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Evidence for Two Nucleotide Sequence Orientations Within the Terminal Repetition of Adeno-Associated Virus DNA

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Duplex adeno-associated virus (AAV) DNA, produced by annealing plus and minus virion single strands, has been digested with several bacterial restriction endonucleases. These studies reveal the existence of alternate secondary structures at the termini of duplex AAV DNA. Analysis of the sites of endo R · *Hpa* II cleavage, the products of complete endo R · *Hpa* II digestion, and the multiple terminal secondary structures leads to the conclusion that there are two possible nucleotide sequences at each end of AAV DNA. A model that attributes the terminal nucleotide sequence heterogeneity to two possible orientations of the first 120 nucleotides at each end of the DNA is proposed; in one case the sequence is 1 to 120; in the other case the sequence is inverted. An origin of the inversion is suggested based on previously described intermediates in AAV DNA replication.

Adeno-associated virus (AAV) is one of a group of animal viruses known as parvoviruses, which are, perhaps, the simplest of all known viruses (2). Like other parvoviruses, AAV contains a small (1.4×10^6 dalton), linear, single-stranded DNA molecule, but unlike most other parvoviruses, both strands of AAV DNA are encapsidated separately so that duplex AAV DNA can be obtained in quantity (3, 6, 20, 22). AAV is also different from other parvoviruses in that it is defective and requires coinfection by a helper adenovirus for a productive infection (1). Because of their simplicity, parvoviruses may be useful probes for the study of intracellular processes such as gene expression and DNA synthesis.

AAV DNA has been found to contain both a natural and an inverted terminal repetition (4, 12, 18). One model that would explain these findings is one involving a terminal symmetrical sequence or palindrome, and we show elsewhere that a terminal palindrome does indeed exist (11). When plus and minus strands of AAV DNA are annealed, duplex molecules with two types of termini are formed. Some of the termini are completely base paired, others have single-stranded regions available for further base pairing, leading to the formation of double-stranded circles and oligomers (12, 18). Consistent with these observations, digestion with bacterial restriction endonucleases produces multiple terminal fragments from each end of AAV DNA (5, 7-10). In this paper we further characterize the structure of the various terminal fragments and propose a model to account for these unusual structures.

MATERIALS AND METHODS

Cells and viruses. AAV-2H (16) was grown on KB cells in suspension culture with adenovirus type 2 helper as described previously (5).

Virus and DNA purification. Virus was purified by banding in CsCl after lysis of infected cells with trypsin and deoxycholate as described (6). DNA labeled with ^3H or ^{32}P was purified by sedimentation through alkaline sucrose, and the resulting single strands were annealed as described (5, 6).

Enzymes. *Hae* II, *Hin*dII, *Hin*dIII, and *Hpa* II were purchased from Bethesda Research Labs, Bethesda, Md. *Hae* III was a gift of D. Brown or was purchased from Bethesda Research Labs. *Bam*HI was a gift of D. Shortle. Bacterial alkaline phosphatase was purchased from Worthington Biochemicals. T4 polynucleotide kinase was purified according to Richardson (21) or purchased from P-L Biochemicals.

Gel electrophoresis. Electrophoresis on 1.4% agarose or 6 or 8% polyacrylamide gels was as described (5, 24). High resolution gels were composed of 20% acrylamide-0.67% bisacrylamide-7 M urea and were run in a buffer of 50 mM Tris-borate (pH 8.3)-1 mM EDTA at 15 V/cm (13, 19). Samples were loaded in 0.05 N NaOH-5 M urea. Gradient gels (3.5 to 7.5% acrylamide) were made as described (17), except that the gel was poured at a 90° angle from the direction in which it was run. Fragments were eluted from gels as described previously (7, 19).

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Terminal labeling. The 5′ ends of the DNA molecules were labeled using polynucleotide kinase in the presence of γ-[^{32}P]ATP (synthesized according to Glynn and Chappell [14] as modified by Maxam and Gilbert [19] after removal of the terminal phosphate with bacterial alkaline phosphatase [14]).

