

Adeno-associated virus Type 2 Rep proteins mediate integration of lentiviral vectors

Victor J. McAlister¹, Anthony T. Craig^{1,2}, and Roland A. Owens^{1*}

Short title: Locus-specific integration of AAV-lentivirus hybrid

¹ Laboratory of Cell and Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland 20892, USA.

² Department of Genetics and Human Genetics, Howard University Graduate School, Washington, DC.

*Correspondence to R. Owens, Bldg. 1, Rm. 158, National Institutes of Health, Department of Health and Human Services, 1 CENTER DR MSC 0151, Bethesda, MD 20892-0151, USA. Phone: (301) 594-7471. Fax: (301) 402-4273. E-mail: owensrol@mail.nih.gov.

E-mail addresses of other authors:

VJM: vmcalist@yahoo.com

ATC: anthony.craig@howard.edu

Keywords: Adeno-associated virus, Rep proteins; Lentivirus Vectors, Integration

Abbreviations: AAV2, adeno-associated virus Type 2; AAVS1, AAV integration Site 1; ITRs, inverted terminal repeats; RRS, Rep recognition sequence

ABSTRACT

Aims: Adeno-associated virus type 2 (AAV2) is a naturally defective human parvovirus that is being developed as a gene therapy vector. In dividing cells, AAV2 DNA persists by integration into the host chromosomes. AAV2 is unique among mammalian viruses in its ability to integrate preferentially into a particular locus within human chromosome 19, designated *AAVS1* (also known as *Mbs 85*). The AAV2 Rep68 and Rep78 proteins mediate this integration. Recent data suggest that Rep68 and Rep78 can mediate integration of non-AAV2 DNA with free ends. To test this hypothesis, we targeted insertion of different lentiviral vectors to *AAVS1*.

Methods: Cells were co-infected with wild-type AAV2, and integrase-proficient or integrase-deficient lentivirus vectors. A highly specific PCR-based assay was used to detect lentivirus integration at *AAVS1*. Similar experiments were performed using lentiviral vectors containing the AAV2 *rep* gene.

Results: All lentiviral vectors tested integrated at *AAVS1*, if the *rep* gene was present either within the lentiviral vector or supplied in *trans*. All that was required for integration at *AAVS1* was the amino acid sequence shared between Rep68 and Rep78. The results were similar with integrase-proficient or integrase-deficient lentiviral vectors.

Conclusions: The inclusion of the *rep* gene with lentiviral vectors may produce more predictable integration patterns.

1 INTRODUCTION

- 2 Genomic integration can be required for the stable transmission of a transgene in cells that are dividing.
- 3 In many cases it can be desirable to have integration at a specific locus. Highly variable transgene
- 4 expression can result from random integration. Random integration can also cause unintended changes
- 5 in cell phenotype, such as insertional activation of proto-oncogenes and acquisition of a tumorigenic
- 6 phenotype (Hacein-Bey-Abina et al., 2008).

7 Adeno-associated virus serotype 2 (AAV2) is unique among mammalian viruses for its ability to
8 integrate into a specific region of the human genome, referred to as *AAVSI* (AAV2 Site 1) (Kotin &
9 Berns, 1989; Kotin et al., 1990; Kotin et al., 1991; Kotin, Linden & Berns, 1992). Most clonal cell
10 lines obtained from AAV2-infected cells contain an AAV2 provirus within this 4 Kb region on
11 chromosome 19 (Kotin et al., 1990; Hamilton et al., 2004; Ward & Walsh, 2012). *AAVSI* has an open
12 chromatin configuration that is compatible with transgene expression (Lamartina et al., 2000). In
13 addition, the presence of an insulator element upstream of *AAVSI* increases the probability that
14 independent integration events involving the same promoter/transgene cassette will result in similar
15 levels of transgene expression (Ogata, Kozuka & Kanda, 2003).

16 Preferential integration at *AAVSI* requires functions encoded by the ~2.2 Kb AAV2 *rep* gene (Shelling
17 & Smith, 1994; Weitzman et al., 1994; Balague, Kalla & Zhang, 1997). The *rep* gene encodes four
18 major proteins from overlapping reading frames (Mendelson, Trempe & Carter, 1986). The larger Rep
19 proteins, Rep68 and Rep78 (Rep68/78) are required for replication and packaging (Im & Muzyczka,
20 1990; King et al., 2001). Rep67/78 recognizes specific sequences at *AAVSI* that are similar to
21 sequences found in the AAV2 inverted terminal repeats (ITRs) (Snyder, Im & Muzyczka, 1990;
22 Chiorini et al., 1994; Weitzman et al., 1994; Urcelay et al., 1995; McAlister & Owens, 2010). Most
23 currently used AAV vectors do not incorporate the AAV2 *rep* gene and are designed to persist
24 episomally in non-dividing cells (Owens, 2002).

