

reduced ability to suppress T-cell proliferation. This hypothesis was further confirmed by the finding that iNOS expression was detected only in MSCs co-cultured with activated T cells.

In our scenario (Fig. 2), when MSCs are administered to the patients with severe acute GVHD, MSCs are considered to accumulate at the site of inflammation. Upon interaction with activated T cells, MSCs express iNOS and produce NO, which suppresses T-cell proliferation via inhibition of STAT5 phosphorylation. Systemic adverse effects of NO do not occur due to local production of NO with very short half-life. This is a very important point, because conventional treatment of acute GVHD causes severe systemic immunosuppression, which sometimes leads to life-threatening infections. Since MSC treatment causes just local immunosuppression, it should be much safer.

4. Interferon- γ and NF- κ B mediate nitric oxide production by mesenchymal stem cells

Human MSCs were reported to suppress Th1 differentiation and augment Th2 differentiation. Therefore, we investigated whether mouse bone-marrow-derived MSCs and the 10T1/2 cell lines have the same effect on Th1 and Th2. We found a reverse correlation between NO production and T cell proliferation in Th1/Th2 conditions, where NO production was highly induced in the presence of MSCs in Th1 but it was only minimally induced in Th2. In particular, primary MSCs and the A54 preadipocyte cell line, which induce strong T cell suppression in Th1, produce high levels of NO in Th1 condition. These results suggest that NO also plays a major

role in the preferential suppression of Th1 proliferation by MSCs.

To determine what inhibits the production of NO in Th2 condition, the two differentiation factors that support Th2 differentiation, anti-IFN- γ antibody and IL-4, were investigated. As a result, anti-IFN- γ antibody clearly inhibited the production of NO, whereas suppression by IL-4 was less evident. These results suggest that IFN- γ is a key regulator of NO production by MSCs.

Interestingly, cell supernatant collected from activated T cells had the ability to induce NO production by MSCs. IFN- γ is critical for NO production; however, in a T cell-free environment, IFN- γ alone does not induce NO production from primary MSCs. IFN- γ in combination with LPS, but not IL-2, stimulates NO secretion from primary MSCs, suggesting that both the IFN- γ and the signal from Toll-like receptor-4 (TLR4) are required for NO induction by MSCs. The addition of flagellin induced NO production in combination with IFN- γ . While, synthetic double strand RNA, poly(I:C), and CpG-oligonucleotide did not induce NO. Flagellin is a protein component of bacteria known to induce NO production from macrophages via TLR5 in the presence of either a TLR4 or IFN- γ signal. In addition to these factors, IL-1 β and TNF- α induce NO when provided in combination with IFN- γ . As NF- κ B is a downstream target of the signaling cascades activated by LPS, flagellin, IL-1 β , and TNF- α , we hypothesized that activation of NF- κ B is required for NO induction by MSCs. Bay-11-7085, a specific inhibitor of NF- κ B, suppressed induction of iNOS in MSCs, thus suggesting that NF- κ B is involved in NO production by MSCs as well as IFN- γ [20].

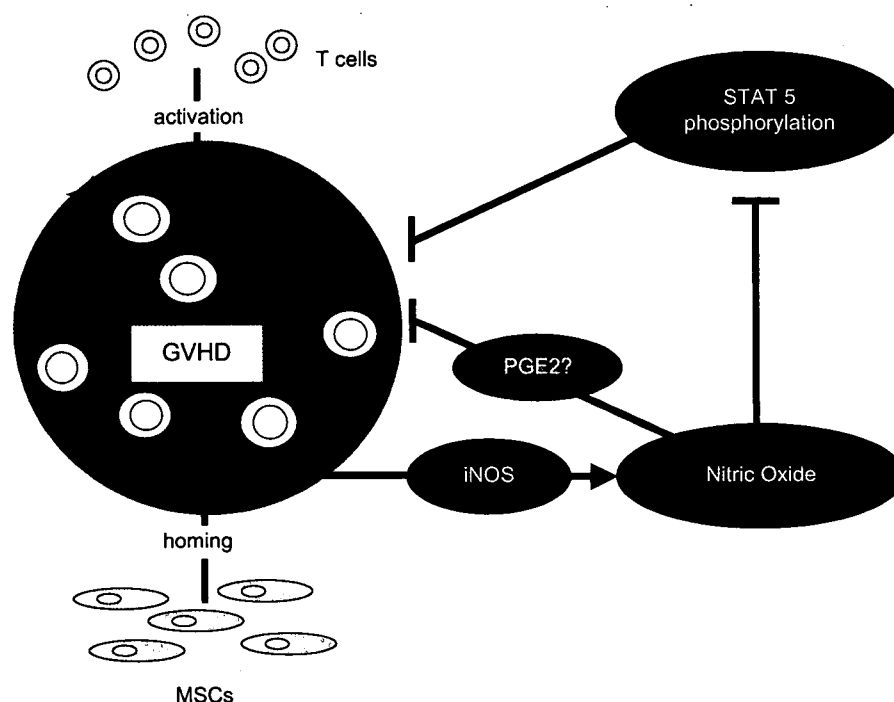


Fig. 2. MSC treatment of acute GVHD and the molecular mechanisms of T-cell suppression. MSCs are considered to accumulate at the site of inflammation and systemic adverse effects may not appear due to the local production of NO, which has very short half-life.

5. Retroviral vector-producing mesenchymal stem cells for tumor tracking and therapeutic gene amplification in suicide cancer gene therapy

MSCs are known to have a tendency to accumulate at the site of tumors, and therefore can be utilized as a platform for targeted delivery of anti-cancer agents [21–23]. The MSC-based targeted cancer gene therapy can enhance the therapeutic efficacy, because MSCs are considered to reach tumors including metastatic lesions and to deliver therapeutic molecules in a concentrated fashion. This targeted therapy can also reduce systemic adverse side effects, because the anti-cancer agents act locally at the site of tumors without elevating their systemic concentrations. We developed genetically-modified MSCs that produce retroviral vectors encoding *HSVtk* aiming at augmenting therapeutic efficacy of systemic suicide cancer gene therapy (Fig. 3). The tumor tropism and anti-tumor effects of vector-producing MSCs (VP-MSCs) were examined by intravascular injection in tumor-bearing nude mice. MSCs isolated from the bone marrow of SD rats were transfected with plasmid DNA expressing luciferase alone (=non-VP-MSCs) or whole retroviral vector components (LTR-Luc or LTR-*HSVtk* with Gag-pol and VSV-G) (=VP-MSCs) by nucleofection. To assess tumor tropism of MSCs, nude mice were subcutaneously inoculated with 9L rat glioma cells or Rat-1 fibroblasts, and were subsequently injected with luciferase-expressing MSCs through the left ventricular cavity. The transgene expression was periodically traced by using an *in vivo* imaging system. As a result, the transgene expression accumulated at the site of subcutaneous 9L tumors, but undetectable at the site of Rat-1 fibroblasts. In addition, the injection of luciferase-expressing VP-MSCs caused much stronger signal of bioluminescence at the site of 9L tumors compared with luciferase-expressing non-VP-MSCs. Immunostaining study showed that luciferase-positive cells (injected MSCs and transduced glioma cells) were detected at the periphery of tumors. To evaluate the therapeutic efficacy, tumor-bearing nude mice were treated with non-VP-MSCs or VP-MSCs combined with *HSVtk*/GCV system and then the size of subcutaneous tumors was periodically measured. In this model experiments, tumor growth was

more efficiently suppressed by injecting VP-MSCs compared with non-VP-MSCs (Uchibori R, et al.: manuscript in preparation). This study suggests the effectiveness of VP-MSCs in suicide cancer gene therapy. The therapeutic benefit of this strategy should be further examined in orthotopic and metastatic tumor models.

