

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

Luisa Pieroni,* Cristina Fipaldini,* Andrea Monciotti,* Daniela Cimini,† Antonella Sgura,† Elena Fattori,*
Olga Epifano,* Riccardo Cortese,* Fabio Palombo,* and Nicola La Monica*¹

*Istituto di Ricerche di Biologia Molecolare "Piero Angeletti," P. Angeletti, Via Pontina Km 30,600, 00040 Pomezia, Italy; and
†Department of Genetics, University of Rome, 00100 Rome, Italy

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Adeno-associated virus (AAV) integrates its genomic DNA into a defined region of human chromosome 19 (AAVS1). The specificity of integration is dependent on the presence of the inverted terminal repeats (ITR) and on expression of the *rep* gene. To develop vectors capable of targeting the insertion of a selected DNA sequence into a specific location of human chromosome, we determined whether the *rep* gene can mediate site-specific integration when cloned outside of an ITR-flanked transgene cassette. HeLa and Huh-7 cells were transfected with a plasmid containing the *rep* gene, as well as the green fluorescent protein (GFP) and neomycin (*neo*) resistance gene inserted between the ITRs of AAV. Southern blot analysis of individual clones detected Rep-mediated site-specific integration of the ITR-flanked DNA in 25% and 12% of the HeLa and Huh-7 clones, respectively. The localization of the GFP-Neo sequence on chromosome 19 also was confirmed by fluorescent *in situ* hybridization analysis of the transfected HeLa clones. Sequence analysis of the ITR-AAVS1 junction of one of the transfected Huh-7 clones indicated that the insertion of the ITR DNA fragment had occurred at nucleotide 1003. These results have implications for the development of AAV-derived vectors capable of directing the site-specific integration of a gene of interest. © 1998 Academic Press

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

¹ To whom reprint requests should be addressed. Fax: 39–6–91093–225. E-mail: Lamonica@irbm.it.

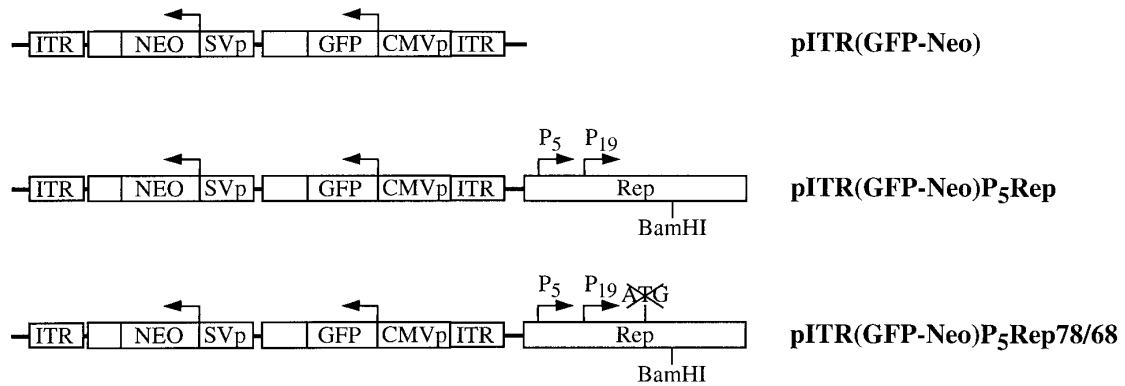


FIG. 1. Schematic representation of the plasmid constructs used in the study. Plasmids pITR(GFP-Neo), pITR(GFP-Neo)P₅Rep, and pITR(GFP-Neo)P₅Rep78/68 contain an GFP and neomycin expression cassette flanked by AAV ITRs. The immediate early CMV promoter and SV40 early promoters as well as the bovine growth hormone (gh) and SV40 polyadenylation signals are indicated. Constructs pITR(GFP-Neo)P₅Rep and pITR(GFP-Neo)P₅Rep78/68 also contain the *rep* gene under the transcriptional control of P₅ and P₁₉ promoters located downstream of the ITR-flanked DNA element. Plasmid pITR(GFP-Neo)P₅Rep78/68 encodes only the two larger Rep polypeptides. Relevant restriction site is indicated. The plasmids were constructed as described in Materials and Methods.

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 (Balagué *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; Koebler *et al.*, 1997; LaFace *et al.*, 1988; Lebkowski *et al.*, 1996; McLaughlin *et al.*, 1988; Phillip *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 AAV 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

