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A Unique Form of Terminal Redundancy in Adenovirus DNA Molecules

(circular molecules/self-annealing/exonuclease/electron microscopy)

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ABSTRACT **A unique form of terminal redundancy has been observed in DNA molecules extracted from several human adenovirus serotypes. Electron microscopic studies reveal that single-stranded circular molecules are formed when native DNA is denatured and then annealed. Temperatures approaching the T_m of native DNA are required to convert circles to linear molecules, indicating a high degree of self-complementarity between terminal base sequences of DNA strands. Single-stranded circles are not generated if a limited number of nucleotides (2–4%) are removed from the 3′ ends of native DNA by digestion with *Escherichia coli* exonuclease III before denaturation and annealing. The length of the redundant segment appears to differ among major serotypic groups, and a possible association between increased length of the redundant segment and increased oncogenic capability of virus serotype is suggested. Evidence for the configuration of the duplex closure region of circular molecules is also presented.**

Human adenovirus DNA is double-stranded and linear, with a molecular weight of 20 to 25 $\times$ 10^6 (1, 2). Because native DNA molecules do not circularize on annealing and form few, if any, circles when annealed after treatment with *Escherichia coli* exonuclease III, it has been thought that the adenovirus genome does not contain a terminal redundancy (3). However, during studies with DNA from an adenovirus 7–SV40 hybrid virus (4), occasional single-stranded circular molecules were seen in preparations of renatured DNA (T. J. Kelly and J. A. Rose, unpublished observations). A self-annealing of single DNA strands into circular molecules would be evidence for terminal redundancy in adenovirus DNA.

In the present report, we show that denatured DNA from several human adenovirus serotypes can be readily self-annealed into single-stranded circular molecules. This finding indicates that adenovirus DNA contains a previously undescribed type of terminal repetition, i.e., the repeated base sequences have exchanged strands, resulting in single-stranded molecules with complementary terminal segments. Based on the extent of exonuclease III digestion required to prevent circle formation, it is estimated that terminal repetitions may represent up to 4% of viral DNA, depending on serotype.

MATERIALS AND METHODS

Viruses. Human adenovirus serotypes 2, 3, 7, and 12 were obtained from W. P. Rowe. Types 1 and 18 were provided by J. C. Hierholzer and type 31 was obtained from H. Shimojo. All are prototype strains except for type 7, which is strain E46$^-$ (4). Stock pools were prepared by passage in human embryonic kidney cells (HEM Research, Inc., Rockville, Md.). Viruses were produced in KB cells in suspension culture and purified as described (5).

Purification and Analysis of Viral DNA. The extraction of intact adenovirus DNA from bands of CsCl-purified virus has been described in detail (4). DNA preparations were stored in a Tris–EDTA buffer [10 mM Tris·HCl (pH 8.5)–1 mM EDTA] at 4°. Ad 2 DNA labeled with ^{32}P (6) was unbroken, as judged by sedimentation in neutral and alkaline sucrose gradients (7). Unless specified, DNA was denatured and renatured as described by Davis *et al.* (8).

Enzymatic Digestion of DNA. Limited removal of nucleotides from the 3′ ends of native adenovirus DNA molecules was achieved by incubation with *E. coli* exonuclease III (a gift of G. Fareed), prepared according to the procedure of Richardson and Kornberg (9). Reaction mixtures (50 μl) contained 0.1 M Tris·HCl (pH 8.0), 3 mM $MgCl_2$, 0.01 M 2-mercaptoethanol, and 1 μg of DNA, essentially as described by Richardson *et al.* (10). Mixtures were chilled in ice, an excess of exonuclease III was added, and reactions were initiated by transfer to a water bath at either 25° or 37°. At specified times reactions were stopped, and DNA was denatured simultaneously by the addition of EDTA and NaOH to final concentrations of 0.04 M and 0.13 N, respectively. Annealing was then done at 35° for 2 hr by dialysis against Tris–EDTA buffer [0.1 M Tris·HCl (pH 8.0)–0.01 M EDTA] containing 50% formamide. Extent of DNA hydrolysis was estimated by the production of cold trichloroacetic acid-soluble radioactivity from ^{32}P-labeled Ad 2 DNA.

