

Original Article

A method mediated AAVS1 recombination with Rep mRNA and homologous arms

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The adeno-associated virus (AAV) genome can be stably integrated into the AAVS1 region of human chromosome 19 (19q13.4-qter) with the assistance of Rep68/78 protein. In the current models of AAV integration in a locus-specific manner, the foreign genes were randomly inserted into the AAVS1 region, which contains several functional genes. As random integration in this region may lead to insertion mutations and disrupt normal gene expression or critical signaling pathways of the host cells, it is necessary to find a precise insertion site in the AAVS1 region. Homologous recombination is the most accurate and versatile mechanism for such site-specific integration. To investigate site-specific integration in the AAVS1 region, a targeted vector containing two homologous arms derived from AAVS1 and a reporter gene was transfected into HeLa cells with or without Rep68/78 mRNA. The results indicated that transient expression of Rep68/78 in HeLa cells improved integration of the gene of interest at the AAVS1 locus in a site-specific manner. Compared with locus-specific integration reported in previous studies, site-specific integration may minimize the risk associated with random DNA integration in the AAVS1 region, which might be helpful for gene therapy.

Keywords adeno-associated virus; homologous recombination; AAVS1; Rep68/78; locus-specific integration; site-specific integration

Received: May 12, 2012 Accepted: August 13, 2012

Introduction

One of the major challenges in gene therapy is the risk of random integration of exogenous DNA into the human genome. Previous studies have attempted to improve gene

transfer systems that permit long-term expression of the gene of interest without disrupting normal gene expression or critical signaling pathways of the host cells. One of the most attractive features of adeno-associated virus (AAV) as a type of gene transfer system is its unique ability to preferentially integrate its genome into a defined locus (termed the AAVS1 region) on human chromosome 19q13.4-ter. A key intermediate step in the integration of AAV appears to be the formation of a complex composed of a large Rep protein (Rep68/78), inverted terminal repeats (ITRs), and AAVS1 [1–5]. A 138-bp region that encompasses the entire p5 promoter, termed the p5 integration efficiency element (P5IEE), has also been shown to be the *cis* element required to mediate Rep-dependent locus-specific integration at the AAVS1 locus [6,7]. Based on these features, a large number of studies have reported transgene integration into the AAVS1 region in a locus-specific manner. The most common approaches to locus-specific integration have involved the creation of hybrid systems including the *Rep* gene and ITR-flanked transgene, which are derived from either non-viral or chimeric viral constructs [4,8–12]. Locus-specific integration decreases the chance of an insertion mutation to some extent, excluding the AAVS1 region, but random integration of exogenous DNA inevitably occurs at the AAVS1 locus, leading to increased risk in gene therapy. For example, single vector integration was found within the *LMO2* locus, associated with aberrant *LMO2* expression during gene therapy for children with X-linked severe combined immune deficiency [13]. Map viewer software analysis shows that there are some functional genes mapped in the AAVS1 region, such as protein phosphatase 1 (regulatory subunit 12C, *ppr112c*), retinol dehydrogenase 13 (*rdh13*), epidermal growth factor receptor pathway substrate 8 (*eps8*), troponin type I (*tnnt1*) and troponin type III (*tnni3*) [14]. These genes encode proteins

that are involved in troponin formation (*tnnt1* and *tnni3*), oxidative stress (*rdh13*), and epidermal growth factor receptor signal transduction (*ppr112c* and *eps8*). Therefore, locus-specific integration still has risk associated with disrupting the normal gene expression or critical signaling pathways of the host cells. To express the genes of interest without an insertion mutation, it is necessary to find a precise insertion site for exogenous DNA in the AAVS1 region. Gene targeting through homologous recombination is the most accurate and versatile mechanism for such site-specific integration [15,16], although homologous recombination occurs in human somatic cells with very low frequencies ($\sim 10^{-7}$) [17].

Problems may arise due to the cytotoxic and/or cytostatic effects associated with Rep proteins when an active *Rep* gene is utilized in this system and allowed to integrate into the host genome. Previous studies have attempted to circumvent this problem by transfecting cells with the Rep protein directly and regulating the expression of the *Rep* gene using a Cre-loxP system [9–12,18,19]. The mRNA transfection was used to achieve transient expression of Rep68/78 protein in the targeted cells [20,21]. Compared with a *Rep* expression plasmid, Rep mRNA transfection is better for facilitating the targeted integration in human cells.

