Targeted Chromosomal Insertion of Large DNA into the Human Genome by a Fiber-Modified High-Capacity Adenovirus-Based Vector System

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Abstract

A prominent goal in gene therapy research concerns the development of gene transfer vehicles that can integrate exogenous DNA at specific chromosomal loci to prevent insertional oncogenesis and provide for long-term transgene expression. Adenovirus (Ad) vectors arguably represent the most efficient delivery systems of episomal DNA into eukaryotic cell nuclei. The most advanced recombinant Ads lack all adenoviral genes. This renders these so-called high-capacity (hc) Ad vectors less cytotoxic/immunogenic than those only deleted in early regions and creates space for the insertion of large/multiple transgenes. The versatility of hcAd vectors is increased by capsid modifications to alter their tropism and by the incorporation into their genomes of sequences promoting chromosomal insertion of exogenous DNA. Adenovirus-associated virus (AAV) can insert its genome into specific human loci designated AAVS1. Trans- and cis-acting elements needed for this reaction are the AAV Rep78/68 proteins and Rep78/68-binding sequences, respectively. Here, we describe the generation, characterization and testing of fiber-modified dual hcAd/AAV hybrid vectors (dHVs) containing both these elements. Due to the inhibitory effects of Rep78/68 on Ad-dependent DNA replication, we deployed a recombinase-inducible gene switch to repress Rep68 synthesis during vector rescue and propagation. Flow cytometric analyses revealed that rep68-positive dHVs can be produced similarly well as rep68-negative control vectors. Western blot experiments and immunofluorescence microscopy analyses demonstrated transfer of recombinase-dependent rep68 genes into target cells. Studies in HeLa cells and in the dystrophin-deficient myoblasts from a Duchenne muscular dystrophy (DMD) patient showed that induction of Rep68 synthesis in cells transduced with fiber-modified and rep68-positive dHVs leads to increased stable transduction levels and AAVS1-targeted integration of vector DNA. These results warrant further investigation especially considering the paucity of vector systems allowing permanent phenotypic correction of patient-own cell types with large DNA (e.g. recombinant full-length DMD genes).

Introduction

Experiments in animal models of various human inherited diseases have shown that high-capacity (hc) adenovirus (Ad) vectors are superior to early region-deleted Ad vectors both in terms of safety and persistence of transgene expression [1–3]. However, at present, extensive use of this gene delivery system in in vivo settings is hampered by the difficulty in obtaining the vast amounts of vector particles needed for large animal experiments and the limited understanding of the physiological effects caused by the interaction(s) between vector capsids and, for instance, the innate immune system. In addition, because Ad genomes normally remain episomal, hcAd vectors are of limited use in applications aiming at stable genetic modification of dividing cell populations. Thus, a high demand exists to endow hcAd vectors with mechanisms that allow exogenous DNA persistence.

A strategy to achieve this goal consists of incorporating into hcAd vector genomes the DNA integration-promoting elements from naturally occurring integration systems such as (i) DNA transposons/transposases, (ii) retroviral long terminal repeats/integrases, (iii) retrotransposons, (iv) DNA recombinases or (v) the Rep78/68-binding sequences/large Rep proteins of the seemingly non-pathogenic helper-dependent parvovirus adeno-associated virus (AAV) [4,5]. Of these integrating hcAd-based vector systems, those that capitalize on the DNA integration machinery of AAV have the advantage of allowing preferential insertion of foreign DNA into the so-called AAVS1 locus on human chromosome 19 (19q13.3-qter). AAV trans- and cis-acting elements needed for this reaction are the Rep78 and/or Rep68 protein and their cognate binding sequences, respectively [6]. The cis-acting elements are located in the AAV inverted terminal repeats (ITRs), which can fold into a palindromic T-shaped hairpin, and in the AAV p5 promoter, which contains a sequence dubbed the p5 integration efficiency element (p5IEE) [7].

Another shortcoming of hcAd-based gene transfer systems relates to the fact that many clinically important cell types (e.g., human hematopoietic stem and progenitor cells, mesenchymal stem cells...
and myoblasts) are refractory to transduction by conventional hcAd vectors, which are based on the species C human Ad (hAd) serotypes 2 and 5. This is explained by the virtual absence of the Ad and Coxsackie B virus receptor (CAR) on the surface of these cells [8–11]. To allow efficient transduction of CAR-negative cell types, others and we have deployed a genetic re-targeting strategy whereby cell receptor-interacting domains of the hAd serotype 5 (hAd5) fiber are substituted by those from other Ads resulting in hcAd particles pseudotyped with chimeric fibers [11–13].

In the present study, we describe the generation of dual hcAd/AAV hybrid vectors (dHVs) [14] with modified capsids and with all the AAV cis- and trans-acting elements needed for locus-specific insertion of exogenous DNA. To avoid the well-described interference of the large AAV Rep proteins (i.e., Rep78 and Rep68) on the Ad-dependent DNA replication [15,16], which is required for vector genome amplification, we devised a FLP recombinase-dependent gene switch module to repress and activate rep68 expression in producer and target cells, respectively. Rep68 synthesis in target cells was induced with the aid of a fiber-modified hcAd vector encoding a thermostable version of the Saccharomyces cerevisiae FLP recombinase (FLP) [17,18]. Long-term cultures and clonal analyses of human cervix carcinoma (HeLa) cells and of myoblasts isolated from a patient with Duchenne muscular dystrophy (DMD) were used to determine stable transduction levels as well as the targeted chromosomal integration frequencies of the large DNA molecules delivered by capsid-modified hcAd vectors (Fig. 1A). The particular design of the resulting dHV genomes with AAV ITR sequences at the axis of symmetry and with the p5IEE at both extremities allows the introduction into target cells of DNA molecules structurally resembling wild-type AAV proviruses [25]. Resolution of the Holliday-like AAV ITR hairpin structures at the axis of symmetry either by cellular factors [14,26] or by Rep68-mediated nicking at the terminal resolution site (tr; Fig. 1B) will generate monomer-sized replicative intermediates that can be reutilized by the Ad replication machinery to produce new dHV genomes. Because they possess a favorable size for efficient packaging into Ad capsids (i.e. between 27 and 38 kb) [14,27] unresolved dimeric replicative intermediates will be preferentially packaged into Ad capsids [14] thus giving rise to dHV particles (Fig. 1B). The localization of the FLP-inducible rep68 gene upstream of the p5IEE in the rep68-positive dHV genomes (Fig. 1C) should impede its Rep68-dependent insertion into the A4V5 locus [14]. To further reduce the likelihood of integrating rep68 sequences into A4V5 locus of target cells, the rep68 expression module was designed in such a way that its FLP-mediated activation is accompanied by its physical separation from the gene(s)-of-interest. Circularization of the FLP recombinase target (FRT)–flanked DNA by FLP–positions a promoter in front of the rep68 open reading frame (ORF) resulting in Rep68 synthesis. Finally, Rep68-mediated interactions between the RBEs in the dHV genomes (i.e., AAV p5IEE and AAV ITR) and in human chromosome 19 (i.e. A4V5 locus) should facilitate targeted insertion of the foreign DNA (Fig. 1C).

