JOURNAL OF VIROLOGY, Sept. 1975, p. 712-719

Vol. 16, No. 3
Printed in U.S.A.

Study of the Fine Structure of Adeno-Associated Virus DNA with Bacterial Restriction Endonucleases

KENNETH I. BERNS,* JAMES KORT, KENNETH H. FIFE, E. WAYNE GROGAN, AND ILENE SPEAR

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

Received for publication 3 April 1975

A physical map of the adeno-associated virus type 2 genome has been constructed on the basis of the five fragments produced by the restriction endonucleases HindII + III from *Hemophilus influenzae*. There are three endo R·HindII cleavage sites and one endo R·HindIII site. Evidence has been obtained to support the existence of two nucleotide sequence permutations in adeno-associated virus DNA, the start points of which have been estimated to be separated by 1% of the genome. The three cleavage fragments produced by endo R·Eco R_I have been ordered and oriented with respect to the endo R·HindII + III cleavage map.

Adeno-associated virus (AAV) is a small, defective, DNA virus which contains a linear single-stranded genome of 1.4×10^6 mol wt. For a productive infection adenovirus is required as a helper (1, 12). AAV is unusual in that there are two types of virions which contain DNA molecules with complementary nucleotide sequences (2, 4, 6, 12, 16). After isolation the complementary polynucleotide chains originating from different virions base pair to form double-stranded DNA if exposed to conditions favorable for annealing. Because the genome contains a limited number of nucleotide sequence permutations, two basic types of linear duplexes may be formed, the first with even ends, and a second with single-stranded cohesive termini. The latter may go on to form hydrogen-bonded circles or concatenates (9).

In an effort to further elucidate the fine structure of the DNA isolated from AAV virions, we have characterized with respect to molecular weight and physical map order the fragments produced by digestion of double-stranded linear AAV DNA of unit length using three bacterial restriction endonucleases, endo R·HindII + III and endo R·Eco R_I, isolated from *Hemophilus influenzae* and *Escherichia coli*, respectively. These enzymes cleave double-stranded DNA at specific nucleotide sequences leading to the production of unique fragments (13, 17). In this paper we report that AAV-2 contains three HindII sites and one HindIII site. The resulting five fragments have been sized and ordered with respect to their locations along the genome. There are two R_I cleavage sites in AAV-2 DNA (5), and the resulting fragments have been oriented with regard to the five Hind restriction fragments.

The endo R·HindII + III digestion was compatible with the presence of two major nucleotide sequence permutations in AAV DNA, the start points of which would be approximately 1% apart on the AAV genome.

MATERIALS AND METHODS

Production of AAV containing radioactively labeled DNA. AAV-2 (AAV-2 H) (10) containing radioactively labeled DNA was grown in human KB cells co-infected with adenovirus type 2 as helper in Eagle medium (8) which had been supplemented with 5% horse serum (Microbiological Associates). The multiplicity of infection for both AAV and adenovirus was 10 infectious units/cell. To produce ^{3}H-labeled AAV DNA, [^{3}H]thymidine (10 μCi/ml; New England Nuclear Corp.) was added 6 to 7 h after infection. AAV containing ^{32}P-labeled DNA were produced in KB cells which had been suspended in low PO_4 (10^{-5} M) Eagle medium 10 to 12 h prior to infection. The ^{32}P (9 μCi/ml; New England Nuclear) was added to the culture 7 h after infection. At 48 h after infection cells were harvested, and virus were purified as previously described (4) by treatment with deoxycholate and trypsin and repeated equilibrium centrifugation in CsCl density gradients. The purification procedure was modified by omission of steps involving sonication. After treatment with deoxycholate and trypsin the crude virus suspension was homogenized with 20 strokes using a Dounce homogenizer before addition of CsCl.

