JOURNAL OF VIROLOGY, June 1970, p. 693–699
Vol. 5, No. 6

Evidence for a Single-Stranded Adenovirus-Associated Virus Genome: Isolation and Separation of Complementary Single Strands

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Received for publication 25 February 1970

Single-stranded adenovirus-associated virus type 2 deoxyribonucleic acid (AAV-2 DNA) has been isolated from the virion after enzymatic pretreatment of the particles by heating at 53 C for 1 hr in 0.015 M NaCl plus 0.0015 M sodium citrate in the presence of 1% sodium dodecyl sulfate. Double-stranded AAV-2 DNA present as a marker is not denatured by this treatment. AAV-2 single-stranded DNA is composed of two complementary species which can be separated in neutral CsCl when 5-bromodeoxyuridine has been substituted for thymidine in the DNA. The present report is the first documented instance of the separation of complementary strands of an animal virus DNA.

The adenovirus-associated viruses (AAV) are small, defective deoxyribonucleic acid (DNA) viruses which require an adenovirus as a helper for production infection (1, 7). Purified AAV DNA was found to be double-stranded with a molecular weight of 3.0×10^6 to 3.6×10^6 (10, 12). Crawford et al. (5), however, suggested that AAV DNA was single-stranded in situ and only formed a double helix during extraction. Rose et al. (11) and Mayor et al. (9) have now demonstrated that AAV DNA is indeed single-stranded in situ, and that the double-stranded form of purified AAV DNA results from the annealing of single strands from different virions during extraction (11).

In this paper we report the isolation of single-stranded AAV DNA from virions and the physical separation of the single strands into two complementary species.

MATERIALS AND METHODS

Materials. Preparation and assay of virus stocks has been described (7, 13). KB cells were from a line originally provided by M. Green. Optical grade CsCl and 5-bromodeoxyuridine (BUDR)-*6-*^{3}*H* (12.7 c/mmole) were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y. Thymidine-*2-*^{14}*C* (52.8 mc/mmole) and thymidine-*methyl-*^{3}*H* (>15 c/mmole) were purchased from New England Nuclear Corp., Boston, Mass. ^{32}P was obtained from Tracer Lab, Waltham, Mass. Crystallized-lyophilized trypsin and 2× crystallized papain were from Worthington Biochemical Corp., Freehold, N.J.; 5-fluorodeoxyuridine (FUDR) from Hoffman-La Roche, Inc., Nutley, N.J.; BUDR from Calbiochem, Los Angeles, Calif.; and Sarkosyl (NL 97) from Geigy Industrial Chemicals, Ardsley, N.Y.

Virus growth and purification. The growth and purification of ^{3}H-BUDR adenovirus type 2 (adenoid 6) has been described (11). AAV-2 (AAV-2H) (7) was produced in KB cells in suspension culture [in Eagle's medium (6) supplemented with 5% horse serum] with adenovirus type 2 as helper. For production of AAV-2 containing either ^{3}H- or ^{14}C-thymidine cells were co-infected with adenovirus type 2 [10 tissue culture infectious doses $(TCID)_{50}$/cell] and AAV-2 (10 $TCID_{50}$/cell), and 6 hr later ^{3}H- or ^{14}C-thymidine was added to a final concentration of 1.0 or 0.1 μc/ml, respectively. The cells were harvested after 48 hr at 37 C. To obtain ^{3}H-BUDR AAV-2, cells were similarly infected. FUDR (0.5 μg/ml) was added 13.5 hr after infection, and ^{3}H-BUDR (0.5 μc/ml) and BUDR (10 μg/ml) 14 hr after infection. The infection was terminated at 48 hr. For production of ^{32}P-BUDR AAV-2, cells were infected as above, and 13 hr after infection the cells were collected, washed, and resuspended in Eagle's medium containing reduced PO_4 (10^{-5} M) supplemented with 5% dialyzed horse serum. FUDR (0.5 μg/ml) was then added, and 30 min later ^{32}P (2 μc/ml) and BUDR (10 μg/ml) were added. The infected cells were harvested at 48 hr. To purify AAV, cell harvests from 1 liter cultures were resuspended in 27 ml of phosphate-buffered saline and sonically treated. The preparations were treated with 2% deoxycholate and 0.02% trypsin at 37 C for 30 min. After debris had been removed by low-speed centrifugation, virus was pelleted by centrifugation in the Spinco model L ultracentrifuge at 22,000 rev/min for 3 hr at 4 C in the SW 25.1 rotor. Sedimented virus was resuspended in 4 ml tris(hy-

droxymethyl)aminomethane (Tris), *p*H 7.9, by sonic treatment and the AAV-2 was purified by CsCl centrifugation as described previously (13, 11).

