Proc. Nat. Acad. Sci. USA
Vol. 73, No. 3, pp. 742–746, March 1976
Biochemistry

Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis*

(DNA replication/hairpin molecules/self-primed DNA synthesis)

STEPHEN E. STRAUS, EDWIN D. SEBRING, AND JAMES A. ROSE

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

Communicated by Wallace P. Rowe, December 9, 1975

ABSTRACT **Replicating DNA molecules of adenovirus-associated virus (AAV) were selectively extracted from KB cells coinfected at 39.5° with a DNA minus, temperature-sensitive mutant of adenovirus 5 (ts125) as helper. Under these conditions AAV DNA replication proceeds normally, but there is little, if any, adenovirus DNA synthesis. An analysis of the replicating molecules in sucrose density gradients reveals that there are AAV DNA intermediates which consist of covalently linked plus and minus DNA strands. Under denaturing conditions, these concatemers are linear single strands whose lengths can reach at least four times the size of the AAV genome. The most abundant concatemeric species is a dimer which presumably exists *in vivo* as a unit length hairpin. Unit length linear duplexes appear to be immediate precursors of plus and minus progeny strands. These findings are compatible with a self-priming mechanism for the synthesis of AAV DNA.**

Adenovirus-associated viruses (AAV) contain single-stranded, linear DNA with a molecular weight of about 1.4×10^6 (1). They are defective parvoviruses that multiply only in cells coinfected with a helper adenovirus that may provide one or more factors required for AAV DNA synthesis (2). There are two unusual genetic features of the AAV: (*a*) plus or minus DNA strands are separately packaged in progeny virions and (*b*) both of these complementary strands possess an inverted terminal repetition 100–200 nucleotides in length (3–6). The function of the repetitious sequences is not definitely known, but their location and the possibility that they contain a terminal palindrome suggest a role in DNA synthesis (5, 7). Furthermore, this repetition may relate to adenovirus dependence, since the adenovirus genome is the only other viral DNA known to possess a similar type of terminal redundancy (8–10).

The present study was undertaken to characterize AAV DNA replicative intermediates (RI). Our work was facilitated by using a DNA minus, temperature-sensitive mutant of adenovirus 5 (ts125) as helper. Ts125 supports normal AAV multiplication at the restrictive temperature (39.5°), but there is little or no detectable adenovirus DNA synthesis (11). Thus, when selectively extracted by a modification of the Hirt procedure, replicating components of AAV DNA are essentially free of contaminating adenovirus DNA (11). In this report we present a detailed analysis of such extracts in neutral and alkaline sucrose density gradients. The results provide evidence for concatemeric DNA intermediates which suggest that AAV DNA replication proceeds by a unique mechanism in which DNA synthesis is initiated by a self-priming terminal sequence.

Abbreviations: AAV, adenovirus-associated virus; Ad, adenovirus; RI, replicative intermediates; $TCID_{50}$, 50% tissue culture infective dose; WT, wild type; SV40, simian virus 40; BND-cellulose, benzoylated-naphtholated DEAE-cellulose; DS, double stranded fraction.

* Presented in part at the meeting of Federation of American Societies for Experimental Biology, April 1975 [(1975) *Fed. Proc.* 34, 639].

MATERIALS AND METHODS

Cells and Viruses. Infections were carried out in KB cell spinner cultures at 39.5°. Both adenovirus 5 (Ad5) wild type (WT) and ts125 were a generous gift of H. S. Ginsberg. Procedures for the growth, purification, and assay of these strains as well as for AAV type 2 (AAV-2) have been described (11, 12). The Ad5 inocula were cell-free concentrates (12); the AAV-2 inoculum consisted of heated, CsCl-purified virus (11). Ad-AAV coinfection multiplicities were 5 $TCID_{50}$ and 2 $TCID_{50}$ units per cell. ($TCID_{50}$ is the 50% tissue culture infective dose.)

