A Helper-Dependent Capsid-Modified Adenovirus Vector Expressing Adeno-Associated Virus Rep78 Mediates Site-Specific Integration of a 27-Kilobase Transgene Cassette[▽]

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Random integration of viral gene therapy vectors and subsequent activation or disruption of cellular genes poses safety risks. Major efforts in the field are aimed toward targeting vector integration to specific sites in the host genome. The adeno-associated virus (AAV) Rep78 protein is able to target AAV integration to a specific site on human chromosome 19, called AAVS1. We studied whether this ability could be harnessed to achieve site-specific integration of a 27-kb transgene cassette into a model cell line for human hematopoietic cells (Mo7e). To deliver *rep78* and the transgene to Mo7e cells, we used helper-dependent adenovirus (Ad) vectors containing Ad serotype 35 fiber knob domains (HD-Ad). An HD-Ad vector containing the *rep78* gene under the control of the globin locus control region (LCR) (Ad.LCR-*rep78*) conferred Rep78 expression on Mo7e cells. Upon coinfection of Ad.LCR-*rep78* with an HD-Ad vector containing a 27-kb globin-LCR-green fluorescent protein (GFP) transgene cassette flanked by AAV inverted terminal repeats (ITRs) (Ad.AAV-LCR-GFP), transduced cells were cloned and expanded (without selection pressure), and vector integration was analyzed in clones with more than 30% GFP-positive cells. Vector integration into the AAVS1 region was seen in 30% of analyzed integration sites, and GFP expression from these integrants was stable over time. Of the remaining integration sites, 25% were within the genomic globin LCR. In almost 90% of sites, transgene integration occurred via the Ad ITR. This indicates that rescue of the AAV ITR-flanked transgene cassette from Ad.AAV-LCR-GFP is not required for Rep78-mediated integration into AAVS1 and that free ends within the vector genome can be created by breaks within the Ad ITRs, whose structure is apparently recognized by cellular “nicking” enzymes. The finding that 55% of all analyzed integration sites were either within the AAVS1 or globin LCR region demonstrates that a high frequency of targeted integration of a large transgene cassette can be achieved in human hematopoietic stem cell lines.

For gene therapy applications, the development of vectors that target transgene integration to specific sites in the host genome is a major focus (7, 32). In this context, the ability of adeno-associated virus (AAV) to integrate into the host genome at a preferred site on chromosome 19q13.4q is of interest (13). This site, called AAVS1, is about 4 kb long and has been mapped to the first exon of myosin binding subunit 85 of the protein phosphatase 1. The AAVS1 site appears to be in an open chromatin conformation in the cell lines tested (31), which is thought to help AAV integration and viral-gene expression (15). Integration into this site is mediated by the large AAV Rep protein Rep68 or Rep78. Rep68/78 demonstrate sequence-specific endonuclease activity and ATP-dependent helicase activity and can mediate rescue/excision of DNA flanked by AAV ITRs in the presence of adenovirus (Ad) superinfection (24). Rep-mediated rescue requires the presence of both a Rep binding site (RBS) and a terminal resolution site (TRS) within the virus AAV ITR and genomic AAVS1 site, and the secondary structures of these sites appear to be crucial for Rep68/78-mediated DNA nicking. Rep-mediated rescue of a cassette flanked by AAV ITRs from double-stranded DNA (dsDNA) substrates and nicking within the

AAVS1 site creates free DNA ends, which allow virus integration via nonhomologous end joining. It is therefore thought that rescue from incoming dsDNA is a prerequisite for integration (2, 36, 37).

The ability of Rep68/78 to mediate integration into AAVS1 has been harnessed for site-specific integration of transgenes from cotransfected dsDNA plasmids. The frequency of AAVS1-specific integration by these plasmid-based methods has differed from 20 to 50% among studies when integration frequency was measured based upon drug selection (2, 8, 14). However, plasmid transfection is not an efficient method for gene transfer into hematopoietic stem cells. The production of Rep-expressing viral-gene transfer vectors (based on adenovirus or herpes simplex virus type 1), however, is problematic, because expression of Rep in packaging cells has been shown to severely reduce vector production (10, 21, 22). The vector yield could be increased by placing the *rep* gene under the control of promoters with low activity in packaging cells (21) or by using Tet- or Cre/Lox-inducible gene expression systems (17, 22). Our goal was to deliver both a transgene under the control of the globin locus control region (LCR) and the *rep78* gene with adenovirus vectors containing Ad group B fiber knob domains. Our group and others have previously shown that these vectors efficiently transduce important gene therapy targets that are refractory to infection with commonly used Ad type 5 (Ad5) vectors, including hematopoietic stem cells (18, 20, 28, 29, 38). Because transduction of primary cells, partic-

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FIG. 1. Structures of helper-dependent Ad vectors. All vectors possess Ad5/35 capsids and contain the β -globin LCR and β -globin 3' HS1 region as stuffer DNA. Ψ , Ad packaging signal; HS, DNase I hypersensitivity region.

ularly primary hematopoietic stem cells, with first-generation recombinant Ad vectors with E1/E3 deleted is associated with toxicity due to viral-gene expression (27, 33), we used helper-dependent (HD) Ad vectors, which are devoid of all viral genes (12), in our studies. We have previously shown that HD vectors containing B-group serotype 35 fiber knob domains (HD-Ad5/35) efficiently transduced human leukemia cells and primary CD34⁺ cells (27, 33). In this study, we analyzed Rep-mediated rescue and site-specific integration upon cotransduction of Mo7e cells with HD-Ad5/35 vectors containing the *rep78* gene and a 27-kb-long transgene cassette.