**Results**

**Experimental strategy**

Since AAV Rep68 contains all the structural information needed to catalyze targeted DNA insertion, we decided to focus on the delivery into target cells of this instead of the larger Rep78 protein. However, because Ad-dependent DNA replication is strongly inhibited by the two large Rep proteins [15,16] rep68 expression during dHV production should be minimized. Indeed, generation of Ad-based vectors encoding Rep78 and/or Rep68 is not a trivial matter since even relatively low concentrations of these proteins in producer cells lead to the emergence and selection of mutant vectors with deletions in the rep sequences [19], and our unpublished results. Another point of concern is the stability of Ad vector genomes containing integration modules consisting of classic recombinant AAV (rAAV) genomes (i.e. ITR-transgene[s]-ITR). Investigations have shown that the presence of pairs of non-terminal inverted repeats in Ad vector genomes leads to replication-induced recombination through these sequences causing deletions/rearrangements in the vector DNA [20,21]. Ad vectors containing both rAAV genomes and rep78 and/or rep68 genes may, in addition, suffer from Rep78/68-mediated rescue (i.e. excision) of the rAAV DNA from a fraction of the vector backbones causing transgene loss. The origin of this problem lies in the fact that, although Rep synthesis can, to a great extent, be inhibited by using stringent inducible gene expression systems, most if not all of them are “leaky” (especially in the context of replicating DNA templates). During the production of rep78/68-positive Ad vectors, producer cells will thus contain not only Ad-derived AAV helper activities (i.e. Ad gene products necessary for AAV DNA rescue and replication [6]) but also Rep78/68 proteins. Finally, permanent expression of the rep78/68 gene at high levels is detrimental for many cell types causing cell cycle arrest and apoptosis [22–24].

Given all these potential pitfalls, we decided to construct hcAd-based vectors containing rep68 genes and Rep78/68-binding elements (RBEs) using a unique vector design in which FLP recombinase-dependent rep68 expression units are incorporated into the non-integrating parts of dHV genomes [14]. Previous results have shown that Ad-dependent DNA replication in concert with AAV ITR-mediated dimerization is the main mechanism responsible for the generation of dHV genomes from input monomeric templates molecularly cloned in shuttle plasmids [14]. These monomeric DNA molecules contain an Ad ITR, Ad packaging signal and AAV p5IEE at one end and an AAV ITR at the other end (Fig. 1A). The particular design of the resulting dHV genomes with AAV ITR sequences at the axis of symmetry and with the p5IEE at both extremities allows the introduction into target cells of DNA molecules structurally resembling wild-type AAV proviruses [25]. Resolution of the Holliday-like AAV ITR hairpin structures at the axis of symmetry either by cellular factors [14,26] or by Rep68-mediated nicking at the terminal resolution site (tr; Fig. 1B) will generate monomer-sized replicative intermediates that can be reutilized by the Ad replication machinery to produce new dHV genomes. Because they possess a favorable size for efficient packaging into Ad capsids (i.e. between 27 and 38 kb) [14,27] unresolved dimeric replicative intermediates will be preferentially packaged into Ad capsids [14] thus giving rise to dHV particles (Fig. 1B). The localization of the FLP-inducible rep68 gene upstream of the p5IEE in the rep68-positive dHV genomes (Fig. 1C) should impede its Rep68-dependent insertion into the A4V5 locus [14]. To further reduce the likelihood of integrating rep68 sequences into the A4V5 locus of target cells, the rep68 expression module was designed in such a way that its FLP-mediated activation is accompanied by its physical separation from the gene(s)-of-interest. Circularization of the FLP recombinase target (FRT)–flanked DNA by FLP–positions a promoter in front of the rep68 open reading frame (ORF) resulting in Rep68 synthesis. Finally, Rep68-mediated interactions between the RBEs in the dHV genomes (i.e., AAV p5IEE and AAV ITR) and in human chromosome 19 (i.e. A4V5 locus) should facilitate targeted insertion of the foreign DNA (Fig. 1C).

**Construction and testing of a FLP-dependent rep68 expression unit**

We started by constructing an inducible rep68 expression unit on the basis of the FLP/FRT site-specific recombination system of Saccharomyces cerevisiae. To this end, the coding sequence for DsRed.T4 in pG5.sPa+.DsRed [28] was replaced by that of Rep68 giving rise to construct pG5.sPa+.Rep68. The functionality of pG5.sPa+.Rep68 was tested in a transient transfection assay. This assay consisted of introducing into pG5.sPa+.Rep68-containing PER.tTA.Cre76 cells, AAV helper functions and an rAAV vector shuttle plasmid through infection with Ad.Boxed.PS.F50 and transfection of pAAV.DsRed, respectively. In the presence of AAV helper functions plus functional AAV Rep68 molecules, rAAV genomes are expected to be rescued (i.e. excised) from plasmid backbones and to originate de novo synthesized replicative intermediates consisting of double-stranded full-length monomeric and dimeric vector DNA, also known as duplex monomers (DMs) and...
in addition, a FLP-inducible AAV former construct only codes for eGFP whereas the latter plasmids have, pdHV differs from that of pdHV.68/5.5 and pdHV.68/3.5 in that the former construct only codes for eGFP whereas the latter plasmids have, in addition, a FLP-inducible AAV rep68 expression module (see below) inserted in between the Ad packaging signal and the AAV p5IEE in the 5’→3’ (pdHV.68/5/3’) or in the 3’→5’ orientation (pdHV.68/3/5’). Solid box, Ad ITR (hAd5 base pairs 1 through 103); ψ, Ad packaging signal-containing sequence (hAd5 genome positions 104 through 454); shaded box with broken line, inducible AAV2 rep68 expression unit; shaded circle, p5IEE (AAV2 genome positions 153 through 291); line with subscript “dystrophin”, stuffer DNA derived from human dystrophin cDNA; solid triangle, human growth hormone gene pA signal; open box, eGFP ORF; solid box with broken arrow, hEF1α promoter; shaded oval, AAV ITR (AAV2 nucleotides 1 through 145); open circle, prokaryotic origin of replication. (B) Schematic representation of the rescue process of capsid-modified and rep68-positive dHV particles. Rescue of dHV DNA into hAd5 capsids containing chimeric fibers is initiated by releasing it from the plasmid backbone at the indicated Pmel restriction sites and transfecting the digestion products into the hAd5 E1-complementing PER.TTA.Cre76 cells. These cells are next infected with the E1-deleted helper Ad vector Ad.floxedY.F50, which supplies in trans all Ad DNA replication activities and Ad structural proteins (including chimeric fiber proteins) needed for the amplification and packaging of dHV DNA, respectively. Replication- and packaging-competent tail-to-tail dimers are assembled from input monomers due to Ad-dependent DNA replication and AAV ITR-mediated dimerization. The resulting dHV particles display on their surface hAd50 fiber domains and can be amplified by serial propagation in Ad.floxedY.F50-infected producer cells prior to purification in CsCl density gradients. The use of a helper Ad vector which has its packaging signal flanked by bacteriophage P1 lopx sites in combination with producer cells expressing the bacteriophage P1 cre gene strongly inhibits the formation of helper Ad particles. (C) Transduction pathway of rep68-positive dHVs. The FLP-inducible rep68 expression unit consists of a direct repeat of two FRT sequences flanking the AAV2 rep68 ORF followed by the herpes simplex virus thymidine kinase gene pA signal and the hGAPDH promoter. Since the transcription-promoting elements of the hGAPDH gene are positioned downstream of the Rep68-coding sequence, the rep68 ORF is not preceded by an active promoter and hence should not get expressed. To suppress rep68 activation by possible cryptic promoter elements located upstream of the rep68 initiation codon, a transcriptional terminator from the murine metallocationine gene was introduced immediately upstream of the 5’ FRT site. The FLP-inducible rep68 expression module is located between the Ad cis-acting elements (i.e., the Ad ITR and the Ad packaging signal [Ψ]) and the AAV p5IEE. Excision and circularization of the FRT-flanked DNA by FLPe will position the hGAPDH promoter in front of the rep68 ORF and lead to the synthesis of Rep68. Finally, Rep68-mediated interactions between the RBEs in the vector DNA (i.e., AAV p5IEE and AAV ITR) and in human chromosome 19 (i.e. AAVST locus) will induce targeted chromosomal insertion of dHV DNA.

