Targeted Integration of Adeno-Associated Virus-Derived Plasmids in Transfected Human Cells

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INTRODUCTION

Adeno-associated virus (AAV) has gained recent interest as a viral vector for gene therapy on the basis of its lack of pathogenicity and its unique capacity of preferentially integrating its viral DNA within a defined region of the cellular genome. AAV is a human parvovirus that usually requires adenovirus (Ad) or herpesvirus as a helper to replicate efficiently (Berns, 1996). In the absence of helper virus, the AAV genome integrates into host-cell genomic DNA and is maintained as a latent provirus. Analysis of flanking sequences from latently infected cells has revealed integration of the AAV genome into a specific locus in 60±70% of cases. The integration locus (AAVS1) has been sequenced and localized to human chromosome 19q13.3-qter (Giraud et al., 1994; Kotin et al., 1990, 1992; Samulski et al., 1991). Other, less-frequent integration sites have been identified in the long arm of chromosome 17 in latently infected HeLa cells (Waltz and Shlehofer, 1992).

The AAV DNA genome is a linear single-stranded DNA molecule of ~4700 nucleotides. The AAV genome has one copy of the 145-nucleotide-long inverted terminal repeat (ITR) located at each end. The ITRs are needed for viral integration, rescue from the host genome, and encapsidation of viral nucleic acids into mature virions (Samulski, 1993; Weitzman et al., 1994). Inserted between the ITRs of AAV is a unique region of ~4470 nucleotides that contains two main open reading frames (ORFs). The right ORF, Cap, encodes the viral structural proteins. The left ORF of the AAV genome encodes the rep gene. Two promoters located at map positions 5 and 19 (promoters P5 and P19, respectively) control the expression of the four polypeptides derived from this ORF. Rep proteins Rep78 and Rep68 are produced from the p5 promoted transcripts, and Rep polypeptides Rep52 and Rep40 are synthesized from the p19 promoted transcripts (Srivastava et al., 1983).

The mechanism of AAV integration is not yet fully understood; however, a model was recently proposed in which the insertion of the AAV genome into chromosome 19 is promoted by the Rep polypeptides and is accompanied by limited DNA synthesis of the AAVS1 site (Linden et al., 1996). This model is based in part on the observation that the larger polypeptides Rep78 and Rep68 have been shown to possess ATP-dependent helicase and strand-specific endonuclease activities (Im and Muzyczka, 1990, 1992). In addition, these proteins can bind DNA in a sequence-specific manner (Ashktorab and Srivastava, 1989; Im and Muzyczka, 1989). A Rep binding site has been identified in both the ITR of AAV and the AAVS1 site, and it has been shown that the Rep polypeptides can interact in vitro with these sequences and promote their nicking and replication (Snyder et al., 1990; Urcelay et al., 1995). Furthermore, it has been demonstrated that the Rep78 and Rep68 polypeptides can individually mediate complex formation between the...
ITR of AAV and the AAVS1 in vitro (Weitzman et al., 1994). In agreement with the involvement of the Rep polypeptides and possibly of other cellular factors in the interaction with the AAV terminal repeats and with AAVS1, the Rep78 protein has been shown to form in vitro multimeric complexes (Hermonat and Batchu, 1997) and to self-associate in the presence of AAV ITRs (Smith et al., 1997). This model of Rep-mediated targeted insertion has been confirmed, at least in part, by experimental evidence indicating that AAV terminal repeats are the required substrate for viral integration in conjunction with the host cellular recombination machinery (Yang et al., 1997) and that expression of Rep proteins is necessary for targeted insertion into the AAVS1 locus proteins (Balague et al., 1997; Kearns et al., 1996; Shelling and Smith, 1994). In addition, it has been shown that expression of only Rep78 or Rep68 is sufficient to obtain highly efficient integration into the AAVS1 site (Lamartina et al., 1998; Surosky et al., 1997).