DNA purification. AAV DNA was isolated by exposure of virions in 1 × SSC (0.15 M NaCl plus 0.015 sodium citrate), 0.15% Sarkosyl to 0.1 N NaOH for 10 min at room temperature and to subsequent sedimentation through a 5 to 20% alkaline sucrose gradient (0.1 N NaOH, 0.9 M NaCl, 0.05 M EDTA, 0.15%

Sarkosyl) using the SW50 rotor in a Beckman model L ultracentrifuge (11). Conditions of sedimentation were 45,000 rpm for 4.5 h at 20 C. Intact AAV single strands were isolated from the gradient and dialyzed against 0.01 M Tris buffer, pH 7.8. To produce double-stranded AAV DNA the complementary single strands thus obtained were exposed to 0.1 N NaOH. After 10 min at room temperature an amount of Tris buffer, pH 7.8, equivalent to the NaOH was added, and the solution was made 49% formamide (Matheson, Coleman, and Bell) and maintained at room temperature for 24 h. DNA concentration in the annealing procedure was 2 to 10 μg/ml. Unit length duplex linear monomers were isolated from neutral sucrose gradients (9).

Lambda bacteriophage DNA was a gift from T. J. Kelly, Jr., and SV40 DNA was a gift from D. Nathans.

Digestion of DNA with restriction nucleases. Endo R·HindII + III and endo R·HindIII which had been prepared as described (7) were kindly provided by W. Scott, N. Muzyczka, and D. Nathans. Endo R·Eco R_I was prepared by P. Geshelin, following the procedures of R. N. Yoshimori (Ph.D. thesis, University of California Medical Center, San Francisco, 1971).

In all enzyme digestions a 0.10 volume of enzyme was used and the DNA concentration was 5 to 7.5 μg/ml. The endo R·HindII + III reaction mixture contained 7 mM β-mercaptoethanol, 8 mM $MgCl_2$, 6.6 mM Tris, pH 7.6, and 50 μg of gelatin per ml. The digestion was allowed to proceed for 1 to 3 h at 37 C. A preparation of endo R·HindII + III enriched for endo R·HindII was also used. The reaction mixture contained 6.6 mM Tris, pH 7.4, and 8.7 mM $MgCl_2$ in addition to enzyme and DNA. Digestion conditions were as above. In the case of endo R·HindIII digestion the reaction mixture contained 7 mM $MgCl_2$, 6.6 mM Tris, pH 7.6, and 50 μg of gelatin per ml. The digestion was for 16 to 18 h at 37 C. The endo R·Eco R_I digestion reaction contained 10 mM $MgCl_2$, 75 mM NaCl, and 15 mM Tris, pH 7.8. Digestion was for 2 h at 37 C.

Agarose gels. Cylindrical 10-cm 1.4% agarose gels were run using a buffer containing 40 mM Tris, pH 7.8, 5 mM Na acetate, and 0.1 mM EDTA in a Buchler Polyanalyst. Seakem agarose was obtained from MCI Biomedical. Electrophoresis was at 10 V/cm for 60 to 70 min at room temperature. A 0.10 volume of bromophenol blue in 50% sucrose and 1 mM EDTA was added to the sample as a marker. DNA bands in the gel were stained by placing the gel after electrophoresis into gel-running buffer containing ethidium bromide (0.5 μg/ml) for 15 to 30 min. The bands were visualized under UV light and photographed using Polaroid type 57 film.

Polyacrylamide gels. Vertical polyacrylamide slab gel electrophoresis and subsequent autoradiography were carried out as described by Danna et al. (7).

Radioactivity assays. Assays of ^3H and ^{32}P radioactivity were done using Triton X-100/toluene (1:2) containing Omnifluor (4 g/liter; New England Nuclear) and 10% water in a Beckman LS 230 scintillation counter. In some cases samples containing ^{32}P were assayed in the same counter by measuring Cerenkov radiation.

^{32}P labeling of 5′ termini. Alkaline phosphatase and polynucleotide kinase (19) were provided by B. Weiss. [γ-^{32}P]ATP was obtained from New England Nuclear. Labeling of the 5′ termini of AAV DNA was done according to the method of Weiss et al. (19).

RESULTS

Digestion with endo R·HindII + III. A complete digestion of linear duplex AAV DNA of unit length with endo R·HindII + III yielded five discrete bands (Fig. 1). Increasing the time of digestion or the amount of enzyme used did not lead to any further fragmentation of the DNA. Decreased time of digestion resulted in the appearance of bands representing larger fragments (Fig. 2) which were considered to be the product of partial digestion. The five fragments resulting from complete digestion have been labeled A to E in order of decreasing size (Table 1). The four fragments resulting from partial digestion were all larger than fragment A and have been labeled 1 to 4 (Table 1).