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duplex dimers (DDs), respectively [6]. To activate the rep68-specific gene switch, we used hAd.FLPe.F50. Negative and positive controls consisted of pGAPDH.Rep78/68- and pAAV.DsRed-transfected PER.TTA.Cre76 cells that were mock-infected and infected with Ad.floxedY.F50, respectively. Results depicted in Fig. 2 clearly show that accumulation of rAAV replicative intermediates in the pGS.pA+Rep68-transfected PER.TTA.Cre76 cells is strongly stimulated by infection with hAd.FLPe.F50 (i.e. in the presence of FLPe; compare lanes 1 and 2 of Fig. 2). In the absence of the recombinase only low amounts of rAAV replicative intermediates were generated (Fig. 2; lane 1). This suggests the existence of “leaky” rep68 expression at a level sufficient to allow limited amplification of rAAV DNA. Indeed, previous research has provided evidence that only few Rep78/68 molecules are required to induce AAV DNA replication [29]. The detection of rAAV-specific DMs and DDs in cells that received pAAV.DsRed, pGAPDH.Rep78/68 and Ad.floxedY.F50 (Fig. 2; lane 3) but not in cells containing exclusively pAAV.DsRed and pGAPDH.Rep78/68 (Fig. 2; lane 4) validated the assay. On the basis of these results, we conclude that pGS.pA+Rep68 is FLPe-responsive and gives rise to the synthesis of functional Rep68 molecules.

Figure 1. Overview of the structure, production and application of dHVs. (A) Diagram of dHV shuttle plasmids. The structure of pdHV differs from that of pdHV.68/5/3’ and pdHV.68/3/5’ in that the former construct only codes for eGFP whereas the latter plasmids have, in addition, a FLP-inducible AAV rep68 expression module (see below)
Rescue, propagation and characterization of rep68-positive and fiber-modified dHV particles

Next, the FLP-inducible rep68 expression unit from pGS.pA+.Rep68 was inserted in either orientation upstream of the p5IEE in the dHV shuttle construct pAd/AAV.EGFP [14], hereafter referred to as pdHV, to generate pdHV.68/5’3’ and pdHV.68/3’5’, respectively (Fig. 1A). pdHV.68/5’3’, pdHV.68/3’5’ and the rep68-negative control plasmid pAAV were digested with PmeI and individually transfected into PER.tTA.Cre76 cells. The PmeI digestion releases the Ad ITRs from almost the complete plasmid backbones turning them into much more efficient substrates for the initiation of Ad-dependent DNA replication than undigested templates. Subsequently, helper activities necessary for the amplification and packaging of dHV genomes were provided in trans by the infection of producer cells with the capsid-modified helper Ad vector Ad.flxed[Y].F50. After exhibiting complete cytopathic effect, the cell cultures were harvested and dHV seed stocks were prepared. These crude vector preparations were used to amplify dHV particles through four successive rounds of infection in Ad.flxed[Y].F50-infected PER.tTA.Cre76 cells. Direct fluorescence microscopy revealed an increase in the frequency of enhanced green fluorescent protein (eGFP)-positive producer cells following each consecutive round of vector amplification (Fig. 3A). Quantification of functional vector particles by eGFP-directed flow cytometry showed that the propagation kinetics of the two different rep68-positive dHVs was very similar to that of their rep68-negative counterpart (Fig. 3B).

Both rep68-negative and rep68-positive fiber-modified dHV particles obtained after four rounds of propagation were purified by isopycnic CsCl density gradient ultracentrifugation, titrated and used to transduce CD46-positive target cells that either do (HeLa cells) or do not (DMD myoblasts) express CAR at their surface. Direct fluorescence microscopy (Fig. 4A) and flow cytometry (Figs. 4B and 4C) showed a clear dose-dependent increase in gene transfer activity in both cell types. Moreover, flow cytometric quantification of the frequency of eGFP-positive cells (Fig. 4C, left graphs) and the average eGFP level per transduced cell (Fig. 4C, right graphs) revealed that, for each given vector dose, dHV.F50 and dHV.68/5’3’.F50 gave similar transduction levels.

To investigate whether dHV.68/5’3’.F50 particles contain FLP-responsive rep68-specific gene switches, we performed Western blot analysis on lysates derived from HeLa cells transduced with CoCl density-gradient-purified dHV.F50 and dHV.68/5’3’.F50 particles using a monoclonal antibody recognizing all four AAV rep gene products. HeLa cells were infected with dHV.F50 or dHV.68/5’3’.F50 alone (Fig. 5A; lanes 2 and 4) or in combination with hcAd.FLP.e.F50 (Fig. 5A; lanes 1 and 3). Co-infection of the target cells with dHV.68/5’3’.F50 and hcAd.FLP.e.F50 resulted in robust induction of rep68 expression (Fig. 5A; lane 3). In contrast, the amount of Rep68 produced in dHV.68/5’3’.F50-infected HeLa cells in the absence of FLP.e was very low (Fig. 5A; lane 4). These results demonstrate dHV.68/5’3’.F50-mediated transfer of functional rep68 gene switch modules into target cells. Subsequently, to examine the synthesis of Rep molecules at the single-cell level we carried out eGFP direct fluorescence microscopy and Rep68 immunofluorescence microscopy of Rep68-specific signals was observed.

Permanent genetic modification of HeLa cells and myoblasts from a DMD patient by rep68-positive and fiber-modified dHV particles