In this study, to minimize the chance of an insertion mutation at the AAVS1 locus, we constructed a targeted vector (pTAVEN) containing two homologous arms from the AAVS1 region. This vector was modified to contain the AAV-derived p5IEE, as well as the expression cassettes of the enhanced green fluorescent protein gene (*EGFP*) and neomycin gene (*neo*) under the control of cytomegalovirus promoter. Following co-transfection with Rep mRNA and pTAVEN in HeLa cells, we demonstrated site-specific integration of the transgene into the AAVS1 region in 29.4% (5/17) clonal cell lines. Similar to targeted integration in a locus-specific manner, Rep proteins can also mediate homologous recombination in the AAVS1 region on ch19q13.4-ter.

Materials and Methods

Targeted vector construction

The AAVS1 region (3.8 kb) was amplified from the BAC clone RP11-700B5 (provided by BACPAC Resources Center, Oakland, USA) using primers AAVSP1, 5'-GCCCTCATCTGGCGATTTCCACTG-3' and AAVSP2, 5'-CCGCCTCCCTAAGACCCAGAAGTC-3', followed by insertion into the pGEM-T easy vector (Promega, Madison, USA). Polymerase chain reaction (PCR) was performed using LA Taq™ kit (TaKaRa, Dalian, China) with the following program: 94°C for 5 min, 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 3 min, and extension at 72°C for 10 min. To obtain homologous arms, AAVS1 was

separated into two arms (AAVS1-a and AAVS1-b) by *NheI* digestion. To generate the targeted vector pTAVEN, the expression cassettes containing *neo* and *EGFP* were inserted into the corresponding sites in pTA (*NheI*^Δ and *MluI*^Δ, respectively).

In vitro transcription of Rep mRNA

The Rep68/78 coding region was amplified from pMTrep with primers RepP1 5'-CATACGATTTAGGTGACACTATA GAATACAGAGATCTCGCAGCCGCCATG-3' and RepP2 5'-(T)₃₀ATAATCTGAGGAGGTCTAG-3', which contain an SP6 promoter (shown in italics) and a poly(T) tract, respectively. PCR products were column purified (Qiagen, Hong Kong, China) and used as templates to generate 5'-end m7GpppG-capped and polyadenylated mRNA encoding the Rep68/78 protein using the SP6 mMessage mMachine *in vitro* transcription kit according to the manufacturer's recommendations (Invitrogen, Carlsbad, USA).

Cell culture, transfection and fluorescence-activated cell sorter analysis

HeLa cells were maintained in continuous culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all reagents provided by HyClone, Utah, USA). Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

HeLa cells were washed twice with serum-free OptiMEM (Invitrogen) and resuspended to a final concentration of 1×10^7 cells/ml. Subsequently, 0.5 ml of the cell suspension was mixed with 10.0 μg of *in vitro* transcribed mRNA (Rep68/78 mRNA) with or without 2.5, 5.0, or 10.0 μg pTAVEN DNA linearized into 7.4-kb DNA fragments by *NotI* (pTAVEN/*NotI*). These cells were electroporated in a 0.4-cm cuvette with a pre-optimized pulse condition (950 μF, 250 V, 30–35 ms, 1 pulse) using a Bio-Rad gene pulser II (Hercules, USA). After electroporation, 20 ml of DMEM supplemented with 10% FBS was added to the cell suspension. Cells were incubated at 37°C in a humidified atmosphere supplemented with 5% CO₂.

After 24 h of transfection, HeLa cells were dissociated into single cells with 0.25% trypsin-EDTA for 3 min, washed twice with phosphate-buffered saline (PBS), resuspended in 500 μl PBS, and run on a fluorescence-activated cell sorter (FACS) Caliber (Becton Dickinson, San Jose, USA). Data were analyzed using Cell Quest Software (Becton Dickinson).

To establish neomycin-resistant cell clones, the GFP⁺ cells were sorted and harvested by FACS 24 h post-transfection. The GFP⁺ cells were plated at 1 cell per well into 96-well plates and in culture media containing G418 (Invitrogen) at a final concentration of 500 μg/ml over a period of 4–6 weeks.

Western blot analysis

Cells were lysed in ice-cold lysis buffer containing protease inhibitors. The cell lysate was centrifuged at 20,000 *g* for 15 min at 4°C and the total protein was estimated using the Bradford assay (Bio-Rad). The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the separated proteins were transferred onto a nitrocellulose membrane, followed by membrane blocking with 5% non-fat milk powder (w/v) in Tris-buffered saline (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20, pH 7.4) for 1 h at room temperature or overnight at 4°C. The membranes were probed with a primary antibody specific to Rep68/78 (Acris Antibodies, Inc., San Diego, USA), followed by anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase (1 : 1000) and enhanced chemiluminescence visualization. The membranes were stripped and re-probed with an anti-actin antibody (Cell Signaling Technology Inc., Danvers, USA) as a loading control.

