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Nucleotide Sequence of the Inverted Terminal Repetition in Adeno-Associated Virus DNA

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The inverted terminal repetition in adeno-associated virus type 2 DNA has been sequenced. The terminal repetition contains 145 nucleotides of which the first 125 nucleotides can self-base pair to form a T-shaped hairpin structure. Both restriction endonuclease analysis with *SmaI* and *BglI* and direct sequence analysis of the *SmaI* fragments provide evidence for two sequences in the region of the terminal repetition between nucleotides 44 and 81. The two sequences represent an inversion of the first 125 nucleotides of the terminal repetition. Based on these data a model for adeno-associated virus DNA replication is presented which agrees in detail with a general model for eucaryotic DNA replication originally proposed by Cavalier-Smith (T. Cavalier-Smith, Nature [London] 18:672-684, 1976).

Adeno-associated virus (AAV) is a defective parvovirus which requires coinfection with adenovirus for multiplication (2, 16). The AAV genome is a linear single-stranded DNA with a molecular weight of 1.4×10^6 (12). Both plus and minus strands are encapsidated with equal frequency but in separate virions (3, 19, 20). Structural studies of the AAV genome demonstrated the existence of an inverted terminal nucleotide sequence repetition (17) which also had the properties of a natural terminal repetition; hence it was suggested that the terminal repetition was palindromic (5, 12). This model was supported by experiments which showed that the DNA in the terminal repetition could fold over on itself to form a stable base-paired hairpin structure which represented approximately 4% of the genome (11).

Because both plus and minus strands are encapsidated, purified AAV DNA can anneal to form duplex molecules. A variety of experiments, primarily involving restriction enzyme analysis, have demonstrated that half of the termini of duplex molecules formed in vitro have a normal double helical structure; the other half have an aberrant secondary structure (4, 6, 9, 24). Fine mapping of the *HpaII* sites within the terminal repetition of AAV-2 DNA by using two methods, complete digestion and the partial digestion technique first described by Smith and Birnstiel (23), yielded paradoxical results (24). The complete digestion data suggested three cleavage sites, but partial digestion revealed six such sites. These data were interpreted as implying the existence of two different nucleotide sequences within the terminal repetition. It was suggested that the two sequences were the result of an inversion of the palindromic region in the terminal repetition.

In this paper, the nucleotide sequence of the terminal repetition has been determined. It is 145 nucleotides long, and the first 125 nucleotides are palindromic. Earlier studies on the 5'-terminal nucleotide sequence of AAV-2 DNA showed heterogeneity in the terminal two nucleotides, but a common sequence thereafter (35% 5'TTGGCCA, 50% 5'TGGCCA, 15% 5'GGCCA [11]). The complete nucleotide sequence of the terminal repetition reveals further heterogeneity in the sequence between nucleotides 44 and 81 which does indeed represent an inversion of the terminal 125 nucleotides. A preliminary description of the overall sequence was given at the Cold Spring Harbor Symposium on DNA Replication, Recombination, and Repair (4).

MATERIALS AND METHODS

Cells and viruses. AAV-2H (16) was grown on KB or HeLa cells in suspension culture with adenovirus type 2 helper as described previously (6).

Virus and DNA purification. Virus was purified by banding in CsCl after lysis of infected cells with trypsin and deoxycholate as described (7). DNA labeled with [^{3}H]thymidine was purified by sedimentation through alkaline sucrose gradients, and the resulting single strands were annealed to form duplex DNA (6, 7).

Enzymes. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. Bacterial alkaline phosphatase was purchased from Worthington Biochemicals Corp., and T4 polynucleotide kinase was purchased from Miles Laboratories, Inc., or Boehringer Mannheim Corp.