Restriction enzyme digestions. Digestions with *Hin*dII, *Hin*dIII, and *Bam*HI were as described previously (5, 7). All digestions were at 37° for 3 h. The *Hpa* II reaction mixture contained 1 to 10 μg of DNA and 1 to 5 U of enzyme in a solution of 6.6 mM $MgCl_2$, 60 mM NaCl, and 20 mM Tris-hydrochloride (pH 7.4) with a total volume of 100 μl. *Hae* II digestions contained 1 to 10 μg of AAV DNA in a volume of 10 to 50 μl in a buffer containing 6 mM Tris-hydrochloride (pH 7.5), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and one-fifth volume of enzyme. DNA (1 to 10 μg) was digested with 1 to 5 U of *Hae* III in a volume of 10 to 50 μl in a buffer of 10 mM Tris-hydrochloride (pH 7.5) and 6 mM $MgCl_2$.

RESULTS

Ordering of restriction endonuclease fragments. We have previously described the physical ordering of fragments produced by digestion of AAV DNA using *Hin*dII, *Hin*dIII, and *Bam*HI (5, 7). Map orders of the fragments produced by *Hpa* II, *Hae* II, and *Hae* III have also been determined. Figure 1 illustrates the maps determined for the cleavage sites of all six of these endonucleases.

Interconversion of different species of terminal fragments. Restriction endonucleases with a terminal cleavage site outside the terminal repetition create two classes of terminal fragments as determined by electrophoresis through polyacrylamide gels (5, 7, 9). *Bam*HI B represents the left 22% of AAV DNA. The separation of the two species of *Bam*HI B is illustrated in Fig. 2. The two species have been designated B_1 and B_2 in order of increasing mobility. The difference in mobilities between the two species might represent either a difference in molecular weight or conformation or both. To resolve this question, *Bam*HI B_1 and -B_2 were separated and individually denatured and reannealed. In both cases the product of renaturation consisted of approximately equal amounts of species migrating as *Bam*HI B_1 and -B_2 (Fig. 2). Thus, the two species are intercon-

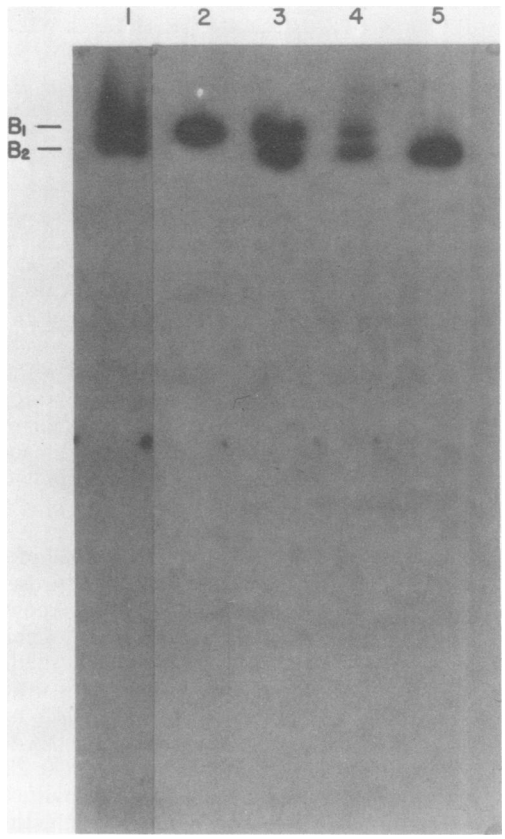

FIG. 2. *Terminally labeled AAV DNA was digested with BamHI and the products were fractionated on a 6% polyacrylamide gel. The two species of B fragments were recovered and then denatured and reannealed and again run on a 6% polyacrylamide gel. Column 1, B_1 denatured and reannealed; column 2, untreated B_1; column 3, mixture of B_1 and B_2; column 4, B_2 denatured and reannealed; column 5, B_2 untreated.*