Determination of the molecular weights of fragments A to E. The molecular weights of the fragments produced by endo R·HindII + III digestion were estimated by comparing their mobilities to the mobilities in parallel gels of fragments of SV40 DNA (form I) produced by the same enzymes (7) and of fragments of adenovirus type 2 DNA produced by digestion with endo R·Eco R_I (15). The molecular weights

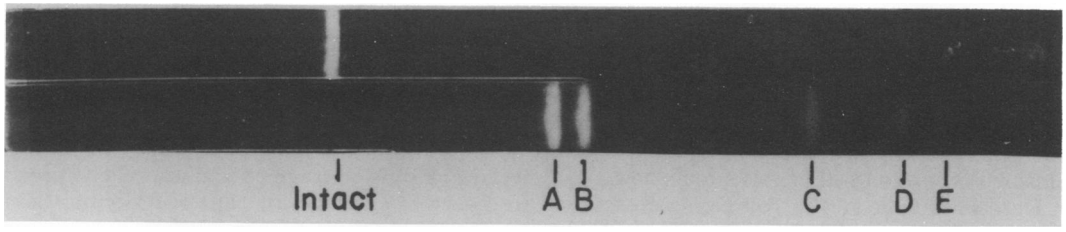

FIG. 1. *A complete endo R·HindII + III digest of unit length linear duplex AAV DNA. The five fragments produced were separated in a 1.4% agarose gel. Fragments have been labeled A to E in order of increasing electrophoretic mobility.*

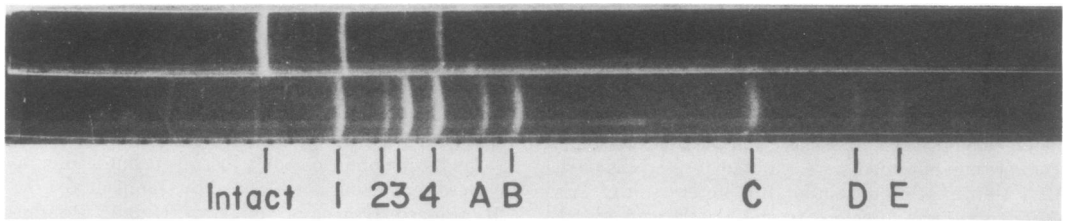

FIG. 2. *A partial digestion of unit-length linear duplex AAV DNA was achieved by decreasing the time of digest. Fragments were separated in a 1.4% agarose gel. The four partial digestion products noted in the bottom gel have been numbered 1 to 4 in order of increasing electrophoretic mobility. The two bands in the top gel corresponding to 1 and 4 represent the endo R·HindIII cleavage products.*

TABLE 1. *Molecular weight estimates of AAV DNA fragments produced by cleavage with endo R·Hind*

HindII + III fragment	Molecular weight ($\times 10^{-6}$)	
	Radiolabel[a]	Relative electrophoretic mobility[b]
1		1.7
2		1.4
3		1.3
4		1.17
A	0.98	0.96
B	0.86	0.86
C	0.40	0.41
D	0.30	0.31
E	0.25	0.27

[a] Uniformly labeled ^{32}P DNA was digested, and the resulting bands cut out from a gel and counted. The fraction of total counts recovered in any one band were converted to molecular weight, assuming a molecular weight of 2.8×10^6 for unit-length duplex DNA. The results are the averages of the data from two independent experiments.

[b] Molecular weights were calculated from relative electrophoretic mobility as described in the text and are the averages of the data from two independent experiments.

for AAV fragments A to E ranged from 9.6×10^5 for A to 2.4×10^5 for E (Table 1). By this method the molecular weight determined for intact duplex linear AAV molecules of unit length was 2.8×10^6 to 2.9×10^6, which is in good agreement with the figure of 2.8×10^6 reported by Gerry et al. (9). The total molecular weight determined for fragments A to E was 2.79×10^6. Because the additive molecular weight of the bands was the same as that of intact DNA, this strongly suggested that each band observed in the gels represented a unique fragment, a conclusion supported by data presented below.