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Purification of fragments for DNA sequencing. AAV DNA was digested with a given restriction endonuclease with the conditions specified by the supplier (Bethesda Research Laboratories), and the cleavage products were separated on 6% polyacrylamide gels as described previously (6), except that the *Hae*III fragment (nucleotides 41 to 85) was separated on a 20% gel by using Tris borate-EDTA (TBE) buffer described by Maxam and Gilbert (18). The fragments were eluted from the gel as described by Maxam and Gilbert (18). To obtain better resolution of the terminal fragments produced by *Sma*I or *Bgl*I, AAV DNA was first digested with *Pst*I and the terminal fragments with normal secondary structure were recovered from a 6% acrylamide gel and then digested with either *Sma*I or *Bgl*I. The complementary strands were usually strand separated on 8% polyacrylamide gels by the methods of Maxam and Gilbert (18) with the following exceptions: *Hae*III (nucleotides 41 to 85) was strand separated on a 20% gel, *Pst-Sma* fragment 1 (nucleotides 1 to 46) on a 15% gel, and *Pst-Sma* fragment 2 (nucleotides 1 to 68) on a 12% gel.

Terminal labeling. The 5' ends of the DNA molecules were labeled, using polynucleotide kinase in the presence of [^{32}P]ATP as described by Maxam and Gilbert (18). The [^{32}P]ATP was either purchased from New England Nuclear Corp. or prepared by the method of Glynn and Chappel (13) as modified by Maxam and Gilbert (18).

DNA sequencing. DNA sequencing was performed by the method of Maxam and Gilbert (18) with modifications (A. Maxam and W. Gilbert, personal communication). The amount of carrier DNA was reduced to 1 μg per reaction. Magnesium acetate was omitted from the "stop" solutions. The RNA concentration in the stop solution was reduced to 100 and 25 μg/ml in the purine and pyrimidine stop solutions, respectively. The piperidine reaction was carried out by using 100 μl of 1 M piperidine at 90°C for 30 min in the original Eppendorf snap-cap tubes which were made airtight with plastic tape.

In earlier experiments, 1.5-mm-thick 20% polyacrylamide–7 M urea sequencing gels were used as described by Maxam and Gilbert (18). In later experiments 0.5-mm-thick 12 and 20% polyacrylamide–8 M urea gels were used as described by Sanger and Coulson (22). In most experiments a voltage of 1,800 to 2,000 was used to run the gel at 50°C, as measured by a flat-back dial thermometer clamped to the gel plate. *Pst-Sma* fragments 2 (nucleotides 1 to 68) and 3 (nucleotides 58 to 400) were electrophoresed at about 70°C (2,300 to 2,500 V) to melt out the internal hairpin structure.

RESULTS

Nucleotide sequence of the terminal repetition. We have determined the nucleotide sequence of the inverted terminal repetition in AAV-2 DNA by using the Maxam-Gilbert technique (18) (Fig. 1). Physical maps of the specific