Labeling and Extraction of DNA. AAV-2 DNA was pulse-labeled with [^{3}H]thymidine (50 Ci/mmol) added to cultures (10 μCi/ml) at 16 hr after infection for intervals specified in individual experiments. [^{3}H]Thymidine chases were performed by pelleting pulse-labeled, infected cells at $600 \times g$ and resuspending cells in warmed growth medium supplemented with thymidine, 100 μg/ml, and 2′-deoxycytidine, 10 μg/ml. These conditions permit continued DNA synthesis without further incorporation of [^{3}H]thymidine (13). Methods for extracting whole cell DNA and DNA from purified virus preparations have been published (14). The selective recovery of viral DNA from infected cells was accomplished by a modification of the Hirt procedure (11). DNA was extracted from pellet fractions as described for whole cell DNA except that pellets were first heated to 80° in 0.5 M NaOH and then neutralized.

Analysis and Recovery of DNA Components. Components of viral DNA synthesis were analyzed in 5–30% neutral sucrose gradients (containing 0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, 0.1% Sarkosyl, pH 8.0) and 10–30% alkaline sucrose gradients (containing 0.3 M NaOH, 0.7 M NaCl, 0.001 M EDTA, 0.1% Sarkosyl) centrifuged in an SW41 rotor at 10° for 8 hr at 40,000 rpm and 12 hr at 40,000 rpm, respectively. Similar gradients were used to recover preparative amounts of specific DNA components. Gradient fractionation and assay of radioactivity were described previously (11).

The fraction of DNA molecules that contained self-complementary regions was assayed by hydroxyapatite chromatography (15), and benzoylated-naphtholated DEAE (BND)-cellulose chromatography (16) was used to isolate the fraction of rapidly reassociating DNA that was completely or nearly completely double-stranded. Isopycnic banding of DNA in ethidium bromide–cesium chloride was performed as reported by Bauer and Vinograd (17).

S1 Nuclease Digestion of DNA. The single-strand specific nuclease, S1, was purified from crude *Aspergillus oryzae* powder (Sigma Chemicals) (18). Reaction mixtures (0.2 ml)

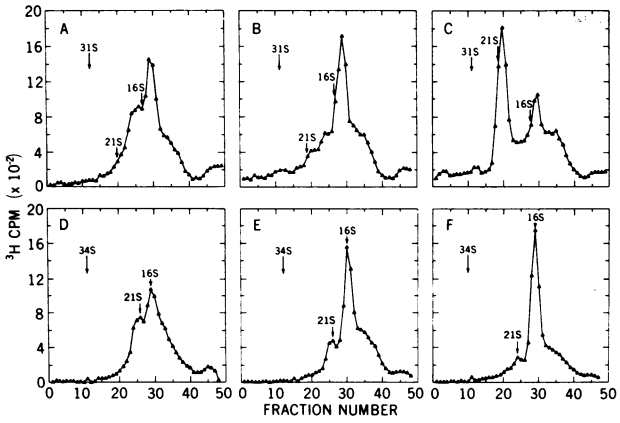

FIG. 1. Pulse-chase kinetics of AAV replicating DNA molecules. Neutral and alkaline sucrose gradient analyses of Hirt extracts of samples obtained after a 2-min pulse (A and D); a 2-min pulse and a 30-min chase (B and E); and a 2-min pulse and 6-hr chase (C and F). Coinfection in this and all subsequent experiments was carried out with ts125 as helper. The arrows at 31 S, 21 S, and 16 S in neutral gradients indicate positions of cosedimented marker Ad5 [^{14}C]DNA, SV40 [^{14}C]DNA I, and SV40 [^{14}C]DNA II. The arrows at 34 S and 16 S in alkaline gradients indicate the positions of marker Ad5 [^{14}C]DNA and SV40 [^{14}C]DNA III.

contained 0.1–0.2 μg of DNA and 3–15 units of nuclease in 30 mM sodium acetate buffer (pH 4.6), 0.5 mM $ZnCl_2$, and 75 mM NaCl. After incubation for 15 min at 37°, the reaction was terminated by addition of 5 μl of 0.4 M EDTA and the extent of cleavage determined by sedimentation in neutral and alkaline sucrose gradients. Digestion conditions were sufficient to completely degrade a 50- to 200-fold excess of single-stranded DNA and to completely convert an equivalent amount of simian virus 40 (SV40) DNA I to DNA III with no detectable single-strand breakage of DNA III.