PCR assay for screening cell clones

The site-specific integration was detected by long-range PCR using specific primers flanked by the homologous arms (60317251–60321048 on ch19q). The forward primer (F), 5'-TGTGAAAGATGCCTGAGGCCTGGG-3' (60317086–60317110 on ch19q), which was upstream of the AAVS1-a (–165 bp). The reverse primer (R), 5'-CTG TCCAGTCGAATTCCTAACTGC-3' (60321183–60321159 on ch19q), which was downstream of the AAVS1-b (+135bp). Genomic DNA derived from neomycin-resistant cell clones was used as a template to perform PCR using the LA Taq™ kit. The amplification protocol consisted of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 4 min and extension at 72°C for 10 min.

Southern blot analysis

Southern blot analysis was performed as described previously [22] with some modifications. Briefly, genomic DNA was harvested from each cell line. Genomic DNA was digested with *Hind*III and separated on 1% agarose gels. After transferring DNA fragments to nylon membranes (Hybond N⁺; GE Healthcare Life Science, Wisconsin, USA), hybridization was performed using [α -³²P] dCTP-labeled neomycin probes. A 420-bp neomycin PCR fragment was generated to use as a DNA probe from primers 5'-ATTCCTGATCGACAAGACCGGC-3' and 5'-GAGAGGCTATTCGGCTATGA-3'. DNA probes were [α -³²P] dCTP labeled with the T7 QuickPrime kit (GE Healthcare Life Science) according to the manufacturer's instructions. The bands were visualized by autoradiography.

Results

Construction of the targeted vector and transfection analysis

To investigate site-specific integration in the AAVS1 region using homologous recombination, we constructed a targeted vector (pTAVEN) containing two homologous arms derived from AAVS1 (3.8 kb, from 60317251 to 60321048 on ch19q). An expression cassette containing a neomycin gene (*neo*) and a reporter gene (*egfp*) was inserted into the corresponding sites shown in **Fig. 1**. RBE (a *cis*-element) remains in the AAVS1-b arm (near the SP6 promoter). This plasmid also contains the P5 integration efficiency element (P5IEE) from AAV.

Due to the cytotoxic and/or cytostatic effects associated with Rep protein, an mRNA approach was used to achieve transient expression of Rep68/78 protein in HeLa cells. *In vitro* transcribed Rep68/78 mRNA (10.0 μ g) was subsequently electroporated into HeLa cells. After 24–72 h of transfection, Rep68/78 protein was detected in transfection cells by Western blot analysis. Rep protein can be effectively expressed in cells 24 h post-transfection. By 36, 48, or 72 h post-transfection, Rep protein expression was significantly reduced or undetectable [**Fig. 2(A)**]. These

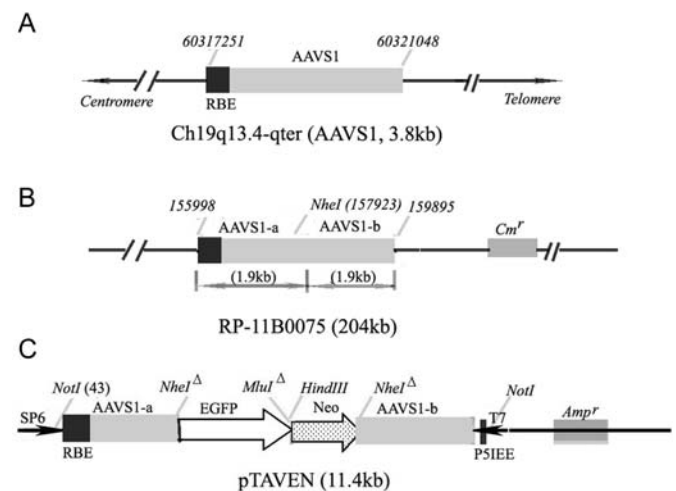


Figure 1 Construction of the pTAVEN targeted vector (A) The genomic organization of the human target site for AAV integration (AAVS1) on human chromosome 19q13.4-ter (60317251–60321048). (B) Schematic representation of the BAC clone (RP11-700B5) containing AAVS1. The length of AAVS1 is ~3.8 kb (155998–159895 on RP11-700B5). The *Nhe*I site locates at 157923. (C) This targeted vector (11.4 kb) contains the AAVS1 fragment used as homologous arms (AAVS1-a and AAVS1-b). The RBE *cis*-element (GCTCGCTC CTCGCTG) is located at 276–291 bp sites on the left homologous arm (AAVS1-a). The expression cassettes for the neomycin gene (*neo*) and reporter gene (*EGFP*) were inserted into the *Nhe*I and *Mlu*I restriction sites, respectively. The vector contains P5IEE flanked by the right homologous arm AAVS1-b. The *Not*I cutting site can be used to generate linearized pTAVEN DNA for transfection. The *Hind*III restriction site was introduced to perform Southern blot analysis.