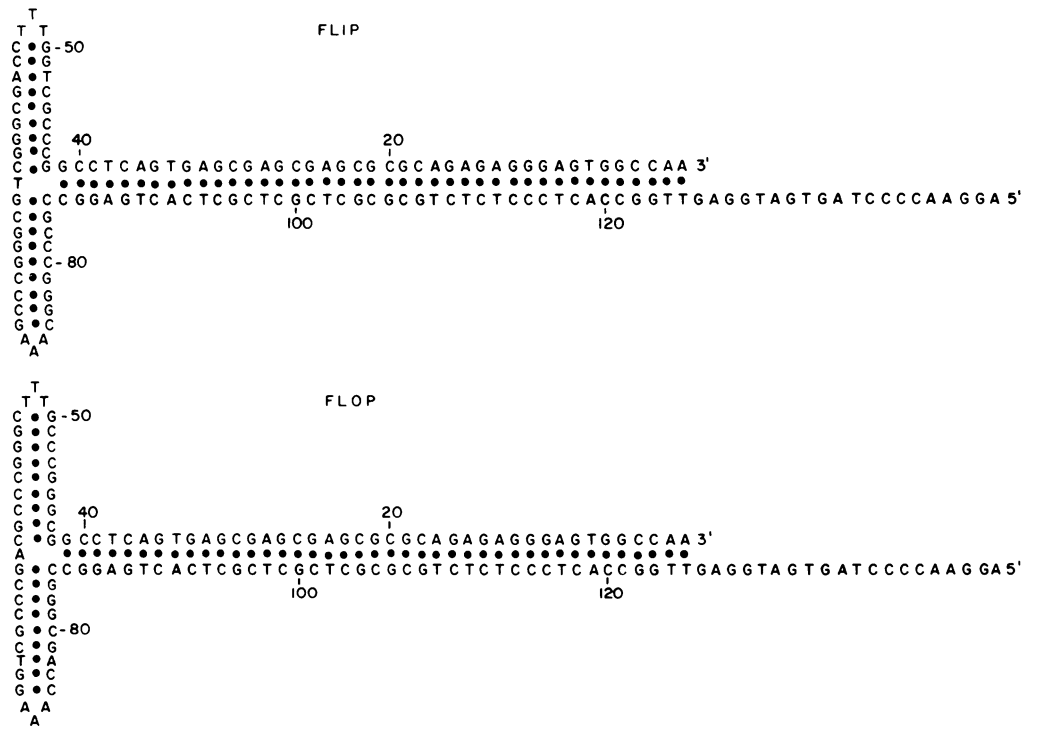

FIG. 1. *Nucleotide sequences of the inverted terminal repetition in AAV-2 DNA. The second sequence (flop) represents an inversion of the first 125 nucleotides. The sequences are represented in the form which contains the maximum amount of self-base pairing.*

fragments used for the sequencing are shown in Fig. 2. Because of the heterogeneity in the number of thymidine residues at the 5′ terminus of the DNA (see above) it was necessary to separate terminal *Hpa*II or *Hae*III fragments differing in length by a single nucleotide on 8% acrylamide gels before the sequence determination for the first 41 nucleotides. Complementary strands were sequenced after strand separation (see above).

The penultimate *Hae*III fragment (nucleotides 41 to 85) yielded an unambiguous sequence. Because this fragment extends beyond the region of heterogeneity and the heterogeneity is caused by an inversion, both strands give an unambiguous sequence. This region contains two short palindromic regions (nucleotides 42 to 62 and 64 to 84) so that hairpinning may occur in some of the longer fragments produced in the sequencing procedure. This may lead to a "compression" in the gel wherein several bands run together. This occurred to a certain extent in both strands of the fragment (Fig. 3), but the bases lost in a compression on one strand are clearly detectable in the sequence of the complementary strand. The sequence of a *Hpa*II

