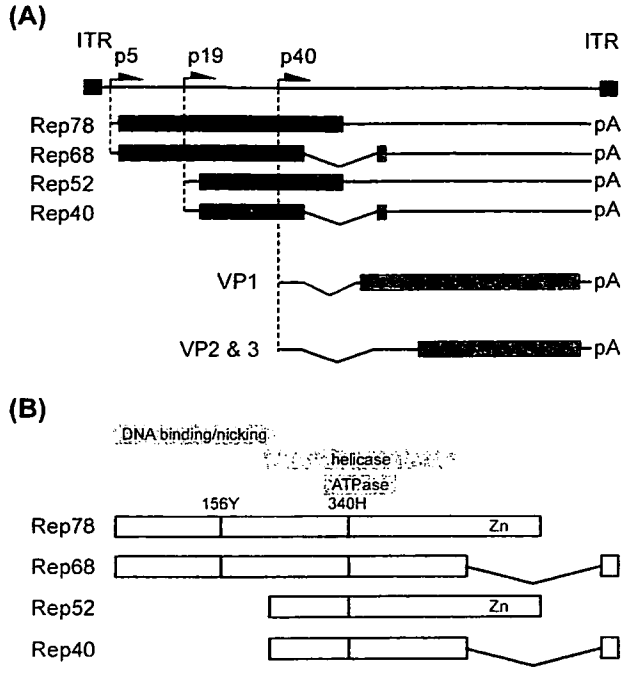

Fig. 2. AAV genome and Rep proteins. **(A)** Map of the AAV genome. Three promoters, p5, p19, and p40 drive transcription of Rep78/68, Rep52/40, and VP proteins, respectively. Spliced transcript from p5 and p19 encode Rep68 and Rep40 proteins, respectively. **(B)** Map of Rep proteins. The domains responsible for DNA binding/nicking, helicase and ATPase domains are indicated. The C-terminal portion of Rep 78 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 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

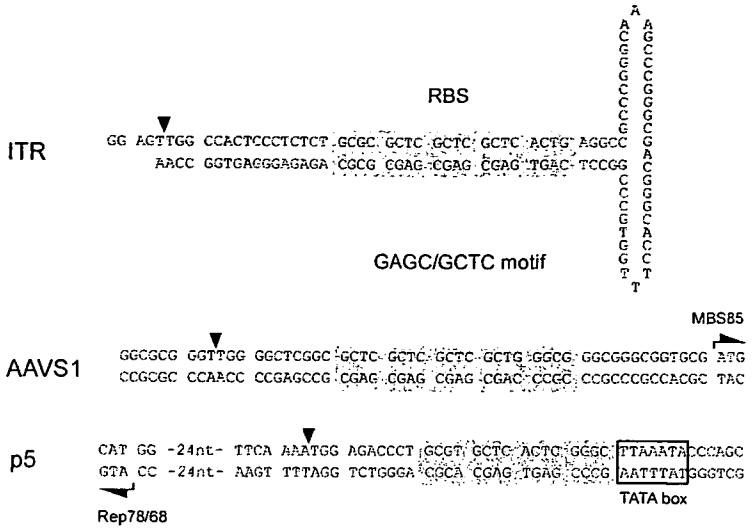

Fig. 3. Comparison of the ITR, AAVS1 and p5 promoter. Terminal resolution site (*trs*) or nicking site is indicated by an arrowhead. A motif consisting of five or four tandem repeats of the GAGC/GCTC tetranucleotide constitutes a Rep binding site (RBS). The initiation codon for myosin binding subunit 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 hexamerization 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 (19q13.4) overlaps with the first exon of the *MBS85* gene encoding myosin binding subunit 85 or protein phosphatase 1 regulatory