25 Lentiviral vectors based on human immunodeficiency virus-1 (HIV-1) are used for many gene transfer
26 applications (Matrai, Chuah & VandenDriessche, 2010). One important aspect of these vectors is that
27 they can integrate in the genome in dividing or non-dividing cells and infect a wide range of cell types

28 including hematopoietic cells (*Holmes-Son, Appa & Chow, 2001*). A packaging plasmid encoding a
29 catalytically inactive lentiviral integrase can be used to package integrase-deficient virus. These
30 viruses are maintained predominantly as transcriptionally active linear and circular forms in non-
31 dividing cells (Wiskerchen & Muesing, 1995; Wu & Marsh, 2001). A number of systems have been
32 used to modify the integration profile of integrase-deficient lentiviral vectors (Staunstrup & Mikkelsen,
33 2011).

34 Recent reports have suggested that the AAV2 *rep* gene products can direct the integration of adenoviral
35 and retroviral vectors into *AAVS1* when the vectors contain key sequences from AAV2 (Wang & Lieber,
36 2006; Huang et al., 2012). In this report we investigate the use of the AAV2 Rep proteins to direct
37 integration of integrase-proficient and integrase-deficient lentivirus vectors to *AAVS1*.

38 MATERIALS AND METHODS

39 Lentivirus production

40 Lentivirus was made by co-transfection (calcium phosphate co-precipitation method) of HEK-293T
41 cells with the pLKO.1 puro vector plasmid (Moffat et al., 2006), pCMV-VSV-G envelope plasmid
42 (Stewart et al., 2003), and packaging vector pCMV- Δ R8.91 (Zufferey et al., 1997). To make virus
43 particles deficient for HIV-1 integrase, pCD/NL-BH* (Zhang et al., 2002) was used in place of pCMV-
44 Δ R8.91. Virus supernatants were cleared of debris by centrifugation for 5 min at 3,000 x g and filtered
45 using a Millex-GV 0.45 μ m PVDF syringe filter (Millipore, Billerica, MA). The virus titer of pLKO.1
46 puro packaged with pCMV- Δ R8.91 was measured by infecting HeLa cells with virus supernatant and
47 selecting for puromycin-resistant colonies. The titer of packaged pLKO.1 puro was 3.5×10^5
48 transducing units/ml.

49 Restriction fragments of the AAV2 *rep* gene from pSub201(-) (Samulski, Chang & Shenk, 1987) were
50 cloned into pLKO.1 puro and used to make plasmids containing hybrid lentivirus genomes. pVM122
51 is a pLKO.1 puro derivative containing the XbaI (4494) to XhoI (2446) restriction fragment from
52 pSub201(-). This *rep* gene fragment was substituted for the SpeI (253) to XhoI (6547) fragment of
53 pLKO.1 puro to make pVM122. pVM123 is a pLKO.1 puro derivative containing the XbaI (4494) to
54 KpnI (2773) restriction fragment from pSub201(-). This *rep* gene fragment was substituted for the
55 pLKO.1 puro SpeI (235) to KpnI (1185) fragment to create pVM123. These virus supernatants were
56 treated with 100 units of Benzonase nuclease (Novagen) per ml of supernatant for 2 hrs at 37°C.

57 **Virus infections and co-infections**

58 The HeLa human cervical carcinoma cell line was grown in Dulbecco's modified Eagle medium
59 (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) to 70% confluency
60 in 25 cm² flasks for virus infections. For lentivirus infections, the culture medium was replaced with 5
61 ml of unconcentrated lentivirus supernatant. The virus supernatant was removed after 12 to 16 hrs and
62 replaced with fresh DMEM with 10% FBS. Cells were harvested 48 hrs post infection for DNA
63 isolation using the DNeasy tissue kit (QIAGEN, Valencia, CA). For AAV2 infections, HeLa cells
64 grown to 50% confluency were infected with approximately 9×10^3 viral genomes per cell in DMEM
65 without FBS for 12 to 16 hrs. AAV2 was prepared as described previously (Carter et al., 1979). AAV2
66 titers were determined by DNA slot blot analysis.

