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

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

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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.

## INTRODUCTION

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



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Adeno-associated virus serotype 2 (AAV2) is unique among mammalian viruses for its ability to integrate into a specific region of the human genome, referred to as *AAVS1* (AAV2 Site 1) (Kotin & Berns, 1989; Kotin et al., 1990; Kotin et al., 1991; Kotin, Linden & Berns, 1992). Most clonal cell lines obtained from AAV2-infected cells contain an AAV2 provirus within this 4 Kb region on chromosome 19 (Kotin et al., 1990; Hamilton et al., 2004; Ward & Walsh, 2012). *AAVS1* has an open chromatin configuration that is compatible with transgene expression (Lamartina et al., 2000). In addition, the presence of an insulator element upstream of *AAVS1* increases the probability that independent integration events involving the same promoter/transgene cassette will result in similar levels of transgene expression (Ogata, Kozuka & Kanda, 2003).

Preferential integration at *AAVS1* requires functions encoded by the ~2.2 Kb AAV2 *rep* gene (Shelling & Smith, 1994; Weitzman et al., 1994; Balague, Kalla & Zhang, 1997). The *rep* gene encodes four major proteins from overlapping reading frames (Mendelson, Trempe & Carter, 1986). The larger Rep proteins, Rep68 and Rep78 (Rep68/78) are required for replication and packaging (Im & Muzyczka, 1990; King et al., 2001). Rep68/78 recognizes specific sequences at *AAVS1* that are similar to sequences found in the AAV2 inverted terminal repeats (ITRs) (Snyder, Im & Muzyczka, 1990; Chiorini et al., 1994; Weitzman et al., 1994; Urcelay et al., 1995; McAlister & Owens, 2010). Most currently used AAV vectors do not incorporate the AAV2 *rep* gene and are designed to persist episomally in non-dividing cells (Owens, 2002).

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



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

Recent reports have suggested that the AAV2 *rep* gene products can direct the integration of adenoviral and retroviral vectors into *AAVS1* when the vectors contain key sequences from AAV2 (Wang & Lieber, 2006; Huang et al., 2012). In this report we investigate the use of the AAV2 Rep proteins to direct integration of integrase-proficient and integrase-deficient lentivirus vectors to *AAVS1* in cells co-infected with AAV2 and in cells infected with lenitiviral vectors expressing Rep68/78 sequences from the AAV2 p5 promoter.

## MATERIALS AND METHODS

### Lentivirus production

Lentivirus was made by co-transfection (calcium phosphate co-precipitation method) of HEK-293T cells with the pLKO.1 puro vector plasmid (Moffat et al., 2006), pCMV-VSV-G envelope plasmid (Stewart et al., 2003), and packaging vector pCMV-ΔR8.91 (Zufferey et al., 1997). To make virus particles deficient for HIV-1 integrase, pCD/NL-BH* (Zhang et al., 2002) was used in place of pCMV-ΔR8.91. Virus supernatants were cleared of debris by centrifugation for 5 min at 3,000 x g and filtered using a Millex-GV 0.45 µm PVDF syringe filter (Millipore, Billerica, MA). The virus titer of pLKO.1 puro packaged with pCMV-ΔR8.91 was measured by infecting HeLa cells with virus supernatant and



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selecting for puromycin-resistant colonies. The titer of packaged pLKO.1 puro was 3.5 x $10^5$ transducing units/ml.

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

**Virus infections and co-infections**

The HeLa human cervical carcinoma cell line was grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) to 70% confluency in 25 $cm^2$ flasks for virus infections. For lentivirus infections, the culture medium was replaced with 5 ml of unconcentrated lentivirus supernatant. The virus supernatant was removed after 12 to 16 hrs and replaced with fresh DMEM with 10% FBS. Cells were harvested 48 hrs post infection for DNA isolation using the DNeasy tissue kit (QIAGEN, Valencia, CA). For AAV2 infections, HeLa cells grown to 50% confluency were infected with approximately $9 \times 10^3$ viral genomes per cell in DMEM without FBS for 12 to 16 hrs. AAV2 was prepared as described previously (Carter et al., 1979). AAV2 titers were determined by DNA slot blot analysis.

