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Adenovirus-2 DNA Contains an Inverted Terminal Repetition

(structure of viral DNA/electron microscopy)

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ABSTRACT **Denaturation and renaturation of the adenovirus-2 chromosome (a duplex rod) generates single-stranded circles of unit length. These circles can be opened into linear DNA molecules by digestion with exonuclease III, indicating that hydrogen bonding between the two ends of an adenovirus strand is responsible for maintaining the rod in a circular state.**

The formation of adenovirus single-stranded circles, and their sensitivity to exonuclease III, indicate that the mature adenovirus-2 DNA molecule contains an inverted terminal repetition. That is, the base sequence at one end of the molecule is inverted and appears again at the other end of the molecule. This is the first example of such a structure, and its function is unknown.

Where it has been possible to isolate chromosomes in an unbroken state, they have proven to be either circles or DNA rods, which are terminally repetitious (1, 2).* These two topological configurations are readily interconvertible. A circle can be opened with staggered nicks (Fig. 1*A–D*). Alternatively, a terminally repetitious rod can form a circle after exonuclease digestion to expose complementary, single-stranded termini (Fig. 1*D–A*).

An exception to this pattern is seen in adenoviruses, a group of more than 40 animal viruses, all of which can be tumorigenic (7). The adenovirus chromosome is a linear double-stranded DNA molecule of molecular weight about 23×10^6 (7). For the chromosomes of adenovirus strains 2, 4, 7, 12, 18, and 21, Green, and his collaborators (8) have shown that digestion with exonuclease III does not allow the formation of double-stranded DNA circles. This means that the single-stranded termini exposed by nuclease digestion cannot base pair and that the ends of the adenovirus chromosome are not terminally repetitious in the conventional manner. It is the purpose of this paper to show that the ends of the adenovirus chromosome possess a repeated base sequence, but the terminal repetition is inverted.

SINGLE-STRANDED ADENOVIRUS CIRCLES

When a solution of adenovirus-2 DNA is denatured and then neutralized, unannealed single-stranded DNA is seen in the electron microscope in two configurations: rods and single-stranded circles (Fig. 2*A*). The single-stranded rods range randomly in length up to unit adenovirus size; they are probably derived from mature viral chromosomes that contained a nick in one DNA strand. In contrast, the single-stranded DNA circles are always of unit length (Fig. 2*B*), indicating that the circles are formed by interaction between the termini of an adenovirus strand of unit length.

To obtain single-stranded circles of adenovirus DNA, a low DNA concentration is used for denaturation and renaturation; this suppresses duplex-rod formation, which is a bimolecular reaction. In a typical experiment, out of a total of 400 molecules observed, only 17% were either fully or partially duplex rods. The remaining DNA was single-stranded: 70% was circular and 30% was linear. Because more than 50% of the single strands of unit length could be recovered as rings, it can be concluded that both the positive and negative strands from

