Proc. Nat. Acad. Sci. USA
Vol. 72, No. 8, pp. 3039–3043, August 1975
Biochemistry

Arrangement of sequences in the inverted terminal repetition of adenovirus 18 DNA

(electron microscopy/circular molecules/terminal repetition/duplex closure projection)

CLAUDE F. GARON, KAREN W. BERRY, AND JAMES A. ROSE

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

Communicated by Robert M. Chanock, June 3, 1975

ABSTRACT **In contrast to the single-stranded circular molecules produced with denatured DNA from other adenoviruses, there was associated with nearly all circular molecules of adenovirus type 18 a visible, duplex projection. These projections had a mean contour length of 0.31 ± 0.12 μm, equivalent to approximately 3% of genome length. Individual projections ranged in size from 0.1 to 2 μm. Alkaline sucrose gradient purification of single-stranded molecules did not affect formation of these projections, and treatment of a preparation of circular molecules with *Neurospora crassa* single-strand specific nucleases yielded 0.34 ± 0.09 μm duplex fragments. Single-stranded circles did not form if a limited number of nucleotides were removed from the 3′ ends of native molecules by *Escherichia coli* exonuclease III digestion prior to denaturation and annealing. In addition, preformed single-stranded circles could be converted to linear molecules by similar treatment. Based on the formation of specific heteroduplex structures when preparations of native DNA were denatured and reannealed and the absence of branches on linear, single-stranded molecules, we conclude that projections are generated by unusually long, inverted terminal repetitions. The repetitious sequences occur in place of rather than in addition to regular sequences. These data provide direct, visual evidence for the arrangement of bases in the inverted terminal repetition of adenovirus DNA.**

DNA molecules extracted from adenovirus particles are linear duplexes and, depending on serotype, have molecular weights reported to range from 20 to 28 × 10^6 (1–3). For all serotypes so far examined, most DNA molecules have been shown to contain an inverted terminal repetition (3–5). At present the DNA of only one other group of viruses, the adenovirus-associated viruses (AAV), has been found to contain a similar type of repetition (6). Because of these repetitious sequences, the ends of single-stranded DNA molecules can self-anneal to form circles closed by a duplex hydrogen-bonded segment. Although the arrangement of sequences within the repetition should be apparent from the morphology of the closure region, in the case of adenovirus DNA this region apparently has been too short to visualize by electron microscopy. We have recently found, however, that preparations of denatured human adenovirus type 18 (Ad 18) DNA can yield up to 80% single-stranded circular molecules which contain a visible projection or "panhandle." In the present report we show that these projections are duplex structures and that they are produced by annealing of the inverted terminal repetition with corresponding complementary sequences at the opposite end of the strand. They thus represent the hydrogen-bonded segments closing circular molecules and consequently provide direct, visual evidence for the arrangement of sequences within the inverted terminal repetition of an adenovirus DNA.

Abbreviations: Ad 18, adenovirus 18; AAV, adenovirus-associated virus.

MATERIALS AND METHODS

Viruses and Viral DNA. Human adenovirus serotypes 18 and 31 were prototype strains and were obtained from Flow Laboratories (Rockville, Md.) and H. Shimojo, respectively. Both viruses had been plaque-purified and were free of AAV contamination. Stock pools were prepared by passage in primary human embryonic kidney cells (HEM Research, Inc., Rockville, Md.). Viruses were produced in suspension cultures of KB cells and purified as described (7). The extraction of intact adenovirus DNA from bands of CsCl-purified virus was carried out as before (8). DNA preparations were stored in a Tris-EDTA buffer [10 mM Tris·HCl (pH 8.5), 1 mM EDTA] at 4°. Conditions for denaturation and renaturation of DNA were the same as used previously (4).