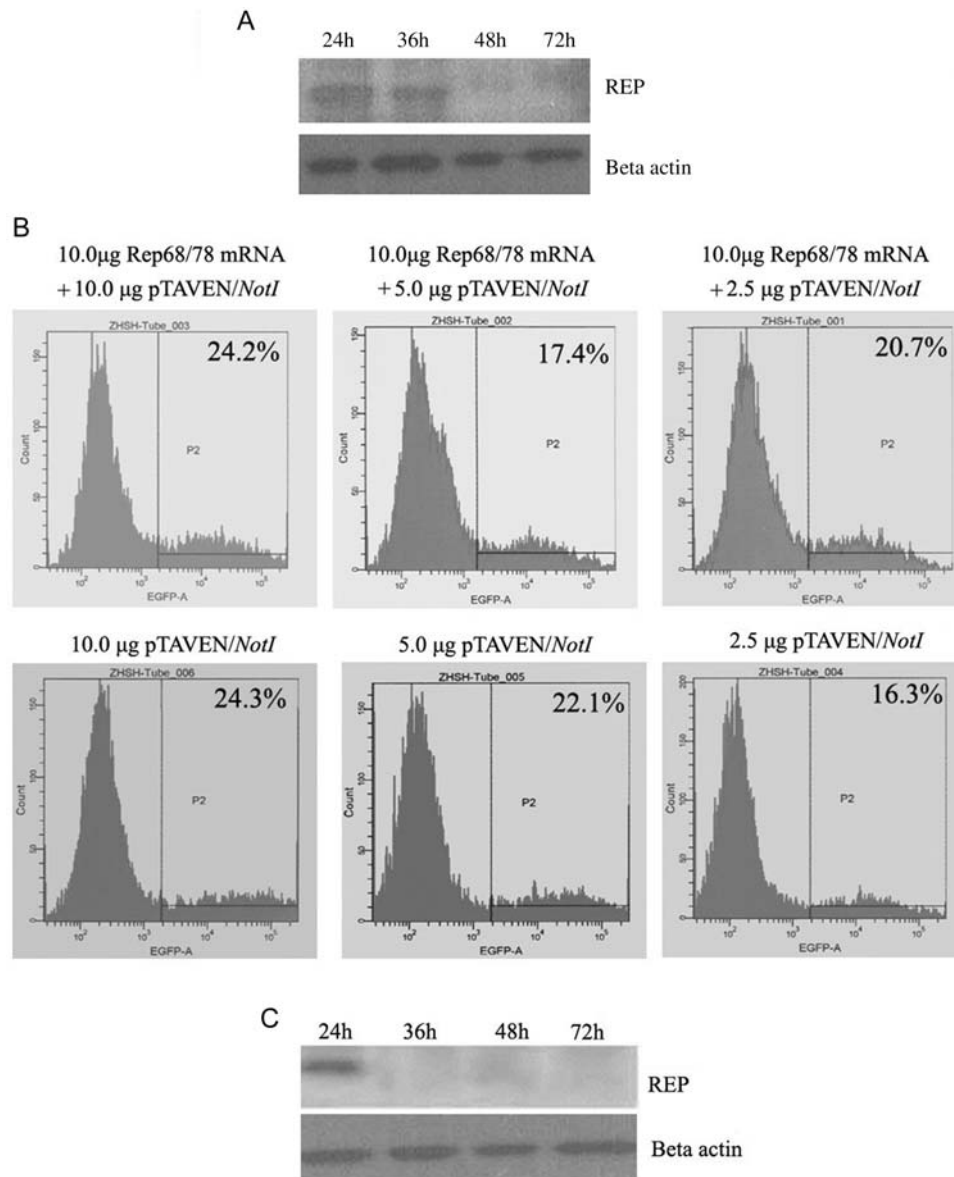


Figure 2 Transfection analysis (A) Analysis of transient expression of Rep68/78 proteins in HeLa cells. Total proteins were detected by western blot analysis at 24, 36, 48, and 72 h post-transfection of Rep68/78 mRNA. (B) FACS analysis of the percentage of GFP⁺ cells 24 h post-transfection. (C) Analysis of transient expression of Rep68/78 protein in the GFP⁺ population. GFP⁺ cells were sorted and harvested by FACS at 24, 36, 48, and 72 h post-transfection. Transient expression of Rep68/78 proteins in GFP⁺ cells was detected by western blot analysis.