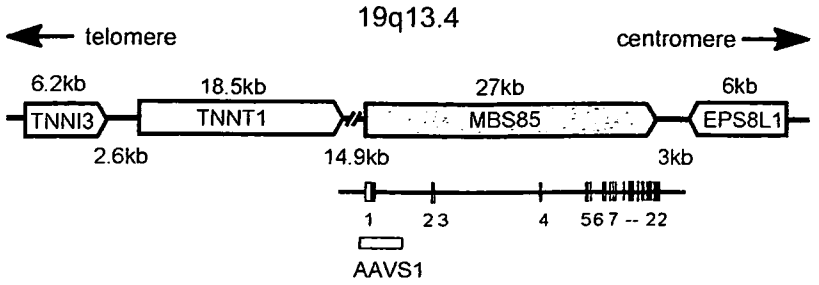

Fig. 4. Location of the AAVS1 site. The AAVS1 site of approximately 2 kb in length overlaps with the first exon and intron of *MBS85*, a hot spot of AAV integration. TNNI3, cardiac troponin I; TNNT1, slow skeletal troponin T1; MBS85, myosin binding subunit 85; EPS8L1, epidermal growth factor receptor 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 phosphrylated 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 inhibiton 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 degradated (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 binds 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 *et al.*	1994	HeLa/293	C (1)	p5 (Rep78/68)	Neo (ITR)	9/12 (75)	Southern
Balagué *et al.*	1997	293	C (1)	p5 (Rep78)	Neo (ITR)	5/9 (45)	Southern
Surosky *et al.*	1997	293	C (2)	RSV (Rep78/68)	LacZ (ITR)	6/7 (86)	Southern/FISH
Pieroni *et al.*	1998	HeLa	C (1)	p5 (Rep78/68)	GFP/Neo (ITR)	15/100 (15)	Southern/FISH
		Huh-7	L (1)	p5 (Rep78/68)	GFP/Neo (ITR)	5/55 (9)	Southern/PCR
Lamartina *et al.*	1998	HeLa	L (1)	CMV (Rep78)	Neo (ITR)	9/37 (24)	Southern
		HeLa	L (1)	CMV (Rep68)	Neo (ITR)	8/37 (22)	Southern
		HeLa	L (1)	Rep68 protein[4]	Neo (ITR)	N.A.[5]	PCR
Tsunoda *et al.*	2000	HeLa	L (1)	p5 (Rep78/68)	Neo (ITR/p5)	22/36 (61)	Southern/PCR
Rinaudo *et al.*	2000	HeLa	C (2)	CMV (Rep78/68-PR[6])	Neo (ITR)	7/28 (25)	Southern
Kogure *et al.*	2001	K562	L (2)	MMTV/IRES (Rep78)	Neo (ITR)	6/17 (22)	Southern/FISH
		K562	E (2)	CMV (Rep78)	Neo (ITR)	8/25 (32)	Southern/FISH
Huttner *et al.*	2003	HeLa	C (rAAV[7])	p5 (Rep78/68)	GFP/Hygro (ITR)	7/10 (70)	Southern/FISH
Urabe *et al.*	2003	293	C (2)	CMV (Rep78)	Neo (ITR)	4/17 (24)	Southern/FISH
		293	C (2)	CMV (Rep68)	Neo (ITR)	8/20 (40)	Southern/FISH
Philpott *et al.*	2004	HeLa	E (1)	p5 (Rep78/68)	GFP (ITR/p5)	82/95 (86)	Southern
		HeLa	E (1)	p5 (Rep78/68)	GFP (p5)	44/47 (94)	Southern
		HeLa	E (2)	T7 (Rep78/68)	CAT (p5)	9/10 (90)	Southern
Wong, Jr. *et al.*	2006	293	C (rAAV2[7])	CMV (Rep78/68-VP22[8])	SEAP (ITR)	N.A.	PCR
Feng *et al.*	2006	293	C (2)	p5 (Rep78/Rep68)	GFP/Neo (RBS)	7/19 (37)	Southern/PCR

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

[2]Number 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.

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

[4]Purified recombinant Rep68 protein was transfected instead of a Rep68 expression cassette.

[5]N.A., not available.

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

[7]AAV vector transduction was used instead of plasmid transfection.

[8]VP22 (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-integrant 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 colocalizaiton 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 (Satoh *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 native p5 promoter is weak enough for Rep expression. However, since the p5 promoter encompasses an imperfect RBS and is thus a *cis* element of AAVS1-specific integration (see above), it is desirable to avoid using the p5 promoter for Rep expression in order to minimize the frequency of *rep* gene integration.

In general, RNA is more labile than DNA. Transfection of RNA encoding Rep proteins may thus reduce the prolonged cytostatic effects of the Rep protein. The delivery of the Rep protein may limit the duration of Rep cytostatic action even more. Lamartina *et al.* transfected HeLa cells with a mixture of a recombinant Rep protein and an ITR-flanked plasmid by the use of a lipid reagent (Lamartina *et al.*, 1998). The analysis of pooled transfected cells by PCR showed that AAVS1 site-specific integration occured in many cells. Three junctions were sequenced and confirmed the site-specific integration.

