VIROLOGY 63, 523–538 (1975)

Specific Cleavage of Adenovirus-Associated Virus DNA by Restriction Endonuclease R·*Eco*RI—Characterization of Cleavage Products

BARRIE J. CARTER*[1] AND GEORGE KHOURY†

* *Laboratory of Experimental Pathology, National Institute of Arthritis, Metabolism and Digestive Diseases and † Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland 20014*

Accepted October 7, 1974

The cleavage of adenovirus-associated virus type 2 (AAV2) DNA by the restriction endonuclease R·*Eco*RI was investigated using neutral and alkaline sucrose sedimentation, electrophoresis in composite agarose-acrylamide gels, and reassociation kinetics. Linear monomeric AAV2 DNA duplexes were cleaved at two specific sites to yield three fragments A, B and C, each of which is equivalent to a unique region of the genome of approximately 57.2, 38.2 and 4.6%, respectively. Fragment C may possibly consist of two smaller fragments, and in this case there would be three, rather than two, specific cleavage sites within a 4.6% region of the genome. Cleavage of oligomeric forms of AAV2 DNA (including linear and circular oligomers and network-like structures) yielded fragments A, B and C and two additional components, AB′ and $\overline{AB}$. In alkaline sucrose, both AB′ and $\overline{AB}$ yielded only A and B strands. Component AB′ had a neutral sucrose sedimentation coefficient and electrophoretic mobility close to those of intact linear monomer AAV duplexes whereas $\overline{AB}$ sedimented more rapidly in neutral sucrose and was specifically trapped close to the electrophoretic origin in composite gels. In conditions expected to dissociate cohesive ends (80° in 1 × SSC), approximately 50% of both AB′ and $\overline{AB}$ released equimolar amounts of fragments A and B. Therefore, at least part of both AB′ and $\overline{AB}$ appear to consist of fragments A and B joined by cohesive ends. The remaining portion of both AB′ and $\overline{AB}$ apparently consists of more bizzare structures such as branched molecules (originating from DNA networks) or they may contain single-stranded regions. The probable physical order of fragments A, B and C in the AAV genome can be deduced.

INTRODUCTION

Virions of the defective parvovirus, adenovirus-associated virus (AAV), contain either a "plus" or "minus" linear DNA strand having a molecular weight of 1.4×10^6. Upon isolation from virions, particularly in the presence of the high salt concentrations and elevated temperature usually employed, the complementary plus and minus strands anneal to form duplex molecules of molecular weight 2.8×10^6 (Rose *et al.*, 1969; Mayor *et al.*, 1969; Koczot *et al.*, 1973; Gerry *et al.*, 1973). The terminal nucleotide sequences of the AAV DNA strands have been studied in some detail. At least 70% of both plus and minus strands possess *inverted*, terminal repetitions (comprising less than 10% of the strand length) such that individual single strands form circles closed by a duplex, hydrogen-bonded segment or "panhandle" (Carter *et al.*, 1972; Koczot *et al.*, 1973; Berns and Kelly, 1974). Two additional types of terminal sequences in AAV DNA have been described by Gerry *et al.* (1973): (a) a *regular* (noninverted) terminal repetition (representing about 1% of the genome) and (b) a limited permutation comprising about 6% of the genome. The consequence

[1] To whom all reprint requests should be addressed.

of the limited permutation is that some AAV duplexes possess single-stranded ("cohesive") termini analogous to those of phage lambda DNA (Hershey *et al.*, 1963) and can therefore form hydrogen-bonded circular and linear oligomeric molecules. Whether or not all three types of sequence arrangement are present in all AAV DNA strands is not yet clear, but all these phenomena occur within less than 10% of the genome length at either strand terminus (Carter *et al.*, 1972; Koczot *et al.*, 1973; Gerry *et al.*, 1973; Berns and Kelly, 1974).

We previously showed that only 70–75% of the AAV DNA minus strand, and none of the plus strand, is represented in the single stable 20S AAV mRNA of molecular weight 0.9×10^6–1.0×10^6 (Carter *et al.*, 1972; Carter and Rose, 1974; Carter and Khoury, manuscript in preparation). Preliminary analysis suggested that the regions of the AAV DNA minus strand that are not represented in the stable RNA included the terminal, inverted repetition. To determine this more precisely, we have first undertaken an analysis of restriction endonuclease cleavage of AAV DNA to provide defined fragments suitable for transcription mapping as well as other studies.

In this report we describe an analysis of the specific cleavage of AAV2 DNA with the restriction endonuclease R·*Eco*RI (Yoshimori, 1971) which cleaves duplex DNA at the sequence

(T/A)G↓AATT C(A/T)
(A/T)C TTAA↑G(T/A)

by breakage of phosphodiester bonds at the sites indicated by arrows to produce cohesive termini of four nucleotides (Hedgpeth *et al.*, 1972). This enzyme makes two (or possibly three) specific breaks in AAV DNA duplexes to yield three (or possibly four) fragments. The probable physical order of these fragments in the duplex genome can be deduced. In addition, this work provides further evidence for the presence of cohesive termini in AAV DNA duplexes as previously indicated by Gerry *et al.* (1973). Elsewhere we describe a rigorous physical proof of the physical order as well as the strand polarity of the AAV DNA R·*Eco*RI cleavage products (Carter, Khoury, and Denhardt, manuscript in preparation).

MATERIALS AND METHODS

Preparation and purification of viral DNA. Adenovirus-associated virus type 2 (AAV2) and adenovirus type 2 (Ad2) were propagated by coinfection of KB-3 cells in Eagles spinner medium at 37° as described before (Carter *et al.*, 1973; Carter and Rose, 1974). Cells were harvested at 40–48 hr post-infection, and AAV and Ad virions were purified by sonication and digestion with trypsin and sodium deoxycholate followed by three cycles of preparative centrifugation in CsCl buoyant density gradients as previously described (Rose *et al.*, 1969; Berns and Rose, 1970).

Isotopic labeling and bromodeoxyuridine (BUdR) substitution of AAV DNA and Ad DNA was carried out using procedures modified from those described by Berns and Rose (1970). To prepare ^{3}H-labeled viral DNA, 5-fluorodeoxyuridine (FUdR) was added to a final concentration of 2×10^{-6} *M* at 8 hr post-infection. Thirty minutes later [^{3}H]methyl-thymidine (43 Ci/mmole) was added to 2–5 μCi/ml (12–28 ng/ml) and also thymidine to 5×10^{-5} *M* and uridine to 1×10^{-5} *M*. To obtain ^{3}H-labeled, BUdR-substituted DNA, BUdR was added to a final concentration of 1.5 μg/ml at the time of addition of [^{3}H]thymidine and cold thymidine was omitted. To prepare ^{32}P-labeled DNA, the cells were resuspended at the time of infection in phosphate-free medium and carrier-free [^{32}P] ($H_3[^{32}P]O_4$; New England Nuclear) to 2–3 μCi/ml. To obtain ^{32}P-labeled, BUdR-substituted DNA, FUdR was added at 8 hr post-infection followed 30 min later by BUdR (final concentration of 1.5 μg/ml), uridine (final concentration 10^{-5} *M*) and thymidine (final concentration 20 ng/ml). These conditions yield approximately 90% substitution of thymidine by BUdR (Baldwin and Shooter, 1963).

Viral DNA was extracted from purified virions using an enzymatic digestion procedure (Rose *et al.*, 1969), including digestion with papain and trypsin followed by incu-

bation with sodium dodecyl sulphate (SDS) at 50° and three successive phenol extractions in ice. For adenovirus DNA, the trypsin digestion was omitted and the SDS incubation was performed at room temperature. The DNA preparations were exhaustively dialysed (at least three changes for 30 hr each) against a buffer containing 10 m*M* Tris-HCl (pH 8.0), 10 m*M* NaCl, and 1 m*M* Na_2EDTA at 4° and were then stored in this buffer at 4°. In some cases, in the first dialysis step the buffer was 1 × SSC (0.15 *M* NaCl, 0.015 *M* sodium citrate). For BUdR-substituted DNA, care was taken to perform all manipulations in subdued light to minimize photochemical damage. DNA concentrations were determined optically assuming 1 optical density unit at 260 nm is equivalent to 50 μg/ml. The specific activity of ^{32}P-labeled DNA was usually 1×10^5–2×10^5 counts/min/μg and for ^3H-labeled DNA was 0.7×10^5–1.4×10^5 counts/min/μg. [^{32}P]SV40 DNA fragments obtained by cleavage with the restriction endonuclease *Hin*d (Danna and Nathans, 1971) were kindly provided by Dr. Peter Howley.

