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# Adeno-Associated Virus Rep78 Protein and Terminal Repeats Enhance Integration of DNA Sequences into the Cellular Genome

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**Two adeno-associated virus (AAV) elements are necessary for the integration of the AAV genome: Rep78/68 proteins and inverted terminal repeats (ITRs). To study the contribution of the Rep proteins and the ITRs in the process of integration, we have compared the integration efficiencies of three different plasmids containing a green fluorescent protein (GFP) expression cassette. In one plasmid, no viral sequences were present; a second plasmid contained AAV ITRs flanking the reporter gene (integration cassette), and a third plasmid consisted of an integration cassette plus a Rep78 expression cassette. One day after transfection of 293 cells, fluorescent cells were sorted by flow cytometry and plated at 1 cell per well. Two weeks after sorting, colonies were monitored for stable expression of GFP. Transfection with the GFP plasmid containing no viral sequences resulted in no stable fluorescent colonies. Transfection with the plasmid containing the integration cassette alone (GFP flanked by ITRs) produced stable fluorescent colonies at a frequency of 5.3% ± 1.0% whereas transfection with the plasmid containing both the integration cassette and Rep78 expression cassette produced stable fluorescent colonies at a frequency of 47% ± 7.5%. Southern blot analysis indicated that in the presence of Rep78, integration is targeted to the AAVS1 site in more than 50% of the clones analyzed. Some clones also showed tandem arrays of the integrated GFP cassette. Both head-to-head and head-to-tail orientations were detected. These findings indicate that the presence of AAV ITRs and the Rep78 protein enhance the integration of DNA sequences into the cellular genome and that the integration cassette is targeted to AAVS1 in the presence of Rep78.**

Adeno-associated virus (AAV) is a nonpathogenic human single-stranded linear parvovirus that replicates only under permissive conditions (i.e., in the presence of a helper virus) (reviewed in reference 3). However, in nonpermissive conditions, AAV can integrate in the host genome and be maintained as a latent provirus. The particular locus where AAV frequently integrates has been sequenced and mapped to chromosome 19q13.3-qter and named AAVS1 (7, 15, 16, 25). Other less frequent integration sites have been identified as well in the long arm of chromosome 17 in latently infected HeLa cells (32).

The mechanism of AAV integration has not been fully elucidated. However, two viral elements have been implicated in this process: the AAV inverted terminal repeats (ITRs) and the larger forms of the Rep viral proteins (Rep78 and Rep68) (23, 33). The AAV ITRs are palindromic sequences, present in both ends of the AAV genome, that fold into hairpin structures and function as origins of replication. The Rep protein family consists of four different members that share sequences of the same open reading frame. Rep78 and Rep68 proteins are products of unspliced and spliced RNAs, respectively, driven by the p5 promoter, whereas Rep52 (unspliced) and Rep40 (spliced) are driven by the downstream promoter p19 (28). Rep52/40 proteins have been implicated in the accumulation of single-stranded DNA. Rep78/68 activities include sequence-specific DNA binding (2, 12), sequence and strand-specific endonuclease activity (11), and ATP-dependent helicase activity (11, 13). These proteins can bind to a specific sequence in the ITR DNA and promote a process called terminal resolution by which the covalently joined ITR is nicked and replicated (27). A Rep-binding motif and a terminal resolution site (*trs*) have been identified in both the AAV ITR (27) and the AAVS1 integration site (30) and demonstrated to promote in vitro DNA replication in the presence of Rep (30). It has also been shown that Rep68 protein can mediate complex formation between AAV ITR DNA and the AAVS1 site in vitro (33). These findings support a role for Rep proteins in targeting AAV integration and have suggested an integration model in which the DNA binding and endonuclease activity of Rep along with limited DNA synthesis at the AAVS1 site would allow targeted integration of AAV sequences flanked by ITRs (18).

Many reports have addressed the use of AAV as a gene transfer vector in which the *rep* and *cap* genes are substituted by exogenous DNA, keeping only the ITRs. The mechanism of persistence of recombinant AAV vectors is still a matter of discussion. In vitro studies have shown that recombinant AAV vectors can either persist as episomal forms (6, 22) or integrate into the cellular genome (19, 31), albeit in a nontargeted fashion, implying that Rep protein expression is necessary for targeted integration. In vivo data on the persistence of AAV-based vectors have only recently become available. Preferential episomal persistence has been demonstrated in monkey lung epithelial cells (1), whereas in muscle tissue of immunocompetent mice, integration seems to be the mechanism underlying long-term expression (34). In another context, studies performed with plasmids have indicated that AAV-plasmid constructs (expressing Rep), when transfected into cells, may be