67 **PCR integration assays**

68 A nested PCR assay was used to detect integration of lentivirus vectors made from the pLKO.1 puro
69 vector. Two primer sets were used in this assay. One set is specific for the pLKO.1 puro LTRs. The
70 other primer set is specific for *AAVS1*. The first round of PCR was done using the primers 5'-AGT

71 TAC CAG AGT CAC ACA ACA GAC GG-3' for pLKO.1 puro and 5'-AAC ATG CTG TCC TGA
72 AGT GGA CAT AGG-3' specific for *AAVSI*. The first reaction contained 100 ng of total cellular
73 DNA. 0.5 µl of the first PCR reaction was used as the template for a second PCR using the primers 5'-
74 ACT ACT TGA AGC ACT CAA GGC AAG CTT T-3' for pLKO.1 puro and 5'-GAA GAC TAG CTG
75 AGC TCT CGG ACC-3' for *AAVSI*. The following PCR parameters were used. 95° for 5 min,
76 followed by 27 cycles of 95°C for 30 s, 61°C for 1 min and 72°C for 1 min, with a final hold at 72° for
77 5 min. Roche FastStart polymerase with High GC buffer (Roche Applied Science, Indianapolis, IN)
78 was used for both amplifications.

79 PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. For DNA
80 sequencing, topoisomerase-mediated ligation was used to insert PCR products into plasmid
81 pCR4TOPO (Invitrogen). The ligation products were then transformed into *Escherichia coli* One Shot
82 TOP10 competent cells (Invitrogen). Plasmids for sequencing were purified using a QIAGEN plasmid
83 mini kit or a QIAprep spin miniprep kit. Sequencing was performed by MWG Biotech, Inc. (High
84 Point, NC), using M13 universal primers.

85 RESULTS

86 Wild-type AAV2 directs integration of a lentiviral vector to *AAVSI* in co-infected cells

87 The cells were infected with the pLKO.1 puro vector and AAV2 on different days. The genomic DNA
88 was used for the PCR integration assay. Single primer controls in which one primer is used in the
89 second amplification are shown Figure 1 B. No junctions were detected when the cells were infected
90 with lentivirus only (Fig. 1 A, lanes 1 and 4). Junctions were also not detected in the single primer
91 controls (Fig. 1 B, lanes 2 and 3). As judged by the number of bands in other lanes, more junctions

were detected when the cells were infected with AAV2 first and then infected with the lentivirus (Fig. 1 A, lanes 3 and 6).

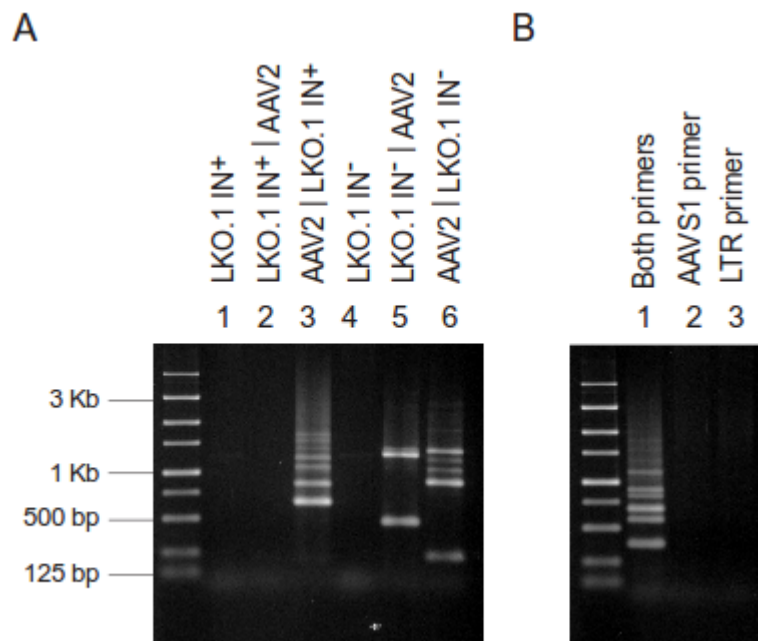
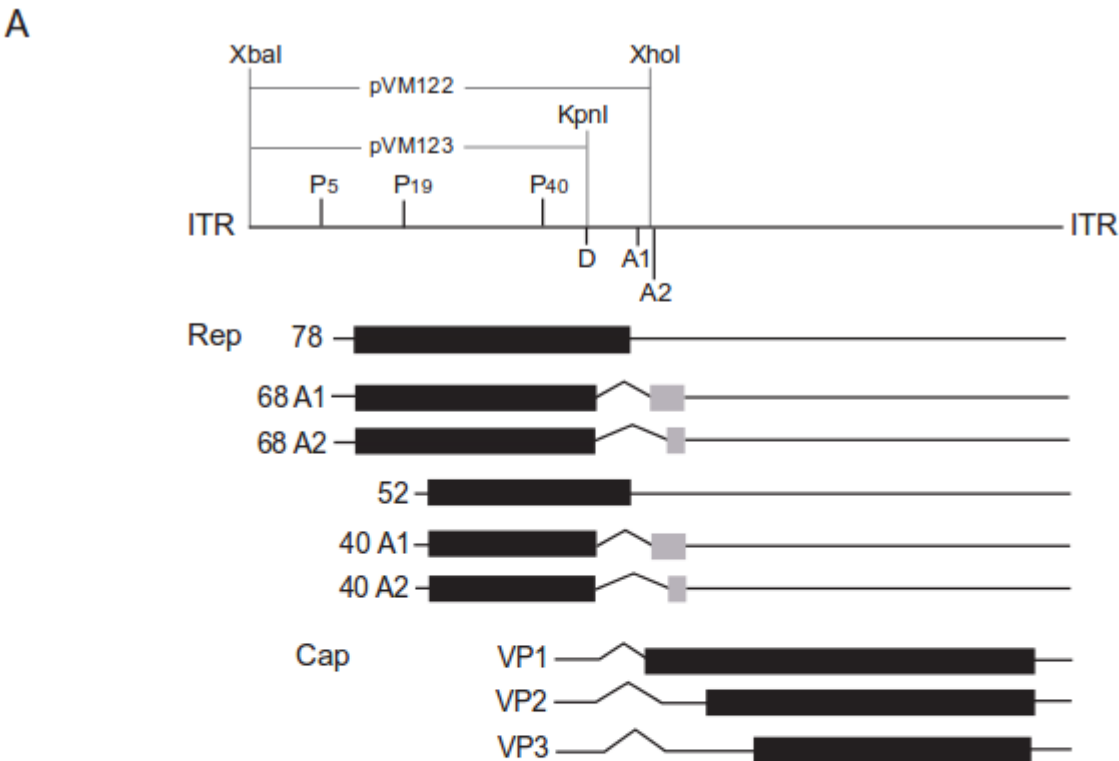