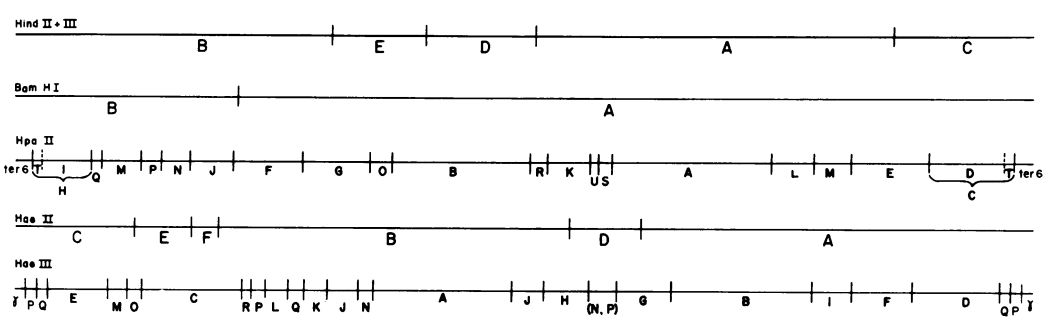

FIG. 1. *Cleavage map of the AAV genome for all restriction endonucleases used in this study: HindII+III (5), BamHI (7), HpaII (Spear, Ph.d. thesis), HaeII (Fife, Ph.d. thesis), HaeIII (Fife, Ph.d. thesis).*

vertible by denaturation and reannealing. These data are most compatible with the difference in mobility between *Bam*HI B_1 and -B_2, reflecting a difference in conformation. If the initial difference in mobility had reflected a difference in molecular weight of normal double helices, denaturation and reannealing of either of the species alone would have resulted in a single product.

***Hpa* II terminal fragments.** Because the terminal cleavage site of *Hpa* II is within the inverted terminal repetition, only a single species of terminal fragment (i.e., the same from both ends) would be expected if double-stranded AAV DNA had a normal double helical structure with a single nucleotide sequence at the ends. Contrary to this expectation, more than six terminal fragments were produced by *Hpa* II digestion. The six terminal fragments reproducibly observed were designated ter 1 to 6 in order of increasing mobility on polyacrylamide gels. Subsequently, it was possible to resolve ter 6 into two species, designated ter 6a and ter 6b on higher percentage gels. To determine the origins of the various *Hpa* II terminal fragments, the right and left ends of duplex AAV DNA were separated before *Hpa* II digestion. Fragments ter 3 through 6 were produced from both ends and thus represent sequences restricted to the terminal repetition (data not shown). Fragment ter 1 comes from the right end and ter 2 from the left end of the molecule, respectively. These species extend beyond the terminal repetition.

As stated above, restriction endonucleases with terminal cleavage sites outside the terminal repetition produce two species of terminal fragments from each end. The relationship of these two species to the various *Hpa* II terminal fragments was determined. *Hind*II +III C_1 and C_2, which represent 0.86 to 1.0 of AAV DNA, were separated, labeled at the 5′ termini with ^{32}P using polynucleotide kinase, and then digested with *Hpa* II (Fig. 3). In spite of some cross contamination, it is clear that *Hpa* II ter 1, 3, 4, and 5 were produced from *Hind*II+III C_1 and that ter 6a and 6b were produced from *Hind*II+III C_2. Thus, the multiplicity of *Hpa* II terminal fragments can be correlated, at least in part, to the conformational differences at the ends of the DNA. *Hae* III also cuts within the terminal repetition and produces multiple terminal fragments. There is a similar correlation of the various *Hae* III terminal fragments with *Hind*II+III C_1 and C_2 (data not shown).