results showed that the electroporation of HeLa cells with Rep68/78 mRNA is an effective approach for achieving transient expression of Rep68/78 protein in HeLa cells. Consistent with a previous study [20], we did not observe a significant difference in transient Rep expression between mRNA transfection and plasmid DNA transfection in preliminary experiments (data not shown).

To determine whether the transfection of HeLa cells with Rep78 mRNA can facilitate DNA homologous recombination at a specific site in the AAVS1 region, HeLa cells were co-transfected with Rep68/78 mRNA and pTAVEN/NotI. Considering the available transfection efficacy of co-transfection, $\sim 5 \times 10^6$ cells were electroporated with

10.0 μg Rep68/78 mRNA and different concentrations of pTAVEN/NotI DNA (10.0, 5.0 or 2.5 μg). As controls, HeLa cells alone were transfected with 10.0, 5.0, or 2.5 μg of pTAVEN/NotI DNA. After 24 h of electroporation, FACS analysis showed no significant difference in transfection efficiency between co-transfected cells and controls [Fig. 2(B)]. For example, 24.3% GFP⁺ cells were observed from co-transfected cells with 10.0 μg of Rep68/78 mRNA and 10.0 μg of pTAVEN/NotI DNA compared with 24.2% GFP⁺ cells from the corresponding control. Therefore, this transfection setup was used in the subsequent experiments.

To investigate whether Rep68/78 mRNA can be effectively expressed in the GFP⁺ population among co-transfected

cells, GFP⁺ cells were sorted and harvested by FACS after 24–72 h of transfection. Total proteins were extracted, and Western blot analysis was performed. We found that REP68/78 protein was expressed in the GFP⁺ FACS-sorted cells 24 h post-transfection. After 36–72 h of transfection, Rep protein expression was significantly reduced or undetectable in the GFP⁺ cells tested [Fig. 2(C)]. These results indicated that GFP⁺ cells co-transfected with Rep68/78 mRNA, and the targeted vector can transiently express Rep68/78 protein.

Determination of homologous recombination in the AAVS1 region

After plating GFP⁺ FACS-sorted single cells into 96-well plates, clonal cell lines were grown for 6 weeks. We obtained 17 neomycin-resistant cell clones following the co-transfection of HeLa cells with pTAVEN and Rep68/78 mRNA, and 19 neomycin-resistant cell clones following the transfection of HeLa cells with pTAVEN alone.

To screen the neomycin-resistant cell clones, PCR was performed using the specific primers. Because one strand exchange occurs between the homologous arms and the targeted locus during homologous recombination, two fragments, including 4.1 and 7.7 kb, were generated by specific PCR [Fig. 3(A)]. In the case of non-homologous recombination in the AAVS1 region on ch19q, only the 4.1 kb

fragment could be generated. As shown in Fig. 3(B,C), homologous recombination occurred in the Clones 2, 4, 5, 6 and 7 and g.

Southern blot analysis was used to further investigate the integration sites of the exogenous DNA in the positive cell clones using neomycin probes labeled with [α -³²P] dCTP. Targeted integration of the plasmid will result in a 4.4-kb band with a *Hind*III restriction site present between the neomycin and the EGFP cassette in the targeted vector [Fig. 3(A)]. Southern blot analysis was performed on all neomycin-resistant cell clones. As shown in Fig. 4, the expected bands at 4.4 kb were generated by *Hind*III digestion of genomic DNA from the co-transfection of HeLa cells with pTAVEN and Rep68/78 mRNA in Clones 2, 4, 5, 6, and 7. In the absence of Rep expression, the expected band was detected only in clone g. The remaining cell clones generated bands with a different molecular weight due to random integration. Homologous recombination occurred at a frequency of 5.3% (1/19) following transfection of the plasmid alone and 29.4% (5/17) following co-transfection of the plasmid with Rep68/78 mRNA (Fig. 5).