**PCR integration assays**



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A nested PCR assay was used to detect integration of lentivirus vectors made from the pLKO.1 puro vector. Two primer sets were used in this assay. One set is specific for the pLKO.1 puro LTRs. The other primer set is specific for *AAVS1*. The first round of PCR was done using the primers 5'-AGT TAC CAG AGT CAC ACA ACA GAC GG-3' for pLKO.1 puro and 5'-AAC ATG CTG TCC TGA AGT GGA CAT AGG-3' specific for *AAVS1*. The first reaction contained 100 ng of total cellular DNA. 0.5 µl of the first PCR reaction was used as the template for a second PCR using the primers 5'-ACT ACT TGA AGC ACT CAA GGC AAG CTT T-3' for pLKO.1 puro and 5'-GAA GAC TAG CTG AGC TCT CGG ACC-3' for *AAVS1*. The following PCR parameters were used. 95° for 5 min, 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 5 min. Roche FastStart polymerase with High GC buffer (Roche Applied Science, Indianapolis, IN) was used for both amplifications.

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

## RESULTS

### Wild-type AAV2 directs integration of a lentiviral vector to *AAVS1* in co-infected cells

The cells were infected with the pLKO.1 puro vector and AAV2 on different days. The genomic DNA was used for the PCR integration assay. Single primer controls in which one primer is used in the



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second amplification are shown Figure 1 B. No junctions were detected when the cells were infected with lentivirus only (Fig. 1 A, lanes 1 and 4). Junctions were also not detected in the single primer 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).

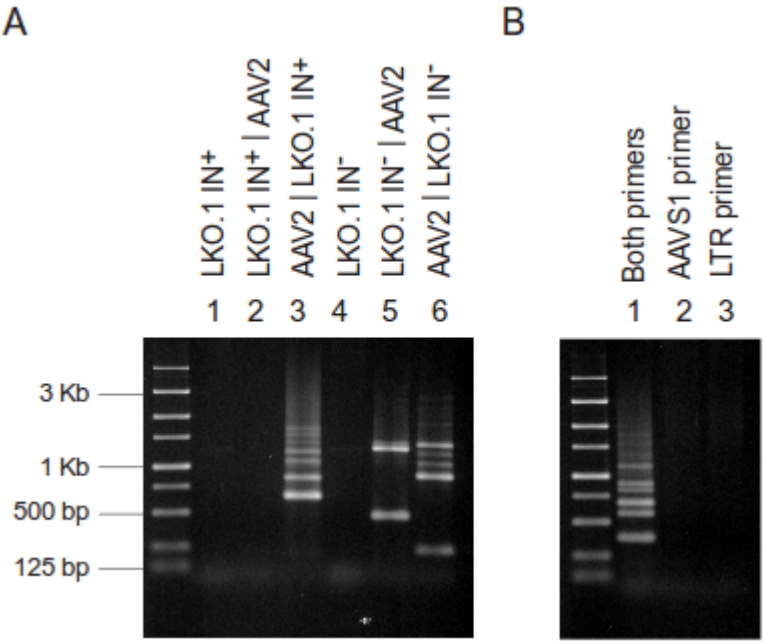


**Figure 1 -** AAV2-lentivirus co-infection experiments demonstrating lentivirus integration at *AAVS1*. A. Human cervical carcinoma cells (HeLa) were infected with lentivirus made from pLKO.1 puro, with or without AAV2, as indicated in the figure, and assayed for integration by nested PCR. ($IN^+$) 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.



