Positive and negative effects of adeno-associated virus Rep on AAVS1-targeted integration

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Adeno-associated virus type 2 integrates preferentially into the AAVS1 locus on chromosome 19 of the human genome. It was reported previously that transfection with two plasmids, one for Rep and the other carrying a transgene flanked by inverted terminal repeats (ITRs), enables preferential integration of the latter into AAVS1. Aiming at increasing the frequency of AAVS1-specific integration, the Rep- to transgene-plasmid ratio necessary to achieve a higher frequency of site-specific integration was examined. 293 cells were co-transfected with the Rep78 plasmid and an ITR-flanked Neo gene at different ratios. G418-resistant clones were selected randomly. Extensive Southern blot analysis showed an optimum range of Rep78 expression. In that range, approximately 20% of clones harboured the Neo gene at AAVS1. Excess Rep expression, however, resulted in ‘abortive’ integration of the Neo gene at AAVS1. Excess Rep expression, however, resulted in ‘abortive’ integration of the Neo gene at AAVS1. Aberrant Rep78 appeared to cause abortive integration more extensively than Rep68. Deleterious effects of the Rep protein on the AAVS1 locus should be considered to develop an improved AAVS1-targeted system.

Retrovirus vectors are used widely for gene therapy applications. However, the random integration of retrovirus vector sequences may cause insertional mutagenesis, and the accidental activation of proto-oncogenes cannot be prevented. Adeno-associated virus type 2 (AAV) is a non-pathogenic parvovirus being considered as a gene transfer vehicle (Berns & Giraud, 1996; Kotin, 1994; Muzyczka, 1992). The AAV genome, a linear single-stranded DNA of 4.7 kb long, integrates preferentially into a defined locus in the human genome, AAVS1, on chromosome 19 (19q13-3qter) (Kotin et al., 1990, 1992; Samulski et al., 1991). AAV can provide a potentially ideal gene delivery system for site-specific integration.

Each end of the AAV genome consists of inverted terminal repeats (ITRs), which are required in cis for AAVS1-specific integration. The AAV rep gene encodes four overlapping non-structural proteins, Rep78, Rep68, Rep52 and Rep40, while the cap gene encodes structural Cap proteins. The unspliced and spliced transcripts from the p5 promoter encode Rep78 and Rep68. Either Rep78 or Rep68 plays a key role in AAVS1-specific integration, binding ITRs (Im & Muzyczka, 1989) and AAVS1 (Weitzman et al., 1994) via tandem repeats of the GAGC tetramer (McCarty et al., 1994). The mechanism of AAVS1-specific integration of AAV has not been elucidated fully. However, a model whereby integration proceeds via a circular intermediate of the AAV genome by a deletion-substitution mechanism has been proposed (Dyall & Berns, 1998; Linden et al., 1996).

A structural difference between Rep78 and Rep68 is that Rep78 possesses a zinc finger-like motif at its carboxyl terminus. Both Rep proteins share essentially the same functions: strand-specific DNA binding (Im & Muzyczka, 1989), site-specific nicking and ATP-dependent helicase activity (Im & Muzyczka, 1990). Either Rep protein alone is sufficient for replication of the AAV genome (Hölscher et al., 1994) and for AAVS1-specific integration (Surosky et al., 1997). The multifunctional Rep proteins inhibit cellular transformation by heterologous genes (Labow et al., 1987; Yang et al., 1992) and suppress heterologous promoters, including the c-fos, c-myc, H-ras and LTR of human immunodeficiency virus type 1 (HIV-1) (Hermonat, 1991, 1994; Oelze et al., 1994). The Rep proteins also modulate cell cycle-regulating proteins (Hermanns et al., 1997). These results indicate that overexpression of Rep proteins has negative effects on cells and is, on occasion, lethal to cells.