Regulation of a protein function by a molecular switch is an attractive tool for Rep protein. Rinaudo *et al.* developed a chimeric protein between Rep and the truncated form of the ligand binding domain (LBD) of the progesterone receptor (Rinaudo *et al.*, 2000). The progesterone receptor (PR) associates with heat shock proteins hsp70, hsp90, and several co-chaperone proteins via its LBD. Binding of progesterone promotes conformational changes in PR, resulting in its release from the chaperone complex, and then its nuclear transport (McKenna *et al.*, 1999). The truncated LBD does not bind endogenous progesterone but a synthetic antagonist, RU486 (Rinaudo *et al.*, 2000). In the absence of RU486, C-terminally truncated Rep (residue 1-491) fused to the LBD is predominantly in the cytoplasm, whereas in the presence of RU485, the fusion protein moves into the nucleus. Following cotransfection of HeLa cells with a Rep-LBD expression plasmid and an ITR-flanked *Neo* plasmid and a 24-hour treatment with RU486, they obtained G418-resistant clones. Southern analysis showed that seven out of 28 clones harbored the *Neo* gene in the AAVS1 site. In addition, generation of AAVS1 rearrangement without insertion of the *Neo* gene was markedly reduced. Another regulation system for the Rep protein was reported. The Rep protein functions as a hexameric complex. The Rep domain responsible for DNA binding and nicking fused to a protein that multimerizes can target an ITR-linked DNA into AAVS1. Oligomerization of Rep molecules on the RBS is a prerequisite for Rep enzymatic activities, including nicking, helicase, and ATPase activities. The N-terminal two thirds portion (1–224) of the Rep protein is able to target site-specific integration when it is fused C-terminally to artificial multimerizing proteins (Cathomen *et al.*, 2000).

8. Vehicles to Deliver the Rep Gene into Cells

A number of strategies have been developed to incorporate DNA into target cells. Plasmid transfection with the calcium phosphate precipitation method or with lipofection is the simplest way to introduce DNA as well as electroporation. For "hard-to-transfect" cells, a viral vector is the second choice.

The Rep proteins are cytostatic as mentioned above and transient or regulated expression of the Rep protein is favored. A number of attempts have been made to create an adenoviral vector harboring the *rep* gene, which turned to be unsuccessful due to low yields and instability of recombinant *rep*-Ad vectors. AAV Rep proteins inhibit the replication of adenovirus at different steps. The Rep78 and Rep68 proteins associate with the single-stranded DNA binding protein, an E2A gene product of adenovirus (Stracker *et al.*, 2004). Rep68 has been shown to bind the E2a promoter region (Casper *et al.*, 2005) and suppresses transcription (Casper *et al.*, 2005; Jing *et al.*, 2001; Nada and Trempe, 2002). In addition Rep78 represses E1a, E2a, E4 promoter activity, but trans-activates E1b and E3 promoters. By contrast, in the presence of E1a protein, Rep78 repressed all the promoters (Jing *et al.*, 2001). Rep78 and Rep68 inhibit the transcription from the Ad major late promoter by the association with the TATA-box binding protein and binding to sites adjacent to the TATA box (Needham *et al.*, 2006). The regulation of *rep* gene expression by the Cre/*LoxP* system (Ueno *et al.*, 2000) or the tetracycline inducible system (Recchia *et al.*, 2004) succeeded in generating an Ad vector with the *rep* gene. In addition, a promoter derived from bacteriophage T7 (Recchia *et al.*, 1999) and the locus control region (LCR) of the human β-globin gene (Wang and Lieber, 2006), which functions very weakly in mammalian cells, have been shown to drive expression levels of the Rep protein compatible with Ad vector production.

Another viral vector tested for incorporation of the Rep expression cassette is herpes simplex virus (HSV) vector. The details are presented by Fraefel *et al.* in the following chapter. HSV is also a helper virus for AAV and support AAV replication. HSV appears to tolerate the anti-viral effect of the Rep protein more than the adenovirus. A p5 promoter-driven Rep cassette could be successfully packaged into an HSV mini-amplicon vector although the titer of the recombinant HSVs was low. By using a hybrid amplicon vector with a *rep* gene and a transgene cassette between the ITR sequences, AAVS1-targeted integration of the transgene was achieved, which was confirmed by amplification of junction sequences in HEK293 cells (Heister *et al.*, 2002) or by Southern blot and FISH analysis in fibroblasts obtained from transgenice mice bearing human AAVS1 sequence (Bakowska *et al.*, 2003).

Baculovirus, an invertebrate virus that is widely used for the production of recombinant proteins is also able to harbor the *rep* gene and to mediate AAVS1-specific integration (Palombo *et al.*, 1998). It was reported that the *rep* gene and the ITRs were stable in the baculovirus genome and that titers of *rep*-baculovirus were comparable to wild-type ones (Urabe *et al.*, 2002).

9. Future Direction

Insertion of foreign DNA into a specific chromosome at a predetermined site will become a prerequisite for human gene manipulation in the future. AAV offers an attractive tool to achieve site-specific integration. Currently it is impossible to insert transgene into AAVS1 in all transfected cells. *Ex vivo* gene therapy is a practical strategy to apply for AAVS1-targeted integration since a cell clone that harbors the therapeutic transgene at the AAVS1 site can be selected and expanded for use.

Some challenges to develop better AAVS1-targeted integration systems include: 1) increasing the frequency of AAVS1-specific integration; 2) decreasing the frequency of disruption of non-AAVS1 sites; and 3) reducing the cytotoxicity of the Rep protein. Better understanding of AAV biology will help us to refine the system.

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