Electron Microscopy. DNA was mounted for microscopy by either the formamide or the aqueous techniques described by Davis *et al.* (8). For formamide mounting, DNA was added to a spreading solution containing 0.1 M Tris·HCl (pH 8.0)–0.01 M EDTA–50% (v/v) formamide and 0.05 mg/ml of cytochrome *c*, then layered over a hypophase containing 0.01 M Tris·HCl (pH 8.0)–1 mM EDTA–17% (v/v) formamide. For isodenaturing mounting at 25° (11), formamide concentrations were increased to 85% and 50% (v/v) in the spreading and hypophase solutions, respectively. For aqueous mounting, the spreading solution contained 0.1 mg/ml of cytochrome *c*– 0.5 M NH_4Ac–1 mM EDTA. The hypophase was 0.25 M ammonium acetate. Grids were examined in a Siemens Elmiskop 101 at 40 kV accelerating voltage. Electron micrographs were taken on Kodak Electron Image Plates, at magnifications of $\times$6,000–12,000. Magnification was calibrated with a grating replica (E. F. Fullam Cat. no. 1000), and contour lengths were measured with a Dietzgen (no. 1718) map measurer.

Abbreviation: Ad, adenovirus.

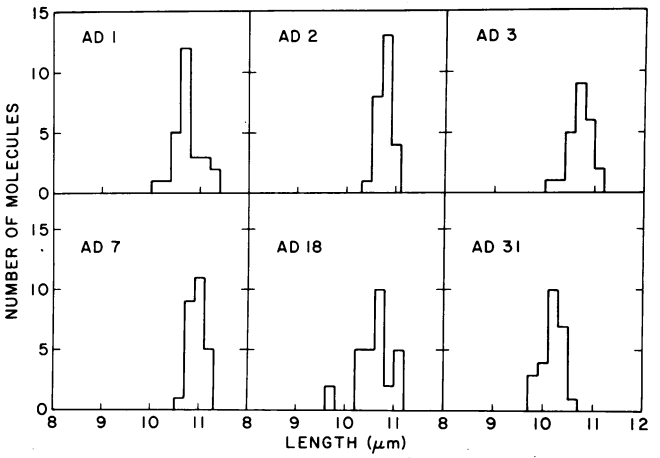

FIG. 1. Length distribution of native DNA molecules from several adenovirus serotypes mounted for microscopy by the formamide technique. Mean lengths were: Ad 1, 10.8 ± 0.3 μm; Ad 2, 10.8 ± 0.1 μm; Ad 3, 10.7 ± 0.2 μm; Ad 7, 11.0 ± 0.2 μm; Ad 18, 10.7 ± 0.4 μm; and Ad 31, 10.2 ± 0.2 μm.

RESULTS

Formation of single-stranded circular DNA molecules

Several human adenovirus serotypes, representing three major groups of oncogenic and transforming viruses (3), were chosen for study: (*a*) types 1 and 2, which are nononcogenic in newborn hamsters, but which morphologically transform rat embryo cells *in vitro;* (*b*) types 3 and 7, which are weakly oncogenic in newborn hamsters; and (*c*) types 18 and 31, which are highly oncogenic in newborn hamsters. Serotypes within each group have a high degree of genetic relatedness, whereas considerable heterology exists between members of different groups (3). Length measurements of native DNA extracted from these viruses are shown in Fig. 1. Mean lengths ranged from 10.2 ± 0.2 μm (Ad 31) to 11.0 ± 0.2 μm (Ad 7). Although a relatively small number of measurements were made in each case, mean lengths and length distributions were in good agreement with measurements of adenovirus DNA reported by others (2, 4, 12, 13).

When DNA samples were alkali denatured, renatured in a formamide solvent, and mounted for electron microscopy by the formamide technique, single-stranded circular molecules could be found (Fig. 2). Shown are photomicrographs of a double-stranded linear molecule with two single-stranded circles (*A*) and a field containing five single-stranded circles (*B*). The characteristic "kinky" appearance of single-stranded DNA, as compared with the smoother appearance of double-stranded DNA, is apparent in these preparations. The mounting of renatured DNA by the aqueous technique also provided evidence that circular molecules are single-stranded. Under these conditions, single-stranded DNA collapses into "bush"-like structures, whereas the contour of double-stranded molecules is unaltered (8). Circular molecules could not be found on grids prepared by the aqueous technique, confirming the single-stranded structure of the circles (Fig. 2) and suggesting, additionally, that adenovirus DNA is not permuted (13, 14). In preparations mounted by the formamide technique, the length ratio between double- and single-stranded molecules was about 1.9 to 1.0, explaining why circles do not appear equivalent in length to the linear duplexes. This apparent contraction of single-stranded molecules has been observed by others, and is presumably due to intrastrand interactions (8). Thus far, circular molecules have been generated with DNA from all the above adenovirus serotypes (and also with Ad 12 DNA).

