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Genome Localization of Adeno-Associated Virus RNA

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In previous work, linear duplex molecules of adeno-associated virus, type 2 (AAV2), DNA were cleaved with the restriction endonucleases R·*Eco*RI, R·*Hind*II, and R·*Hind*III. The physical order of the specific fragments obtained was deduced and oriented with respect to the DNA strand polarity and the direction of transcription. Stable AAV RNA is transcribed only from 70% of the minus DNA strand. We report here RNA-DNA hybridization experiments using these restriction fragments to obtain a more accurate map of the portion of the AAV genome represented in stable RNA. The data obtained with several sets of restriction fragments annealed to either whole-cell RNA or poly(A)-containing RNA were internally consistent. The AAV RNA annealed with a continuous region of the AAV DNA, beginning at 0.18 map units (18%) from the left end of the molecule and ending at 0.88 map units. In addition, the restriction endonuclease *Bam*HI was found to make one specific cleavage in AAV2 DNA at 0.22 map units, which is 0.04 map units (i.e., 160 nucleotides) to the right ("down stream") of the point corresponding to the 5′ end of the viral mRNA.

The DNA of adeno-associated virus (AAV) is a well-defined viral genome of relatively low genetic complexity which can be fairly readily obtained in milligram quantities. This DNA is a potentially useful probe for studying some aspects of gene function in mammalian cells. Pertinent to this objective is a detailed analysis of the structure and function of AAV DNA.

Single strands of AAV DNA have a molecular weight of 1.4×10^6, and strands of opposite polarity (i.e., plus and minus) are separately encapsidated in individual virions (5, 8, 17, 21, 26, 30). After purification, these strands can anneal to yield a variety of duplex monomer and oligomer molecules having either a linear or circular configuration (21, 24). The duplex circular or oligomer molecules apparently arise as a consequence of a limited permutation in the nucleotide sequence of AAV DNA. The ends of AAV DNA have properties attributable to both a natural nucleotide sequence repetition and an inverted nucleotide sequence repetition, suggesting the possibility of a terminal palindrome representing 1 to 2% of the genome (6, 11, 21, 24).

The complementary plus and minus strands of AAV DNA can be preparatively separated (8), and this facilitated previous studies that showed that only one of the DNA strands (called the "minus" strand) is stably transcribed in infected cells (13, 15, 31). A region comprising 70% of the minus-strand length is transcribed in the nucleus to yield a 20*S* mRNA species, which is polyadenylated and transported to polysomes without any further cleavage (13, 16; B. J. Carter, Virology, in press). Indirect evidence suggested that the stably transcribed region of AAV DNA did not include the terminal repetitions (13).

Recently, duplex AAV type 2 (AAV2) DNA has been cleaved with the restriction endonucleases *Eco*RI, *Hind*II, and *Hind*III, and the specific DNA fragments produced have been ordered on the linear genome (7, 11, 12). In addition, these restriction fragment maps can be oriented in relation to the DNA strand polarity and the direction of transcription on the minus strand (12). We now describe experiments using these restriction fragments to determine a precise transcription map of the AAV2 genome. The data obtained with several sets of restriction fragments and either whole-cell RNA or poly(A[+]) RNA from infected cells were internally consistent. The stable AAV RNA is comprised of a single continuous transcript of 70% of the DNA minus strand, beginning at 0.17 to 0.19 map units from the left end of the conventional AAV2 DNA map and terminating at 0.88 to 0.90 units. This supports the suggestion that the terminal nucleotide sequence repetitions in AAV DNA are not represented in stable AAV RNA. We also report that

the restriction endonuclease *Bam*HI cleaves AAV DNA at a single specific site located at 0.22 map units from the left-hand terminus.

MATERIALS AND METHODS

Cells and viruses. AAV2 was grown in KB3 cells in suspension culture using adenovirus type 2 (Ad2) as a helper, as described previously (14, 21).

Purification of viral DNA. The production and purification of ^{32}P-labeled viral DNA has been fully described before (7, 11). Briefly, AAV virions were purified from infected cells by digestion with trypsin and deoxycholate and three cycles of centrifugation in CsCl density gradients (8). DNA was extracted from AAV2 virions by treatment with 0.1 N NaOH and sedimentation through an alkaline sucrose density gradient (24). The DNA was reannealed in 50% formamide (7) and linear, monomer duplex AAV DNA was purified by sedimentation in a neutral sucrose gradient.

Preparation of RNA from infected cells. At 20 h after infection of KB cells with AAV2 and Ad2, RNA was isolated from the whole cells using a hot-phenol-detergent procedure as described before (16). The poly(A)-containing fraction of the RNA was obtained by chromatography on oligo(dT)-cellulose (2; Carter, in press). All RNA preparations were treated with pancreatic DNase (15).