MATERIALS AND METHODS

Ad and plasmid vectors. Ad.AAV-LCR-GFP was generated earlier and is described elsewhere (33). It is an HD-Ad vector carrying the β -globin LCR, a cytomegalovirus (CMV) promoter, and the green fluorescent protein (GFP) gene; the LCR-CMV-GFP cassette is flanked by AAV ITRs. This vector contains the Ad 35 fiber knob. Ad.LCR-GFP is derived from Ad.AAV-LCR-GFP and lacks the AAV ITRs. The PCR primers used for the construction of vectors were described before (33). The pAd-3'HS1 plasmid contains the 5' ITR (nucleotides 1 through 436) and 3' ITR (nucleotides 35741 through 35938) of Ad5. To generate pAd-3'HS1, a PCR fragment of the Ad5 5' ITR and packaging signal was digested with EcoRV and inserted into the EcoRV site of pBluescript II KS(-) (pBS-Ad5ITRpsi). A PCR fragment of the Ad5 3' ITR was digested with PmeI and EcoRV and inserted into the PmeI site of pBS-Ad5ITRpsi (pBS-Ad5). pWE15c (33) was digested with EcoRI, blunted, and ligated with the EcoRV fragment from pBS-Ad5 (pWE15c-Ad5). A 6.7-kb BamHI fragment from pHCA (33) was inserted into the BglII site of pWE15c-Ad5. A 3-kb fragment containing the 3' HS1 region of the human β -globin locus was inserted between the two Ad ITRs (pAd.3'HS1). Next, a 21.5-kb human β -globin LCR derived from the

β -globin minilocus (Sall/ClaI fragment) was inserted in front of the 3' HS1 in pAd-3'HS1 (XhoI/ClaI) to make pAd.LCR. To generate pAd.LCR-GFP (Fig. 1), a CMV-GFP-polyadenylation signal (pA) cassette was inserted into the ClaI site of pAd.LCR (33). The resulting plasmids were packaged into phages using Gigapack III Plus Packaging Extract (Stratagene, La Jolla, CA) and propagated. To construct the Ad.LCR-Rep78 vector, we replaced the GFP gene in Ad.LCR-GFP with a modified *rep78* gene. The *rep* cassette is designed to express only Rep78 with the P5 promoter completely removed. The Rep 52/40 start codon (the ATG at position 993) was mutated, and the splice donor site for Rep68 was destroyed (position 1905), resulting in individual Rep78 expression. pBS- β -GFP (33) was digested with NcoI and EcoRI, blunted with mung bean nuclease, and religated (pBS- β). The Rep78-simian virus 40 (SV40) pA fragment derived from pBKS.rep78.SV40pAKS(-) (3) by ClaI digestion was blunted with T4 DNA polymerase and inserted into pBS- β downstream of the β -globin promoter. The β -Rep78-pA cassette was inserted into the ClaI site of pAd.LCR-GFP instead of the CMV-GFP-pA cassette. Notably, in pAd.LCR-rep78, Rep expression is under the control of the β -globin promoter/LCR, which minimizes expression in 293 cells and Rep-mediated inhibition of Ad replication. Ad.Co (Ad.LCR-MSCV-ecoR) was constructed by replacing the β -Rep cassette in Ad.LCR-Rep78 with a mouse stem cell virus (MSCV)-ecoR cassette that expresses the ecotropic retroviral receptor (ecoR). The MSCV-ecoR-pA cassette (30) was inserted into the ClaI site of pAd.LCR-GFP instead of the CMV-GFP-pA cassette.

HD-Ad vectors were rescued, amplified, and purified as described earlier (33). Ad genome concentrations were determined by both quantitative Southern blotting and quantitative PCR as described earlier (33). Titers of Ad vectors were in the range of 3×10^{12} to 5×10^{12} genomes/ml. Helper vector contamination in final preparations was less than 1% (based on quantitative PCR).