At concentrations of DNA used (5 μg/ml) during studies of adenovirus 7–SV40 hybrid DNA (4), single-stranded circular molecules were seen infrequently. If these molecules arose from a self-annealing of terminal base sequences, it would be predicted that their proportion should increase by a decrease in DNA concentration in the annealing mixture to favor intramolecular interactions. Several concentrations of Ad 2 DNA were therefore denatured and renatured, and the resulting molecular forms were classified (Table 1). With decreasing DNA concentration the proportion of duplex molecules fell, whereas the proportion of single-stranded circles increased. While the use of even lower concentrations of DNA virtually abolished the formation of duplex molecules, these low concentrations provided too few molecules, which made scoring difficult. A DNA concentration of 1 μg/ml was thus used in subsequent experiments to produce a high proportion of single-stranded circles in numbers sufficient to permit easy scoring. The percentage of circular molecules at this concen-

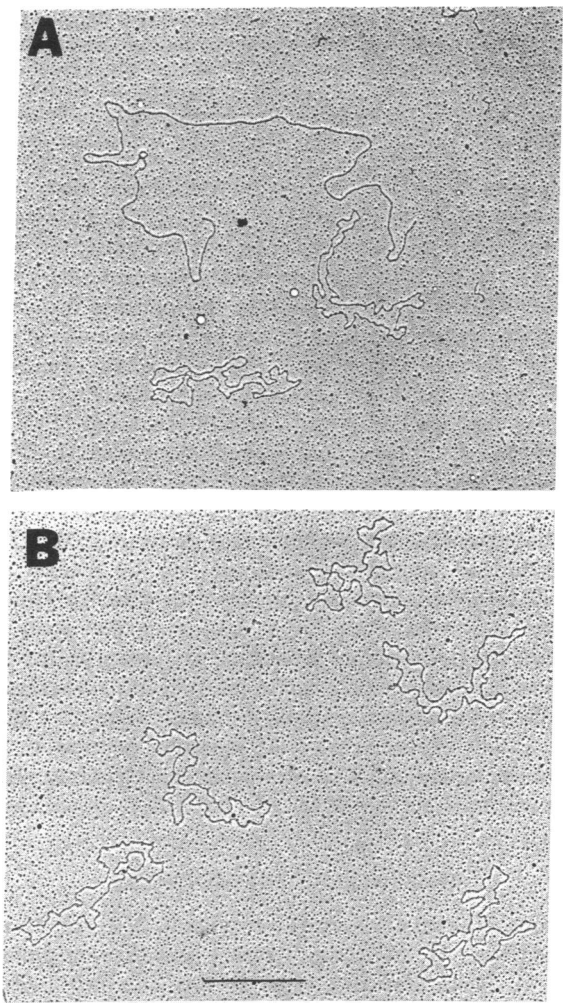

FIG. 2. Ad 31 DNA molecules after denaturation and renaturation. DNA was mounted for electron microscopy by the formamide technique. (*A*) Two single-stranded circular molecules, with a linear duplex molecule. (*B*) a field containing five single-stranded circles. The *bar* represents 1 μm.

tration was usually somewhat greater than that indicated by the data in Table 1. Up to 90% circular forms could be generated with DNA from the various serotypes, suggesting that most, if not all, viral genomes contain a terminal repetition.

Thermal stability of single-stranded circular molecules

Table 2 shows that circular molecules or, more specifically, the segments closing these molecules, have considerable thermal stability. Samples of DNA from several adenovirus serotypes were denatured and renatured to provide a high proportion of circular molecules (25°). Aliquots were removed, incubated at different temperatures, then mounted immediately so that the fraction of circles remaining could be determined for each temperature. The circular molecules from all serotypes tested were essentially melted at 55°. Percentages of circles present at 51° (83% or greater) and 53° (44% or greater) were, however, relatively high, as well as roughly similar among the serotypes. At the concentration of formamide present in incubated samples (8), the melting temperature (T_m) of adenovirus DNA was equivalent to 50–55°, depending on serotype. This experiment demonstrates that significant opening of circular structures does not occur until temperatures approaching the T_m for native adenovirus DNA are reached. The thermal stability of circles is thus consistent with a highly ordered base-pairing between sequences involved in cyclization of DNA strands.