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AAV vectors lacking the rep gene fail to integrate into AAVS1, showing apparent random integration into the host chromosomal DNA (Kearns et al., 1996). A non-viral plasmid-based system capable of integrating a transgene specifically into AAVS1 has been described; this was achieved by transferring the transgene flanked by the ITRs with transient expression of Rep78 or Rep68 (Balague et al., 1997; Pieroni et al., 1998; Shelling & Smith, 1994; Surosky et al., 1997; Tsunoda et al., 2000). Thus, this system is safer than integrating retrovirus and AAV vectors randomly. A strategy utilizing two plasmids, one harbouring the transgene cassette between the ITR sequences and the other for Rep expression, allows only the transgene plasmid to integrate into the AAVS1 locus (Surosky et al., 1997). This method successfully introduced the transgene into AAVS1 in haematopoietic K562 cells (Kogure et al., 2001).

The frequency of AAVS1-specific integration by the plasmid-based methods has differed among studies. Shelling & Smith (1994) reported that 9 of 12 cell clones (75%) obtained by transfecting HeLa or 293 cells with an AAV vector plasmid on which the Neo gene was placed under the control of the p40 promoter, the original promoter for Cap proteins, had rearranged AAVS1 and mentioned that approximately 50% of the rearranged bands also hybridized to an AAV probe. Another strategy using one plasmid on which both a Rep cassette and an ITR-flanked transgene cassette were placed has targeted the transgene to AAVS1 in 6 of 21 (29%) 293 cell clones (Balague et al., 1997). Similar methods applied to other cell lines, HeLa and Huh-7 cells, have been able to insert the transgene to AAVS1 in up to 20% of clones (Lamartina et al., 1998; Pieroni et al., 1998). All the studies mentioned here used a one plasmid system and the p5 promoter for Rep expression.

Aiming at increasing the frequency of AAVS1-directed integration, we first examined whether AAVS1-specific integration depended on the levels of Rep protein expressed in cells. To control the expression of the cytotoxic Rep proteins, we chose to vary the amount of Rep plasmid DNA. 293 cells were transfected using the calcium phosphate precipitation method with 2, 0.2, 0.04, 0.02 or 0 µg pCMVR78, which expresses Rep78 under the control of the CMV promoter (Surosky et al., 1997), and 2 µg pWNeo (Rep : Neo ratio of 1, 0.2, 0.1, 0.02, 0.01 or 0). pWNeo bears a Neo gene under the control of the CMV promoter between the ITRs. To monitor the amount of plasmid DNA incorporated, extrachromosomal DNA was analysed by Southern blot with a plasmid backbone probe (Fig. 1a). As the amount of Rep plasmid decreased, signal intensities corresponding to pCMVR78 decreased gradually, whereas those corresponding to pWNeo changed little, indicating that the amount of plasmid DNA incorporated into the cells correlated with that used for transfection. Western analysis of the transfected 293 cells confirmed that the expression level of the Rep protein was a function of the amount of pCMVR78 (Fig. 1b).