The main limitation of AAV for gene therapy is constituted by the packaging limit of the AAV virion, which cannot exceed 4.5 kb. Thus, most of the AAV-derived vectors have been generated by deleting the Rep and Cap genes and replacing them with the gene to be delivered. These vectors can persist for a prolonged period of time as episomal forms or they can integrate randomly, thus supporting the role of the Rep polypeptides in targeting to chromosome 19 (Afione et al., 1996; Fisher et al., 1997; Flotte et al., 1994; Hermonat and Muzyczka; 1984; Kaplitt et al., 1994; Kearns et al., 1996; Kessler et al., 1996; Koehler et al., 1997; LaFace et al., 1988; Lebkowski et al., 1996; Mclaughlin et al., 1988; Philip et al., 1994; Reed Clark et al., 1997; Russel et al., 1994; Tratschin et al., 1985; Xiao et al., 1996).

In an effort to develop a reliable delivery system that will allow us to achieve targeted integration of foreign DNA, we examined the contribution of AAV rep gene on the efficiency of site-specific integration of transfected plasmids. We analyzed the efficiency of site-specific integration in transfected cells of constructs carrying the rep gene located outside of the AAV ITRs and compared it with that of a similar plasmid lacking the rep gene. The results obtained indicate that integration of a specific DNA fragment into the AAVS1 site occurs at a significant frequency and in different cell types.

RESULTS

Construction of AAV-derived plasmids

To determine whether the rep gene of AAV can be used as a means of directing the site-specific integration of a gene of interest into the DNA of target cells, plasmid pITR(GFP-Neo)P5Rep and the control plasmid pITR(GFP-Neo) were constructed (Fig. 1). In both plasmids, the neo gene and the green fluorescent protein (GFP) gene were inserted between the 5' and 3' ITRs of AAV. In addition, in plasmid pITR(GFP-Neo)P5Rep, the rep gene under the transcriptional control of P5 and P19 promoters was cloned outside the two ITRs but next to the 3' terminal repeat. To verify that the expression of Rep52 and Rep40 is not required for site-specific integration, plasmid pITR(GFP-Neo)P5Rep78/68 was also constructed, in which the ATG located downstream of promoter P19 had been mutated.

The functionality of these plasmids in determining the replication of the ITR-flanked DNA was assessed by transfection in Ad5-infected 293 cells. As control, pITR(GFP-Neo) was cotransfected with an helper plasmid, pAd/AAV. This construct encodes the Rep and Cap polypeptides but is incapable of replicating itself due to the absence of AAV ITRs (Mclaughlin et al., 1988; Samulski et al., 1989). The replication pattern of these plasmids was compared with that of plasmid pSub201. Low-molecular-weight DNA was isolated 48 h posttransfection.
and fractionated on an agarose gel after digestion with restriction enzyme DpnI to degrade unreplicated input plasmid DNA. DpnI distinguishes between the newly replicated DNA and input plasmid DNA based on resistance to digestion due to the loss of methylation (Wobbe et al., 1985). The digested DNA was then subjected to Southern blot analysis using a probe specific for the ITR sequence (Fig. 2). Plasmid pSub201 produced DpnI-resistant bands of ~4.5 and ~9.0 kb corresponding to the monomeric and dimeric forms of the AAV genome, indicating that viral genome had undergone successful rescue and replication (Fig. 2, lane 1). In the absence of the AAV helper, plasmid pITR(GFP-Neo) was DpnI sensitive, and no bands could be detected with the ITR-specific probe (Fig. 2, lane 2). In contrast, when transfected with the helper plasmids pAd/AAV, a band of 3.5 kb resistant to DpnI digestion was apparent. This band corresponds to the monomeric GFP-Neo fragment inserted between the ITRs of AAV, thus indicating that the ITR DNA fragment had undergone successful rescue and replication (Fig. 2, lane 3). Similarly, constructs pITR(GFP-Neo)\(\Phi_5\)Rep and pITR(GFP-Neo)\(\Phi_5\)Rep78/68 transfected alone displayed the same 3.5-kb DpnI-resistant band that hybridized to the ITR-specific probe (Fig. 2, lanes 4 and 5).

Thus, the results indicate that the plasmids contain functional ITR sequences and that in agreement with published data (Samulski et al., 1989), the Rep polypeptides are fully capable of determining the rescue and replication of the ITR DNA segment when expressed outside of the ITR context.