6. Site-specific insertion of a therapeutic gene into the AAVS1 locus (19q13.4) in human mesenchymal stem cells by using adeno-associated virus integration machinery

Hematopoietic stem cells, ES cells, and MSCs are attractive targets for gene therapy and regenerative medicine, since they replicate themselves and differentiate into various cell lineages. To introduce genes in these stem cells, it is especially important to utilize a system that results in a minimal risk of insertional mutagenesis. To date, only one animal virus, the adeno-associated virus (AAV), is able to integrate into a defined site in human chromosome, AAVS1 (19q13.4), which is mediated by the activity of specific replicase/integrase protein, Rep. The Rep78 or Rep68 protein recognizes the GAGC motif on the viral inverted terminal repeat (ITR) sequence and a similar motif in AAVS1, leading to the site-specific integration of the AAV genome.

We and others have reported that a plasmid transfection system utilizing AAV derived components, the *rep* gene and ITR, could integrate the gene of interest preferentially into AAVS1 in epithelial or adherent cells (e.g., 293, HeLa, Huh-7 cells) [24–26]. Our system uses two plasmids, one harboring the transgene cassette flanked by the ITR sequences, and the other for *rep* expression, allowing only plasmid DNA harboring the ITR to integrate into the AAVS1 locus. In addition, this system can deliver DNA segments larger than the 4.5-kb packaging limit of AAV. As a first step toward establishing a method capable of integrating therapeutic DNA into the AAVS1 locus in MSCs, we tested this strategy in KM-102 cells, a cell line derived from human marrow stromal cells. KM-102 cells were co-transfected with a bicistronic plasmid containing a humanized GFP gene and a blasticidin S resistance gene (*bsr*) between the ITRs and a Rep68 plasmid. After transfection, single cell clones were grown in the presence of

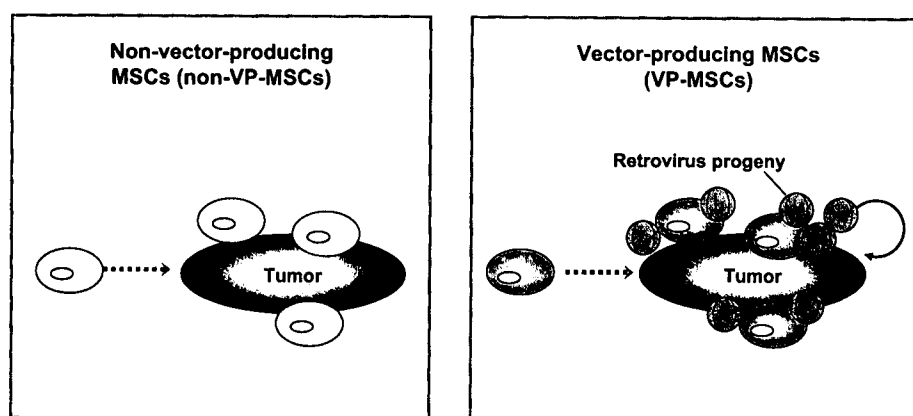


Fig. 3. Development of vector-producing tumor-tracking MSCs to augment suicide cancer gene therapy.

blasticidin S. Southern blot analysis of their genomic DNA revealed that three out of eight blasticidin S resistant clones showed site-specific integration of transgene into the AAVS1 site and that these clones had the GFP gene only at AAVS1. These results indicated that foreign DNA linked with ITR sequence could be targeted specifically into AAVS1 in KM-102 cells.

It is reported that the genome of myosin binding subunit 85 (MBS85) overlaps with the AAVS1 site [27]. To identify the junction between the transgene plasmid and the AAVS1 site, PCR was conducted using a transgene- and an AAVS1-specific primers. In two of the three clones the integration site was identified. In one clone the GFP gene was inserted at the first intron of MBS85 gene. The other clone had insertion of the GFP gene upstream of the first exon. Quantification of mRNA for MBS85 by real time PCR showed that the mRNA level decreased in these two KM-102 clones. The MBS85 is involved in the assembly of actin cytoskeleton. Although the outcome of allelic disruption of the MBS85 genome should be carefully evaluated, the system for AAVS1-specific integration of therapeutic DNA using AAV integration machinery is particularly valuable for *ex vivo* gene therapy applications for stem cells, such as ES cells and MSCs. For additional readings on the use of bone marrow cells for the treatment of autoimmunity, the reader is referred to companion papers published herein in this special issue of the Journal of Autoimmunity [28–38].

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Chapter 2

Targeted Insertion of Transgene into A Specific Site on Chromosome 19 by Using Adeno-Associated Virus Integration Machinery

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Akihiro Kume and Keiya Ozawa†

Site-specific integration of the therapeutic transgene is favorable for gene therapy applications since it minimizes the risk of insertional mutagenesis and thereby prevents target cells from developing tumors. Adeno-associated virus (AAV), a member of parvovirus, is unique in that it integrates its genome into a specific site termed the AAVS1 locus (19q13.4) in the human genome. A non-structural replication initiator protein of AAV, Rep78 or Rep68, binds the inverted terminal repeat (ITR) sequence at either end of the AAV genome via tandem repeats of the GAGC/GCTC motif. A homologous sequence exists at the AAVS1 site. The Rep protein recognizes it and drives the integration of the AAV genome into AAVS1. The ITR is a cis element sufficient for AAVS1-specific integration. The incorporation of the ITR sequence into plasmid DNA is thus discussed in terms of Rep-mediated site-specific integration and of AAVS1 as a hazard-free target for transgene integration. Therefore, the use of the AAV integration machinery should allow us to develop a safer gene delivery system.

Keywords: Site-specific transgene integration; adeno-associated virus; Rep protein; AAVS1 locus.

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1. Introduction

Stem cells such as hematopoietic stem cells, embryonic stem (ES) cells and mesenchymal stem cells (MSCs) are attractive targets for gene therapy since they replicate themselves and differentiate into various cell lineages. To manipulate genes in these cells, it is especially important to utilize a system to introduce therapeutic DNA with a minimal risk of insertional mutagenesis. Insertion of the gene of interest into a defined site in the human chromosome is desirable. However, current strategies that achieve the integration of transgene into host chromosomal DNA insert it randomly, which is an insertional mutagenic/oncogenic hazard as shown with retroviral vectors. It is undoubtedly a milestone in gene therapy that 10 patients with X-linked severe combined immune deficiency, a lethal inherited disease characterized by an early block in T and natural killer lymphocyte differentiation due to mutations of the gene encoding the γ c cytokine receptor subunit, were treated by infusion of autologous CD34⁺ hematopoietic stem cells transduced with a murine retrovirus vector encoding the common γ chain (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2002). Unfortunately T-cell leukemia developed in four patients approximately three years after gene therapy (Hacein-Bey-Abina *et al.*, 2003; Baum, 2007). The vector sequence was integrated into the upstream region or the first intron of the LMO2 gene, which is known to be a T-cell proto-oncogene. The activation of the LMO2 gene by chromosomal translocation has been reported in patients with T-cell acute leukemia (Nam and Rabbitts, 2006). The integrated vector promoter, the long terminal repeat (LTR) enhanced the activity of the LMO2 promoter and resulted in aberrant LMO2 expression and premalignant cell proliferation (Hacein-Bey-Abina *et al.*, 2003). To prevent such an adverse event, it is absolutely necessary to employ a strategy that introduces foreign DNA specifically into a predefined safe region of chromosomal DNA.

