JOURNAL OF VIROLOGY, Feb. 1972, p. 394-396

Vol. 9, No. 2
Printed in U.S.A.

Separation of Two Types of Adeno-Associated Virus Particles Containing Complementary Polynucleotide Chains

K. I. BERNS AND S. ADLER

Department of Microbiology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received for publication 20 October 1971

An adeno-associated virus containing bromodeoxyuridine-substituted deoxyribonucleic acid has been fractionated by equilibrium centrifugation in CsCl into two classes of virions which contain complementary polynucleotide chains.

Purified adeno-associated virus (AAV) deoxyribonucleic acid (DNA) has been characterized as a double-stranded molecule with a molecular weight of 3.0 to 3.6 $\times$ 10^6 daltons (5, 7). However, AAV DNA is single-stranded within the virion (1, 4) and, after release, complementary single strands from different particles base-pair to form a double helix (6). For these reasons, and because of evidence that the AAV virion is too small to accommodate the mass of a double-stranded AAV DNA molecule (2), it has been inferred that there are two types of AAV particles, each of which contains one of the complementary single strands. In this paper we report the separation of the two types of virions.

Normally, the two types of AAV virions have the same buoyant density in CsCl. Our basic plan was to create a difference in the buoyant densities of the two particle types by labeling the DNA with bromodeoxyuridine (BUdR). The complementary strands of AAV DNA contain different amounts of thymidine (26 and 21%, respectively; Rose and Koczot, *personal communication*). Therefore, if BUdR is substituted for thymidine, the complementary strands differ in buoyant density in neutral CsCl. When there is 85 to 90% replacement of the thymidine by BUdR, this difference is approximately 30 mg/cm^3 (1). Since unsubstituted AAV particles are 20% DNA by mass, the two types of AAV virions containing BUdR-substituted single strands should differ in density by 6 mg/cm^3.

AAV 2 (AAV 2H) containing BUdR-substituted DNA was grown in KB cells which had been coinfected with adenovirus type 2 (as the helper). The cells were collected 13 hr after infection, washed, and suspended in Eagle medium (3) containing reduced PO_4 (10^{-5} M) supplemented with 5% dialyzed horse serum. Flurodeoxyuridine (0.5 μg/ml) was then added, and 30, min later, ^{32}P (2 μCi/ml) and BUdR (10 μg/ml) were added. The infection was terminated after 48 hr at 37 C, and the AAV was purified (1).

Two classes of ^{32}P-BUdR-substituted AAV particles differing in buoyant density were obtained by successive fractionation of the original AAV preparation on the basis of density in two consecutive CsCl gradients. The virus peak in the initial gradient is shown in Fig. 1A. Fractions 9 to 12 (heavy) and fractions 16 to 19 (light) were pooled. The two virus pools were then sedimented in separate equilibrium CsCl gradients (Fig. 1B, C), and the fractions from the dense side of the heavy particles were pooled (fractions 11 and 12, Fig. 1B) as were the fractions from the light side of the light particles (fractions 16 and 17, Fig. 1C). To demonstrate that the two virus preparations did indeed differ in density, samples of each were sedimented again in separate equilibrium CsCl gradients containing ^{3}H-BUdR-substituted, unfractionated AAV as a density marker (Fig. 1D, E). As shown in Fig. 1, the two classes of AAV particles differed in density by 6 mg/cm^3.

To demonstrate that the heavy particles contained the heavy complementary strand and the light particles the light strand, DNA was purified from each of the preparations separately by exposure to papain and trypsin, by heating at 50 C in the presence of 1% sodium dodecyl sulfate in 2$\times$ SSC (0.30 M NaCl, 0.03 M sodium citrate), and by phenol extraction (6). When both complementary strands are present, this method results in the formation of double-stranded DNA. Purified ^{32}P-BUdR DNA from the heavy particles was mixed with ^{3}H-BUdR double-stranded AAV DNA and sedimented in an equilibrium CsCl