Cleavage of DNA with restriction endonuclease. The restriction endonuclease R·*Eco*RI, purified from *Escherichia coli* RY13 according to Mulder and Delius (1972), was kindly provided by Dr. George Fareed. For cleavage of DNA, reaction mixtures contained 0.1 *M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$, DNA up to several micrograms, and R·*Eco*RI restriction enzyme (1 unit/50 ng of DNA) in final volume of 100 to 300 μL. Reactions were incubated for 45 min at 37° and stopped by chilling to 0° and by addition of Na_2EDTA to 10 m*M*. One unit of enzyme activity is the amount needed to convert 0.1 μg SV40 DNA I to DNA III under the assay conditions described (Martin *et al.*, 1973). To ensure that the enzyme cleavage of AAV DNA went to completion, Ad2 DNA or SV40 DNA was included in some reactions (see Results section).

The products of the reaction were then analysed directly on acrylamide gels or neutral sucrose gradients. For analysis in alkaline sucrose, it was necessary to dialyse briefly the reaction mixture against 10 m*M* Tris (pH 8.0), 10 m*M* NaCl, 1 m*M* EDTA to avoid precipitation of some of the DNA which otherwise occurred at alkaline pH.

Sedimentation analysis of DNA. Neutral gradients (11.3 ml) were formed in nitrocellulose tubes and contained a 5–20% sucrose gradient in 1 *M* NaCl, 50 m*M* Tris-HCl (pH 8.0) and 1 m*M* Na_2EDTA.

Alkaline gradients (11.3 ml) were formed in nitrocellulose tubes and contained a 5–20% sucrose gradient in 0.3 *M* NaOH, 0.7 *M* NaCl and 0.15% sarkosyl.

All gradients were centrifuged in the SW41 rotor of a Beckman L2-65B ultracentrifuge at 20° and 35,000 rpm (149,800 g_{av}). The gradients were then fractionated by piercing the bottom of the tube and collecting drops using a constant volume device. Usually, approximately 52 equal fractions (225 μL) were collected. Either the entire fraction or aliquots (20 μL) were taken and counted for radioactivity in 10 ml of a toluene-triton × 100—liquiflor scintillation cocktail. Recovery of isotope in the gradients was 85–95%.

Gel electrophoresis. DNA preparations were analyzed electrophoretically in composite agarose-acrylamide gels. Cylindrical gels containing 0.5% agarose and 2.0% acrylamide were formed in 0.6 × 12 cm glass tubes to a height of 10–11 cm essentially according to Dingman and Peacock (1968) except that the buffer contained 50 m*M* Tris, 40 m*M* sodium acetate and 1 m*M* Na_2EDTA adjusted to pH 7.6 with acetic acid, and 0.1% SDS was included in gels and buffer. Gels were pre-run for 1 hr, then the DNA sample was adjusted to 1% SDS, 50 m*M* Tris (pH 7.6), 40 m*M* sodium acetate, 1 m*M* Na_2EDTA and 10% sucrose and layered on top of the gel column. Electrophoresis was then performed initially for 20 min at 1 mA/gel and then at 4–6 mA/gel for 4–20 hr. Following this, gels were cut into 1.25 mm slices using an "egg-slicer." Each slice was dissolved at 60° in 50 μL of H_2O_2 and radioactivity was determined in 10 ml of toluene-triton-scintillation cocktail. Recovery of isotope in the gel was at least 90%.

Reassociation kinetics of viral DNA. Reassociation of viral DNA fragments was measured as described before (Carter *et*

al., 1972). Briefly, DNA solutions were adjusted to 0.14 *M* phosphate buffer (pH 6.8) and 0.4% SDS and heat denatured for 5 min at 100°C. The mixtures were then incubated at 68°C and aliquots were removed at appropriate times for determination of the proportion of DNA in duplex molecules by hydroxyapatite chromatography at 60°. Reassociation data is plotted as the proportion of duplex DNA as a function of C_0t (product of initial DNA concentration and time of incubation).

RESULTS

Sedimentation Analysis of AAV DNA R·*Eco*RI *Cleavage Products*

In order to obtain specific duplex fragments of AAV DNA that could then be separated into complementary strands (for use in transcription mapping experiments), we have used mainly BUdR-substituted AAV DNA. For intact molecules, this allows preparative separation of the complementary strands by equilibrium density gradient centrifugation in CsCl (Berns and Rose, 1970). Preparations of BUdR-substituted or non-substituted AAV DNA labeled with either ^3H or ^{32}P were used interchangeably and gave similar results. Substitution of BUdR for thymidine to the extent of about 90% in the preparations used causes an increase in sedimentation rate by a factor of 1.09–1.1. In 1 *M* Na^+ (using SV40 DNA components I, II and III as reference markers), AAV2 DNA has *S* values of 14.2 and 15.5 at neutral and alkaline pH respectively, while BUdR-substituted AAV2 DNA has sedimentation values of 15.5S at neutral pH and 17.0S at alkaline pH.

The action of the specific restriction endonuclease R·*Eco*RI on [^3H]BUdR AAV2 DNA was first studied by sedimentation as shown in Fig. 1. Prior to incubation with the enzyme, about one half of the AAV DNA molecules sedimented in neutral sucrose as linear monomers at 15.5 *S* and the other half sedimented more rapidly (Fig. 1a). This sedimentation profile is characteristic of AAV DNA. The faster sedimenting DNA contains several species including circular monomers, linear and circular dimers, and other oligomers and large aggregated networks (Gerry *et al.*, 1973). As shown by sedimentation at alkaline pH (Fig. 1b), all the AAV duplex DNA species contained only linear strands of unit length (17.0 *S*), at least 90% of which were intact.

After incubation of the AAV DNA with R·*Eco*RI, neutral sucrose sedimentation (Fig. 1c) revealed three cleavage products with *S* values of 12.6, 11.0 and 6.6 and designated A, B and C respectively according to the convention proposed by Smith and Nathans (1973). Components A and B jointly contained about 65% and fragment C about 5.5% of the total ^3H label. The neutral sucrose gradient of Fig. 1c also shows that about 15% of the total ^3H label (component AB′) still sedimented in the position of intact monomers (15.5S) and an additional 14% (component $\overline{AB}$) still sedimented more rapidly than 15.5 *S*. Analysis of the cleaved DNA preparation in alkaline sucrose (Fig. 1d) showed only the three single strand populations A, B and C with *S* values of 13.6, 11.8 and 6.0 and containing approximately 53, 40.5 and 5–6% respectively of the total ^3H label. The complete absence of any AAV strands sedimenting at 17.0 *S* in alkaline sucrose following incubation with the R·*Eco*RI enzyme (Fig. 1d) indicates that all strands had been cleaved at least once. Thus, the two molecular species AB′ and $\overline{AB}$ observed in neutral sucrose following enzyme cleavage (Fig. 1c) do not represent molecules which were resistant to the R·*Eco*RI enzyme, but must represent molecules in which all the component strands had been cleaved but remained associated by some non-covalent structure.

Electrophoretic Analysis of AAV DNA R·*Eco*RI *Cleavage Products*

The cleavage of AAV DNA with R·*Eco*RI was also analysed by electrophoresis in composite agarose-acrylamide gels (Fig. 2). In these gels, uncleaved [^{32}P]AAV DNA showed two major components (Fig. 2a); about 50% of the DNA (presumably duplex monomers) migrated as a discrete peak and about 50% remained close to the electrophoretic origin. Following cleavage with