DNA extraction. The extraction of adenovirus DNA and the double-stranded form of AAV DNA have been described (11).

DNA sedimentation. DNA was sedimented through preformed, linear, neutral sucrose gradients containing 1 M NaCl (2). CsCl solutions for isopycnic centrifugation were 0.05 M Tris (*p*H 7.9) and 0.001 M (ethylenedinitrilo) tetraacetic acid and contained 0.15% Sarkosyl in a final volume of 4.5 ml. The specific conditions for each gradient are described in the figures.

Denaturation and annealing of DNA. Denaturation of DNA in alkali and the neutralization of alkali-denatured DNA have been described (2). DNA was annealed in 0.15 M NaCl plus 0.015 M sodium citrate (1× SSC) for 1 hr at 70 C.

RESULTS

Isolation of single-stranded DNA from AAV. AAV DNA is single-stranded in situ (9, 11), but the purified form is double-stranded (10, 13). We were interested in isolating single-stranded DNA under conditions which would (i) not denature the double-stranded form of the DNA, and (ii) not permit annealing of single strands into the double-stranded form. To do this experiment, it is necessary to be able to distinguish single-stranded AAV-2 DNA from the double-stranded form. The two forms of AAV-2 DNA may be separated by isopycnic CsCl centrifugation (12) and by zonal sedimentation through a neutral sucrose gradient (Fig. 1). AAV DNA which had been denatured in alkali and neutralized sedimented more rapidly than double-stranded DNA in a neutral sucrose gradient containing 1 M NaCl (Fig. 1A and B). The ratio of distances traveled was 1.57 (Fig. 1C). AAV single-stranded DNA rapidly anneals to form double-stranded DNA (9, 11), and it is assumed that the trailing material observed in Fig. 1B represents AAV DNA which has annealed at room temperature between the time of neutralization and the completion of the sucrose run (5 hr). The reported method for the purification of AAV DNA (11, 13) releases DNA from the particles after enzymatic pretreatment by heating the virus at 50 C in 1× SSC in the presence of 1% sodium dodecyl sulfate (SDS). These conditions of salt and temperature allow annealing of AAV single strands (11). Alteration of conditions to prevent annealing (e.g., decreased ionic strength or increased temperature, or both) would result in the release of single strands into solution (9). Enzymatically pretreated AAV particles containing ^{3}H-DNA were heated together with purified double-stranded ^{14}C-AAV DNA at 53 C for 1 hr in 0.1× SSC in the presence