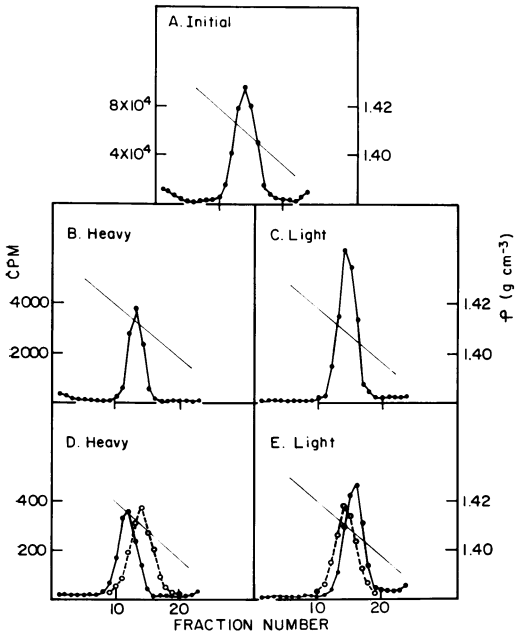

FIG. 1. *Sedimentation in equilibrium CsCl gradients of unfractionated and fractionated ^{32}P-bromodeoxyuridine(BUdR-)-substituted adeno-associated virus (AAV). ^{32}P-BUdR-substituted AAV was sedimented in 3.5 ml of CsCl containing phosphate-buffered saline in a no. 50 fixed-angle rotor in a Beckman model L ultracentrifuge at 33,000 rev/min and 4 C for 48 hr. A, Unfractionated virus; B, fractions 9 to 12 (heavy) from A were pooled and resedimented; C, fractions 16 to 19 (light) from A were pooled and resedimented; D, fractions 11 and 12 (heavy) from B were pooled, and a sample was resedimented in the presence of unfractionated ^{3}H-BUdR AAV. Symbols: ^{32}P (●); ^{3}H (○). E, fractions 16 and 17 (light) from C were pooled and a sample was resedimented in the presence of unfractionated ^{3}H-BUdR AAV. Symbols: ^{32}P (●); ^{3}H (○).*

gradient. Because the buoyant density of heavy BUdR-substituted single strands is approximately 30 mg/cm³ greater than that of either light BUdR single strands or native BUdR DNA, it is possible to estimate directly from the pattern in CsCl (Fig. 2A) that 60% of the ^{32}P-BUdR DNA banded as heavy single strands and 40% as double-stranded DNA. We assume that one-half of the native DNA present in the gradient is heavy strand and that, in the case of excess heavy strands, no free light strands remain. (We know from Fig. 2B that no free heavy strands are present in the case of excess light strands.) Thus, 80% (60% + 0.5 × 40%) of the particles in the heavy virus preparation contained the heavy single strand.

The light particle DNA was also extracted under conditions which permitted annealing of complementary strands and was sedimented in an equilibrium CsCl gradient (Fig. 2B). In this case, as noted above, no free heavy single strands were observed, but it is not possible to estimate directly the fraction of light single strands in the peak observed in Fig. 2B, because light BUdR single strands differ in density from native BUdR DNA by only 4 mg/cm³ (1). Therefore, fractions 7 to 14 from Fig. 2B were pooled, dialyzed, and sedimented through a linear, neutral, 5 to 20% sucrose gradient containing 1 M NaCl (Fig. 3). Under these conditions, single-stranded BUdR-substituted AAV DNA sediments 1.58 times as fast as double-stranded BUdR-substituted AAV DNA (1). Approximately 80% of the DNA was single-stranded; therefore, 90% of the DNA from the light particle preparation was of the light single-strand type. In several experiments of this type, the preparations ranged from 75 to 90% pure.