Discussion

One of the major concerns with many current gene therapy protocols is the risk associated with random integration of

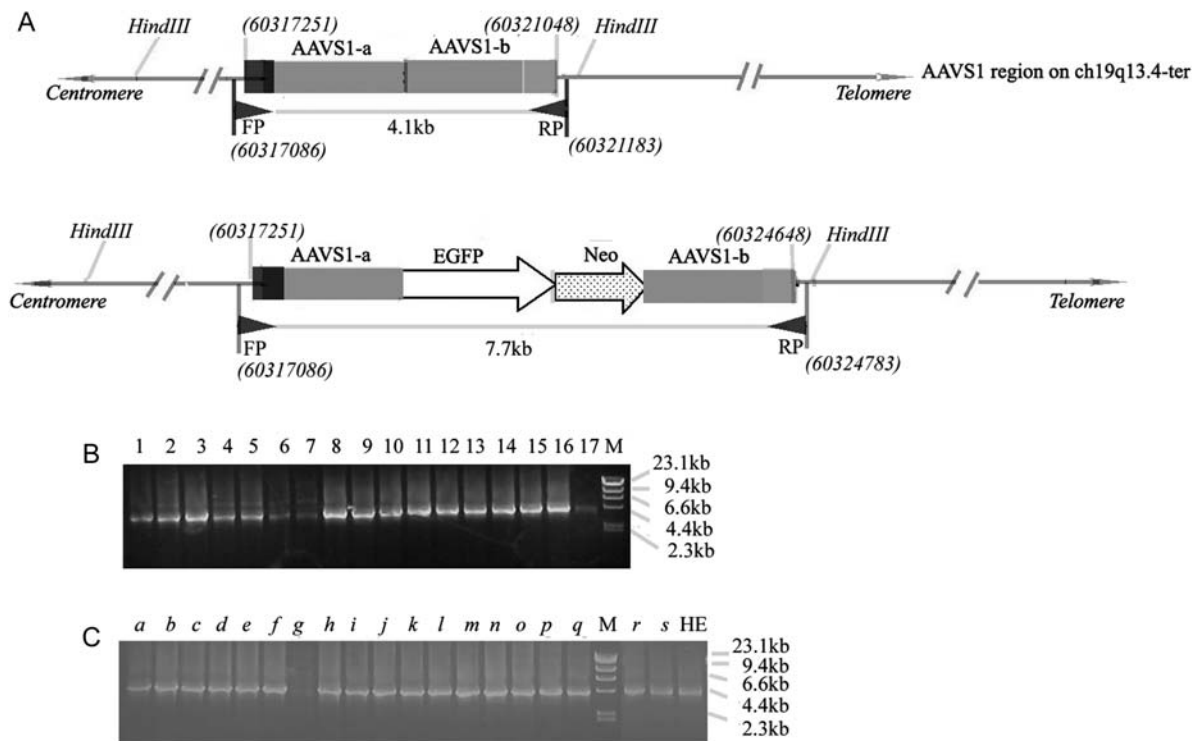


Figure 3 Illustration of screening PCRs (A) Illustration of non-homologous recombination (top) and homologous recombination (bottom) on ch19q13.4-ter. Black arrows represent primers. (B) PCR assay for screening the co-transfected cell clones with pTAVEN/*Not*I and mRNA of Rep68/78 (nos. 1–17). ‘M’ represents DNA marker (λ -DNA/*Hind*III). (C) PCR assay for screening the transfected cell clones with pTAVEN/*Not*I only (italic lower case letter a–s). ‘M’ represents DNA marker (λ -DNA/*Hind*III). HE indicates genomic DNA from HeLa cells.