Site-specific integration of AAV-derived constructs

The efficiency of site-specific integration of pITR(GFP-Neo)\(\Phi_5\)Rep and pITR(GFP-Neo)\(\Phi_5\)Rep78/68 was compared with that of pITR(GFP-Neo) by transfection of HeLa cells. In addition, in view of the fact that liver-specific gene transfer has received a great deal of attention because of the large biosynthetic capacity of this organ and because many genetic diseases result from the absence or deficiency of a hepatocyte-derived gene product (Ledley, 1993), the human hepatoma cell line Huh-7 was also used to assess the efficiency of targeted integration of the AAV-derived plasmids.

Neomycin-resistant clones were isolated and expanded, and high-molecular-weight DNA was extracted and subjected to Southern blot analysis. The rearrangement of the AAVS1 site resulting from the integration of the transfected plasmid was verified using AAVS1- and neo-specific probes. The hybridization pattern of selected clones was compared with that of mock-transfected cells. Site-specific integration of the transfected plasmid was scored when the two following conditions were met: (1) The hybridization pattern of the genomic DNA using the AAVS1 probe was different from that of the mock-transfected cells DNA in that it contains an additional restriction fragment recognized by the AAVS1 probe. (2) The same additional fragment revealed with the genomic probe was detected with the neo-specific probe.

As shown in Figure 3, in 3 of 25 (12%) Huh-7 selected clones that had been transfected with plasmid pITR(GFP-Neo)\(\Phi_5\)Rep, the AAVS1 probe hybridized to a fragment ranging in size of 4.5±10 kb that was not detected in the mock-transfected Huh-7 genomic DNA (Fig. 3A, compare lanes 3, 7, and 12 with lane 1). The same band was hybridized by the neo-specific probe, indicating that integration of the ITR-flanked DNA into chromosome 19 also had occurred in these cells (Fig. 3B, lanes 3, 7, and 12). The site-specific integration of the ITR-flanked DNA was revealed with both the neo- and GFP-specific probes, indicating that both markers had integrated into the AAVS1 site (data not shown).

The summary of results obtained in the transfection of both HeLa and Huh-7 cells are shown in Table 1. No site-specific integration was detected in 39 selected HeLa cell clones that had been transfected with plasmid pITR(GFP-Neo). In contrast, targeted integration in the AAVS1 site was detected in 8 of 32 (25%) clones transfected with plasmid pITR(GFP-Neo)\(\Phi_5\)Rep and in 7 of 38
(18%) clones transfected with pITR(GFP-Neo)P5Rep78/68. Transfection of Huh-7 cells with plasmid pITR(GFP-Neo)P5Rep78/68 resulted in site-specific integration of the ITR-flanked DNA into 2 of 26 clones (7%).

The selectivity of integration of the ITR-flanked DNA fragment into the AAVS1 site was assessed by hybridization of the BamHI-digested genomic DNA of the transfected clones with a probe specific for the rep gene. No band could be hybridized by the rep-specific probe, and no Rep activity could be detected by performing rescue...
and replication assays (data not shown). Thus, the lack of correlation in the hybridization pattern of the probe with the neo and AAVS1 probes suggests that no insertion of the DNA sequences located outside of the ITRs has occurred into the AAVS1 site.

The site-specific integration of AAV genome into chromosome 19 has mostly been mapped within the first 1.5 kb of the AAVS1 site (Kotin et al., 1992). To determine whether in the Huh-7 cells transfected with the AAV-derived plasmids integration had occurred in the same region of AAVS1 as observed in AAV-infected cells, the DNA from a transfected clone was analyzed using two sets of nested primers that span the potential junction between the ITR and chromosome 19 sequences (Goodman et al., 1994). As a comparison, the DNA from AAV-infected Huh-7 cells was subjected to the same PCR amplification. These cells were infected with AAV at an m.o.i. of 20 and passaged nine times before DNA extraction. The amplification products were identified as the junction fragments by virtue of their hybridization to AAVS1- and ITR-specific probes (data not shown) and thus were cloned and sequenced. As shown in Figure 4, the junction fragment derived from clone H6, which was selected from cells transfected with plasmid pITR(GFP-Neo)P<sub>5</sub>Rep, indicated that the insertion of the transfected ITR-flanked DNA had occurred at nucleotide 1003 of AAVS1 and that a deletion of 92 nucleotides of the ITR had taken place. In addition, an insertion of 9 nucleotides was detected at the ITR<sub>5</sub>AAVS1 crossover. Similarly, the insertion of viral DNA was mapped at nucleotides 870, 1091, and 1116 of AAVS1 with a concomitant partial deletion of the ITR sequence of 61, 60, and 66 nucleotides, respectively (Fig. 4, samples AAV/3, AAV/12, and AAV/26). In one of the viral–cellular junction analyzed, a short 2-bp homology was found at the crossover. Also, in two other AAV<sub>5</sub>AAVS1 junctions, an insertion of 3 or 12 nucleotides was detected at the viral–cellular crossover.