Enzymatic Digestion of DNA. Adenovirus DNA samples were treated with *Escherichia coli* exonuclease III (obtained from M. Gottesman) essentially as described by Richardson *et al.* (9). The substrate [1 μg/ml, either native DNA or preformed single-stranded circles (4)] was treated with an excess of enzyme in a solution containing 0.1 M Tris·HCl (pH 8.0), 3 mM $MgCl_2$, 0.1 M 2-mercaptoethanol and incubated at 37°. The reaction was stopped either by the addition of EDTA or by heating the sample to 65° for 5 min to inactivate the enzyme (9). *Neurospora crassa* single-strand specific exonuclease (provided by K. Bartok) was prepared as described by Rabin *et al.* (10); the purified enzyme preparation contained 120 units/ml. One unit of nuclease activity is defined as that amount of enzyme which causes the release of 1.0 A_{260} nm unit of acid-soluble material from 600 μg of heat-denatured calf-thymus DNA per ml (Worthington Biochemical Corp.) in 30 min at 37° in 0.1 M Tris·HCl (pH 8.0), 0.01 M $MgCl_2$. The *N. crassa* nuclease preparation was mostly exonucleolytic in character, with a small amount of endonucleolytic activity. Both activities were highly single-strand specific in the presence of 0.1 M NaCl (11). Adenovirus DNA was treated with 2 units/ml of nuclease for 15 min at 37° in a solution containing 0.1 M Tris·HCl (pH 8.0), 0.01 M $MgCl_2$, and 0.1 M NaCl.

Electron Microscopy. DNA was mounted for microscopy using the formamide technique essentially as described by Davis *et al.* (12). 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 6,000–12,000×. The magnification was calibrated for each set of plates with a grating replica (E. F. Fullam no. 1000). Negatives were projected (5×) onto a Rand Tablet, where contour lengths were measured (PDP-10 Computer-Digital Equipment Corp.).

RESULTS

Visualization of projections on single-stranded circular molecules

Shown in Fig. 1 are several characteristic single-stranded circular molecules produced when a low concentration (1 μg/ml) of DNA extracted from Ad 18 was alkali-denatured and renatured in a formamide solvent. A single projection is clearly visible on almost every circle in this preparation and, based on their morphology, these projections appear to be double-stranded. Similar projections were also seen on most single-stranded, circular molecules prepared from eight other preparations of Ad 18 DNA. Previous examination of DNA from six other human adenovirus serotypes, as well as from an avian adenovirus, had failed to reveal such structures (3–5). The preparations of Ad 18 DNA contained no detectable contamination with AAV DNA, nor did the addition of AAV DNA produce the observed projections. Furthermore, alkaline sucrose gradient purification of Ad 18 DNA strands did not alter subsequent formation of projections, and projections or branches were not seen on single-stranded, linear molecules. Thus, the projections arise from sequences covalently linked to the viral genome but do not appear to represent self-annealed internal sequences.

Contour length measurements of projections seen in a number of DNA preparations are summarized in Table 1. Depending on the preparation, some variation was found in the total percentage of molecules with projections as well as in the proportions of molecules with relatively short or long projections. The reason for these variations is not known, and certain factors that might be involved are presently under study (e.g., virus multiplicity, cell line). It was found that projections occasionally represented up to 20% (rarely >20%) of genome length (Fig. 2), but in most instances projections were equivalent to 3–4% of mean molecular length.

