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Self-Complementarity of Terminal Sequences Within Plus or Minus Strands of Adenovirus-Associated Virus DNA*

(circular DNA/electron microscopy/length measurements/exonuclease III)

FRANK J. KOCZOT, BARRIE J. CARTER, CLAUDE F. GARON, AND JAMES A. ROSE

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **At least 70% of plus or minus strands of adenovirus-associated virus DNA contain self-complementary sequences at or near their termini. Self-annealing of these sequences generates circular molecules that are closed by duplex, hydrogen-bonded segments. The self-annealed segments are sensitive to exonuclease III and have a thermal stability comparable to that of double-stranded DNA molecules. Length measurements of double-stranded adenovirus-associated virus DNA molecules show a bimodal distribution, with the larger component being 10% shorter than SV40 DNA.**

The presence of self-complementary terminal sequences in single-stranded molecules of viral DNA has been observed previously only with DNA from adenoviruses. It is thus especially notable that adenovirus-associated virus replication is unconditionally dependent on a helper adenovirus. A possible role for terminal self-complementary sequences in viral DNA replication is suggested.

On the basis of hydroxyapatite chromatography data and their resistance to degradation by nuclease S_1, we recently concluded that minus strands of adenovirus-associated virus (AAV) DNA contain self-complementary nucleotide sequences (1). These studies suggested that over 50% of the strands contained self-annealed regions, estimated to represent up to 20–25% of the total DNA sequences. Although plus strands were not similarly studied, they also would be expected to contain self-complementary sequences.

In this report the presence of self-complementary sequences in both plus and minus strands of AAV DNA is confirmed by electron microscopy. After denaturation, plus or minus strands rapidly form stable, hydrogen-bonded circles. These circular structures indicate that the complementary sequences are located at or near the ends of the DNA strand. Depending on the method used to extract DNA from virions, the percentage of self-annealed molecules in minus or plus strand preparations ranged from 50 to 70%.

MATERIALS AND METHODS

Viral DNA. The preparation of ^{3}H-labeled, bromodeoxyuridine (BrdU)-substituted AAV type 2 (AAV-2) DNA and separation of the plus and minus strands of AAV DNA in CsCl gradients has been described (2). For extraction of DNA from virions, prolonged incubations with papain (12 hr) and trypsin (3 hr) have been used (3). To avoid possible breakage of DNA during these incubations, an alternative extraction procedure was used. DNA was efficiently released when purified virions were centrifuged into alkaline sucrose gradients. Fractions containing DNA were pooled and dialyzed against 0.2 M Tris·HCl (pH 8.5)–0.01 M EDTA at 4°. Comparison of alkaline sucrose sedimentation profiles of AAV-2 DNA extracted with NaOH (Fig. 1*A*) or with proteolytic enzyme (Fig. 1*B*) demonstrates that the latter technique produces considerable strand breakage.

DNA was digested with *Escherichia coli* exonuclease III (free of endonuclease activity) as before (4), except that the incubation temperature was increased to 45° (5, 6).

Electron Microscopy. Samples of DNA were mounted by either the formamide or aqueous techniques described by Davis *et al.* (7). Thermal denaturation of DNA was performed in the presence of 12% formaldehyde essentially as

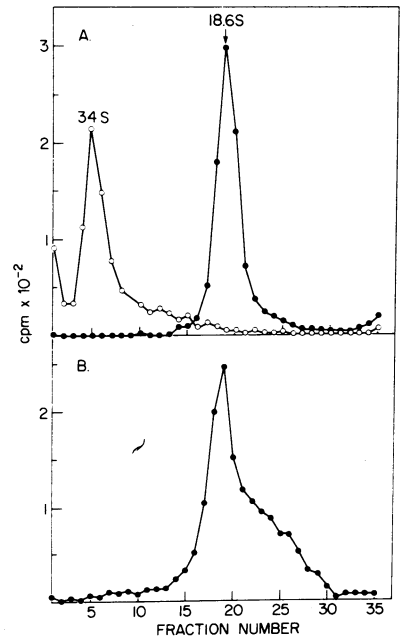

FIG. 1. Sedimentation of [^{3}H]BrdU-labeled DNA from AAV through 5–20% alkaline sucrose gradients. Gradients contained 0.3 N NaOH, 0.7 M NaCl, 1 mM EDTA, and 0.15% Sarkosyl. Centrifugation was for 3 hr at 42,000 rpm and 20° in a Spinco model 50.1 rotor. (*A*) Alkali-extracted DNA. (*B*) Enzyme-extracted DNA. (○) ^{32}P-labeled adenovirus type-2 DNA marker. BrdU-substituted AAV DNA sediments more rapidly than AAV DNA without BrdU (2).

