Effects of Adeno-Associated Virus DNA Hairpin Structure on Recombination‡

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Received 12 November 2004/Accepted 28 December 2004

Hairpin DNA ends are evolutionarily conserved intermediates in DNA recombination. The hairpin structures present on the ends of the adeno-associated virus (AAV) genome are substrates for recombination that give rise to persistent circular and concatameric DNA episomes through intramolecular and intermolecular recombination, respectively. We have developed circularization-dependent and orientation-specific self-complementary AAV (scAAV) vectors as a reporter system to examine recombination events involving distinct hairpin structures, i.e., closed versus open hairpins. The results suggest that intramolecular recombination (circularization) is far more efficient than intermolecular recombination (concatemerization). Among all possible combinations of terminal repeats (TRs) involved in intermolecular recombination, the closed-closed TR structures are twice as efficient as the open-open TR substrates for recombination. In addition, both intramolecular recombination and intermolecular recombination exhibit the common dependency on specific DNA polymerases and topoisomerases. The circularization-dependent and orientation-specific scAAV vectors can serve as an efficient and controlled system for the delivery of DNA structures that mimic mammalian recombination intermediates and should be useful in assaying recombination in different experimental settings as well as elucidating the molecular mechanism of recombinant AAV genome persistence.

Hairpin (HP) DNA structures are recombination intermediates in systems ranging from bacterial transposons to mammalian V(D)J recombination (for reviews, see references 8, 10, and 11). The proteins that carry out the processing of terminal HPs are evolutionarily conserved in bacteria, yeast, plants, and animals (10, 12–14, 21). The linear single-strand DNA genome of adeno-associated virus (AAV) forms similar HP structures at its ends, through base-pairing of inverted terminal repeats (TRs), and also undergoes extensive recombination, forming DNA circles and concatamers after infection (17, 25, 31). Thus, AAV provides a useful model for studying mammalian DNA recombination involving HP structures.

Self-complementary AAV (scAAV) is a derivative of conventional recombinant AAV which we have recently developed as an enhanced-efficiency gene delivery vector (15). While conventional single-stranded recombinant AAV (rAAV) requires second-strand synthesis before genes can be expressed, scAAV bypasses this step by delivering a duplex genome. This was achieved by deleting a minor portion (28 bases of 145 bases) of the sequence of one TR such that it no longer serves as a replication origin but still forms the wild-type (wt) AAV TR HP structure (16). Rolling HP replication from the remaining wt TR creates single-stranded, dimeric inverted-repeat genomes, with the altered HP sequence situated in the middle of the molecule and a wt TR at each end (see Fig. 1A, B, and C).

When the DNA is folded to a double-stranded molecule, a closed HP end (C) is formed from the altered TR, and an open end (O) from the two wt TRs (see Fig. 1C). The scAAV genome not only mimics the structure of the single-stranded rAAV genome after second-strand synthesis but also creates a novel feature: controlled orientation of the coding sequences with respect to the closed and open HPs (see Fig. 1C). These unique features allowed us to devise a marker rescue system to investigate the contributions of the HP structures to the relative frequencies of intramolecular and intermolecular recombination. In addition, we were able to investigate the cellular factors involved in TR HP recombination. In designing our system, we employed a previously described orientation-non-specific split-gene vector strategy (1, 6, 18, 23, 28, 33). For intramolecular recombination, we engineered an orientation-specific split-gene vector that would depend on circularization for functional transgene expression (see Fig. 1A). For intermolecular recombination, we cloned two half-gene segments into separate scAAV vectors with defined orientation of coding sequences with respect to the open and closed TR HP (see Fig. 1B). Using these reagents, we were able to specifically study the intramolecular recombination frequency between closed and open TRs on the same genome as well as score specific intermolecular recombination events between two split vectors (i.e., closed-to-closed [C-C], open-to-open [O-O], and closed-to-open [C-O] HP ends) by functional expression of the transgene product (see Fig. 1B).