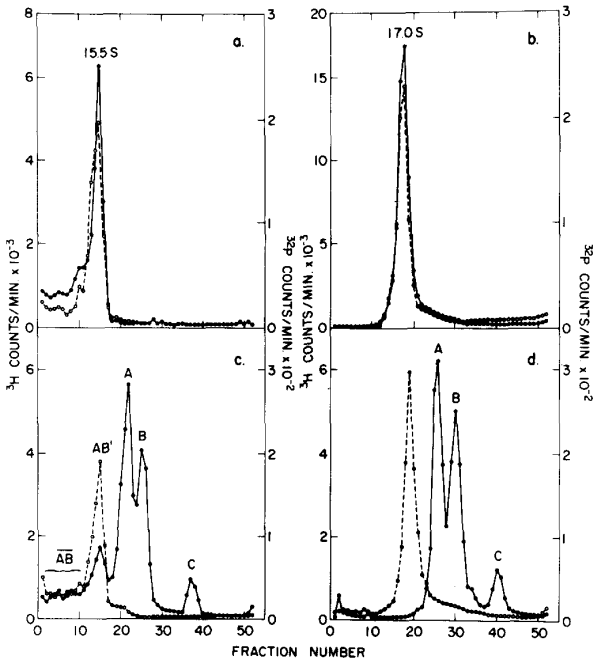

FIG. 1. Sedimentation analysis of cleavage of AAV DNA by nuclease R·*Eco*RI. [^{3}H]BUdR-AAV2 DNA (specific activity 1.99 × 10^5 counts/min/μg) was incubated with the R·*Eco*RI enzyme, and aliquots of the reaction mixture were analysed in neutral and alkaline sucrose gradients as described in Materials and Methods. (a) Neutral sucrose and (b) alkaline sucrose sedimentation profile of [^{3}H]BUdR-DNA prior to cleavage. (c) Neutral sucrose and (d) alkaline sucrose profile of [^{3}H]DNA following enzyme cleavage. Gradients were centrifuged for 11.5 hr. To each sample layered on the gradients was added a trace amount of [^{32}P]BUdR-AAV DNA (specific activity 1.08 × 10^5 counts/min/μg). ●——●, [^{3}H]BUdR-AAV2 DNA. O- - -O, [^{32}P]BUdR-AAV2 DNA.

R·*Eco*RI (Fig. 2b), two new species (A and B) were observed migrating more rapidly than intact AAV DNA. There was, however, a significant proportion of material remaining close to the electrophoretic origin and also a component which migrated approximately in the position of linear SV40 DNA. To reveal fragment C in the cleaved DNA, the gel electrophoresis was performed for a shorter period as shown for [^{3}H]BUdR-AAV DNA in Fig. 2c. In this gel, fragments A and B were still resolved from components AB′ and $\overline{AB}$, and fragment C was observed to migrate much more rapidly. Also included in the gel shown in Fig. 2c were the purified fragments G and K obtained by cleavage of [^{32}P]SV40 DNA with the restriction endonuclease *Hin*d (Danna and Nathans, 1971). The SV40 *Hin*d-K fragment comigrated with the AAV *Eco*·RI fragment C. Other experiments (not shown) employing 5% acrylamide gels also showed comigration of AAV fragment C with SV40 *Hin*d-K.

R·*Eco*RI *Cleavage of "15.5 S" and "Oligomer" Fractions of AAV DNA*

Since the population of AAV DNA duplex molecules formed upon extraction from virions is a mixture of species, several questions arise from the cleavage experiments described thus far. Although every AAV duplex is apparently cleaved by R·*Eco*RI, does each topological species yield the same population of cleavage products? What is the correlation between the DNA species observed in neutral sucrose gradients and those in gels? What is the

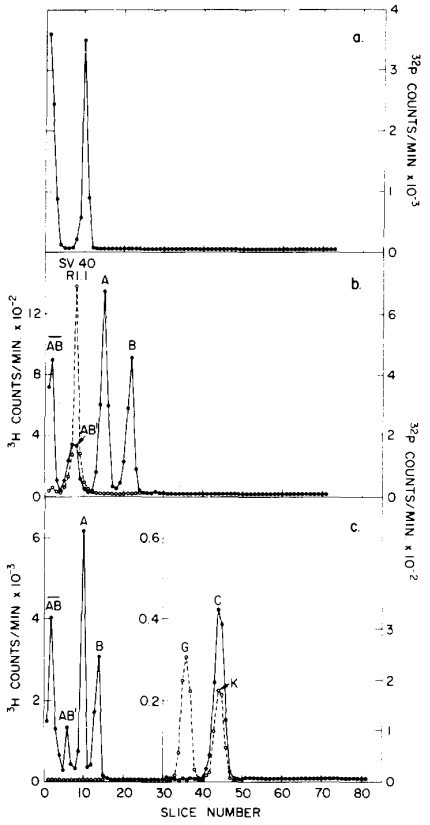

FIG. 2. Electrophoretic analysis in composite 2.0% acrylamide–0.5% agarose gels of the cleavage of AAV2 DNA by nuclease R·*Eco*RI. (a) [^{32}P]AAV2 DNA (0.05 μg; specific activity 2.1 × 10^5 counts/min/μg) analysed prior to enzyme cleavage. (b) [^{32}P]AAV2 DNA (0.02 μg) analyzed following incubation with R·*Eco*RI. [^{3}H]SV40 DNA component I, in which R·*Eco*RI makes one site-specific break (Morrow and Berg, 1972; Mulder and Delius, 1972), was included in the cleavage reaction. (●——●), [^{32}P]AAV DNA. (O- - -O), Linear [^{3}H]SV40 DNA. (c) Analysis of [^{3}H]BUdR-AAV2 DNA (0.15 μg; specific activity 2 × 10^5 counts/min/μg) following cleavage with the R·*Eco*RI enzyme. A trace amount of purified [^{32}P]SV40 *Hin*d-G and [^{32}P]SV40 *Hin*d-K fragments were added as molecular weight standards prior to electrophoresis. (●——●), [^{3}H]BUdR-AAV DNA. (O- - -O), [^{32}P]SV40 DNA. Note the tenfold change of scale for [^{3}H]DNA beginning at fraction 30. The gels shown in (a) and (b) were electrophoresed for 10.0 hr at 3.0 mA/gel. The gel shown in (c) was run for 6 hr at 3 mA/gel. Migration in the gels is from left to right in order of decreasing molecular weight.

structure of components AB′ and $\overline{AB}$? Are fragments A, B and C unique regions of the AAV duplex genome? The experiments described next are addressed to the first two of these problems. The latter two questions are considered in succeeding sections.

[^{3}H]BUdR-AAV DNA was sedimented in a neutral sucrose gradient (Fig. 3), and DNA from the two regions designated "15.5 *S*" and "oligomers" were separately pooled. The "15.5 *S*" pool should contain predominantly linear duplex molecules as well as hydrogen-bonded circles (which are expected to sediment approximately 10% more rapidly than linear monomers) whereas the "oligomer" pool should comprise circular and linear oligomers and network-like structures as well as some

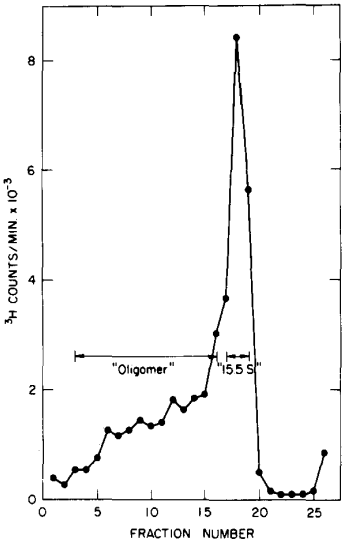

FIG. 3. Fractionation of AAV DNA by neutral sucrose sedimentation. 5 μg of [^{3}H]BUdR-AAV2 DNA (specific activity, 7.5 × 10^4 counts/min/μg) was sedimented in a neutral sucrose gradient for 5 hr. Fractions (450 μL) were collected and 10 λ aliquots of each were taken for radioactivity determinations by scintillation counting. The remainder of each fraction from the two regions designated "oligomer" and "15.5 *S*" were separately pooled, concentrated by pressure dialysis, then dialysed against 0.01 *M* Tris pH 8.0, 0.01 *M* NaCl. Following this the pools were further analysed as described in Fig. 4.

hydrogen-bonded monomer circles (Gerry *et al.*, 1973). Portions of each pool were then electrophoresed in composite agarose-acrylamide gels (Fig. 4). The "oligomer" pool (Fig. 4a) was almost totally comprised (>85%) of molecules which remained near the gel origin whereas the "15.5 *S*" fraction (Fig. 4b) consisted of molecules about 65% of which migrated as a discrete peak, 30% that remained at the origin, and 5% which migrated intermediate distances. The proportion of molecules remaining near the origin in the "15.5 *S*" fraction presumably indicates the extent of contamination with the oligomer fraction. As shown in Fig. 4d, cleavage of the "15.5 *S*" DNA with R·*Eco*RI produced mainly fragments A and B (45 and 33% respectively fo the total ^{3}H label) as well as some component AB′ (13% of total) and only a very minor amount of $\overline{AB}$. Cleavage of the "oligomer" fraction (Fig. 4c) produced also fragments A and B (22 and 15% of total) and component AB′ (13% of total) but in addition a larger proportion of component $\overline{AB}$ (22% of total). In these gels, fragment C migrated off the end of the gel and was therefore not observed.