Taken together, these data indicate that integration of the transfected plasmid DNA into the AAVS1 site occurs with significant efficiency in different cell types and is dependent on the presence of the rep gene. Furthermore, in agreement with published observations (Lamartina et al., 1998; Shelling and Smith, 1994; Surosky et al., 1997), targeted integration of the ITR-flanked DNA does not require the expression of Rep52 and Rep40. Last, in analogy to what has been observed on AAV infection of cultured cell lines (Giraud et al., 1994; Kotin et al., 1990, 1992; Samulski et al., 1991), the transfected plasmid DNA has integrated in the same region of the AAVS1 site.

In situ chromosome analysis of neomycin-resistant HeLa cell clones

The site-specific integration of the AAV-derived plasmid was confirmed by fluorescent in situ hybridization.
(FISH) analysis of cytogenetically prepared HeLa cell chromosomes derived from HeLa cell clones, where targeted integration into the AAVS1 site had been demonstrated by Southern blot analysis (data not shown). For this purpose, two probes were used: a 3.5-kb DNA fragment corresponding to the entire GFP-Neo sequence and a 80-kb DNA fragment isolated by screening of a genomic DNA library using the 3.5-kb DNA fragment of AAVS1 as a probe. Figure 5 demonstrates the presence of the GFP-Neo sequence on each sister chromatid of a single chromosome of HeLa cell clone P5z12 that had been transfected with pITR(GFP-Neo)P5Rep. In addition, the same region of the chromosome was hybridized by the AAVS1-specific probe. Three other chromosomes present in this HeLa cell clone annealed to the AAVS1-specific probe, which is in agreement with the polyploid nature of this cell line. However, no GFP-Neo sequence could be detected, thus indicating that integration had occurred only in one of the available AAVS1 sites. A similar chromosomal staining was observed with two other cell clones, whereas no GFP-Neo hybridization was detected with mock-transfected cells (data not shown). Thus, the data from the FISH analysis provide further evidence for the targeted insertion of the AAV-derived plasmid into the AAVS1 site of transfected cells.

DISCUSSION

In this study, we examined whether the rep gene of AAV can direct the site-specific integration of a transgene flanked by the ITRs of AAV. The results obtained indicate that the targeted integration of a specific DNA fragment to human chromosome 19 occurs at a significant frequency and in different cell types.

The strategy devised in the construction of the plasmids used for the transfection experiments is based on the assumption that the rep gene is required for site-specific integration and that by inserting the Rep coding sequences outside of the ITRs, the integration event is limited to the gene of interest flanked by the AAV terminal repeats. In addition, because the rep gene is not expected to be cointegrated with the ITR-flanked DNA, the Rep coding sequence should be lost with time. Thus, the transient nature of the Rep coding sequence may contribute to the stability of the inserted DNA fragment by limiting the possibility of further recombination events.
Our hypothesis is based on the observation that the ITRs function as signals for the initiation of viral replication and for integration (Snyder et al., 1990; Urcelay et al., 1995) and that the events leading to integration may involve selective amplification of the ITR-flanked DNA mediated by the Rep polypeptides (Linden et al., 1996). The excision and replication of the ITR-flanked DNA can be observed on transfection of plasmids containing the AAV terminal sequences in Ad-infected cells as a function of Rep expression, in analogy to what is observed on transfection of plasmid pSub201 (Fig. 2) (Samulski et al., 1987). Last, in the selected HeLa and Huh-7 cell clones, integration into the AAVS1 site of the transfected plasmid is limited, at least in most cases, to the ITR-flanked DNA sequence; this observation suggests that a mechanism of selective excision and replication is likely to occur before site-specific integration into chromosome 19 (data not shown; Balagå et al., 1997).