Abbreviation: AAV, adenovirus-associated virus.

* Paper no. X in the series, "Adenovirus-Associated Virus Multiplication." Paper no. IX is ref. 1.

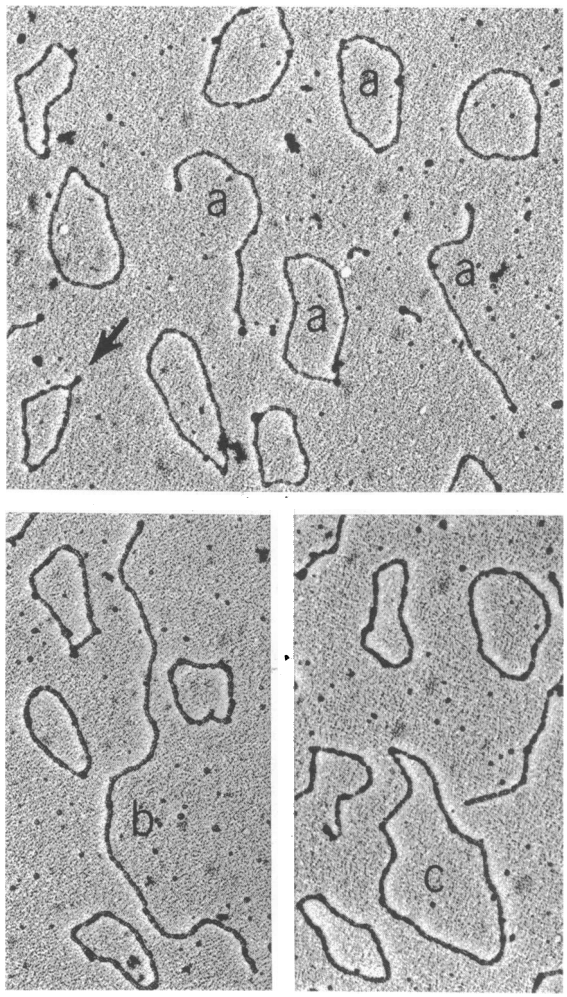

FIG. 2. Minus strand DNA. After preparative purification in CsCl, minus strands were dialyzed into 0.01 M Tris·HCl (pH 8.5)–1 mM EDTA and mounted for electron microscopy by the formamide technique, which permits visualization of extended single-stranded molecules. DNA concentration, 5 μg/ml. (*a*) Circular and linear monomers, (*b*) linear dimer, and (*c*) circular dimer. *Arrow* indicates projection. Magnification ×43,000. When DNA from the same preparation was mounted under conditions that promote collapse of single-stranded DNA (aqueous technique), linear or circular structures were not observed.

described by Doerfler and Kleinschmidt (8). Grids were examined in a Siemens Elmiskop 101 and photographed as described (4). Contour lengths of molecules were measured with a Rand Tablet and PDP-10 computer (Digital Equipment Corporation, Maynard, Mass.).

RESULTS

Electron microscopy of minus and plus strands

The apparent extent of minus strand self-annealing (1), which readily occurs during preparative separation in CsCl gradients, would be expected to produce distinctive structures that could be identified by electron microscopy. When molecules from minus strand preparations are mounted for electron microscopy by the formamide technique, both linear and circular structures are observed (Fig. 2). Identical results are also obtained with plus strand preparations. However, since minus strands are more easily purified than plus strands (2), studies were performed mainly with minus strands. DNA preparations shown in Fig. 2 were extracted from virions in 0.3 N NaOH, and the proportion of circular molecules is relatively high (70–75%). When DNA was extracted with proteolytic enzymes, the proportions of circular molecules was diminished (50–55%). This difference is probably due to less strand breakage during alkali extraction (Fig. 1*A* and *B*). It is notable that with minus strands prepared from enzyme-extracted DNA, the fraction of strands containing self-annealed sequences based on hydroxyapatite chromatography (1) is similar to that found to be circular by electron microscopy. The circular molecules present in plus or minus strand preparations thus appear to be generated by *intramolecular* annealing of complementary sequences located at or near each end of the DNA strand.