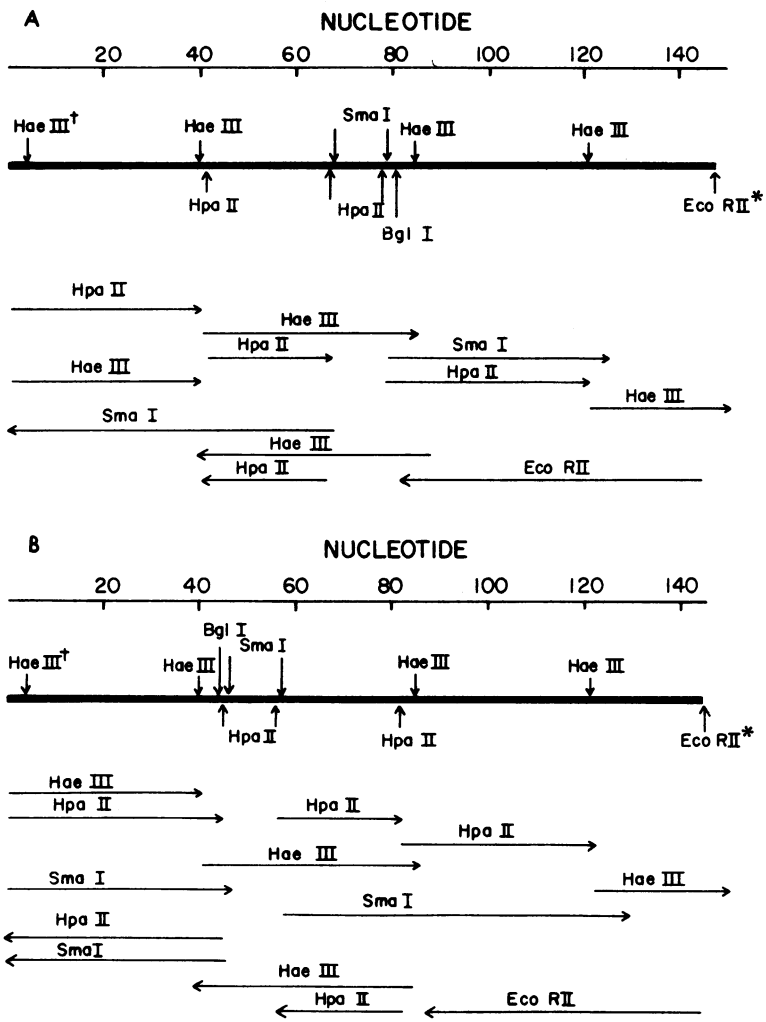

FIG. 2. *Restriction maps for the two AAV nucleotide sequences in the terminal repetition. HaeIII sites are outside the region of heterogeneity (nucleotides 44 to 81) and are at identical positions in the two orientations. BglI, HpaII, and SmaI sites are all within the region of heterogeneity and thus depend on the orientation. The arrows define the strand of each fragment that was sequenced (always in the 5′ → 3′ direction).* †, *The HaeIII site at nucleotide 4 is only cleaved in molecules with the sequence 5′ TTGGCC.* *, *The EcoRII site at nucleotide 145 is present only at the left terminus because part of the recognition sequence is outside the terminal repetition.*

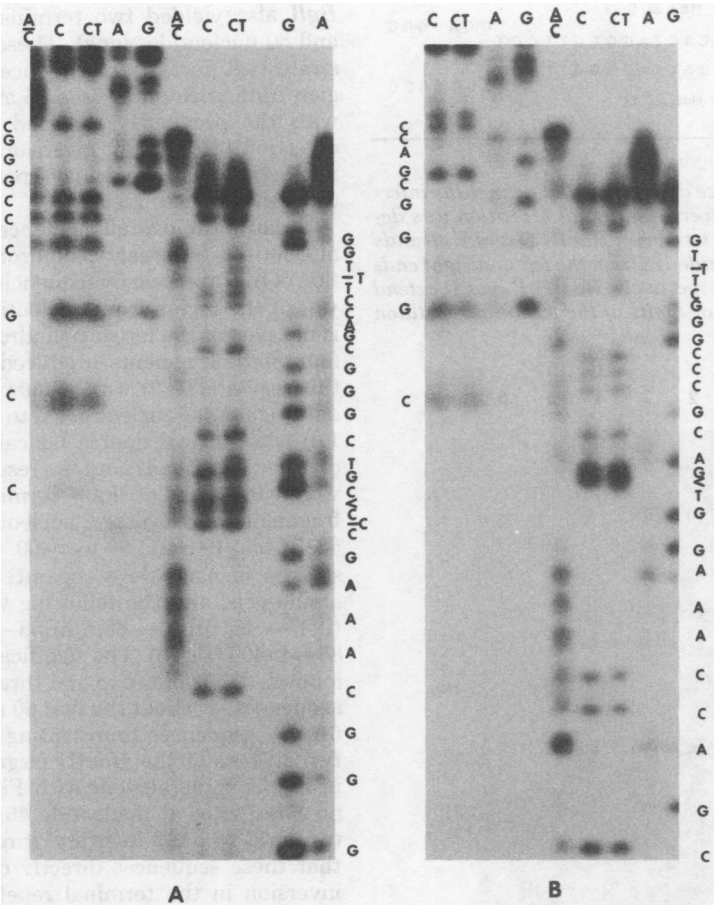

FIG. 3. *Sequencing gel (20% polyacrylamide) of both complementary strands of the HaeIII fragment from nucleotides 41 to 85 which contains the region of heterogeneity. Because this fragment extends beyond the region of heterogeneity and the heterogeneity is caused by an inversion, both strands give an unambiguous sequence. Sequences in each strand which are not determinable because of compression (caused by hairpin formation at internal palindromic sequences) are indicated by (<). (A) The sequence from 85 → 49 in the flip orientation (Fig. 1) or the sequence complementary to 41 → 77 in the flop orientation (Fig. 1). (B) The sequence from 84 → 50 in the flop orientation (Fig. 1) or the sequence complementary to 42 → 76 in the flip orientation (Fig. 1).*