Among the novel findings we report are that the majority of vector genomes undergo intramolecular recombination (circularization) within 24 h. Preexisting free DNA 5’ and 3’ ends are not required for intramolecular or intermolecular scAAV genome recombination, and recombination between C-C genome ends is preferred. Furthermore, both intramolecular and
which were not resolved (see arrow), while the same digest on the single-cut enzyme, SacI, created fragments with 1.1 kb and 1.8 kb cassette, for example, digestion of the GFP2 genome (lane e) with the denaturing conditions. For determining the direction of the transgene twice the size of the fragment excised from digested plasmid (P) under vertical arrowhead). The undigested hairpinned virion DNA (V) is genomes (unique restriction enzyme recognition site indicated with a (D) Alkaline gel electrophoresis of undigested and single-cut viral vectors in concatemerization and circularization recombination assays. Illustrated. Both serve as positive controls for comparison to the split vectors in concentamerization and circularization recombination assays. (D) Alkaline gel electrophoresis of undigested and single-cut viral genomes (unique restriction enzyme recognition site indicated with a vertical arrowhead). The undigested hairpinned virion DNA (V) is twice the size of the fragment excised from digested plasmid (P) under denaturing conditions. For determining the direction of the transgene cassette, for example, digestion of the GFP2 genome (lane c) with the single-cut enzyme, SacI, created fragments with 1.1 kb and 1.8 kb which were not resolved (see arrow), while the same digest on the intermolecular recombinations require similar host cell factors, such as DNA polymerases and topoisomerases. In addition to the utility of scAAV as a general model for DNA recombination, the elucidation of the principles governing rAAV vector recombination and persistence is important for its use as a therapeutic vector in humans.

MATERIALS AND METHODS

Maintenance of cells. HeLa and 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma). C12 cells used for infectious centers assay (2, 38) were grown under the same conditions as HeLa cells with the addition of 0.5 mg/ml G418 (Invitrogen). Cells were grown at 37°C in a 5%-CO2 humidified incubator.

Plasmid constructs. The expression cassette of the split vectors and circularization-dependent scAAV-GFP(c-d) vector contain a cytomegalovirus promoter, a green fluorescent protein (GFP) transgene split into a 5’, left half (GFP-L) and a 3’, right half (GFP-R), and an hCG intron (29) inserted between GFP-L and GFP-R, followed by a polyadenylation site after GFP-R. The GFP-L and GFP-R fragments were PCR generated using primers containing restriction sites: GFP-Ltop (Acc65I, MluI), 5’GGTACCACGCCGCTGCTACATAACTTACGGTTAA; GFP-Lbot (BglII, MluI), 5’TCTGACCTCTTTCTTACGTCTCTACAGG; GFP-Rtop (PstI), 5’TCTGACGGATCCACGCTTCCTACAGGGAGGCGCAACA; and GFP-Rbot (SalI, MluI), 5’GTCGACACCGCGTTTAAAATAACCTCCCCAACA CTTCCC. GFP-Lbot also contained the 5’ splice donor site of the intron. The remainder of the hCG intron was amplified from pCMV-Laz-Int (28) using the following primers: IntKPtop (Acc65I, BglIII), 5’GGTACCAGATCTTCTTACCTTCAGCTCGATGCGGTTCAC; IntKPtrb (PstI), 5’GCCCTGCAAGGTGGAAACCAAGA CAAG. The GFP-L fragment or the intron plus GFP-R fragment were cloned into both orientations into the AAV construct, pHpa7 (26), between Hpal and Xbal by blunt-end ligation. Lambda phage M13III stuffer fragments of 956 bp and 1,256 bp were cloned into the MluI sites of GFP-L and GFP-R, respectively. To generate the scAAV-GFPced vector, the GFP-L fragment was placed downstream of the GFP-R fragment and cloned into pHpa7 as above. As positive controls, the intact expression cassette with intron was cloned into pHpa7 as above.