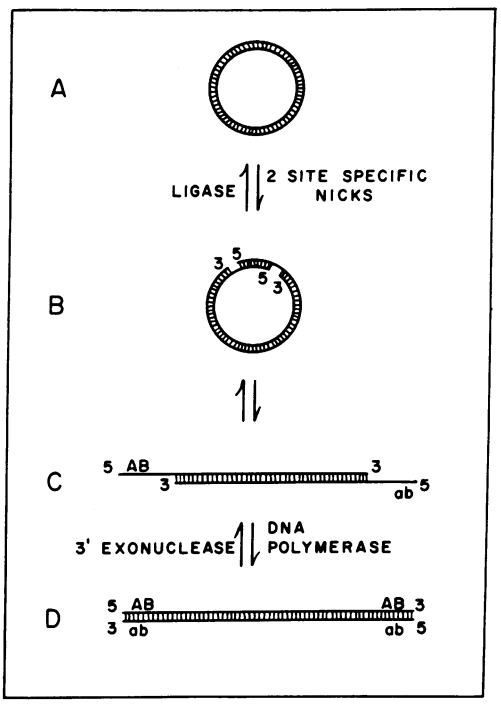
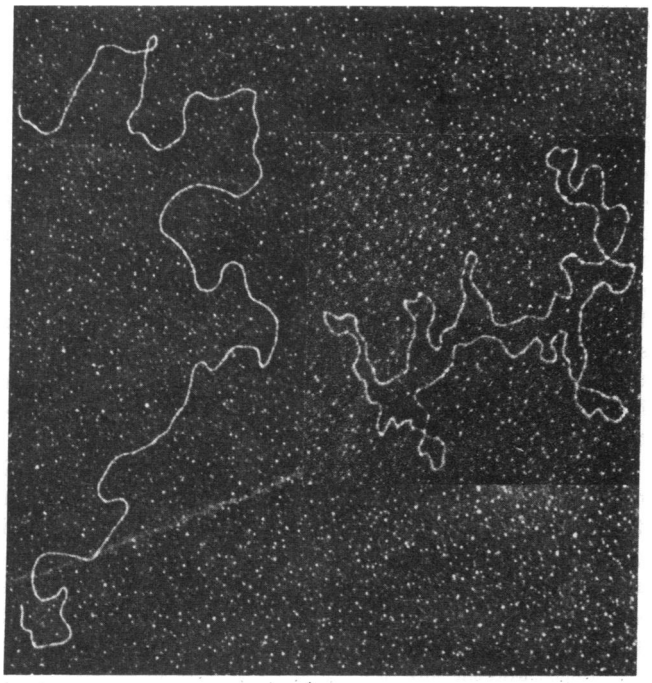


FIG. 1. Interconversion of a DNA circle and a terminally repetitious rod. Fig. 1 shows the series of events involved in the interconversion of a DNA circle (*A*) and a terminally repetitious rod (*D*). Staggered nicks are introduced into the duplex circle (*B*). If the hydrogen bonds in the region between the nicks melt out, the circle can unfold into a DNA rod (*C*). This rod contains single-stranded and complementary termini, and therefore is, in essence, terminally repetitious. If the single-stranded termini of the rod are used as templates by a DNA polymerase, a fully duplex DNA rod is formed (*D*). The whole process can be reversed. An exonuclease III-type enzyme (3) can expose the complementary termini (*C*). After hydrogen bonding occurs to form a circle (*B*), the staggered nicks can be sealed by ligase. Thus, a fully covalent, double-stranded circle is generated (*A*). The key intermediate in the interconversion process is the linear molecule with single-stranded and complementary ends (*C*). A linear DNA molecule with exactly this structure is seen in the lambda chromosome isolated from the mature phage particle (4–6). Except for adenovirus chromosomes, all DNA molecules thus far characterized are either circles or duplex, terminally repetitious rods (1, 2) capable of this interconversion.

* A terminally repetitious DNA rod contains the same base sequence at both ends.



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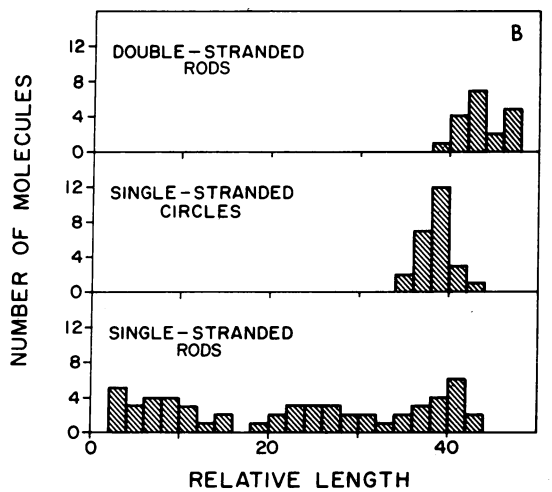

FIG. 2. Observation of single-stranded adenovirus DNA circles. The electron micrograph (*A*) shows a mature adenovirus duplex rod and a single-stranded adenovirus circle. The adenovirus single-stranded circles were made in the following way: a solution of viral DNA (10 μg/ml) was dialyzed against 0.07 M Tris·HCl (pH 7.6) and then mixed with an equal volume of formamide (Matheson, Coleman, and Bell). The DNA solution was then denatured at 70° for 60 sec and cooled to 0°.