Figure 1 - AAV2-lentivirus co-infection experiments demonstrating lentivirus integration at *AAVS1*.

A. Human cervical carcinoma cells (HeLa) were infected with lentivirus made from pLKO.1 puro, with or without AAV2, as indicated in the figure, and assayed for integration by nested PCR. (IN⁺) Lentivirus particles containing an active version of the lentiviral integrase. (IN⁻) Lentivirus particles containing an inactive lentiviral integrase. For the samples in lanes 2 and 5, the cells were exposed to the lentivirus vector before being exposed to AAV2. For the samples in lanes 3 and 6, the cells were exposed to the lentivirus vector after being exposed to AAV2. B. Single primer controls using only the *AAVS1* or lentivirus primer in the second round of the nested PCR (lanes 2 and 3, respectively). Lane 1 is using both primers. The DNA template is same as lane 6 of part A.

Lentiviral vectors designed to express truncated versions of the AAV2 *rep* gene integrate at *AAVS1*

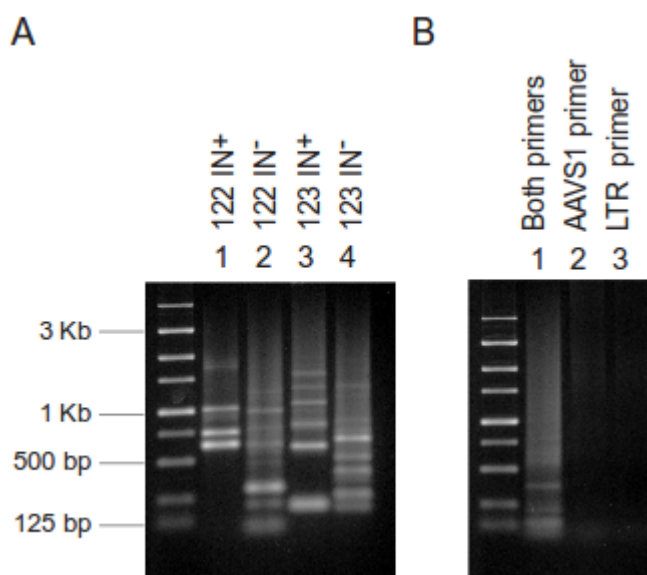
105 pVM122 and pVM123 are lentiviral vectors that contain different versions of the *rep* gene (Figure 2A).
106 pVM122 encodes the reading frame for Rep78 and part of the second exon for Rep68. Rep68 protein
107 made using the second exon in pVM122 contains a three amino acid truncation at the C-terminus.
108 pVM123 encodes a 91 amino acid C-terminal deletion of Rep78 with one missense amino acid at the
109 C-terminus. The C-terminal sequences of Rep68/78 and the Rep proteins encoded by pVM122 and
110 pVM123 are shown in Figure 2 B.