Determination of the relative amounts of endo R·HindII + III fragments A to E. In order to directly ascertain the relative amounts of the five endo R·HindII + III fragments observed, a preparation of AAV DNA uniformly labeled with ^{32}P was digested and electrophoresed through a 1.4% agarose gel. The bands were stained with ethidium bromide, cut out, and counted (Table 1). The relative radioactivities of the five fragments were in good agreement with the molecular weights determined for each of the fragments by relative electrophoretic mobility.

Digestion with endo R·Eco R_I. Digestion of linear duplex AAV DNA of unit length with endo R·Eco R_I produces three fragments A to C, two of which are large and one of which is about the same length or slightly smaller than endo R·HindII + III fragment K of SV40 DNA (1.3×10^5) (Fig. 3). The faint band observed to migrate to a position between SV40 A and B is tentatively attributed to contamination of the endo R·Eco R_I preparation with a second enzyme endo R·Eco R_I. The relative electrophoretic mobilities indicated molecular weights of R_I A and B of 1.57×10^6 and 1.1×10^6, respectively. Partial digestion with endo R·Eco R_I produced two fragments slightly larger than the two large fragments A and B produced by complete digestion, establishing a map order of BCA (Fig. 4). These data are in agreement with those of Carter and Khoury (5).

Alignment of the physical orders of the fragments produced by endo R·HindII + III and endo R·Eco R_I. In order to determine the two terminal endo R·HindII + III fragments, unit-length duplex linear monomers were labeled at the 5′ termini with ^{32}P using polynucleotide kinase. After phenol extraction the recovered DNA was cleaved with endo R·HindII + III. Bands A to E were cut out, dissolved in concentrated perchloric acid, neutralized with NaOH, and counted. Only the bands corresponding to fragments B and C contained radioactivity (Table 2), thus identifying these as the terminal fragments.

With the knowledge that endo R·HindII + III fragments B and C were terminal, the five

fragments A to E were assigned to the products of partial digestion with endo R·HindII + III, fragments 1 to 4, on the basis of molecular weight, i.e., combinations of complete digestion fragments A to E whose additive molecular weights most closely agreed with the molecular weights determined for partial digestion fragments 1 to 4 (Table 3). From this a tentative physical order of BEDAC was determined.

Endo R·HindIII cleaved unit length duplex linear monomers at only one site, producing endo R·HindII + III partial digestion fragments 1 and 4 (Fig. 2). These two fragments were separated on a neutral sucrose gradient and digested with endo R·HindII + III. The composition of fragment 1 was endo R·HindII + III fragments ACD, and the composition of fragment 4 was endo R·HindII + III fragments EB (Table 3).

Similarly, endo R·Eco R_I fragments A and B were separated and digested with endo R·HindII + III. Endo R·Eco R_I A contained endo R·HindII + III fragments A and C and a fragment slightly smaller than D. Endo R·Eco R_I B contained endo R·HindII + III fragment B and a fragment slightly smaller than E (Table 3).

Cleavage of AAV DNA with an endo R·Hind preparation enriched for endo R·HindII produced a new band between bands B and C (Fig. 5). The relative position of the new band corresponded to a molecular weight of 6×10^5, about what would be expected (5.8×10^5) for a fragment containing endo R·HindII + III fragments D and E.

From these experiments we concluded that the physical order along the AAV DNA molecule of the endo R·HindII + III fragments is BEDAC (Fig. 6). From a knowledge of the physical order and molecular weights of the products of digestion with endo R·Eco R_I, it was possible to align these fragments on the endo R·HindII + III cleavage map (Fig. 6).