Double-strandedness of projections

Several lines of experimental evidence strongly supported the visual impression (Figs. 1 and 2) that projections represent double-stranded segments. First, measurements of the projections on intact single-stranded circles were compared to measurements of duplex fragments that remained after digestion of these molecules by single-strand specific nucleases of *N. crassa*. The results are shown in Fig. 3. Contour length measurements gave a mean value of 0.31 ± 0.12 μm for the apparently duplex projections on intact single-stranded circles and a mean value of 0.34 ± 0.09 μm for the duplex fragments observed after nuclease treatment. For the circular molecules all projection lengths are plotted (Fig. 3B), but the occasional fragments greater than 1 μm (<1% of fragments) in the nuclease-treated sample (Fig. 3A) were not scored because of the possibility that they might represent intermolecular reassociations generated during circle formation. The similarity of sizes of projections and fragments resistant to digestion by the *N. crassa* nucleases suggests that the observed projections are duplex structures. In a control experiment fragments were not seen when denatured Ad 18 DNA was treated with nuclease without prior reannealing. Second, grids of circular molecules were prepared using the isodenaturing technique of Davis and Hyman (13), and at 35° and a formamide concentration of 88.5% a denatured region could be seen in some projections (Fig. 4). In addition, the stability of the duplex segments closing circular molecules was relatively great, as indicated by the fact that formamide concentrations as high as 85% failed to melt open the circles, whereas homoduplex molecules showed considerable melting [>50% under identical conditions (Fig. 5)]. It is also of interest that under these conditions terminal segments in homoduplexes, which were at least equivalent to mean projection length, remained unmelted (Fig. 5), a situation that might be expected if projections represented self-annealed strand ends. The finding that both circles and terminal segments of homoduplex molecules resisted denaturation would suggest that the terminal sequences in Ad 18 DNA contain a proportionately high concentration of G + C base pairs. Third, it was observed con-

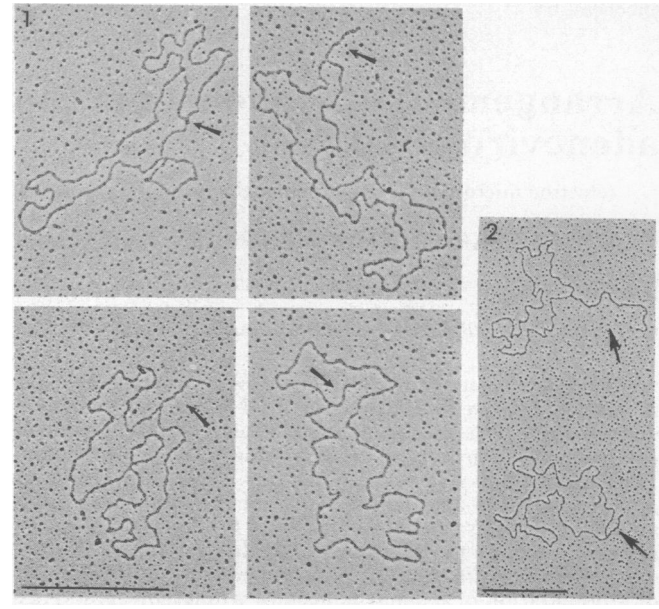

FIG. 1. Ad 18 DNA molecules after denaturation and renaturation at a concentration of 1 μg/ml. DNA was mounted for electron microscopy by the formamide technique. Typical projections (*arrows*) associated with each single-stranded circle are shown. The bar represents 1 μm.

FIG. 2. Electron micrograph of single-stranded circular molecules after denaturation and reannealing of Ad 18 DNA. Differences in size of the duplex projections associated with two of the single-stranded circles are evident (*arrows*). Mounted by the formamide technique. The bar represents 1 μm.

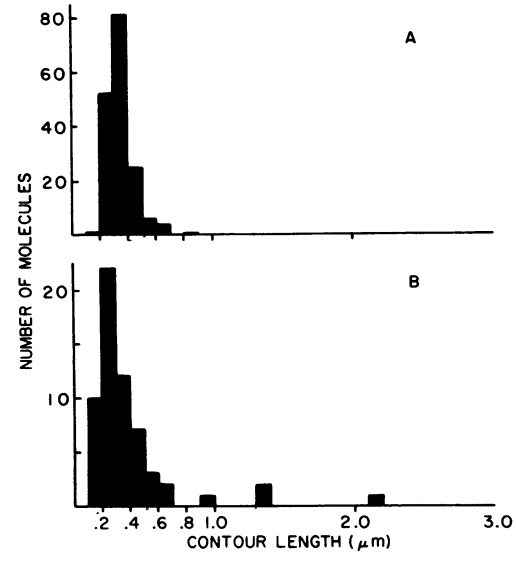

FIG. 3. Contour length measurements of (A) duplex fragments remaining after digestion with *N. crassa* single-strand specific nuclease of Ad 18 single-stranded circular molecules and (B) projections on intact single-stranded circles.