RESULTS

Extraction of AAV DNA. When Hirt extracts of [^{3}H]thymidine-labeled viral DNA are sedimented in neutral sucrose gradients after coinfection with AAV and ts125 or Ad5 WT (at 39.5°), the sedimentation profiles are identical with the exception that adenovirus DNA components (≧31 S) are absent when ts125 is the helper (11). With ts125 as helper, labeled DNA sediments in a multicomponent pattern ranging from 10 to 21 S (ref. 11; Figs. 1A, 2, and 4A). Hybridization analyses of radioactive virus-specific DNA in both the Hirt supernatant and pellet fractions after a 1-hr pulse revealed that about 80% of total labeled AAV (or Ad WT) DNA was present in the supernatant fraction. Furthermore, in coinfections with AAV and ts125, the ^{3}H-labeled DNA in Hirt extracts that sedimented from 10 to 21 S in neutral sucrose gradients was found to be over 95% AAV-specific both by filter hybridization (11) and by free solution annealing reactions monitored by hydroxyapatite chromatography (15).

Because progeny AAV DNA molecules consist of single plus and minus strands, the analysis of AAV DNA replication requires methods that preserve these specific molecular forms (i.e., duplex molecules must not be artifactually generated from progeny strands during extraction or subsequent sedimentation in neutral sucrose gradients). To be certain that methods used did not foster annealing of plus and minus progeny strands, we added either alkali-denatured or annealed (double-stranded) AAV [^{3}H]DNA, obtained from purified virions, to unlabeled AAV-ts125 coinfected KB cells (16 hr at 39.5°) and then recovered DNA by the modified

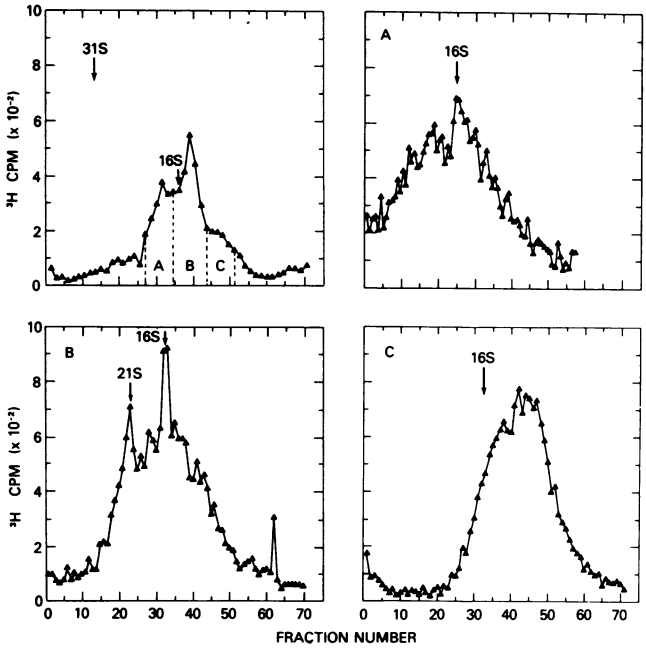

FIG. 2. Alkaline sucrose sedimentation of AAV replicating DNA previously fractionated in a neutral sucrose gradient. DNA from the 2-min pulse-labeled preparation analyzed in Fig. 1 (A and D) was sedimented through neutral sucrose (upper left panel) and pools A, B, and C were collected as indicated. Each pool was then cosedimented through alkaline sucrose with a SV40 [^{14}C]DNA III marker (16 S). Pool A (A); pool B (B); and pool C (C).

Hirt procedure. In neutral sucrose gradients over 90% of the denatured AAV [^{3}H]DNA remained single stranded (20 S), whereas over 90% of the annealed AAV [^{3}H]DNA sedimented as unit length duplexes (15 S) (data not shown). In addition, an extract was prepared from cells infected for 16 hr with Ad5 WT alone, pulsed for 10 min with [^{3}H]thymidine, and then chased with thymidine for 6 hr. On alkaline sucrose sedimentation, the extract contained no radioactive DNA in molecules shorter than unit length (34 S). Thus, significant single-strand breakage of DNA does not occur during the extraction procedure (data not shown).