Finally, we tested the ability of dHV.68/5’3’.F50 to permanently transduce target cells by locus-specific transgene integration using two different human cell types, i.e., HeLa cells and DMD myoblasts. HeLa cells were chosen for this purpose because they constitute a reference cell line on which both wild-type AAV and Rep78/68-dependent DNA integration have been evaluated both in terms of stable transduction levels and targeted DNA insertion efficiencies. Myoblasts are being deployed in clinical trials for DMD [30]. One of the problems associated with this treatment modality is that it depends on strong and durable immunosup-
pression to prevent graft rejection. The use of gene-corrected autologous myoblasts may obviate the need to severely immunosuppress the patients. We previously showed that dystrophin-defective myoblasts are very well transduced {\textit{ex vivo}} by non-integrating hcAd-based vectors carrying the receptor-interacting domains of hAd serotype 50 (hAd50) fibers [11]. This makes it relevant to test the ability of dHV.68/5'3'.F50 to stably transduce DMD myoblasts through targeted transgene integration. Moreover, contrary to HeLa cells, the DMD myoblasts used in this study possess a normal, diploid karyotype [31]. HeLa cells and DMD myoblasts were independently exposed to equal doses of dHV.F50 or dHV.68/5'3'.F50, either alone or together with a constant amount of hcAd.FLPe.F50. Three days later the initial levels of target cell transduction were determined by eGFP-directed flow cytometry. The frequencies of eGFP-positive cells were within a relatively narrow range, i.e., between 72 and 78\% for the HeLa cells and between 76 and 81\% for the DMD myoblasts. Next, the stable transduction frequencies (STFs) reached by dHV.F50 and by dHV.68/5'3'.F50 were determined by extensively subculturing both types of dividing cells and by measuring the percentages of eGFP-positive cells through flow cytometry as a function of time. This experimental setup does not introduce any bias towards the selection of cells carrying chromosomally integrated vector DNA and prevents that cells containing only episomal vector genomes are included in the STF calculations, which is a concern given the relatively long half-life of eGFP [32]. Stable transduction levels were reached at 27 days postinfection in HeLa cells whereas it took 56 days before the percentage of eGFP-positive DMD myoblasts stabilized, most likely reflecting the lower division rate of the latter cell type. The STFs obtained with dHV.68/5'3'.F50 in the presence of hcAd.FLPe.F50 were 0.6\% (n = 2) and 0.4\% (n = 1) for HeLa cells and DMD myoblasts, respectively (n events acquired = 10,000). In the absence of FLPe, dHV.68/5'3'.F50 yielded an STF of 0.05\% (n = 2) for HeLa cells and of 0.1\% (n = 1) for DMD myoblasts (n events acquired = 10,000). Thus, rep68 gene activation increased by 12- and 4-fold the STF of dHV.68/5'3'.F50-transduced HeLa cells and DMD myoblasts, respectively. Importantly, infection of both target cell types with the control vector dHV.F50 alone or together with hcAd.FLPe.F50 gave rise to similar STFs as observed for HeLa cells and DMD myoblasts transduced exclusively with dHV.68/5'3'.F50.

To determine the frequencies of targeted DNA integration, stably transduced HeLa cells and DMD myoblasts were isolated from cell cultures initially exposed to both dHV.68/5'3'.F50 and hcAd.FLPe.F50 and individually expanded. HeLa cell clones derived from cultures infected exclusively with dHV.68/5'3'.F50 served as controls. Genomic DNA was extracted from the various cell lines and subjected to Eco32I digestion and Southern blot analysis. The transferred DNA was incubated first with an A4FSI-
Figure 4. Transduction of target cells by CsCl density gradient-purified dHV.F50 and dHV.68/5'3' -F50 particles. (A) Direct fluorescence microscopy analysis of HeLa cells and DMD myoblasts that were either mock-infected or infected with 4, 20 or 100 HTUs of dHV.F50 or dHV.68/5'3' -F50 per cell. (B) Representative flow cytometry histograms corresponding to HeLa cells and DMD myoblasts transduced with different amounts of dHV.F50 or dHV.68/5'3' -F50 (i.e., 0, 4, 20 or 100 HTUs/cell). The % of maximum refers to the ratio between the number of events analyzed in each case and the number of events in the sample with the largest number of events. (C) Quantification by flow cytometry of gene transfer into HeLa cells and DMD myoblasts exposed to 4, 20 or 100 HTUs of dHV.F50 or dHV.68/5'3' -F50 per cell (n = 3). Gene transfer activity is expressed both in terms of the frequency of eGFP-positive cells and the mean fluorescent intensity (MFI) of the transduced cells (which reflects mean intracellular eGFP levels). Error bars represent standard deviations. All measurements were performed at 72 hours postinfection.

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whereas the latter is confined to the nucleus. Original magnification: Rep68. Note that the former protein is distributed throughout the cell arrowheads in the right panels point to cells containing both eGFP and from the green and red channels are overlaid in the lower panels. The fluorescent signals derived (50 GSAUs/cell) and either dHV.F50 (5 HTUs/cell; left column) or fluorescence microscopy on HeLa cells infected with hcAd.FLPe.F50 control. (B) eGFP direct fluorescence microscopy and Rep68 immuno-products. An antibody directed against actin served as a loading incubated with a monoclonal antibody specific for all four AAV extract, separated in an SDS-10% polyacrylamide gel and transferred 5

Figure 5. Testing the inducible rep68 expression unit in dHV.68/5′.F50 particles purified by CsCl density gradient ultracentrifugation. (A) Western blot analysis of FLPe-induced Rep68 synthesis. HeLa cells were co-transduced with dHV.F50 and hcAd. FLPe.F50 (lane 1) or with dHV.68/5′.F50 and hcAd.FLPe.F50 (lane 3) or were transduced exclusively with dHV.F50 (lane 2) or with dHV.68/5′.F50 (lane 4). Three days postinfection, cellular proteins were extracted, separated in an SDS-10% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. Next, the membrane was incubated with a monoclonal antibody specific for all four AAV rep gene products. An antibody directed against actin served as a loading control. (B) eGFP direct fluorescence microscopy and Rep68 immuno-fluorescence microscopy on HeLa cells infected with hcAd.FLPe.F50 (50 GSAUs/cell) and either dHV.F50 (5 HTUs/cell; left column) or dHV.68/5′.F50 (5 HTUs/cell; right column). The pictures are derived from representative microscopic fields. The fluorescent signals derived from the green and red channels are overlaid in the lower panels. The arrowheads in the right panels point to cells containing both eGFP and Rep68. Note that the former protein is distributed throughout the cell whereas the latter is confined to the nucleus. Original magnification: 400×.

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dDV- and dHV.68/5′.F50 (5 HTUs/cell). The pictures are derived from representative microscopic fields. The fluorescent signals derived from the green and red channels are overlaid in the lower panels. The arrowheads in the right panels point to cells containing both eGFP and Rep68. Note that the former protein is distributed throughout the cell whereas the latter is confined to the nucleus. Original magnification: 400×.

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specific probe (Fig. 6A; upper panel) and, after membrane stripping, was exposed to a mixture of two probes binding immediately downstream of the rep68 gene and close to the right end of the monomeric vector genome (Fig. 6A; lower panel). Examples of autoradiograms derived from these experiments are shown in Fig. 6B (HeLa cell clones) and Fig. 6C (DMD myoblast clones). Hybridization of a particular Eco32I fragment with both the AAVS1- and dHV.68/5′.F50-specific probes signifies inser-