fragment 26 nucleotides long (nucleotides 42 to 67, flip orientation; 59 to 84, flop orientation; Fig. 2) provided independent confirmation for a large portion of this region. Finally, sequences determined from *Sma*I fragments were also in agreement (see below).

*Hpa*II and *Sma*I fragments from both orientations (Fig. 2) were used to sequence in an inward direction the region from nucleotide 85 to 125 which was complementary to 1 to 41. A terminal *Eco*RII fragment yielded most of the sequence of the complementary strand in this region. *Hae*III E and D start inside (nucleotide 122) and extend beyond the terminal repetition on the left and right ends of the molecule, respectively. *Hae*III E and D have a common 5′ sequence at one end which extends for 24 nucleotides before diverging (Fig. 4). In this way it was determined that the terminal repetition in AAV-2 DNA contains 145 nucleotides (in the form with two thymidine residues at the 5′ terminus).

Evidence for inversion in the terminal repetition. The *Hae*III fragment containing nucleotides 41 to 85 has two *Sma*I sites and one *Bgl*I site (Fig. 3). These are the only sites for these two enzymes within the terminal repetition. However, partial digestion of terminally ^{32}P-labeled AAV-2 DNA revealed four sites for *Sma*I and two sites for *Bgl*I within the terminal repetition (Fig. 5). Complete digestion with *Sma*I yielded two terminal fragments (46 and 68 nucleotides long), and complete digestion with

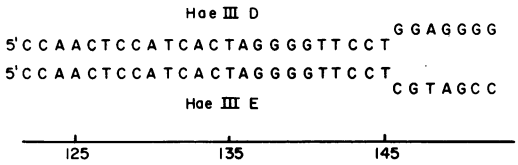

FIG. 4. *Sequence divergence that defines the internal limit of the inverted terminal repetition was determined by the 5' termini of HaeIII D and E strands starting at nucleotide 122 from the left and right ends of the molecule, respectively. HaeIII E and D extend beyond the internal limits of the terminal repetition (24).*

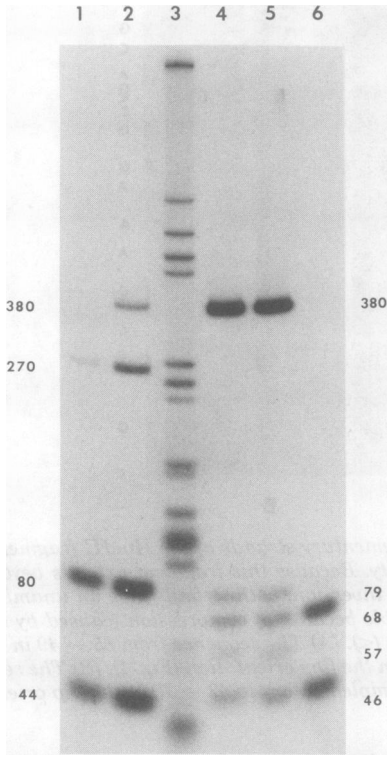

FIG. 5. *Digestion of 5' ^{32}P-labeled AAV-2 DNA with BglI or SmaI. AAV-2 DNA labeled at the 5' termini with ^{32}P by using polynucleotide kinase was digested with PstI. The right terminal fragment (380 nucleotides) with normal secondary structure was isolated from a 6% polyacrylamide gel and digested with either BglI or SmaI and electrophoresed on a 10% polyacrylamide gel. Lane 1, complete digestion with BglI. Lane 2, partial digestion with BglI. The species at 380 represents an undigested PstI fragment. The species at 270 represents a BglI site outside of the terminal repetition. Lane 3, marker lane. Intact AAV-2 DNA was digested with HaeIII, and the fragments were labeled at the 5' termini with ^{32}P by using polynucleotide kinase. Lanes 4 and 5, partial digestion with SmaI. Lane 6, complete digestion with SmaI.*