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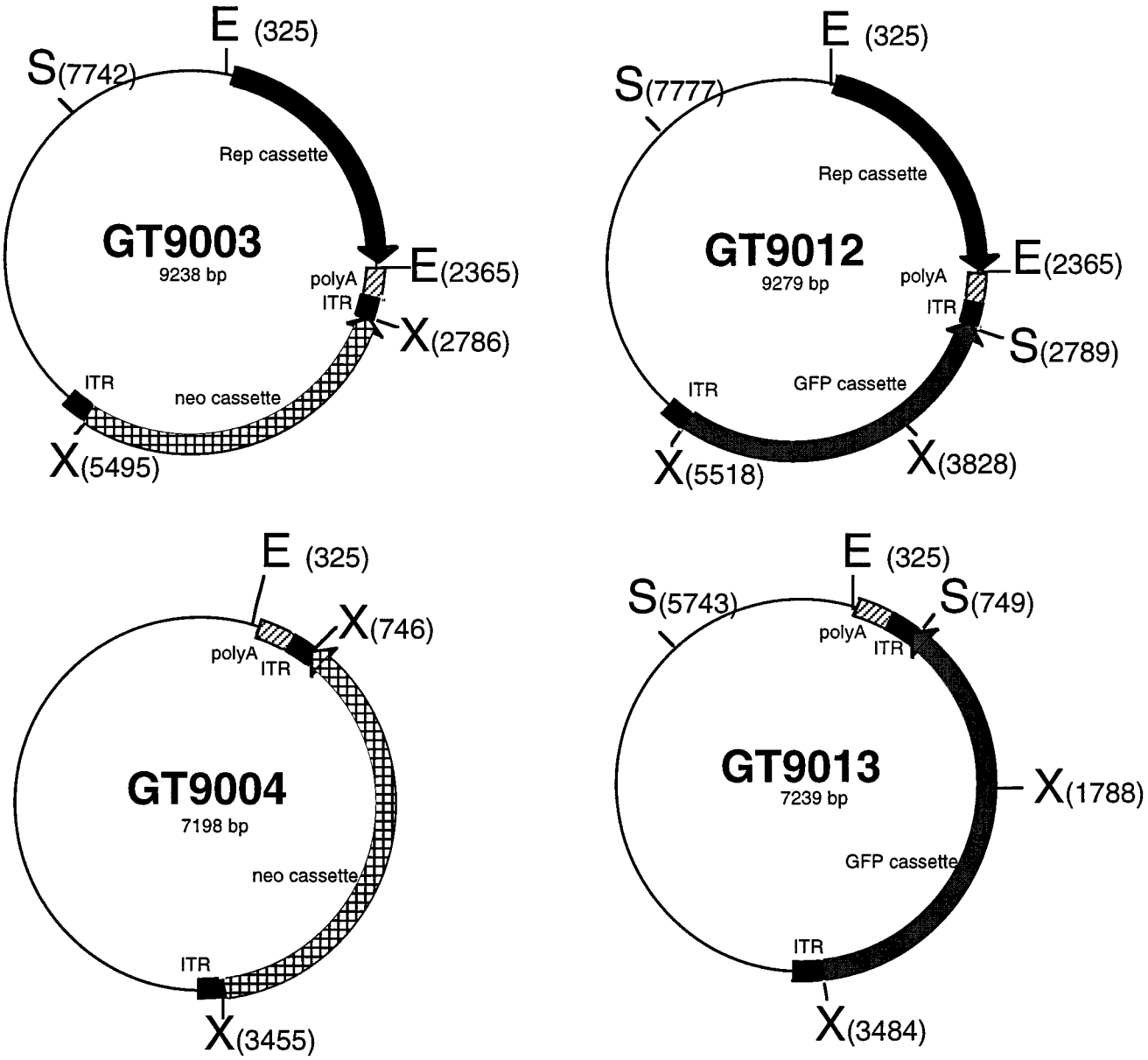


FIG. 1. Schematic representation of the plasmid constructs used in this study. Plasmids GT9003 and GT9004 contain a *neo* expression cassette flanked by AAV ITRs; GT9012 and GT9013 contain a GFP expression cassette flanked by AAV ITRs; GT9003 and GT9012 also contain a Rep78 expression cassette upstream of the integration cassette. Relevant restriction sites are indicated: S, *Sca*I; E, *Eco*RI; X, *Xba*I. See text for details on the construction.

targeted to the AAVS1 site (26). It was also reported that cells transfected with plasmids containing AAV ITRs show sustained transgene expression, although the mechanism accounting for this observation was not thoroughly elucidated (21). Altogether, these findings suggest a complex mechanism of persistence in which AAV ITRs, Rep proteins, and the target cell or tissue are undoubtedly involved.

We have studied the AAV elements necessary for integration in a plasmid transfection assay by comparing the integration efficiency of two different reporter genes (*neo* and GFP) in the presence or absence of AAV ITRs and Rep78 as well as in the presence or absence of selective pressure. This study provides further evidence that AAV ITRs and the Rep78 protein increase the integration frequency of a reporter gene when placed between two AAV ITRs, in contrast to plasmids that do not contain viral sequences. We also demonstrate that in the presence of Rep78 the integration is site specific such that 50% of the clones analyzed show targeted integration to the AAVS1 site on chromosome 19.