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

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





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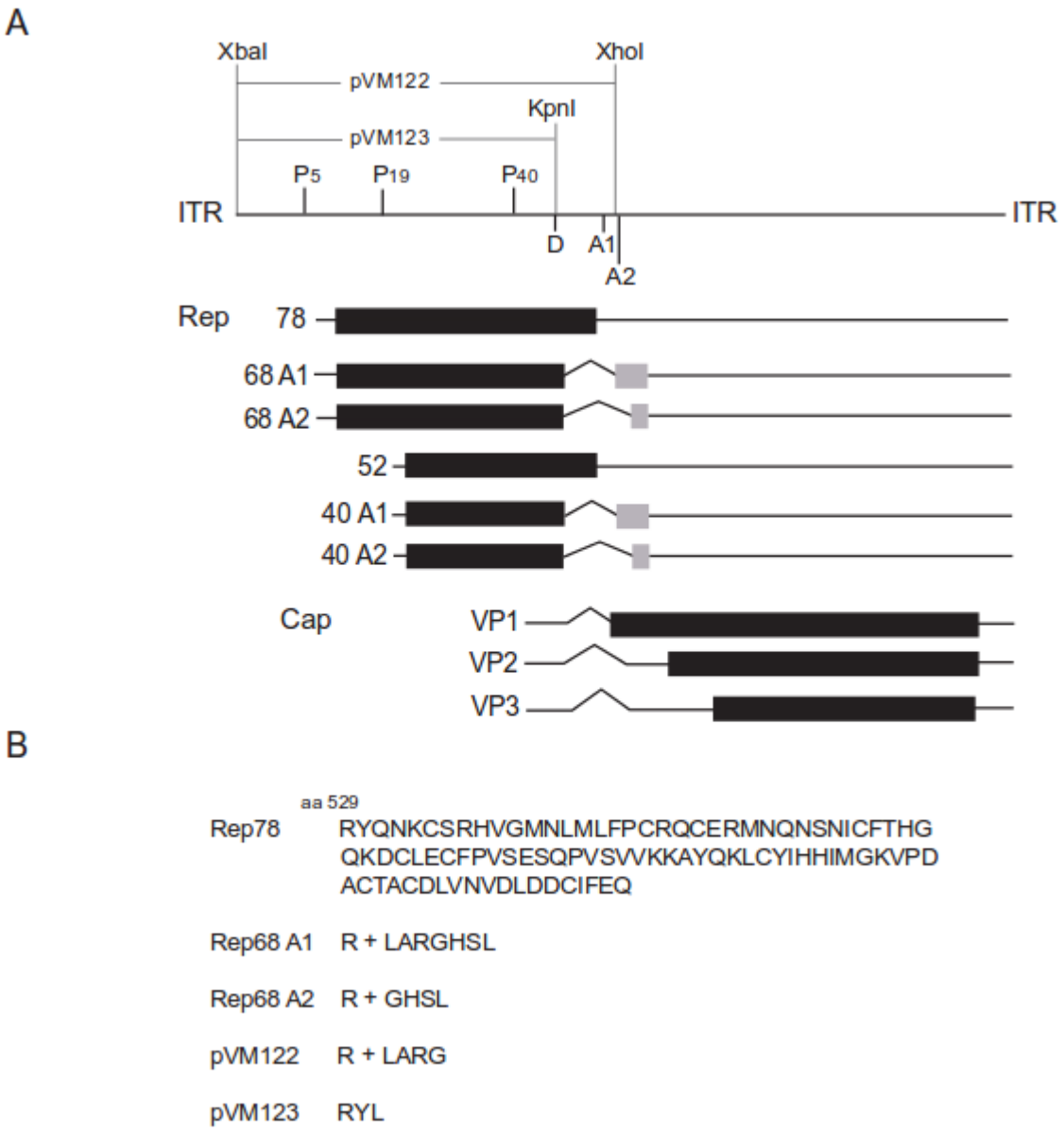


113 **Figure 2 -** Map of the AAV2 genome in pSub201(-) and *rep* gene fragments used in the construction of
114 hybrid lentiviral vectors expressing AAV2 Rep proteins. A. Map of the AAV2 genome contained in
115 pSub201(-). Rep proteins are translated from alternately spliced RNAs transcribed from the $P_5$ and $P_{19}$
116 promoters. pVM122 contains the pSub201(-) XbaI-XhoI fragment. pVM123 contains the pSub201(-)
117 XbaI-KpnI fragment. Both constructs contain the $P_5$ and $P_{19}$ promoters. B. The predicted amino acid
118 sequences of the wild-type and truncated Rep proteins. pVM122 is predicted to express full-length



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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.