B

Rep78	aa 529 RYQNKCSRHVGMNLMFLPCRQCERNQNSNICFTHG QKDCLECFPVSESQPVSVVKKAYQKLCYIHIMGKVPD ACTACDLVNVDLDDCIFEQ
Rep68 A1	R + LARGHSL
Rep68 A2	R + GHSL
pVM122	R + LARG
pVM123	RYL

Figure 2 - Map of the AAV2 genome in pSub201(-) and *rep* gene fragments used in the construction of hybrid lentiviral vectors expressing AAV2 Rep proteins. A. Map of the AAV2 genome contained in pSub201(-). Rep proteins are translated from alternately spliced RNAs transcribed from the P₅ and P₁₉ promoters. pVM122 contains the pSub201(-) XbaI-XhoI fragment. pVM123 contains the pSub201(-) XbaI-KpnI fragment. Both constructs contain the P₅ and P₁₉ promoters. B. The predicted amino acid sequences of the wild-type and truncated Rep proteins. pVM122 is predicted to express full-length Rep78 and a three amino acid truncation of Rep68 made from the first splice acceptor site A1. pVM123 is predicted to express a 91 amino acid C-terminal truncation of Rep78 and no Rep protein translated from alternatively spliced RNA because the KpnI site overlaps the splice donor site (D). Integration assays performed using total DNA from cells infected with lentivirus made using the pVM122 and pVM123 constructs are shown in Figure 3. For both constructs the results of the integration assays are similar. Integration junctions were not detected with the single primer controls (Figure 3 B). The same DNA sample was used as the amplification template in Fig. 3 A, lane 4 and Fig. 3 B, lanes 1-3.



126 **Figure 3** - Integration assays of hybrid lentiviral vectors designed to express the AAV2 *rep* gene. A.
127 Human cervical carcinoma cells (HeLa) were infected with lentivirus made using pVM122 and
128 pVM123. (IN⁺) Lentivirus particles containing an active version of the lentiviral integrase. (IN⁻)
129 Lentivirus particles containing an inactive lentiviral integrase. B. Single primer controls using only
130 the *AAVSI* or lentivirus primer in the second round of the nested PCR (lanes 2 and 3, respectively).
131 Lane 1 is using both primers. The DNA template is same as lane 4 of part A.

132 The presence of lentiviral vector-*AAVSI* junctions within the PCR products of the integration assays
133 was confirmed by DNA sequencing. Eight products were sequenced. Six unique junctions were
134 identified. The sequences are shown in Figure 4 A. Most were characterized by one or more bases of
135 homology between the vector DNA and *AAVSI*. Consistent with our previous observations (McAlister
136 & Owens 2007), most of the junctions were at or near polypyrimidine tracts of five or more bases
137 within *AAVSI*.

A

pLKO.1 pLKO.1 806 AAVS1 1744 AAVS1
*CCTTCCAGTACCACTCTTCTCT*cTGCCATCTCTCGTTTCTTAG

pLKO.1 pLKO.1 3292 AAVS1 1678 AAVS1
*CTTTTAAAAAGTGGCTAAG*aCCTCCCTCACCCAACCCCATG

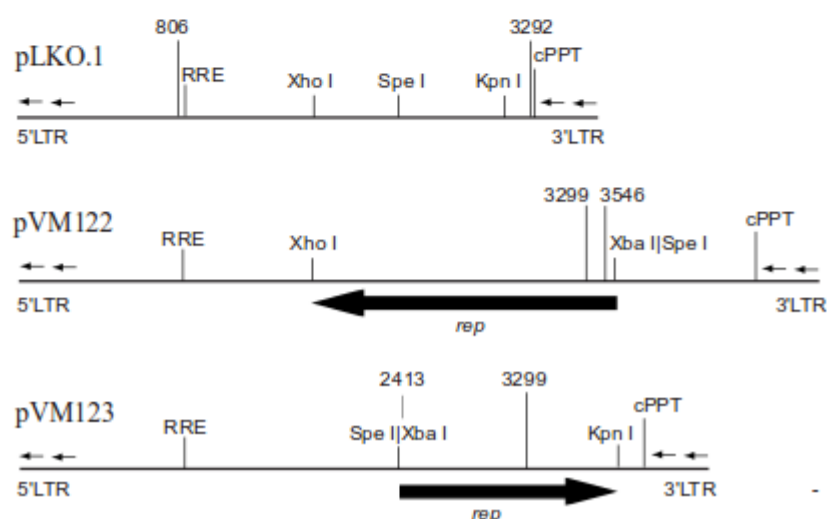
pLKO.1 pLKO.1 3358 AAVS1 1775 AAVS1
*CAGTACAAGCAAAAAGCAGAT*CTCCGACGGATGTCTCCCTTG