The experiments shown in Figs. 3 and 4 thus served to correlate the neutral sucrose components $\overline{AB}$, AB′, A and B of AAV DNA with those observed in composite gels. This correlation was confirmed also both by elution of these components from composite gels and subsequent analysis in sucrose gradients and by their purification from neutral sucrose gradients and subsequent electrophoresis in gels (data not shown; Carter, Khoury, and Denhardt, in preparation).

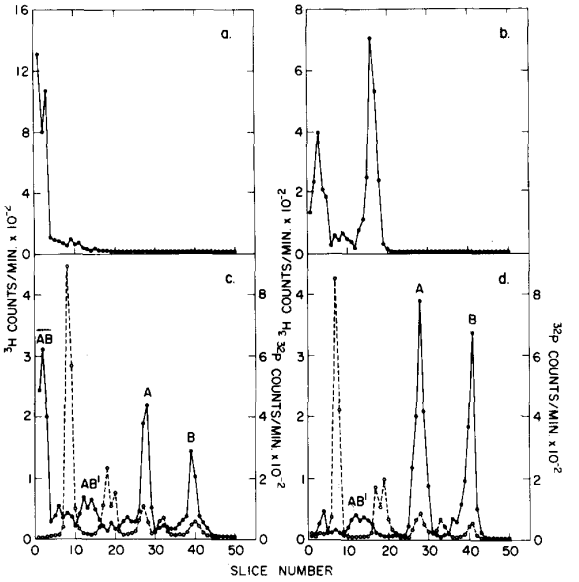

FIG. 4. Electrophoretic analysis in composite gels of R·*Eco*RI cleavage of "oligomer" and "15.5 *S*" fractions of AAV DNA. Portions of the "oligomer" and "15.5 *S*" fractions of [^{3}H]BUdR-AAV2 DNA obtained from the neutral sucrose gradient as described in Fig. 3 were analyzed in composite gels without any additional treatment. (a) "oligomer" fraction (b) "15.5 *S*" fraction. The remainder of each fraction was mixed with tracer amounts of [^{32}P]BUdR-Ad2 DNA and incubated with nuclease R·*Eco*RI. (c) Electrophoresis of "oligomer" fraction of [^{3}H]BUdR-AAV2 DNA following enzyme cleavage. (d) "15.5 *S*" [^{3}H]BUdR-AAV2 DNA following enzyme cleavage. In gels (c) and (d) the AAV DNA components are designated $\overline{AB}$, AB′, A and B. The six Ad2 DNA R·RI cleavage products in order of decreasing molecular weight from left to right are A, B, C, D, E and F (Pettersson *et al.*, 1973). The Ad2 fragments B and C are incompletely resolved in these gels. Electrophoresis was performed for 16 hr at 3 mA/gel. ●——●, [^{3}H]BUdR-AAV2 DNA. O- - -O, [^{32}P]BUdR-Ad2 DNA.

In the cleavage reactions analyzed in Figs. 4c and 4d, [^{32}P]BUdR-Ad2 DNA was included to provide an internal molecular weight calibration (Pettersson *et al.*, 1973). Gel electrophoresis of the cleavage products (Fig. 4) showed that the AAV R·RI-A fragment migrated close to the Ad2 R·RI-D fragment and the AAV R·RI-B fragment migrated close to the Ad2 R·RI-F fragment. The ^{32}P label showed the expected distribution among the six Ad2 R·RI fragments (Pettersson *et al.*, 1973) which indicates that the enzyme digestion was complete and also that the substitution of BUdR for thymidine did not alter the specificity of the R·*Eco*RI enzyme. An additional point, illustrated by the experiment shown in Fig. 4, is that the trapping of part of the AAV DNA at or near the gel origin is probably not solely a function of high molecular weight since no Ad2 DNA remained at the origin. In these composite gels, linear DNA duplex molecules larger than about 3×10^6 molecular weight (up to the value of at least 22×10^6) all migrate with the same electrophoretic mobility in the position of the Ad2 R·RI-A fragment shown in Figs. 4c and 4d (Pettersson *et al.*, 1973; see also Fig. 8).

The gel analysis of the "15.5 *S*" and "oligomer" fractions in Fig. 4 did not reveal AAV fragment C since it had migrated beyond the end of the gel. To determine if both fractions of AAV DNA yielded fragment C upon cleavage with R·*Eco*RI, the reaction mixtures were analysed in neutral sucrose gradients. As shown in Fig. 5, both the "oligomer" fraction (Fig. 5a) and the "15.5 *S*" fraction (Fig. 5b) yielded upon cleavage approximately 5.5% of the ^{3}H-labeled AAV DNA in fragment C. Thus, apparently all AAV duplexes in the population yield the same proportion of fragment C upon cleavage. These gradients show also that, in addition to fragment C, the "15.5 *S*" fraction yielded only fragments A and B whereas the "oligomer" yielded fragments A and B and also components AB′ and $\overline{AB}$, consistent with the gel analysis in Fig. 4. (The "oligomer" and "15.5 *S*" pools used in the experiment of Fig. 5 were taken from a gradient similar to that shown in Fig. 3 but

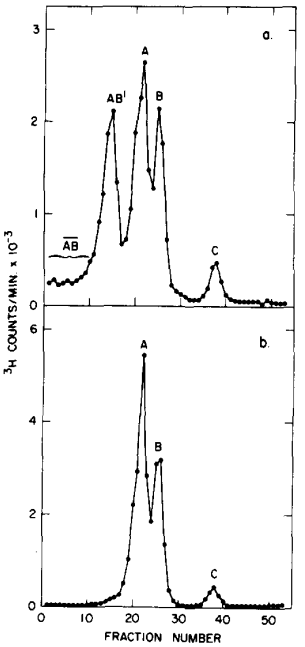

FIG. 5. Neutral sucrose sedimentation analysis of "oligomer" and "15.5 *S*" fractions of [^{3}H]BUdR-AAV2 DNA following cleavage by R·*Eco*RI. [^{3}H]BUdR-AAV2 DNA (specific activity 8.4×10^4 counts/min/μg) was separated into "oligomer" and "15.5 *S*" fractions by neutral sucrose sedimentation in a similar fashion to the separation shown in Fig. 3, but with one minor modification as detailed in the text. The separated fractions were incubated with R·*Eco*RI and sedimented through neutral sucrose gradients for 12 hr. (a) "oligomer" fraction cleaved by R·*Eco*RI. (b) "15.5 *S*" fraction cleaved by R·*Eco*RI.

in which only the very peak fractions of the monomer region were taken as "15.5." Thus, hydrogen-bonded circular monomers should be almost completely contained in the "oligomer" fraction in contrast to the fractionation shown in Fig. 3 in which the hydrogen-bonded circular monomers were probably distributed between both fractions. This accounts for the fact that, in the gradients in Fig. 5, the "15.5 *S*" peak yielded little or no component AB′ whereas some component AB′ was produced from "15.5 *S*" in the gels of Fig. 4d.)

The Structure of Components AB′ and $\overline{AB}$

The components AB′ and $\overline{AB}$ arise from cleavage of the "oligomer" fraction of AAV

DNA. Furthermore, the alkaline sucrose gradient analysis (Fig. 1) suggested that these two components apparently consisted of two or more of fragments A, B or C held together by some non-covalent (alkali-sensitive) structure. To determine directly the single-strand composition of components AB′ and $\overline{AB}$, the experiment shown in Fig. 6 was performed. [^{3}H]BUdR-AAV DNA, cleaved with R·RI, was sedimented in neutral sucrose (Fig. 6a) and regions corresponding to components $\overline{AB}$, AB′, A, B and C respectively were separately pooled and analyzed subsequently in alkaline sucrose gradients. This analysis showed that fragments A, B and C each contained a discrete class of single-strands of the size expected if each fragment is a discrete duplex molecule containing few if any nicks (Figs. 6d–f). Both components AB′ (Fig. 6c) and $\overline{AB}$ (Fig. 6b) in alkali released approximately equimolar proportions of single-strands equal in length to those of both fragments A and B, but there were no strands released equal in size to those of fragment C. There was some heterogeneous material sedimenting more slowly than peak B in the alkaline profile of $\overline{AB}$ (Fig. 6b), but this material sedimented ahead of the expected position of C strands. Thus, $\overline{AB}$ and AB′ both appear to comprise fragments A and B held together by some non-covalent structure. One possible structure is a "cohesive end" or single-strand complementary termini. Such cohesive ends are generated by R·*Eco*RI cleavage but are only four nucleotides in length with T_m of 6° (Mertz and Davis, 1972) and would be unstable under the conditions of sedimentation or gel electrophoresis at 20°. Also, if this were the structure responsible for formation of $\overline{AB}$ and AB′, fragment C should also be present in such structures since it must also have at least one R·RI generated cohesive terminus. Furthermore, the linear monomer AAV DNA as well as the oligomer fraction would be expected to yield components $\overline{AB}$ and AB′ after R·RI cleavage. A more probable explanation is the presence of cohesive termini in some AAV DNA duplexes (Gerry *et al.*, 1973) which accounts for the presence of the circular and linear oligomers in duplex