Identification of Replicating AAV DNA Components. Fig. 1 depicts the results of a pulse-chase experiment after AAV-ts125 coinfection. Shown are the neutral sucrose sedimentation patterns of AAV DNA after a 2-min pulse (A), a 2-min pulse plus a 30-min chase (B), and a 2-min pulse plus a 6-hr chase (C). Panels D, E, and F show the corresponding alkaline sucrose sedimentation patterns. The 2-min pulse-labeled DNA in the 16–21S region of gradient A chases into part of unit length linear duplex DNA (15 S) (B), which then chases into single-stranded progeny DNA (20 S) (C). In both neutral and alkaline gradients the amount of labeled DNA sedimenting more slowly than unit length DNA (10–12 S) appears to be relatively unchanged throughout the chase and probably represents fragments of duplex DNA. In addition to unit length DNA, the alkaline gradients contain a peak of faster sedimenting DNA that can be chased into unit length molecules (16 S in alkaline sucrose). Analysis of the fast sedimenting DNA by electron microscopy (E. Sebring, unpublished results) has failed to reveal covalently closed single- or double-stranded circles. Furthermore, radioactivity could not be detected in closed duplex circular molecules when DNA in the 2-min pulse or 6-hr chase extracts was banded in ethidium bromide-CsCl. Thus, the fast sedimenting DNA apparently consists of linear molecules that are

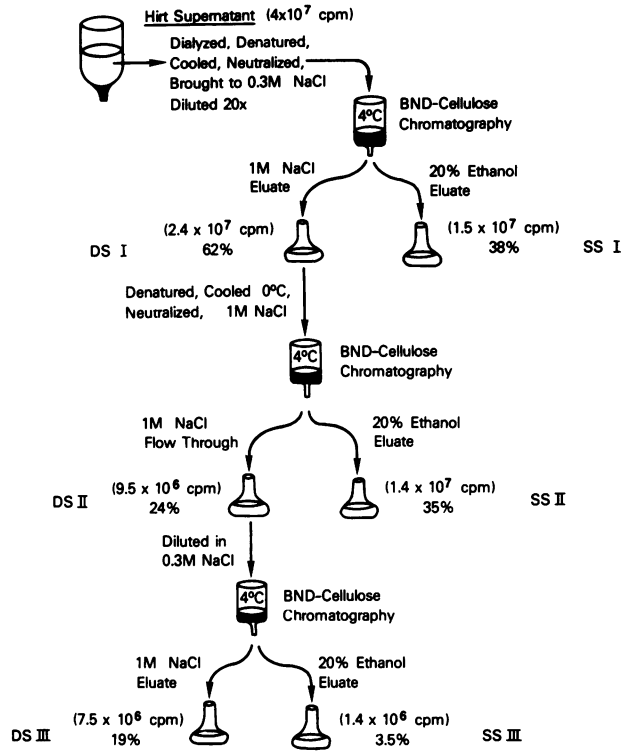

FIG. 3. Purification of AAV DNA replicating snap-back molecules.

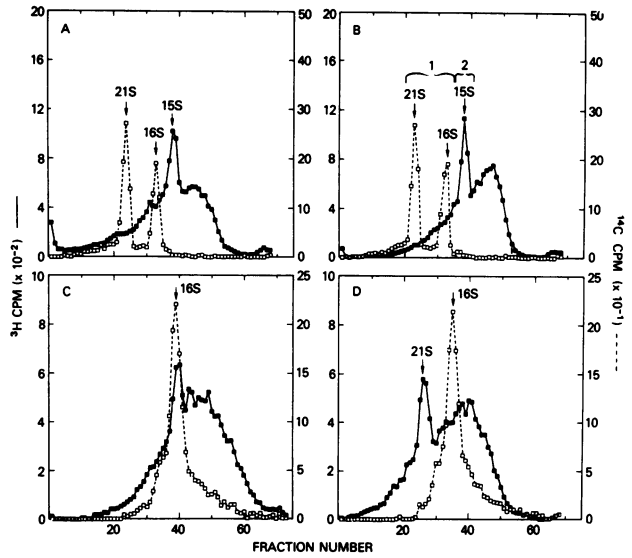

FIG. 4. Sucrose gradient analysis of rapidly reassociating DNA. Hirt supernatant AAV replicating DNA and the fraction of rapidly reassociating DNA (DS III), isolated as depicted in Fig. 3, were each analyzed in neutral and alkaline gradients. Neutral sucrose sedimentation of (A) Hirt supernatant DNA and (B) DS III DNA; alkaline sucrose sedimentation of (C) Hirt supernatant DNA and (D) DS III DNA. Cosedimented marker SV40 [^{14}C]DNA radioactivity is plotted with a dashed line. Preparative neutral sucrose gradients of DS III DNA were also run and divided into two pools (1 and 2) as shown in panel (B).