When examined in the electron microscope, the solution had only single-stranded rods, most of which were of unit length. The DNA solution was then allowed to anneal at 4° for 72 hr. During this time the DNA, which was originally present entirely as single-stranded rods, changed to the following assortment of forms: out of 400 molecules, 100 (25%) were single-stranded rods, 232 (58%) were single-stranded circles, and 68 (17%) were rods of unit length that were either fully or partially duplex. The DNA was prepared for viewing in the electron microscope by the basic-protein film technique of Kleinschmidt and Zahn (9) as modified by Davis, Simon, and Davidson (10). An additional change was made to enhance the difference between the appearance of single-stranded and double-stranded DNA in the electron microscope: a sodium carbonate buffer (pH 9.3) was used in the spreading solution instead of the Tris·HCl buffer (11).

the mature adenovirus chromosome are intrinsically capable of forming circles.

The formation of single-stranded circles is most easily explained if the ends of positive and negative strands of the mature adenovirus chromosome can hydrogen bond to form a small double helical segment, as shown in Fig. 3. In this case, the adenovirus chromosome would contain an inverted terminal repetition (see Fig. 4).

The region of assumed hydrogen bonding in the single-stranded circles is probably between 200 and 500 base pairs in length. The hydrogen-bonded region is not likely to be much longer than 500 base pairs; otherwise the rings observed in the electron microscope would contain a visible duplex projection. On the other hand, the double helical region cannot be much shorter than 200 base pairs (20) since the rings are very stable: under conditions that approach the denaturation of DNA [with high concentrations of formamide (12)], the rings are not converted to rods until the ends of duplex adenovirus DNA within the same sample begin to denature. The stability of the single-stranded circles in high concentrations of formamide also rule out the possibility of circle formation by hydrogen bonding between only partially homologous DNA sequences (13, 14).

AN EXPERIMENTAL TEST OF THE PROPOSED STRUCTURE

A direct method to show that hydrogen bonding is involved in the formation of adenovirus single-stranded circles uses exonuclease III. This enzyme sequentially removes mononucleotides from the 3'OH ends of double-helical DNA (3). Single-stranded adenovirus rings with a structure such as that shown in Fig. 3*A* or *B* are expected to be digested by exonuclease III in a way that will remove the critical region of hydrogen bonding, and thus convert the single-stranded circles to single-stranded rods. Single-stranded rings with the structure shown in Fig. 3*C* should be refractory to exonuclease III digestion. Rings with the structure shown in Fig. 3*D* would also be refactory, provided that the terminal region of non-base pairing is at least 5 bases long (15).

When the adenovirus circles were exposed to exonuclease III, 86% of the single-stranded circles were converted to rods. This greater than 50% susceptibility of the single-stranded circles to exonuclease III indicates the involvement of a hydrogen bonded 3'OH terminus in the formation of both positive- and negative-strand circles, and supports the structures shown in Fig. 3*A* and *B*.

The sample was spread as follows: 4 μl of a stock cytochrome *c* (Calbiochem) solution (1 mg/ml in 1 M carbonate buffer, pH 9.3) was added to 15 μl of double-distilled H_2O. Then, 6 μl of the DNA sample was added, and the volume was doubled by the addition of 25 μl of formamide. This solution was immediately spread onto a fresh hypophase of 15 mM Tris·HCl and 1.5 mM EDTA (pH 8.5). A portion of the cytochrome film containing the DNA was picked up on a grid coated with Parlodian, stained with Ur_2OAc, and examined in the electron microscope (Philips 300) (12). Molecules were photographed, projected, and traced with a map measurer (Keuffel and Esser 620300). The three histograms (*B*) show the lengths of fully duplex rods (*upper*), single-stranded circles (*middle*), and single-stranded rods (*lower*). The length ratio of single-stranded DNA to duplex DNA was 0.91, as determined with single-stranded and double stranded DNA of phage ϕX. The average projected lengths of the duplex rods and the single-stranded circles were 43.7 and 38.4 cm, respectively.