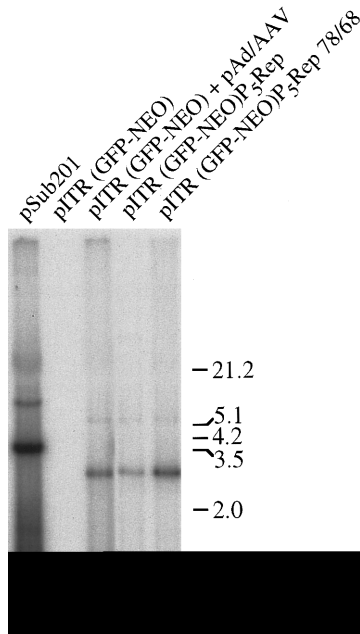


FIG. 2. Southern blot analysis of rescue of AAV genome and of recombinant plasmids from transfected constructs in human 293 cells. Five micrograms of each of the recombinant plasmids were transfected in Ad5-infected 293 cells. Low-molecular-weight DNA was isolated 40 h posttransfection using published protocols (Hirt, 1967), digested extensively with *DpnI*, and analyzed on Southern blots using a ^{32}P -labeled probe specific for the ITR sequence. Rescue and replication of the wild-type AAV from plasmid pSub201 (lane 1) is readily apparent. Similarly, rescue of the ITR-flanked DNA fragment from plasmids pITR(GFP-Neo) P_5Rep (lane 4) and pITR(GFP-Neo) $\text{P}_5\text{Rep}78/68$ (lane 5) is readily apparent. No rescue and replication occurs on transfection of plasmid pITR(GFP-Neo) (lane 2) unless it is cotransfected with helper plasmids pAd/AAV (lane 3).

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) P_5Rep and pITR(GFP-Neo) $\text{P}_5\text{Rep}78/68$ transfected alone displayed the same 3.5-kb *DpnI*-resistant band that hybrid-

ized 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) P_5Rep and pITR(GFP-Neo) $\text{P}_5\text{Rep}78/68$ 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) P_5Rep , 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) P_5Rep and in 7 of 38

A



FIG. 3. Southern blot analysis of Huh-7 *neo*-resistant clones derived from transfection of plasmid pITR(GFP-Neo) P_5 Rep. The conditions of transfection and DNA analysis are described in Materials and Methods. (A) Hybridization to an AAVS1-specific probe. (B) Same member after rehybridization to a *neo* probe. (Arrowhead) Upshifted bands that are annealed to both probes are considered to be indicative of site-specific integration. Positions of molecular weight standards (in kb) are indicated.

(18%) clones transfected with pITR(GFP-Neo) P_5 Rep78/68. Transfection of Huh-7 cells with plasmid pITR(GFP-Neo) P_5 Rep78/68 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 *Bam*HI-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

TABLE 1

Site-Specific Integration in Transfected and Selected Cell Clones

Plasmid	AAVS1-targeted integration in HeLa clones ^a	AAVS1-targeted integration in Huh-7 clones ^a
pITR(GFP-Neo)	0/39 (0%)	ND
pITR(GFP-Neo)P ₅ Rep	8/32 (25%)	3/29 (12%)
pITR(GFP-Neo)P ₅ Rep78/68	7/68 (18%)	2/26 (7%)

Note. Integration into the AAVS1 site was assessed by DNA hybridization analysis with AAVS1- and *neo*-specific probes as described in the text. ND, not determined.

^a Number in parentheses indicates the efficiency of site-specific integration.

and replication assays (data not shown). Thus, the lack of correlation in the hybridization pattern of the *rep* 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)P₅Rep, 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-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-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 (Lamarina *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

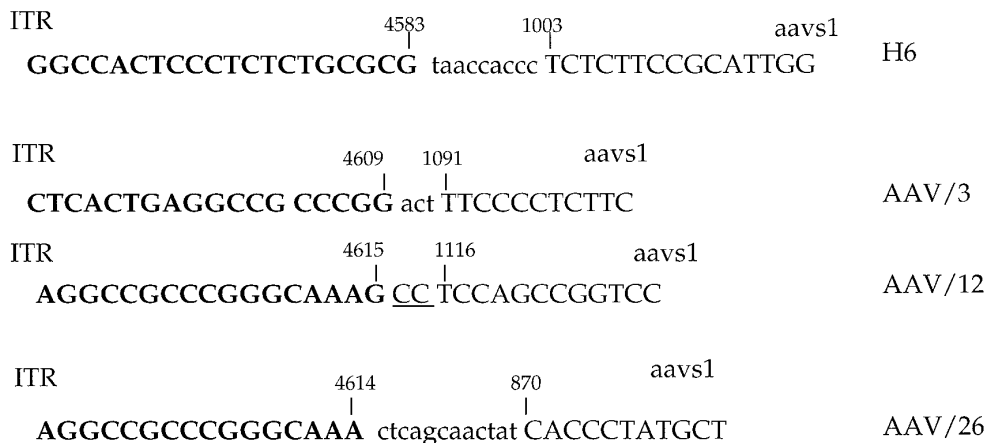


FIG. 4. Analysis of PCR-amplified ITR-chromosome 19 junctions. Genomic DNA from Huh-7 cells infected with AAV virus and from the transfected stable cell line H6 was subjected to PCR amplification using AAVS1- and ITR-specific primers. Clone H-6 was selected from Huh-7 cells transfected with plasmid pITR(GFP-Neo)P₅Rep. Amplified DNA was cloned, and the sequence of the H6 ITR-AAVS1 crossover was compared with three different clones derived from the AAV-infected cells (AAV/3, AAV/12, and AAV/26) (Left) Part of the ITR-derived sequence. (Right) The cellular sequence. (Underlining) DNA overlap between ITR and AAVS1 sequence. (Top numbers) Nucleotide position on the last identifiable viral and AAVS1 sequence. (Lowercase letters) Amplified sequences that cannot be directly associated to either the ITR or the AAVS1.