A schematic representation of the plasmid vectors used in this study is shown in Fig. 1. To construct plasmid GT9003, *rep* sequences from nucleotides 193 to 2216 in the AAV genome (28) were amplified by PCR with *Pfu* polymerase Stratagene) from plasmid pSUB201 (24) and cloned in pCRII (Invitrogen). This fragment starts right after the ITR and extends through the p5 promoter and the Rep78 coding sequence. The resulting plasmid (GT9000) was digested with *Not*I and *Xho*I, and a fragment containing a simian virus 40 (SV40) poly(A) signal (*Not*I-*Sal*I) was cloned. The resulting plasmid (GT9001) was digested with *Xba*I and filled in with Klenow. A *Pvu*II fragment

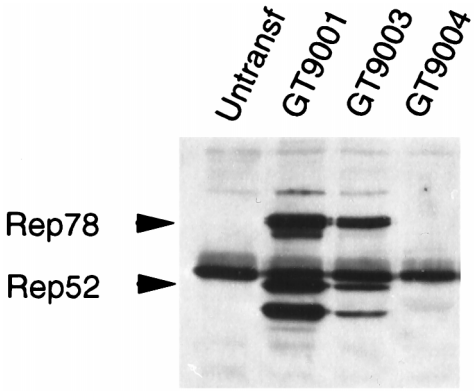


FIG. 2. Analysis of Rep expression from plasmid GT9001, GT9003, and GT9004 in 293 cells. GT9001 contains a Rep78 expression cassette alone. GT9003 contains the same cassette as in GT9001 plus a *neo* integration cassette. GT9004 contains a *neo* integration cassette and no Rep cassette. Cells were transiently transfected by the modified calcium phosphate precipitation method with either plasmid. Immunoprecipitation of Rep proteins was performed with monoclonal antibody 226.7 (American Research Products, Belmont, Mass.) in transfected as well as in untransfected cells. Rep proteins were detected by Western blot analysis combined with chemiluminescence (ECL kit; Amersham). Bands corresponding to Rep78 and Rep52 proteins are indicated.

containing the whole AAV genome was obtained from pSUB201 and subcloned in the blunted *Xba*I site in GT9001. This plasmid (GT9002) was then cleaved with *Xba*I, which removes the AAV coding sequences, keeping only the two AAV ITRs. A *neo* expression cassette (*Bam*HI) was then subcloned in GT9002 by using adaptors *Xba*I-*Bam*HI, giving rise to plasmid GT9003. In this cassette, *neo* expression is driven by the SV40 promoter and contains an SV40 poly(A) signal. Plasmid GT9004 was generated by excising the *rep* promoter plus coding sequences in GT9003 with *Eco*RI. Plasmid GT9012 and GT9013 were generated by replacing the *neo* sequences in GT9003 and GT9004, respectively, by a green fluorescent protein (GFP) expression cassette, consisting of a CA promoter driving GFP expression and an SV40 poly(A) signal.

An analysis of *rep* expression from plasmid GT9003 in transiently transfected 293 cells by immunoprecipitation plus Western blotting revealed two bands corresponding to Rep78 and Rep52 proteins, products of the viral p5 and p19 promoters, respectively (Fig. 2). Two other bands of sizes around 68 and 40 kDa were also detected. Rep68 and Rep40 are products of spliced mRNAs from the p5 and p19 promoters, respectively, that occur when a major splice acceptor site located at position 2228 of the AAV-2 genome is utilized. However, since this major acceptor site was not included in our design, these bands may be products of a minor alternative splice event that utilizes an acceptor site at position 2203 (29).