Virral production and characterization. Viruses were generated using the triple-transfection method as described previously (32). The virus was purified by discontinuous iodixanol gradient separation and heparin chromatography (27). Five independently prepared batches of split scAAV vectors, three purified preparations and two cleared freeze/thaw lysates, were characterized by dot blot hybridization and infectious center assay (2, 38) and tested for recombination. The structure and orientation of the scAAV genomes were confirmed by alkaline gel electrophoresis and Southern blotting of virion DNA digested with single-cut restriction enzymes. Vector sequences were excised from the plasmid using restriction enzymes to serve as size markers on Southern blots.

Recombination assays. Circularization assays were performed by infecting cells at a low multiplicity of infection (MOI) with equal infectious units of scAAV-GFPced or the intact cassette vector. For concatemerization assays, equal infectious units of L1 or L2 and R1 or R2 split scAAV vectors were coinfectcd into HeLa, HEK 293, and CHOK1 cells at the indicated multiplicities for 3 to 4 h and then replaced with fresh medium. Cells were harvested 24 h postinfection (p.i.) and scored by fluorescence microscopy or by FACScan (Becton-Dickinson) fluorescence-activated cell sorting as indicated.

Drug treatment. HeLa cells were treated with either 20 mM hydroxyurea (HU), 10 μg/ml aphidicolin (Aph), 1 μM etoposide (Etopo), or 25 mM campothecin (CPT) (Sigma) 24 h prior to and during virus infection. The topoisomerase inhibitors (Etopo and CPT) were characterized on HeLa cells by a dose-response curve to ensure that apoptosis was not induced at these concentrations. Virus was added in a minimal volume and left on the cells for 3 h. The medium and unbound virus were removed and replaced with fresh medium containing the same concentration of drug. Cells were harvested 24 h p.i., and GFP expression was scored by FACScan (Becton-Dickinson).

GFP1 genome (lane f) created fragments with 3.4 kb and 0.7 kb (see arrows), which could be resolved on the gel. The digested virion DNA mobilities were consistent with the predicted sizes of fragments having the hairpin at either the upstream or downstream end. Over 90% of virion DNA was dimeric and orientation specific. a, L1; b, L2; c, R1; d, R2; e, GFP2; f, GFP1; g, scAAVGFP-cd.
RESULTS

A novel system for characterizing hairpin recombination. We created a scAAV reporter system to measure the efficiency of intramolecular and intermolecular recombination between AAV terminal repeat HP structures, which mechanism involves the utilization of free DNA ends (or generation of free ends from a DNA strand) and strand processing, followed by ligation to covalently join the DNA ends. The assay utilizes a CMV-GFP cassette split into halves (5′, left and 3′, right half) which can only be reconstituted by DNA recombination. An intron sequence spanning the recombination junction allows protein expression from the reconstituted gene regardless of the recombination junction (Fig. 1).

The vector used to measure intramolecular recombination (circularization) in this assay was made by situating the 5′/H11032 the recombination junction (Fig. 1). Reconstitution of gene expression will result from recombination between the two ends of a single vector molecule or by joining two molecules in the correct orientation. We established conditions (low MOI) to preclude interactions between two molecules, such that only circularization was measured (see Methods).

Similarly, for measurement of intermolecular recombination, separate scAAV vectors were constructed, carrying either the 5′ or the 3′ half of the GFP cassettes (as described above) in fixed orientation with respect to altered or wt terminal repeats (Fig. 1B). This resulted in a total of four split-vector substrates. In coinfections using different combinations of these vector substrates, transgene expression is observed after intramolecular recombination takes place between specific TR substrates, designated closed-closed (C-C), open-open (O-O), closed-open (C-O), and open-closed (O-C) (Fig. 1B). Two control vectors that can lead to gene expression without the requirement of DNA recombination were constructed with the intact expression cassette (including the exogenous intron) cloned in each orientation with respect to the viral open and closed TR structures (Fig. 1C). Using these reagents, maximum levels of gene expression as well as potential influence of orientation with respect to TR structures on GFP expression could be measured.