The following plasmids were employed as rescue substrates. pAd.AAV-LCR-GFP (which was used to generate the Ad.LCR-CMV-GFP virus) is a 42.7-kb plasmid yielding a 27-kb rescue product. pAd.AAV(2)-HS5 (10.3 kb) and pAAV-HS5 (10.5 kb) both yield a 6.5-kb rescue product. pAAV(2)-HS5 contains the HS5 fragment of the β -globin LCR, the β promoter-GFP cassette, and the 3'

FIG. 2. Rep78 protein expression and activities of LCR- β and CMV promoters in 293 cells. (A) Detection of Rep78 protein expression upon HD-Ad infection. 293 and Mo7e cells were infected with Ad.LCR-rep78 virus and control virus (Ad.AAV-LCR-GFP) at an MOI of 400 genomes/cell. At 60 h postinfection, cells were collected and subjected to immunoprecipitation and Western blot analysis with Rep78-specific antibodies. (B) Transient GFP expression in 293 cells. 293 cells were infected with Ad.LCR- β -GFP and Ad.HCA-CMV-GFP (33) HD vectors at increasing MOIs; 48 h postinfection, the percentage of GFP-expressing cells (top) and the mean fluorescence (bottom) were analyzed by flow cytometry. The error bars represent standard deviations. (C) Rep78 expression in 293 cells after plasmid transfection. 293 cells were transfected with pCMV-Rep and pBS β -Rep plasmids (see Materials and Methods). Cells were harvested 72 h after transfection and analyzed by Western blotting.

ITR of AAV2. p3'HS1-AAV (24) was digested with SalI and XhoI and ligated with the PstI/SalI fragment containing the β -GFP-pA cassette from pBS- β -GFP (33) and the SalI/PstI fragment containing the HS5 region from pAd.LCR-GFP. The 5' ITR of AAV2, derived from pAE1.IRS.RSV.hAAT.ApoE (4), was inserted downstream of the GFP gene (pAAV-HS5- β -GFP-AAV). pAd.AAV(2)-HS5 was generated by inserting a ClaI/FseI fragment of pHS5- β -GFP-AAV into the ClaI/FseI sites of pBS-Ad-5'AAV (33).

Cells. Culture media (Dulbecco's modified Eagle's medium, Iscore's modified Dulbecco's medium, and RPMI 1640) were purchased from GIBCO/BRL (Gaithersburg, MD). Fetal calf serum (FCS) was from HyClone (Logan, UT). The human leukemic cell lines Mo7e, HEL, and K562 (ATCC 45506) were maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. For culturing Mo7e cells, 0.1 ng/ml of granulocyte-macrophage colony-stimulating factor (Avigen, Seattle, WA) was added to the medium. 293 (human embryonic kidney; Micro-biex, Toronto Canada) and C7-cre (9) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM glutamine, and 1 $\times$ penicillin-streptomycin solution (Invitrogen, Carlsbad, Calif). C7-cre cells were used for propagation of HD-Ad vectors; 0.5 mg/ml G418 and 100 μ g/ml hygromycin were added to maintain Cre, polymerase, and pre-terminal protein expression.

Cloning of transduced Mo7e cells. A total of 2×10^5 Mo7e cells were infected with HD-Ad vector at the indicated multiplicities of infection (MOI). Twenty-four hours postinfection, the cells were diluted and seeded onto 96-well plates at 0.5 cell/well. Two to 3 weeks later, when the number of cells in the colonies reached 2×10^3 to 3×10^3 , the wells containing GFP-positive cells were counted and the percentage of GFP-positive cells in each clone was estimated. Clones with more than 30% GFP-positive cells were sorted by fluorescence-activated cell sorter. The sorted cells were further expanded to $\sim 10^7$ cells for Southern analysis.

Western blot analysis of Rep78 expression. To detect the Rep78 protein, lysates of transduced 293 or Mo7e cells were incubated with a mouse monoclonal anti-AAV-2 Rep78 antibody (catalog no. 03-61073; American Research Products, Inc., Belmont, MA). Complexes were pulled down with protein G-Sepharose beads (Sigma, St. Louis, MO) and separated by polyacrylamide gel electrophoresis. The proteins were blotted onto nitrocellulose membranes and probed with the mouse monoclonal anti-AAV-2 Rep78 antibody (catalog no. 03-61071;

American Research Products), followed by anti-mouse immunoglobulin horse-radish peroxidase-conjugated antibody.

Southern blot analyses. Total cellular DNA was extracted from cultured cells by pronase digestion, followed by phenol-chloroform extraction and ethanol precipitation, and 10 μ g of total DNA was subjected to Southern blot analysis as described before (33). 32 P-labeled DNA probes were used for hybridization and are described in the figure legends. The HS5 probe was a 972-bp MscI/SacI fragment derived from the human β -globin LCR HS5; a 3' HS1 probe was the 938-bp BglII/HindIII fragment derived from the human β -globin 3' HS1. The AAVS1 probe was a 1.6-kb EcoRI/BamHI fragment from pRE2 (24).