Fig. 1. (a) Quantification of plasmid DNAs incorporated into 293 cells. 293 cells (2 x 10⁵ cells per well) in 6-well plates were transfected with 2 µg pWNeo and various amounts of pCMVR78 (0, 2, 0.4, 0.2, 0.04 or 0.02 µg) at a Rep to Neo plasmid ratio of 0, 1, 0.2, 0.1, 0.02 or 0.01 by the calcium phosphate precipitation method. The amount of plasmid DNA transfected per well was made up to a total of 4 µg with a plasmid devoid of a Rep cassette (pCMV). Following transfection, extrachromosomal DNA was isolated and treated with BsmHI. BsmHI digestion generates a 5.9, 3.4 or 4.0 kb band, derived from pCMVR78, pWNeo or pCMV, respectively, that hybridizes to a plasmid backbone probe. (b) Expression of Rep78 in 293 cells transfected with various amounts of pCMVR78. The Rep to Neo ratio is indicated above each lane. pM45 harbours the AAV rep and cap genes (McCarty et al., 1991). Anti-Rep antibody 294.4 (a gift from J. Kleinschmidt) was used. (c) Comparison of the number of G418-resistant colonies obtained using various amounts of pCMVR78. Following transfection with pCMVR78 and pWNeo at different Rep to Neo ratios (1, 0.2, 0.1, 0.02, 0.01 or 0 µg), a 1/500 fraction of transfected cells was replated onto a 10 cm dish in triplicate and cultured for 10 days in the presence of G418. (d) Representative comparison of the number of G418-resistant colonies generated using pCMVR78 or pCMVR68. After transfection with 0.8 µg pCMVR78, pCMVR68 or pCMV and 3.2 µg pWNeo or pCMVNeo, a 1/500 fraction of transfected cells was replated onto 6-well plates in triplicate and cultured for 10 days in the presence of G418. When either Rep plasmid was co-transfected with pWNeo, a larger number of colonies was formed.
Fig. 1(c) compares G418-resistant colonies grown after transfection with pCMVR78. The number of colonies increased significantly when pCMVR78 was added to the transfection solution. The number of colonies observed did not differ significantly at the Rep to Neo ratios of 0-2–0-01. On transfection at the Rep to Neo ratio of 1, however, the number of colonies decreased, probably due to the strong cytotoxicity of Rep78. To estimate the frequency of integration of the Neo gene to AAVS1, we extensively analysed clones by Southern blot. From each group, 14–20 clones were expanded and their genomic DNA was digested with HindIII or EcoRV, enzymes that do not cleave plasmid pWNeo or the proximal portion of AAVS1 where integration of the AAV genome occurs predominantly (Giraud et al., 1994; Kotin et al., 1992). The presence of co-migrating bands that hybridized to both AAVS1 and Neo probes on both HindIII- and EcoRV-blots was a criterion to conclude that the Neo gene was integrated into AAVS1. Table 1 summarizes the result of Southern blot analysis of the 293 cell clones. When the pCMVR78 to pWNeo ratio was 1 or 0-2, approximately 95 % of clones showed rearrangement of AAVS1. The frequency of rearrangement of AAVS1 decreased gradually as the amount of pCMVR78 was reduced. Unexpectedly, integration of the Neo gene into AAVS1 was observed only in 1 of 16 clones (6 %) at the Rep to Neo ratio of 1. In contrast, transfection at the Rep to Neo ratio of 0-2–0-02 produced approximately 20 % of clones that delivered the Neo gene to AAVS1. These results indicated that a high-level expression of the Rep proteins increased the frequency of AAVS1 rearrangement and rather decreased the frequency of AAVS1-specific integration of the transgene.

In the second experiment, we compared Rep78 with Rep68 by transfecting 0-8 μg pCMVR78, pCMVR68 or pCMV along with 3-2 μg pWNeo or pCMVNeo (Rep to Neo ratio of 0-25). pCMVR68 expresses Rep68 alone (Surosky et al., 1997) and pCMVNeo is the same as pWNeo except for the absence of ITRs. Fig. 1(d) is a representative comparison of G418-resistant colony formation. The results of the Southern blot analysis of clones selected randomly are summarized in Table 1. Rep78 generated rearrangement of AAVS1 in approximately 90 % of clones and 24 % of clones had the Neo gene at AAVS1. The frequency of rearrangement of AAVS1 is 65 % with the use of pCMVR68 and 40 % of clones integrated the Neo gene to AAVS1. This result suggested that Rep78 appeared to cause more ‘abortive’ integration of the Neo gene, rearrangement of AAVS1 without integration of transgene, although the difference between Rep78 and Rep68 was not statistically significant.

Fig. 2 shows Southern blot analysis of representative clones with the Neo gene at AAVS1. Fig. 2(a, b) is the HindIII- or EcoRV-digest probed with an AAVS1-specific probe (upper panel) or a Neo probe (lower panel). Each clone presented here has an upshifted band(s) other than a basal band (arrow). Common bands that hybridized to both AAVS1 and Neo probes are indicated by arrowheads. Fluorescent in situ hybridization (FISH) analysis confirmed the integration of the Neo gene into chromosome 19 in 11 of 12 clones. A representative chromosomal analysis is shown in Fig. 2(c). The 293 cells used in the present study have four copies of chromosome 19 labelled with Cy3-conjugated chromosome 19-specific probe (arrowheads). The left panel shows a metaphase spread of clone C6/6. Fluorescein Neo signals are localized to chromosome 19 and another unidentified site (arrows). In the right panel showing analysis of clone C6/18, one chromosome 19 harbours the Neo signals at its terminal portion.