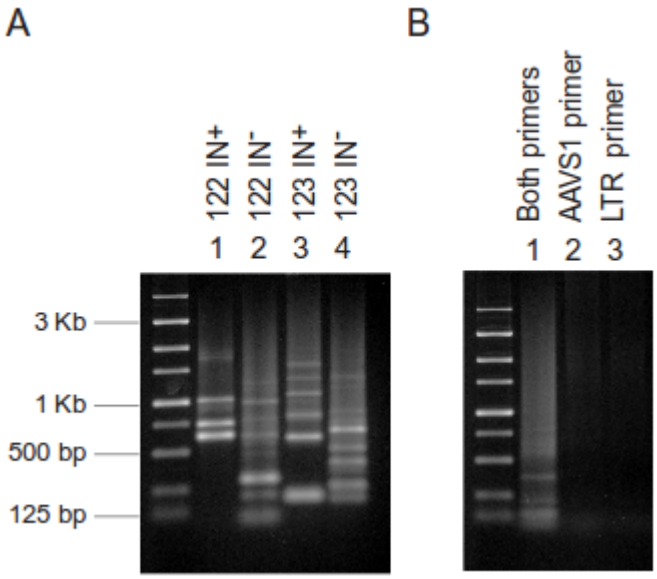


**Figure 3 -** Integration assays of hybrid lentiviral vectors designed to express the AAV2 *rep* gene. A. Human cervical carcinoma cells (HeLa) were infected with lentivirus made using pVM122 and pVM123. ($IN^+$) Lentivirus particles containing an active version of the lentiviral integrase. ($IN^-$) Lentivirus particles containing an inactive lentiviral integrase. 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 4 of part A.



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The presence of lentiviral vector-*AAVS1* junctions within the PCR products of the integration assays was confirmed by DNA sequencing. Eight products were sequenced. Six unique junctions were identified. The sequences are shown in Figure 4 A. Most were characterized by one or more bases of homology between the vector DNA and *AAVS1*. Consistent with our previous observations (McAlister & Owens 2007), most of the junctions were at or near polypyrimidine tracts of five or more bases within *AAVS1*.





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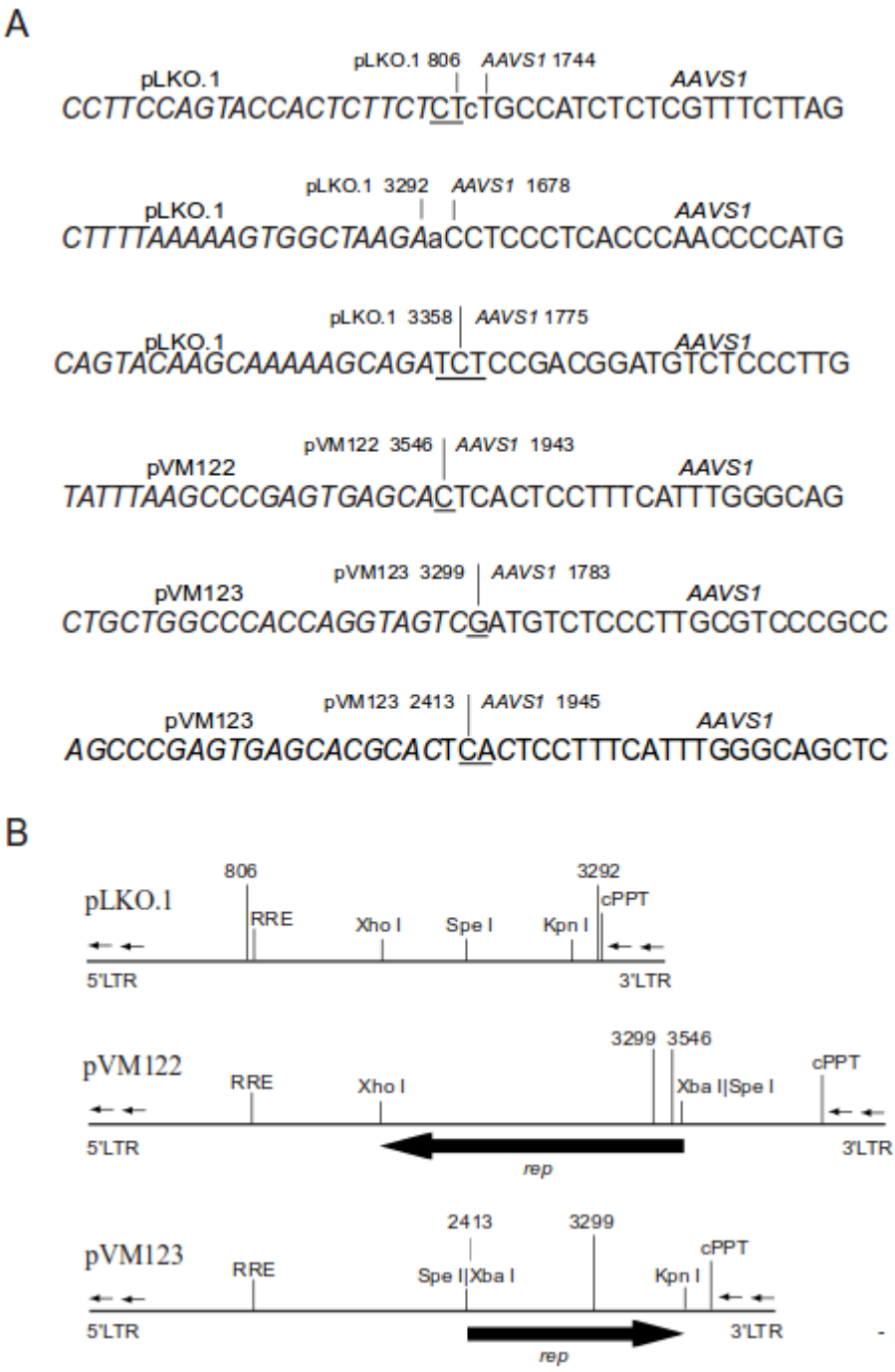