Prokaryotic site-specific recombinases such as Cre (Sauer and Henderson, 1988) or Fli (Andrews *et al.*, 1985) are widely used to introduce DNA into a site that their respective recognition sequence in eukaryotic cells as well as prokaryotic cells. However, the recognition sequence must be inserted into target chromosomal DNA in advance. Phage phiC31 integrase recognizing phage *attP* and *attB* sites has been shown to mediate site-specific DNA integration in human genome at native "pseudo" *attP* sites (Thyagarajan *et al.*, 2001).

AAV integrates its genome into a particular site in human chromosome 19, termed AAVS1 (19q13.4) (Kotin *et al.*, 1992; Samulski *et al.*, 1991), through the activity of a specific replicase/integrase protein, Rep. Taking advantage

of the AAV integration machinery, systems for AAVS1-specific integration of therapeutic DNA have been developed (Balague *et al.*, 1997; Surosky *et al.*, 1997). The systems are particularly valuable for *ex vivo* gene therapy applications for stem cells. Here the authors will review the current trend of the development of Rep-mediated AAVS1-targeted integration as well as basic biology of the site-specific integration of AAV.

2. Overview of AAV

The adeno-associated virus (AAV) belongs to the family *Parvoviridae* and is classified into the genus *Dependovirus*. A number of AAV serotypes have been reported so far. The AAV serotype 2 was sequenced more than 20 years ago and has been most extensively studied (for general review, see: Muzyczka, 2001; Smith, 2002). The type 2 is referred here as AAV. The AAV depends for its replication and propagation on a helper virus such as adenovirus, herpes virus, papilloma virus and vaccinia virus. When the AAV infects cells alone, it enters a latent infection phase and integrates into the human genome preferentially into the AAVS1 locus on chromosome 19 (19q14.2) (Kotin *et al.*, 1992; Samulski *et al.*, 1991). The integrated genome can be activated and rescued by subsequent superinfection by a helper virus (Fig. 1). There is no disease reported associated with AAV infection. More than 80% of adults are seropositive for antibodies against the AAV. The AAV is used as a gene-transfer vector particularly for long-term gene expression in livers, neurons and muscles. However, AAV vectors that are devoid of the *rep* gene fail to integrate into AAVS1 (see below for details).

The wild-type AAV has been reported to integrate into the AAVS1 site in immortalized cell lines at a frequency of 68% (Kotin *et al.*, 1990) or 94% (Kearns *et al.*, 1996). The insertion of AAV DNA into the AAVS1 site was also reported in human testis tissue (Mehrlé *et al.*, 2004). The AAV vector genome has also been shown to persist extrachromosomally in cells (Afione *et al.*, 1996; Duan *et al.*, 1998; Nakai *et al.*, 2001). A recent study reported that the AAV genome in humans appears to persist as episomal forms, not as integrated forms; an attempt to detect a junction DNA sequence between the AAV genome and host chromosome by PCR from clinical tonsil-adenoid samples obtained from children was made. The junction sequence, however, could not be amplified, suggesting that the AAV genome existed in the respiratory tissues as an episomal form (Schnepp *et al.*, 2005). Thus, the mode of persistence of the AAV genome may be different among cell types.

The AAV genome is a single-stranded DNA of 4.8 kb in size. Either end of the genome shows a unique T-shaped hairpin configuration, which is

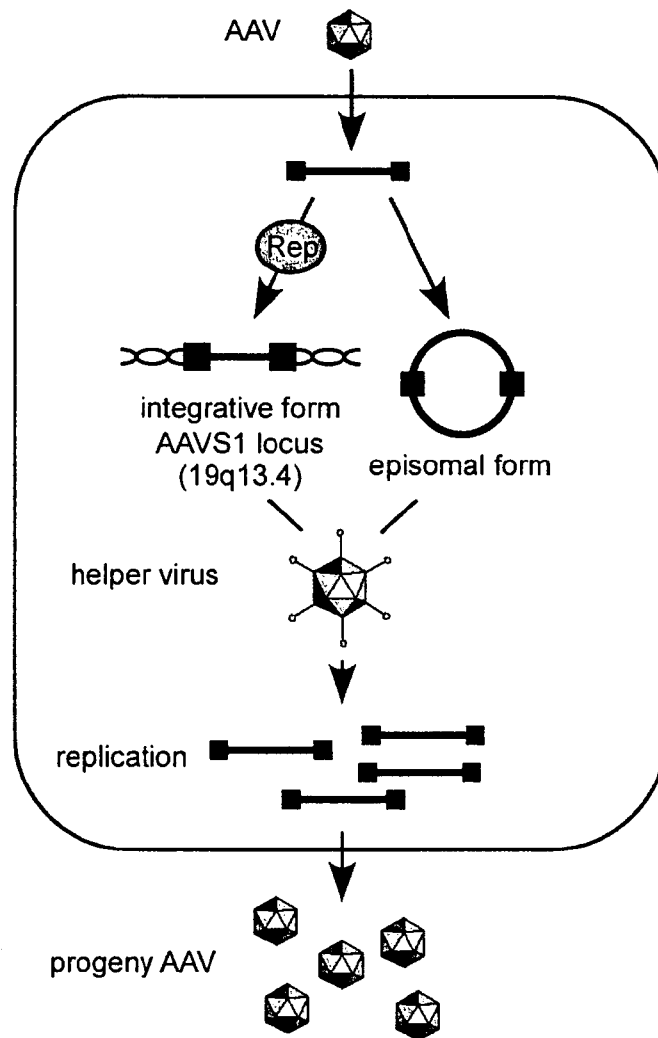


Fig. 1. Life cycle of AAV. Following infection, the AAV genome persists as an integrative or episomal form. The AAV genome preferentially integrates into the AAVS1 site (19q13.4) in the presence of the Rep protein. When AAV and a helper virus (e.g., adenovirus) coinfect cells or a helper virus superinfects the cells harboring the AAV genome, burst replication of AAV occurs.

called an inverted terminal repeat (ITR). The ITR serves as an origin of AAV genome replication. Between the ITRs are two open reading frames corresponding to *rep* and *cap* [Fig. 2(A)]. The *rep* gene encodes four overlapping nonstructural proteins, Rep78, Rep68, Rep52 and Rep40. The *cap* gene codes for structural proteins, VP1, VP2 and VP3. On the genome are three promoters, p5, p19, and p40, designated according to their map positions. The unspliced and spliced transcripts from the p5 promoter encode Rep78 and Rep 68, respectively, while Rep52 and Rep40 are translated from p19-unspliced and -spliced transcripts. Rep78 or Rep68 is a replication initiator of the AAV genome, which possesses site-specific, strand-specific endonuclease activity, ATP-dependent helicase activity (Im and Muzyczka, 1990), and ligase activity (Smith and Kotin, 2000). The Rep78 and Rep68 proteins bind