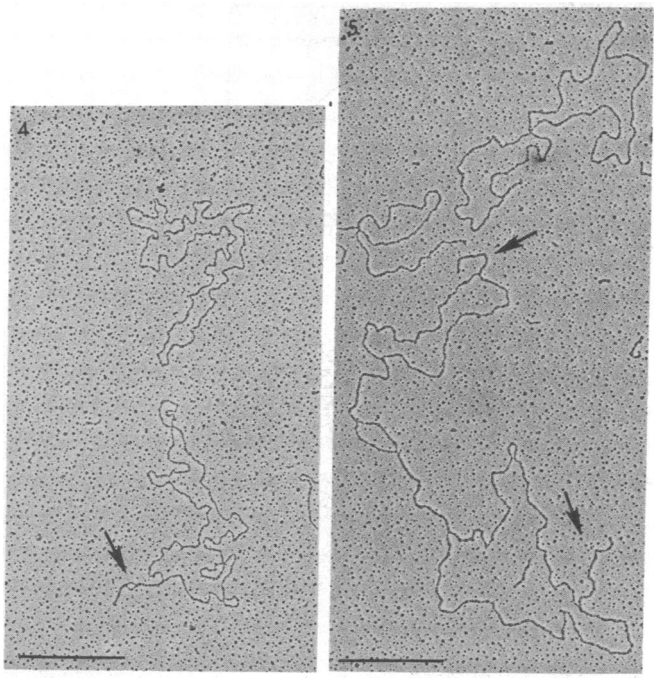

FIG. 4. Two Ad 18 single-stranded DNA circles mounted for electron microscopy under denaturing conditions (88.5% formamide at 35°). The duplex projection on one of the circles appears to be partially denatured (*arrow*).

FIG. 5. Ad 18 DNA mounted for electron microscopy under denaturing conditions (85% formamide at room temperature). Shown is a duplex molecule with greater than 50% melting along its length, whereas a single-stranded circle with duplex projection remains intact. The terminal segments of the melting duplex molecule remain unmelted (*arrows*). The bar represents 1 μm.

sistently that, for individual circular molecules, the sum of circular contour length plus twice the projection length approximated the mean length of linear duplexes. This observation also indicates that the complementary sequences responsible for projections would occur in place of rather than in addition to regular sequences. Finally, if projections represent double-stranded segments that have arisen from a

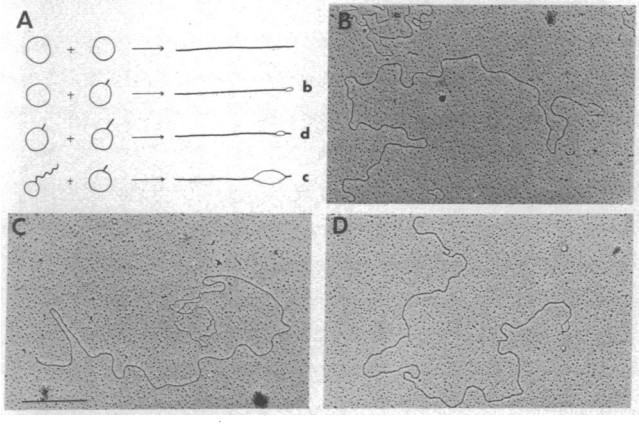

FIG. 6. Heteroduplexes found after denaturation and renaturation of native Ad 18 DNA (5–10 μg/ml). (A) Schematic representation of how specific heteroduplexes would be generated from strands having differences in length of terminal repetitions. These differences are reflected in variations of projection length seen on single-stranded circular molecules (circles without projections are closed by segments too short to be visible in the electron microscope). Heteroduplexes expected from pairings of linear forms of such molecules are indicated. (B), (C), and (D) Observed heteroduplexes corresponding to (b), (c), and (d) in panel (A). The bar equals 1 μm. Formamide spread.