Previous studies have shown that half of the termini of double-stranded AAV DNA have a normal double helical structure (12). To determine which *Hpa* II terminal fragments have aberrant secondary structures, an *Hpa* II digest

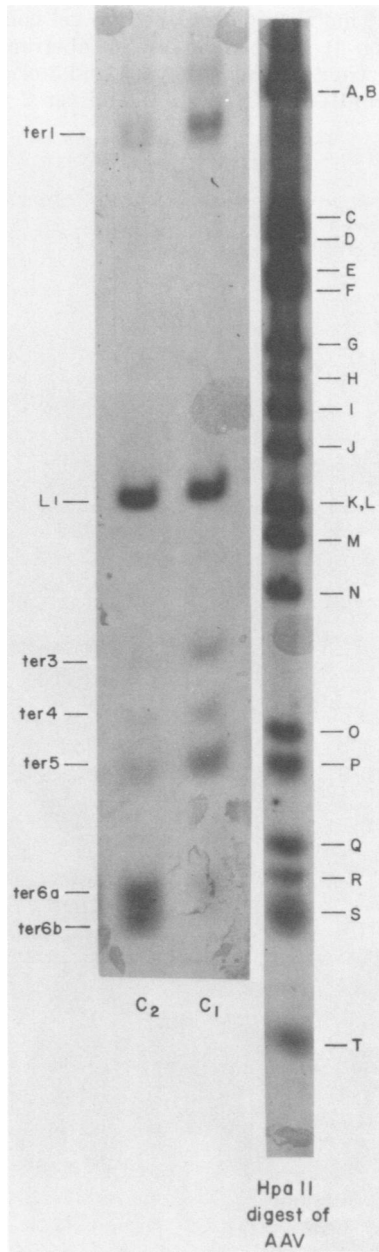

FIG. 3. *AAV DNA was digested with HindII+III and then labeled at the 5′ ends with ^{32}P by using polynucleotide kinase. Fragments were separated on 6% polyacrylamide gels and recovered. The two HindII+III C fragments were each recovered and redigested with HpaII. ^{32}P-labeled AAV DNA digested with HpaII was used as marker. L′ is the internal terminal fragment of both HindII+III C_1 and C_2.*

was analyzed by electrophoresis through a transverse gradient gel of 3.5 to 7.5% acrylamide. *Hpa* II ter 1 and ter 2 and, possibly, ter 3, have an

aberrant mobility dependence on gel composition (Fig. 4) and presumably an aberrant secondary structure. Because ter 1 and 3 originate from *Hin*dII+III C_1 (Fig. 3) and ter 2 and 3 originate from *Hae* II C_1 and *Bam*HI B_1 (data not shown), the decreased mobilities of *Hin*dII+III C_1, *Bam*HI B_1, and *Hae* II C_1 reflect an aberrant secondary structure.