Length of terminal repetition

E. coli exonuclease III specifically cleaves nucleotides from the 3′ ends of polynucleotide chains in duplex molecules (9, 10). A terminal repetition in the DNA of certain bacteriophages is evidenced by an ability to anneal linear duplex molecules into double-stranded circles after limited exonuclease III digestion (14, 15). It was observed (2) that native adenovirus DNA molecules form few, if any, circles on annealing after treatment with exonuclease III. This would be expected, since, unlike the terminal repetition in bacteriophage DNA, the terminal repetition in adenovirus DNA appears to be "inverted," i.e., the repeated base sequences have exchanged strands, and exonuclease digestion would, therefore, not expose complementary single-stranded ends. On the other hand, exonucleolytic removal of a limited number of nucleotides from the 3′ ends of native adenovirus DNA would be expected to prevent subsequent formation of single-stranded circles, because required terminal sequences are removed from each strand. Fig. 3 shows that the formation of single-stranded circular molecules can be prevented by treatment with exonuclease III before denaturation and annealing. Furthermore, differences clearly exist among serotypes tested with respect to enzyme treatment required to abolish circles. Circle formation with Ad 18 and Ad 31 DNA was unaffected by conditions that almost completely prevented circle formation with DNA from Ad 1 and Ad 2. Interestingly, there seems to be a correlation between length of terminal repetition and serotypic grouping. Nononcogenic types 1 and 2 appear to have the shortest terminal redundancy, whereas highly oncogenic types 18 and 31 have the longest. Weakly oncogenic types 3 and 7 fall between.

TABLE 1. *Effect of DNA concentration on formation of single-stranded circles*

Ad 2 DNA concentration (μg/ml)	Molecular forms (%)* DS linear	SS linear	SS circular
15.00	84	16	0
7.50	59	37	4
3.75	31	45	24
0.75	2	63	35

DS, double-stranded; *SS*, single-stranded.
* 200 Molecules were scored at each DNA concentration.

TABLE 2. *Thermal stability of single-stranded circles*

Adenovirus serotype	% Single-stranded circles* 25°	51°	53°	55°
1	76	63	43	0
2	73	64	32	0
3	62	—	30	0
7	68	59	—	0
18	73	68	39	2
31	67	—	36	0

DNA samples contained 0.1 M Tris·HCl (pH 8.0)–0.01 M EDTA–50% (v/v) formamide, and were incubated for 15 min at the temperatures indicated.
* 100 Molecules were scored in each sample.

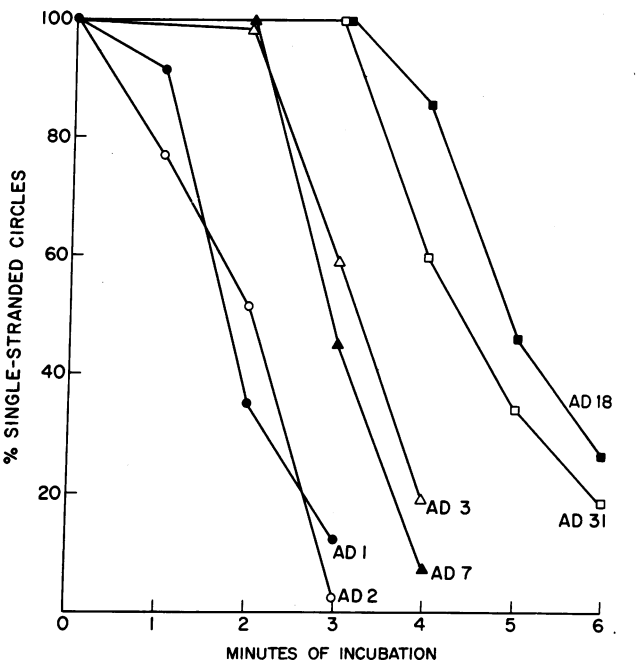

FIG. 3. The effect of exonuclease III digestion on single-stranded circle formation with DNA from several adenovirus serotypes. Samples of DNA were incubated for the indicated times at 25°, with a nonlimiting amount of exonuclease III. After denaturation and annealing, the fraction of single-stranded circles present in DNA samples was determined by electron microscopy, and percentages relative to initial fractions were plotted as % of single-stranded circles. There was no significant reduction in the initial fraction of circles when incubations were done without enzyme. In these control preparations, single-stranded circles accounted for 60–80% of molecules on the grid. Percentages were based on counts of 100–200 molecules.