The results obtained on transfection of HeLa cells indicate that insertion into the AAVS1 site is dependent on the expression of the Rep polypeptides because no site-specific integration is observed on transfection of plasmid pITR(GFP-Neo) (Table 1). Thus, our results are in agreement with the role of the Rep polypeptides in determining the insertion of the AAV genome into the AAVS1 site (Balagå et al., 1997; Linden et al., 1996; Shelling and Smith, 1994; Surosky et al., 1997). In addition, these results support the observation based on FISH analysis of infected cells that current AAV vectors lacking the rep gene will integrate into chromosome 19 only on coinfection with wild-type AAV, thus indicating that expression of Rep proteins in trans-targeted integration in AAVS1 does occur (Ponnazhagan et al., 1997). Interestingly, in latently infected cell lines, the AAV insertion points have been located at AAVS1 nucleotides 1026±1030 and 1144±1146 (Kotin et al., 1992), in a region of nucleotides 713±1303 (Samulski et al., 1991), and at position 727 (Goodman et al., 1994). Infection of Huh-7 cells with AAV virus results in the insertion of the viral DNA into the same general area of AAVS1. Similarly, the junction fragment derived from PCR amplification of the genomic DNA of the Huh-7 cell clone H6 transfected with plasmid pITR(GFP-Neo)P3Rep indicates that the insertion of the ITR-flanked DNA has occurred in the same region of chromosome 19 (Fig. 4). The detection of a partial deletion of the ITRs and of the insertion of a short stretch of nucleotides between the ITR-cellular junction is not unprecedented (Leonard and Berns, 1994; Kotin and Berns, 1989; Palombo et al., 1998; Samulski et al., 1991; Yang et al., 1997), and it may be a consequence of the limited replication and rearrangement associated to Rep-mediated integration.

The localization of the ITR-flanked DNA fragment within the 5'-end region of AAVS1 is also corroborated by Southern blot analysis of all of the HeLa and Huh-7 clones with a genomic probe specific for nucleotides 1±1600. In these clones, the upshifted AAVS1 band was detected with the 5'-end probe of AAVS1, thus indicating that the insertion of the ITR integration cassette had occurred within this region (data not shown). Therefore, the mechanism involved in the integration of the transduced DNA is likely to be very similar to that responsible for the insertion of the wild-type AAV genome into the same region of chromosome 19.

An important requirement of the gene transfer strategy outlined above is that the integration of the ITR-flanked DNA into AAVS1 is not accompanied by rearrangements of the inserted DNA that could render it nonfunctional. With a significant frequency, transfection of plasmid pITR(GFP-Neo)P3Rep in both HeLa and Huh-7 cells results in the single integration of the ITR-GFP-Neo DNA fragment into the AAVS1 site, as shown by the Southern blot analysis of the genomic DNA of selected clones. This conclusion also is supported by FISH analysis of HeLa cell clones that showed the presence of the GFP-Neo sequence colocalized with the AAVS1 site and no other metaphase chromosome (Fig. 5). Nevertheless, the size of the upshifted bands indicative of site-specific integration varies from one clone to the other and does not match the sum of the size of the ITR-flanked DNA and of the AAVS1 BamHI restriction fragment (~7 kb) (Fig. 3). In addition, sequence analysis of the ITR-AAVS1 junction of both infected and transfected Huh-7 cells revealed that partial deletion of the AAV terminal repeat has taken place on integration (Fig. 4). Thus, partial deletion or rearrangement of both the inserted DNA and the AAVS1 site probably have occurred on integration. Nevertheless, although we cannot provide any evidence on GFP expression simply because the gene used in this construct is not functional (data not shown), the hybridization data suggest that it is conceivable to obtain site-specific integration of multiple functional genes, which can be readily expressed after integration. In support of this hypothesis, we recently observed that transduced 293 cells that carry a 6-kb DNA fragment consisting of the hygromycin resistance as well as the β-galactosidase gene integrated into the AAVS1 site are indeed resistant to hygromycin and express significant amounts of β-galactosidase (Palombo et al., 1998).