longer than progeny strands. The location of long molecules with respect to the neutral sucrose sedimentation profile proved to be interesting (Fig. 2). When fractions in the 15S region from a preparative neutral sucrose gradient of the DNA after the 2-min pulse were pooled and sedimented in alkaline sucrose, about half of this DNA (unit length duplex DNA in neutral sucrose) sedimented faster than 16S unit length single strands, with a distinct peak at 21S which is twice unit length size (Fig. 2B). Additionally, at least half of the 16–21S DNA from the neutral gradient also sedimented faster than unit length strands (Fig. 2A), whereas most of the 10–12S DNA sedimented more slowly (Fig. 2C). These data indicate that (*a*) there are AAV DNA RI that contain continuous linear strands whose lengths exceed that of single-stranded progeny molecules and (*b*) the long strands may be folded into partially or completely duplex structures (e.g., hairpin-like molecules). The specific nature of the rapidly labeled but seemingly "dead end" 10–12S DNA is uncertain.

Concatemeric DNA Molecules. When a Hirt extract of AAV-ts125 coinfected KB cells, pulse-labeled for 10 min, was denatured with 0.1 M NaOH, chilled to 0°, neutralized, and chromatographed on hydroxyapatite, 55% of the labeled DNA eluted as double-stranded DNA. Thus, a large portion of the pulse-labeled AAV DNA contains self-complementary regions that rapidly reassociate to form duplex DNA, a result consistent with postulation (*b*) above. To further characterize these "snap-back" molecules, BND-cellulose, which selectively retains DNA with single-stranded regions, was used to isolate the fraction of rapidly reassociating DNA that is completely or almost completely double-stranded. Extracted DNA that had been labeled for 1 hr with [^{3}H]thymidine was denatured with 0.1 M NaOH. After neutralization, double-stranded DNA was isolated and the procedure repeated a second time. Approximately 19% of the starting radioactivity was recovered in the final double-stranded fraction (DS III) (Fig. 3). Fig. 4 shows neutral and alkaline sucrose sedimentation patterns of AAV DNA in the Hirt supernatant (A and C) and the DS III BND-cellulose fraction (B and D). The DS III alkaline gradient (D) demonstrates a marked enrichment for DNA sedimenting faster than unit length with a pronounced peak sedimenting as molecules that were twice unit length (21 S). To define more clearly the nature of these long molecules, we pooled fractions from the neutral sucrose gradient of rapidly reassociated DNA, as indicated in Fig. 4B, and each pool was sedimented in alkaline sucrose, as shown in Fig. 5. Double-stranded DNA that sedimented ahead of unit length duplexes in neutral sucrose (pool 1) mostly consisted of strands greater than unit length (Fig. 5A), and about two-thirds of molecules that sedimented in the region of unit length duplexes (pool 2) were also longer than unit length strands (Fig. 5B). Approximately 40% of this latter DNA sedimented as molecules that were

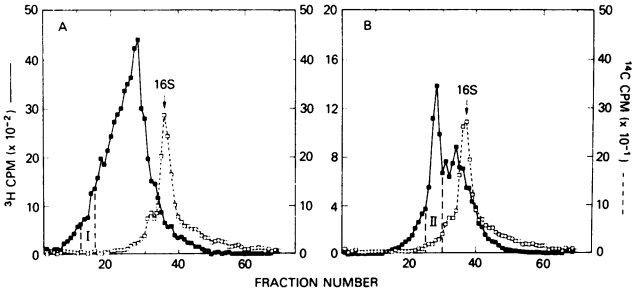

FIG. 5. Alkaline sucrose sedimentation of DS III DNA pools 1 and 2 which were fractionated as shown in Fig. 4B. Pool 1 (A); pool 2 (B). Cosedimented marker SV40 [^{14}C]DNA III radioactivity is plotted with a dashed line. Fractions corresponding to a tetramer (27 S) and peak fractions corresponding to a dimer (21 S) were collected from similarly run preparative gradients and designated pool I and pool II, as shown in (A) and (B).