Evidence for two nucleotide sequence per-

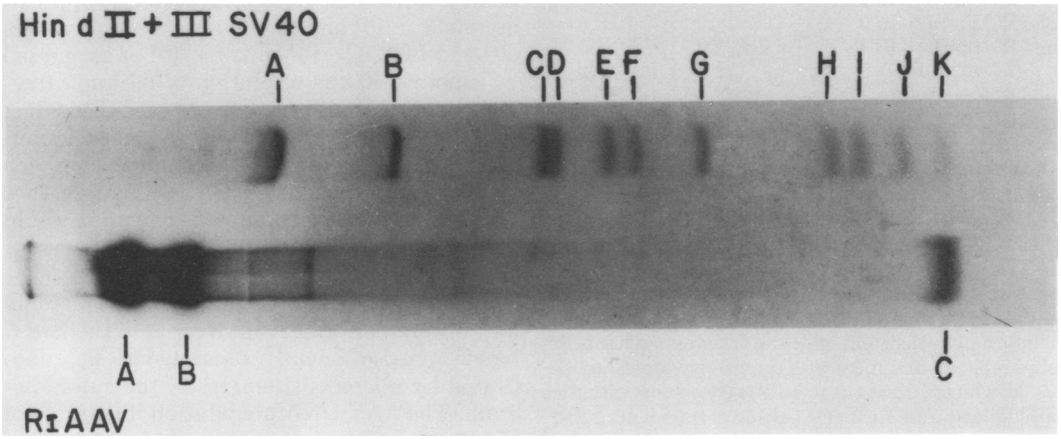

FIG. 3. *A complete endo R·Eco R_I digest of unit-length linear duplex AAV DNA. The three fragments produced were separated in a 4% polyacrylamide gel. Fragments have been labeled A to C in order of increasing electrophoretic mobility. As a molecular weight standard, a parallel gel containing an endo R·HindII + III digest of SV40 DNA was present.*

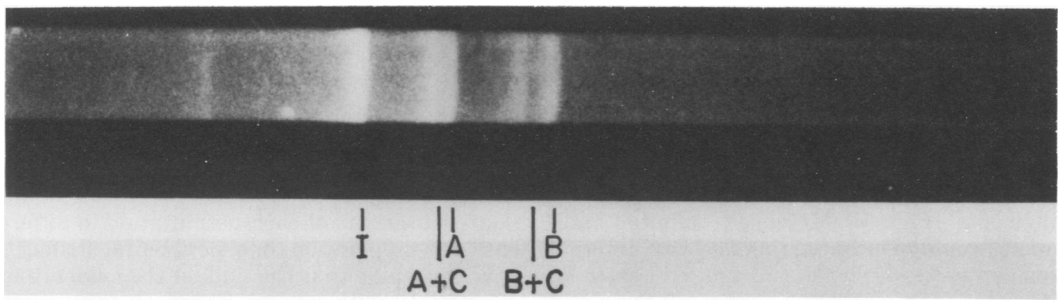

FIG. 4. *A partial endo R·Eco R_I digestion of unit-length linear duplex AAV DNA. Intact DNA, partial, and complete digestion products were separated on a 1.4% agarose gel. Fragment C is not visible.*

TABLE 2. *Labeling of endo R HindII + III terminal fragments with ^{32}P by using polynucleotide kinase*[a]

Fragment	Counts/min
A	19
B	232
C	240
D	9
E	2

[a] Fragments were visualized in a 1.4% agarose gel using ethidium bromide, and were cut out, dissolved, and counted.

TABLE 3. *Composition of endo R·HindII + III partial digestion fragments 1 to 4 and endo R·Eco R_I fragments A and B*

Fragment	Composition	
	Digestion with endo R·HindII + III	Molecular weight[a]
endo R·HindII + III 1	ACD	ACD
endo R·HindII + III 2		AC
endo R·HindII + III 3		AD
endo R·HindII + III 4	BE	BE[c]
endo R·Eco R_I A	ACD'[b]	
endo R·Eco R_I B	BE'[b]	

[a] Determination of composition by molecular weight is described in the text.

[b] D' and E' had slightly greater mobilities than D and E.

[c] The molecular weight of partial digestion fragment 4 is actually closer to the additive molecular weights of fragments B + D than B + E. However, to be internally consistent with the other assignments in column 3, this fragment was tentatively identified as B + E, which turned out to be also consistent with the data derived from further digestion of fragments 1 and 4 (column 2).

mutations in AAV DNA. Gerry et al. (9) concluded that AAV DNA contained a small number of nucleotide sequence permutations, possibly only two, all of whose start points occurred within a limited region representing less than 6% of the genome. This conclusion has been supported by the work of Koczot et al. (11) and Carter et al. (5). Digestion of a population of DNA molecules containing permuted nucleotide sequences with a bacterial restriction enzyme should generate more than two terminal fragments (Fig. 7). Moreover, terminal fragments would not be equimolar with internal fragments. However, if the limited region of permutation in AAV DNA were small compared to the average size of the terminal fragments generated by the restriction enzyme, resolution might prove to be difficult. With 1.4% agarose gels, as illustrated above, only five bands were visible after digestion of unit-length duplex AAV DNA with endo R·HindII + III. Furthermore, all five bands were present in equimolar amounts including the two terminal fragments (B and C).