tion of vector DNA into the AAVS1 locus on human chromosome 19 (Figs. 6B and 6C, open arrowheads). Subsequently, the membrane was once again stripped and incubated with a probe specific for a 1.9-kb Eco32I fragment in the vector genome (Fig. 6A; lower panel). The autoradiograms corresponding to hybridizations using the AAVS1-specific probe showed that 50% of the 38 randomly selected HeLa cell clones yielded DNA fragments with larger and, less often, smaller sizes than that of the 5.6-kb Eco32I fragment characteristic for undisrupted AAVS1 alleles (Fig. 6B; upper panel and Table 1). This observation is consistent with the well-known capacity of Rep78 and/or Rep68 to locally amplify the AAVS1 region due to the usage of its RBE and trs as an origin of replication [33]. Thus, the absence of detectable changes to the AAVS1 loci in the other 50% of the HeLa cell clones most likely reflects the presence of no or few Rep68 molecules in their founder cells probably due to a lack of FLPe. In agreement with this interpretation, no disruptions of the AAVS1 alleles were found after Southern blot analysis of chromosomal DNA extracted from 40 randomly selected clones of HeLa cells exposed exclusively to dHV.68/5′.F50 (Fig. 6D; Table 1), indicating that although “leaky” rep68 expression may occur (Fig. 5A) in most cases the amount ofRep68 does not suffice to amplify the AAVS1 region. Consistent with this finding, Southern blot hybridization using the vector-specific probes revealed that these clones did not have dHV.68/5′.F50 DNA integrated into the AAVS1 region (data not shown). Conversely, in the presence of the FLPe-encoding hcAd vector, integration of dHV.68/5′.F50 DNA into the AAVS1 locus could be demonstrated (Fig. 6B; middle panel and Table 1). The targeted DNA integration efficiency, as determined on the basis of the total number of HeLa cell clones analyzed was 24% (9/38), whereas if one considers exclusively clones with discernible changes in the AAVS1 locus (as a surrogate indicator for cells that originally contained enough Rep68) it reached 47% (9/19; Fig. 6B; Table 1). The same analysis applied to autoradiograms corresponding to DMD myoblast clones revealed that these cells were targeted at the AAVS1 locus with an efficiency of 28% (5/18) or 100% (5/5; Fig. 6C; Table 1), respectively.

Molecular characterization of AAVS1-foreign DNA junctions following dHV.68/5′.F50-mediated gene transfer

To confirm by an independent method the ability of rep68-positive dHV particles to accomplish locus-specific foreign DNA insertion, we performed experiments aiming at the isolation of junctions between AAVS1 and vector DNA. To this end, DMD myoblasts were transduced with dHV.68/5′.F50 alone or together with hcAd.FLPe.F50. Subsequently, chromosomal DNA was extracted from the transduced cells and AAVS1-vector DNA junctions were analyzed by PCR (Fig. 7A). Chromosomal DNA derived from human cells stably transduced by dHV.68/5′.F50 DNA in a non-targeted (i.e. HeLa cell clone N+2) and AAVS1-specific (i.e. DMD myoblast clone 3C5) manner served as negative and positive controls, respectively. The DNA derived from the DMD myoblast clone yielded a discrete PCR fragment whereas no amplification product was obtained with the DNA from the HeLa cell clone (Fig. 7B, lower panel, compare lanes 1 and 2). Nested PCR products were also detected in samples corresponding to reactions performed on DNA from DMD myoblast cultures transduced with dHV.68/5′.F50 alone (Fig. 7B, lower panel, lane 3) or together with hcAd.FLPe.F50 (Fig. 7B, lower panel, lane 4). Importantly, however, a much broader range of PCR product sizes was present in the sample derived from the cells that had been exposed to both vectors (Fig. 7B, lower panel, compare lanes 5 and 4). These results indicate dHV.68/5′.F50 DNA

![Figure 5](image-url)

Figure 5. Testing the inducible rep68 expression unit in dHV.68/5′.F50 particles purified by CsCl density gradient ultracentrifugation. (A) Western blot analysis of FLPe-induced Rep68 synthesis. HeLa cells were co-transduced with dHV.F50 and hcAd. FLPe.F50 (lane 1) or with dHV.68/5′.F50 and hcAd.FLPe.F50 (lane 3) or were transduced exclusively with dHV.F50 (lane 2) or with dHV.68/5′.F50 (lane 4). Three days postinfection, cellular proteins were extracted, separated in an SDS-10% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. Next, the membrane was incubated with a monoclonal antibody specific for all four AAV rep gene products. An antibody directed against actin served as a loading control. (B) eGFP direct fluorescence microscopy and Rep68 immuno-fluorescence microscopy on HeLa cells infected with hcAd.FLPe.F50 (50 GSAUs/cell) and either dHV.F50 (5 HTUs/cell; left column) or dHV.68/5′.F50 (5 HTUs/cell; right column). The pictures are derived from representative microscopic fields. The fluorescent signals derived from the green and red channels are overlaid in the lower panels. The arrowheads in the right panels point to cells containing both eGFP and Rep68. Note that the former protein is distributed throughout the cell whereas the latter is confined to the nucleus. Original magnification: 400×.

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Figure 6. Examination of targeted versus random dHV.68/5’3’;F50 DNA insertion through Southern blot analysis of genomic DNA.

(A) Upper panel, Eco32I restriction map of the AAV DNA preintegration region (i.e. the AAVS1 locus) on human chromosome 19 at 19q13.3-qter. Vertical line and solid oval, trs and RBE, respectively. The vertical solid arrows indicate Eco32I recognition sites delimiting a 5.6-kb DNA segment. The solid horizontal bar denotes the AAVS1-specific probe drawn in relation to its target sequence (diagram not on scale). Lower panel, Eco32I restriction map of dHV.68/5’3’;F50 DNA showing the various hybridization probes (horizontal solid bars) drawn in relation to their cognate target sequences (drawing not on scale). For an explanation of symbols and abbreviations, see the legend of Fig. 1. (B) Ten micrograms of total cellular DNA from 10 representative clones derived from HeLa cells co-infected with dHV.68/5’3’;F50 and hcAd.FLPe.F50 were digested with Eco32I. Following agarose gel electrophoresis, the resolved DNA fragments were subjected to Southern blot analysis with the AAVS1-specific probe (AAVS1 probe; upper panel). After removal of the AAVS1-specific probe, the transferred DNA was incubated simultaneously with a probe derived from the AAV p5IEE plus the rIG gene pA signal and a probe specific for the eGFP ORF (5’ and 3’ vector probes; middle panel). Finally, following a second stripping step, the transferred DNA was exposed to a fourth probe corresponding to the 1.9-kb Eco32I fragment in the dHV.68/5’3’;F50 genome (internal vector probe;
integration into the *AAVS1* locus and, consistent with the Southern blot data (Fig. 6 and Table 1), show that targeted integration events are more frequent in cells in which *rep68* expression is up-regulated by FLPe than in cells in which the *rep68* gene switch is not activated. AAV Rep78/68-dependent DNA integration into the *AAVS1* locus is not site-specific in the strict sense since it does not take place at a single nucleotide position but within a relatively long stretch of DNA. As a result, *AAVS1*-exogenous DNA junctions generated through the AAV machinery are usually present as single copies within a population of stably transduced cells. Thus, the smear detected in Fig. 7B, lane 4 most likely reflects the amplification of a mixture of *AAVS1*-dHV.68.5′.F50 DNA junctions with various sizes as observed following wild-type AAV DNA integration. To investigate this, PCR products from the smear (Fig. 7B, lane 4) were inserted into the cloning vector pCR-Blunt II-TOPO. The PCR fragment corresponding to the DMD myoblast clone 3C5 (Fig. 7B, lane 2) was cloned in parallel. Plasmid DNA purified from randomly selected colonies was digested with EcoRI to release the inserts. As expected, agarose gel electrophoresis revealed differently sized inserts reflecting the variation in vector DNA insertion sites within the *AAVS1* locus (data not shown). Finally, DNA sequencing of these inserts representing individual integration events revealed the precise nucleotide location at which target site and vector DNA covalently joined each other. Two examples are depicted in Fig. 7C. Altogether, these experiments complement the Southern blot data presented in Fig. 6 and firmly establish the capacity of *rep68*-positive dHV particles to accomplish insertion of large DNA at a specific locus in the human genome.