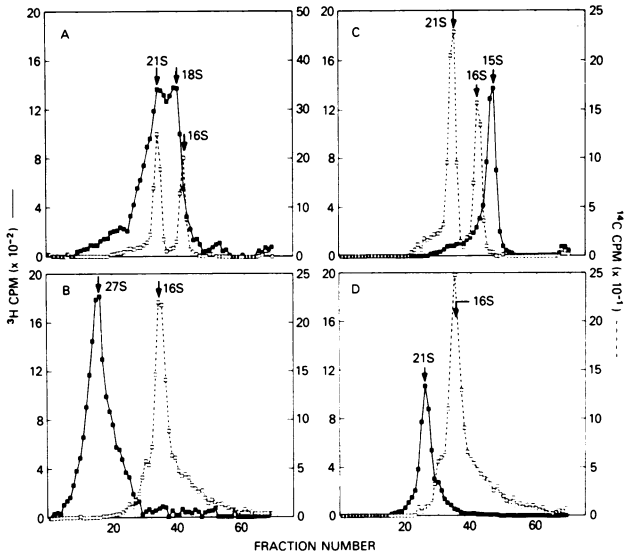

FIG. 6. Neutral and alkaline sucrose sedimentation analyses of tetramer and dimer length AAV DNA strands (pools I and II, Fig. 5). (A) Neutral and (B) alkaline sedimentation of tetramer pool; (C) neutral and (D) alkaline sedimentation of dimer pool. Cosedimented marker SV40 [^{14}C]DNA radioactivity is plotted with a dashed line.

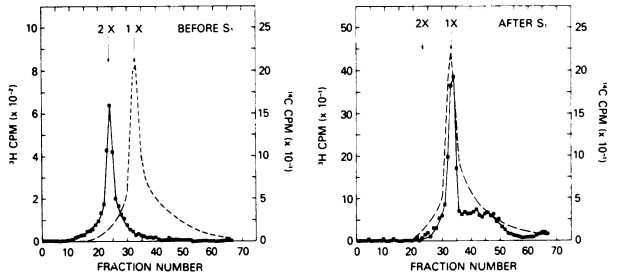

FIG. 7. Treatment of dimer length strands of rapidly reassociating AAV replicating DNA with S1 nuclease. Dimer strands obtained as in Fig. 6D were further purified by sedimentation through neutral sucrose. The unit length duplex peak was collected and dialyzed into 0.01 M Tris–0.01 M NaCl, pH 7.5. Portions of this DNA were treated with 2, 5, or 10 μl of S1 nuclease (1600 units/ml), and the samples as well as untreated DNA were analyzed in alkaline sucrose. Only the sample treated with 5 μl of S1 nuclease is shown, since all three enzyme concentrations gave identical sedimentation patterns. Cosedimented marker SV40 [^{14}C]DNA radioactivity is plotted with a dashed line.

twice unit length. Finally, fractions from the first gradient corresponding to a tetramer (27 S) were combined (Fig. 5A, pool I) as well as peak fractions from the second gradient corresponding to a dimer (Fig. 5B, pool II) and each pool was resedimented in neutral and alkaline sucrose (Fig. 6.). Comparison of the respective neutral and alkaline sedimentation patterns indicates that on neutralization the denatured molecules can fold into duplex structures of approximately half single strand length. The most likely structure of molecules with these sedimentation properties would be linear strands composed of covalently end-to-end linked plus and minus strands of AAV DNA. The bimodal sedimentation pattern observed in the neutral gradient of pool I DNA (Fig. 6A) may have arisen because of different folding configurations possible with a tetramer composed of alternating plus and minus strands (i.e., the 18S component would represent a linear hairpin twice unit length, whereas the 21S component might represent a branched duplex form).

Further evidence that replicating DNA can exist as a hairpin structure was obtained by S1 nuclease digestion of the rapidly reassociated unit length duplexes. A hairpin molecule would necessarily have several unpaired bases in the hairpin loop, and S1 nuclease, which is highly specific for single-stranded DNA, would be expected to cleave this loop. Denaturation of treated molecules should then give rise to unit length single strands. Fig. 7 shows alkaline sucrose gradients of rapidly reassociated unit length duplexes before and after S1 nuclease treatment. Clearly, molecules that sediment in alkaline sucrose as 21S dimers are cleaved by S1 nuclease to molecules that sediment as 16S unit length strands.