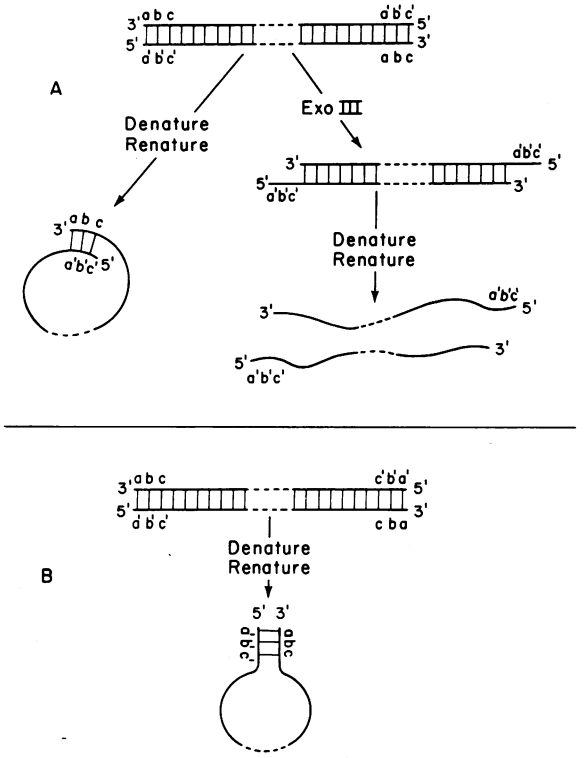

FIG. 4. A schematic representation of possible arrangements of the complementary terminal base sequences in adenovirus DNA. Circles generated from native DNA molecules containing the terminal base sequences depicted in (*A*) would be closed by an in-line duplex segment. If the order of bases at one end of the DNA molecule shown in (*A*) is reversed as depicted in (*B*), circular molecules arising after denaturation and annealing would contain a duplex projection or panhandle.

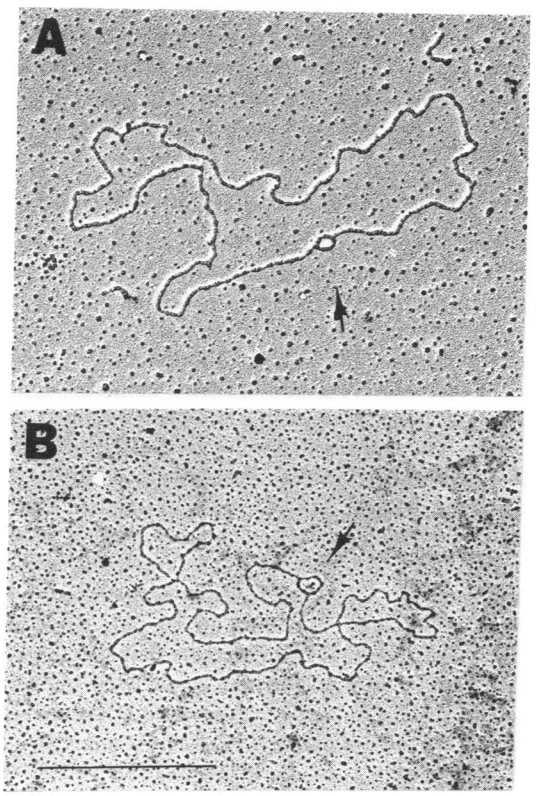

FIG. 5. Single-stranded circular DNA molecules showing a single, small region of denaturation (*arrows*). Single-stranded circles prepared from Ad 18 DNA were mounted at the approximate Tm of native DNA by the isodenaturing technique of Davis and Hyman (11), which maintains precise denaturing conditions throughout the mounting procedure. The *bar* represents 1 μm.

The amount of hydrolysis required to prevent circle formation was used to provide an estimate of the length of terminal repetition. Under conditions that produced a mean DNA digestion of about 2% (Table 3), the ability to form circles with DNA from types 1, 2, 3, and 7 was abolished, whereas about half of the initial quantity of circles could still be generated with Ad 31 DNA. Based on data given in both Fig. 3 and Table 3, it seems likely that terminal repetitions in DNA molecules from the viruses studied are at least 1%, and not more than 4%, of the viral genome, depending on serotype.