If minus or plus strands contain self-complementary terminal sequences, they should also be capable of producing specific structures as a result of *intermolecular* annealing reactions. As shown in Fig. 2, both linear and circular dimers can be seen. It must be noted, however, that linear and circular molecules either smaller or larger than those taken to represent the predominant monomeric species are also present in these preparations. This size variation may be due to technical factors, to the presence of true subspecies, or to both. The significance of the small projections (shown by the arrow in Fig. 2) seen on some linear and circular molecules is not clear. We have observed similar projections on single-stranded linear and circular SV40 DNA molecules spread under identical conditions, a result that suggests that these projections may represent preparational artifacts. Neither the size nor frequency of the projections was altered with higher concentrations of formamide.

Since the formation of circular molecules apparently results from self-annealing during strand purification in CsCl, it should be possible to open circles by alkali denaturation and to subsequently reform them under conditions of renaturation. Furthermore, the ability to efficiently denature circular molecules would indicate that covalently closed strands are absent. The experiment shown in Fig. 3 demonstrates that circular molecules are completely denaturable and that they are rapidly and efficiently reformed during renaturation (50% reformation within 10 min). In addition, the initial fraction of circles did not significantly increase during incubation under annealing conditions, suggesting that maximal self-annealing is achieved during preparative separation of the strand species.

The above data indicate that circular molecules are closed by a duplex, hydrogen-bonded segment. As further evidence for this mechanism of closure, it was observed that circular molecules were converted to linear forms when they were

TABLE 1. *Treatment of minus strand circles with exonuclease III*

Incubation time (min)	% Circular molecules*	
	+Enzyme	−Enzyme
0	67	67
2	37	63
4	29	66

* 200 Molecules were scored in each sample.

treated with exonuclease III (Table 1), which specifically cleaves nucleotides from the 3′ ends of polynucleotide chains in duplex molecules (5). There was a 57% reduction in circular molecules after 4 min of incubation. The use of exonuclease III in confirming the duplex, hydrogen-bonded closure of circular, single-stranded adenovirus DNA molecules has been described in detail (4, 6).

The stability of the hydrogen-bonded closure of single-stranded AAV DNA circles was tested by thermal denaturation in 12% formaldehyde. Temperatures that produced a 50% or greater denaturation of double-stranded AAV DNA molecules that contained BrdU (54° and 56°) were required to convert single-stranded circles to linear forms (conversions of 53 and 96%, respectively). This finding is consistent with a highly ordered base-pairing between sequences involved in cyclization of DNA strands.

Electron microscopy of double-stranded AAV DNA

Double-stranded AAV DNA is formed by an annealing of plus and minus strands after their release from virions (9). When these preparations of extracted DNA are mounted for electron microscopy by either the aqueous or formamide procedures, aggregated masses of DNA strands, branched and unbranched molecules, and occasional circular structures are observed. However, denaturation and reannealing of this DNA, as described in Fig. 4, prevents molecular aggregations and produces about equivalent proportions of single- and double-stranded molecules. An electron micrograph of reannealed DNA, mounted by the aqueous procedure, is shown in Fig. 4. Since, as previously noted, only duplex molecules remain extended during aqueous mounting, the circular molecules seen must be double-stranded. Double-stranded circles could be found in all preparations of reannealed DNA mounted by the aqueous technique, and accounted for 5–15% of the total molecules. The presence of covalently closed, double-stranded circular molecules in extracted AAV DNA is argued against by the absence of a fast-sedimenting component in alkaline sucrose gradients (J. A. Rose *et al.*, unpublished experiments). Therefore, it seems likely that the observed double-stranded circles arise from annealing of intact strands with either strands missing some or all of their terminal sequences, or with a subpopulation of permuted strands.