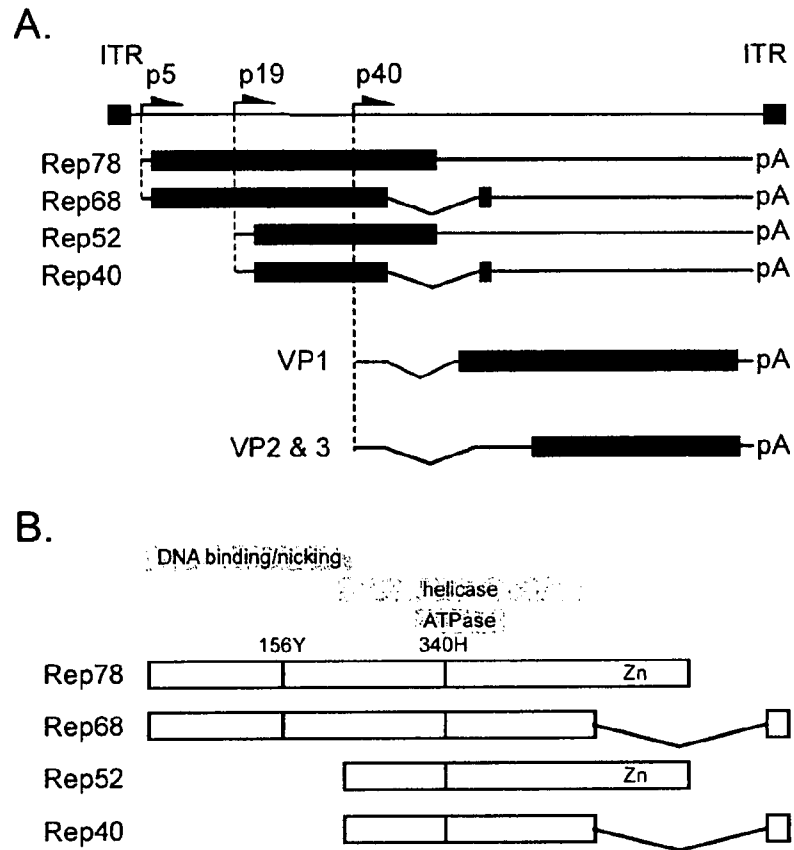


Fig. 2. AAV genome and Rep proteins. **(A)** Map of the AAV genome. Three promoters, p5, p19, and p40 drive transcription of Rep78/68, Rep52/40, and VP proteins, respectively. Spliced transcript from p5 and p19 encode Rep68 and Rep40 proteins, respectively. **(B)** Map of Rep proteins. The domains responsible for DNA binding/nicking, helicase and ATPase domains are indicated. The C-terminal portion of Rep78 or Rep52 comprises a Zn-finger like motif. The tyrosine and histidine residues at position 156 and 340 are indicated, respectively.

the ITR via the Rep binding site (RBS) consisting of five tandem repeats of the GAGC/GCTC tetranucleotide, self-associate to form a hexameric complex (Hickman *et al.*, 2004; Smith *et al.*, 1997), extrude the terminal resolution site (*trs*), thereby forming a stem-loop structure via their helicase activity (Brister and Muzyczka, 1999) and nicking at the *trs* between the thymidine residues. The protrusion of the *trs* is a prerequisite for nicking at this site by the Rep protein. The site-specific nicking event is followed by unwinding of the terminal hairpin. The smaller Rep52 and Rep40 proteins also have a helicase activity (Collaco *et al.*, 2003; Smith and Kotin, 1998) and are involved in the packaging of the AAV genome into viral capsids (King *et al.*, 2001).

A number of mutational studies and the determination of the three-dimensional structure of the Rep protein (Hickman *et al.*, 2002 and 2004) revealed that the N-terminal half of the large Rep polypeptide is responsible for DNA binding and endonuclease activity. The central portion is essential for helicase activity, ATPase activity and multimerization of Rep proteins.

The lysine residue at position 340 associates with ATP. The tyrosine residue at position 156 covalently links to the 5'-end of single stranded DNA, a product derived from the nicking reaction mediated by the Rep protein [Fig. 2(B)]. Charged amino acids that are important for the site-specific integration were identified in the N-terminal half of Rep78 (Urabe *et al.*, 1999).

Either large Rep protein is capable of supporting the replication of the AAV genome (Holscher *et al.*, 1995). However, functional differences between the two have been reported. Rep68 also shows a stronger nicking activity than its counterpart and is more efficient in processing dimers to monomer duplex DNA (Ni *et al.*, 1998 and 1994), which is an intermediate replicative form of the AAV genome. The helicase activity of Rep78 has been reported to be stronger (Wollscheid *et al.*, 1997). Rep78 suppresses CREB-dependent transcription by the interaction of a domain unique to Rep78 with protein kinase A (PKA) (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998) which implies that Rep78 (or Rep52) indirectly inhibits adenoviral nuclear transport by PKA (Suomalainen *et al.*, 2001) and CREB-dependent adenovirus promoters E1A, E2 and E4 (Leza and Hearing, 1989). Rep68, but not Rep78, associates with 14-3-3 proteins through phosphorylated serine at position 535 and its interaction may affect the life cycle of AAV (Han *et al.*, 2004).

3. Mechanism of AAVS1-Targeted Integration of the AAV Genome

There are similar RBS and *trs* sequences in the AAVS1, which correspond to the first exon and intron of the *MBS85* gene (Fig. 3). The Rep protein binds the RBS, which is the first event during the replication and amplification of the AAV genome. The Rep protein has been shown to mediate complex formation between AAV DNA and AAVS1 DNA (Weitzman *et al.*, 1994). It has been reported that the Rep protein asymmetrically amplifies the AAVS1 sequence (Urcelay *et al.*, 1995), suggesting that a similar event observed on the ITR during the replication of the AAV genome takes place on the AAVS1 locus as well. A model of deletion-substitution mechanism has been proposed for the mechanism of the site-specific integration of the AAV genome (Linden *et al.*, 1996a).

The ITR sequence alone appears to enhance the integration into host chromosomal DNA (Lieber *et al.*, 1999; Philip *et al.*, 1994), albeit not site-specifically. The minimal *cis* element for AAVS1-specific integration is controversial. A series of analyses of AAV integration events on an EBV-based episomal vector harboring the human AAVS1 sequence in the HEK293 cell

