EVIDENCE FOR A SINGLE-STRANDED ADENOVIRUS-ASSOCIATED VIRUS GENOME: FORMATION OF A DNA DENSITY HYBRID ON RELEASE OF VIRAL DNA

By James A. Rose, Kenneth I. Berns, M. David Hoggan, and Frank J. Koczot

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

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Abstract.—Extracted adenovirus-associated virus DNA is known to be double-stranded, and, therefore, it has been assumed that these virus particles contain a double-stranded genome. Recent findings, however, have suggested that the DNA in virus particles is equivalent to only half the molecular weight of extracted molecules. A density analysis of DNA extracted from a mixture of virus particles containing either bromodeoxyuridine-substituted or unsubstituted DNA shows that virions contain single-stranded DNA which, when released, forms duplex structures. A similar circumstance is as yet unknown among other viruses.

Introduction.—Deoxyribonucleic acid (DNA) extracted from adenovirus-associated viruses (AAV) has been shown to be double-stranded and to have a molecular weight of 3.0 to 3.5 $\times$ 10^6 daltons.[1-3] Because the extracted DNA is double-stranded, AAV particles were considered to contain a double-stranded genome.[3] However, Mayor and Melnick have reported that purified AAV preparations react with acridine orange in a manner characteristic for single-stranded DNA.[4] Recently, Crawford *et al.* compared physical properties of minute virus of mice, phage ϕX 174, and AAV-type 1 (AAV-1).[5] They concluded that the molecular weight of AAV-1 DNA in the particles should not exceed that of minute virus of mice and ϕX 174 DNA (1.7 $\times$ 10^6 daltons) which, in effect, is equivalent to half the molecular weight of extracted AAV DNA. To explain this discrepancy, these investigators considered the possibility that single, complementary strands of DNA are present in different AAV particles. Double-stranded DNA molecules, each composed of strands from two particles, would then be formed after DNA was released from virions. If true, this would be the first observed instance of such an occurrence. The density labeling of viral DNA with bromodeoxyuridine (BUdR) provides a means by which the proposed hypothesis can be tested.[6] It can be predicted from this hypothesis that single polynucleotide chains released from virus particles should hybridize to form a DNA density hybrid when DNA is extracted from a mixture of virions which contain either BUdR-substituted (heavy) or unsubstituted (light) DNA. The present experiments demonstrate that this expectation is, in fact, the case.

Experimental Procedure.—Materials: Primary monolayer cultures of human embryonic kidney cells in 32-oz bottles were purchased from HEM Research, Inc., and KB cells were from a line originally provided by M. Green. Optical grade CsCl, 5-bromodeoxyuridine-6-^{3}H (12.7 c/mM) and thymidine-2-^{14}C (47.9 mc/mM) were obtained from Schwarz BioResearch, Inc. Crystallized-lyophilized trypsin and 2X crystallized papain were

purchased from Worthington Biochemical Corp.; 5-fluorodeoxyuridine (FUdR) was from Hoffman-La Roche, Inc., and 5-bromodeoxyuridine (BUdR) from Calbiochem.

Virus production and purification: AAV-3 was grown in human embryonic tissue cells with adenovirus type 7, substrain E46$^-$, as helper, and adenovirus type 2 (adenoid 6) was propagated in KB cells. The preparation and assay of viral stocks and conditions for infection have been described.[1, 7] For production of AAV-3 containing either ^{3}H-BUdR-substituted or ^{14}C-thymidine-labeled DNA, cells were first infected with E46$^-$ at a multiplicity of ~5 $TCID_{50}$/cell. After 9 hr, cells were superinfected with AAV-3 at a multiplicity of ~8 $TCID_{50}$/cell. The AAV-3 inoculum was initially heated 10 min at 56°C to inactivate contaminating adenovirus.[7] At 4.5 hr following AAV infection, FUdR (0.5 μg/ml) was added to all cultures. Additions of BUdR (2.4 μc ^{3}H-BUdR/ml and 10 μg BUdR/ml as carrier) or ^{14}C-thymidine (0.3 μc/ml) were made 30 min later and incubation continued for an additional 30 hr. Virus was then purified from cells according to the method detailed previously.[1] Adenovirus type 2 was produced in 500 ml suspension cultures of KB cells infected at a multiplicity of ~100 $TCID_{50}$/cell. At 6 hr after infection, FUdR (0.5 μg/ml) was added and 30 min later additions of BUdR or ^{14}C-thymidine were made as above. Cells were harvested 48 hr following infection, and virus was purified as before.[1] The AAV and adenovirus preparations were free of AAV contaminants.[7]