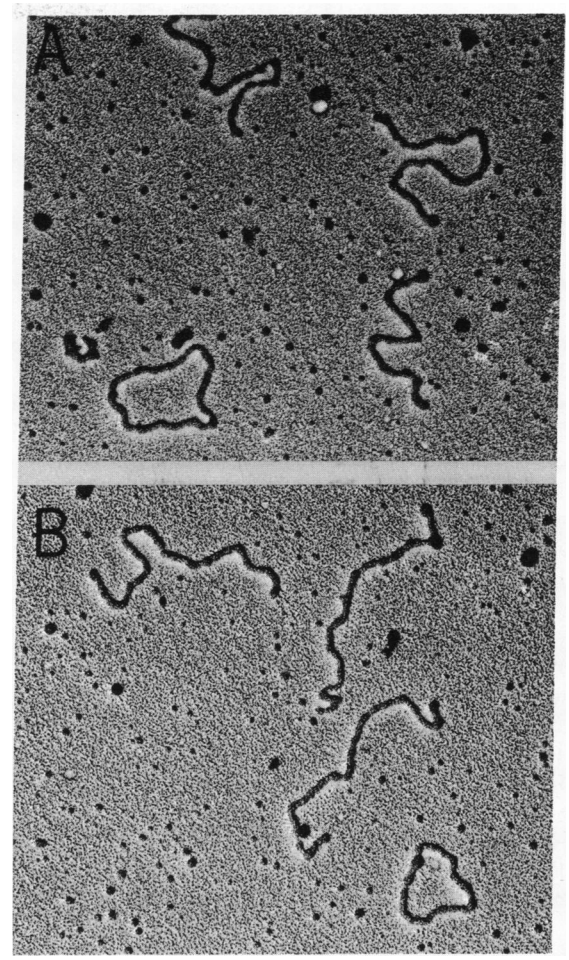

FIG. 4. Double-stranded AAV DNA. DNA was extracted from virions with NaOH. Conditions for denaturation and renaturation of DNA (5 μg/ml) as in Fig. 3. Renaturation was done for 30 min, and DNA was mounted by the aqueous technique. Small circular molecules (panel *B*) were infrequent. Magnification ×38,000.

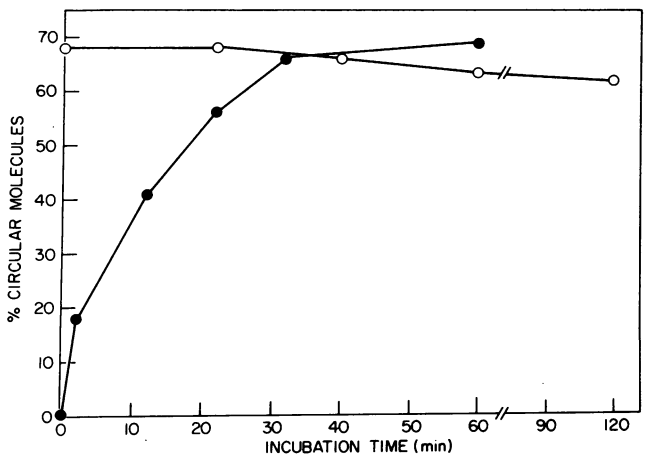

FIG. 3. Reformation of single-stranded circles after denaturation. Minus strands were denatured with alkali and renatured in 50% formamide according to conditions described by Davis *et al.* (7). DNA was mounted by the formamide technique at the indicated times. The "zero time" was obtained by neutralization of a denatured DNA sample at 4° before spreading. (O) Incubated, nondenatured strands. 200 Molecules were scored in each sample.

Lengths of duplex AAV DNA molecules mounted by the aqueous technique were measured in samples that also contained added SV40 component-II DNA. The length distributions of both AAV and SV40 DNA molecules are shown in Fig. 5. The SV40 DNA molecules (Fig. 5*B*) were 97% circular, with a mean contour length of 1.55 ± 0.04 μm. This value agrees well with data published by others (10). SV40 DNA molecules are readily resolved from AAV DNA molecules (Fig. 5*A* and *B*), which are shorter. A bimodal distribution of the lengths of AAV molecules (components with means of 1.25 and 1.39 μm) is seen, and the few AAV circular forms (5% of total molecules) have lengths in the range of the larger linear component (Fig. 5*A* and *B*). Also, smaller circular structures were occasionally found in these DNA preparations (Fig. 4*B*).

DISCUSSION

We conclude that a self-annealing of complementary sequences at or near the ends of plus or minus strands of AAV DNA generates the observed single-stranded circular molecules (Fig. 2). On the basis of nuclease S_1 data (1), the self-annealed region appears to contain 400–500 base-pairs. Two possible arrangements of the terminal complementary sequences in plus or minus strands are shown diagrammatically in Fig. 6. In model *A*, self-annealed strands would be closed by an "in-line" duplex segment, whereas strand closure in model *B* produces a duplex projection or "panhandle." The terminal sequence arrangement depicted in model *A* must be considered highly unlikely (though not completely excluded), because self-annealing would result in a base-paired, *parallel* structure that is not known to occur. However, if the self-annealed region contains 400–500 base-pairs, then the panhandles predicted by model *B* should be visible. Alternatively, it may be that the length of self-annealed sequences has been over-estimated and that panhandles are simply too small to be seen. Although small projections can be seen on occasional single-stranded molecules (Fig. 2), their significance is not clear.