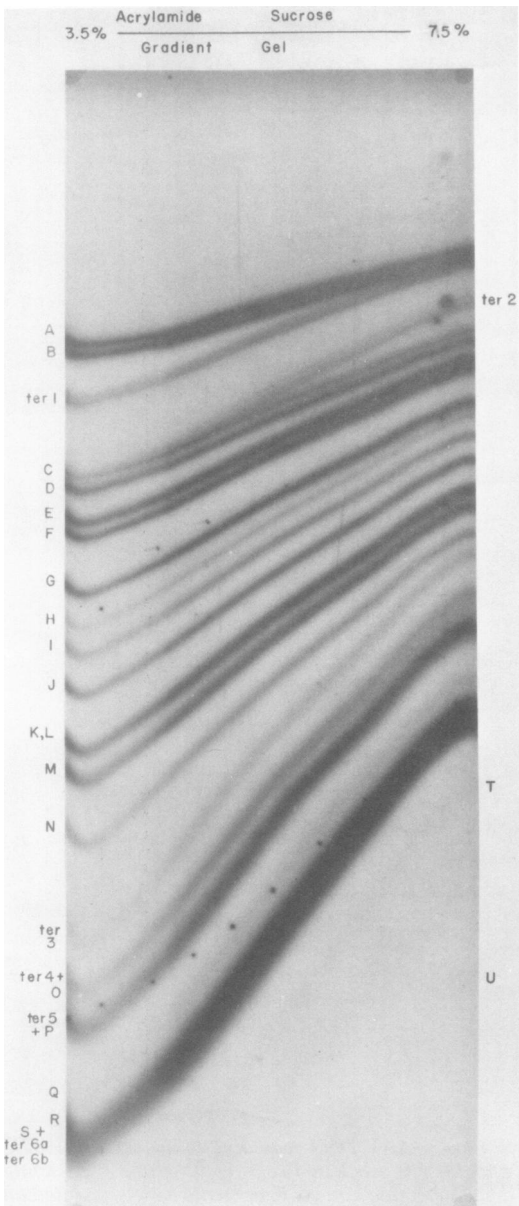

FIG. 4. *Transverse gradient gels of 3.5 to 7.5% acrylamide of ^{32}P-labeled AAV DNA and DNA labeled on the 5′ end with γ-[^{32}P]ATP by T4 polynucleotide kinase and then digested with Hpa II. The specific activity of minor species of terminal fragments are increased by this method and aberrant migration patterns (ter 1 and ter 2) are clearly evident. ter 3 may also have an aberrant migration pattern.*

To further document aberrant secondary structure in various *Hpa* II terminal fragments, ter 1 through 6 were denatured and electrophoresed under denaturing conditions through polyacrylamide (Fig. 5). Fragments with a normal double helical structure should produce a single species of single strands in terms of length and, thus, mobility under these conditions. The existence of multiple single-stranded species in a terminal fragment is strong evidence for an aberrant secondary structure. The analysis is somewhat complicated by the fact that 35% of AAV single strands have the sequence TT at the 5′ terminus, whereas 50% have only a single T in this position (11) and that a one-nucleotide difference in length is detectable in this experiment. Nevertheless, the results were straightforward. *Hpa* II ter 6a and 6b each produced only one single-stranded species (actually two differing in length by a single nucleotide), and ter 6a proved to be four nucleotides longer than ter 6b. The presence of a minority of shorter ter 6b species in the ter 6a channel is considered to represent initial contamination of 6a by 6b. *Hpa* II ter 1 through 5 all produced multiple species of single strands with discrete mobilities. We conclude that all *Hpa* II terminal fragments other than ter 6a and 6b reflect aberrant secondary structures at the ends of double-stranded AAV DNA. Additionally, we conclude that ends with a normal double helical structure produce two terminal fragments, ter 6a and 6b, which differ in length by four nucleotides. Possible reasons for this are considered below.

***Hpa* II cleavage sites within the terminal repetition.** The method of Smith and Birnstiel (23) was used to determine the positions of *Hpa* II cleavage sites within the terminal repetition. Double-stranded AAV DNA labeled at the 5′ termini with ^{32}P was digested with *Bam*HI. *Bam*HI B_2 (0 to 0.22), which has a normal secondary structure at the terminus, was subjected to a partial digestion with *Hpa* II. Those partial digestion products containing the original 5′ terminus of the DNA were detected by autoradiography after polyacrylamide gel electrophoresis, and the relative lengths of these species were determined. These lengths corresponded to the distance of the various *Hpa* II cleavage sites from the end of the DNA. Cleavage sites were found 42, 46, 56, 66, 76, and 80 nucleotides from the end (Fig. 6). Analysis of the products of a complete *Hpa* II digestion showed that *Hpa* II T was the penultimate fragment from DNA with a normal secondary terminal structure (Fig. 1).