Extraction of viral DNA: DNA was extracted from purified AAV and adenovirus preparations by a method previously described for the extraction of AAV DNA.[1] The sodium dodecyl sulfate incubation was carried out at 50°C. DNA concentration was measured by absorbance at 260 mμ, and radioactivity of DNA was determined in a Triton X-100 (Rohm and Haas) emulsion[8] in a Beckman LS-250 scintillation counter. ^{3}H-BUdR-substituted AAV and adenovirus DNA had spec. act. of 7,240 and 21,180 cpm/μg, respectively. The spec. act. of ^{14}C-thymidine-labeled AAV and adenovirus DNA were 19,050 and 21,600 cpm/μg.

Equilibrium density centrifugation: Radioactive viral DNA was centrifuged to equilibrium in 3.5 ml of a CsCl solution (0.01 *M* Tris, pH 8.6, ρ = 1.730 gm/ml) overlaid with paraffin oil. Centrifugations were performed in the SW 50 rotor in the Spinco model L ultracentrifuge at 25°C and 40,000 rpm for 40 hr. Gradients were fractionated through a puncture in the bottom of the centrifuge tube, and the density of selected fractions determined as before.[1] ^{3}H and ^{14}C counts in each fraction were assayed in the Triton X-100 emulsion and were corrected for channel spill. The equilibrium density gradient of mixed heavy and light particles was similarly analyzed.

Zonal centrifugation in sucrose gradients: Samples of radioactive AAV DNA were sedimented in 5–20% sucrose gradients at neutral and alkaline pH. Fractions were collected and counted as above. To prevent chemiluminescence, 0.15 ml of 4% $SnCl_2$ in 0.1 *N* HCl was added to counting vials that contained fractions from the alkaline gradient. The 20*S* marker used in neutral gradients is ^{14}C-thymidine-labeled SV40 DNA (component I), kindly supplied by M. Martin. Sedimentation coefficients and the molecular weight of duplex DNA were estimated according to the calculations of Burgi and Hershey.[9] The relationship between sedimentation coefficient and molecular weight of denatured DNA was based on Studier's data.[10]

Results.—Buoyant density of virus particles: The buoyant density of adenovirus-associated virus particles synthesized in the presence or absence of BUdR was measured in a CsCl density gradient (Fig. 1). Particles which contained ^{3}H-BUdR-substituted or heavy DNA (heavy particles) were well resolved from those which contained ^{14}C-thymidine-labeled or light DNA (light particles). Each species appeared in a single, sharp band with a density of 1.42 gm/ml for heavy particles and 1.40 gm/ml for light particles.

Buoyant density of viral DNA: DNA extracted from purified heavy and light AAV particles was centrifuged to equilibrium in CsCl density gradients (Fig. 2). When heavy DNA and light DNA were extracted from heavy and light particles,

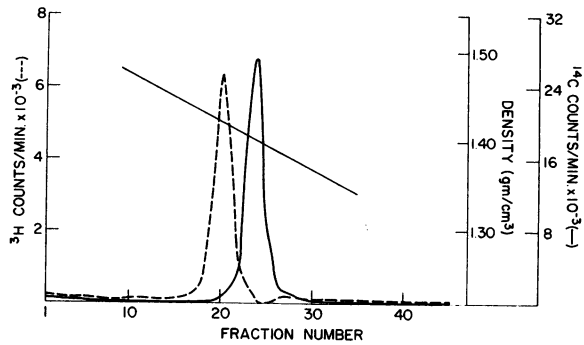

FIG. 1.—Pattern of radioactivity in 0.1 ml fractions from a CsCl equilibrium density gradient of mixed heavy and light AAV-3 particles. Portions of principle viral bands from initial CsCl gradients were mixed and again centrifuged to equilibrium.