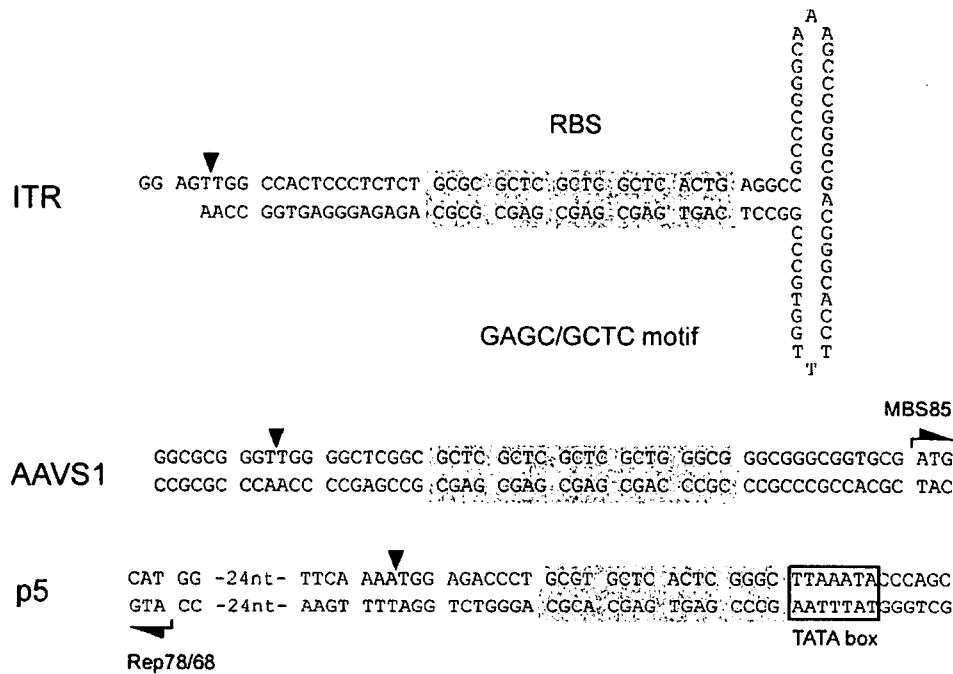


Fig. 3. Comparison of the ITR, AAVS1 and p5 promoter. Terminal resolution site (*trs*) or nicking site is indicated by an arrowhead. A motif consisting of five or four tandem repeats of the GAGC/GCTC tetranucleotide constitutes a Rep binding site (RBS). The initiation codon for myosin binding subunit 85 (MBS85) or for Rep78/68 is indicated by an arrow. The TATA box on the p5 promoter is indicated by a box.

line expressing EBNA-1, revealed that both the RBS and the *trs* sequences are required for AAVS1-targeted integration (Linden *et al.*, 1996a and 1996b). Removal of the sequence upstream of the *trs* appears to result in a decrease in the frequency of AAVS1 specific-integration (Linden *et al.*, 1996a and 1996b). The eight-base spacer sequence between the RBS and *trs* also appears important for site-specific integration, as partial replacement with unrelated nucleotides in the spacer sequence greatly reduces the integration frequency (Meneses *et al.*, 2000). On the other hand, a recent study reported that the RBS motif alone is sufficient for the Rep-mediated AAVS1-specific insertion of a GFP/Neo plasmid into HEK293 cells (Feng *et al.*, 2006). Seven out of 19 (37%) G418-resistant clones were considered to have the GFP/Neo transgene integrated at the AAVS1 site by Southern analysis. The RBS motif within the p5 promoter could direct the GFP/Neo gene into AAVS1 albeit with a lower frequency (2 of 13 clones).

Although the RBS in the ITR is the primary binding site for the Rep protein, a tip of the T-shaped hairpinned structure of the ITR is identified as another Rep association site (Ryan *et al.*, 1996). It has been shown that the affinity of the Rep protein for the RBS in the ITR is higher than for the RBS analog in the p5 promoter, as revealed by electrophoretic mobility shift assay

(Glauser *et al.*, 2005). The ITR thus is the perfect substrate for Rep binding and seems to mediate AAVS1-specific integration most efficiently. The Rep protein is capable of associating with transcription factor Sp1 (Hermonat *et al.*, 1996; Pereira and Muzyczka, 1997) and the TATA binding protein (TBP) (Francois *et al.*, 2005; Hermonat *et al.*, 1998; Su *et al.*, 2000), in both cases binding to the p5 promoter region. The association of Rep with Sp1 and TBP may stabilize the binding of Rep to the p5 element, compensate for Rep's lower affinity for the DNA substrate, and enhance the site-specific integration.

Besides driving transcription of Rep78 and Rep68 mRNA, the p5 promoter sequence also serves as an origin of replication that promotes the amplification of integrated AAV genome (Francois *et al.*, 2005; Nony *et al.*, 2001). The p5 sequence element also enhances the AAVS1-specific integration and is sufficient for the site-specific integration (Philpott *et al.*, 2002a and 2002b). The p5 promoter comprises a RBS and *trs* homolog like the ITR and the AAVS1 site. The Rep protein binds the p5-RBS (Kyostio *et al.*, 1995) and nicks at the *trs* homolog (Francois *et al.*, 2005). The analysis of junction sequences between Rep-mediated integrants and host AAVS1 site revealed that some break points occur at the p5 promoter (Tsunoda *et al.*, 2000), which corroborates the presence of a *cis* element for AAVS1-specific integration.

Cellular factors involved in Rep-mediated integration into AAVS1 have not been fully identified although the Rep protein is a major player. A protein capable of associating with the Rep protein may be involved in the targeted integration. The DNA-dependent protein kinase, which is involved in the repair of double-stranded DNA break and in V(D)J recombination by nonhomologous end-joining, is responsible for the circularization and concatemerization of the AAV genome in cells and inhibits the site-specific integration into host DNA (Song *et al.*, 2004). Recently the TAR RNA-binding protein of 185 kDa (TRP-185), which was identified as a protein binding to the TAR RNA loop of human immunodeficiency virus type 1 (Wu *et al.*, 1991), has been shown to bind to the RBS region. Inhibiting the integration at the RBS of the AAV genome, TRP-185 appears to promote the hexamerisation of the Rep protein and to enhance the integration downstream of the RBS (Yamamoto *et al.*, 2007).

4. AAV Target Site: AAVS1 Locus (19q13.4)

The AAVS1 locus (19q14.2) overlaps with the first exon of the *MBS85* gene encoding myosin binding subunit 85 or protein phosphatase 1 regulatory

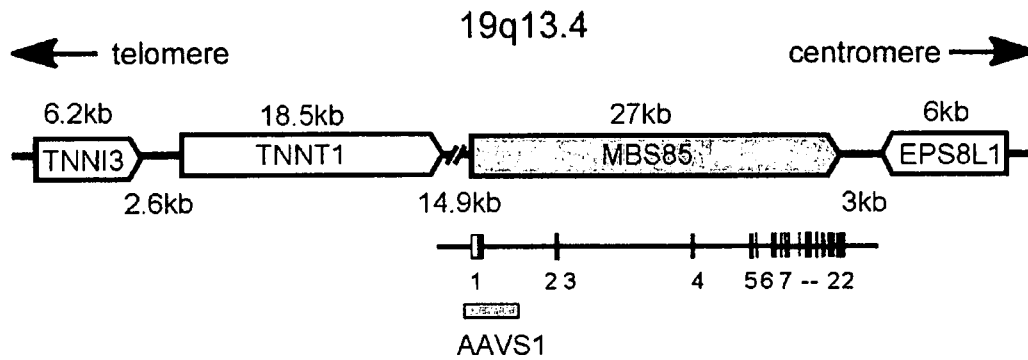


Fig. 4. Location of the AAVS1 site. The AAVS1 site of approximately 2 kb in length overlaps with the first exon and intron of *MBS85*, a hot spot of AAV integration. *TNNI3*, cardiac troponin I; *TNNT1*, slow skeletal troponin T1; *MBS85*, myosin binding subunit 85; *EPS8L1*, epidermal growth factor receptor pathway substrate 8-like protein 1. (Adapted from a figure by Dutheil *et al.* 2004).

subunit 12C (Fig. 4). *MBS85* is one of the effector molecules in Rho signaling pathway and is involved in the assembly of myosin chains (Tan *et al.*, 2001). Rho is a small molecule with GTPase activity (Bishop and Hall, 2000). RhoA associates with a kinase and the kinase phosphorylates *MBS85*. The phosphorylated *MBS85* inhibits the myosin light chain phosphatase and a net increase in myosin light chain phosphorylation activates myosin and finally induces stress fiber formation. A region upstream of the initiation codon for *MBS85* is homologous to the AAV origin of replication, at which are two elements essential for AAV integration: a RBS homolog and a *trs* homolog.