BglI also yielded two terminal fragments (45 and 80 nucleotides long). These results demonstrate two nucleotide sequences within the region from nucleotide 45 to 85 and are in accord with the previously reported data for *HpaII* digestion (24). Again, inversion of the first 125 nucleotides of the terminal repetition would predict exactly these results.

Because the overall sequence of the first 125 nucleotides is largely palindromic, demonstration of the inversion by sequencing requires analysis of the region from nucleotide 44 to 81 that is not part of the larger palindrome. To this end four *SmaI* fragments produced by cleavage in this region (Fig. 2) were sequenced. *PstI* terminal fragments (nucleotides 0 to 0.10 and 0.89 to 1.0) with normal double helical structure were digested with *SmaI*, and the resultant fragments were ^{32}P-labeled at the 5' termini. Four labeled fragments were isolated: nucleotides 1 to 46, 1 to 68, 58 to >400, and 80 to >400. Complementary strands of each were separated on polyacrylamide gels, and the following were sequenced: (i) 1 ← 46, (ii) 1 ← 68, (iii) 58 → >400 and (iv) 80 → >400 (Fig. 6). The significant results are as follows. (i) Strands two and three gave identical sequences for about the first 60 nucleotides (Fig. 6A, B), sequences representing only one of the two strands of the *HaeIII* fragment sequenced in Fig. 3. In agreement with Fig. 3, there were no *SmaI* sites at nucleotide 46 or 57 in strand two or at 68 or 79 in strand three. We conclude that these sequences directly demonstrate the inversion in the terminal repetition of AAV-2 DNA. (ii) Strands 1 and 4 also gave identical sequences for about 40 nucleotides (Fig. 6C, D). These data correspond with the overall structure in Fig. 1 and also support (over the first three nucleotides) the existence of the inversion. (iii) Strands 2 and 3 gave sequences identical to those obtained for 1 and 4 for nucleotides beyond 43 toward the terminus of the molecule and beyond 82 toward the interior of the molecule. Thus, the sequence heterogeneity in the terminal repetition is restricted to the region between nucleotides 44 and 81 and is the result of an inversion.

Symmetry within the terminal repetition. The first 125 nucleotides of the terminal repetition are highly symmetrical. Nucleotides 1 to 41 are complementary to 125 to 85, nucleotides 42 to 50 are complementary to 62 to 54, and nucleotides 64 to 72 are complementary to 84 to 76. Thus, all but 7 of the first 125 nucleotides can be base paired to form the structures shown in Fig. 1. Interestingly, at least six of the seven unpaired bases are required for structural reasons (to fold the chain back on itself) and all seven are adenine or thymidine residues; the exact number of adenine versus thymidine resi-