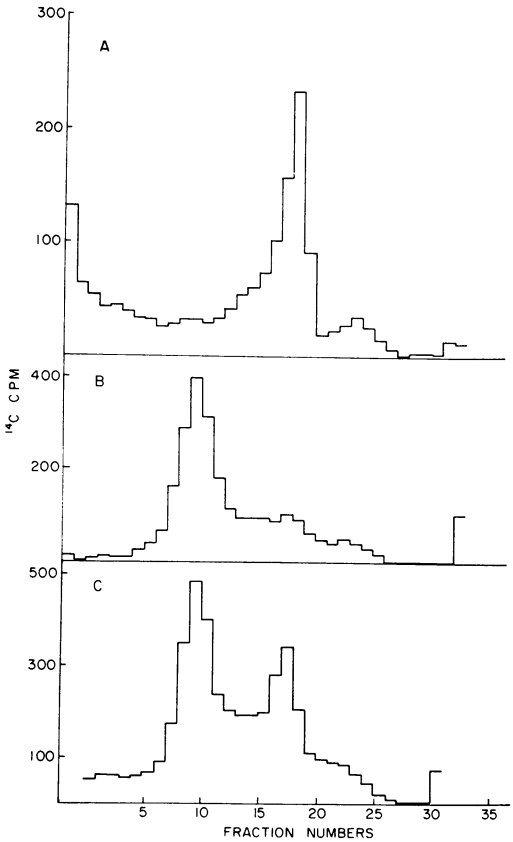

FIG. 1. *Sedimentation of ^{14}C-AAV-2 DNA through linear 5 to 20% neutral sucrose gradients. Centrifugation was for 4 hr at 40,000 rev/min and 20 C in Spinco model SW 50 rotor. (A) Double-stranded AAV-2 DNA. (B) Single-stranded AAV-2 DNA. Double-stranded AAV-2 DNA was alkali-denatured and then neutralized. (C) A mixture of single- and double-stranded AAV-2 DNA. The single-stranded AAV-2 DNA sediments more rapidly than the double-stranded form.*

of 1% SDS. The DNA was then sedimented through neutral sucrose (Fig. 2). Although the ^{14}C-AAV DNA still sediments as double-stranded DNA, the newly released ^{3}H-AAV DNA sediments as single strands. The ratio of distances traveled was 1.58. There is trailing ^{3}H-DNA (approximately 25%) but no more than seen in the case of purified AAV DNA which has been alkali-denatured (Fig. 1B). Thus, under conditions which do not denature purified double-stranded AAV DNA, single-stranded DNA is recovered from the particles. The same preparation was banded in CsCl (Fig. 3). The ^{14}C-double-stranded DNA bands at a density of 1.714 g/cm^{3}. The ^{3}H-DNA peak (fraction 17) is 12 mg/cm^{3} more dense. This is the density difference ob-

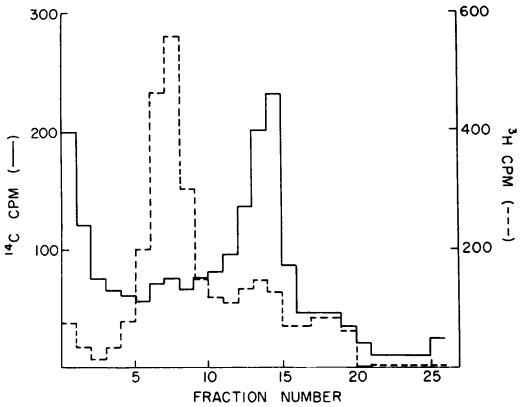

FIG. 2. *Sucrose sedimentation of newly released ^{3}H-AAV-2 DNA and double-stranded ^{14}C-AAV-2 marker DNA. After enzymatic pretreatment, AAV-2 particles containing ^{3}H-DNA were mixed with double-stranded ^{14}C-AAV-2 DNA and heated at 53 C 1 hr in 0.1× SSC in the presence of 1% SDS. Conditions of centrifugation were those described in the legend to Fig 1.*

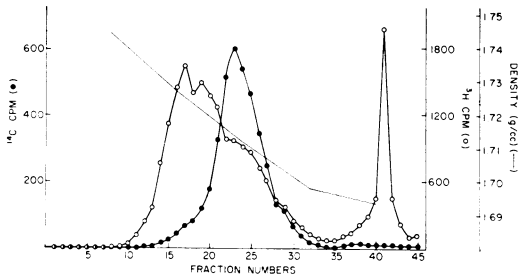

FIG. 3. *Isopycnic CsCl centrifugation for 72 hr at 33,000 rev/min at 20 C in Spinco model 40 rotor. Newly released ^{3}H-AAV-2 DNA and double-stranded ^{14}C-AAV-2 marker DNA. The conditions of DNA release were those described in the legend to Fig. 2.*

served between native and denatured AAV-2 DNA in the analytical ultracentrifuge (12). There is skewing of the ^{3}H band toward lighter density. This result has been repeatedly obtained, and we believe it represents annealing which has occurred during the course of centrifugation (72 hr at 20 C; reference 3). The sharp peak of ^{3}H near the top of the gradient probably represents DNA still associated with capsid protein.