self-annealing of strand ends, then differences in projection length should be due to differences in length of the inverted terminal repetition within some molecules. Thus, denaturation and renaturation of DNA at a high concentration (>5 μg/ml) would be expected to yield specific heteroduplexes owing to the reassociation of strands that contain terminal repetitions of different length. This expectation was borne out, as shown in Fig. 6. The several types of heteroduplexes predicted from differences in observed lengths of projections could be readily found, and no molecules were encountered with bubbles at both ends.

Terminal location of sequences producing projections

Both the heteroduplex data (Fig. 6) and the fact that branches were not seen on linear, single-stranded molecules strongly indicate that projections are generated by annealing of the inverted terminal repetition with its corresponding complementary segment at the opposite end of the strand. A schematic representation showing how projections would be formed from these terminal sequences is given in Fig. 7. As noted above, each of the illustrated heteroduplex molecules (Fig. 6) can best be explained on the basis of a pairing of two strands that contain terminal repetitions of different lengths. Heteroduplexes with forked or open ends were not detected, suggesting that most, if not all, molecules in these DNA preparations contain an inverted terminal repetition. Occasionally, annealing of two single-stranded circular molecules apparently occurred, and interesting, partially duplex structures were produced (Fig. 8). In the example shown, the projection associated with each single-stranded circle is clearly visible, and their similar locations within each circular molecule are implied by their close proximity.

Further evidence that duplex projections represent self-annealed strand ends was obtained by treatment of DNA molecules with *E. coli* exonuclease III. Exonuclease III specifically cleaves nucleotides from the 3′ ends of polynucleotide chains in duplex molecules (9) and was used to abolish single-stranded circle formation when native molecules were treated prior to denaturation and reannealing (Fig. 9A) or to open previously formed single-stranded circles (Fig. 9B) by digesting away a length of sequences needed to effect closure of single-stranded circular molecules (4, 5). In both cases Ad 31 DNA molecules were similarly treated for comparison. It can be seen that an equivalent amount of exonuclease digestion was required either to open or to prevent formation of single-stranded circular molecules of Ad 18. However, Ad 31 DNA circles (where no duplex projections were visible) were considerably more susceptible to exonuclease digestion. It should be noted that in the Ad 18 DNA samples there remained a portion (approximately 20%) of single-stranded circles that was far more resistant to exonuclease digestion (Fig. 9A and B). This fraction presumably represents those molecules that contain long inverted repetitions (1–2 μm).

DISCUSSION

An inverted terminal repetition is responsible for the cyclization of single-stranded adenovirus DNA (3–5). Distinct from circular molecules formed with DNA from other adenovirus serotypes, however, most single-stranded Ad 18 circles were found to contain a visible projection. The essential problem, then, was to determine whether these projections represent the double-stranded segment that closes circular molecules, i.e., whether they are generated by annealing of the inverted terminal repetition with corresponding complementary sequences at the opposite end of the strand. In the