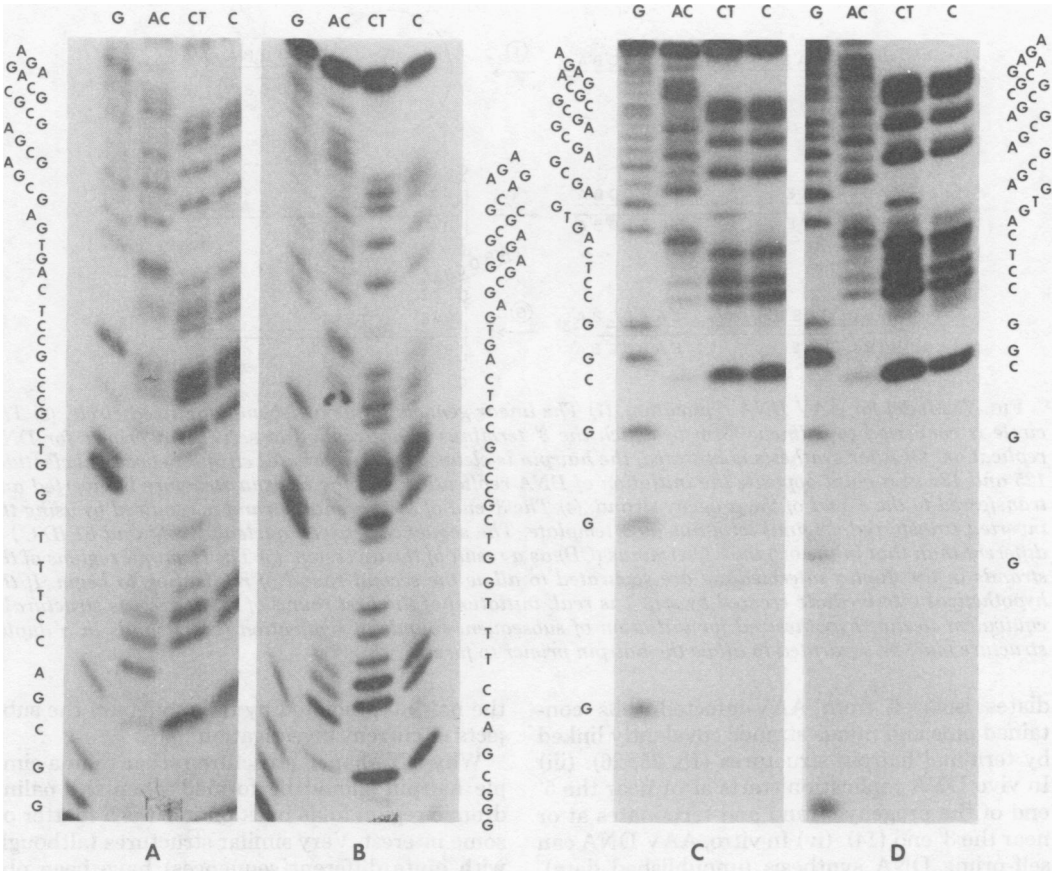

FIG. 6. *Sequencing gels of SmaI fragments. (A) 58 → >300. The sequence shown starts at nucleotide 66. (B) 1 ← 68. The sequence shown starts at nucleotide 61. (C) 1 ← 46. (D) 80 → >300.*

dues depends upon the form of the inversion used for the structure.

DISCUSSION

The AAV-2 genome is a linear DNA molecule which replicates in the nuclei of human cells. We have concentrated much of our study of AAV DNA replication on the nucleotide sequence organization of the mature genome. The rationale has been that as the virion DNA is the final product, any proposed mechanism of DNA replication must be able to account for the structural details of the mature genome. Because all known DNA polymerases require a primer, as well as a template, the synthesis of the 5′ ends of linear DNAs presents a special problem (8, 28). In the case of linear bacteriophage DNAs, the problem is circumvented by circle or concatemer formation during the replicative process by means of the natural terminal nucleotide sequence repetitions found in all linear bacteriophage DNAs (27, 28). From genetic and cytological data, it would appear that eucaryotic cellular DNA is linear, yet neither circle nor concatemer formation is observed during DNA replication. To account for this, Cavalier-Smith (8) originally proposed that linear eucaryotic DNAs contain a palindromic terminal sequence to allow the accurate replication of the 5′ ends of progeny strands. A sequence of this type at the 3′ terminus of a parental strand could fold over and be covalently joined to the shorter progeny strand. The parental strand would then be cleaved at a point opposite the joint between the original 3′ end and the newly synthesized progeny strand, allowing transfer of the original 3′ sequence to the 5′ end of the progeny strand and repair synthesis of the 3′ end of the parental strand.