In a first round of experiments, we analyzed the integration of the *neo* sequences flanked by AAV ITRs in plasmids GT9003 and GT9004. 293 cells were transfected by the modified BBS-calcium phosphate precipitation method (4) with 2 μg of plasmid GT9003 or GT9004 in six-well plates and then selected for 12 days with medium containing G418 (0.5 mg/ml) (Life Technologies). During the selection period, we observed that those cells transfected with GT9003 (Rep-expressing plasmid) grew more slowly but did not show an increased mortality rate, indicating that there was no overt toxicity due to Rep protein expression. In spite of their slower growth, the GT9003-transfected cells generated a higher number of resistant colonies than GT9004-transfected cells. The ratio of GT9003- to GT9004-resistant colonies ranged in different experiments from 3:1 to 12:1. We believe that this variability is due to the transfection efficiency, and it was overcome in subsequent experiments performed without selective pressure (see below). We further tested whether the *neo* gene was targeted to the AAVS1 site. For this purpose, *neo*-resistant colonies were isolated and expanded and genomic DNA was extracted by the salt precipitation method (20). Genomic DNA was digested with *Eco*RI and analyzed by Southern blotting with a probe specific for AAVS1 obtained in our laboratory. *Eco*RI was chosen because the AAVS1 preintegration site is wholly contained within an 8-kb *Eco*RI fragment (15). As shown in Fig. 3A, the expected 8-kb band for the AAVS1 site was detected in all the colonies analyzed as well as in the parental cell line. In addition, five of eight GT9003 transfectants (i.e., 3L1, 2L2, 3L3, 1S3, and 4L5) showed upshifted bands indicating rearrangements of the AAVS1 site. No rearrangements were observed in the GT9004 transfectants, suggesting that this phenomenon is dependent on the expression of Rep. The same Southern blot was stripped and rehybridized with a specific probe for *neo* (Fig. 3B). The presence of *neo*-containing bands of sizes inconsistent with solely AAVS1 site-specific integration suggested that random integration events also took place. Only clone GT9003 2L2 showed a *neo* band matching the one obtained with hybridization to AAVS1 probe. We believed that the lack of correspondence between *neo* bands and AAVS1 bands could be an effect secondary to the selective pressure applied. For this reason, experiments were designed in which selective pressure was not applied. We constructed plasmids GT9012 and GT9013 (Fig. 1) that contain the reporter gene GFP (*Aequorea victoria* green fluorescent protein). This reporter makes cells suitable for single-cell sorting by flow cytometry, thereby eliminating effects of metabolic stress imparted by the geneticin selection. Monolayers of 293 cells were transfected with either plasmid as described above. Twenty hours after transfection, cells falling into a set range of fluorescence were sorted by flow cytometry at 1 cell per well in 96-well plates. By selecting cells in a specific window of fluorescence, variability due to differences in transfection efficiency was eliminated. Two to three weeks after sorting, colonies were scored for fluorescence. This time frame was enough for the single cell to become a visible colony as well as to develop stable fluorescent transfectants. Three independent experiments were performed, and the results are shown in Table 1. The cloning efficiency (number of colonies per total number of seeded wells) reflects the ability of single cells to develop colonies plus a systematic error due to the flow cytometer cloning procedure. The cloning efficiencies observed for cells transfected with either the GT9012 or GT9013 plasmids indicated that, under the conditions used in these experiments, Rep expression did not have a detrimental effect on the colony formation ability. After 2 weeks in culture, 47% ± 7.5% of the colonies derived from plasmid GT9012 were fluorescent, as opposed to 5.3% ± 1% of those derived from plasmid GT9013. The fluorescence intensity for stable transfectants was dimmer than for cells shortly after transfection, an observation that is compatible with the integration of one or a few copies of the GFP expression cassette. Some colonies showed a mosaic pattern of GFP expression; i.e., there was a mixed population of fluorescent and nonfluorescent cells within the same colony. One explanation for this could be that the integration event occurred after the sorted cell started division, giving rise to different populations. For GT9012-derived cells, the fluorescent population within the same colony ranged from 50 to 100% of the cells and was constant in subsequent passages, indicating that the GFP sequences were not lost. For GT9013-derived colonies, we also observed mosaicism, but in this case