Enzymes. Endonuclease R·*Eco*RI was obtained from Miles Laboratories. Endonuclease R·*Hind*II+III prepared as described (18) was provided by W. Scott, N. Muzyczka, and D. Nathans. Endonuclease R·*Bam*HI was purified from *Bacillus amyloliquefaciens* by the method of Wilson and Young (37) and was a gift from D. Shortle. Bacterial alkaline phosphatase was purchased from Worthington Biochemicals Corp. T4 polynucleotide kinase was purified according to Richardson (28). Nuclease S_1, purified according to Ando (1), was obtained from Miles Laboratories.

Cleavage of DNA with restriction enzymes. AAV2 DNA was cleaved with R·*Eco*RI or R·*Hind*II+III as described before (7, 11). For R·*Bam*HI, digestion mixtures containing AAV DNA (10 to 50 μg/ml), 6 mM Tris-hydrochloride (pH 7 to 4), 6 mM $MgCl_2$, 6 mM mercaptoethanol, and one-fifth volume of enzyme were incubated at 37°C for 30 to 60 min. For all restriction enzymes and the DNA fragments produced, the nomenclature system of Smith and Nathans has been used (34).

Labeling of 5′ terminus of DNA. The DNA was first treated with bacterial alkaline phosphatase and then incubated with T4 polynucleotide kinase and [γ-^{32}P]ATP. The [γ-^{32}P]ATP was either purchased from New England Nuclear Corp or synthesized according to Glynn and Chappell (22). Reactions with T4 polynucleotide kinase were performed as described (36), except that 0.1 mM spermidine was included (25).

Electrophoresis of DNA. DNA fragments were subjected to electrophoresis in either 1.4% agarose gels (23) or 6% acrylamide gels (18). DNA bands in the gels were detected either by staining with ethidium bromide and viewing under UV illumination (32) or by autoradiography. Fragments were eluted from agarose gels by dissolving the appropriate portion of the gel in saturated KI solution (9). The DNA was then purified from the KI and agarose by chromatography on hydroxyapatite. DNA fragments were recovered from 6% acrylamide gels by crushing the gel slice in an elution solution (0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1 mM EDTA, 0.1% sodium dodecyl sulfate) for 20 to 72 h at 37°C, filtering the solution through siliconized glass wool, and then precipitating the DNA with 2.5 volumes of ethanol (Allan Maxam, personal communication).

Sucrose gradient sedimentation. DNA was analyzed in 12.0-ml sucrose gradients containing 5 to 20% sucrose, 1 M NaCl, 50 mM Tris-hydrochloride (pH 8.0), and 0.5 mM EDTA. Gradients were centrifuged using the Beckman SW41 rotor at 30,000 to 35,000 rpm for 16 h at 20°C. Fractions were collected through a hole pierced in the bottom of the tube.

Nucleic acid hybridization. Fragments of ^{32}P-labeled AAV DNA were denatured by boiling for 10 min in 0.01 M Tris-hydrochloride (pH 8.0) and were then quenched in ice. Samples of the denatured DNA were then mixed with appropriate amounts of RNA from infected cells, adjusted to 0.21 M NaCl and 0.01 M Tris-hydrochloride (pH 8.0), and boiled for 3 to 4 min in a sealed vessel. The mixtures were then incubated at 67°C for appropriate times, as indicated for individual experiments. After this incubation, the mixtures were chilled in ice and the proportion of ^{32}P-labeled DNA present in duplex molecules was determined using nuclease S_1 (1, 13, 35). One-half of the reaction was precipitated directly with an equal volume of 10% trichloroacetic acid in the presence of carrier yeast RNA at 0 to 4°C. The other half of the reaction was first digested with nuclease S_1 before acid precipitation. Reactions (2.0 ml) for nuclease digestion contained up to 0.4 ml of the RNA-DNA incubation mixture, fragmented, heat-denatured salmon sperm DNA (12.5 μg/ml), 0.03 M sodium acetate (pH 4.5), 0.15 M NaCl, 10^{-5} M $ZnSO_4$, and sufficient S_1 nuclease to completely digest all the single-stranded DNA present. Incubation was for 75 min at 48°C. Acid-precipitable material was collected on nitrocellulose filters, dried at 80 to 90°C, and counted in toluene-Liquifluor scintillation cocktail. Under the conditions used, less than 2% of duplex DNA and more than 98% of single-stranded DNA became acid soluble. For all the data reported, the proportion of ^{32}P-labeled DNA in duplex molecules after annealing has been corrected for "zero time," i.e., the proportion of DNA resistant to digestion by S_1 nuclease after heat denaturation and before any additional annealing. In most cases, the zero-time value was less than 1%, except where specifically noted.