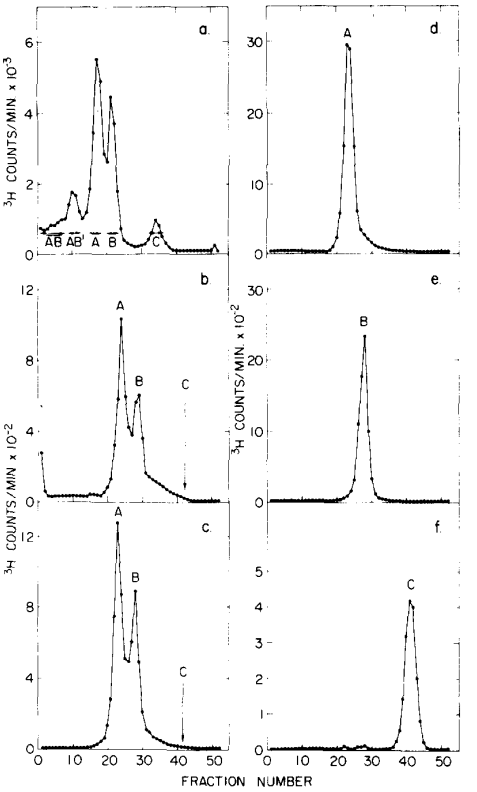

FIG. 6. Analysis of separated AAV DNA R·*Eco*RI cleavage products in alkaline sucrose. [^{3}H]BUdR-AAV2 DNA (6 μg; specific activity 8.4 × 10^4 counts/min/μg) was cleaved with R·*Eco*RI enzyme, and the reaction mixture sedimented through a neutral sucrose gradient for 12 hr as shown in panel (a). Aliquots (10 μL) of each fraction from the gradient were taken for radioactivity counting. Fractions were then pooled as indicated in a) to yield components $\overline{AB}$, AB′, A, B and C. Each pool was dialyzed against 0.01 *M* Tris, pH 8.0, 0.01 *M* NaCl, 0.001 *M* EDTA, and concentrated by pressure dialysis. Aliquots of each pool were then taken and sedimented in alkaline sucrose gradients for 12 hrs as shown in panels (b)-(f). (b) Alkaline sedimentation profile of component $\overline{AB}$, (c) component AB′, (d) fragment A, (e) fragment B, (f) fragment C.

In (b) and (c) the arrows indicate the expected position in the gradient of fragment C.

AAV DNA. These cohesive ends can be melted by heating at 80° in 1 × SSC which is equivalent to 12° below the T_m of AAV2 DNA (Gerry *et al.*, 1973).

As shown in Fig. 7, incubation at 80°

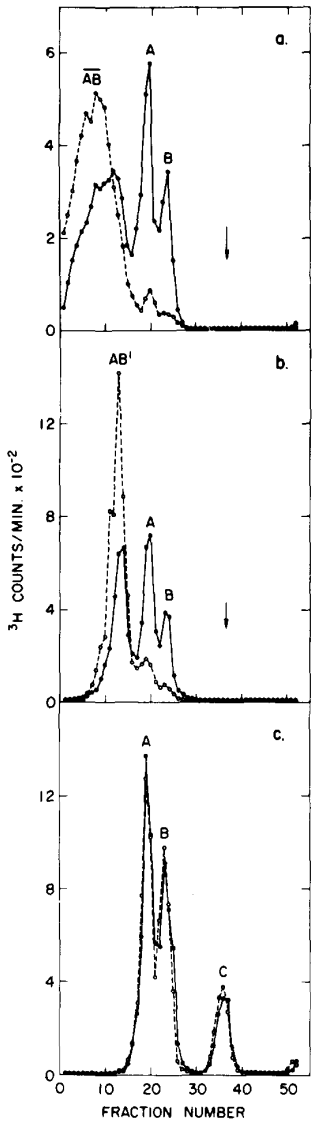

FIG. 7. The effect of heating on AAV DNA R·*Eco*-RI cleavage products. Aliquots of the DNA fractions from the neutral sucrose gradient in Fig. 6a were adjusted to 1 × SSC, and one-half of the mixture was heated at 80° for 10 min and rapidly cooled by quenching in ice-water, then analyzed in neutral sucrose gradients. The remaining half of the mixture, which was not heated, was analysed on a parallel gradient. To facilitate visual comparison, the gradient profiles of both the heated and unheated portions are plotted in one frame. (a) component $\overline{AB}$, (b) component AB', (c) fragments A, B and C. Note that for analysis of A, B and C, portions of the respective pools from Fig. 6a were mixed together in the proportions shown prior to heat treatment. In panels (a) and (b) the arrows indicate the expected position of fragment C. O- - -O, unheated. ●——●, heated prior to sedimentation.

converted about 40% of both $\overline{AB}$ (Fig. 7a) and AB' (Fig. 7b) to equimolar amounts of fragments A and B, whereas the sedimentation rate of fragments A, B and C (Fig. 7c) was not altered by such treatment. The melting experiment in Fig. 7 also shows that upon heating $\overline{AB}$ or AB' there was no detectable release of any fragment C which is consistent with the alkaline sedimentation analysis in Figs. 6a and b. Components $\overline{AB}$ and AB' obtained by elution from gels also showed approximately 50% conversion to fragments A and B when melted under conditions identical to those described in Fig. 7. In addition, melting of unfractionated intact AAV DNA under similar conditions also resulted in conversion of about 40–50% of the oligomer fraction to linear monomers. Finally, we note that, under the same melting conditions, Gerry *et al.* (1973) also observed about 50% conversion of an AAV DNA oligomer fraction (their peak III material) to monomeric molecules. Thus, at least part of components $\overline{AB}$ and AB' are apparently comprised of fragments A and B joined noncovalently by cohesive termini.

Molecular Weight Determination for the AAV DNA Fragments

The molecular weights of the AAV DNA fragments can be determined from sedimentation in neutral and alkaline sucrose, from relative electrophoretic mobility in composite gels, or from the distribution of isotope in each fragment (Table 1). For fragments A and B, sedimentation analyses in either neutral or alkaline sucrose yielded estimated molecular weights of approximately 1.6×10^6 and 1.1×10^6 respectively. To obtain molecular weight estimations for A and B from electrophoresis, the gel system was calibrated as shown in Fig. 8 using the Ad2 DNA R·RI fragments as reference standards (Pettersson *et al.*, 1973). This yielded values for the AAV fragments A and B of 1.65×10^6 and

TABLE 1

MOLECULAR WEIGHT OF AAV-2 DNA R·*Eco*RI FRAGMENTS[a]

DNA	Neutral sucrose		Alkaline sucrose		Gel electrophoresis	Isotope distribution	
	S	MW	*S*	MW	MW	% ^{32}P	MW
Intact	14.2	2.8 × 10^6	15.5				
RI-A	11.7	1.59 ± .1 × 10^6	12.5	1.63 ± .1 × 10^6	1.65 ± .1 × 10^6	57.2	1.6 × 10^6
RI-B	10.4	1.12 ± .06 × 10^6	10.8	1.1 ± .1 × 10^6	1.10 ± .1 × 10^6	38.2	1.05 × 10^6
RI-C	5.5	1.7 ± .1 × 10^5 0.7 ± .1 × 10^5	5.0	1.65 ± .1 × 10^5	1.3 × 10^5 0.74 × 10^5	4.6	1.3 × 10^5

[a] The molecular weights were calculated from sucrose gradients using the relationships derived by Studier (1965). The sedimentation constants are those for non-BUdR containing AAV DNA. Molecular weights determined from alkaline sucrose are reported as those for the equivalent duplex DNA molecule. The error is 2σ. For fragment C, the value of 1.7 × 10^5 was determined using the Studier relationship, and the value of 0.7 × 10^5 using the modified relationship proposed by Danna and Nathans. The two values for C from gel electrophoresis are based on comigration with SV40 *Hin*-K and taking the molecular weight of *Hin*-K as 0.74 × 10^5 (Danna and Nathans, 1971) or 4% of SV40 DNA (molecular weight 3.2 × 10^6) = 1.3 × 10^5 (Nathans *et al.*, 1974). Values based on isotope distribution were computed from analysis of [^{32}P]DNA in gels. The amount of isotope in A and B was normalized to account for that present as $\overline{AB}$ and AB′ assuming both these components have equimolar proportions of A and B.