Isopycnic centrifugation of denatured BUDR AAV DNA. Rose et al. (11) had noted that density hybrid AAV double-stranded DNA molecules (i.e., molecules composed of BUDR- and non-BUDR-substituted single strands) banded in a bimodal distribution in CsCl. It was suggested that the components might represent two complementary species which contain different amounts of thymidine. An extensive random substitution of thymidine by BUDR would result, therefore, in two single-stranded species with substantially different densities in CsCl and thus account for the bimodal hybrid. (Non-BUDR-substituted, denatured AAV DNA bands in CsCl as a single uniform band) (12). Double-stranded ^{3}H-BUDR AAV DNA banded in CsCl as a uniform component and was 75 mg/cm^{3} more dense than unsubstituted ^{14}C-double-stranded AAV DNA (Fig. 4). When ^{3}H-BUDR DNA was denatured in alkali, neutralized, and banded in CsCl, two bands were observed (Fig. 5A). If these bands represented two single-stranded species, the heavier band would be expected to have more radioactivity, because it would contain more ^{3}H-BUDR. Under our conditions this is not the case. The light band contains more radioactivity and, in addition, is skewed toward light density. These results could be attributed to the fact that the peak of the light band is close to the density of the original double-stranded ^{3}H-BUDR DNA so that the light band of single polynucleotide chains would be difficult to distinguish from double-stranded DNA which had formed during the experiment (i.e., the two bands would overlap). To test this possibility, double-stranded ^{3}H-BUDR DNA was added as a marker to the denatured, neutralized ^{3}H-BUDR single strands (Fig. 5B). The heavy band is again present, and a portion of the light band is seen as a shoulder on the dense side of the double-stranded marker DNA. An estimate of the counts/min representing light strands can be made from the skew of the double-stranded DNA band in Fig. 5B. The light strands would contain 40% fewer counts/min than the heavy strands. The heavy band was approximately 40 mg/cm^{3}, and the light band-shoulder was about

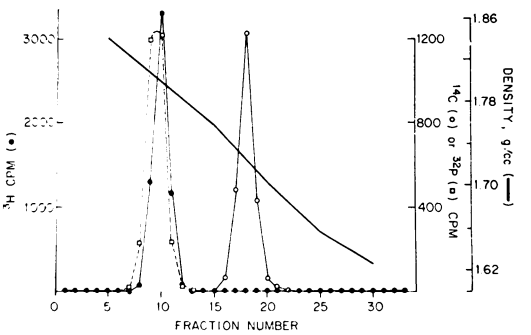

FIG. 4. *Isopycnic CsCl centrifugation for 48 hr at 40,000 rev/min at 25 C in Spinco model SW 50 rotor of double-stranded ^{14}C-thymidine AAV-2 DNA, double-stranded ^{3}H-BUDR AAV-2 DNA, and double-stranded ^{32}P-BUDR AAV-2 DNA.*

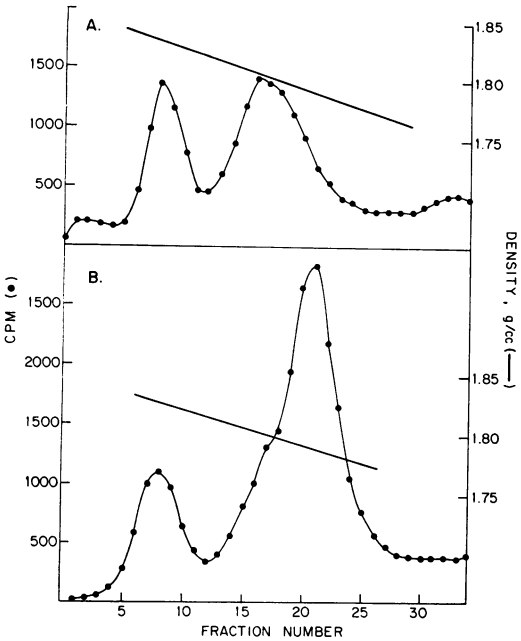

FIG. 5. *Isopycnic CsCl centrifugation for 48 hr at 40,000 rev/min at 25 C in Spinco model 65 rotor of ^{3}H-BUDR AAV-2 DNA. (A) ^{3}H-BUDR AAV-2 DNA was alkali-denatured and then neutralized. (B) Double-stranded ^{3}H-BUDR AAV-2 DNA was added to the solution described in part A as a density marker.*

8 mg/cm^{3} more dense than the double-stranded form of the DNA.