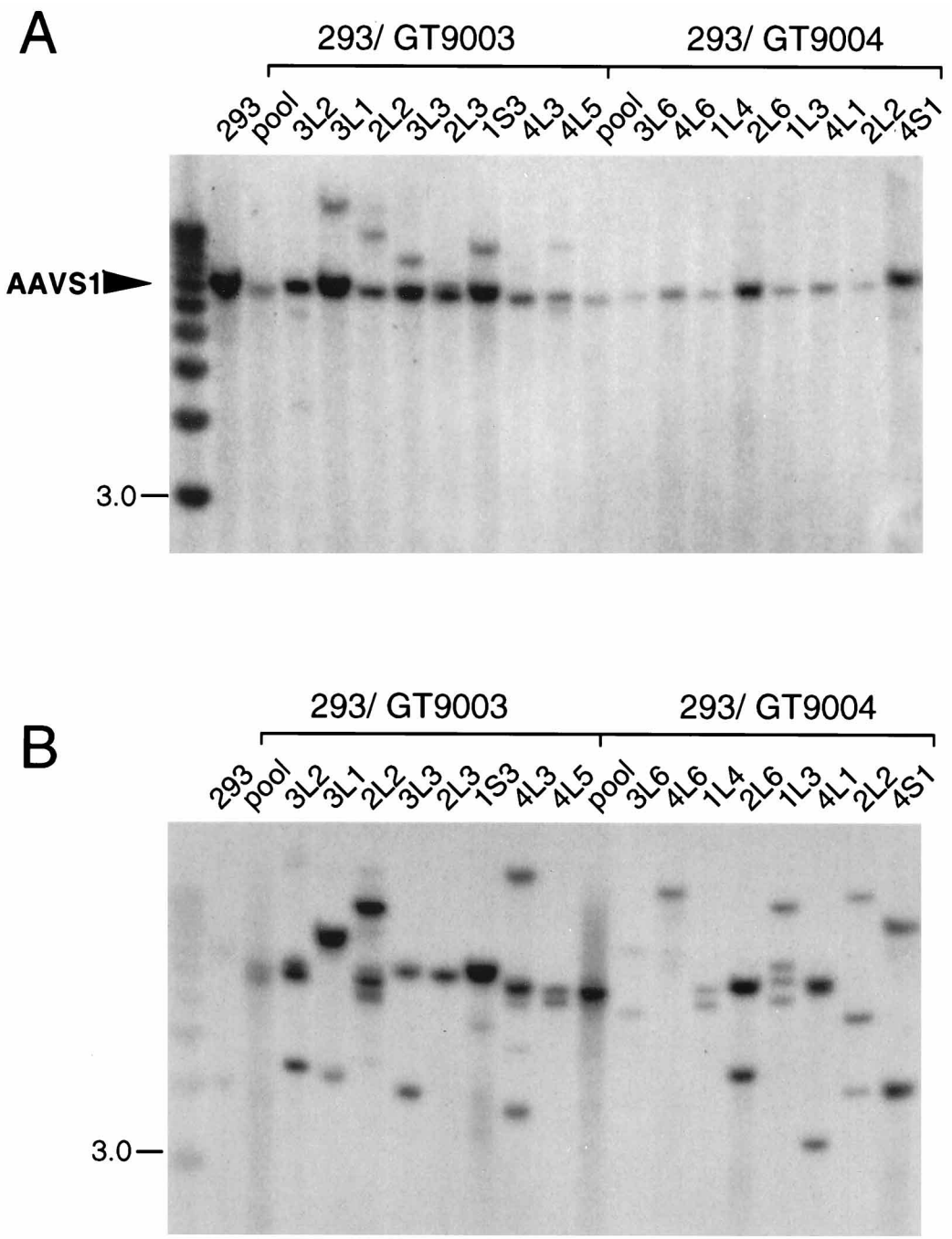


FIG. 3. Southern blot analysis of 293 *neo*-resistant clones derived from transfections with plasmid GT9003 and GT9004. Fifteen micrograms of genomic DNA from each clone was digested with *Eco*RI, electrophoresed in 1% agarose gel, and transferred to a nylon membrane (Hybond-N+, Amersham). Lanes labeled pool indicate a heterogeneous *neo*-resistant population. (A) Hybridization to an AAVS1 probe. An 8-kb band is indicated that corresponds to the AAVS1 preintegration site. Upshifted bands are detected in five GT9003-derived clones corresponding to rearrangements of AAVS1. (B) Same membrane after rehybridization to a *neo* probe. M, molecular size markers (1-kb ladder).

the fluorescent population was never higher than 10%. In a parallel experiment, cells were transfected with a plasmid that contains a GFP expression cassette alone (no viral sequences). No fluorescent colonies were detected 2 weeks after sorting plus single-cell cloning (Table 1). Taken together, these results indicate that the efficiency of integration is enhanced by the presence of AAV ITRs and that it is around nine times higher when Rep is expressed. To further analyze whether the integration of GFP was targeted to AAVS1, several colonies were expanded and genomic DNA was extracted and digested as described above. Figure 4 shows results obtained by Southern blotting with a probe for AAVS1. In 9 out of 13 colonies (i.e., 2D11, 2A11, 2A7, 2C3, 2F12, 2C6, G9, B1, and C1) derived from plasmid GT9012, rearrangements of AAVS1 were detected, whereas no AAVS1-specific rearrangement was observed in GT9013-derived colonies. The same Southern blot

TABLE 1. Integration frequency of plasmids GT9012, GT9013 and pGFP in 293 cells

| Plasmid | Expt 1 | | Expt 2 | | Expt 3 | | Total integration frequency (%) |
|---|---|---|---|---|---|---|---|
| | Cloning efficiency (%)[a] | Integration frequency (%)[b] | Cloning efficiency (%) | Integration frequency (%) | Cloning efficiency (%) | Integration frequency (%) | |
| GT9012 ($Rep^+$) | 92/192 (48) | 43/92 (47) | 42/96 (44) | 23/42 (55) | 93/192 (48) | 37/93 (40) | 47 ± 7.5 |
| GT9013 ($Rep^-$) | 116/192 (60) | 5/116 (4) | 26/96 (27) | 2/26 (8) | 25/192 (13) | 1/25 (4) | 5.3 ± 1.0 |
| pGFP | | | | | 51/96 (53) | 0/51 (0) | 0 |

[a] Number of wells containing colonies/total number of seeded wells. No more than one colony per well was observed.
[b] Number of colonies showing fluorescent cells 2 weeks after sorting/total number of colonies.

was then reprobed with GFP to check for the presence of GFP sequences within the AAVS1 rearranged bands (Fig. 4B). Five of the nine colonies (55%) (2A11, 2F12, G9, B1, and C1) that revealed AAVS1 rearrangements showed bands over 8 kb matching those obtained with an AAVS1 probe, indicating specific integration of GFP. Hybridization to the GFP probe also revealed a 7-kb band in several colonies. This band is not present in the parental cell line DNA, thus ruling out the possibility of a cross-hybridization with human-related sequences. An integration event occurring through any sequence contained between the two *Eco*RI sites in plasmid GT9012 (depicted in Fig. 1) would render a 7-kb band under the conditions used in this Southern blot (i.e., *Eco*RI digestion, GFP probe hybridization). Therefore, in another Southern blot we determined whether this band could be the *Eco*RI digestion product of plasmid GT9012 integrated through *rep* plasmid sequences. For this purpose, genomic DNA from colonies 2A11, 2D11, B1, and C1 that showed the 7-kb band in the previous analysis was digested with *Sca*I (Fig. 1). Nonspecific integration through *rep* sequences would render a GFP-containing 5-kb band when DNA is digested with *Sca*I. Hybridization with a GFP probe revealed the presence of a 5-kb band (Fig. 5A) in all of the colonies analyzed. We also found that the presence of additional bands of 6.0 and 3.0 kb in colonies B1 and C1 was compatible with concatemerization of the integration cassette. We found evidence for tandem arrays placed in both orientations (head to tail and head to head) (Fig. 5B). This observation was reinforced by digestion with *Xba*I, which also cuts inside the integration cassette (Fig. 5C). Clones B1 and C1 showed two bands with sizes of around 1.4 and 2.4 kb that could account for head-to-tail and tail-to-tail concatemers, respectively. These bands were not observed when the membrane was rehybridized with a plasmid backbone probe, indicating that only GFP sequences were present (data not shown).