Analysis of integration sites. Integration into AAVS1 was analyzed by nested PCR of cellular DNA isolated from GFP-expressing clones. The following primers were used: vector-specific primers, AAV-1 (5'-GATACCGTCGACCTCGA TCTCAGGAAC-3') and AAV-0 (5'-CGATCTCAGGAACCCCTAGTGATG GAGTTG-3'); AAVS1-specific primers, P1a (5'-GCTGTCCAGTCGAATTCC TAACTGC-3') and P1b (5'-GGCAGTCTGCTATTCATCCCTTTAC-3'), P2a (5'-AGAGTAGGTCGAAGGGGAATGGTAAGGAG-3') and P2b (5'-CAGA GTGGTCAGCACAGAGTGGCTAAG-3'), and P3a (5'-GTCTCTCTCTCTGA GTCCGGACCACTTTG-3') and P3b (5'-ACCACTTTGAGCTCTACTGGCT TCTGC-3').

PCR was performed with one primer specific to the vector (universal for both the 5' end and the 3' end) and the other primer specific to the AAVS1 region, with the following conditions: 95°C for 2 min and 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The PCR products were diluted 1:100, and nested PCR was performed under the same conditions using a second set of primers. PCR fragments obtained from different clones were cloned into pGEM-T Easy (Promega, Madison, WI), and junctions were sequenced using vector-specific primers.

RESULTS

Construction and characterization of Rep78-expressing HD-Ad vector. All HD vectors contained Ad serotype 35 fibers (Ad5/35). As outlined above, Ad5/35 vectors efficiently transduce human CD34⁺ cells, particularly subsets with potential

FIG. 3. Rep78-mediated rescue in 293 cells. (A) 293 cells were transfected with pAd.AAV(2)-HS5 (a 10.3-kb plasmid yielding a 6.5-kb rescue product), pAAV-HS5 (a 10.7-kb plasmid yielding a 6.5-kb rescue product), or pAd.AAV-LCR-GFP (a 42.7-kb plasmid yielding a 27-kb rescue product), together with pBS- β -Rep and pHelper (an AAV helper-free system; Stratagene) plasmids. Seventy-two hours after transfection, cells

FIG. 4. Rep-mediated rescue in human leukemia cell lines. Mo7e, K562, and HEL cells were infected with Ad.AAV-LCR-GFP (300 genomes/cell) and Ad.LCR-rep78 (300 genomes/cell). Seventy-two hours after virus infection, cells were harvested and total cellular DNA was extracted. A total of 10 μ g DNA was digested with *Sac*I and subjected to Southern blotting with an HS5 probe.

stem cell capacity. Previously, we constructed an Ad5/35-based HD adenovirus vector containing a 27-kb globin LCR-GFP expression cassette flanked by AAV ITRs (Ad.AAV-LCR-GFP) (33) (Fig. 1). Our primary goal was to test whether transient Rep78 expression would rescue this large expression cassette from the HD-Ad genome and mediate its site-specific integration into AAVS1. We planned to use HD-Ad5/35 vectors to deliver the *rep78* gene to our target Mo7e cells, a human erythroleukemic cell line. However, construction of Rep78-expressing Ad vectors is problematic, because trace amounts of Rep78 inhibit Ad replication (5). To minimize Rep78 expression in 293 cells (the cell line that is used to generate Ad vectors), we placed the *rep78* gene under the control of the human β -globin LCR (Fig. 1). We produced this vector (Ad.LCR-rep78) at a titer of 2.45×10^{12} genomes per ml with less than 0.5% helper virus contamination.

Rep78 expression from Ad.LCR-rep78 virus after infection of 293 and Mo7e cells was analyzed by Western blotting using Rep-specific antibodies (Fig. 2). Rep78 was clearly detectable in both cell lines. The presence of a less intense band with a molecular mass of ~ 50 kDa indicated that the mutation of the Rep52/40 start codon (see Materials and Methods) did not completely abolish Rep52 expression. Notably, the relatively high Rep78 expression levels in 293 cells could be due to coreplication of Ad.LCR-rep78 genomes by residual first-generation helper virus present in the HD-Ad vector preparation. Furthermore, we were not able to produce HD-Ad vectors that contained

the *rep78* gene under the control of the phosphoglycerate kinase or CMV promoter, and we speculate that Rep78 levels expressed from these promoters affect Ad replication/packaging. Significant differences in levels of transgene expression were demonstrated when 293 cells were infected with Ad.AAV-LCR-GFP (containing the GFP gene under the control of the LCR- β -globin promoter) and Ad.HCA-CMV-GFP (containing the GFP gene under the control of the CMV promoter) (Fig. 2B). Furthermore, analysis of Rep78 levels by Western blotting upon transfection of 293-Cre7 cells with pCMV-Rep78 and pBS β -Rep78 (which contains the 1.6-kb beta promoter and not the LCR) revealed higher Rep78 levels in pCMV-Rep78-transfected cells (Fig. 2C).