Targeted integration of AAV-derived plasmids has been described by Balagå et al. (1997), who reported that transfection of 293 cells with a construct expressing Rep78 and Rep52 results in the site-specific integration of a GFP gene flanked by the AAV ITRs in 50% of the clones sorted on the basis of GFP expression. Interestingly, the authors did not obtain the same result when they used the neomycin resistance gene as a selectable marker. By Southern blot analysis, they observed extensive rearrangement of the AAVS1 locus associated with the transfection of Rep-positive plasmid, which, however, could not be ascribed to insertion of the neo gene. The authors conclude that the lack of correspondence be-
between the neo and AAVS1 bands is an effect secondary to the selective pressure applied. This conclusion, however, seems to be limited only to the 293 cells because as stated above, we have not observed such rearrangements in most of the transfected HeLa and Huh-7 clones selected with neo. However, in view of the fact that resistance to neomycin was used as selective pressure for the isolation of transfected clones, it is possible that the efficiency of integration detected in transfected HeLa and Huh-7 cells is actually an underestimate of the real efficiency of site-specific insertion in these cell lines. The biological significance of these different experimental results is not clear. However, taken together, these data suggest that the overall ability of AAV to determine site-specific integration into AAVS1 may be affected by differences among human aneuploid cell lines. Such differences may be related to the concentration of host factors present in the cell required for integration and to the level of Rep expression obtained in each cell line. In particular, the constitutive expression of E1A in 293 cells could determine an higher level of expression from the P5 promoter, and this could, at least in part, influence the efficiency of site-specific integration detected in this cell line. Thus, it would be of interest to determine whether the use of strong constitutive promoters for the expression of the Rep gene may affect the overall targeting efficiency to the AAVS1 locus in E1A-negative cell lines.

The integration of therapeutic genes into specific locations of the DNA of nondividing cells accompanied by prolonged expression is the optimal strategy for somatic gene therapy. The results presented in this work indicate that it is possible to devise a strategy aimed at the insertion of specific DNA sequences at a predetermined location of the human chromosomes. However, the functionality of AAV-derived constructs must be properly addressed in a variety of different cell types and in primary cultures in particular. Furthermore, the relevance of site-specific integration in the in vivo persistence and expression of therapeutic genes awaits the development of suitable animal models in which AAV DNA insertion into the AAVS1 site can be examined.

MATERIALS AND METHODS

Construction of recombinant plasmids

For pITR(GFP-Neo), pSub201 (Samulski et al., 1987) was cut with XbaI to remove the Rep and Cap coding sequences of AAV, and the 4.0-kb DNA fragment containing the ITR of AAV then was ligated to a 1.7-kb DNA fragment carrying an Nhel restriction site at either end. This fragment contains the cDNA of the GFP (Chalfie et al., 1994) flanked at its 5′-end by the CMV immediate early promoter and enhancer and by the bovine growth hormone (bgh) polyadenylation signal at its 3′-end. The GFP fragment was obtained by PCR amplification with sequence-specific primers using as template a deriva-

tive of vector pCDNA-3 (Invitrogen) in which the GFP cDNA had been cloned [pCD3(GFP)]. The primer specific for the bgh polyadenylation signal was designed so it contains an SacII site located near the Nhel site at the 3′-end. The construct so obtained was named pITR-GFP. Plasmid pITR(GFP-Neo) was produced by inserting the blunted EcoRl-BamHI fragment from pRc/RSV (Invitrogen), which contains the SV40 early promoter, neomycin resistance gene, and SV40 polyadenylation signal, into the blunted SacII site of pITR(GFP).

pITR(GFP-Neo)P5Rep was derived from PCR amplification of nucleotides 138±2234 of the AAV genome with sequence-specific primers using plasmid pTAV-2 (Heilbronn et al., 1990) as template. The amplified DNA fragment was digested with ClaI and cloned into the ClaI site of plasmid pITR(GFP-Neo).

pITR(GFP-Neo)P5Rep78/68 was constructed by inserting into the ClaI site of plasmid pITR(GFP-Neo) a DNA fragment extending from nucleotides 138±2234 of the AAV genome in which the internal ATG for Rep52 and Rep40 (nucleotides 993±995) had been changed to GGA.