140 **Figure 4 -** Lentivirus-*AAVS1* integration junctions. A. DNA sequences of the integration junctions.

141 Lentiviral vector sequences are in italicized lettering. *AAVS1* base positions are the number of bases

142 from the Rep nicking site. Bases that are shared by two sequences are underlined. Bases not present in

143 either sequence are in lowercase. B. Map of Integration junctions shown in part A. (RRE) rev-



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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 *AAVS1*. 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 *AAVS1* is a free DNA end or nick from which a free end can be generated.

Rep68/78-mediated targeting of lentivirus vectors to *AAVS1* 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 *AAVS1* 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 *AAVS1* 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 *AAVS1* 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.



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Our results help to refine models of the mechanism of Rep68/78-mediated preferential integration. It was first thought that the inverted terminal repeats (ITRs) of the AAV2 genome were required for preferential integration (Weitzman et al., 1994; Balague, Kalla & Zhang, 1997), but more recent work has shown that Rep proteins can mediate integration of a hybrid adenovirus vector into *AAVS1,* often with the adenovirus ITRs, rather than the AAV2 ITRs found at the junction with *AAVS1* (Wang & Lieber, 2006). Another recent report shows that an integrase-deficient murine stem cell retrovirus vector, containing a key integration-enhancing sequence from AAV2, can also integrate into *AAVS1* when Rep68/78 is present (Huang et al., 2012). Combined with the results we present here, a new hypothesis emerges that all that is required of the inserting DNA is that it contains DNA from which free ends (single-stranded or double-stranded) can be generated and that it contains a binding site for Rep68/78. Binding sites are predicted to be quite common, since the sequences bound by Rep68/78 are quite degenerate (McCarty et al., 1994; Chiorini et al., 1995; Wonderling & Owens, 1997), and weak binding sites have been identified within the HIV-1 long terminal repeat (Kokorina et al., 1998) (our lentiviral vector is based on HIV-1 HXBIII isolate) and within the *rep* gene (McCarty et al., 1994).

The absence of ITRs in our *rep* gene-containing lentiviral vectors creates two advantages over ITR-containing hybrid vectors. First, it lowers the probability that the Rep proteins would disrupt the vector DNA during packaging, since the ITRs contain the two major Rep68/78 nicking sites within AAV2 (Im & Muzyczka, 1990). The absence of ITRs also lowers the probability of disruption of the vector after integration into *AAVS1*. Having the *rep* gene under the control of its natural promoter, p5, reduces the probability of Rep-mediated rearrangement of the vector, since Rep proteins down-regulate the p5 promoter (Beaton, Palumbo & Berns, 1989; Kyostio, Wonderling & Owens, 1995). The use of C-



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

## CONCLUSIONS

The ability of AAV2 Rep proteins to mediate integration into the *AAVS1* locus of human chromosome 19 may allow the creation of safer gene therapy vectors. The observation that these proteins target integration of a lentiviral vector provides further evidence for the hypothesis that Rep-mediated 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.

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