rep expression construct (pKS.P5.Rep) and, to enhance AAV-dependent replication (12), an Ad E2a expression plasmid (pCMV.E2a). Next, the cells were infected with Ad.floxedΨ to provide in trans early Ad gene products that support AAV replication and late Ad proteins required for capsid assembly. Control experiments consisted of transfections performed without the AAV rep expression plasmid pKS.P5.Rep. The cell cultures were harvested after detection of complete CPE, and cleared lysates were used to infect HeLa indicator cells. After 48 h, EGFP gene transfer efficiencies were determined by fluorescence-activated cell sorter (FACS) analyses. Reporter gene transfer activity was exclusively present in lysates of cell cultures that had been transfected with pKS.P5.Rep (Fig. 4A). Control samples derived from Rep-negative cell cultures did not exhibit EGFP gene transfer activity above background levels (Fig. 4A). Characteristic histograms corresponding to one of these experiments are depicted in Fig. 4B. Thus, in the presence of AAV rep expression, the EGFP gene-positive hybrid vector AAV/Ad.EGFP was efficiently rescued in PER.tTA.Cre cells. In addition, PCR assays using oligonucleotides targeting different AAV ITR sequences together with oligonucleotides recognizing either the Ad packaging signal or the EGFP ORF (Fig. 5A) demonstrated packaging of both AAV ITRs and Ad packaging signal-containing sequences. The numbers above the bars indicate the average titers from three independent infection experiments.

FIG. 3. Schematic representation of the high-capacity AAV/Ad hybrid vector shuttle constructs pHV.EGFP (A), pHV.lacZ (B), and pHVΔ18.lacZ (C). These constructs contain AAV/Ad chimeric genomes. The core functional elements of these genomes consist of AAV ITRs and Ad packaging elements. Solid arrows, AAV ITRs; shaded boxes, left Ad5 TR and packaging signal-containing sequences. The numbers below the shaded boxes correspond to Ad5 genome positions. The numbers in parentheses correspond to the sizes (in kilobases) of the AAV/Ad chimeric genomes after rescue from the shuttle plasmid backbones (i.e., the lengths from the left to the right AAV ITRs). pHV.lacZ and pHVΔ18.lacZ have the gene for a nuclear-targeted E. coli β-galactosidase (lacZ) under the transcriptional control of an IE-CMV promoter and a simian virus 40 polyadenylation signal (SV40), whereas pHV.EGFP contains the EGFP gene. This reporter gene is located between the promoter, first exon, first intron, and the 5' portion of the second exon of the human elongation factor 1α gene and the human growth hormone gene transcriptional terminator (hGH). To enhance transgene expression, a SUR element (30) is located between the truncated second exon of the EF1α gene and the 5' end of the EGFP ORF (solid box). The Ad cis-acting elements present in pHV.lacZ and pHV.EGFP are the left TR and contiguous packaging elements AI through AVII (27). In pHVΔ18.lacZ, the 5' terminal 18 bp of the Ad TR corresponding to the core origin of replication are deleted. The antibiotic resistance gene (ampR) and the prokaryotic origin of replication (ori) are also indicated.