The expression of the rep gene in plasmids pITR(GFP-Neo)P5Rep and pITR(GFP-Neo)P5Rep78/68 was determined by Western blot analysis of Ad5-infected 293 cell lysate that had been transfected with these constructs. The expression of Rep78 and Rep68 was found to be comparable to the level of Rep poly peptides expression detected on transfection of the original construct pSub201 (Samulski et al., 1987), which carries the full-length genome of AAV, whereas no expression of Rep52 and Rep40 could be detected in cells transfected with plasmid pITR(GFP-Neo)P5Rep78/68.

The replication capacity of the constructed plasmids was assessed by transfecting 5 μg of plasmid DNA onto 293 cell monolayers that had been infected 1 h before transfection with wild-type Ad2 at an m.o.i. of 10. Low-molecular-weight DNA was isolated according to published protocols (Hirt, 1967) from the cultures at 40 h after transfection/infection, digested with restriction enzyme DpnI for 4 h at 37°C, and analyzed by Southern blot analysis using a probe specific for the ITR sequence.

Cell transfections

The 293, Huh-7, and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in 10-cm dishes (Falcon) at 37°C in 5% CO2. Stock cells were routinely passaged every 3 days by treatment with trypsin (0.05%) and EDTA (0.53 mM) and replated at cell densities appropriate for exponential growth.

The 293 and HeLa cells were transfected according to the calcium phosphate precipitate method using the calcium phosphate mammalian cell transfection kit (5
precipitated in ethanol, resuspended in 0.5 ml of H2O, chloroform (1:1 ratio), and chloroform. The DNA was precipitated with 10 mM Tris–Cl, pH 8.0, 1 mM EDTA, phenol and three consecutive extractions with phenol (equilibrated in 12 h, the medium was replaced, and the cells were incubated for an additional 32 h.

Transfection of the hepatoma cell line Huh-7 was performed by using Lipofectin according to the manufacturer's instructions (GIBCO BRL). The cell monolayer was incubated in the presence of the DNA–Lipofectin mix for 12 h, the medium was replaced, and the cells were incubated for an additional 32 h.

To isolate stable cell clones, the transfected cells were then treated with trypsin and EDTA, and the cell suspension derived from a single 10-cm plate was diluted 1:3 in selection medium (20 ml DMEM, 10% FCS, and 500 µg/ml G418) and plated onto three 15-cm plates (Falcon). Neomycin-resistant clones were isolated after 10 days of selection, expanded, and processed for genomic DNA extraction, Southern blot, and FISH analyses.

**Southern blot analysis of transfected clones**

Cell clones resistant to neomycin were grown in 10-cm plates, and the cell monolayer was detached by scraping and washed twice with 2 ml of PBS. The cell suspension was transferred to Eppendorf tubes, and the cells were pelleted and resuspended in 0.5 ml of TEN (50 mM Tris±Cl, pH 7.5, 150 mM NaCl, 10 mM EDTA). SDS and Proteinase K were added to each sample to final concentrations of 1% and 1 mg/ml, respectively. The cell lysate was incubated for 4 h at 56°C and subjected to three consecutive extractions with phenol (equilibrated with 10 mM Tris±Cl, pH 8.0, 1 mM EDTA), phenol and chloroform (1:1 ratio), and chloroform. The DNA was precipitated in ethanol, resuspended in 0.5 ml of H2O, and incubated overnight at 4°C. Ten micrograms of high-molecular-weight chromosomal DNA were incubated with 40 units of the restriction enzyme BamHI (New England Biolabs) in a 0.1-ml volume for 12 h at 37°C. The digested DNA was electrophoresed on a 0.8% agarose gel, transferred to nylon membrane (Hybond N+; Amersham) as recommended by the manufacturer, and hybridized overnight at 65°C in Church buffer (7% SDS, 0.25 M NaPi, pH 7.2, 1 mM EDTA, pH 8.0, 0.1 g/ml bovine serum albumin) with random-primed 32P-labeled probes. Filters were washed in 40 mM NaPi, pH 7.2, 1 mM EDTA, pH 8.0, and 1% SDS at 65°C three times for 20 min and then in 0.1× SSC and 0.1% SDS at 65°C for 20 min. Filters were exposed to x-ray film with an intensifying screen for 1 week. To determine site-specific integration of the ITR DNA fragment, filters were first hybridized with a probe specific for the neomycin gene, the hybridized probe then was removed by boiling the filters in 0.2× SSC and 1% SDS for 10 min, and the same filters then were hybridized to a probe specific for the AAVS1 site. The probe specific for the chromosome 19 was obtained by random priming reaction using as template a DNA fragment derived from plasmid pRVK (K. Berns, Cornell Medical School, New York, New York) covering nucleotides 1±3525 of AAVS1 (Kotin et al., 1992). DNA fragments of 630 and 750 bp were used as templates in random-priming reactions for the synthesis of neo- and GFP-specific probes, respectively.