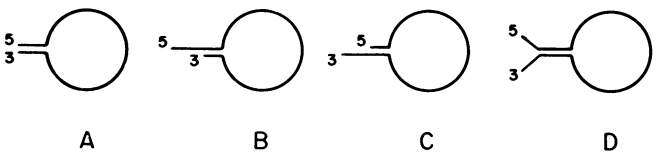

FIG. 3. Four possible structures for single-stranded DNA rings from adenovirus. In each case, the two ends of a positive or negative single-strand base pair to form a region of duplex DNA, and thus the single-stranded rod is converted to a circle. In Panel *A*, the ends of the strand are perfectly base paired out to the 5′ and 3′ termini. In Panel *B*, the region of base pairing ends before the 5′ end of the single strand, leaving a small single-stranded tail. In Panel *C*, the region of base pairing ends before the 3′ end of the strand, again leaving a short single-stranded tail. In Panel *D*, the precise termini of the single strand are not involved in the region of base pairing. The region of presumed base pairing is less than about 500 bases long (see *text*), and thus, it is not possible to distinguish between these four structural alternatives by electron microscopy. However, it is possible to eliminate certain of these structures by the use of DNA nucleases. Specifically, exonuclease III (an enzyme that releases mononucleotides from the 3′ end of a DNA strand in a double helix) (3) should remove the critical region of hydrogen bonding in structures A and B, and thus convert the rings to rods. The molecules shown in Panels *C* and *D* should not be susceptible to digestion by exonuclease III. Single-stranded adenovirus DNA circles were dialyzed to remove the formamide (four 6-hr changes against 0.07 M Tris·HCl, pH 7.6). The solution was then made 7 mM in $MgCl_2$ and 10 mM in 2-mercaptoethanol. To 50 μl of the solution, an equal mass of single-stranded DNA circles of phage φX was added. One unit of exonuclease III was then added, the reaction mixture was incubated at 45° for 20 min, and then a second unit of enzyme was added. 20 min later, the reaction was stopped by transferring the mixture to 0°. Digestion was at 45° to inhibit an exonuclease activity (directed against the 3′ ends of single-stranded DNA), which is present in exonuclease III preparations (3). The material was processed for electron microscopy as described in the legend of Fig. 2. The results show that, while the single-stranded DNA circles from phage φX were virtually untouched during the digestion by the enzyme (indicating the absence of single-stranded endonuclease activity), a preparation with 66% (157/238) single-stranded circles was, after digestion, 10% (23/239) single-stranded rings. That is, about 86% of the single-stranded adenovirus circles had been converted to rods. It is thus concluded that a region of hydrogen bonding, such as that shown in Panels *A* or *B*, is involved in the formation of the single-stranded rings of adenovirus DNA.

As a control, to rule out the conversion of the adenovirus circles to rods by a contaminating single-stranded endonuclease, an equal mass of single-stranded DNA rings of phage φX were included in the adenovirus–enzyme mixture. Before digestion, 87% (872/1000) of the phage φX DNA was in the form of single-stranded circles, and after digestion, 86% (862/1000) of the DNA was in the form of circles. Therefore, since the phage φX rings were essentially untouched during digestion with exonuclease III, the adenovirus circles were not converted to rods by a single-stranded endonuclease.