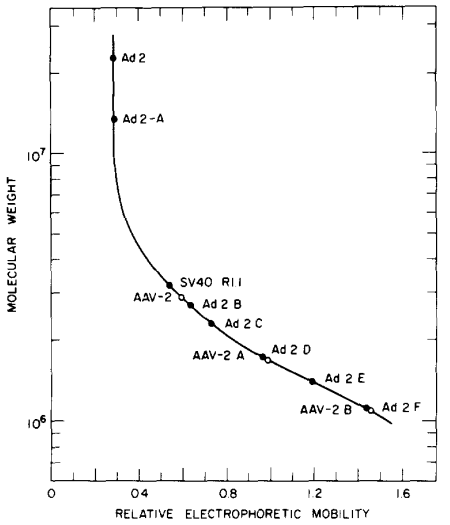

FIG. 8. Molecular weight calibration of 2.0% acrylamide-0.5% agarose gels for duplex DNA. The calibration curve was drawn using the following DNA's as standards: Intact Ad2 DNA (22.9 × 10^6), Ad2 R·RI-A (13.6 × 10^6), Ad2 R·RI-B (2.7 × 10^6), Ad2 R·RI-C (2.3 × 10^6), Ad2 R·RI-D (1.7 × 10^6), Ad2 R·RI-E (1.4 × 10^6), and Ad2 R·RI-F (1.1 × 10^6) (Pettersson *et al.*, 1973), SV40 R·RI (3.2 × 10^6) and intact AAV2 duplex monomer (2.8 × 10^6) (Gerry *et al.*, 1973). For each fragment, the relative electrophoretic mobility was computed relative to that of AAV R·RI-A which was taken as 1.0. All values for the standards and the AAV R·RI fragments A and B are the mean of at least seven individual measurements except that for SV40 R·RI which is a mean of three determinations. Substitution of AAV2 or Ad2 DNA with BUdR caused no alteration in the relative or absolute mobility of either intact DNA or the R·RI fragments. The Ad2 and SV40 DNA standards are indicated by solid circles. Intact AAV2 duplex monomer and the AAV2 R·RI fragments A and B are indicated by open circles.

1.1 × 10^6 which are in good agreement with the sedimentation data. The estimated molecular weights of A and B, based upon the distribution of isotopic label ^{32}P and assuming a random distribution of the isotope, were also in excellent agreement with the sedimentation and electrophoretic estimates. Thus A and B respectively represent approximately 57.2 and 38.2% of the AAV duplex monomer DNA.

The values obtained for the molecular weight of the AAV fragment C showed some variation depending upon the procedure used. Based upon the proportion of ^{32}P label in C, this fragment is equivalent to 4.6% of the AAV genome which yields a calculated molecular weight of 1.3 × 10^5. This value is in good agreement with the observed comigration of C with the SV40 *Hin*d-K fragment which comprises 4% of the SV40 genome (Nathans *et al.*, 1974) equivalent to a molecular weight of 1.3 × 10^5 (assuming 3.2 × 10^6 for the entire SV40 genome). Estimation of the size of fragment C in neutral or alkaline sucrose

gradients yielded somewhat higher values of 1.7×10^5 and 1.65×10^5. Danna and Nathans (1971) suggested that the Burgi and Hershey (1963) relationship used to determine molecular weights by neutral sucrose sedimentation should be modified for DNA species smaller than 1×10^6. Using this modification, a value of 0.71×10^5 is obtained for the molecular weight of the AAV C fragment which is equivalent to Danna and Nathans' estimate for SV40 *Hin*d-K obtained by extrapolation of molecular weight analyses in acrylamide gels. All these procedures are somewhat unreliable for the estimation of molecular weights of DNA much smaller than 1×10^6, but for the present we have taken 1.3×10^5 as a reasonable estimate of the size of fragment C. Thus C is equivalent to 4.6% of the AAV duplex genome. It is possible that C might represent two smaller fragments each equivalent to about 2.3% of the AAV genome and having molecular weights of about 0.65×10^5–0.7×10^5, although reassociation experiments described below might argue against this.

Reassociation Rates of AAV DNA Fragments

The reassociation kinetics of the AAV *Eco*RI fragments were analysed as shown in Fig. 9. Fragments A, B and C reassociated in 0.14 *M* phosphate buffer (0.21 *M* Na^+) at 68° with $C_0t_{1/2}$ values of 5.4×10^{-4}, 4.5×10^{-4} and 1.7×10^{-4} respectively, and when fragments A and B were mixed in equimolar proportions and reassociated, the $C_0t_{1/2}$ of this reaction was 9.5×10^{-4}. Under the same conditions of reassociation, the $C_0t_{1/2}$ for intact AAV strands is 7.5×10^{-4}, and for AAV strands fragmented by sonication to 1/8 to 1/10 genome size, the $C_0t_{1/2}$ is 1.2×10^{-3} (Carter *et al.*, 1972). These data therefore indicate that each of the R·RI AAV fragments contains only a portion of the AAV genome.

If the R·RI fragments each contain a unique region of the AAV genome, then their expected reassociation rates relative to that for intact AAV DNA can be calculated according to the relationship deduced by Wetmur and Davidson (1968) that the rate of reassociation is proportional to

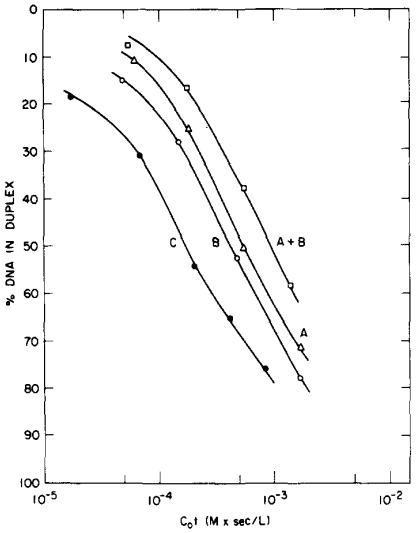

FIG. 9. Reassociation analysis of purified AAV2 DNA R·RI fragments. Fragments A, B and C obtained from cleavage of [^{3}H]BUdR-AAV2 DNA by R·*Eco*RI were purified from neutral sucrose gradients as described in Fig. 6a. Subsequent analysis of the purified fragments in alkaline sucrose gradients (Figs. 6d–f) and neutral sucrose gradients and composite gels (Carter, Khoury, and Denhardt, in preparation) showed that each of the fragments was at least 95% pure, and more than 90% of each preparation was free of single strand nicks. Reassociation reactions were performed in 0.14 *M* phosphate buffer pH 6.8 (i.e., 0.21 *M* Na^+) at 68° and analyzed as described in Materials and Methods. The data was not corrected for "zero-time" values (i.e., the proportion of duplex DNA following heat denaturation, but prior to reassociation) which was less than 2–3% in all cases. ●——●, Reassociation of fragment C. O——O, Reassociation of fragment B. Δ——Δ, Fragment A. □——□, Fragments A + B mixed in equimolar proportions.