Thus far, only one other virus species has been found that contains DNA with self-complementary terminal sequences within its strands. Their presence in single-stranded molecules of adenovirus DNA was reported by this laboratory (4, 11), and subsequently confirmed by others (6). Denatured adenovirus DNA can also self-anneal to form single-stranded circles. These circles are closed by duplex segments, which may be similar in size (4) to those that close plus and minus AAV circles. As is the case with AAV circles, the structure of the self-annealed region of adenovirus circles has not been determined definitively.

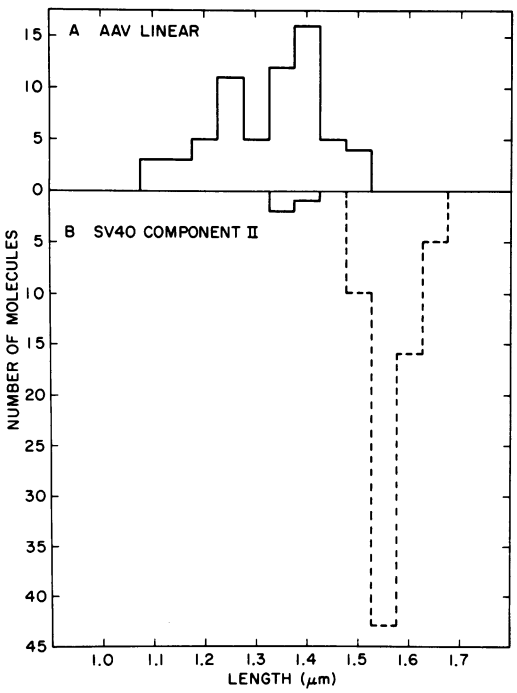

FIG. 5. Length distributions of double-stranded AAV DNA molecules and SV40 component II DNA molecules. Double-stranded AAV DNA molecules were prepared as described in Fig. 4. The *solid line* in *B* represents double-stranded AAV circles.

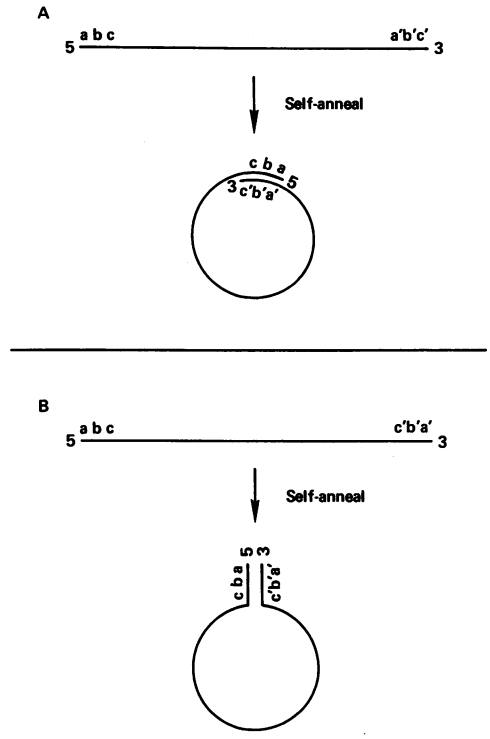

FIG. 6. Two possible models for the arrangement of terminal sequences in plus or minus strands of AAV DNA. (*A*) Self-annealing results in an in-line duplex segment. (*B*) Self-annealing produces a duplex panhandle. The model depicted in (*A*) must be considered highly unlikely (see text).

The occurrence of self-complementary terminal sequences in both AAV and adenovirus DNA strands is of great interest in view of the fact that AAV replication is unconditionally dependent on a helper adenovirus (12), and that adenoviruses appear to provide a factor(s) necessary for AAV DNA synthesis (13). That these self-complementary sequences may play a role in DNA replication is suggested by recent evidence that adenovirus DNA is replicated from an end of the template molecule (14), as well as by data that indicate that the self-complementary sequences in AAV DNA are not transcribed *in vivo* (1). Furthermore, if we assume the existence of a duplex replicative form of AAV DNA, it is interesting that the most likely order of terminal sequences (model *B*) would yield double-stranded AAV molecules having identical ends. The same conclusion also holds true for adenovirus duplexes (4, 6). This circumstance might provide a structural basis for initiating DNA replication from either end of the parent molecule. It would not be surprising if a bidirectional mechanism was involved in the synthesis of plus or minus strands of AAV DNA. For instance, if AAV plus or minus strands are synthesized separately by strand displacement from a linear duplex molecule, then a bidirectional replication mechanism would seem necessary. However, whether or not the terminal sequences in AAV or adenovirus DNA play a role in DNA replication, there is a good probability that they have some function in common.

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