Rescue of a 27-kb expression cassette. We next tested whether Rep78 expressed from our Ad.LCR-rep78 vector was able to rescue an AAV ITR-flanked transgene cassette. Initial studies were performed in 293 cells with plasmids containing AAV ITR-flanked transgene cassettes of 6.5 kb [pAd.AAV(2)-HS5 and pAAV-HS5] and 27 kb (pAd.AAV-LCR-GFP) (Fig. 3A). The plasmids were transfected into 293 cells, followed by infection with Ad.LCR-rep78. After 48 h, cellular DNA was analyzed by Southern blotting using a vector-specific probe. Rescue products of the predicted size appeared in monomeric and dimeric forms. The amounts of rescue products (relative to input plasmid DNA) were comparable for the two plasmid templates, indicating that Rep78-mediated rescue of a 27-kb transgene cassette is as efficient as rescue of a 6.5-kb cassette (Fig. 3A). Next, we analyzed Ad.LCR-rep78-mediated rescue

were collected, and 15 μ g total cellular DNA was digested with *Sac*I and analyzed using an HS5-specific probe. M, monomeric rescue product (5' *Sac*I fragment); D, dimeric rescue product (5' *Sac*I fragment). (B) (Lower left) 293 cells were infected with Ad.AAV-LCR-GFP at an MOI of 200 genomes/cell and Ad.LCR-rep78 at increasing MOIs of 0, 10, 25, 50, 100, and 200 genomes/cell (Ad.Co was used to bring the total MOI to 200 genomes/cell). Forty-eight hours after infection, cells were harvested and total cellular DNA was extracted; 15 μ g DNA was digested with *Sac*I for Southern blot analysis using an HS5 probe. (Lower right) 293 cells were infected with Ad.AAV-LCR-GFP at an MOI of 200 genomes/cell and coinfecting with Ad.LCR-rep78 at an MOI of 100 genomes/cell (lanes 2, 4, and 6) or coinfecting with control virus (Ad.Co) at an MOI of 100 genomes/cell (lanes 1, 3, and 5). Forty-eight hours after infection, cells were harvested, total cellular DNA was extracted, and 15 μ g of DNA was digested for Southern blot analysis with a 3' HS1 probe or an HS5 probe (see the vector scheme above). Lanes 1 and 2 show DNA digested with *Bgl* II; lanes 3 and 4 show DNA digested with *Eco*RI; lanes 5 and 6 show DNA digested with *Sac*I.

FIG. 5. Roles of Ad proteins in rescue. (A) Mo7e cells were infected with Ad.AAV-LCR-GFP (300 genomes/cell) and Ad.LCR-rep78 (300 genomes/cell). First-generation Ad5/35 virus was added at increasing MOIs: 0, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, and 1,200 genomes/cell. Forty-eight hours postinfection, total cellular DNA was extracted and digested with *SacI*. Southern blotting was performed with an HS5 probe. M, monomeric rescue product (5' *SacI* fragment); D, dimeric rescue product (5' *SacI* fragment). (B) Mo7e cells were infected with Ad.AAV-LCR-GFP virus (MOI, 300 genomes/cell), Ad.LCR-rep78 virus (MOI, 300 genomes/cell), or Ad5/35 (first-generation) virus (MOI, 1,000 genomes/cell). One set of cells was incubated with 10 mM hydroxyurea (HU) 1 h after infection to block viral DNA replication; 48 h after infection, cells were harvested and total cellular DNA was extracted. A total of 10 μ g DNA was digested with *SacI* and subjected to Southern blotting with an HS5 probe. (C) 293 cells were transfected with plasmids pAd.AAV(2)-HS5 and pBS- β -Rep and with one of the following plasmids: pCMV-DBP, pCMV-E4orf6, pCMV-pol, pCMV-pTP, or pHelper (an AAV helper-free system; Stratagene). At 72 h after transfection, total cellular DNA was extracted, digested with *SacI*, and subjected to Southern blotting using an HS5 probe.

from a coinfecting Ad.AAV-LCR-GFP virus (Fig. 3B). We showed that the rescue efficiency correlated with the MOI of Ad.LCR-rep78 vector used for infection (Fig. 3B, left). Rescue of the complete 27-kb cassette was demonstrated by Southern blotting using DNA probes that were specific to the extreme ends of the cassette (Fig. 3B, middle and right). While previously published studies reported the rescue of <5-kb cassettes (3), this is the first demonstration of an efficient Rep78-mediated rescue of a 27-kb cassette from an Ad vector. We have also found that the rescue efficiency from plasmids containing

two AAV ITRs was not significantly higher than that from plasmids containing only one AAV ITR (data not shown). In contrast to the rescue study in 293 cells, similar rescue studies in Mo7e cells and other leukemic cell lines (HEL and K562) did not yield detectable rescue products (Fig. 4). Notably, the transduction efficiency of these cells 18 h after infection with Ad.AAV-LCR-GFP based on GFP fluorescence was as great as that of 293 cells. We hypothesize that rescue in 293 cells could have been enhanced by the presence of low levels of helper virus. Because 293 cells complement the E1 deletion,