$L^{0.5}/N$ where L is the strand length and N is the complexity. For a unique DNA sequence, the complexity is directly proportional to molecular weight. Shown in Table 2 are the *expected* relative reassociation rates of the AAV DNA fragments, and these values are compared to the *observed* relative reassociation rates calculated as being inversely proportional to the $C_0t_{1/2}$. Assuming that A and B are unique fragments of molecular weight 1.6×10^6 and 1.1×10^6 yields excellent agreement between the expected and observed reassocia-

TABLE 2

RELATIVE REASSOCIATION RATE OF AAV-2 DNA R·*Eco*RI FRAGMENTS

DNA	$C_0t_{\frac{1}{2}}$[a]	Observed relative reassociation rate	Molecular weight	Expected relative reassociation rate
A + B	9.5×10^{-4}	0.78	2.7×10^6	0.71
Intact	7.4×10^{-4}	1.00	2.8×10^6	1.00
A	5.4×10^{-4}	1.37	1.6×10^6	1.33
B	4.5×10^{-4}	1.64	1.1×10^6	1.63
C	1.7×10^{-4}	4.45	1.7×10^5	4.05
			1.3×10^5	4.60
			0.71×10^5	6.30

[a] $C_0t_{\frac{1}{2}}$ values were measured in 0.14 *M* phosphate buffer at 68° (see legend to Fig. 9). The observed relative reassociation rate was calculated as the reciprocal of the $C_0t_{\frac{1}{2}}$ value and normalized to that of intact duplex (1.0). The expected relative reassociation rate was calculated according to Wetmur and Davidson (1968) that $k_r = L^{0.5}/N$ where L is the single-strand length (molecular weight) of the reassociating species and N is the complexity (molecular weight of the double-stranded species). For the reassociation of equimolar amounts of A + B, L was taken as the mean of L for A and B, and N was taken as 2.7×10^6 (molecular weight of A duplex + molecular weight of B duplex).

tion rates. Mixing of A and B in equimolar amounts resulted in an observed reassociation rate in good agreement with the rate expected if A and B are separate, unique regions of AAV DNA and together account for nearly the entire genome.

The expected reassociation rate for fragment C is more difficult to predict because of the uncertainty in the molecular weight estimates. Shown in Table 2 are the expected rates if C is a unique fragment of molecular weight 0.71×10^5, 1.3×10^5 or 1.7×10^5. The best fit with the observed rat is obtained if the molecular weight of C is 1.3×10^5. Therefore, C is probably not one piece as small as 0.71×10^5, but it might be two pieces of this molecular weight. The relative reassociation rate would be 6.3 if both pieces were identical sequences and 3.15 for nonidentical sequences. Neither of these two assumptions gives a good fit of the observed rate and expected rate. These arguments relating to the size of fragment C depend upon the validity of the Wetmur and Davidson relationship for this size range which, for a molecular weight of 0.7×10^5, is approximately 100 nucleotide pairs. Below this value, the rate of reassociation is proportional to L rather than $L^{0.5}$ (Wetmur and Davidson, 1968). In this case, the relative reassociation rate for C, taking a molecular size of 0.7×10^5 would be 6.3.

From these considerations, the best estimation for the size of C at present appears to be about 1.3×10^5 which is equivalent to approximately 4.6% of the AAV genome and about 200 nucleotide pairs. Fragments A and B are equivalent to 57.2 and 38.2% and 2400 and 1600 nucleotide pairs respectively.

DISCUSSION

The experiments described in this study show that the R·*Eco*RI nuclease makes two site-specific breaks in linear, duplex monomers of AAV2 DNA, thus producing three fragments, A, B and C, equivalent in size to 57.2, 38.2 and about 4.6% respectively of the intact genome. The distribution of isotope in AAV DNA randomly labeled with ^{32}P, together with molecular weight estimates from sedimentation and electrophoretic analyses, indicated that all three fragments were produced in equimolar proportions, and that each probably contains a unique region of the duplex genome. This latter conclusion is supported further by measurement of the rate of reassociation for each of the purified fragments. Because of some uncertainty in the molecular weight estimates of C, it is possible that this fragment might represent two fragments of 2.5% of the genome rather than one 5% fragment. A consideration of data from the reassociation analysis argues against this possibility, but this argument rests upon uncertain assumptions regard-

ing the molecular weight range in which the excluded volume of a single DNA strand affects the rate of reassociation as described by Wetmur and Davidson. Thus we cannot rigorously exclude the possibility of three, rather than two, R·*Eco*RI sites within a single 200 nucleotide region of AAV DNA, although this would represent a remarkable clustering of cleavage sites. Also, we cannot completely exclude the occurrence of additional R·*Eco*RI cleavage sites in AAV DNA since fragments less than 1% of the genome (40 nucleotides long) might have escaped detection. However, this would also require additional sites to be located near to the region from which C arises because we show elsewhere that the A and B fragments each contain one terminus of the DNA duplex and fragment(s) C arises from a single contiguous 5% region of the AAV genome (Carter, Khoury, and Denhardt, in preparation). Thus, at least for the purposes of establishing the physical map of the R·*Eco*RI fragments or their use in transcription mapping, fragment C can be regarded as a single fragment.

Gerry *et al.* (1973) demonstrated in AAV duplex DNA the presence of cohesive ends apparently resulting from a limited permutation such that the starting point of each strand may vary within a 6% region of the genome sequence. The presence of cohesive ends thus results in formation of circular and linear duplex oligomers. A direct prediction of this model is that cleavage of the oligomeric molecules with a restriction enzyme should yield two fragments (the terminal fragments obtained from cleavage of linear monomers) which will be joined by cohesive ends provided, of course, that the cleavage does not occur within the region of the permutation. This prediction is fulfilled by the experiments described here which showed that cleavage of AAV duplex oligomers yielded, in addition to fragments A, B and C, two components AB′ and $\overline{AB}$, both of which consist of equimolar amounts of A and B strands present in structures which depend at least in part upon annealing of cohesive ends. This is illustrated in Fig. 10b which shows the

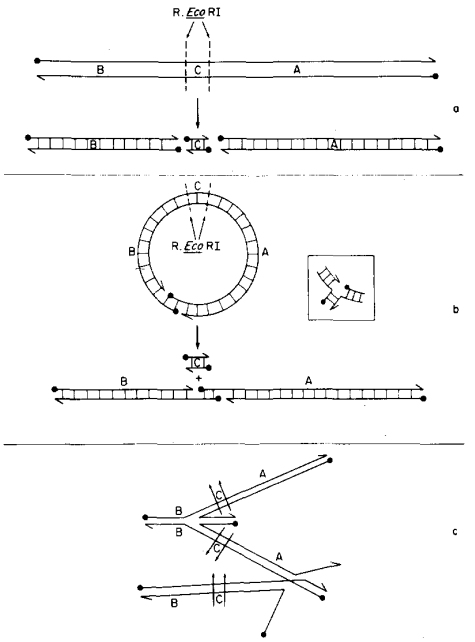

FIG. 10. Schematic diagrams of cleavage of AAV2 DNA by R·*Eco*RI nuclease. For individual DNA strands, the 5′ terminus is indicated by the solid circle (●——) and the 3′ terminus by the arrow(—). The physical order and strand polarity of the fragments A, B and C as shown in this figure was unambiguously determined by labeling the 5′ termini with radioactive phosphorus as will be detailed elsewhere (Carter, Khoury, and Denhardt, manuscript in preparation). (a) Cleavage of linear duplex monomer AAV2 DNA. (b) Cleavage of hydrogen-bonded, duplex circles of AAV2 DNA. As drawn, the molecule is shown containing a cohesive end resulting from the presence of a limited permutation. The cohesive end might contain single-stranded gaps or single-stranded tails as noted in the text (see Discussion). The inset shows a possible alternative structure for formation of hydrogen-bonded circles in which one strand does not contain the inverted repetitious sequences (see Discussion). (c) A possible hypothetical model of an AAV DNA duplex aggregate or "network." R·*Eco*RI cleavage sites are indicated by the arrows. Cleavage of such networks might result in complexes of A strands or B strands which could account for some of the observed sedimentation and electrophoretic properties of component $\overline{AB}$. It should be noted that the network structures may also contain cohesive ends as discussed in the text. Finally, the models shown in these diagrams represent only the most simple hypotheses, and more complex models can be drawn.

most likely structure of component AB′ as that of one fragment A and one fragment B held by a cohesive end. This structure would be expected to have a neutral sucrose sedimentation coefficient and electrophoretic mobility close to that of intact linear monomers which are 5% larger. That the component AB′ has electrophoretic mobility which is slightly *lower* than linear monomers may reflect the presence of single strand gaps or tails in the annealed cohesive ends which would also be predicted if the limited permutation is variable and which would decrease the electrophoretic mobility (Dingman *et al.*, 1974). Recently Tye *et al.* (1974) have demonstrated the presence of a limited but variable permutation in the DNA of phage P22.