Complementarity of the heavy and light components. The sedimentation characteristics of both single- and double-stranded ^{3}H-BUDR DNA in neutral sucrose were determined. Double-stranded ^{3}H-BUDR AAV DNA sediments 1.16 times as fast as double-stranded ^{14}C-thymidine AAV DNA (Fig. 6A). ^{3}H-BUDR AAV DNA which has been alkali-denatured and neutralized sediments 1.85 times as fast as the double-stranded ^{14}C-AAV DNA (Fig. 6B). When the denatured ^{3}H-BUDR AAV DNA is annealed at 70 C in 1 × SSC for 1 hr, it sediments as double-stranded ^{3}H-BUDR DNA (Fig. 6C). Thus, ^{3}H-BUDR AAV single polynucleotide chains which have been annealed into double-stranded DNA can be readily distinguished from the original single strands in a neutral sucrose gradient. If the two bands seen in Fig. 5A primarily represent the separation of two species of AAV single strands which are complementary, neither population should form double-stranded DNA when exposed to annealing conditions, but double-stranded DNA should be formed when the two species are mixed under the same annealing conditions. The results of this experiment are shown in the sucrose sedimentation data illustrated in Fig. 7. Annealing conditions were the same as for Fig. 6. DNA from the heavy band in Fig. 5A does not form double-stranded DNA when self-annealed (Fig. 7A). DNA from the dense side of the light band remains predominantly single-stranded, but there is a significant amount of trailing material which we assume to be double-stranded DNA resulting from contamination of the light single strands with double-stranded

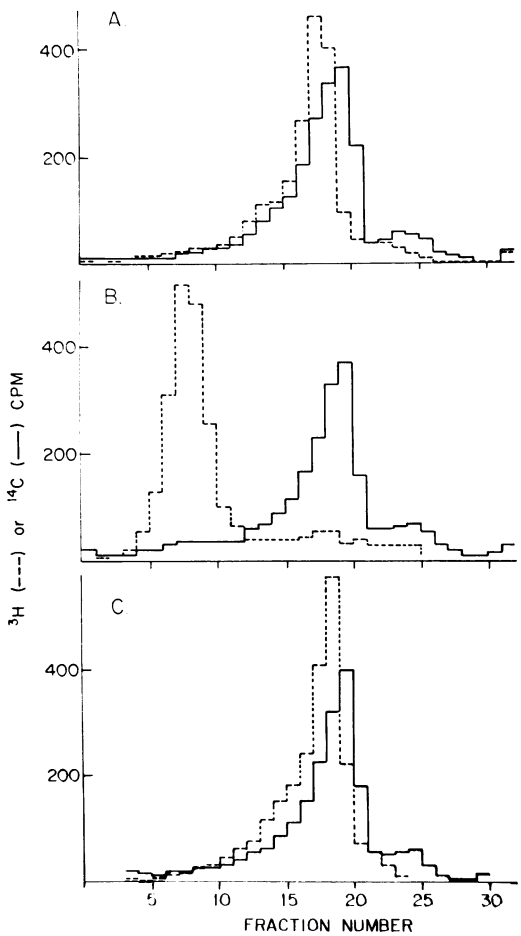

FIG. 6. *Sucrose sedimentation of ^{3}H-BUDR AAV-2 DNA and ^{14}C-thymidine AAV-2 DNA. Conditions of centrifugation were those described in the legend to Fig. 1. (A) Double-stranded ^{3}H-BUDR AAV-2 DNA and double-stranded ^{14}C-thymidine AAV-2 DNA. (B) Single-stranded ^{3}H-BUDR AAV-2 DNA derived from double-stranded DNA by alkali-denaturation and subsequent neutralization and double-stranded ^{14}C-thymidine AAV-2 DNA. (C) The single-stranded ^{3}H-BUDR AAV-2 DNA from part B was annealed at 70 C for 1 hr in 1× SSC. Double-stranded ^{14}C-thymidine AAV-2 DNA present as a marker.*