FIG. 6. Analysis of transduction and integration in Mo7e clones infected with Ad.LCR-GFP and Ad.LCR-rep78 or Ad.Co. (A) Mo7e cells were infected with Ad.AAV-LCR-GFP at an MOI of 300 genomes/cell and Ad.LCR-rep78 (gray bars) or control virus, Ad.Co (black bars), at an MOI of 300 genomes/cell. Thirty-four hours after infection, the infected cells were diluted and plated as single cells in 96-well plates. Three weeks later, GFP-positive colonies that stably expressed GFP were analyzed. (B) Mo7e GFP-positive cell clones from cells infected with Ad.AAV-LCR-GFP and Ad.Co. A total of 15 μ g genomic DNA was digested with EcoRI and analyzed by Southern blotting with an AAVS1-specific probe (a 1.6-kb EcoRI/BamHI fragment from pRE2). (C) Mo7e GFP-positive cell clones derived from cells infected with Ad.AAV-LCR-GFP and Ad.LCR-rep78. The encircled bands marked with stars appear in both plots hybridized with the AAV-S1 and HS5 probes. A total of 15 μ g genomic DNA from each GFP⁺ clone was digested with EcoRI and hybridized with an AAVS1-specific probe or HS5 and 3'HS1 probes.

helper virus replicates in infected cells, which results on one hand in the production of viral proteins and, on the other hand, in coreplication of HD-Ad genomes. To test this hypothesis, we infected Mo7e cells with Ad.LCR-rep78 and Ad.AAV-LCR-GFP and added increasing amounts of first-generation Ad5/35 vector (Fig. 5A). Addition of a first-generation Ad5/35 vector (of ≥ 200 particles per cell) resulted in detectable rescue in Mo7e cells in a dose-dependent manner. This effect of coinfecting first-generation Ad was nullified by the addition of hydroxyurea, an inhibitor of adenoviral DNA replication (Fig. 5B). Coinfection of first-generation Ad could increase rescue due to (i) coreplication of Ad.AAV-LCR-GFP and generation of single-stranded vector genome intermediates, which are potentially better targets for Rep78-mediated rescue; (ii) coreplication of Ad.LCR-rep78 genomes, resulting in increased

Rep78 expression; and/or (iii) expressed viral proteins (expressed from first-generation Ad vectors upon transduction) that act *in trans* to support rescue or to stabilize Rep78. In an attempt to distinguish between these possibilities, we performed plasmid transfection experiments (which exclude viral DNA replication). The helper functions of Ad for AAV replication are well characterized and are supplied by E1a, E1b, E2, E4, and the virus-associated (VA) RNA (23). Since we observed rescue using Ad vectors with E1 deleted as "helpers" in Mo7e cells, we formally excluded the possibility that E1 proteins support rescue. We obtained or generated plasmids that express DNA-binding protein (DBP) (39), polymerase (26), or E4orf 6 (25) under the control of the CMV promoter. We also used the pHelper plasmid from Stratagene (35), which expresses a combination of E4, E2A, and VA RNA genes

under the control of the endogenous viral promoters. We transfected 293 cells with pAd.AAV(2)-HS5 and a Rep78-expressing plasmid in combination with different plasmids that express Ad proteins (Fig. 5C). We found that Rep-mediated rescue was enhanced by coexpression of viral proteins from pCMV-DBP and pCMV-E4orf6. The greatest effect, however, was seen with pHelper that expressed a combination of Ad proteins.

Transduction and integration analyses. We next performed transduction and integration studies with Ad.LCR-rep78 and Ad.AAV-LCR-GFP vectors. Mo7e cells were infected with Ad.AAV-LCR-GFP at an MOI of 300 genomes/cell and Ad.LCR-rep78 or Ad-control at an MOI of 300 genomes/cell. (We determined in preliminary studies that infection of Mo7e cells at this MOI results in an average of one integrated Ad.AAV-LCR-GFP vector copy per Mo7e cell genome [33].) Twenty-four hours after infection, cells were seeded into 96-well plates with 0.5 cell per well. Three weeks after limited dilution of infected Mo7e cells, GFP expression was analyzed in all colonies (Fig. 6A). Under these infection conditions, the overall frequencies of stable transduction with the GFP-expressing vector were 25.8% and 18.9% upon coinfection with Ad.LCR-rep78 and Ad.Co, respectively. The difference between the two groups was not significant ($P = 0.2$). Notably, the majority of integration events in the presence of Rep78 occurred in AAVS1.

We then performed detailed integration studies in colonies with >30% GFP⁺ cells. GFP⁺ cells were sorted from GFP-negative cells and expanded, and the genomic DNA was analyzed by Southern blotting (33), inverse PCR (33), and AAVS1 PCR (see Materials and Methods) for vector integration. Southern blot analysis using an AAVS1-specific probe revealed no chromosomal DNA rearrangements in clones from Ad.Co-coinfected cells (Fig. 6B). In contrast, disruption of the AAVS1 region was found in 10 out of 20 Mo7e clones infected with Ad.LCR-rep78 (Fig. 6C). Bands hybridizing to both AAVS1 probe and viral probes that could be considered AAVS1 site-specific vector integration were found in clones 6, 11, 13, 14, 16, and 19 (30% of all clones). GFP expression from the LCR-GFP cassette upon integration into AAVS1 was significantly higher than expression from other integration sites ($P < 0.05$) (Fig. 7). We then cloned and sequenced the vector/AAVS1 integration junctions using PCR with primers that bind 0.9 kb upstream and 0.36 kb downstream of the AAVS1 RBS/TRS site (Fig. 8A). In agreement with previous studies using recombinant AAV vectors (16), integration sites were not in the direct vicinity of RBS/TRS sites but were scattered over the analyzed AAVS1 region of 5.3 kb (Fig. 8B). Interestingly, in five out of six junctions within AAVS1, the vector DNA broke near the terminal ends of the Ad ITRs, while only one integration site involved the AAV ITR(s) (Fig. 8C). To analyze vector integration sites in other genomic regions, we used inverse PCR. In agreement with an earlier study, we found vector integration tethered to the globin LCR at chromosome 11 (Fig. 8D); 25% of analyzed integrants were within the LCR.