Whether Fig. 3*A* and/or *B* represents the correct structure of the adenovirus single-stranded circles is not certain. However, if one assumes that the mature viral chromosome is entirely duplex (as in Fig. 4*A* and *B*), then the structure shown in Fig. 3*B* can be eliminated: denaturation of the duplex rod shown in Fig. 4*B* produces a pair of complementary single-stranded rings, only one of which is susceptible to exonuclease III digestion. The finding that more than 85% of the single-stranded rings can be digested with exonuclease III thus rules out the imperfectly symmetric inversion shown in Fig. 4*B*, but is consistent with the structure shown in Fig. 4*A*. If, on the other hand, one assumes that the mature adenovirus-2 chromosomes are not entirely duplex (but, for example, contain an unpaired 5′ nucleotide) (Fig. 4*C*), then the observed result of greater than 50% conversion by exonuclease III would, as in the case of Fig. 4*A*, be predicted. At this time, it is not possible to distinguish between the structures shown in Fig. 4*A* and *C*. But this information should come from termini sequencing studies on the adenovirus-2 DNA molecule. The studies have thus far identified guanine as the 5′ terminal nucleotide for both strands of the adenovirus-2 chromosome (Byron Burlingham, personal communication).

In summary, the linear duplex chromosome of adenovirus-2 contains an inverted terminal repetition. A consequence of this novel structure is that from both ends inward the termini of the adenovirus DNA molecule are identical. This property might be used in a packaging mechanism, in a protective capacity, for DNA replication, or for RNA transcription. At present, it is not possible to distinguish between these and other uses, and the function of the inverted terminal repetition remains a mystery.

It should be noted that the adenovirus inverted terminal repetition structurally eliminates the potential of forming, by any known mechanism, a covalently closed duplex ring. This structural inability might at first appear to be a disadvantage, since both the processes of DNA replication and lysogeny are usually pictured as occurring through circular intermediates. However, in each case, there is an exception: for bacteriophage T7, DNA replication takes place in an intermediate that is linear, not circular (12, 17), and for coliphage P1, lysogeny occurs without integration: the viral DNA molecule maintains itself as a plasmid (19), and a circular state presumably is not necessary for the purpose of recombining into the host chromosome. Thus, the inability of the adenovirus chromosome to form a double-stranded circle does not leave the virus at any apparent disadvantage with respect to DNA replication or lysogeny.

Originally, the phenomenon of formation of adenovirus single-stranded DNA circles was independently observed for adenovirus-7 and -2 by Dr. Thomas Kelly and by us. Since that time, adenovirus-3 circles have been seen by Dr. Philip Sharp (personal communication), and adenovirus-12 and -18 circles have been seen by Dr. Clark Tibbets (personal communication). The formation of adenovirus single-stranded DNA circles has been also studied by Garon, Berry, and Rose (16). Thus far, all adenovirus DNA molecules tested have been found to be capable of forming single-stranded circles.