By virtue of its small size, the linear AAV genome is amenable to detailed structural analysis as a potential model system to test the validity of the Cavalier-Smith hypothesis. To date, all experimental data support the model. (i) The first 125 nucleotides of the terminal repetition can self-base pair to form a hairpin structure. (ii) Presumptive replication interme-

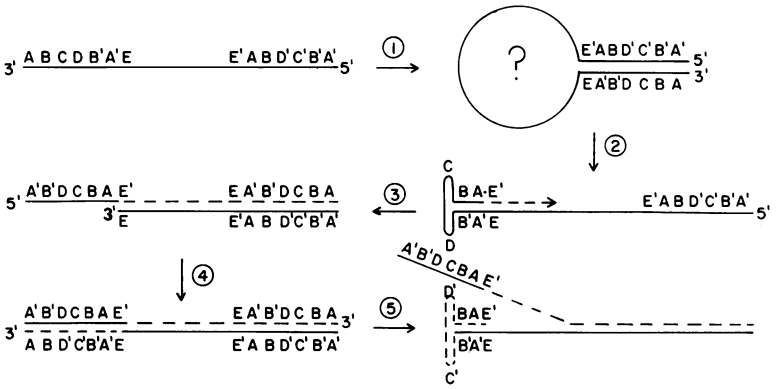

FIG. 7. *Model for AAV DNA replication. (1) The linear genome forms a H-bond stabilized circle. (2) The circle is converted to a linear form in which the 3' terminus is hairpinned to serve as a primer for DNA replication. (3) After synthesis is initiated, the hairpin is cleaved on the parental strand between nucleotides 125 and 126 at a point opposite the initiation of DNA replication and the hairpin structure is inverted and transferred to the 5' end of the progeny strand. (4) The 3' end of the parental strand is repaired by using the inverted transferred original terminus as a template. The sequence between nucleotides 44 and 81 (D'C') is different than that in the original 3' terminus (CD) as a result of the inversion. (5) The terminal regions of the strands in the duplex intermediate are separated to allow the second round of replication to begin. If the hypothetical intermediate created by step 1 is real, initiation of the first round of replication is structurally equivalent to that hypothesized for initiation of subsequent rounds of replication (i.e., strands in a duplex structure must be separated to allow the hairpin primer to form).*

diates isolated from AAV-infected cells contained plus and minus strands covalently linked by terminal hairpin structures (15, 25, 26). (iii) In vivo DNA replication starts at or near the 5' end of the progeny strand and terminates at or near the 3' end (14). (iv) In vitro, AAV DNA can self-prime DNA synthesis (unpublished data). (v) In this paper a detailed structural prediction of the model has been fulfilled. Because the first 125 nucleotides are not a perfect palindrome, the inversion resulting from the transfer of the hairpin structure from the parental strand to the progeny strand, predicted by the model, should lead to two possible sequences between nucleotides 44 and 81. The heterogeneity resulting from such an inversion has been documented above. Thus, in all respects, the Cavalier-Smith model proves to be an accurate description of the process of the AAV DNA replication.

A detailed model for AAV DNA replication is given in Fig. 7. During the first round of replication, the 3' end could directly form a hairpin to initiate DNA synthesis. In subsequent rounds, the ends of the putative duplex intermediate would first have to be separated, presumably by specific protein-DNA interaction, to allow 3' hairpin formation. If the parental genome first forms a "panhandled" circle, as has been observed in vitro, the initiation of the first round of synthesis would be equivalent to that required for subsequent rounds. Proteins potentially required for the separation of the ends of duplex intermediates and the site-specific cleavage of the hairpin predicted by the model are the subjects of current investigation.

Why a T-shaped structure rather than a simple hairpin should be formed when the palindromic region folds back on itself is a matter of some interest. Very similar structures (although with quite different sequences) have been observed for the autonomous parvoviruses (1, 21). Possibilities include one or both of the following: a specific tertiary structure requirement for protein-DNA interactions during replication; the structure is the result of simple probability during evolution for a polydeoxyribonucleotide chain 125 nucleotides long in which the maximal degree of self-base pairing is advantageous as has been suggested by Eigen and Schuster (10).

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