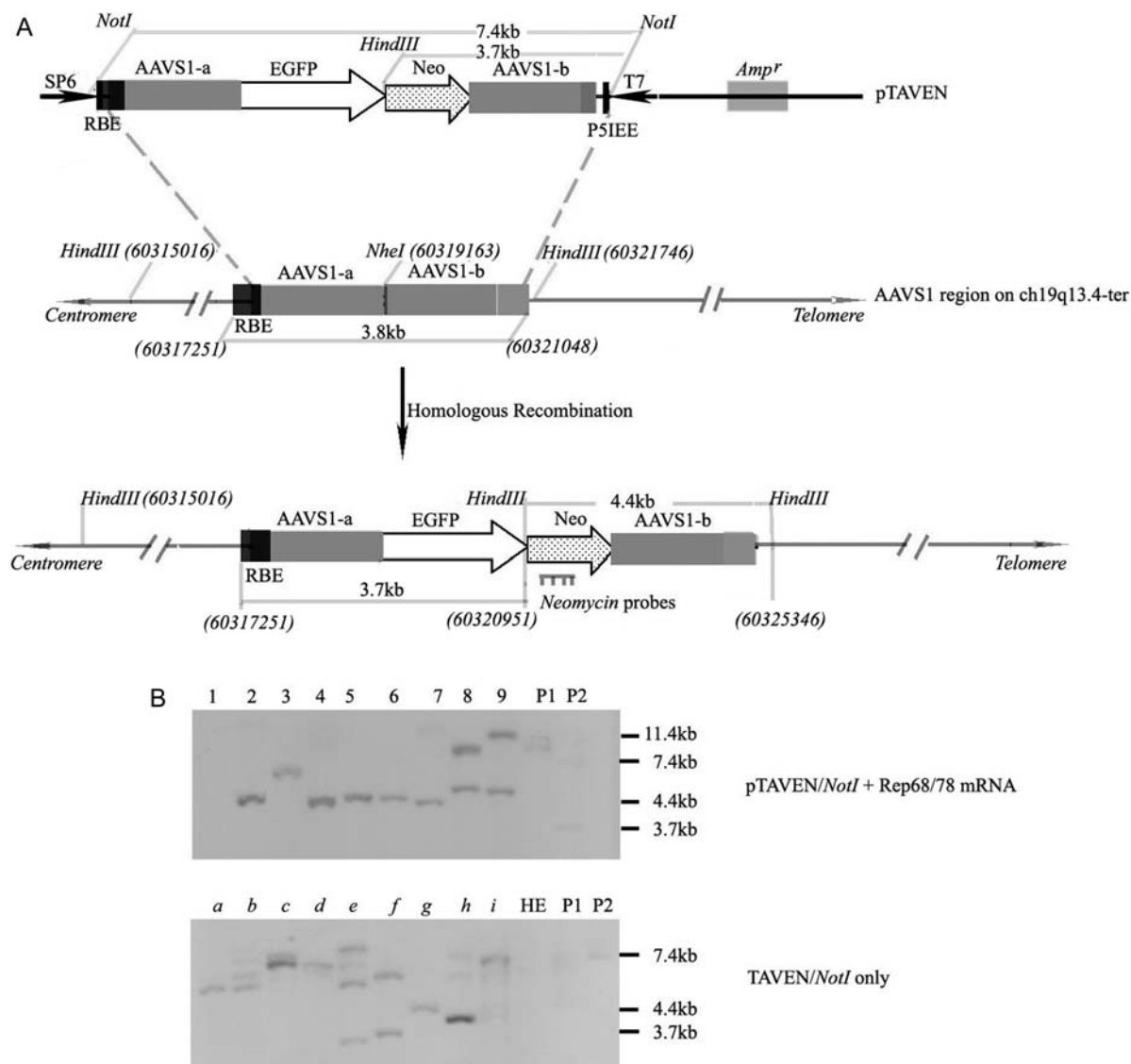


Figure 4 Southern blot analysis of REP-mediated homologous recombination (A) Schematic representation of the restriction endonuclease recognition sites in the targeted vector and the AAVS1 region on ch19q13.4-ter. The expected sizes of the DNA fragments following digestion with the restriction endonuclease *HindIII* are shown. (B) Southern blot analysis of genomic DNA isolated from clones following digestion with the restriction endonuclease *HindIII*. The DNA was fractionated by electrophoresis, transferred to nylon membranes, and hybridized with [α - 32 P] dCTP-labeled probes specific to neomycin. Clones 1–9 (Arabic numbers) represent the co-transfected cell clones with pTAVEN/*NotI* and mRNA of Rep68/78 (top). Clones *a–i* (lower case, italics) represent the transfected cell clones with pTAVEN/*NotI* only (bottom). HE indicates the genomic DNA isolated from HeLa cells following digestion with the restriction endonuclease *HindIII*. P1 represents the targeted vector pTAVEN following digestion with *HindIII* (generating a 11.4-kb fragment). (The 11.4-kb *HindIII* band in the bottom figure is unclear.) P2 represents the mixture of pTAVEN/*NotI* (7.4 kb) and pTAVEN/*NotI* + *HindIII* (3.7 kb). Specific bands were not detected in the remaining clones, including Clones 10–17 and Clones *j–s* (**Supplementary Fig. S1**).

exogenous DNA into the human genome. Thus, the development of gene transfer systems that can facilitate the targeted integration of DNA to specific sites in the host genome is of significant importance. As AAV is able to preferentially integrate its genome into the AAVS1 region with the assistance of Rep protein, many of the previous studies have shown that a transgene can be integrated into the AAVS1 region in a locus-specific manner. However, random integration of exogenous DNA inevitably occurs at the AAVS1 locus.