pVM122 pVM122 3546 AAVS1 1943 AAVS1
*TATTTAAGCCCGAGTGAGCA*CTCACTCCTTTCATTGGGCAG

pVM123 pVM123 3299 AAVS1 1783 AAVS1
*CTGCTGGCCCCACCAGGTAGT*CGATGTCTCCCTTGCGTCCCGCC

pVM123 pVM123 2413 AAVS1 1945 AAVS1
*AGCCCGAGTGAGCACGCA*CTCACTCCTTTCATTGGGCAGCTC

B



138 **Figure 4 -** Lentivirus-*AAVS1* integration junctions. A. DNA sequences of the integration junctions.
 139 Lentiviral vector sequences are in italicized lettering. *AAVS1* base positions are the number of bases
 140 from the Rep nicking site. Bases that are shared by two sequences are underlined. Bases not present in
 141 either sequence are in lowercase. B. Map of Integration junctions shown in part A. (RRE) rev-

responsive element, (cPPT) central polypurine tract. Small arrows indicate the location of the lentivirus primer set used to amplify integration junctions. The scale is approximate.

DISCUSSION

Our results indicate that the Rep68/78 proteins of AAV2 can direct integration of a lentiviral vector to *AAVSI*. This has both practical and theoretical implications. From a practical standpoint, the ability to produce more predictable patterns of integration with lentiviral vectors could improve the safety of gene therapies targeted at diseases such as hematopoietic disorders, the treatment of which requires the long-term expression of a therapeutic gene in dividing cells. From a theoretical standpoint, our results contribute to a model in which the main requirement for Rep-mediated integration at *AAVSI* is a free DNA end or nick from which a free end can be generated.

Rep68/78-mediated targeting of lentivirus vectors to *AAVSI* appears to improve when the lentivirus integrase is catalytically inactive. This is based on the observation that there are more bands of PCR products in the *AAVSI* integration assay when the integrase is catalytically inactive compared to the appropriate integrase-positive controls (Fig. 3A). We hypothesize that when a functional integrase is present, there is some competition between Rep-mediated integration at the *AAVSI* locus and random integration mediated by the lentiviral integrase. Consistent with this competition hypothesis is our observation that Rep68/78-mediated integration of an integrase-positive lentiviral vector into *AAVSI* is more efficient when the *rep* gene is delivered to the cell before the lentiviral vector (Fig. 1A, lanes 2 and 3). However, the results using the integrase-positive and integrase-deficient lentiviruses are similar enough that any such competition cannot be overwhelming. It is also possible that any competition by the lentiviral integrase is counterbalanced by the integrase slowing the rate at which viral cDNA is converted to non-integrating circular forms.

164 Our results help to refine models of the mechanism of Rep68/78-mediated preferential integration. It
165 was first thought that the inverted terminal repeats (ITRs) of the AAV2 genome were required for
166 preferential integration (Weitzman et al., 1994; Balague, Kalla & Zhang, 1997), but more recent work
167 has shown that Rep proteins can mediate integration of a hybrid adenovirus vector into *AAVSI*, often
168 with the adenovirus ITRs, rather than the AAV2 ITRs found at the junction with *AAVSI* (Wang &
169 Lieber, 2006). Another recent report shows that an integrase-deficient murine stem cell retrovirus
170 vector, containing a key integration-enhancing sequence from AAV2, can also integrate into *AAVSI*
171 when Rep68/78 is present (Huang et al., 2012). Combined with the results we present here, a new
172 hypothesis emerges that all that is required of the inserting DNA is that it contains DNA from which
173 free ends (single-stranded or double-stranded) can be generated and that it contains a binding site for
174 Rep68/78. Binding sites are predicted to be quite common, since the sequences bound by Rep68/78 are
175 quite degenerate (McCarty et al., 1994; Chiorini et al., 1995; Wonderling & Owens, 1997), and weak
176 binding sites have been identified within the HIV-1 long terminal repeat (Kokorina et al., 1998) (our
177 lentiviral vector is based on HIV-1 HXBIII isolate) and within the *rep* gene (McCarty et al., 1994).