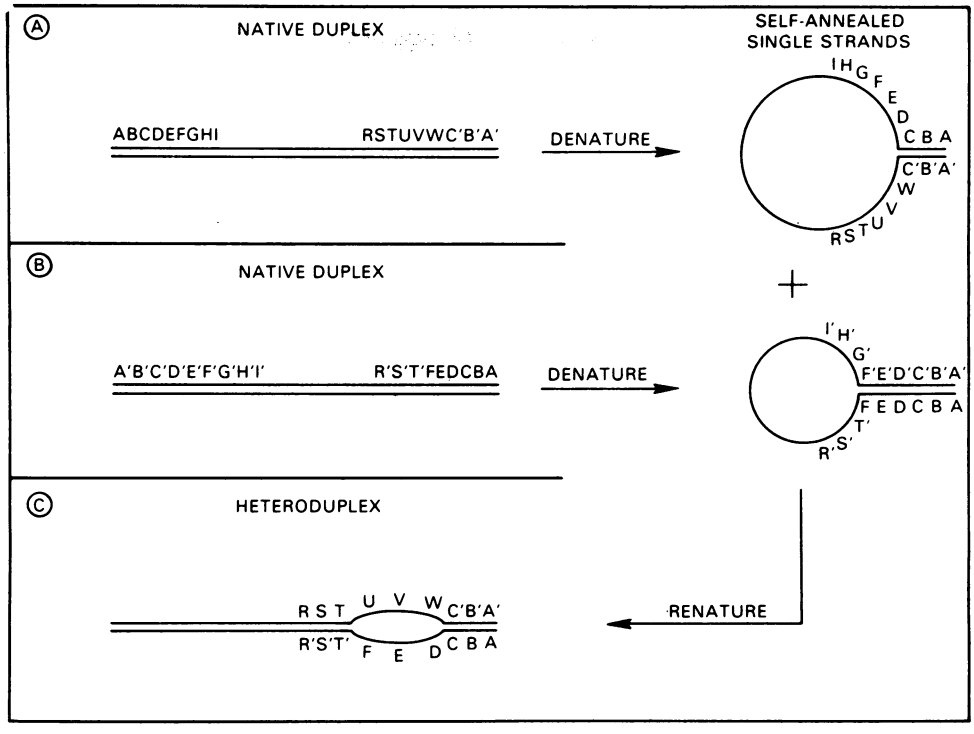

FIG. 7. Schematic representation of possible arrangements of terminal sequences in Ad 18 single-stranded circles and heteroduplex molecules. Shown in panels (A) and (B) are native, duplex molecules which, when denatured and allowed to reanneal under dilute conditions, form single-stranded circular molecules with projections having varying lengths. Panel (C) shows a heteroduplex molecule that would be formed as a result of reannealing of linear forms of the two depicted circular molecules. Complementary sequences are designated by primed letters.

DNA preparations examined, projections had a mean length of about 0.3 μm, which is equivalent to 3% of genome length. Individual projections, however, ranged in size from 0.1 to 2 μm and could thus represent up to 20% of genome length. Alkaline sucrose gradient purification of DNA strands did not abolish formation of the projections, demonstrating that their nucleotide sequences are covalently linked to the DNA strand. That projections are double-stranded is indicated by their morphology, apparent resistance to *N. crassa* nucleases, denaturation in 87% formamide, and the formation of specific heteroduplexes. Furthermore, these heteroduplex structures as well as the absence of branches on linear single-stranded molecules provide good evidence that projections result from a self-annealing of sequences at the ends of the strand and hence do represent the duplex segment that closes single-stranded circles. Finally, because the overall lengths of all circular molecules (i.e., circular contour length plus twice projection length) are approximately the same, it also can be concluded that the inverted terminal repetition (which is responsible for the observed projections) occurs in place of rather than in addition to regular sequences. It should be noted, however, that only virion DNA was examined, and that differences could exist between these molecules and unpackaged, intracellular DNA molecules.

The finding that single-stranded circles are closed by duplex segments that appear as projections is consistent with an antiparallel, base-pairing of sequences from each end of the DNA strand. This, in turn, indicates the general order of bases in the inverted terminal repetition of Ad 18 DNA (Fig. 7). It seems probable that inverted terminal repetitions in the genomes of other adenoviruses (3–5) will also contain the same general base order. This conclusion is supported by recent analyses of short nucleotide sequences at the 3′-termini of Ad 2 and Ad 5 DNA (14) and restriction enzyme cleavage fragments of terminal portions of Ad 2 DNA (15). In addition, it is notable that DNA from AAV also contains an inverted terminal repetition which apparently possesses a similar general base order (16, 17).