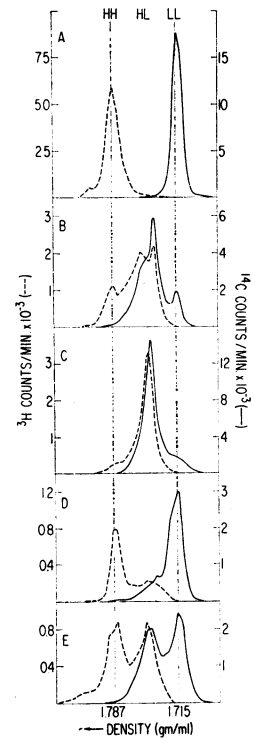

FIG. 2.—Equilibrium density centrifugation of AAV DNA. Fractions of two drops each were collected.

(*A*) A mixture of heavy and light DNA.

(*B*) DNA extracted from a mixture of heavy and light particles.

(*C*) Sample of DNA shown in (*B*) which was denatured in 0.1 *M* NaOH and reannealed in 4 × SSC at 67°C for 4 hr.

(*D*) A mixture of heavy and light DNA which had been put through extraction procedure.

(*E*) Sample of DNA shown in (*B*) after breakage in the French press.

respectively, then mixed and centrifuged, each DNA was separate in the gradient (Fig. 2*A*). Light DNA formed a single band, and at least 90 per cent of the heavy DNA appeared as one component. Heavy DNA had a density of 1.787 gm/ml based on a calibration of the density gradient with light DNA which has a known density of 1.715 gm/ml.[3] AAV DNA extracted by the method used is double-stranded.[1, 3] The DNA obtained from heavy particles is thus composed of two strands (HH) in which BUdR has replaced thymidine, while light particle

DNA contains two strands (LL) labeled with ^{14}C-thymidine. Heavy DNA would have an expected density of 1.815 gm/ml if thymidine were completely substituted by BUdR.[11] The observed density increase of 0.072 gm/ml is therefore equivalent to a 72 per cent substitution.

The centrifugation pattern of DNA extracted from a mixture of heavy and light particles is shown in Figure 2*B*. Most of the DNA was shifted to a density range intermediate between the densities of heavy and light DNA. This DNA is a density hybrid which consists of heavy and light DNA linked together during the extraction procedure. The average density of the hybrid was similar to that of hybrid which formed when DNA extracted from a mixture of heavy and light particles was denatured and reannealed (Fig. 2*C*).

Heavy and light DNA were mixed and cycled back through the extraction procedure to rule out the possibility that double-stranded AAV DNA could be denatured and reannealed during extraction (Fig. 2*D*). Most of the heavy and light DNA were resolved as was seen for the untreated DNA mixture (Fig. 2*A*). Although some hybrid appeared, the amount formed was much less than that observed when DNA was prepared from a mixture of heavy and light particles (Fig. 2*B*). This difference was unaffected by the concentration of DNA during the extraction procedure. It is clear that AAV DNA is not simply denatured and reannealed as a result of extraction conditions. The hybrid seen in Figure 2*D* was likely generated from polynucleotide sequences which were incompletely base-paired during the first extraction procedure.

The formation of a density hybrid (Fig. 2*B*) on release of viral DNA was predicted from the suggested hypothesis. However, the observed hybrid could consist of either laterally paired H and L strands (lateral hybrid) or HH and LL duplexes joined end-to-end (end-to-end hybrid) by short lateral linkages as can occur with bacteriophage DNA.[12-14] To be consistent with the hypothesis, the hybrid must be lateral (HL); an end-to-end hybrid would be indicative of double-stranded DNA in the virion. These alternatives can be distinguished on the basis of the density distribution of fragmented hybrid when the extent of breakage is known.[12] The molecular weight of extracted DNA was therefore estimated before and after shear in the French press. DNA from the preparation shown in Figure 2*B* was sedimented through a sucrose gradient (Fig. 3*A*). Most of the DNA had an average sedimentation coefficient ($S^{\circ}_{20,w}$) of 15 which corresponds to a molecular weight of $\sim 3.0 \times 10^6$ daltons for double-stranded, linear DNA.[9] Smaller species were not evident. After shear, the DNA sedimented homogeneously with an average sedimentation coefficient of 9 (Fig. 3*B*). This corresponds to about one quarter the molecular weight of the 15*S* DNA. Unsheared DNA was also sedimented in an alkaline sucrose gradient (Fig. 3*C*) to ascertain the molecular weight of single strands. Denatured DNA sedimented homogeneously and had an average sedimentation coefficient of 15 which corresponds to a molecular weight half that of double-stranded, 15*S* DNA.[10] These findings indicated that the molecular unit of duplex DNA was 3.0×10^6 daltons, and that shearing had produced an average of three breaks per duplex unit. Considering both hybrid and nonhybrid associations, this number of breaks would be expected to reduce the hybrid component to 10 per cent or less of total