### Discussion

The ability of wild-type AAV to insert its DNA into a specific genomic region in cultured cells of higher primates is unique among all known eukaryotic viruses. However, the AAV DNA integration mechanism remains sketchy, partially owing to its complexity [34]. For example, it is not known whether the pre-integration complexes consist of DNA molecules with linear, circular or both types of topologies. There is evidence, nonetheless, for genomic insertion through circular intermediates from studies showing (i) the capacity of rAAV plasmids to integrate into the *AAVS1* locus, (ii) the generation of circular double-stranded DNA via AAV ITR-mediated intermolecular recombination and (iii) the presence in cells latently infected with wild-type AAV of head-to-tail proviral tandem repeats [34]. The unique structure of dHV genomes consisting of linear DNA molecules that lack AAV ITRs at one or both ends in their monomeric and dimeric configuration, respectively, precludes the generation of circular integration substrates via AAV ITR-mediated recombination. Our finding that Rep68 can catalyze the insertion of dHV DNA into the *AAVS1* region thus suggests that linear templates are also amenable to the AAV Rep78/68-dependent integration process.

Although detailed knowledge about the AAV DNA integration mechanism is lacking, researchers are actively pursuing the development of gene delivery systems that exploit the desirable feature of locus-specific integration provided by AAV elements [4,5,35,36]. When compared to non-targeted integrating vectors, these gene transfer systems might impose a lower risk of insertional oncogenesis caused by the inactivation of tumour suppressor genes or, as observed in clinical trials using gammaretroviral vectors, by the dysregulation of proto-oncogenes [37]. Among the many viral and non-viral vector systems capitalizing on the AAV integration machinery are those based on hAd vectors [14,38,39]. Recchia and colleagues incorporated rAAV DNA encoding GFP and hygromycin B phosphotransferase into an hAd vector genome [38]. Co-infection of Hep3B cells with the resulting vector and another hAd vector containing a bacteriophage T7 promoter-driven *rep78* gene at a multiplicity of infection (MOI) of 10 transducing units per cell each followed by hygromycin B selection revealed that the overall STF was 0.4% (i.e. 400 hygromycin B-resistant clones/10⁵ target cells). According to the authors, similar results were obtained in other human hepatic cell lines [38]. Of note, the STF was not enhanced by AAV *rep78* expression. However, Rep proteins did promote the insertion of the AAV

### Table 1. Frequencies of targeted foreign DNA integration events derived from Southern blot analysis of dHV.68.5′.F50-transduced HeLa cell and DMD myoblast clones.

|                  | HeLa cells | DMD myoblasts |
|------------------|------------|---------------|
|                  | dhHV.68.5′.F50 | dhHV.68.5′.F50 |
|                  | +hAd.FLPe.F50  | +hAd.FLPe.F50  |
| Randomly selected eGFP⁺ clones (N) | 40          | 38            |
| Clones with AAVS1 disruptions (D)/N [%] | 0/0 [0]    | 19/38 [50]    |
| Targeted integration events/N [%]      | 0/0 [0]    | 9/38 [24]     |
| Targeted integration events/D [%]      | 0/0 [0]    | 9/19 [47]     |

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ITR-flanked DNA into the AAVS1 locus in 14/39 (i.e. 35%) clones of stably transduced HepG2 cells [38]. Similar results were obtained with a single-particle version of this hcAd/AAV hybrid vector system accommodating a 3.8-kb rAAV genome and an inducible rep78 expression unit regulated by a doxycycline-controlled transcriptional silencer [40]. Recently, Wang and Lieber generated capsid-modified hcAd vectors containing a GFP-encoding rAAV genome significantly larger than those tested by Recchia and co-workers [39]. In parallel, they succeeded in rescuing and propagating a Rep78-encoding hcAd vector by relying on a strategy based again on a transcriptional regulatory element with low basal activity in producer cells (i.e. the human β-globin locus control region). Co-infection of human megakaryoblastic leukemia (Mo7e) cells with the rAAV- and rep78-containing hcAd vectors at an MOI of 300 and 800 genomes per cell, respectively, followed by their clonal expansion yielded 25.8% cell...
clones containing stably transduced Mo7e cells. However, the percentage of GFP-positive cells in individual Mo7e clones varied greatly, which makes it very hard to accurately determine the actual STF. Nonetheless, from data presented in Fig. 9 of the study by Wang and Lieber [39] one can deduce that the STF at best was in the order of a few percent. Substitution of the sup978-positive for a rep78-negative hcAd vector resulted in somewhat lower stable transduction levels [39]. Finally, in 30% of the stably transduced clones derived from Mo7e cells co-infected with the rAAV- and rep78-negative containing hcAd vectors, the foreign DNA was found to be inserted into the A141S1 region [39].

In this study, we report the generation of tropism-modified dHV containing AAV rep68 expression units and show their ability to mediate integration of DNA fragments of at least 10.3 kb into the A141S1 locus on human chromosomes 19 in HeLa and muscle progenitor cells. Side-by-side rescue and propagation experiments revealed that the growth properties of the rep68-positive dhV are very similar to that of the rep68-negative control vector. These results signify that the amounts of Rep68 made during the rescue and propagation of the rep68-positive dhVs were not enough to markedly inhibit their production. Transduction of rapidly proliferating human cells with dHV 68/5′3′.F50 particles followed by the activation of the rep68 gene switch incorporated in the vector genome led to the insertion at 19q13.3-qter of large DNA molecules that included a functional transgene expression unit and human dystrophin-coding sequences. Significantly, in HeLa cells exposed exclusively to dHV 68/5′3′.F50 (i.e., hcAd. FLPc.F50-negative cells), the basal Rep68 levels did not suffice to disrupt A141S1 loci and, in agreement with this observation, no targeted DNA insertion could be detected in any of the 40 clones that were analyzed (Fig. 6). However, by using a more sensitive PCR-based assay, evidence for low-level A141S1-targeted vector DNA insertion in FLPc-negative cells was found (Fig. 7B, lane 3).

An interesting aspect of the present study is the side-by-side comparison of the performance of an AAV Rep78/68-dependent DNA targeting system in cell types with very different genetic stabilities. HeLa cells are notorious for their aneuploidy and widespread translocations, whereas the human myoblasts used in this study remain diploid even after many cell divisions [31]. If one considers only those cells in which Rep68 induced unambiguous A141S1 disruptions, the targeted DNA insertion efficiency reached 47% in HeLa cells (9/19 clones) and a remarkable 100% in DMD myoblasts (5/5 clones). It is thus tempting to speculate that the lower targeted DNA integration proficiency of hybrid vector genomes in HeLa cells is a consequence of the higher genetic instability of these tumor cells causing a substantial fraction of vector genomes to insert randomly at double-stranded breaks possibly through non-homologous end-joining as observed with rep-negative rAAV DNA [41]. This would suggest that in the presence of appropriate amounts of rep78 and/or Rep68 molecules, dHV DNA might indeed display a marked preference for locus-specific insertion in the genomes of therapeutically relevant (i.e., non-transformed) cell types. Further experiments that might include tests on a larger panel of cell types are warranted to establish or disprove this postulate.