Configuration of region involved in cyclization

A diagrammatic representation of two possible modes of terminal repetition in adenovirus DNA is shown in Fig. 4. When native DNA (panel *A* or *B*) is denatured and renatured under the conditions described, single-stranded circular molecules are formed. If the native molecules are treated with exonuclease III before the denaturation and renaturation procedure (as shown in panel A), single-stranded circle formation is prevented due to removal of required sequences from the 3′ end of each strand. Complementary strands can still anneal to form linear molecules, but double-stranded circles would not be generated. The arrangement of terminal base sequences depicted in panel *A* would result in circles with the indicated duplex closure. An alternative arrangement of terminal base sequences is illustrated in Fig. 4*B*. If the order of bases at one end of the duplex molecule shown in Fig. 4*A* were reversed, the self-annealed segment of circles would be constructed as shown in Fig. 4*B*. These two possibilities might be distinguished by morphology of the double-stranded region of the circle. Based on exonuclease III data, this double-stranded region may consist of 350–1400 base pairs (depending on serotype)—a segment that might be visible as a "panhandle" if the model illustrated in Fig. 4*B* is correct. Although survey of many preparations containing single-stranded circles failed to reveal panhandles (Fig. 4*B*), neither could an "in-line" duplex segment (Fig. 4*A*) of expected length be convincingly demonstrated. An attempt was then made to locate the double-stranded region of circles by partially denaturing the self-annealed segment. Using the isodenaturing technique of Davis and Hyman (11), we were able to produce the structures shown in Fig. 5. They appear to be single-stranded circular

TABLE 3. *Amount of digestion by exonuclease III required to abolish circle formation with DNA from adenovirus serotypes*

Incubation (min at 37°)	Mean DNA digestion (%)*	95% Confidence interval for mean	Circle formation (% of initial fraction)	
			Type 31	Types 1,2,3,7
1	2.2	1.1–3.3	54	0
2	3.8	3.1–4.5	1	—
3	5.0	4.3–5.7	0	—

* Acid-soluble radioactivity was not released in the absence of enzyme.

molecules, with a small region of denaturation within the duplex segment of the circle. Under the conditions of denaturation used, linear duplex molecules were 20–50% melted, whereas circles having more than one "bubble" could not be found. While molecules containing a "bubble" were infrequent in isodenatured preparations, they have been detected with DNA from 4 serotypes (1, 3, 18, and 31), representing the three groups of adenoviruses studied. The location of a single, denatured region in line with the circular contour of these molecules supports the terminal sequence arrangement depicted in Fig. 4*A*. Furthermore, a greater difficulty in demonstrating these structures in DNA from types 1 and 3 is consistent with exonuclease data (Fig. 3) that suggests that their terminal repetitions are shorter than those of types 18 and 31.

DISCUSSION

Terminally repetitious nucleotide sequences have been found in the DNA of several bacteriophages. Native DNA molecules from the lambda-related phages of *E. coli* have short, complementary single-stranded ends, which can self-anneal to form duplex circles (16). DNA from phages T2, T3, T7, and P22 contain double-stranded terminal repetitions, and self-annealing into circular duplexes requires preliminary treatment with exonuclease III to expose complementary, single-stranded ends (16). In addition, the cyclically permuted DNA from phages T2, T4, and P22 can also be annealed into double-stranded circles after denaturation and renaturation (16). Adenovirus DNA, however, contains a type of terminal redundancy that differs from that found in bacteriophage DNA. Native adenovirus DNA cannot be self-annealed into circular molecules, nor does limited digestion with exonuclease III promote subsequent circle formation. The nature of the terminal redundancy in adenovirus DNA is indicated by findings that (*a*) denatured DNA can readily form thermally stable, single-stranded circles and (*b*) the capability for generating these circular molecules is abolished by treatment of native DNA with exonuclease III. Therefore, we conclude that the terminally repeated base sequences in adenovirus DNA have exchanged strands, resulting in an "inversion" of the redundant duplex segment. An absence of panhandles, and the possible detection of an in-line duplex segment within circular molecules (Fig. 5) is consistent with the order of repeated base sequences illustrated in Fig. 4*A*.

The differential effect of exonuclease digestion on circle formation (Fig. 3) seems related to serotypic groups that have distinct differences in oncogenic capability (3). The apparent correlation between increasing length of the redundant DNA segment and increasing oncogenic potency suggests an interesting possibility. The observed terminal repetition in adenovirus DNA could provide a mechanism for circularizing viral DNA within the cell. If a required integration of the viral genome proceeds via a circular intermediate, the stability of this intermediate and, hence, the frequency of integration, might be enhanced by an increased length of redundant base sequences.

Finally, it can be considered that the terminal repetition in adenovirus DNA might play a role in the replication of the viral genome. Either a linear or circular intermediate structure is possible. An annealing of complementary terminal base sequences could provide a short, duplex primer segment from which DNA synthesis could be initiated (17).

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