Taken together, our results indicate that the introduction of AAV terminal repeat sequences in a plasmid vector enhances integration into the cellular genome. Furthermore, the integration efficiency is enhanced in the presence of AAV Rep78 protein and is also targeted to the AAVS1 site in human chromosome 19.

In our system, integration of the sequences flanked by AAV ITRs into the cellular genome may require either excision from the plasmid or replication. Although we did not directly address this question, it has already been shown that rescue of the AAV genome integrated in a pBR322 construct can occur under conditions that are not permissive for AAV replication (i.e., absence of helper virus coinfection) (8, 10). For this process to occur, a complete AAV ITR sequence is necessary, since a 55-bp deletion of the ITR impairs excision (10) and it requires cellular enzymatic activities (8). Thus rescue by excision of the sequences flanked by AAV ITRs is likely to occur in the conditions used in this study.

We have observed correspondence of AAVS1 rearranged bands with reporter sequences in several colonies derived from GT9003 and GT9012 (Rep-expressing plasmids) indicating AAVS1 targeted integration; however, we have also observed AAVS1 rearranged bands that did not show a positive hybridization for reporter sequences. Since these rearrangements are not detected in clones derived from GT9004 and GT9013 plasmids (no Rep cassette), we conclude that they are dependent on Rep expression. It is possible that Rep by itself can promote rearrangements of AAVS1. Alternatively, the sequences integrated in AAVS1 could present some kind of instability in the presence of Rep.

Although the number of clones analyzed was low, two colonies derived from the Rep-positive GFP plasmid showed the presence of integrated tandem arrays; this resembles a wild-type-like mechanism of integration. Different authors have reported head-to-tail tandems for latent wild-type AAV (5, 17, 19), and only one study reported tail-to-tail arrangements of integrated AAV DNA in cloned populations of Detroit 6 cells (14). AAV DNA replication presents head-to-head and tail-to-tail intermediates (9), whereas integrated AAV sequences present mostly head-to-tail arrangements, which indicates that this tandem array is not a consequence of replication during or before the process of integration. Since we have found evidences for the presence of integrated sequences in both head-to-tail and head-to-head tandems in the absence of DNA replication, the current models for AAV integration cannot account for the presence of these tandem arrays and therefore, the question of how these tandem arrays are generated remains to be resolved.

Some biological changes have been reported in cell lines containing stably integrated AAV DNA (14, 32). These changes have been attributed to the expression of Rep. Although we did not address this question extensively, we did observe a slower growth rate of the GT9003-transfected 293 cells during the selection period. Once selection was complete and colonies were isolated, the majority of colonies resumed normal growth rate and were morphologically normal. In our constructs the *rep* gene was driven by the endogenous p5 promoter which functions as a constitutive promoter. Yang et al. (35) have reported that 293 cell lines that express Rep under control of an inducible promoter have a lower growth rate in the induced state and that the expression of Rep increases the population of cells stopped in the S phase of the cell cycle. Our results are consistent with this report, indicating that Rep was expressed during the selection period, promoting a growth delay. The fact that the GT9003-derived colonies resumed a normal growth rate following selection suggests a subsequent loss of Rep expression, either by loss of the *rep* gene or by inactivation of the *rep* gene upon integration. Similarly, no biological alterations have been observed in most of the GT9012-derived colonies, albeit some did show a lower growth rate. Consistent with these observations, Western blot analysis of the GT9012-derived colonies B1, 2A11, and 2C6 failed to