‘Abortive’ integration into AAVS1, rearrangement of AAVS1 without foreign gene insertion, has been described in 293 or HeLa cells (Balagué et al., 1997; Shelling & Smith,

Table 1. Summary of Southern blot analysis

<table>
<thead>
<tr>
<th>Rep plasmid</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pCMVR78</td>
<td>pCMVR68</td>
</tr>
<tr>
<td>Rep to Neo</td>
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<td>0-2</td>
</tr>
<tr>
<td>No. of clones analysed</td>
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<td>20</td>
</tr>
<tr>
<td>Enzyme used</td>
<td>HindIII EcoRV</td>
<td>HindIII EcoRV</td>
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<tr>
<td>Rearranged AAVS1 band*</td>
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<td>14</td>
</tr>
<tr>
<td>Common band†</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>AAVS1 rearrangement (%)‡</td>
<td>15 (94)</td>
<td>19 (95)</td>
</tr>
<tr>
<td>Neo at AAVS1(%)§</td>
<td>1 (6)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Neo signal on chromosome 19‖</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

*Number of clones with rearrangement of AAVS1.
†Number of clones with common bands hybridizing to both AAVS1 and Neo probes.
‡Number of clones with rearrangement of AAVS1 on either the HindIII or the EcoRV blot.
§Number of clones with common bands on both HindIII and EcoRV blots.
‖Number of clones with the Neo signal on chromosome 19.
1994; Surosky et al., 1997). A similar disruption of AAVS1 has been detected in cell lines latently infected with wild-type AAV (Kotin et al., 1990). This phenomenon may be explained in three ways. First, the integrated transgene or AAV genome is disrupted during or after an integration event such that Southern blot analysis cannot detect it. The instability of the integrated AAV genome over passages in a latently infected cell line was described (Cheung et al., 1980). An additional rearrangement can occur in the rearranged AAVS1 region (Shelling & Smith, 1994). Second, recombination between the AAVS1 region and other sites may cause rearrangement of AAVS1 without integration of the transgene at AAVS1. Third, the Rep protein may excise the integrated plasmid DNA or AAV genome, resulting in the loss of the preintegrated sequences.

The 293 cells used in the present study have four copies of chromosome 19. Southern blot analysis showed that some clones had more than three upshifted bands besides a basal band. We used a relatively large probe (3-0 kb) for detecting AAVS1 bands. It is possible that Rep-mediated disruption of the AAVS1 region can produce the multiple bands hybridizing to the AAVS1 probe. Another explanation is as follows: at 24 h post-transfection, we replated transfected cells to isolate clones derived from single cells. At this time-point, the Rep protein was still being expressed in cells and an additional integration event might occur in some cells after cell division.

Lamartina et al. (1998) reported no apparent difference between Rep78 and Rep68 in the ability to deliver foreign DNA to AAVS1 in HeLa cells. Several studies have reported the functional differences between Rep78 and Rep68. Rep68 is more efficient in processing dimers to monomer duplex DNA and possesses a stronger nicking activity (Ni et al., 1994, 1998), while the helicase activity of Rep78 is stronger (Wollscheid et al., 1997). The differential effects of Rep78 and Rep68 on the p5, p19 and p40 promoters were described (Weger et al., 1997). In addition, Rep78 inhibits CREB-dependent transcription by interacting with protein kinase (Chiorini et al., 1998; Di Pasquale & Stacey, 1998). None of these findings explains why Rep78 appears to cause more abortive integration. Rep68 may be more suitable for the AAVS1-targeted integration system. To confirm the usefulness of Rep68 in the AAVS1-targeted integration system, further analysis of a larger population of cell clones would be required. Also, the exact functions of the Rep protein in AAVS1-specific integration should be elucidated.

The results presented here have important implications for developing an AAVS1-directed integration system as well as for understanding the mechanism of AAVS1-specific integration by the Rep proteins.

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