Importantly, we demonstrated that transduction of human cells with capsid-modified dHV particles can lead to long-term transgene expression in an AAV Rep68-dependent manner. However, notwithstanding the diverse experimental setups used, a common feature of this and other Rep78/68-dependent integrating hcAd vector-based gene carriers is their overall low STFs. The low STFs reached by these hybrid vector systems are at odds with those reported for wild-type AAV in human cells in culture. For instance, using Southern blot analyses, Hamilton and colleagues deduced that wild-type AAV can stably transduce up to 50% of infected HeLa cells [42]. In addition, by deploying a quantitative real-time PCR assay, other investigators showed that wild-type AAV DNA integrates at 19q13.3-qter in at least 13% of infected HeLa cells [43,44]. Recchia and colleagues found for their monopartite hcAd/AAV hybrid vector system that the efficiency of A141S1-specific integration was directly proportional to the Rep78 level in the target cells [40]. However, as mentioned previously, above a certain concentration, which might be different for different cell types, the two largest AAV Rep proteins have cytostatic or even cytotoxic properties. The low STFs achieved by the Rep78/68-dependent integrating hcAd vectors in long-term experiments deploying rapidly proliferating cells may thus relate to uncontrolled Rep78/68 synthesis. According to this hypothesis, many of the cells that have vector DNA integrated into the A141S1 locus will display a strong growth disadvantage due to (persistent) high Rep78/68 levels. This will result in the selection of cells that contain no or few Rep78/68 molecules and are therefore not stably transduced or have the vector DNA integrated outside of the A141S1 region.

The idea of a strong selection against cells displaying high Rep68 activity is consistent with the observation that although most cells that initially received dHV 68/5′3′.F50 were also transduced by hcAd.FLPc.F50, a significant proportion of the randomly isolated HeLa cell and DMD myoblasts clones did not show evidence of Rep68-induced A141S1 disruptions (i.e., 50 and 72%, respectively). Future hcAd/AAV hybrid vector systems should therefore be designed in such a way as to ensure that the Rep78/68 activity is minimal in producer cells and high but short-lived in target cells. Inducible gene expression cassettes encoding Rep78 and/or Rep68 variants whose stability or subcellular localization can be controlled by cell-permeable small-molecule drugs may help to fulfill these requirements. Such controllable gene expression may, in addition, allow the production of rep68-positive dHV particles that are not dependent on the introduction of FLPc or on any other foreign protein into target cells.

Materials and Methods

Cells

Culturing conditions for the human cervical carcinoma (HeLa) cells (American Type Culture Collection), the hAd5 early region 1 (E1)-deleted and bacteriophage P1 cre-expressing PER.tTA.Cre76 cells and the DMD myoblasts have been described elsewhere [11,31,45].

DNA constructions

Recombinant DNA techniques were performed by using established methods [46] or following the instructions supplied with specific reagents. Large-scale plasmid DNA purifications were carried out using JetStar 2.0 kits (Genomed) according to the manufacturer’s recommendations. All DNA constructs as well as their nucleotide sequences are available upon request.

Viral vectors

The production and titration of stocks of the E1-deleted and capsid-modified helper Ad vector Ad.floxedY.F50 have been previously described [11]. Ad.floxedY.F50 particles display at their surface chimeric fiber proteins consisting of basal shaft sequences from hAd5 and apical shaft and knob domains from hAd50 [11]. The rescue and propagation of dHV.F50, dHV.68/5′3′.F50 and dHV.68/5′3′.F50 were carried out essentially as described elsewhere [11,14]. The titers of dHV.F50, dHV.68/5′3′.F50 and dHV.68/5′3′.F50 stocks were determined by flow
cytometry [11,14] on the basis of eGFP expression and are expressed as HeLa-cell transducing units (HTUs) per ml. The fiber-modified hAd vector hAd.FLP.e.F50 served as source of FLPe [17]. hAd.FLP.e.F50 particles were produced in PER. tTA.Cre76 cells using the hAd vector shuttle plasmid pAd. FLP.e.dAgel together with Ad.floxed.P.F50. pAd.FLP.e.dAgel was derived from construct pAd.FLP.e [10] by removal of a 3.3-kb Agel fragment comprising stuffer sequences derived from the human hypoxanthine-guanine phosphoribosyltransferase 1 gene. As a consequence, the FLP-encoding hAd.FLP.e.F50 vector particles contain a 27.4-kb genome. Titration of the hAd.FLP.e.F50 preparation was performed on HeLa cells containing the FLP-responsive construct pGS.pA.Rep68 and of pAAV.DsRed at an MOI of 5 infectious units per cell served as positive control. pGAPDH.Rep78/68 directs the constitutive expression and are gene switch-activating units (GSAUs) per ml.

Flow cytometry
Quantification of eGFP- or DsRed.T4-positive cells was carried out by using a BD LSRII flow cytometer (Becton Dickinson). In all instances, 10,000 events were acquired. Data was analysed with the aid of BD FACSDiva Software version 5.0.1 (Becton Dickinson).

DNA transfections
Plasmid DNA transfections were carried out with Lipofectamine (Invitrogen) as specified before [14].

AAV-dependent DNA replication assay
The functionality of the FLP-activatable AAV serotype 2 (AAV2) rep68 expression plasmid pGS.pA.+Rep68 and of hAd.FLP.e.F50 were evaluated as follows. PER.tTA.Cre76 cells were seeded in 6-well plates (Greiner) at a density of 2.5 \times 10^5 cells per well. The next day, these cells were transfected with 1.5 \mu g of pGS.pA.+Rep68 and 1.5 \mu g of the rAAV vector shuttle construct pAAV.DsRed. pAAV.DsRed contains an expression unit consisting of a chimeric human elongation factor 1α (hEF1α) gene promoter [47], the DsRed.T4 ORF and the rabbit \( \beta \)-globulin (rBG) gene polyadenylation (pA) signal. The following day, the transfected cells were co-infected with Ad.floxed.P.F50 (5 infectious units [IU]/cell) and hAd.FLP.e.F50 (50 GSAUs/cell). PER.tTA.Cre76 cells transfected with 1.5 \mu g of pAAV.DsRed and 1.5 \mu g of the AAV2 rep78/68 expression plasmid pGAPDH.Rep78/68 and infected with Ad.floxed.P.F50 at an MOI of 5 IU/cell served as positive control. pGAPDH.Rep78/68 directs the constitutive synthesis of Rep78 and Rep68 from the promoter of the human glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene [14]. Negative controls consisted of pGS.pA.+Rep68- and pAAV.DsRed-transfected PER.tTA.Cre76 cells infected with Ad.floxed.P.F50 alone and of pGAPDH.Rep78/68- and pAAV.DsRed-transfected PER.tTA.Cre76 cells that were not exposed to hAd.FLP.e.F50 or Ad.floxed.P.F50. Three days posttransfection, the cells were harvested and processed for extrachromosomal DNA extraction.