178 The absence of ITRs in our *rep* gene-containing lentiviral vectors creates two advantages over ITR-
179 containing hybrid vectors. First, it lowers the probability that the Rep proteins would disrupt the vector
180 DNA during packaging, since the ITRs contain the two major Rep68/78 nicking sites within AAV2 (Im
181 & Muzyczka, 1990). The absence of ITRs also lowers the probability of disruption of the vector after
182 integration into *AAVSI*. Having the *rep* gene under the control of its natural promoter, p5, reduces the
183 probability of Rep-mediated rearrangement of the vector, since Rep proteins down-regulate the p5
184 promoter (Beaton, Palumbo & Berns, 1989; Kyostio, Wonderling & Owens, 1995). The use of C-

185 terminally truncated Rep proteins may also reduce the probability of adverse effects, since the C-
186 terminal end of Rep78 contains a kinase inhibitor domain (Di Pasquale & Stacey, 1998).

187 CONCLUSIONS

188 The ability of AAV2 Rep proteins to mediate integration into the *AAVS1* locus of human chromosome
189 19 may allow the creation of safer gene therapy vectors. The observation that these proteins target
190 integration of a lentiviral vector provides further evidence for the hypothesis that Rep-mediated
191 integration can work with any linear DNA that contains a Rep68/78 binding site.

DECLARATION OF COMPETING INTERESTS

R.A.O. is a co-inventor on several patents involving AAV vectors. To the extent that this work will increase the value of those patents, he has a competing interest.

AUTHORS' CONTRIBUTIONS

VJM was the primary contributor to project conception, overall experimental design, plasmid construction, virus production, cell infection, integration assays, data analysis and writing of manuscript. He also performed all experiments.

ATC assisted with plasmid construction and experimental design.

RAO was overall project coordinator, and contributed to experimental design, data analysis and writing of the manuscript.

All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We thank Dr. John Tisdale and Dr. John Hanover for their critical reading of the manuscript. We thank Dr. R. Jude Samulski for the kind gift of pSub201 (-). We thank Dr. Tyler Lahusen for the kind gifts of pLKO.1 puro and pCMV-VSV-G. We thank Dr. Tshaka Cunningham for the kind gift of pCMV-ΔR8.91, and for useful discussions. We thank Dr. Jakob Reiser for the kind gift of pCD/NL-BH*. This research was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases.

REFERENCES

Balague C, Kalla M, Zhang WW. 1997. Adeno-associated virus Rep78 protein and terminal repeats enhance integration of DNA sequences into the cellular genome. *Journal of Virology* 71:3299-3306.

Beaton A, Palumbo P, Berns KI. 1989. Expression from the adeno-associated virus p5 and p19 promoters is negatively regulated in trans by the rep protein. *Journal of Virology* 63:4450-4454.

Carter BJ, Laughlin CA, de la Maza LM, Myers M. 1979. Adeno-associated virus autointerference. *Virology* 92:449-462.

Chiorini JA, Wiener SM, Owens RA, Kyostio SR, Kotin RM, Safer B. 1994. Sequence requirements for stable binding and function of Rep68 on the adeno-associated virus type 2 inverted terminal repeats. *Journal of Virology* 68:7448-7457.

Chiorini JA, Yang L, Safer B, Kotin RM. 1995. Determination of adeno-associated virus Rep68 and Rep78 binding sites by random sequence oligonucleotide selection. *Journal of Virology* 69:7334-7338.

Di Pasquale G, Stacey SN. 1998. Adeno-associated virus Rep78 protein interacts with protein kinase A and its homolog PRKX and inhibits CREB-dependent transcriptional activation. *Journal of Virology* 72:7916-7925.

Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, et al. 2008. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *Journal of Clinical Investigation* 118:3132-3142.

Hamilton H, Gomos J, Berns KI, Falck-Pedersen E. 2004. Adeno-associated virus site-specific integration and AAVS1 disruption. *Journal of Virology* 78:7874-7882.

Holmes-Son ML, Appa RS, Chow SA. 2001. Molecular genetics and target site specificity of retroviral integration. *Advances in Genetics* 43:33-69.

Huang S, Kawabe Y, Ito A, Kamihira M. 2012. Adeno-associated virus Rep-mediated targeting of integrase-defective retroviral vector DNA circles into human chromosome 19. *Biochemical and Biophysical Research Communications* 417:78-83.

Im DS, Muzyczka N. 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. *Cell* 61:447-457.

King JA, Dubielzig R, Grimm D, Kleinschmidt JA. 2001. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. *European Molecular Biology Organization Journal* 20:3282-3291.

Kokorina NA, Santin AD, Li C, Hermonat PL. 1998. Involvement of protein-DNA interaction in adeno-associated virus Rep78-mediated inhibition of HIV-1. *Journal of Human Virology* 1:441-450.

Kotin RM, Berns KI. 1989. Organization of adeno-associated virus DNA in latently infected Detroit 6 cells. *Virology* 170:460-467.