The parent plasmids used to generate scAAV viral vectors for this study have been described previously (26). To ensure that the split and intact vector orientations were maintained after vector production, purified virion DNA was extracted and analyzed by alkaline agarose electrophoresis with and without restriction digestion (Fig. 1D). In this analysis, all fragments migrated with the correct mobility as expected in each construct (Fig. 1D). The split-vector constructs were designated L1 (5′, left half of GFP with closed hairpin at downstream end), L2 (5′, left half of GFP with closed hairpin upstream), R1, and R2, respectively. The two intact GFP control vectors were identified as GFP1 and GFP2. The titer of each vector control (GFP2 and GFP1) at 0.2 IU/cell (A) or 0.5 IU/cell (B). Drug treatments were the same as described in the legend to Fig. 4.

Circularization of vector genomes. Intramolecular recombination yielding a circular genome would utilize the closed and open HP of a single DNA molecule. In order to measure only intramolecular and not intermolecular recombination (concatemerization), a low MOI was used in these experiments (0.2 IU/cell or 0.5 IU/cell). Surprisingly, up to 97% of cells infected with the scAAV-GFPcd vector resulted in GFP expression compared to results with intact GFP vectors at the same MOI 24 h postinfection (Fig. 2A and 2B). This suggested that the linear DNA forms of the infected genomes are not stable intermediates and are rapidly converted to circles by recombination between the two HP substrates. In addition, the numbers of cells transduced by control intact vectors, GFP1 and GFP2, were equivalent and not greatly influenced by the juxtaposition of the transgene cassette relative to the open and closed TRs. Since a hallmark of AAV genome persistence in animals is the accumulation of a high-molecular-weight concatemeric DNA vector substrate, the requirement of TR HP structures for such intermolecular recombination was explored.

Closed-end TR is preferred over open-end TR for rAAV intermolecular recombination. A time course measuring intermolecular recombination using the orientation-specific split vector pairwise combinations was performed at 10 IU per cell in a 1:1 ratio at 13, 18, 24, 36, and 42 h p.i. (Fig. 3A). At all time points, the C-C combination was scored as the most efficient substrate, while O-O was least. Consistent with this observation, the frequency of joining C-O and O-C ends was always intermediate (see Fig. 3 and Discussion regarding recombination probabilities). Recombination events plateau at 48 h p.i.,
suggested that most of the substrate molecules were either no longer available or were diluted as a result of cell division.

To test whether increased substrate concentrations influenced intramolecular recombination or if the phenomenon was unique to this cell type, we performed the assay at a higher MOI (20 and 50 IU/cell/virus) in HeLa (Fig. 3B), HEK293, and CHOK1 cells (data not shown). Again, the C-C intramolecular recombination events were consistently twofold greater than the O-O combination (Fig. 3B), with O-C and O-C scored as intermediate (Fig. 3A and 3B). Analyses of intramolecular recombination in HEK293 and CHOK1 cells were nearly identical (Fig. 3A and 3B). The cellular factors or activities required for processing the open and closed HP ends of all four combinations of split scAAV vectors to similar levels that that in the untreated cells. These results suggest that host cell DNA synthesis has a significant role in the recombination process required for AAV TR HP substrate circularization.

Unwinding of DNA, either in conjunction with DNA synthesis or through generation of single-stranded intermediates, introduces conformational stress on closed double-stranded DNA molecules, which can be relieved by topoisomerases (20, 30). We tested the role of DNA topoisomerases I and II in recombination between rAAV TR HP structures by inhibition with CPT and Etopo, respectively (Fig. 2B). The two intact GFP control vectors were unaffected by either topoisomerase inhibitor. In contrast, scAAV-GFPcd was slightly inhibited by Etopo and strongly inhibited by CPT treatment. This suggests that topoisomerase I plays an important role in intramolecular recombination between the open and closed HP ends.