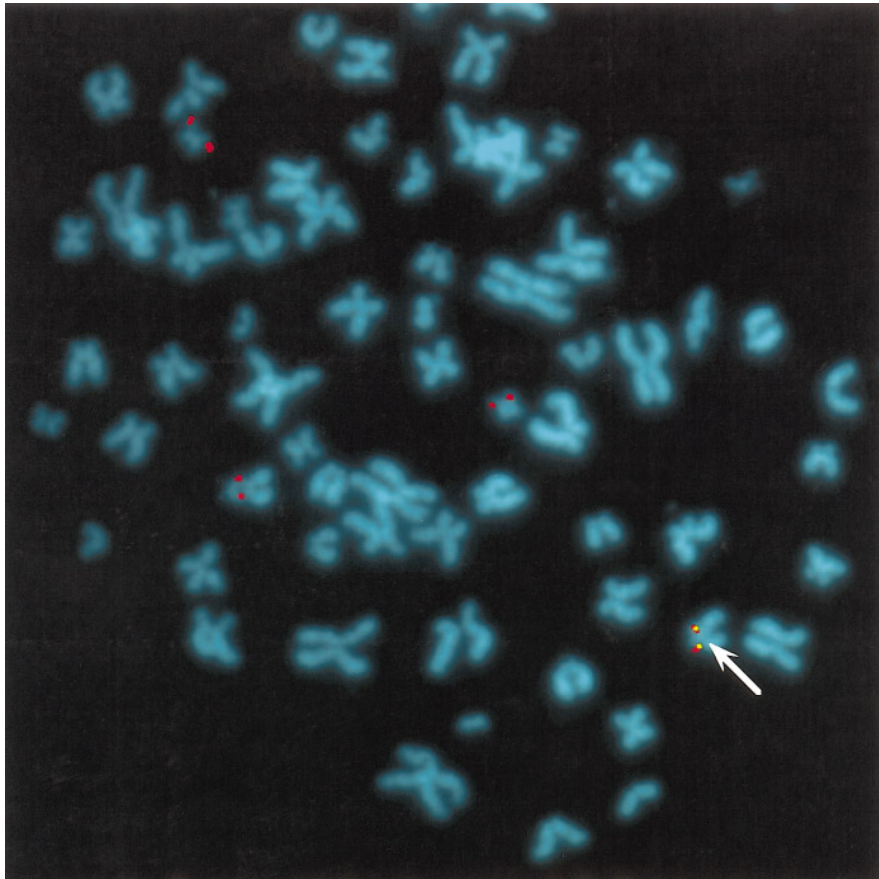


FIG. 5. FISH of GFP-Neo sequences in transfected HeLa cell clone. Chromosome preparation was hybridized with GFP-Neo- or AAVS1-specific probes as described in Materials and Methods. (White arrow) Colocalization of the AAVS1-specific probe (red) and of the GFP-Neo-specific probe (yellow).

(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 P5–12 that had been transfected with pITR(GFP-Neo)_{P5}Rep. 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; Balagué *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 (Balagué *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)_{P₅}Rep 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 transfected 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)_{P₅}Rep 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 *Bam*HI 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 Balagué *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-

tween 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 a 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 await 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 pTR(GFP-Neo), pSub201 (Samulski *et al.*, 1987) was cut with *Xba*I 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 *Nhe*I 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 *Sac*II site located near the *Nhe*I site at the 3'-end. The construct so obtained was named pTR(GFP). Plasmid pTR(GFP-Neo) was produced by inserting the blunted *Eco*RI-*Bam*HI fragment from pRc/RSV (Invitrogen), which contains the SV40 early promoter, neomycin resistance gene, and SV40 polyadenylation signal, into the blunted *Sac*II site of pTR(GFP).

pTR(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 *Cla*I and cloned into the *Cla*I site of plasmid pTR(GFP-Neo).

pTR(GFP-Neo)P₅Rep78/68 was constructed by inserting into the *Cla*I site of plasmid pTR(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 pTR(GFP-Neo)P₅Rep and pTR(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 polypeptides 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 pTR(GFP-Neo)P₅Rep78/68.

The replication capacity of the constructed plasmids was assessed by transfecting 5 μ g 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 *Dpn*I 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 μ g/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

Prime-3 Prime) according to the manufacturer's instructions. Twenty micrograms of plasmid DNA (10 μg of the indicated plasmid plus 10 μg of carrier DNA) were transfected in 1×10^6 cells. Sixteen hours after transfection, 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 $\mu\text{g}/\text{ml}$ 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 *Bam*HI (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'-GCGCGCATAAGCCAG-TAGAGC 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'-TTAACTACAAGGAACCCCTA) and Cr2 (AAVS1) (5'-ACAATGGCCAGGGCCAGGCAG). 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 metaphases, and photographic images were taken with a CCD camera (Photometrics) using green (FITC signal) or blue violet (Cy3 or rhodamine) illumination. Images were processed using Adobe Photoshop on an Apple Quadra computer.

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