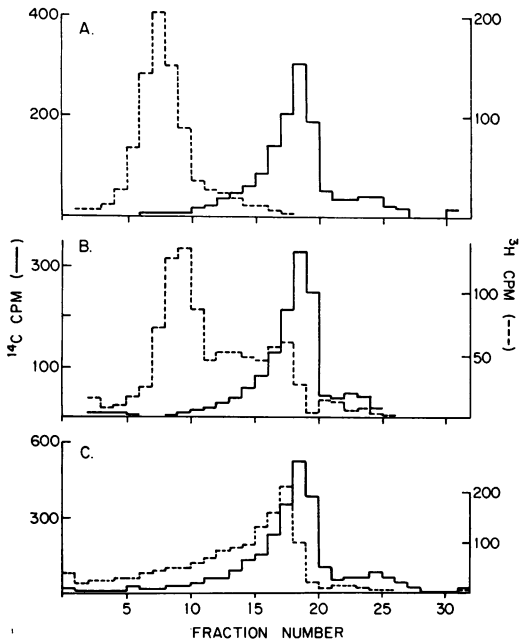

FIG. 7. *Sucrose sedimentation of ^{3}H-BUDR AAV-2 single polynucleotide chains after attempting annealing. Conditions of annealing were those described in Fig. 6C. Conditions of centrifugation were those described in the legend to Fig. 1. Double-stranded ^{14}C-thymidine AAV-2 DNA was present in all runs as a marker. (A) ^{3}H-BUDR AAV-2 heavy strands from Fig. 5A were exposed to annealing conditions. (B) ^{3}H-BUDR AAV-2 light strands from the heavier side of the light-density peak in Fig. 5A were exposed to annealing conditions. (C) The single strands from parts A and B were mixed and then annealed.*

DNA that formed during the CsCl run (Fig. 7B). When equivalent amounts of the heavy and light strands are annealed together, a pattern similar to that obtained with the original ^{3}H-BUDR double-stranded DNA is seen (Fig. 7C). There is no peak of single-stranded DNA. We conclude that we have physically separated the complementary single polynucleotide chains of AAV DNA, although the light strands are contaminated with double-stranded DNA. Confirmation of this conclusion was obtained by repeating the preceding experiment and measuring the extent of formation of double-stranded DNA in CsCl density gradients. ^{32}P-BUDR double-stranded AAV DNA was used as a density marker. This DNA preparation banded at a slightly greater density than the double-stranded ^{3}H-BUDR AAV DNA (~4 to 5 mg/cm^{3}), indicating 4% greater BUDR incorporation (Fig. 4 and 8A; reference 11). The heaby ^{3}H-BUDR band formed no double-stranded DNA under annealing conditions (Fig. 8B). The light ^{3}H-BUDR band was slightly more dense than double-stranded ^{3}H-BUDR DNA and was broad, again indicating that the preparation is not completely free of double-stranded DNA (Fig. 8C). A mixture of the heavy and light bands does reform double-stranded DNA under annealing conditions (Fig. 8D). The annealed DNA appears as a homogeneous peak at a density about 4 mg/cm^{3} lighter than the ^{32}P-BUDR marker, exactly as observed in Fig. 8A.

DISCUSSION

That AAV DNA is single-stranded in situ has now been demonstrated in this and previous communications (9, 11). Rose et al. (11) showed that double-stranded AAV DNA is formed by the annealing of single strands released from different particles during the DNA extraction procedure. In this paper we demonstrated that extracted AAV DNA is composed of two species of single polynucleotide chains which do not self-anneal but do form double-stranded DNA when mixed under annealing conditions, i.e., they are complementary. The heavy BUDR single strands can be separated from the light BUDR strands in one operation. However, the light strands are contaminated with double-stranded DNA; therefore, an additional purification step is necessary to obtain a unique preparation of light strands. These data are compatible with the suggestion that an AAV particle contains one single polynucleotide chain (+ or −) which has a molecular weight of approximately 1.5×10^{6} (5, 9, 11).

In light of our present knowledge of the structure and composition of AAV DNA, it is not surprising that the resolution of the structure of AAV DNA in situ has proven difficult. The existence of complementary strands, the small size of the DNA, the relatively high overall guanine plus cytosine content, the extraction procedure, and the experimental conditions required to study the DNA would all contribute to rapid formation of double-stranded DNA (18, 19). The magnitude of the problem is illustrated in this paper. In the relatively short experiments involving sucrose gradients, about 25% of the single strands have annealed (Fig. 1, 2, 6); however, over 50% of the same single-stranded DNA centrifuged for 72 hr in CsCl at 25 C has annealed (Fig. 3). Even under conditions where the complementary single strands have different densities in CsCl (i.e., BUDR labeling), approximately 40% of the DNA has annealed (Fig. 5).