**Inhibition of circularization by drug treatment.** Based on the above results, significant processing of vector DNA would be required prior to recombination, i.e., nicking, unwinding, and/or degradation of the closed HP and/or the open end. Prior studies have determined that recombination between AAV TRs results in nonhomogenous junctions with various size deletions (4, 24, 35). Whether TR recombination involves only breakage and rejoining of preexisting TR HP DNA substrates or requires DNA synthesis is not clear. To further explore this, we assayed DNA recombination in the presence of DNA synthesis and topoisomerases inhibitors.

Two different kinds of inhibitors were used to treat HeLa cells 24 h before and during the infection with the scAAV-GFPcd and control GFP vectors. HU inhibits host cell ribonucleotide reductase and broadly inhibits DNA synthesis. As shown in Fig. 2A, HU treatment completely abolished circularization of scAAV-GFPcd but transduction of neither of the control vectors was inhibited. In contrast, Aph specifically inhibits the cellular DNA polymerases α, δ, and ε. While Aph enhanced transduction with the control vectors, as we had previously described (Fig. 2A) (15), Aph treatment inhibited the efficiency of scAAV-GFPcd to about threefold lower than that in the untreated cells. These results suggest that host cell DNA synthesis has a significant role in the recombination process required for AAV TR HP substrate circularization.

**Differential inhibition of concatemerization by drug treatment.** The study on intramolecular recombination using scAAV-GFPcd is independent of the concentration of vector ends and only assayed recombination between a close-open combination of TRs carried on the same molecule. Intramolecular recombination assays permitted the measurement of drug inhibition effects on the open and closed TR HP structures in the four vector combinations (C-C, O-O, C-O, and O-C). The cellular factors or activities required for processing the closed and open TR structure could thus be inferred.

The HU treatment reduced the concatemerization efficiency of all four combinations of split scAAV vectors to similar levels (Fig. 4A). Likewise, the effect of Aph on recombination between specific TR structures closely paralleled the HU treatment (Fig. 4B). It should be noted that C-C combination showed the greatest sensitivity to these drug treatments. Also, there appeared to be no preference for specific HP substrates for recombination under these conditions, which contrast the results in the absence of drug. This supports the idea that DNA
synthesis is required for recombination between rAAV TR HP substrates. Moreover, these studies suggest additional processing of closed HP ends and further suggest the potential involvement of α, δ, or ε polymerase complexes.

The role of topoisomerases on each combination of TR HP structures was also evaluated. Etopo treatment resulted in significant, but nonspecific, inhibition of each recombination pair (Fig. 4C). In contrast, the CPT treatment generated a pattern of HP substrate preference that was opposite to that seen in the untreated cells (Fig. 4D). The O-O combination was favored for recombination over the C-C combination by 10-fold in CPT-treated cells. The O-C recombinations were also consistently lower than O-O under CPT treatment, in contrast to untreated cells. This suggests that there is a stringent requirement for topoisomerase I in processing closed-end TR HP structures for both intramolecular and intermolecular recombination.

The effect of each inhibitor on intermolecular recombination between O-C pairs of TRs was generally similar in magnitude to the inhibition of intramolecular recombination. This again is consistent with the hypothesis that intramolecular recombination was mechanistically the same as O-C intermolecular recombination but more efficient due to the close proximity of the two ends and the independence from DNA substrate concentration.

DISCUSSION

We used circularization-dependent and orientation-specific split scAAV vectors as a model system for the characterization of recombination between different DNA HP structures. This virus-based delivery system permitted controlled and quantifiable transport of DNA substrates to the nucleus. By taking advantage of the unique properties of the scAAV genome, we could control the orientation of the reporter gene segments between the open and closed TR HP ends. This, in turn, allowed the measurement and characterization of recombination events between different kinds of HP structures, represented by AAV termini. We observed that both the open and closed HP scAAV ends can serve as substrates for circularization and concatemerization. The two TR HP ends are not equivalent in recombination efficiency or sensitivity to drug inhibition. Indeed, it was surprising to discover that the joining of two closed HP ends was more efficient than any combination involving an open TR in concatemerization. However, this advantage was lost under conditions of DNA synthesis inhibition.