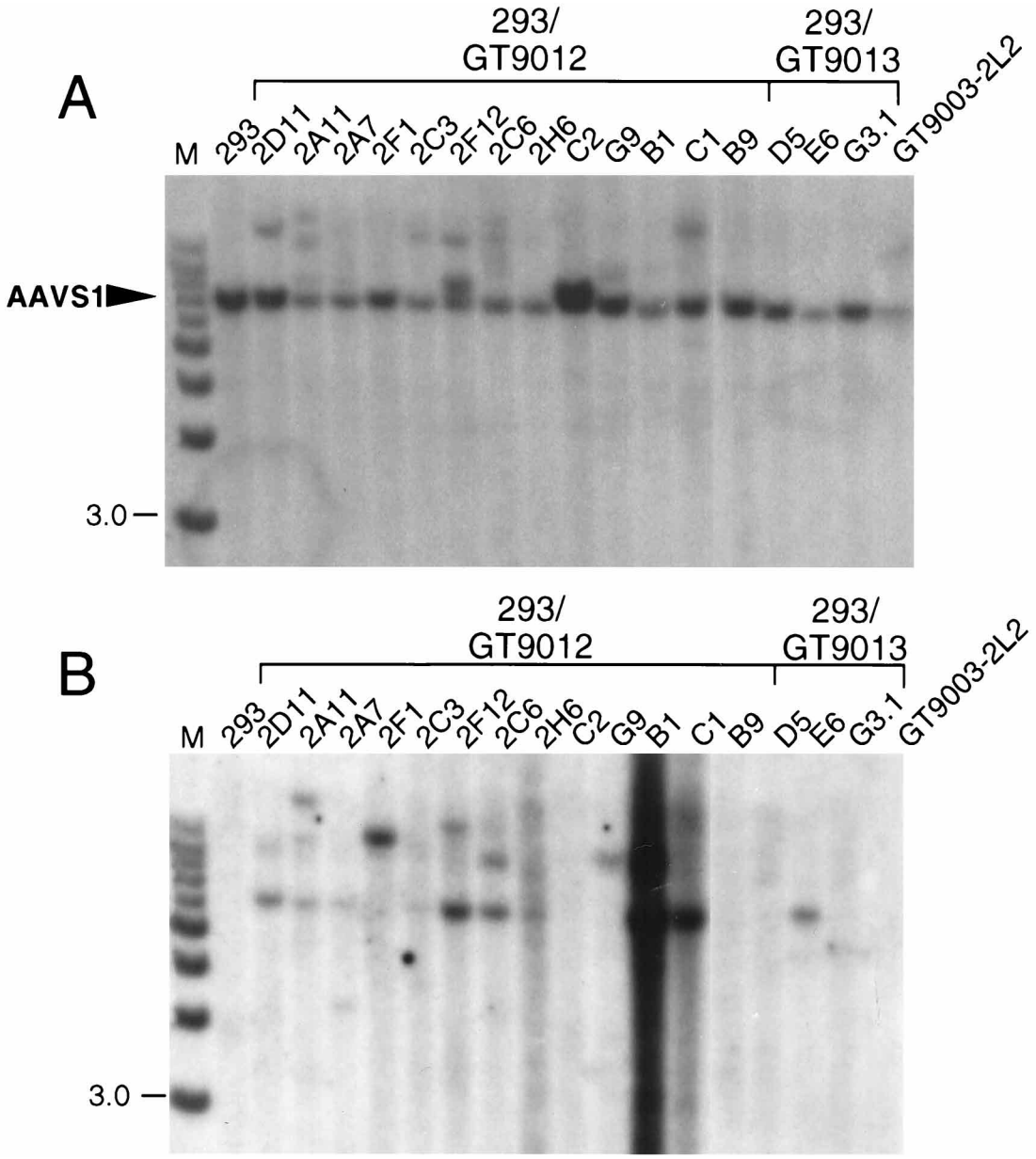


FIG. 4. Southern blot analysis of stable fluorescent colonies derived from plasmids GT9012 and GT9013. Conditions are the same as for Fig. 3. (A) Hybridization to an AAVS1 probe. The band corresponding to the AAVS1 preintegration site is indicated. Upshifted AAVS1-positive bands are detected in several colonies. (B) Same membrane after rehybridization to GFP sequences. M, Molecular size markers (1-kb ladder).

demonstrate Rep expression in these colonies (data not shown). At the DNA level, as shown in the experiments performed with GFP, the finding of integration events that occurred through the Rep expression cassette is also consistent with the impairment of Rep expression. This pattern of integration appears to be very common throughout the clones studied. Since constitutive Rep expression may slow down growth, it is possible that cells expressing Rep are underrepresented in a given colony. Another possibility is that unknowingly we may have picked fast-growing colonies for further analysis, therefore selecting for those having impaired Rep expression. Alternatively, there may be DNA sequences (other than ITRs) playing a role in integration.

It is important to note that the present experiments were performed in 293 cells, in which Rep is likely to be expressed at higher levels from the p5 promoter due to constitutive E1a expression. For this reason, it would be of great interest to analyze whether these results can be extended to other E1a-negative cell lines. Preliminary experiments performed in Chang liver cells indicate that there still may be an enhancement of integration due to Rep expression, albeit to a lesser degree (data not shown). The lower integration frequency in these cells may correlate with a lower level of Rep expression. Further investigation on the Rep-mediated integration in these cells is warranted.