We were able to separate the complementary AAV single strands because, apparently, they do not contain equal amounts of thymidine. Thus,

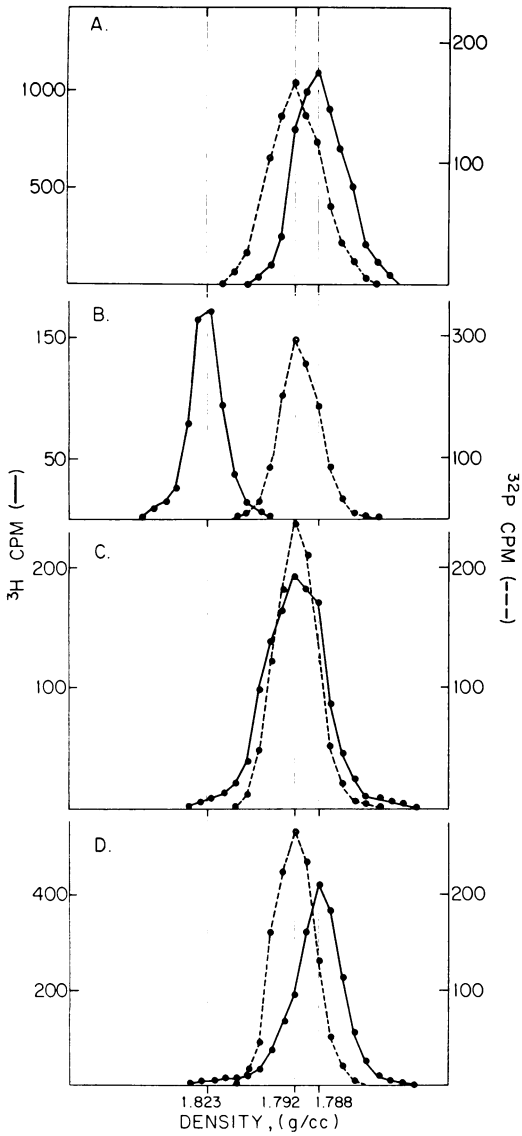

FIG. 8. *Isopycnic CsCl centrifugation of ^{3}H-BUDR AAV-2 single polynucleotide chains after attempted annealing. Conditions of annealing were those described in Fig. 6C. Conditions of centrifugation were those described in the legend to Fig. 5. ^{32}P-BUDR AAV-2 double-stranded DNA was used as a density marker in all gradients. (A) ^{3}H-BUDR AAV-2 double-stranded DNA. (B) ^{3}H-BUDR AAV-2 heavy strands from Fig. 5A were exposed to annealing conditions. (C) ^{3}H-BUDR AAV-2 light strands from the heavier side of the light density peak in Fig. 5A were exposed to annealing conditions. (D) The single strands from parts B and C were mixed and then annealed.*

when BUDR is substituted for thymidine, there is a difference in density between the two-strand species. Assuming a 75% substitution of thymidine by BUDR (11), the difference in thymidine content of the two strands would be about 40% (i.e., the heavy strand would be 26% T and the light strand 18% T), if the expected density for single strands with equal thymidine content would be the average of the two densities observed. Differences in thymidine content of complementary, single polynucleotide chains equal to those assumed here have been reported for the replicative form of ϕX 174 DNA (15, 16) and for HeLa cell light satellite DNA (14). We attempted the separation of the complementary strands of BUDR adenovirus DNA, but there was only one peak of denatured DNA in neutral CsCl.

BUDR-substituted double-stranded AAV DNA sediments more rapidly in neutral sucrose than non-BUDR-substituted DNA. The difference observed is too great to be accounted for simply by the expected increase in mass caused by BUDR (4). Interestingly, the ratio of distances sedimented by single- and double-stranded BUDR labeled AAV DNA in neutral sucrose is the same as that observed for the unlabeled DNA (1.58). This is in reasonable agreement with the data of Studier (17) which would predict a ratio of 1.66 for a double-stranded DNA of molecular weight 3×10^6 which is composed of two intact complementary single strands.

The present report is the first documented instance of the separation of complementary strands of an animal virus DNA. These findings will permit a more detailed study of AAV DNA infectivity (8) and allow analysis of strand-specific transcription in vivo. Information derived from such studies may help reveal the molecular basis for AAV defectiveness.

ACKNOWLEDGMENT

We thank C. Silverman for technical assistance.

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