We conclude that this separation directly

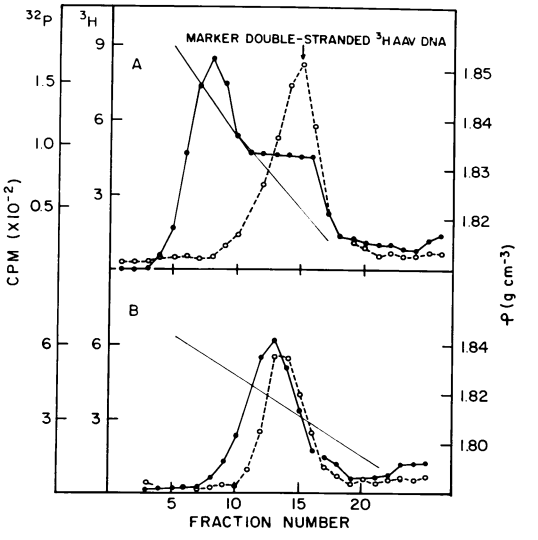

FIG. 2. *Equilibrium CsCl gradients of deoxyribonucleic acid (DNA) extracted from separated heavy and light particles. DNA was extracted under conditions which anneal complementary single strands. The purified DNA was sedimented in 3.5 ml of CsCl containing 0.05 M tris(hydroxymethyl)aminomethane (pH 7.9), 0.001 M ethylenediaminetetraacetic acid, and 0.15% Sarkosyl in a no. 50 fixed-angle rotor in a model L ultracentrifuge at 40,000 rev/min and 20 C for 48 hr. ^{3}H-BUdR-substituted, double-stranded AAV DNA was present as a marker. Symbols: ^{32}P (●); ^{3}H (○). A, DNA purified from heavy particles; B, DNA purified from light particles.*

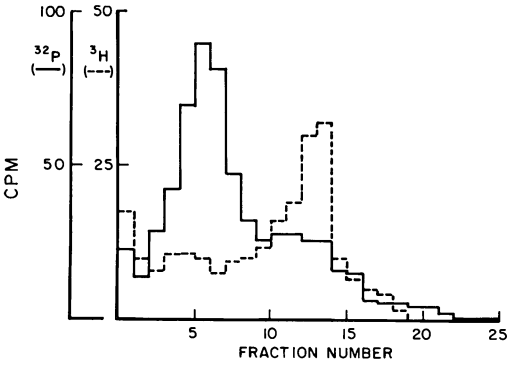

FIG. 3. *Sedimentation through a linear 5 to 20% sucrose gradient of deoxyribonucleic acid (DNA) purified from light particles. Fractions 12 to 17 of the CsCl gradient shown in Fig. 2B, which contained the ^{32}P-bromodeoxyuridine DNA from light particles and the marker ^{3}H-bromodeoxyuridine double-stranded marker DNA, were pooled and dialyzed against SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.2). Sucrose solutions contained 1 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane, pH 7.9, and 0.001 M ethylenediaminetetraacetic acid. Centrifugation was for 4 hr at 40,000 rev/min and 20 C in an SW50 rotor (Beckman). Fractions were collected directly into 10 ml of Triton-toluene fluor containing 10% water and were counted in a Beckman LS 230 scintillation counter. Solid line, ^{32}P; dashed line, ^{3}H.*

demonstrates that there are two types of AAV particles, each of which contains only one type of complementary single strand. Because AAV containing BUdR-substituted DNA is infectious (Rose, *personal communication*; Berns and Adler, *unpublished data*), we can now determine whether one or both of the AAV virions are infectious and thus begin to explore the role of each strand in virus development.

This work was supported by Public Health Service grant CA-11895 from the National Cancer Institute and grant E-645 from the American Cancer Society. K. I. B. is a Howard Hughes Medical Investigator.

LITERATURE CITED

1. Berns, K. I., and J. A. Rose. 1970. Evidence for a single-stranded adenovirus-associated virus genome: isolation and separation of complementary single strands. J. Virol. 5:693–699.
2. Crawford, L. V., E. A. C. Follett, M. G. Burdon, and D. J. McGeoch. 1969. The DNA of a minute virus of mice. J. Gen. Virol. 4:37–46.
3. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science 130:432–437.
4. Mayor, M. D., K. Torikai, J. L. Melnick, and M. Mandel. 1969. Plus and minus single-stranded DNA separately encapsidated in adeno-associated satellite virions. Science 166: 1280–1282.
5. Parks, W. P., M. Green, M. Piña, and J. L. Melnick. 1967. Physicochemical characterization of adeno-associated satellite virus type 4 and its nucleic acid. J. Virol. 1:980–987.
6. Rose, J. A., K. I. Berns, M. D. Hoggan, and F. Koczot. 1969. Evidence for a single-stranded adenovirus-associated virus genome: formation of a DNA density hybrid on release of viral DNA. Proc. Nat. Acad. Sci. U.S.A. 64:863–869.
7. Rose, J. A., M. D. Hoggan, and A. J. Shatkin. 1966. Nucleic acid from an adeno-associated virus: chemical and physical studies. Proc. Nat. Acad. Sci. U.S.A. 56:86–92.