It has been postulated that the inverted terminal repetition might play a role in viral DNA synthesis or oncogenesis

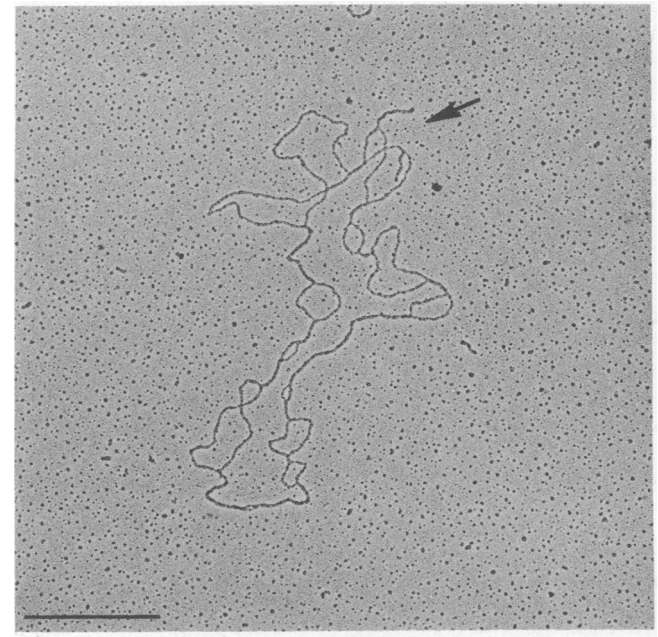

FIG. 8. Annealing of two single-stranded circular molecules of Ad 18. The fixed circular structure of each molecule prevents full helical winding, and this constraint presumably results in unpaired regions. Duplex projections lie in close proximity (*arrow*). Mounted by the formamide technique. The bar represents 1 μm.

Table 1. Occurrence of projections on single-stranded Ad 18 circles*

Type of projection	Projection length (μm)	% Molecules with projections	No. of base pairs†	% Genome length†
None	—	10–50	—	—
Short	0.1–0.7 (0.31)‡	50–80	300–2000	1–6
Long	0.7–2	2–20	3000–6000	10–20

* Summary of data from 9 individual preparations of Ad 18 DNA.
† Calculation based on genome molecular weight of 22×10^6 (4).
‡ Mean value.

or both (4), but the biochemical and biological significance of these sequences is still uncertain. Of interest, however, are experiments recently described by Graham *et al.* (18) in which both the transforming and infectious capabilities of Ad 5 DNA were determined after sequential treatment with *E. coli* exonuclease III and S1 nuclease. This treatment had the effect of digesting both strands of the DNA molecules inward from both ends. The results indicated that transforming activity was more resistant to loss of end sequences than was infectivity, i.e., DNA transforming activity was unaffected until approximately 1% of sequences from each end had been removed, whereas DNA infectivity was eliminated by as little as 0.2% total digestion. Assuming that the inverted terminal repetition in Ad 5 DNA is less than 1% of genome length, these data suggest that terminal repetition sequences are not involved in oncogenesis, but that they could be required for virus replication.