Our results obtained with the GFP vectors also indicate that the AAV terminal repeat sequences can enhance integration. Although the percentage of GT9013-derived cells for a given

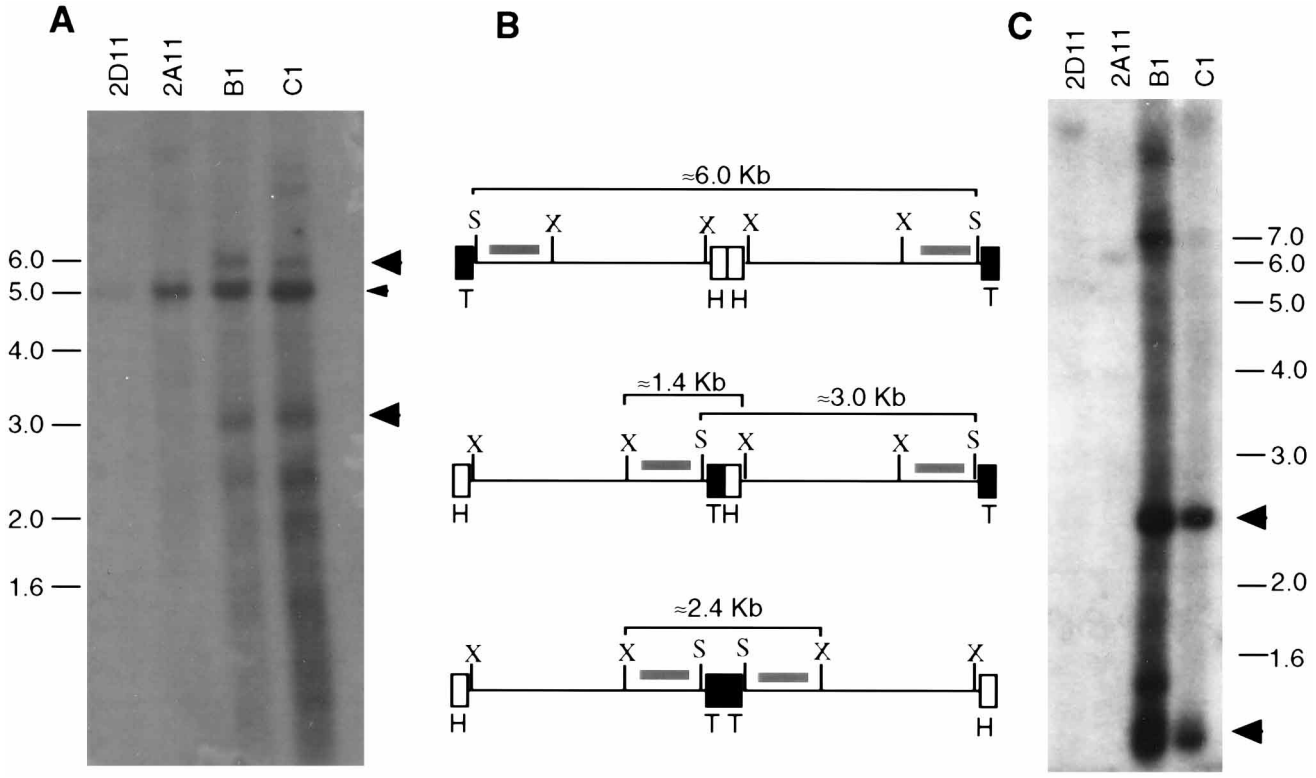


FIG. 5. Analysis of genomic DNA from GT9012-derived colonies. Conditions are the same as for Fig. 3. (A) Genomic DNA was digested with *Sca*I, and membrane was hybridized to a GFP probe. A 5-kb band (small arrowhead) is detected in all four clones analyzed and corresponds to integration of GT9012 through *rep* sequences. Bands with sizes of 3 and 6 kb are also detected (big arrowheads) and can be explained as tandem arrays. (B) Possible tandem arrangements and expected fragments from digestion with *Sca*I (S) or *Xba*I (X). For clarity, each ITR in a cassette is depicted as an empty box (head, H) or filled box (tail, T), solely to indicate the orientation. The gray solid bar encompasses the GFP probe used in this experiment. (C) Same clones as in panel A digested with *Xba*I. Expected bands with sizes of 1.4 and 2.4 kb are indicated.

colony showing fluorescence was never higher than 10%, this frequency of fluorescent colonies was higher than for a vector that does not contain any viral sequence. We have not found any evidence of AAVS1-targeted integration in these Rep-negative clones, although the number of clones analyzed was small. Nevertheless, the fact that AAV ITRs per se can enhance integration, albeit in a random fashion, suggests that ITR sequences present some features that make them more prone to recombination with genomic DNA. A cellular enzymatic activity like endo R (8) might rescue the sequences flanked by ITRs, which along with the secondary structure of the AAV ITR could account for this increase in recombination.

In summary, the presence of AAV ITRs and the Rep78 expression cassette in a plasmid increases its integration frequency into the cellular genome, and the integration is targeted to AAVS1 in the presence of Rep78. At this point, it would be of great interest to test whether these AAV elements can be introduced in other vectors like adenovirus and be used for in vivo stable integration. This hybrid vector would combine the advantages of the adenovirus vector (i.e., high titer, infectivity, and large capacity) and the integration capability of the AAV vector. Whether a hybrid system like this can work requires further investigation.

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