Using homologous recombination, we have developed the targeted vector (pTAVEN) to induce targeted integration in a site-specific manner at the AAVS1 locus in this study. The targeted integration of the AAV genome is mediated by Rep proteins, specifically the Rep68 and Rep78 variants, which bind RBEs located within the AAV genome and the AAVS1 region. Previous studies also proved that a 16-bp of the RBE was sufficient for mediating Rep-dependent locus-specific integration [6,23]. So Rep68/78 functions in the same way in AAV site-specific

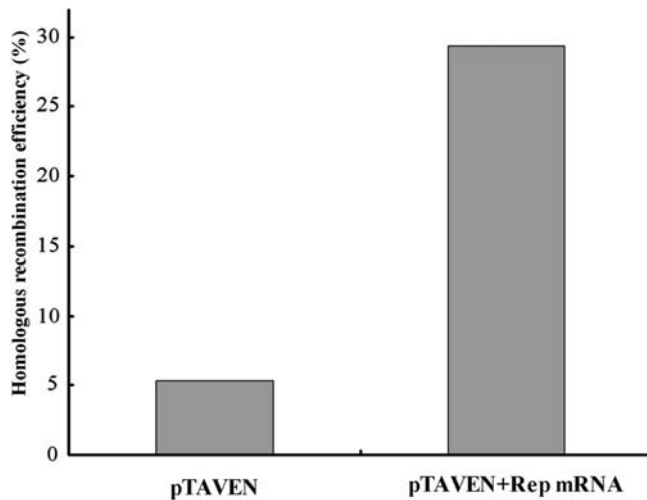


Figure 5 Illustration of homologous recombination efficiency The homologous recombination efficiency, measured as the percentage of targeted integration cell clones, following electroporation of pTAVEN in HeLa cells was compared with that observed following the co-electroporation of pTAVEN and Rep68/78 mRNA.

integration and is sufficient for integration *in vitro*. To minimize the deleterious effects associated with long-term and/or overexpression of Rep, Rep mRNA transfection was used to facilitate transient expression of Rep68/78 protein in human cells. This key feature of mRNA transfection makes an extremely effective approach for transgene expression in the majority of cells [8]. To determine whether the transfection of HeLa cells with Rep78 mRNA can facilitate DNA homologous recombination at a specific site in the AAVS1 region, cells were co-transfected with Rep68/78 mRNA and pTAVEN/*NotI*. FACS analysis demonstrated that transfection efficiency (GFP⁺ cells) was not significantly different between co-transfected cells and control cells. Western blot analysis confirmed that Rep68/78 mRNA was effectively expressed in the GFP⁺ population among cells co-transfected for 24 h. Compared with transfection with pTAVEN/*NotI* alone, transient expression of the Rep68/78 protein following mRNA transfection resulted in site-specific integration in 29.41% (5 of 17) of analyzed clones. Therefore, the Rep protein/DNA complex may also enhance homologous recombination in the AAVS1 region [24,25]. Prior to our studies, it was unknown whether this integration occurred before or after transgene integration, or the integration event itself may cause the translocation event.

Similar to targeted integration in a locus-specific manner, Rep protein can also mediate homologous recombination in the AAVS1 region on ch19q13.4-ter, if a plasmid vector carrying the RBE and homologous arms was used *in vitro*. Interestingly, a previous study showed that transient expression of Rep78 increased the degradation of SP100, a member of the promyelocytic leukemia nuclear bodies. On the contrary, SP100 inhibited the Rep-dependent non-viral integration [26]. It may lead to an improvement in the

efficiency of Rep-mediated homologous recombination. However, one of the challenges with this system is that it is difficult to find a safe integration site in the AAVS1 region. However, this system may be beneficial to help understand the site-specific integration of exogenous DNA in this region in human cells. This technique also helps to minimize the risk associated with random integration in the AAVS1 region in gene therapy.

Supplementary Data

Supplementary data are available at *ABBS* online.

Acknowledgement

We are grateful to Professor Dusty Miller (Human Biology Division, Fred Hutchinson Cancer Research Center, USA) for providing the pMTrep plasmid.

Funding

This work was supported by the grants from the General Program of National Natural Science Foundation of China (81172591), Anhui Provincial Natural Science Foundation (090413074), the Open Funds of the State Key Laboratory of Medical Molecular Biology (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) (2060204), and Scientific Research of BSKY (XJ200801) from Anhui Medical University.

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