Prior to the current study, investigations of recombination between AAV TR HP substrates had relied on amplification of the end products in bacteria or by PCR followed by Southern blot analysis. This approach has limitations (i.e., rearrangement of TR products) but most importantly cannot distinguish between the different starting substrates (3–5, 24, 33, 35). The oriented split vectors allowed us to distinguish between starting HP substrates. In any coinfection, there are four possible combinations of TR HPs that can lead to recombination products (see Fig. 1B). The design of this recombination system allowed us to specifically measure the four possible combinations independently (Fig. 3). This includes the highly efficient circu-
larization of monomeric genomes, which can be considered a competing intramolecular O-C end recombination. While it is possible that some concatemeric genomes arise through intermolecular recombination between these monomeric circles, we do not expect this to be a major contributor to concatemers formed within the time frame of our experiments (24 h) (36), because in vivo data suggest that the generation of concatemeric products from circles form over weeks (31, 35). From our studies, the majority of the recombination that takes place utilizes these HP substrates to form intramolecular products (97%) (Fig. 2). Despite this very efficient reaction, we were able to detect intermolecular recombination at high substrate concentration and to specifically measure the influence of starting HP substrate on efficiency (i.e., C-C) (Fig. 3). These studies have provided for the first time the ability to test the requirement for HP substrate in both intra- and intermolecular recombination.

The importance of using our reporter system to study the efficiency of concatemeration is that the four possible combinations of TR HP substrates can be quantitatively determined. To calculate the overall concatemeration efficiency of any two scAAV vectors used in our study, we can combine the four measured recombination events that lead to GFP expression and those that do not but can be inferred statistically. Total intermolecular recombination efficiency \( E_{\text{total}} \) is the sum of intermolecular recombination efficiencies of all the HP combinations (Fig. 1C); \( E_{\text{total}} = E^{\text{C-C}} + E^{\text{O-O}} + E^{\text{C-O}} + E^{\text{O-C}} \), where \( E_{\text{total}} \) is defined as the percentage of the number of intermolecular recombination events per total number of input genomes. Given the experimental conditions used in this assay and based on the measurements of percentage of GFP-expressing cells for each pair of HP substrates (Fig. 3B, dosage 20 IU/virus/cell), we obtained the following: \( E^{\text{C-C}} = 0.75\% \); \( E^{\text{O-O}} = 0.3\% \); \( E^{\text{C-O}} = 0.45\% \); \( E^{\text{O-C}} = 0.55\% \). Therefore, the total efficiency of intermolecular recombination is determined to be \( E_{\text{total}} = 2.05\% \) (refer to supplemental material for detailed calculation). The remainder of the genomes that did not participate in concatemeration, but as closely as we can measure by using scAAV-GFPed, had formed circles.

While it would be of great interest to examine the structures arising through recombination between specific TR open and closed HP substrate ends, the detailed structure of TR junctions between AAV concatemers has been difficult to determine due to the high GC content and secondary structure of the TR sequence (22, 34). Our calculations suggest that the C-C and O-O products comprise less than 1% of the vector genomes in the nucleus, making direct observation of these junctions by Southern blotting unlikely. Analysis of AAV TR HP structures using current methods, such as PCR amplification, is highly prone to artifacts. This can be attributed to the inefficiency of amplification of junctions containing two copies of the TR compared to that for those with one copy (37; D. M. McCarty, unpublished data).