Extrachromosomal DNA extraction and analysis
The purification of extrachromosomal DNA was performed as previously described [48], To selectively degrade the prokaryotic input DNA, the samples were treated for 1 h at 37°C with DpnI. After agarose gel electrophoresis through 1% agarose gels in 1× Tris-acetate-EDTA buffer, the DNA was transferred by capillary action onto nylon membranes (Amersham Hybond-XL; GE Healthcare). These membranes were subsequently incubated with blocking solution, exposed to the DNA probe specified below and repeatedly washed using a standard Southern hybridization procedure. Finally, the Southern blots were exposed to autoradiography films (Amersham Hyperfilm MP; GE Healthcare).

rAAV-specific replication products were detected with the aid of the 0.7-kb Neol×Eco52I fragment from pAAV.DsRed, which includes the entire DsRed.T4 ORF. This DNA probe was labeled with [\( ^{32}P \)]dATP (GE Healthcare) by using the HexaLabel DNA Labeling Kit (Fermentas).

Transduction experiments
HeLa cells and DMD myoblasts were seeded at densities of 1.0 \times 10^5 and 4.0 \times 10^5 cells per well of 24-well plates (Greiner), respectively. After an overnight incubation period the cells were exposed for 24 hours to dHV.50 and dHV.68/53F50. At 4, 20 and 100 HTUs/cell in total volume of 500 \mu l. Direct fluorescence microscopy and eGFP-directed flow cytometry were carried out 72 hours postinfection. Mock-infected HeLa cells and DMD myoblasts processed in parallel served to set the background levels of each assay.

Light microscopy and immunostaining
eGFP direct fluorescence microscopy and AAV Rep immunofluorescence microscopy were performed with an Olympus IX51 inverse fluorescence microscope. Images were captured by a ColorView II Pellet-cooled charge-coupled device camera and were archived using analySIS software (Soft Imaging Systems). Cells were processed for immunostaining as described before [11] except that the primary antibody solution consisted of a 1:50 dilution of murine monoclonal antibody 303.9 (PROGEN Biotechnik; catalog number: 61069), which recognizes all four AAV Rep proteins.

Western blot analysis
Two-cm^2 monolayers of HeLa cells were washed once with phosphate-buffered saline and dissolved in 100 \mu l ice-cold lysis buffer consisting of 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% sodium deoxycholate (SDS), 0.5% sodium deoxycholate, 1% Nonidet P40 and 10% glycero1 plus a cocktail of protease inhibitors (Complete Mini, Roche Applied Science). The protein lysates were spun for 10 min at 4°C in a minicentrifuge at 25,000 \times g. Fifteen \mu l of the resulting supernatants were mixed with 3 \mu l of 6× sample buffer (360 mM Tris-HCl (pH 6.7), 60% glycerol and 12% SDS), which was supplemented immediately prior to use with 150 \mu l of \( \beta \)-mercaptoethanol and 150 \mu l of a saturated bromophenol blue solution per 2.5 ml. Next, the samples were heated for 5 min at 100°C and subjected to electrophoresis through an SDS-10% polyacrylamide gel. Resolved proteins were subsequently transferred onto an Immobilon-P membrane (Millipore) by electroblotting. After incubation for 2 h at room temperature (RT) with blocking solution (10 mM Tris-HCl [pH 8.0], 150 mM NaCl and 0.05% Tween-20 [TBST]) containing 10% non-fat dry milk powder (Elk, Campina), the Western blot was incubated overnight at 4°C with monoclonal antibody 303.9 diluted 1:50 in blocking solution. Next, the Immobilon membrane was washed 3 times for 5 min each with TBST at RT and subjected to a 1-h incubation with horseradish peroxidase-conjugated goat-anti-mouse IgG secondary antibodies (Santa Cruz Biotechnology; catalog number: sc-2005) diluted 1:10,000 in blocking solution. After three 20-min washes with TBST, the Western blot was incubated for 5 min with chemiluminescence substrate solution (100 mM Tris-HCl [pH 8.6], 250 \mu g/ml 5-aminoo-2,3-dihydroxy-1,4-phenylazinedione [Sigma-Aldrich]) which was supplemented immediately prior to use with 20 \mu l of a 30% H2O2 solution and 1 ml of a 0.33% 3-(4-
hydroxynaphthyl-2-propenoic acid (Sigma-Aldrich) solution in dimethylsulfoxide per 100 ml. The chemiluminescent signal was visualized by exposing the Immobilon membrane to an Amersham Hyperfilm MP film. Actin served as loading control and was visualized by exposing the Immobilon membrane to an Amersham hydroxyphenyl)-2-propenoic acid (Sigma-Aldrich) solution in 24 hours after which the inocula were substituted by fresh culture medium. At 72 hours postinfection, genomic DNA was extracted from these cells as described above. Chromosomal DNA was also extracted from HeLa cell clone N+2 and DMD myoblast clone 3C5, which were derived from cells stably transduced with dHV.68/5’3’.F50 DNA through random and AAVS1-targeted DNA insertion, respectively. Next, samples containing 160 ng of genomic DNA were subjected to PCR with the AAVS1-specific primer # 211 (5’-GAGGGTCACGCTCTGCTG-3’), together with primer # 651 (5’-TTGCTAAAGCCAAGCTTGGC-3’) targeting the vector DNA (i.e. kEF1z promoter sequence). PCR amplifications with the AAVS1-specific oligodeoxyribonucleotides # 211 and # 196 (5’-GGCTCAAGACGGAGG-3’) were carried out in parallel to serve as a control for template integrity and are expected to give rise to a 354-bp PCR product. PCR mixtures of 50 µl containing 200 µM dNTPs, 0.2 µM of each primer, 0.012 U/µl of Phusion High-Fidelity DNA polymerase (Finnzymes) and 1 × GC buffer (Finnzymes) were placed in a DNA Engine Tetrad 2 thermal cycler (Bio-Rad) and a touchdown PCR program was initiated by a 3-min incubation at 98°C, followed by 19 cycles of 98°C for 20 sec, an annealing period of 30 sec with the temperature decreasing by 0.5°C every cycle from 62 to 52°C, and a 2-min extension step at 72°C. When the PCR program reached the lower annealing temperature of 52°C, 28 additional cycles were performed by using the same cycling conditions except for the use of a constant annealing temperature of 52°C. This was followed by a 5-min incubation at 72°C. Next, 1 µl of each PCR sample was subjected to a nested PCR with the AAVS1- and vector DNA-specific primers #212 (5’-GGCTTGGCACCCTATGGT GAC-3’) and # 188 (5’-GGATCTAGGACCCCTAGTGAGTGG-3’), respectively. The cycling conditions applied to the nested PCR were as described above except for the use of a maximal annealing temperature of 69°C and a final touchdown temperature of 59°C. Samples from all PCR amplifications were subjected to agarose gel electrophoresis. Subsequently, PCR products were purified from the gel with the aid of the JetSorb gel extraction kit (GENOMED) and the recovered DNA was incorporated into cloning vector pCR-Blunt II-TOPO (Invitrogen) according to the instructions supplied by the manufacturer. Finally, the nucleotide sequence of several of the cloned PCR fragments was determined as previously described [43] by using the M13 forward (5’-GAGCTGTTGAAACCGCGCCAGT-3’) or reverse (5’-GAGGGAACGCACTGATGAATG-3’) primer.

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Author Contributions

Conceived and designed the experiments: MAFVG AAfIDV. Performed the experiments: MAFVG MH GPN AAfIDV. Analyzed the data: MAFVG MH AAfIDV. Contributed reagents/materials/analysis tools: MAFVG MH GPN RW MGP AAfIDV. Wrote the paper: MAFVG. Edited the paper: AAfIDV.

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