DISCUSSION

Little is known concerning the actual DNA synthetic mechanism of any parvovirus, because the structures of replicating DNA molecules have not been sufficiently characterized. In the case of minute virus of mice and Kilham rat virus (both nondefective parvoviruses) there is evidence for a linear, duplex replicating intermediate (19, 20). For minute virus of mice, some of these molecules were shown to be capable of spontaneous, intramolecular renaturation, but the specific structural feature responsible for renaturation was not defined. This observation, however, prompted the speculation that minute virus of mice DNA synthesis might be mediated by a self-primed hairpin intermediate (19).

Our results indicate that AAV DNA RI contain continuous linear strands whose lengths may reach or possibly exceed four times the length of progeny strands, and that these long molecules appear to be concatemers composed of alternating plus and minus strands. Of the population of concatemers capable of self-annealing into completely or almost completely double-stranded molecules, the relatively largest and most discrete fraction consists of dimers that presumably exist as linear hairpin structures *in vivo* (Fig. 2B). Longer DNA concatemers might also possess this same conformation during DNA replication. However, other structures are possible, and branched molecules can be found when preparations of RI isolated by BND-cellulose chromatography are examined in the electron microscope (E. Sebring, unpublished results). Although we are not yet able to exclude a rolling circle mechanism for AAV DNA synthesis, electron microscopic studies of AAV DNA RI do not support this possibility.

The observations that (*a*) the immediate precursor of progeny strands is a unit length duplex (Fig. 1), (*b*) a unit length hairpin is a major DNA intermediate (Figs. 2 and 4), and (*c*) RNA sequences do not appear to be covalently joined to replicating DNA molecules (stability of concatemers in alkali; S. Straus, unpublished results) are compatible with a replication scheme in which DNA synthesis is initiated by a self-priming mechanism. A model for the formation of a unit length hairpin molecules as an intermediate in AAV DNA replication is shown in Fig. 8A. The self-complementary terminal segments of AAV DNA strands (5) are postulated to contain a palindromic sequence (7) which could fold back on itself to form a short duplex region. The 3′ end of one or both strands could then serve as a primer for synthesis of the complementary strand, generating a hairpin. Based on this mechanism, one of several possible models for synthesis of single-stranded progeny molecules is depicted in Fig. 8B and C. Fig. 8B shows how the hairpin molecule (Fig. 8A) might be converted to a unit length duplex with complete ends according to steps recently proposed by Cavalier-

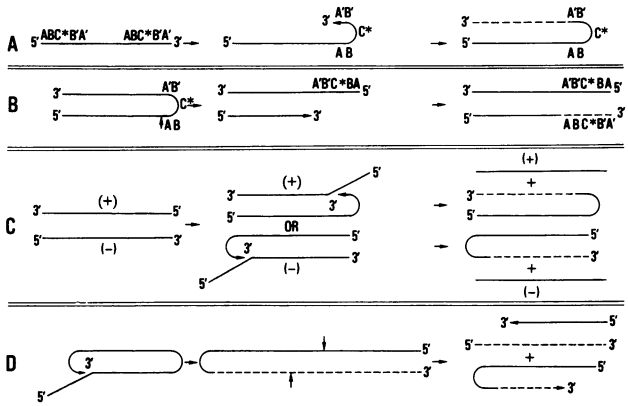

FIG. 8. Model for replication of AAV DNA. In (A) and (B) letters represent terminal sequences equivalent to a total of 100–200 nucleotides. Sequence complementarity is indicated by corresponding primed and unprimed letters. The letter C* represents a sequence that contains unpaired bases in the hairpin loop. To provide identical molecular ends, this sequence would have to be a palindrome (as shown).

Smith (21). After a nick (arrow) near the closed end of the hairpin, the 5′-ended segment, containing a full complement of terminal sequences, becomes available as a template for completing the other strand by 5′–3′ synthesis. A plus or minus strand could then be generated from the resulting duplex intermediate by displacement synthesis, which again involves self-priming (Fig. 8C). Initiation of displacement synthesis by either strand in the duplex template may relate to the presence of identical molecular ends, a consequence of the inverted repetition (5). The unit length hairpins produced along with single strands could be processed (Fig. 8B) to provide more templates for displacement synthesis. Consistent with the expectation that a connecting loop would occur at either end of the hairpin molecule (Fig. 8C), we have recently found that when unit length hairpins are cleaved by endonuclease R·*Eco*RI (22), and then denatured, approximately half of both right- and left-end fragments undergoes rapid renaturation (unpublished results). Although hairpin molecules have not been identified as intermediates in adenovirus DNA replication, it is notable that these AAV helpers also utilize displacement synthesis to produce their own DNA (23).