Kotin RM, Linden RM, Berns KI. 1992. Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *European Molecular Biology Organization Journal* 11:5071-5078.

Kotin RM, Menninger JC, Ward DC, Berns KI. 1991. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. *Genomics* 10:831-834.

Kotin RM, Siniscalco M, Samulski RJ, Zhu XD, Hunter L, Laughlin CA, et al. 1990. Site-specific integration by adeno-associated virus. *Proceedings of the National Academy of Sciences USA* 87:2211-2215.

Kyostio SRM, Wonderling RS, Owens RA. 1995. Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. *Journal of Virology* 69:6787-6796.

Lamartina S, Sporeno E, Fattori E, Toniatti C. 2000. Characteristics of the adeno-associated virus preintegration site in human chromosome 19: open chromatin conformation and transcription-competent environment. *Journal of Virology* 74:7671-7677.

Matrai J, Chuah MKL, VandenDriessche T. 2010. Recent advances in lentiviral vector development and applications. *Molecular Therapy* 18:477-490.

McAlister VJ, Owens RA. 2007. Preferential integration of adeno-associated virus type 2 into a polypyrimidine/polypurine-rich region within *AAVS1*. *Journal of Virology* 81:9718-9726.

McAlister VJ, Owens RA. 2010. Substitution of Adeno-Associated Virus Rep Protein Binding and Nicking Sites with Human Chromosome 19 Sequences. *Virology Journal* 7:218.

McCarty DM, Pereira DJ, Zolotukhin I, Zhou X, Ryan JH, Muzyczka N. 1994. Identification of linear DNA sequences that specifically bind the adeno-associated virus Rep protein. *Journal of Virology* 68:4988-4997.

Mendelson E, Trempe JP, Carter BJ. 1986. Identification of the trans-acting Rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. *Journal of Virology* 60:823-832.

Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepper AM, Hinkle G, et al. 2006. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* 124:1283-1298.

Ogata T, Kozuka T, Kanda T. 2003. Identification of an insulator in *AAVS1*, a preferred region for integration of adeno-associated virus DNA. *Journal of Virology* 77:9000-9007.

Owens RA. 2002. Second generation adeno-associated virus type 2-based gene therapy systems with the potential for preferential integration into *AAVS1*. *Current Gene Therapy* 2:145-159.

Samulski RJ, Chang LS, Shenk T. 1987. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication. *Journal of Virology* 61:3096-3101.

Shelling AN, Smith MG. 1994. Targeted integration of transfected and infected adeno-associated virus vectors containing the neomycin resistance gene. *Gene Therapy* 1:165-169.

Snyder RO, Im DS, Muzyczka N. 1990. Evidence for covalent attachment of the adeno-associated virus (AAV) rep protein to the ends of the AAV genome. *Journal of Virology* 64:6204-6213.

Staunstrup NH, Mikkelsen JG. 2011. Integrase-defective lentiviral vectors--a stage for nonviral integration machineries. *Current Gene Therapy*, 11:350-62.

Stewart SA, Dykxhoorn DM, Palliser D, Mizuno H, Yu EY, An DS, et al. 2003. Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 9:493-501.

Urcelay E, Ward P, Wiener SM, Safer B, Kotin RM. 1995. Asymmetric replication in vitro from a human sequence element is dependent on adeno-associated virus Rep protein. *Journal of Virology* 69:2038-2046.

Wang H, Lieber A. 2006. A helper-dependent capsid-modified adenovirus vector expressing adeno-associated virus rep78 mediates site-specific integration of a 27-kilobase transgene cassette. *Journal of Virology* 80:11699-11709.

Ward P, Walsh CD. 2012. Targeted integration of a rAAV vector into the AAVS1 region. *Virology* 433:356-66.

Weitzman MD, Kyostio SRM, Kotin RM, Owens RA. 1994. Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proceedings of the National Academy of Sciences USA* 91:5808-5812.

Wiskerchen M, Muesing MA. 1995. Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. *Journal of Virology* 69:376-386.

Wonderling RS, Owens RA. 1997. Binding sites for adeno-associated virus Rep proteins within the human genome. *Journal of Virology* 71:2528-2534.

Wu Y, Marsh JW. 2001. Selective transcription and modulation of resting T cell activity by reintegrated HIV DNA. *Science* 293:1503-1506.

Zhang XY, La Russa VF, Bao L, Kolls J, Schwarzenberger P, Reiser J. 2002. Lentiviral vectors for sustained transgene expression in human bone marrow-derived stromal cells. *Molecular Therapy* 5:555-565.

Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature Biotechnology* 15:871-875.