**Huh-7 infection and PCR amplification of ITR±AAVS1 junction**

Wild-type AAV-2 virus was prepared by transfection of Ad5-infected 293 cells with plasmid pSub201 according to published protocols (Samulski et al., 1982, 1987). Semiconfluent Huh-7 cells were infected at an m.o.i. of 20 infectious units per cell. After absorption for 1 h at 37°C, the medium was replenished, and the cells were incubated for 2 days until they reached confluence. Cells were harvested and split 1:5 and incubated again at 37°C for several days. After nine passages, the presence of AAV virus in the cell culture was confirmed by performing a rescue and replication assay (Samulski et al., 1982), the cells were harvested, and genomic DNA was extracted. Integration of AAV-2 viral genome was compared with that of transfected ITR-flanked DNA fragment by PCR using nested primer pairs that flank the AAV±chromosome junction as previously described (Goodman et al., 1994): primers 16s (AAV) 5’-GTAGCATGGCG-GGTTAATCA and 15a (AAVS1) 5’-GCGCGCATAAGCCAGTAGAGC were used in the first round of PCR amplification with 0.5 µg of genomic DNA as substrate. After an initial incubation for 4 min at 94°C, the reaction mixture was subjected to 30 cycles of PCR amplification with following parameters: 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. Twenty percent of the amplification product was diluted into a new reaction mixture containing a set of nested primers with the following sequences: 17s (AAV) 5’-TTAATCAAAGGAACCCCTA and Cr2 (AAVS1) 5’-ACATGGCCAGGCCAGGCAG. The PCR parameters were the same as for the first amplification. The products were resolved on a 1% agarose gel, transferred to Hybond N+ paper (Amersham), and probed with ITR (nucleotides 4563±4670) and AAVS1 (nucleotides 210±1207) restriction fragments labeled by Amersham MegaPrime DNA labeling system. For molecular cloning of the amplified junction fragments, the product of the second round of amplification was purified on a 1% agarose gel and subcloned by blunt-end ligation into plasmid pZERO-2.1 (Invitrogen). Sequencing was performed using standard chain termination protocols.

**In situ hybridization**

A 3.5-kb DNA fragment corresponding to the entire GFP-Neo sequence and an 80-kb AAVS1 DNA isolated by screening a genomic DNA library were labeled using
the Nick Translation Kit (Boehringer-Mannheim) according to the manufacturer's instructions and used as probes in chromosome analysis.

The chromosome spreads from selected clones were prepared according to typical cytogenetic techniques (Lawrence et al., 1988). Cytogenetic preparations were pretreated with 0.05% pepsin solution and dehydrated through cold 70%, 90%, and 100% ethanol. The preparations then were denatured using a 70% formamide solution. Hybridization conditions were as previously described (Palombo et al., 1998). Visualization of the biotin-labeled probe was carried out by repeated incubations with Cy3-avidin (Amersham), biotinylated anti-avidin D (Vector Laboratories), and Cy3-avidin. The digoxigenin-labeled probe was detected using mouse anti-digoxigenin, digoxigenin-labeled anti-mouse, and FITC-labeled anti-digoxigenin antibodies (Boehringer-Mannheim). Alternatively, FITC-avidin (Vector Laboratories) and rhodamine-labeled anti-digoxigenin (Boehringer-Mannheim) antibodies were used. After immunodetection, slides were counterstained with 200 ng/ml 4',6'-diamidino-2-phenylindole. UV excitation was used to locate metadamine-labeled anti-digoxigenin (Boehringer-Mannheim) and FITC-avidin signal or blue violet (Cy3 or rhodamine) illumination. Images were processed using Adobe Photoshop on an Apple Quadra computer.

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