FIG. 2. Determination of Cre activity in PER.tTA.Cre cell clones by titration of Ad.floxedΨ. Ad.Ψ is a first-generation Ad vector with E1 deleted and with the same genetic composition as Ad.floxedΨ (see Fig. 1A) except that it contains a wild-type nonfloxed packaging signal. Six million PER.tTA.Cre76 and PER.tTA.Cre43 cells were infected (+) for 24 h with Ad.floxedΨ or Ad.Ψ at an MOI of 5 IU/cell. The amounts of Ad vectors with E1 deleted produced in both cell lines were determined by endpoint titrations on E1-complementing 911 cells (8). The numbers above the bars indicate the average titers from three independent infection experiments.
FIG. 4. Effect of AAV rep gene expression on the ability to rescue AAV/Ad.EGFP hybrid vectors in PER.tTA.Cre76 cells. (A) pHV.EGFP was transfected into PER.tTA.Cre76 cells together with the Ad E2a expression construct pCMV.E2a alone (−) or with pCMV.E2a plus the AAV rep expression plasmid pKS.P5.Rep (+). Subsequently, the PER.tTA.Cre76 cells were infected with Ad.foxedΨ at an MOI of 2.5 IU/cell (n = 4). Upon the emergence of complete CPE, the cells were harvested and lysed by three cycles of freezing and thawing. Cellular debris was removed by low-speed centrifugation, and the recovered supernatants were filtered and used to infect HeLa indicator cells. Forty-eight hours p.i., the number of EGFP-positive cells was determined by FACS analysis. AAV/Ad.EGFP hybrid vector titers were assayed for the presence of functional AAV/Ad hybrid vector particles. Flow cytometric analyses of EGFP expression in HeLa indicator cells at 48 h p.i. revealed an AAV/Ad hybrid vector titer of 9.7 × 10^5 IU/ml (Fig. 7). In a parallel experiment, extrachromosomal DNA extracted from PER.tTA.Cre76 cells transfected with the AAV rep and Ad E2a expression plasmids and coinfectected with AAV/Ad.EGFP and Ad.foxedΨ was subjected to agarose gel electrophoresis.

AAV rep expression did not give rise to DNase I-resistant templates, confirming the absence of encapsidated hybrid vector genomes in the corresponding cell lysate (Fig. 5B, lanes 9 and 10). Furthermore, the inability to amplify AAV ITR-specific PCR products after spiking the Rep-negative cell lysate with pHV.EGFP DNA demonstrated the effectiveness of the nuclease treatment in eliminating unpackaged template DNA (Fig. 5B, lanes 9 and 10). This control strengthens the conclusion that the PCR fragments originated from templates packaged in intact particles and not from shuttle plasmid DNA. The titer of Ad.foxedΨ in the AAV/Ad.EGFP vector stocks was 6.2 ± 3.9 × 10^5 IU/ml (n = 4). This corresponds to approximately 0.02% of the amounts routinely detected in AAV/Ad hybrid vector preparations generated in parental PER.C6 cells using helper Ad vectors with a nonflexed packaging signal (12). From these experiments, we conclude that it is possible to greatly reduce (by up to 4 orders of magnitude) helper Ad vector assembly through Cre/loxP-mediated formation of packaging-deficient templates without compromising high-capacity AAV/Ad hybrid vector rescue.