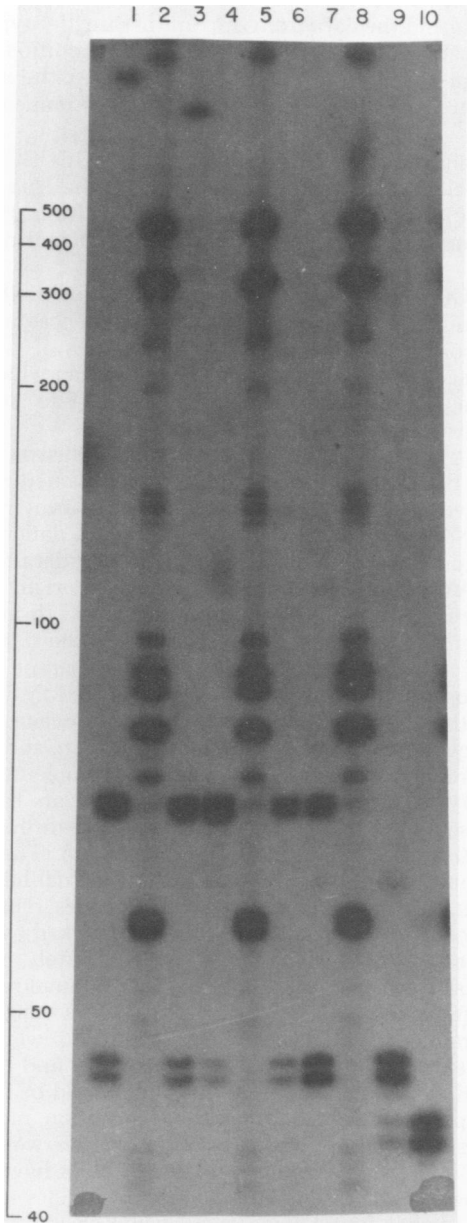

FIG. 5. *Sizes of the strands of the various terminal species. Terminally labeled AAV DNA was digested with Hpa II, and the various terminal species were located and recovered from an 8% polyacrylmamide gel. These fragments were then denatured and loaded onto a high resolution denaturing gel (20% polyacrylamide, 7 M urea) along with marker fragments of known size. The scale at left gives the approximate chain length in nucleotides. Column 1, ter 1; column 3, ter 2; column 4, ter 3; column 6, ter 4; column 7, ter 5; column 9, ter 6a; column 10, ter 6b; columns 2, 5, and 8, marker fragments consisting of a mixture of Hpa II F, O, and Q and Hae III C and L.*

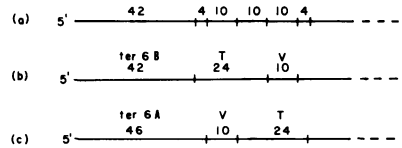

FIG. 6. *(a) Hpa II cleavage sites within the terminal repetition as determined by the Smith-Birnstiel partial digestion technique (23). (b) and (c) Interpretation of the origins of the pattern in (a) assuming an inversion of the first 120 nucleotides in the terminal repetition.*

Hpa II T is 24 nucleotides long and would not be predicted from the results of the Smith-Birnstiel type of analysis. Reasons for this apparent paradox are discussed below.

DISCUSSION

In this paper we have further documented the existence of two types of secondary structure at the ends of double-stranded AAV DNA, one with the properties of a linear double helix, the other with an aberrant secondary structure. These two types of terminal structures are interconvertible by denaturation and reannealing. We had previously suggested that the heterogeneity in terminal structure was the result of a limited number of nucleotide sequence permutations of the type seen in various phage DNAs (12), but the present data do not seem compatible with this hypothesis. It is more likely that there is some mechanism by which complete base pairing between the complementary strands of AAV DNA is prevented in the terminal regions about 50% of the time. The problem with base pairing is confined to the terminal repetition because only *Hpa* II and *Hae* III fragments containing sequences within the terminal repetition are affected (i.e., have alternative secondary structure [K. Fife, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1977; I. Spear, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1977]).

One possibility is that the palindromic nature of the terminal repetition (11) prevents interstrand base pairing. This seems unlikely for two reasons. (i) *Bam*HI B_1 produces equal amounts of B_1 and B_2 upon denaturation and renaturation. If the hairpin caused by self annealing within the terminal repetition were stable enough to resist branch migration, the above result would not be anticipated. (ii) If the extent of self base pairing within the aberrant terminal structure were constant, only one *Hpa* II terminal fragment would be produced from this structure, yet there are several reproducible *Hpa* II terminal fragments of this type.