In order to improve resolution, the cleavage products of an endo R·HindII + III digestion of AAV DNA were separated by electrophoresis through a 4% polyacrylamide slab gel (Fig. 8). Under these conditions the bands representing fragments B and C were clearly resolved into two species. Additionally, on the original autoradiogram partial digestion fragments 1, 2, and 4, which contain C or B, could also be resolved into doublets, whereas fragment 3 was still single. (In Fig. 8 only the 2 and 4 doublets are still visible.) Assuming that the resolution of B and C into doublets represents species differing in molecular weight rather than base composition or conformation, the two species of fragment B differed in molecular weight by approximately 3×10^4 and the two fragment C species differed by 2.8×10^4 to 2.9×10^4 as estimated by mobility. If the resolution of terminal fragments B and C into two species each did reflect the existence of two nucleotide sequence permutations in AAV DNA, it would have been expected that the molecular weight difference between the two species of fragment B would have been equal to the difference between the species of fragment C as was the case.

Five experiments of the type illustrated in Fig. 8 were performed. In every case the two C bands were sufficiently separated to be quantitated by microdensitometry of the autoradiogram. The AAV DNA preparation illustrated in Fig. 8 was used twice. The ratio of larger to smaller C band species (as estimated by relative mobility) was 0.67:1 in the experiment shown in Fig. 8 and 0.88:1 in a second experiment. Three more experiments with three different AAV DNA preparations yielded ratios of 1:1. In each case the presence of two B bands was evident but separation was insufficient to allow a similar analysis. If we assume that the presence of two B bands and two C bands on polyacrylamide electrophoresis does indeed represent the existence of two permutations, the variation in the ratio may either reflect differences in the relative amounts of each permutation in different DNA preparations or experimental artifact.

We conclude that the bulk of the evidence is compatible with the existence of two major nucleotide permutations in AAV DNA, al-

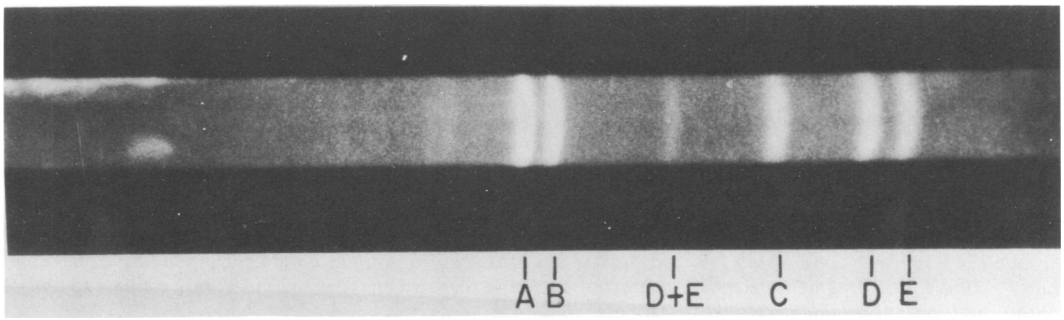

FIG. 5. *AAV linear duplex DNA was digested with an endo R·HindII + III preparation enriched for endo R·HindII. A new fragment corresponding to endo R·HindII + III D + E in molecular weight was observed on electrophoresis through a 1.4% agarose gel.*

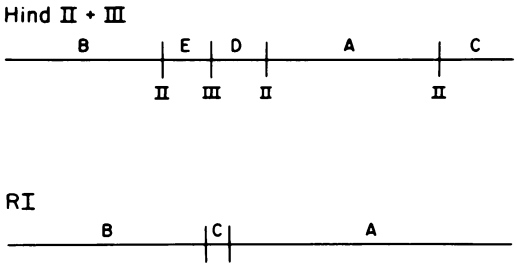

FIG. 6. *A cleavage map of the AAV genome. The relative position of endo R·Eco R_I C was determined from the molecular weight determinations on 1.4% agarose gels for fragments A and B (see Fig. 3).*

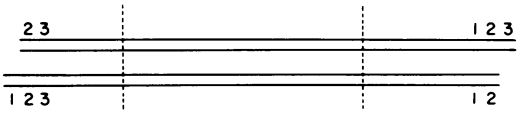

FIG. 7. *A model to demonstrate that a collection of linear duplex DNA molecules containing two nucleotide sequence permutations would yield two species of terminal fragments with respect to molecular weight from each end upon digestion with a restriction endonuclease.*

though additional structural complexity cannot be absolutely ruled out.