The Cre/loxP system enables effective amplification of high-capacity AAV/Ad hybrid vectors. After having determined the ability to generate high-capacity AAV/Ad hybrid vectors in PER.tTA.Cre cells infected with Ad.foxedΨ, we investigated whether the same production system could be used for their propagation. Earlier, it was shown that AAV/Ad hybrid vector particles contain bona fide AAV-dependent replicative intermediates with functional AAV ITRs (12). The functionality of the AAV origins of replication was demonstrated by the ability to propagate hybrid vector particles in the presence of AAV Rep proteins introduced into E1-complementing cells by plasmid transfection. In this set of experiments, hybrid vector amplification was dependent on the use of very small amounts of the primary AAV/Ad hybrid vector preparation. As a consequence, no absolute increase in hybrid vector titers was achieved. Most likely, the very large excess of Ad vector with E1 deleted hampered efficient hybrid vector propagation due to severe competition between AAV/Ad chimeric genomes and helper Ad replications for limiting viral and/or cellular trans-acting factors or due to the very fast emergence of CPE in the producer cells. Thus, we hypothesized that the Cre/loxP-mediated suppression of Ad.foxedΨ assembly would allow the effective amplification of AAV/Ad hybrid vector particles. To test this hypothesis, the experimental design as depicted in Fig. 6 was followed. PER.tTA.Cre76 cells were transfected with the expression plasmids pKS.p5.REP and pCMV.E2a. Next, 2 ml, corresponding to one-fifth, of a preparation containing 3.0 × 10^4 EGFP transducing units (GTU) of AAV/Ad.EGFP/ml and 4.2 × 10^5 IU of Ad.foxedΨ/ml was added to the transfected cells together with Ad.foxedΨ supplemented at an MOI of 2.5 IU/cell. The producer cells were not harvested until complete CPE was detected. The cleared lysates derived from these cells were assayed for the presence of functional AAV/Ad hybrid vector particles. Flow cytometric analyses of EGFP expression in HeLa indicator cells at 48 h p.i. revealed an AAV/Ad hybrid vector titer of 9.7 × 10^5 GTU/ml (Fig. 7). This corresponds to a 323-fold titer increase and to a 1,617-fold hybrid vector amplification (i.e., the total yield divided by the input). Conversely, Ad.foxedΨ showed only a 1.8-fold increase in titer from 4.2 × 10^5 to 7.5 × 10^5 IU/ml (Fig. 7).
and Southern blot analyses. After autoradiography, characteristic AAV-dependent replicative intermediates consisting of duplex monomers (14.3 kb) and duplex dimers (28.6 kb) were readily detected (data not shown). To confirm that the efficient hybrid vector propagation achieved with the present system is strictly dependent on the AAV DNA replication machinery and is not caused by the use of the left Ad TR by the Ad DNA replication complex, we compared the propagation of AAV/Ad chimeric genomes derived from pHV.lacZ and pHV/H900418.lacZ. The only difference between these two DNA molecules is the deletion in the latter of the first 18 bp of the left Ad TR (Fig. 3B and C, respectively). The Ad DNA region located between positions 9 and 18 is conserved among all human Ad serotypes and is the binding site for the precursor terminal protein-DNA polymerase heterodimer. Together with the first 8 nucleotides, this region constitutes the core origin of replication of the Ad genome (31). Thus, the 18-bp deletion at the Ad TR terminus prevents any possible interaction between the Ad DNA replication initiation complex and the template DNA. The rationale to originally incorporate the left Ad TR in the pHV.EGFP shuttle construct was based on previous results indicating a contribution of sequences located within the 103-bp Ad TR to the Ad encapsidation process (12, 28). Once again, the experimental setup was as depicted in Fig. 6. First, AAV/Ad chimeric genomes were rescued into Ad capsids by transfecting pHV.lacZ or pHV/H900418.lacZ into PER.tTA.Cre76 cells together with pKS.P5.Rep and pCMV.E2a. Helper functions were provided by Ad.floxed/H9023 infection at an MOI of 2.5 IU/cell. After the development of full CPE, cell lysates were prepared and used to infect HeLa indicator cells. pHV.lacZ and pHV/H900418.lacZ gave rise to AAV/Ad.lacZ and AAV/Ad/H900418.lacZ hybrid vector preparations with titers of 6.3 × 10^4 and 2.0 × 10^4 LTU/ml, respectively. Subsequently, 1/10 (i.e., 1 ml) of each preparation was added to pKS.P5.Rep- and pCMV.E2a-transfected PER.tTA.Cre76 cells together with Ad.floxedΨ at an MOI of 2.5 IU/cell. Control experiments were performed with PER.tTA.Cre76 cells that had not been transfected with the AAV rep expression construct pKS.P5.Rep. Cleared lysates from these cells contained 1.2 × 10^7 and 3.2 × 10^7 LTU of AAV/Ad.lacZ and AAV/
AdΔ18.lacZ/ml, respectively (Fig. 8). Control propagations carried out in the absence of AAV rep expression did not result in hybrid vector amplification (data not shown). With these experiments, we confirmed that propagation of high-capacity AAV/Ad hybrid vectors is dependent on the AAV DNA replication machinery. Accordingly, the resulting hybrid vector DNA should have retained the AAV origins of replication. Indeed, this was proven correct through the detection of AAV ITR-specific DNA fragments after PCR amplification of the 5′ termini of hybrid vector genomes extracted from propagated AAV/Ad.lacZ and AAV/AdΔ18.lacZ particles (Fig. 9, compare lanes 3 and 4 with lanes 7 and 8). Once again, rescue experiments performed in the absence of AAV rep expression did not yield DNase I-resistant templates (Fig. 9, lanes 5 and 6). Taken together, these experiments demonstrate the ability to produce high-titer AAV/Ad hybrid vector preparations.
through the use of Cre/loxP-mediated genetic selection against the propagation of the trans-complementing Ad vector.