The analysis of vector-AAVS1 junctions indicated that vector integration in the vast majority of cases did not occur via the AAV ITR, indicating that Rep78-mediated nicking within the vector AAV ITR and rescue of the AAV ITR-flanked

FIG. 7. Comparison of mean fluorescence for GFP⁺ clones that did or did not integrate into AAVS1. The integration copy number of each GFP⁺ clone was determined by Southern blotting as described earlier (33). Only clones with one integrated vector copy are shown.

cassette is not required for integration into AAVS1. To further confirm this, we constructed a vector that was identical to Ad.AAV-LCR-GFP but that lacked both AAV ITRs (Ad.LCR-GFP) and performed transduction and integration studies as described above. The vectors conferred stable GFP expression and integration into AAVS1 at comparable levels for the two vectors (Fig. 9). Analysis of integration sites by Southern blotting using AAVS1- and vector-specific probes (analysis was done as for Fig. 6C) (data not shown) revealed that for both vectors 11 out of 20 integration sites were in AAVS1. (The higher AAVS1 integration frequency is probably due to a higher MOI of Ad.LCR-rep78 used in this study [800 viral particles/cell]). These data demonstrate that the AAV ITRs are not a structural requirement for Ad.LCR-rep78-mediated transgene integration.

DISCUSSION

In this study, we show that a helper-dependent Ad5/35 vector expressing Rep78 under the control of a promoter with low activity in 293 cells (β -globin LCR) can be generated. Recently, Recchia et al. reported on an HD-Ad vector containing the *rep* gene under the control of a doxycycline-inducible system for site-specific transgene integration. Although Rep expression from these vectors was greatly reduced in 293 cells in the noninduced stage, yields of the *rep*-containing vector were significantly lower than those of our Ad.LCR-rep78 vector. Other novel features of our vector system include the use of Ad5/35 fibers for efficient infection of hematopoietic stem cells and the incorporation of the complete globin LCR for cell-type-specific gene expression that is protected against epigenetic silencing. We demonstrate that Ad.LCR-rep78 mediates the rescue of a 27-kb-long transgene cassette from coinfecting Ad.AAV.LCR-GFP genomes in transduced cells. With this dual-vector system, LCR-GFP transgene integration into AAVS1 was seen in 30% of analyzed integration sites, and transgenes integrated into AAVS1 expressed GFP at high and stable levels. Of the remaining integration sites, 25% were within the genomic globin LCR. This implies that the majority (55%) of integrations occurred in two defined sites within the human genomes, which theoretically should reduce the risk of insertional mutagenesis by the vector. In almost 90% of the sites, transgene integration occurred via the Ad ITR, indicating that the rescue of the AAV ITR-flanked transgene cassette

FIG. 8. Analysis of integration sites. (A) Schematic representation of nested PCR. AAV-0 and AAV-1 are vector-specific primers; P1a, P1b, P2a, P2b, P3a, and P3b are AAVS1 region-specific primers. (B) Schematic representation of integration sites found on chromosome 19. Nested PCR was performed with vector-specific primers and an AAVS1-specific primer. (C) Summary of AAVS1 integration sites. Nested PCR was performed with one primer specific to the vector (universal for both the 5' end and the 3' end) and a second primer specific to the AAVS1 region. XX, additional nucleotides that were not homologous to the viral genome or chromosomal DNA. (D) Analysis of integration sites outside the AAVS1 region by inverse PCR was performed as described before (33). The UCSC Genome Browser (<http://genome.ucsc.edu/>) was used to perform alignments of identified integration sites.

from Ad.AAV-LCR-GFP is not required for Rep78-mediated integration into AAVS1. Notably, integration studies were performed using colonies with >30% GFP-positive cells. The possibility that the level of site-specific integration is lower in colonies with less than 30% GFP-expressing cells cannot be excluded.