A sequence homologous to human AAVS1 is also found in the simian (Amiss *et al.*, 2003) as well as rodent genome (Dutheil *et al.*, 2004). Integration of the AAV genome into the simian AAVS1 ortholog was identified in a cell line, COS7 (Amiss *et al.*, 2003). Rep68 that are derived from serotype 4 AAV, which was originally isolated from monkey, has a higher affinity for simian RBS than AAV2 Rep68. A survey in genomic banks revealed the presence of an AAVS1 ortholog in the mouse genome too (Dutheil *et al.*, 2004). *In vitro* studies showed that a recombinant type 2 Rep68 can bind to mouse RBS and nick at *trs*, suggesting that the AAV genome would target the mouse AAVS1 locus if the genome is delivered to the nucleus.

The integration of the AAV genome occurs mostly within approximately 2 kb downstream of the RBS (Linden *et al.*, 1996a). The integration of the AAV genome or a transgene into AAVS1 is accompanied by deletion of the target site. After nicking at the *trs*, the Rep protein unwinds target DNA progressively and the DNA with the ITR sequences replaces the existing host DNA, which is contrasted to the microdeletion accompanying the retrovirus-mediated integration event.

5. Multifunctional Rep Protein

The Rep proteins are “sticky” and have been reported to associate with a number of cellular proteins, including TBP (Hermonat *et al.*, 1998; Su *et al.*, 2000); Sp1 (Hermonat *et al.*, 1996; Pereira and Muzyczka, 1997); E2F-1 (Batchu *et al.*, 2001), p53 (Batchu *et al.*, 1999), pRB (Batchu *et al.*, 2002); a topoisomerase I binding protein (Topors) (Weger *et al.*, 2002); protein kinase A (PKA); a protein kinase (PrKX) (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998); transcription-positive cofactor 4 (PC4) (Muramatsu *et al.*, 1998; Weger *et al.*, 1999); 14-3-3 proteins (association with Rep68 via phosphorylated serine at position 535) (Han *et al.*; 2004); UBC9 (E2 conjugating enzyme for the small ubiquitin-related polypeptide SUMO-1) (Weger *et al.*, 2004); TRP-185 (Yamamoto *et al.*, 2007); and high mobility group chromosomal protein 1 (HMG1) (Costello *et al.*, 1997).

The anti-proliferative action of the Rep protein is partially explained by the association of the Rep protein with TBP (Hermonat *et al.*, 1998; Su *et al.*, 2000), which is required for the assembly of the transcription initiation complex. The CREB protein (cAMP responsive element binding protein) is a transcription factor and is activated by PKA, which plays a central role in cell growth and development. Thus, the inhibition of PKA by the Rep protein eventually suppresses the CREB-dependent transcriptional activation (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998). PC4 is involved in the downregulation of the p5, p19, and p40 promoters in the absence of a helper virus (Weger *et al.*, 1999). Coinfection of adenovirus relieves the suppression of the AAV promoters (Weger *et al.*, 1999) and enhances the replication of the AAV genome (Muramatsu *et al.*, 1998). UBC9 is involved in the attachment of small ubiquitin-like modifier (SUMO-1), which protects a protein from ubiquitin-mediated degradation. A prolonged life span of the Rep protein (being protected by UBC9) may establish AAV latency in cells (Weger *et al.*, 2004). The association of adenovirus E1b with p53 induces ubiquitin-mediated degradation of the p53 tumor suppressor gene product and this disturbs the cell cycle pathway (Ciechanover *et al.*, 1994). AAV Rep78 associates with p53 and prevents it from being degraded (Batchu *et al.*, 1999). Topors appears to enhance the expression of Rep78, Rep52 and capsid proteins as a transcriptional regulator in the absence of a helper virus (Weger *et al.*, 2002). The 14-3-3 proteins are associated with a number of cellular proteins and implicated in their modification (Fu *et al.*, 2000). The interaction between Rep68 and 14-3-3 proteins results in reduced DNA binding activity of Rep68. However, the significance of the interaction has to be elucidated (Han *et al.*, 2004). A transcription factor E2F-1 is suppressed by its interaction

with the retinoblastoma protein (pRB). Rep78 associates with E2F-1 and stabilizes the E2F-1-pRB complex (Batchu *et al.*, 2002). The Rep protein has been reported to interact with Sp1 and to inhibit a promoter bearing an Sp1 binding motif (Hermonat *et al.*, 1996). The Rep protein also binds to the E2 transactivator of human papilloma virus (HPV) type 16, disrupts the binding of E2 to the cellular transcriptional coactivator p300, and inhibits the replication and transforming activities of HPV (Marcello *et al.*, 2000).

Although the consensus Rep binding site on the ITRs is a five tandem repeat of the GAGC/GCTC tetranucleotide, the Rep proteins can bind to imperfect RBS motifs (Chiorini *et al.*, 1995). Within the AAV p5, p19, and p40 promoter regions, incomplete RBS motifs were identified and the Rep protein has been shown to bind there (Kyostio *et al.*, 1995). The Rep protein regulates the AAV p5, p19, and p40 promoter activity. In latent infection, the large Rep protein suppresses the p5 promoter, which is suitable for persistence of the AAV genome. In the presence of adenovirus, the large Rep protein trans-activates the p19 and p40 promoters.

Systematic *in vitro* studies indicated that the Rep protein can bind to many cellular promoter regions, including the *c-sis* proto-oncogene, glucose transporter, and KIP2 (cyclin-dependent kinase inhibitor) (Wonderling and Owens, 1996 and 1997). The Rep polypeptides have been shown to down-regulate the promoters of a number of genes such as *c-H-ras*, *c-fos*, *c-myc* (Batchu *et al.*, 1994; Hermonat, 1991 and 1994); *c-sis* (Wonderling and Owens, 1996), E2F-1 (Batchu *et al.*, 2001); and the LTR promoter of the human immunodeficiency virus 1 (Batchu and Hermonat, 1995; Oelze *et al.*, 1994). A detailed study showed that Rep suppresses the HPV type 16 p97 promoter by inhibiting the binding of TBP to the p97 promoter (Su *et al.*, 2000) or by directly binding the p97 promoter region (Zhan *et al.*, 1999). While most promoter activities are down-regulated by Rep78 or Rep68, some promoters, such as the human cytomegalovirus (CMV) immediate early promoter (Wonderling *et al.*, 1997) and the *c-sis* promoter (Wonderling and Owens, 1996) are trans-activated. The Rep protein also suppresses translation (Takeuchi *et al.*, 2000).