Evidence that most of the radioactivity in unit length hairpins moves into unit length single strands during a chase indicates that these hairpin molecules are directly converted to templates for displacement synthesis. An alternative pathway may account for plus-minus concatemers of greater than twice unit length (Fig. 8D). The 3′ end of a unit length hairpin would displace the 5′ end of its complementary strand, priming the synthesis of a hairpin twice unit length (or longer).† Such molecules might then be cleaved by a staggered cut (arrows) as proposed for T7 DNA (24), and the 3′ ends regenerated by 5′–3′ synthesis giving a unit length linear duplex (with complete ends) and hairpin.

Processing of concatemers to unit length single strands (Fig. 1) implies the presence of a sequence-specific endonuclease(s) which might act as suggested in Fig. 8B and D. This activity could reside in an AAV, adenovirus, or cellular protein and is specially notable because a restriction-like endonuclease has not yet been isolated from an animal cell.

1. Rose, J. A. (1974) in *Comprehensive Virology*, eds. Fraenkel-Conrat, H. & Wagner, R. (Plenum Press, New York), Vol. 3, pp. 1–61.
2. Carter, B. J., Koczot, F. J., Garrison, J., Dolin, R. & Rose, J. A. (1973) *Nature New Biol.* **244**, 71–73.
3. Rose, J. A., Berns, K. I., Hoggan, M. D. & Koczot, F. J. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 863–869.
4. Berns, K. I. & Rose, J. A. (1970) *J. Virol.* **5**, 693–699.
5. Koczot, F. J., Carter, B. J., Garon, C. F. & Rose, J. A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 215–219.
6. Berns, K. I. & Kelly, T. J., Jr. (1974) *J. Mol. Biol.* **82**, 267–271.
7. Gerry, H. W., Kelly, T. J., Jr. & Berns, K. I. (1973) *J. Mol. Biol.* **79**, 207–225.
8. Garon, C. F., Berry, K. W. & Rose, J. A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2391–2395.
9. Wolfson, J. & Dressler, D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3054–3057.
10. Garon, C. F., Berry, K. W. & Rose, J. A. (1975) *Proc. Nat. Acad. Sci. USA* **72**, 3039–3043.
11. Straus, S. E., Ginsberg, H. S. & Rose, J. A. (1976) *J. Virol.* **17**, 140–148.
12. Ensinger, M. J. & Ginsberg, H. S. (1972) *J. Virol.* **10**, 328–339.
13. Lavelle, G., Patch, C., Khoury, G. & Rose, J. A. (1975) *J. Virol.* **16**, 775–782.
14. Rose, J. A. & Koczot, F. J. (1972) *J. Virol.* **10**, 1–8.
15. Thoren, M. M., Sebring, E. D. & Salzman, N. P. (1972) *J. Virol.* **10**, 462–468.
16. Sedat, J. W., Kelly, R. B. & Sinsheimer, R. L. (1967) *J. Mol. Biol.* **26**, 537–540.
17. Bauer, W. & Vinograd, J. (1968) *J. Mol. Biol.* **33**, 141–171.
18. Vogt, V. (1973) *Eur. J. Biochem.* **33**, 192–200.
19. Tattersall, P., Crawford, L. V. & Shatkin, A. J. (1973) *J. Virol.* **12**, 1446–1456.
20. Salzman, L. A. & White, W. L. (1973) *J. Virol.* **11**, 299–305.
21. Cavalier-Smith, T. (1974) *Nature* **250**, 467–470.
22. Carter, B. J. & Khoury, G. (1975) *Virology* **63**, 523–538.
23. Sussenbach, J. S., van der Vliet, P. C., Ellens, D. J. & Jansz, H. S. (1972) *Nature New Biol.* **239**, 47–49.
24. Watson, J. D. (1972) *Nature New Biol.* **239**, 197–201.

† AAV DNA synthesis would thus be moving back and forth in a boustrophedonic manner, i.e., like the method of ancient writing in which lines were written from right to left and from left to right.