Another possibility that seems more likely is that there are two nucleotide sequences possible within the terminal repetition. In this case, complementary strands with appropriate nucleotide sequences in the terminal repetition would have ends with a normal double helical structure, whereas a mismatch in this region would result in an aberrant terminal secondary structure. The results illustrated in Fig. 2 are compatible with this possibility. The occurrence of two *Hpa* II terminal fragments (ter 6a and 6b) from ends with a normal secondary structure are also compatible with this idea, although two adjacent *Hpa* II sites might yield the same results. Probably the best evidence for nucleotide sequence heterogeneity is the apparent paradox between the results of complete *Hpa* II digestion and the mapping of *Hpa* II sites within the terminal repetition by the Smith-Birnstiel partial digestion technique (23). No two *Hpa* II sites within the terminal repetition are greater than 10 nucleotides apart, yet complete *Hpa* II digestion yields *Hpa* II T, which is 24 nucleotides long and within the terminal repetition. It might be argued that *Hpa* II cannot cleave a second site within 24 nucleotides of an initial site of cleavage, but we know this is not true because the smallest fragments seen in Fig. 4 are about 10 base pairs in length. The problem is simply resolved if there are two possible nucleotide sequences.

The extent of possible nucleotide sequence heterogeneity within the terminal repetition is limited. We have reported that the first seven nucleotides are the same at the 5′ termini of all AAV strands, with the exception of the number of 5′ terminal Ts (0, 1, or 2) (11). By using the Maxam-Gilbert method of DNA sequencing (19) we have extended this analysis (Fife, Ph.d. thesis). The unique 5′ terminal nucleotide sequence extends for 41 nucleotides. At this point the sequence diverges. Similarly, nucleotides 79 through 140 are constant and, tentatively, 79 through 120 are palindromic to 1 through 41. Thus, any heterogeneity must occur from positions 42 through 78. Interestingly, all the *Hpa* II sites are within this region.

Therefore, it appears that there are two possible nucleotide sequences within the terminal repetition and that the actual heterogeneity occurs from positions 42 through 78. We would like to suggest that the heterogeneity results from two possible orientations of nucleotides 1 through 120. Half of the time these nucleotides would be in the reverse orientation (i.e., an inversion or flip flop). This model would be in agreement with both the nucleotide sequence data and the data from the Smith-Birnstiel-type experiment, as illustrated in Fig. 6. Additional sequencing data support this model. At the inboard end of ter 6b the *Hpa* II site is immediately adjacent to an *Hae* III site (5′GGCCGG3′, nucleotides 40 through 45), whereas the *Hpa* II site creating ter 6a is separated from the same *Hae* III site by four nucleotides (5′GGCCGCCGG3′, nucleotides 40 through 48). The complements of these two sequences are resolvable at the outward 5′ terminus of two species of *Hpa* II I (the fragment within which the left terminal repetition ends) (Fife and Berns, manuscript in preparation).

A possible origin for the inversion is illustrated in Fig. 7. Terminal covalent hairpin structures have been postulated in models of parvovirus DNA replication based on both in vitro data for minute virus of mice (25) and in vivo data for AAV (15, 24) as possible sites of the origin or termination of DNA synthesis. Hauswirth and Berns (15) suggested the possibility of the transfer of the terminal repetition from parent to progeny strand. In the model presented in Fig. 7, the numbers 1 through 5 represent nucleotide sequences within the terminal repetition, and 1′ through 5′ represent the respective complementary sequences. Positions 1 through 41 are represented by 1,2; 42 through 78 by 3,4; 79 through 120 by 2′,1′; and 121 through 140 by 5. There would be an unpaired region at the actual hairpin, which would involve the 37 bases represented by 3,4. The hairpin could be nicked by a site-specific endonuclease approximately 20 bases from the inboard end of the terminal repetition between 1 and 5′ creating a single-stranded 5′ terminus. The resultant gap would then be filled in by DNA polymerase, and the individual plus and minus strands would be encapsidated. A second round of replication, nicking, gap filling, and strand separation would result in half the encapsidated strands having one orientation of the first 120 nucleotides and the other half having the reverse orientation. Purification of this DNA and annealing of plus and minus strands would lead to half the termini having a normal double helical structure and the other half having mismatching of some bases.