AAV inhibits the replication of viruses (Bantel-Schaal and zur Hausen, 1988) and cellular transformation by SV40 or Ad E1a plus *ras* oncogene (Khleif *et al.*, 1991). Establishment of cellular transformants by plasmid transfection was also inhibited (Labow *et al.*, 1987). A cellular factor induced by AAV infection that suppresses cell proliferation has been reported (Bantel-Schaal, 2001). It is reported that AAV infection results in an increase in p21 level, a cyclin-dependent kinase inhibitor (Hermanns *et al.*, 1997). Rep68 and Rep78 induce cell cycle arrest in G1 and G2 phases and, in addition,

Rep78 inhibits S-phase progression by accumulating active hypophosphorylated pRb (Saudan *et al.*, 2000), a negative regulator of transcription factors (Weinberg, 1995). Infection with AAV suppresses indirectly the HPV18 promoter activity in transgenic mice (Walz *et al.*, 2002). The Rep protein has been reported to suppress the expression of *c-myc*, *c-myb*, and pRb whereas it up-regulates *c-fos* expression (Klein-Bauernschmitt *et al.*, 1992).

6. The use of the AAV Integration Machinery to Achieve Site-Specific Integration

Since the elements required for AAVS1-specific integration of AAV are Rep78 or Rep68, and the ITR sequences, it is possible to insert any DNA sequence linked to an ITR sequence into AAVS1 if the Rep78 or Rep68 protein is expressed simultaneously. Early studies demonstrated that a reporter gene was efficiently inserted into the AAVS1 site in cultured cells such as HeLa cells and HEK293 cells, although the frequency of the site-specific integration differed (Balague *et al.*, 1997; Shelling and Smith, 1994; Surosky *et al.*, 1997). Table 1 summarizes the studies of AAVS1-targeted integration in cultured cells.

Shelling and Smith inserted a *Neo* gene downstream of the p40 promoter, the original promoter for capsid protein expression (Shelling and Smith, 1994). The plasmid construct harbored the coding sequence for the p5-, p19-*rep* genes and a p40-driven *Neo* gene flanked by the ITRs (Tratschin *et al.*, 1985). Since the open reading frames (ORFs) of *rep78* and *rep68* extend over the p40 promoter, the Rep proteins expressed from this plasmid were C-terminally truncated. After transfecting it into HeLa cells and HEK293 cells, they analyzed G418-resistant clones by Southern blot and showed that nine out of 12 clones had the *Neo* gene integrated in AAVS1. They also generated an AAV vector with the same *rep* and *Neo* genes. The AAVS1-specific integration efficiency was 82% (nine out of 11 clones).

Balague *et al.* adopted a similar strategy, but the difference is that a *rep78* gene was placed outside the ITR-flanked *GFP* transgene portion on the plasmid. One day after transfecting HEK293 cells, they sorted fluorescent cells and plated them at 1 cell per well, thereby isolating single-cell clones without selection for stable transgene expression analysis. Forty five percent (103 out of 227) of the clones were GFP-positive as compared to eight out of 167 with control *rep78*-free plasmid. Southern analysis showed that in five out of nine (55%) clones integration of the *GFP* gene was into AAVS1. The results indicated that Rep78 enhances the chromosomal integration of an ITR plasmid both site-specifically and randomly (Balague *et al.*, 1997).

Table 1 Studies of AAVS1-targeted Integration in Cultured Cells

Authors	Year	Cells	Transfection ¹ (n Plasmid ²)	Rep Expression: Promoter (Protein)	Transgene (cis Element)	AASV1 / Total ³ (% Targeted)	Analysis of Integration
Shelling <i>et al.</i>	1994	HeLa/293	C (1)	p5 (Rep78/68)	Neo (ITR)	9/12 (75)	Southern
Balagué <i>et al.</i>	1997	293	C (1)	p5 (Rep78)	Neo (ITR)	5/9 (45)	Southern
Surosky <i>et al.</i>	1997	293	C (2)	RSV (Rep78/68)	LacZ (ITR)	6/7 (86)	Southern/FISH
Pieroni <i>et al.</i>	1998	HeLa Huh-7	C (1) L (1)	p5 (Rep78/68) p5 (Rep78/68)	GFP/Neo (ITR) GFP/Neo (ITR)	15/100 (15) 5/55 (9)	Southern/FISH Southern/PCR
Lamartina <i>et al.</i>	1998	HeLa HeLa HeLa	L (1) L (1) L (1)	CMV (Rep78) CMV (Rep68) Rep68 protein ⁴	Neo (ITR) Neo (ITR) Neo (ITR)	9/37 (24) 8/37 (22) N.A. ⁵	Southern Southern PCR
Tsunoda <i>et al.</i>	2000	HeLa	L (1)	p5 (Rep78/68)	Neo (ITR/p5)	22/36 (61)	Southern/PCR
Rinaudo <i>et al.</i>	2000	HeLa	C (2)	CMV (Rep78/68-PR ⁶)	Neo (ITR)	7/28 (25)	Southern
Kogure <i>et al.</i>	2001	K562 K562	L (2) E (2)	MMTV/IRES (Rep78) CMV (Rep78)	Neo (ITR) Neo (ITR)	6/17 (22) 8/25 (32)	Southern/FISH Southern/FISH
Hutner <i>et al.</i>	2003	HeLa	C (rAAV ⁷)	p5 (Rep78/68)	GFP/Hygro (ITR)	7/10 (70)	Southern/FISH
Urabe <i>et al.</i>	2003	293 293	C (2) C (2)	CMV (Rep78) CMV (Rep68)	Neo (ITR) Neo (ITR)	4/17 (24) 8/20 (40)	Southern/FISH Southern/FISH
Philpott <i>et al.</i>	2004	HeLa HeLa HeLa	E (1) E (1) E (2)	p5 (Rep78/68) p5 (Rep78/68) T7 (Rep78/68)	GFP (ITR/p5) GFP (p5) CAT (p5)	82/95 (86) 44/47 (94) 9/10 (90)	Southern Southern Southern
Wong, Jr. <i>et al.</i>	2006	293	C (rAAV2 ⁷)	CMV (Rep78/68-VP22 ⁸)	SEAP (ITR)	N.A.	PCR
Feng <i>et al.</i>	2006	293	C (2)	p5 (Rep78/Rep68)	GFP/Neo (RBS)	7/19 (37)	Southern/PCR

¹method used : C, calcium phosphate method; L, lipofection; E, electroporation.

²number of plasmids used for transfection. "1" indicates that a *rep* gene and a transgene cassette were on the same plasmid. "2" indicates that a *rep* plasmid and a transgene plasmid were cotransfected.

³number of clones with AAVS-targeted integration / total number of clones analyzed.

⁴purified recombinant Rep68 protein was transfected instead of a Rep68 expression cassette.

⁵N.A., not available.

⁶PR (ligand binding domain of the progesterone receptor) was fused to Rep78/68.

⁷AAV vector transduction was used instead of plasmid transfection.

⁸VP22 (a tegument protein of herpes simplex virus) was fused to Rep78/68.

In order to reduce the frequency of the chromosomal integration of the *rep* gene, Surosky *et al.* used a two-plasmid system: one plasmid is for the expression of the Rep protein and the other is an ITR-linked transgene plasmid (Surosky *et al.*, 1997). The idea is that the frequency of integration of a *rep* plasmid is lower than that of an ITR-plasmid when the Rep protein is supplied *in trans*. They concluded that six out of seven LacZ-positive colonies had the LacZ transgene into the AAVS1 site as revealed by Southern blot analysis. Fluorescent *in situ* hybridization (FISH) confirmed that two out of six clones had a LacZ signal on chromosome 19. They also showed that Rep78 or Rep68 alone was sufficient for promoting AAVS1-specific integration and that one ITR sequence could target integration to the AAVS1 site. The full sequence of one AAVS1-integrand was determined and it was thus shown that the whole plasmid was integrated at AAVS1 in tandem array.