In this study, we used an LCR-containing HD-Ad vector to express Rep78. Considering data from a previous study, this

FIG. 9. Analysis of stable GFP expression in clones. Mo7e cells were infected with Ad.AAV-LCR-GFP (gray bars) or Ad.LCR-GFP (black bars) at an MOI of 300 genomes/cell and Ad.LCR-rep78 at an MOI of 800 genomes/cell. Thirty-six hours after infection, the cells were diluted and plated as single cells in 96-well plates. Three weeks later, GFP-positive colonies that stably expressed GFP were analyzed.

type of vector integrates into the host genome, preferentially into chromosome 11 (33). However, in all GFP-expressing clones that were used for integration studies, we did not detect Rep78 expression by Western blotting. We speculate that clones with high-level Rep78 expression died off due to Rep-mediated toxicity. This is supported by the fact that the total number of clones that developed was two- to threefold lower for Ad.LCR-rep78-infected cells than in settings with Ad.Co. Furthermore, the percentage of GFP-expressing cells in all surviving clones was constant over time, underscoring that these cells did not have a proliferative disadvantage. Taking into account the fact that Ad.LCR-GFP-transduced cells died due to Rep expression, the overall stable Rep78-mediated transduction frequencies might be even higher than shown in Fig. 6A. Clearly, future efforts have to focus on controlling the timing and level of Rep78 expression. Furthermore, the Rep78-expressing vectors should contain scrambled fragments of X-chromosomal DNA. We have shown recently that this reduces the integration frequency about fourfold compared to HD-Ad vectors containing the globin LCR (33).

Based on previous AAV integration studies (2, 36, 37), we assumed that Rep-mediated nicking within the chromosomal AAVS1 site and within the vector AAV ITRs is necessary for efficient site-specific transgene integration. However, our hypothesis proved to be wrong. Our integration site analysis indicates that free ends within the vector genome can be created by single-strand or double-strand breaks within the Ad ITRs, whose structure is apparently recognized by cellular “nicking” enzymes. Nicking within the Ad ITR appears to occur more often (and more efficiently) than nicking within the AAV ITRs, and an Ad.LCR vector without AAV ITRs integrates as efficiently into AAVS1 as an Ad.AAV-LCR vector. Finally, we observed efficient vector integration into AAVS1 in Mo7e cells in the absence of detectable Rep78-mediated rescue. The only function of Rep78 in our system appears to be to mediate DNA breaks within the AAVS1 site. Our finding is in agreement with studies by Hamilton et al. and Philpott et al. reporting that the presence of AAV ITRs and nicking within these ITRs is not required and that an integration efficiency

element (p5IEE) located in the AAV p5 promoter sequence is the sole *cis* requirement for high-efficiency integration of plasmid DNA into AAVS1 (8, 19). However, the role of p5IEE is not absolute, as our study has shown. It remains to be tested whether p5IEE could increase the integration frequency of our Ad.LCR vectors into AAVS1. It is also notable that omitting AAV ITRs in Ad.LCR vectors greatly simplifies vector construction and high-titer vector production, as AAV ITRs often tend to rearrange and induce vector genome instability. Importantly, it is thought that the ability of Rep78 to stimulate double-strand breaks within chromosomal DNA is a prerequisite to achieving vector integration in (quiescent) hematopoietic stem cells.

Although Ad.LCR-rep78-mediated rescue was efficient in 293 cells, no rescue products were detectable in Mo7e cells in Southern blot analyses. We attributed this observation to the presence of low levels (<1%) of helper virus (with E1/E3 deleted) in HD-Ad preparations. Because 293 cells complement the E1 deletion, helper virus replicates in infected cells, which results on one hand in the production of viral proteins and on the other hand in coreplication of HD-Ad genomes. Our studies indicate that expression of Ad proteins, specifically DBP (E2a) or E4orf6, is sufficient to mediate Rep78-mediated rescue. This finding is not novel, as the helper functions of Ad have been well characterized and shown to be mediated by E1a, E1b, E2a, E4, and the VA RNA (23). It was thought that their predominant role was in regulation of gene expression for AAV proteins. Our data contribute to a better understanding of the functions of these proteins in Ad rescue. It is also interesting that DBP expression is sufficient for efficient rescue. DBP is a single-stranded DNA-binding protein encoded in the Ad E2a region. It is possible that DBP enhances binding by bringing Rep into contact with its substrate or by promoting multimerization of Rep (34). Interestingly, there are a number of cellular proteins with similar functions. These include RPA (replication protein A), a heterotrimeric cellular complex that is involved in both replication and repair of cellular DNA (reviewed in reference 11). We speculate that the amount/activity of RPA vary between cell types, and it would be interesting to test whether the transient overexpression of Ad DBP would increase the Rep78-mediated integration frequency of the Ad.LCR-GFP vector.

Integration of wild-type AAV has been associated with rearrangements in the AAVS1 locus, including both duplications and deletions (6). In our study, we found transgene integration spread over the 5.3-kb AAVS1 site. Though the inaccuracy of integration and genomic rearrangements is a concern, epidemiological studies show widespread wild-type AAV infection (80% of humans are AAV seropositive), while no known pathologies, including neoplastic malignancies, have been reported (1).

This study is a proof of principle that targeted integration of a 27-kb globin LCR-driven transgene can be achieved at high frequency. It has implications for stem cell gene therapy.

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