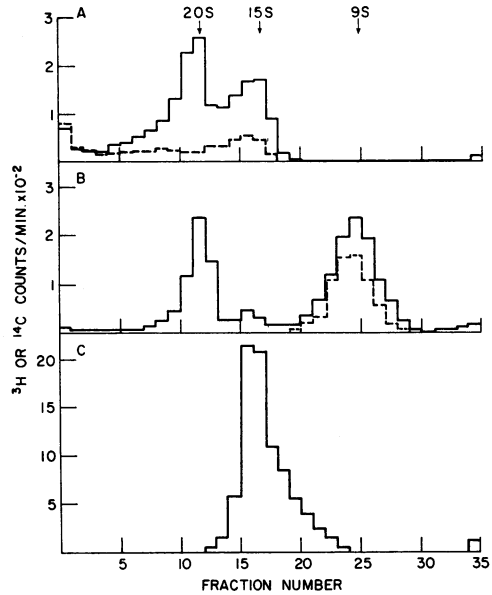

FIG. 3.—Sedimentation of radioactive AAV DNA in sucrose gradients. Samples of DNA in 0.2 ml 0.1 × SSC were layered onto 4.6 ml gradients and centrifuged at 42,000 rpm in the SW 50 rotor in the Spinco model L ultracentrifuge for 4 hr at 20°C. Fractions of four drops each were collected. Dashed line, counts ^{3}H; solid line, counts ^{14}C.

(*A*) Unsheared and (*B*) sheared DNA (~0.1 μg each) sedimented through a gradient of 5 to 20% sucrose in 1.0 *M* NaCl, 0.15% sarkosyl, 0.05 *M* Tris pH 7.9. DNA was sheared by two passages through a French press at 10,000 psi.

(*C*) ^{14}C-thymidine-labeled DNA (~0.4 μg) sedimented through a gradient of 5 to 20% sucrose in 0.9 *M* NaCl, 0.1 *M* NaOH, 0.15% sarkosyl.

The 20*S* marker preparation used in (*A*) and (*B*) contained a small amount of 16*S* DNA (SV40 component II DNA) which is apparent in (*B*).

DNA if the hybrid is constructed end-to-end, while up to 50 per cent of total DNA should remain in the hybrid if it is formed laterally.[12] The equilibrium density pattern of sheared DNA is shown in Figure 2*E*. Approximately half the total DNA has remained in the hybrid, and it is thus concluded that the hybrid is lateral, i.e., formed by annealing of complementary H and L strands.

An additional, although remote, possibility can also be considered. Virions could contain two DNA strands with complementary base sequences unpaired except for a relatively small region. A combined lateral and end-to-end hybrid might then result, and most of the 15*S* DNA shown in Figure 3*A* would be expected to separate into heavy and light DNA when centrifuged in a density gradient. However, the centrifugation pattern of 15*S* DNA was similar to that shown in Figure 2*E* and is the predicted distribution for duplex molecules formed by lateral associations of heavy and light DNA strands. Furthermore, this finding also indicates that the hybrid is lateral, since heavy and light DNA are joined in the basic duplex unit.

Because of the unusual finding with AAV DNA, DNA from another virus, adenovirus type 2, was tested in the same way. The DNA from this adenovirus is double-stranded, has a density in CsCl of 1.717 gm/ml, and is about six times larger than AAV DNA.[1, 15] ^{3}H-BUdR-substituted adenovirus type 2 DNA had a density of 1.795 gm/ml (Fig. 4*A*) and an estimated 72 per cent BUdR substitution.[11] The centrifugation pattern of combined heavy and light adenovirus type 2 DNA (Fig. 4*A*) was similar to that obtained with DNA extracted from mixed heavy and light virus particles (Fig. 4*B*).