The work by Pieroni *et al.* described the transfection of HeLa cells or Huh-7 cells with a plasmid harboring the *p5-rep* gene and an ITR-flanked GFP/Neo cassette, with the resulting generation of clones with the transgene integrated in AAVS1 (7 to 25%), which were then analyzed by Southern blot. AAVS1-specific integration was confirmed in one Huh-7 clone by PCR amplification of the ITR-AAVS1 junction sequence and in three HeLa cell clones by FISH colocalization of the GFP/Neo and AAVS1 signals (Pieroni *et al.*, 1998).

By using an AAV vector plasmid similar to that of Shelling and Smith, Tsunoda *et al.* tested site-specific integration in HeLa cells (Tsunoda *et al.*, 2000). In addition, their plasmid had a hygromycin resistance gene beside the ITR-flanked *p5-rep* and *Neo* gene cassette. Southern blot analysis showed that 22 out of 36 (61%) clones had the *GFP/Neo* gene in AAVS1. The junction sequence between the ITR and AAVS1 was amplified by PCR and this showed that the junction occurred in the p5 promoter region. A RBS homolog and *trs* homolog exist in the p5 promoter region, where Rep78 or Rep68 binds and regulates the p5 promoter activity. The Rep-mediated amplification of the AAV genome occurs via the RBS homolog (Nony *et al.*, 2001; Tessier *et al.*, 2001; Tullis and Shenk, 2000), which is important for efficient production of AAV progeny. In addition, the p5 element has been shown to be sufficient for AAVS1-specific integration (Philpott *et al.*, 2002a and 2002b). All the junction sequences amplified had a partially deleted p5 portion, not an ITR sequence (Tsunoda *et al.*, 2000). This is probably partially because cell survival depends on the inactivation of the *rep* gene. The Rep protein is cytotoxic and anti-proliferative (see above). Tsunoda *et al.* also speculated that the plasmid backbone was also simultaneously inserted into AAVS1 as

well as the ITR-flanked *rep/Neo* cassette, an observation consistent with the result of Surosky *et al.* (Surosky *et al.*, 1997).

Kogure *et al.* applied the AAVS1-targeting system to K562 cells, a hematopoietic cell line (Kogure *et al.*, 2001). To limit the expression of Rep78, they used a weak promoter derived from the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) driving bicistronic expression of GFP and Rep78. They transfected the *rep* plasmid and an ITR-Neo plasmid into K562 cells, analyzed 17 clones by Southern blot and found that six clones (22%) harbored the *Neo* gene in AAVS1. FISH analysis confirmed that five out of six clones had a *Neo* gene on chromosome 19.

Urabe *et al.* used a CMV-driven *rep78* gene plasmid for AAVS1-specific integration in HEK 293 cells (Urabe *et al.*, 2003). To regulate the expression level of Rep78, they used decreasing amounts of *rep78* plasmid. They observed that approximately 20% of the clones analyzed by Southern blot showed integration of the *Neo* gene into AAVS1. They also compared the efficiency of targeted integration mediated by Rep78 and Rep68, and found that Rep68 was superior to Rep78 in their experiment. Southern analysis of G418-resistant clones obtained from transfection with the *rep68* plasmid showed that eight out of 20 clones (40%) had the integration of the *Neo* gene into AAVS1. The presence of the *Neo* gene on chromosome 19 was confirmed in all six clones.

Philpott *et al.* utilized the p5 promoter instead of the ITR as a *cis* element for AAVS1-specific integration and successfully introduced the *GFP* gene into AAVS1 at a frequency of 94% (out of 47 HeLa cell clones), a result comparable to data obtained with an ITR-plasmid (86%) (Philpott *et al.*, 2004). They observed that clones obtained by transfection of a plasmid harboring both a *rep* cassette and a *GFP* cassette showed a gradual decrease in the transgene expression over 18 weeks, while clones obtained from transfection with two plasmids (one for Rep expression, and the other for transgene delivery) stably expressed the transgene over time.

Recombinant AAV vector is widely used as a gene transfer vector and is being evaluated for some human applications including coagulation factor IX deficiency, lipoprotein lipase deficiency, and Parkinson's disease. Conventional AAV vectors harbor the gene of interest flanked by the ITRs and are thus devoid of the *rep* gene. Since the *rep* gene product is essential to AAVS1-specific integration, these AAV vectors are not able to preferentially integrate into AAVS1. To achieve the AAVS1-specific integration of the AAV vector genome, Huttner *et al.* infected HeLa cells with an AAV vector after

transfection with a Rep-expression plasmid and reported that seven out of 10 clones showed site-specific integration of the AAV vector genome (Huttner *et al.*, 2003). Wong, Jr. *et al.* also performed Rep-mediated insertion of rAAV by using a fusion protein consisting of Rep and VP22, a tegument protein of herpes simplex virus (Wong *et al.*, 2006). VP22 traffics intercellularly and spreads over adjacent cells (Elliott and O'Hare, 1997). The Rep protein has a nuclear localization signal (NLS) sequence, which inhibits the spread of the Rep-VP22 fusion protein. Removal of the NLS allowed the fusion protein to traffic intercellularly. The advantage of this strategy is that it can be applied to cells easy to transduce with AAV vectors and hard to transfect with plasmid DNA.

7. How to Regulate Rep Expression

The Rep protein affects numerous cellular phenomena as mentioned above. In addition, infection with wild-type AAV or Rep-directed transgene insertion into AAVS1 appears to accompany the disruption and rearrangement of the AAVS1 locus (Balague *et al.*, 1997; Hamilton *et al.*, 2004; Kotin *et al.*, 1990; Shelling and Smith, 1994; Urabe *et al.*, 2003). Thus, it is necessary to regulate the expression level of the Rep protein to as low as possible for applications aimed at AAVS1-specific integration.

The regulation of Rep protein expression at the DNA level includes the bacterial Cre/*loxP* system (Sauer and Henderson, 1988) and the yeast FLP system (O'Gorman *et al.*, 1991). The former was applied to the regulation of the Rep protein (Sato *et al.*, 2000). The p5 promoter was moved downstream of the *rep* ORF on a plasmid and a *loxP* sequence was placed upstream of the *rep* ORF and another was placed downstream of the p5 promoter. Cre removes a stuffer sequence between the two *loxP* sites and the *rep* cassette is circularized such that the p5 promoter is placed just upstream of the *rep* ORF. Co-transfection of HEK293 cells was performed with a Rep-expression plasmid, a Cre expression plasmid, and an AAV vector plasmid on which a *Neo* gene and an expression cassette for a secreted form of alkaline phosphatase (SEAP) were placed between the ITRs. Only by the use of a *Cre-expression* plasmid could they obtain G418-resistant clones where the *Neo/SEAP* gene was targeted into the AAVS1 locus.

Regulation of the *rep* gene at the transcription level includes the use of a weak promoter. The bacteriophage T7 promoter (Recchia *et al.*, 1999) and the combination of the mouse mammary tumor virus (MMTV) LTR promoter and internal ribosome entry site (IRES) sequence of the encephalomyocarditis virus (Kogure *et al.*, 2001) have been used for Rep expression. The