We are now investigating further the *Hpa* II partial digestion products to more rigorously test the inversion hypothesis.

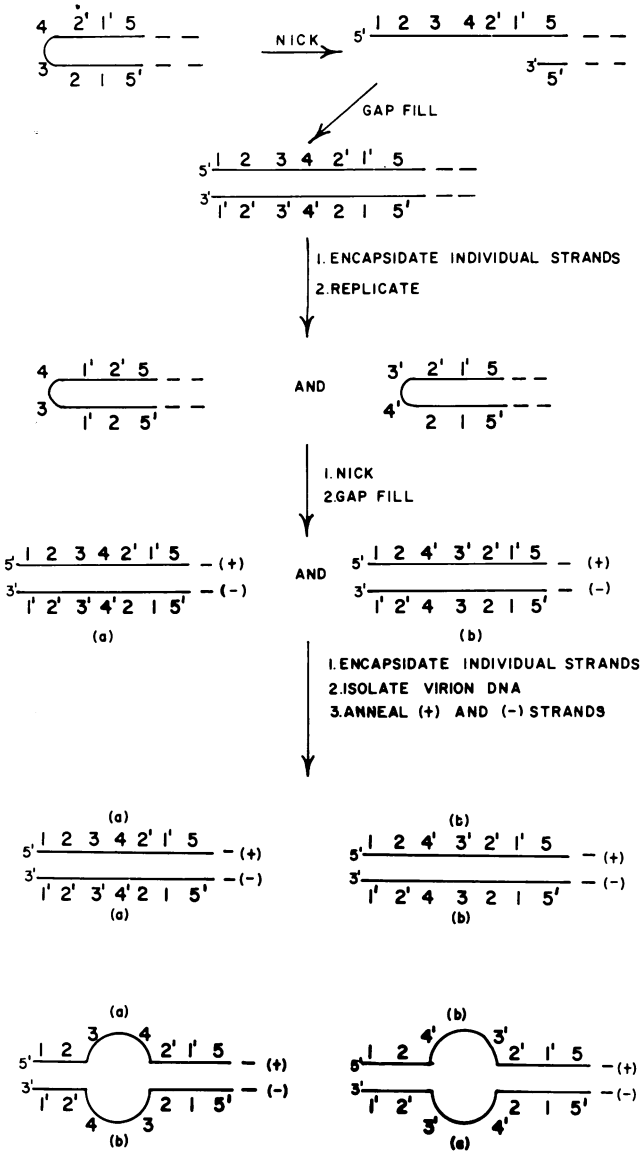

FIG. 7. *Model for the origin of two nucleotide sequence orientations (inversion) within the terminal repetition of AAV DNA. Nucleotide sequences within the terminal repetition are represented by 1 through 5, and 1' through 5' are the complementary sequences. The proposed sequence for the terminal repetition is 12342'1'5 (see text for details). During replication, a covalent hairpin structure forms to link plus and minus strands. The hairpin is nicked by a site-specific endonuclease between 1 and 5', and the resultant gap is filled by DNA polymerase. After strand separation and encapsidation, a second round of replication, hairpin formation, nicking, gap filling, and strand separation results in equal numbers of both nucleotide sequence orientations in the terminal repetition of encapsidated DNA. Isolation of virion DNA and annealing of complementary strands results in four classes of duplex ends; two fully double helical and two incomplete duplexes with non-based-paired regions within the terminal repetition.*

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