DISCUSSION

The major contribution of this paper with regard to the fine structure of AAV DNA is evidence compatible with the existence of nucleotide sequence permutations as demonstrated by the high-resolution polyacrylamide gels of endo R·HindII + III digests. Similar data might result from two species of linear duplex AAV DNA, one of which had a 1% deletion at each end. This would imply a 2% difference in molecular weight between the two species. We have been unable to resolve unit-length duplex linear AAV DNA into more than one species under conditions with 0.6% agarose gels where two lambda bacteriophage DNA endo R·Eco R_I fragments of approximately AAV size which differ by as little as 2% in molecular weight may be resolved (N. Muzyczka, personal communication). A deletion of 1% at only one or the other end of the DNA molecule would be the equivalent of the suggested permutation model. No evidence for subterminal or more internal deletions of 1% or more has been obtained from high-resolution electron microscopy (Berns and Kelly, unpublished data). However, a 1% deletion (30 to 40 nucleotides) would be close to the borderline of detectability in our experience. Single-strand termini on some molecules might contribute to heterogeneity in terminal fragments, but there is no evidence for such termini in unit length duplex linear AAV DNA molecules isolated from neutral sucrose gradients (9).

It is not completely clear why we are unable to resolve the two endo R·HindII + III AAV C species in 1.4% agarose gels. However, we have been able to separate the two species of C using 0.6% and 0.4% agarose gels although overall resolution suffers and diffusion of the bands increases (data not shown).

Again assuming that the resolution of the terminal endo R·HindII + III fragments into two species each on polyacrylamide gels reflects primarily difference in molecular weight, these gels have enabled us to measure the separation on the AAV genome of the starting points for the two permutations. The figure of 1% of the AAV genome is especially intriguing because this is the same value determined for the length of the natural terminal nucleotide sequence repetition by Gerry et al. (9) and is close to the value of 1.5% reported for the length of the inverted terminal repetition by Berns and Kelly (3). This is the figure which might be expected if AAV DNA replicated via a concatenate and were

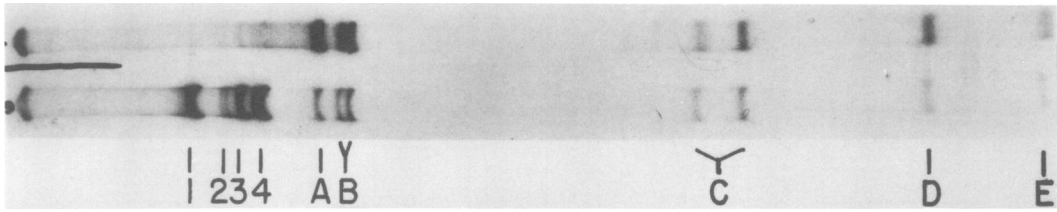

FIG. 8. *An endo R·HindII + III digest of unit length linear duplex AAV DNA molecules was electrophoresed through a 4% polyacrylamide slab gel. A complete digest displayed doublet bands corresponding to terminal endo R·HindII + III fragments B and C. A partial digestion revealed that partial digestion products (2 and 4) containing terminal fragments B and C were also doublets.*

packaged via a "headful" mechanism as has been suggested for some bacteriophage DNAs (18). However, the existence of only two permutations would imply the longest concatenate to be only a dimer or trimer.

We have been interested in the fine structure of AAV DNA because of the implications of such studies with regard to events at the molecular level during viral multiplication. The ability to obtain unique fragments representing specific segments of the mature genome should be useful with regard to transcriptional mapping and the sequencing of interesting parts of the AAV genome. Segments of particular interest would include the termini which have been reported to have properties consistent with both a natural terminal nucleotide sequence repetition (12----12) and an inverted terminal nucleotide sequence repetition (12----2′1′, where 2′ and 1′ represent nucleotide sequences complementary to 2 and 1, respectively) (3, 9, 11), and potential initiation and termination sites of transcription.