**Role of host factors.** In previous studies, the junctions in bacterial amplified episomal AAV have been described as one or two imperfect double-strand copies of the TR carrying various crossover points (3–5, 24, 33, 35). Although the exact role *E. coli* may play in the stable isolation of these junctions is unknown, eukaryotic cellular activities that would be predicted to carry out the steps required to form these junctions include an endonuclease to nick the closed HP, an exonuclease to create single-stranded regions, DNA polymerase to fill gaps, and ligase to covalently join the DNA strands. One interpretation of the differential drug inhibition of the various kinds of recombination is that some are more dependent on specific processing steps than others. For example, the C-C combination, which is most affected by both DNA synthesis and topoisomerase I inhibition, may be more dependent on gap filling because this HP must be nicked or otherwise degraded prior to joining. Alternatively, we note that under DNA synthesis inhibition and consequent G1/S-phase (Aph) or S-phase (HU) cell cycle arrest, C-C recombination is reduced to the same base level as the other HP substrate combinations. This raises the possibility that an alternate pathway may utilize all HP substrates equivalently and that additional pathway(s) may selectively utilize C-C substrates which would result in an overall increase in recombination products (Fig. 3). Because DNA replication is tightly coupled and regulated in the cell cycle, the selective pathway identified by our drug studies may also participate in the cell cycle.

We also note that while topoisomerases I and II both appear to participate in the recombination reactions, the former has a far greater effect on closed HP ends and the latter appears nonspecific. It has been proposed that topoisomerase I is involved in Holliday junction-like resolution and in recruiting recombination proteins to the recombination site (30). Camptothecin binds reversibly to the complex of topoisomerase I and nicked DNA but not to the free protein or DNA alone, suggesting that there is a direct enzymatic role for topoisomerase I in processing the HP end (7). This raises the interesting possibility that topoisomerase I mediates an efficient concerted nicking-and-joining reaction using HP substrates, as has been observed in TnI0 transposition (9) and mammalian V(D)J recombination (36). While the CPT treatment also arrests cells in the G1/S phase, the C-C recombination is decreased to a level below that of either O-O or O-C under these conditions. This inverse pattern of preference suggests a more specific requirement for topoisomerase I in the processing of closed-HP substrates and begins to distinguish a mechanism for how different HP substrates are utilized by the host cell recombination machinery.

In a previous study (13), the circularization efficiency of transfected blunt-end DNA substrate was determined to be fivefold greater than that of DNA with HP ends in nonlymphoid mammalian cells. In a separate study (19), DNA fragments with or without AAV TR sequence at the ends were introduced into mouse hepatocytes by transfection. This study concluded that the AAV TR sequence is not required for circularization and concatemeration. However, the AAV TR sequence was left blunt rather than in hairpinned structures, and all the substrate analyzed was delivered by transfection. In contrast, our results suggest that essentially all AAV TR HP substrates utilized in this system participate in recombination events within 24 h of entering the nucleus. It is difficult to determine if AAV TR HP structures are extremely recombinogenic due to a different route of substrate delivery (viral versus nonviral) or due to the availability for forming AAV TR HP conformation. Regardless, our results suggest that the HP structures can participate efficiently in recombination. In addition, another recent report has demonstrated the ability of
scAAV vectors to carry a non-AAV HP substrate efficiently into cells. This creates a new opportunity to systematically study the specific role of HP sequence and/or structure on intra- and intermolecular recombination by viral delivery (X. Zhu, personal communications).

The efficient and predictable delivery of defined DNA substrates to nuclei using this viral system is likely to have broad relevance for mammalian DNA recombination. The circular-strand break repair-deficient transgenic animals), leading to new insights into the role of HP structures in recombination and DNA damage repair.

ACKNOWLEDGMENTS

We thank Dale Ramsden and John Olsen for helpful discussions and Dawn Bowles for critically reading the manuscript. This article is dedicated to Jerri Coleman for her tireless contribution to the UNC Gene Therapy Center.

This work was supported in part by NIH grants HL051818, HL06673, P30DK65088, and GM059299 and NIH grant AI048074 awarded to Douglas McCarthy.

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