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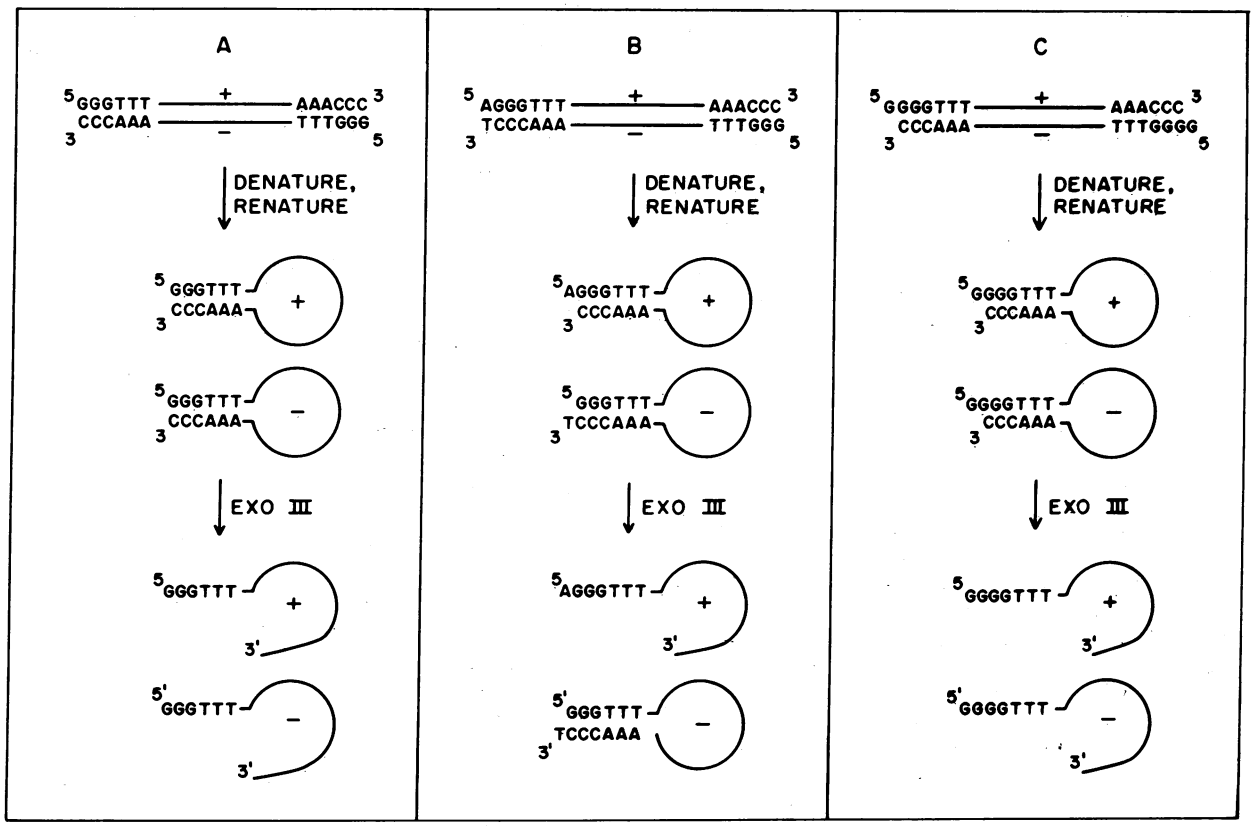

FIG. 4. Three possible structures for the mature adenovirus chromosome with an inverted terminal repetition. The types of single-stranded circles produced upon denaturation of the viral DNA, and their sensitivity to exonuclease III are also shown. As is discussed in the text, both the simpler structure of panel *A* and the more complex structure of panel *C* are conistent with the data.

NOTE ADDED IN PROOF

In a recent communication [*Proc. Nat. Acad. Sci. USA* **69**, 2391–2395 (1972)], Garon, Berry, and Rose, in reporting the formation of single-stranded circles by the chromosomes of adenoviruses 1, 2, 3, 7, 18, and 31, offered two possible strand substructures for the adenovirus single-stranded circles (Fig. 4*A* and *B*, page 2394). Their Fig. 4*B* structure is the same as that shown in our Fig. 3*A*. We wish to rule out their Fig. 4*A* structure by noting that, in the formation of the single-stranded circle, the ends of an adenovirus strand are held together in a double helix with the *complementary* strands aligned in a *parallel* rather than an *antiparallel* orientation. The formation of a DNA double helix with complementary and parallel strands has never been reported as structurally possible.

Conceivably, one could explain the adenovirus single-stranded circles as being held together by a double-helical segment with *parallel* and *noncomplementary* DNA strands. For instance, at least at pH values near 5, two poly(A) (Rich, A., Davies, D., Crick, F. & Watson, J. (1961) *J. Mol. Biol.* **3**, 71–86) or two poly(C) strands (Langridge, R., & Rich, A. (1963) *Nature* **198**, 725–728; Hartman, K. & Rich, A. (1965) *J. Amer. Chem. Soc.* **87**, 2033–2039) can interact to form a parallel double helix. However, the possible relationship of such special structures to any *in vivo* process remains a matter for conjecture.

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