ACKNOWLEDGMENTS

We wish to thank J. Lopresti Olsen for technical assistance and H. O. Smith and D. Nathans for helpful suggestions with regard to the manuscript.

This work was supported by grants from the U.S. Public Health Service (1 RO1 AI 10843 from the National Institute of Allergy and Infectious Diseases) and the National Science Foundation (GB 37950). K. I. B. is a Howard Hughes Medical Investigator. J. K. and K. H. F. were supported by the Hughes Medical Institute, and I. S. was supported by Public Health Service grant 5 TO1 GM 00795 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Atchison, R. W., B. C. Casto, and W. McD. Hammon. 1965. Adenovirus-associated defective virus particles. Science **194**:754–756.
2. Berns, K. I., and S. Adler. 1972. Separation of two types of adeno-associated virus particles containing complementary polynucleotide chains. J. Virol. **9**:394–396.
3. Berns, K. I., and T. J. Kelly, Jr. 1974. Visualization of the inverted terminal repetition in adeno-associated virus DNA. J. Mol. Biol. **82**:267–271.
4. 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.
5. Carter, B. J., and G. Khoury. 1975. Specific cleavage of adenovirus-associated virus DNA by restriction endonuclease R·Eco R_I-characterization of cleavage products. Virology **63**:523–539.
6. Crawford, L. V., E. A. C. Follet, M. G. Burdon, and D. J. McGeoch. 1969. The DNA of a minute virus of mice. J. Gen. Virol. **4**:37–46.
7. Danna, K. J., G. H. Sack, Jr., and D. Nathans. 1973. Studies of simian virus 40 DNA. VII. A cleavage map of the SV40 genome. J. Mol. Biol. **78**:363–376.
8. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.
9. Gerry, H. W., T. J. Kelly, Jr., and K. I. Berns. 1973. Arrangement of nucleotide sequences in adeno-associated virus DNA. J. Mol. Biol. **79**:207–225.
10. Hoggan, M. D., N. R. Blacklow, and W. P. Rowe. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological and immunological characteristics. Proc. Natl. Acad. Sci. U.S.A. **55**:1467–1471.
11. Koczot, F. J., B. J. Carter, C. F. Garon, and J. A. Rose. 1973. Self-complementarity of terminal sequences within plus or minus strands of adenovirus-associated virus DNA. Proc. Natl. Acad. Sci. U.S.A. **70**:215–219.
12. 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.
13. Morrow, J., and P. Berg. 1972. Cleavage of simian virus 40 DNA at a unique site by a bacterial restriction enzyme. Proc. Natl. Acad. Sci. U.S.A. **69**:3365–3369.
14. Old, R., K. Murray, and G. Roizes. 1975. Recognition sequence of restriction endonuclease III from *Hemophilus influenzae*. J. Mol. Biol. **92**:331–340.
15. Pettersson, U., C. Mulder, H. Delius, and P. Sharp. 1973. Cleavage of adenovirus type 2 DNA into six unique fragments by endonuclease R·R_I. Proc. Natl. Acad. Sci. U.S.A. **70**:200–204.
16. Rose, J. A., K. I. Berns, M. D. Hoggan, and F.J. Koczot. 1969. Evidence for a single-stranded adenovirus-associated virus genome: formation of a DNA density hybrid on release of viral DNA. Proc. Natl. Acad. Sci. U.S.A. **64**:863–869.
17. Smith, H. O., and K. W. Wilcox. 1970. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. J. Mol. Biol. **51**:379–392.

18. Thomas, C. A., Jr., and L. A. MacHattie. 1967. The anatomy of viral DNA molecules, p. 485-518. *In* P. D. Boyer (ed.), Annual review of biochemistry, vol. 36. Annual Reviews, Inc., Palo Alto, Calif.
19. Weiss, B., T. R. Live, and C. C. Richardson. 1968. Enzymatic breakage and joining of deoxyribonucleic acid. V. End group labeling and analysis of deoxyribonucleic acid containing single strand breaks. J. Biol. Chem. **243**:4530-4542.