Discussion.—These experiments demonstrate that AAV-3 contains single-stranded DNA which hybridizes into duplex structures when DNA is released from virions. The results support the hypothesis of Crawford and his associates, although it is still uncertain whether the DNA complements consist of one or

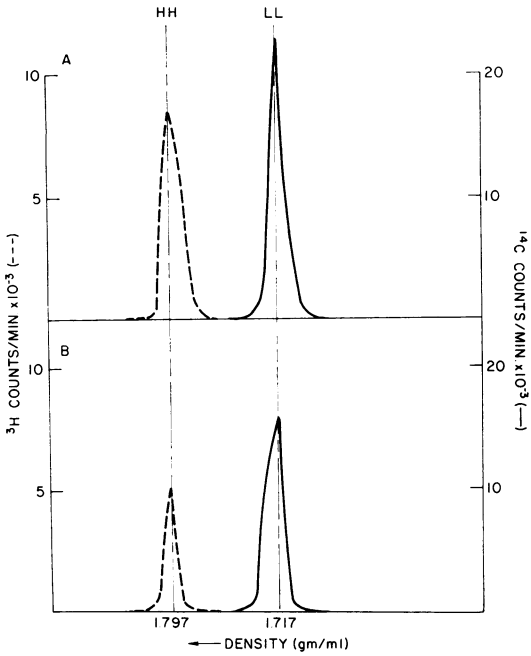

FIG. 4.—Equilibrium density centrifugation of adenovirus DNA. Fractions of four drops each were collected.

(*A*) A mixture of heavy and light DNA.

(*B*) DNA extracted from a mixture of heavy and light particles.

two strand species. Double-stranded DNA could be formed from pairs of identical strands that contain large regions of self-complementarity. Self-annealed single strands would also be expected to occur, but there was no indication of their presence in sucrose gradient studies (Fig. 3*A*). It may be significant that a lack of complete coincidence between the H and L components of hybrid DNA was observed repeatedly (Fig. 2*B*, *C*, and *E*). This could be due to two strand species with differing thymidine contents. Alternatively, a different amount of BUdR substitution in a portion of heavy DNA might account for the separation, but BUdR substitution was predominantly homogeneous in heavy DNA as judged by equilibrium density centrifugation (Fig. 2*A*). Present evidence would then seem to favor the possibility that AAV virions carry different DNA complements.

Comparative studies with other virus particles strongly suggest there is only one DNA strand per AAV virion.[5] It is thus likely that all duplex molecules consist of strands originating from different virus particles. It should be noted, though, that a random pairing of heavy and light strands (Fig. 2*E*) might also occur if virions contained more than one single-stranded DNA molecule.

The estimated molecular weights of duplex DNA units and their single-stranded subunits are in agreement with previously reported values,[1, 2] but the proportions of heavy and light DNA in the unsheared hybrid were greater than expected for hybrid molecules composed of single heavy and light strands (Fig. 2*B*). There was a further decrease in relative amounts of HH and LL (Fig. 2*C*) when DNA, extracted from a mixture of heavy and light particles, was denatured and reannealed. In addition, sedimentation of extracted DNA in sucrose gradients (Fig. 3*A*) has consistently revealed 30–50 per cent of the DNA hetero-

geneously distributed ahead of the 15*S* component.[1] These findings indicate that DNA strands may also form hydrogen-bonded aggregrates when subunits are hybridized during extraction or under the specified conditions of higher salt and temperature.

It is not now apparent how the described peculiarity of AAV DNA might relate to AAV defectiveness.[1, 3, 7, 16–19] If, indeed, there are two strand species, the mechanism for their synthesis would be of considerable interest.

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The following abbreviations are used in this paper: DNA, deoxyribonucleic acid AAV, adenovirus-associated virus; BYdR, bromodeoxyuridine; HH, DNA in which BYdR has been subsituted for thymidine in both strands; LL, DNA composed of strands which do not contain BYdR; HL, DNA consisting of one unsubstituted and one BYdR-containing strand.

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