The structure of component $\overline{AB}$ is less certain, but it may arise from cleavage of DNA "networks" formed as a result of extraction of AAV DNA from virions at high concentration (30–100 μg/ml in typical preparations). These networks presumably arise from participation of more than two strands in duplex formation causing branching and also because of the presence of cohesive ends. Thus, as shown in Fig. 10c, cleavage might yield, in addition to fragments A, B, C and AB′, branched molecules consisting of several A or B strands. Such branching might also result in single-strand tails. Since branching would occur less frequently in the C region, and also because A and B might also be involved in cohesive end joining, cleavage of networks would generally release fragment C, consistent with our observations. The configuration of the branched cleavage products would be expected to lead to a heterogenous range of sedimentation rates in neutral sucorse, and in composite gels might be responsible for the specific trapping which we observe close to the origin, as recently described by Dingman *et al.* (1974) for the replication point of *E. coli* DNA.

The melting experiments designed to detect cohesive ends showed that at 80°C in 1 × SSC (equivalent to 12° below the T_m of intact AAV duplex DNA) not more than 55% of either $\overline{AB}$ or AB′ could be converted to equimolar amounts of A and B. Several possibilities might account for this. (a) As discussed above, $\overline{AB}$ may be in part comprised of branched structures containing only A or B strands. These structures would be expected to melt at a temperature approximately equivalent to the T_m for intact AAV DNA. Also, some of these branched molecules might sediment at the same rate as the structure proposed in Fig. 10b for component AB′. (b) Some AAV duplex circles (as well as linear and circular oligomers) may result from the annealing of one strand with a complementary strand which has lost the inverted repetitious sequences from its termini. These duplex circles would then arise from annealing of the inverted terminal repetitions within one strand to form a "panhandle" (inset, Fig. 10b). The T_m of these panhandles is at least equivalent to that of intact AAV duplex molecules (Koczot *et al.*, 1973) and therefore would probably not melt under the conditions of our experiments. (c) Part of $\overline{AB}$ and AB′ may result from cleavage of molecules in the "oligomer" population which contained broken or incomplete DNA strands, thus resulting in regions of single-strandedness. This possibility seems likely for part of component $\overline{AB}$, at least, since sedimentation in alkali of this component revealed some strands shorter in length than those of A or B though longer than those of C. (d) The conditions employed in the experiments described in Fig. 7 may not be optimal for the complete melting of all AAV cohesive ends. Further, experiments are currently in progress to evaluate these possibilities.

Finally, we wish to note that the presence of a limited permutation of up to 6% of the genome length will cause a range in size of both fragments A and B of this amount. Thus, the observed molecular weights of A and B must be considered as mean values with a range of ±3% of the AAV genome length. The size estimation of fragment C is not affected by the permutation. In experiments to be described elsewhere (Carter, Khoury, and Denhardt, manuscript in preparation), we show that the complementary strands of fragments A

and B can be separated, thus yielding a preparative purification of each of the four single-strand termini and allowing a more precise measurement of the AAV transcription map.

REFERENCES

BALDWIN, R. L., and SHOOTER, E. M. (1963). The alkaline transition of BU-containing DNA and its bearing on the replication of DNA. *J. Mol. Biol.* **7,** 511-526.

BURGI, E., and HERSHEY, A. D. (1963). Sedimentation rate as a measure of molecular weight of DNA. *Biophys. J.* **3,** 309-321.

BERNS, K. I., and ROSE, J. A. (1970). Evidence for a single-stranded adenovirus-associated virus genome: Isolation and separation of complementary single strands. *J. Virol.* **5,** 693-699.

BERNS, K. I., and KELLY, T. J., JR. (1974). Visualization of the inverted terminal repetition in adeno-associated virus DNA. *J. Mol. Biol.* **82,** 267-271.

CARTER, B. J., and ROSE, J. A. (1972). Adenovirus-associated virus multiplication. Analysis of *in vivo* transcription induced by complete or partial helper virus. *J. Virol.* **10,** 9-16.

CARTER, B. J., KHOURY, G., and ROSE, J. A. (1972). Adenovirus-associated virus multiplication. Extent of transcription of the viral genome *in vivo*. *J. Virol.* **10,** 1118-1125.

CARTER, B. J., KOCZOT, F. J., GARRISON, J., DOLIN, R. W., and ROSE, J. A. (1973). Separate helper functions provided by adenovirus for adenovirus-associated virus multiplication. *Nature New Biol.* **244,** 71-73.

CARTER, B. J. (1974). Analysis of parvovirus message RNA by sedimentation and electrophoresis in aqueous and non-aqueous solution. *J. Virol.* **14,** 834-839.

CARTER, B. J., and ROSE, J. A. (1974). Transcription *in vivo* of a defective parvovirus: Sedimentation and electrophoretic analysis of RNA synthesized by adenovirus-associated virus and its helper adenovirus. *Virology* **61,** 182-199.

DANNA, K., and NATHANS, D. (1971). Specific cleavage of simian virus 40 DNA by restriction endonuclease of Hemophilus Influenzae. *Proc. Nat. Acad. Sci. USA* **68,** 2913-2917.

DINGMAN, C. W., and PEACOCK, A. C. (1968). Analytical studies on nuclear ribonucleic acid using polyacrylamide gel electrophoresis. *Biochemistry* **7,** 659-668.

DINGMAN, C. W., FISHER, M. P., and ISHIZAWA, M. (1974). DNA replication in *Escherichia coli:* Physical and kinetic studies of the replication point. *J. Mol. Biol.* **84,** 275-295.

GERRY, H. W., KELLY, T. J., JR., and BERNS, K. I. (1973). The arrangement of nucleotide sequences in adeno-associated DNA. *J. Mol. Biol.* **79,** 207-225.

HEDGPETH, J., GOODMAN, H. M., and BOYER, H. W. (1972). DNA nucleotide sequence restricted by the RI endonuclease. *Proc. Nat. Acad. Sci. USA* **69,** 3448-3452.

HERSHEY, A. D., BURGI, E., and INGRAHAM, L. (1963). Cohesion of DNA molecules isolated from phage lambda. *Proc. Nat. Acad. Sci. USA* **49,** 748-755.

KOCZOT, F. J., CARTER, B. J., GARON, C. F., and ROSE, J. A. (1973). Self-complementarity of terminal sequences within plus or minus strands of adenovirus-associated virus DNA. *Proc. Nat. Acad. Sci., USA* **70,** 215-219.

MARTIN, M. A., GELB, L. D., FAREED, G. C., and MILSTEIN, J. B. (1973). Reassortment of simian virus 40 DNA during serial undiluted passage. *J. Virol.* **12,** 748-757.

MAYOR, H. D., TORIKAI, K., MELNICK, J. L., and MANDEL, M. (1969). Plus and minus single-stranded DNA separately encapsidated in adeno-associated satellite virions. *Science* **166,** 1280-1282.

MERTZ, J. E., and DAVIS, R. W. (1972). Cleavage of DNA by RI restriction endonuclease generates cohesive ends. *Proc. Nat. Acad. Sci. USA* **69,** 3370-3374.

MORROW, J. F., and BERG, P. (1972). Cleavage of simian virus 40 DNA at a unique site by a bacterial restriction enzyme. *Proc. Nat. Acad. Sci. USA* **69,** 3365-3369.

MULDER, C., and DELIUS, H. (1972). Specificity of the break produced by restricting endonuclease RI in simian virus 40 DNA as revealed by partial denaturation mapping. *Proc. Nat. Acad. Sci. USA* **69,** 3215-3219.

NATHANS, D., ADLER, S. P., BROCKMAN, W. W., DANNA, K. J., LEE, T. N. H., and SACK, G. H., JR. (1974). Use of restriction endonucleases in analysing the genome of simian virus 40. *Fed. Proc.* **33,** 1135-1138.

PETTERSSON, U., MULDER, C., DELIUS, H., and SHARP, P. A. (1973). Cleavage of adenovirus type 2 DNA into six unique fragments by endonuclease R·RI. *Proc. Nat. Acad. Sci. USA* **70,** 200-204.

ROSE, J. A., BERNS, K. I., HOGGAN, M. D., and KOCZOT, F. J. (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. USA* **64,** 863-869.

SMITH, H. O., and NATHANS, D. (1973). A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. *J. Mol. Biol.* **81,** 419-423.

STUDIER, F. W. (1965). Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* **11,** 373-390.

TYE, B. K., HUBERMAN, J. A., and BOTSTEIN, D. (1974). Non-random circular permutation of phage P22 DNA. *J. Mol. Biol.* **85,** 501-532.

WETMUR, J. G., and DAVIDSON, N. (1968). Kinetics of renaturation of DNA. *J. Mol. Biol.* **31,** 349-370.

YOSHIMORI, R. N. (1971). Ph.D. Thesis, University of California at San Francisco Medical Center.