**DISCUSSION**

In the present study, we reveal the ability to rescue and efficiently amplify high-capacity AAV/Ad hybrid vectors using Cre/loxP-mediated negative selection against the assembly of the trans-complementing helper Ad. The proof of principle that these vectors can be produced without the simultaneous assembly of significant numbers of helper Ad particles indicates that further improvements in reducing or completely eliminating helper Ad escape will not have deleterious effects on high-capacity AAV/Ad hybrid vector production. In addition, we also illustrate the flexibility of the hybrid vector design by showing the packaging and delivery of different combinations of transgenes and regulatory elements. These results strengthen the basic principle of the hybrid vector system as being dependent on AAV ITRs and Ad packaging elements in cis and on AAV replicative functions and Ad structural proteins in trans. At another level, the use of AAV/Ad hybrid vector preparations with a low content of Ad particles with E1 deleted indicated that the cellular transduction process is mediated by hybrid vector particles per se without a direct role of or significant contribution by helper Ad functions.

Despite the reliance on transfection to provide producer cells with key AAV replicative functions, high-capacity AAV/Ad hybrid vectors were amplified efficiently, i.e., $10^2$ to $10^3$-fold after a single passage. Indeed, it is unlikely that the delivery of AAV replicative functions through transfection was limiting, since control cell cultures receiving a lacZ reporter construct and subjected to β-Gal histochemistry consistently showed a transfection efficiency of 80 to 90% (data not shown). This suggests that the limiting process in hybrid vector production is instead the initial rescue of AAV/Ad chimeric genomes from the shuttle construct. Once hybrid vector particles are generated, this barrier is overcome. Next, the Ad capsid-mediated delivery of AAV-dependent replicative intermediates introduces into producer cell nuclei templates that can be readily recognized and amplified by the AAV DNA replication machinery. Interestingly, AAV/Ad hybrid vectors could be amplified more efficiently than high-capacity Ad vectors, since equivalent titer were reached without the numerous serial propagations normally required for high-capacity Ad vector production. This observation is based both on published data (22) and on our own results using the present PER.tTA.Cre/Ad.foxedΨ production system to rescue and propagate high-capacity Ad vectors with a genetic makeup similar to that of the high-capacity AAV/Ad hybrid vectors described above (i.e., containing the same transcription units and stuffer DNA fragments [unpublished data]). The adenoviral sequences retained in high-capacity Ad vectors are the origins of replication and the packaging elements, whereas the minimal viral sequences kept in high-capacity AAV/Ad vectors are the AAV origins of replication and the Ad packaging elements. Thus, the fundamental difference between high-capacity Ad and AAV/Ad hybrid vectors is their dependence on the Ad and AAV DNA replication mechanisms, respectively. Consequently, in a coinfected producer cell, high-capacity Ad and helper Ad replicons compete at all times for the same replicative functions in contrast to high-capacity AAV/Ad and helper Ad replicons. As an example, AAV DNA replication is dependent on cellular polymerase(s), whereas Ad encodes its
AAV ITRs delivered by rAAV vector particles can mediate permanent genetic modification of target cells in vivo through either host chromosomal DNA integration (21) or formation of circular double-stranded high-molecular-weight concatemers (5). Moreover, it has previously been shown that double-stranded AAV-dependent replicative intermediates introduced into rapidly proliferating cells by Ad capsids gave rise to prolonged lacZ expression as opposed to Ad genomes with E1 deleted encoding the same reporter gene cassette (12). In addition, by using high-capacity AAV/Ad hybrid vectors generated with the present Cre/loxP system, we have been able to transduce target cells without triggering cellular toxicity. In the same study, we demonstrated that stable transgene expression was achieved through host chromosomal DNA integration of full-length AAV/Ad chimeric genomes (unpublished data). Future work will be devoted to the evaluation of high-capacity AAV/Ad hybrid vector transgene delivery ex vivo and in vivo.

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