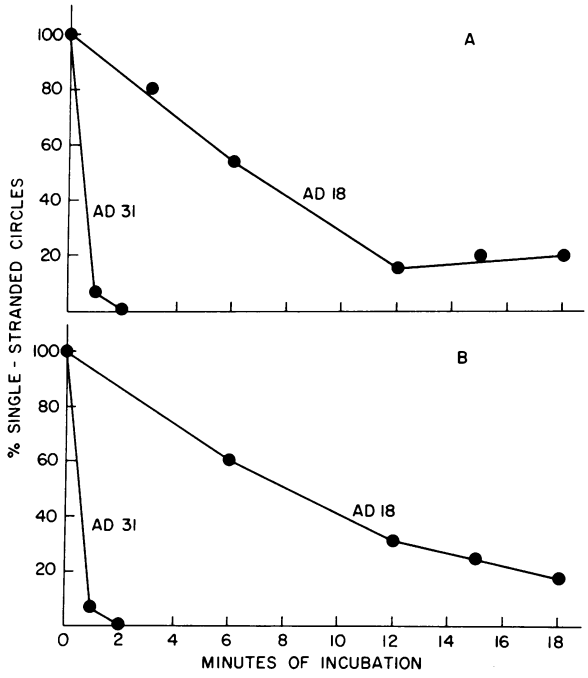

FIG. 9. The effect of exonuclease III digestion on (A) formation of single-stranded Ad 18 or Ad 31 circles when native molecules were treated prior to denaturation and reannealing or (B) previously formed single-stranded Ad 18 or Ad 31 circles. In control experiments no significant reduction in the initial fraction of circles was noted when incubations were performed without enzyme. Percentages are based on counts of 200 molecules.

It is curious that the terminal repetitions in Ad 18 DNA may achieve far greater length than those so far found in any other adenovirus DNA. Considering that up to a fifth of observed molecules have repetitions equivalent to 10–20% of genome length (Table 1) and that the repetitions substitute for regular sequences, a moderate proportion of Ad 18 DNA molecules should be defective. This may in part explain the relatively low infectious yields obtained with Ad 18 (J. A. Rose, unpublished results). Furthermore, although it seems unlikely, there is still a possibility that virus multiplication may depend upon a relatively small proportion of genomes without repetitions.

We thank J. W. Garrison for invaluable technical assistance.

1. Green, M., Piña, M., Kines, R., Wensink, P. C., MacHattie, L. A. & Thomas, C. A., Jr. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 1302–1309.
2. Van Der Eb, A. J., Van Kesteren, L. W. & Van Bruggen, E. F. J. (1969) *Biochim. Biophys. Acta* **182**, 530–541.
3. Robinson, A. J. & Bellett, A. J. D. (1975) *J. Virol.* **15**, 458–465.
4. Garon, C. F., Berry, K. W. & Rose, J. A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2391–2395.
5. Wolfson, J. & Dressler, D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3054–3957.
6. Koczot, F. J., Carter, B. J., Garon, C. F. & Rose, J. A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 215–219.
7. Rose, J. A. & Koczot, F. (1971) *J. Virol.* **8**, 771–777.
8. Kelly, T. J., Jr. & Rose, J. A. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1037–1041.
9. Richardson, C. C., Lehman, I. R. & Kornberg, A. (1964) *J. Biol. Chem.* **239**, 251–258.
10. Rabin, E. Z., Preiss, B. & Fraser, M. J. (1973) *Prep. Biochem.* **1**, 283–307.
11. Mills, C. & Fraser, M. J. (1973) *Can. J. Biochem.* **51**, 888–895.
12. Davis, R. W., Simon, M. & Davidson, N. (1971) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. XXI, pp. 413–428.
13. Davis, R. W. & Hyman, R. W. (1971) *J. Mol. Biol.* **62**, 287–301.
14. Steenbergh, P. H., Sussenbach, J. S., Roberts, R. J. & Jansz, H. S. (1975) *J. Virol.* **15**, 268–272.
15. Roberts, R. J., Arrand, J. R. & Keller, W. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3829–3833.
16. Berns, K. I. & Kelly, T. J., Jr. (1974) *J. Mol. Biol.* **82**, 267–271.
17. Rose, J. A. (1974) in *Comprehensive Virology*, eds. Fraenkel-Conrat, H. & Wagner, R. (Plenum Press, New York), Vol. 3, pp. 1–61.
18. Graham, F. L., Van Der Eb, A. J. & Heijneker, H. L. (1974) *Nature* **251**, 687–691.