RESULTS

Transcription mapping with AAV DNA fragments. Duplex fragments of ^{32}P-labeled AAV2 DNA were denatured and then annealed in the presence of unlabeled RNA extracted from KB-3 cells 20 h after infection with AAV2 and Ad2 as helper. Annealing was done under

conditions in which there was little or no annealing of the DNA in the absence of RNA. Under these conditions, the fraction of DNA that was protected by excess RNA from subsequent degradation by the single-strand-specific nuclease S_1 is a measure of the fraction of the DNA sequences represented in the RNA population. Since the plus strand of AAV DNA does not anneal with AAV RNA transcripts from infected cells, the theoretical maximum extent of protection of any DNA fragment by RNA is 50% (i.e., 100% of the minus-strand component). The fragments used were those derived by cleavage of linear, duplex AAV DNA with endonucleases R·*Eco*RI, R·*Hind*II+III, or R·*Bam*HI. The size of each of these fragments is listed in Table 1 and their physical order in AAV2 DNA is shown in Fig. 1.

Annealing of AAV RNA with *Eco*RI fragments. The annealing of denatured, ^{32}P-labeled AAV2 DNA R·*Eco*RI fragments with varying amounts of AAV RNA is shown in Fig. 2. Fragments A and B show plateaus at values of approximately 38 and 26%, respectively. Annealing with fragment C did not reach a plateau but it is apparent that a level of 50% is approached. These data indicate that C, which is the internal fragment, is completely transcribed, whereas only portions of B or A (the terminal fragments) are transcribed.

It was previously shown that the minus and plus strands of the AAV RI-A and RI-B fragments can be separated and that only the minus-strand components annealed with AAV RNA (12). The estimates of the extent of transcription that were derived were only approxi-

TABLE 1. *Annealing of AAV DNA fragment with AAV RNA*[a]

AAV DNA restriction fragment[b]		Mol wt (× 10^{-6})[c]	% AAV2 genome	% Reaction with RNA[d]	Fraction of fragment transcribed[e]	Location on DNA minus strand of AAV RNA[f] (map units)	
						5′ end	3′ end
*Eco*RI	B	1.1	38.9	26,25	0.52	0.187	
	C	0.13	4.6	>47	1.00		
	A	1.6	56.5	37,40	0.77		0.871
Hind	B	0.86	30.6	20,21	0.41	0.178	
	E	0.27	9.6	47,45	1.00		
	D	0.31	11.0	46,>41	1.00		
	A	0.96	34.2	45,>41	1.00		
	C	0.41	14.6	15,19	0.34		0.899
*Bam*HI	B1	0.62	{22.0}	11.0	0.22	0.174	
	B2	0.61		11.6	0.24	0.170	
	A	2.2	78.0	42.0	0.84		0.876
*Eco*RI	B(−)			57.0	0.57	0.170	
	A(−)			79.3	0.79		0.883

[a] Denatured ^{32}P-labeled AAV DNA restriction fragments were annealed with AAV RNA as described in Fig. 2, 3, 4, and 8.

[b] *Hind* indicates *Hind*II+III. B(−) and A(−) indicate the minus-strand component of *Eco*RI-B and *Eco*RI-A, respectively.

[c] Molecular weights of *Eco*RI and *Hind* fragments were determined previously (7, 11). The molecular weights of the two left-hand *Bam* B components (*Bam*B1 and *Bam*B2) were obtained from data reported here and by electron microscopy to be reported elsewhere. The braces around these components in column 3 indicate that each is equivalent in length to approximately 22% of the AAV genome

[d] Values listed are the averages of points at hybridization plateaus as determined in experiments such as those in Fig. 2, 3, 4, and 8. Pairs of numbers indicate estimations from different experiments using separate DNA and RNA preparations. The symbol > indicates that a plateau was not completely attained but that the curve apparently approached a plateau level close to 50%.

[e] For duplex fragments these values were obtained by multiplying the values in column 4 by 2 since only the minus-strand component was transcribed. Pairs of estimates were averaged. For *Eco*RI-C and *Hind* E, D, and A, the data are taken to represent complete transcription, which is consistent with the internal map position of these fragments (Fig. 1).

[f] The values were calculated from those in column 5 and the known map position of the DNA fragment (see Fig. 1). The data are presented in terms of map units (Fig. 1) and were calculated assuming a single continuous transcript and the direction of transcription determined previously (12). Average map units: 5′ end, 0.176; 3′ end, 0.882.

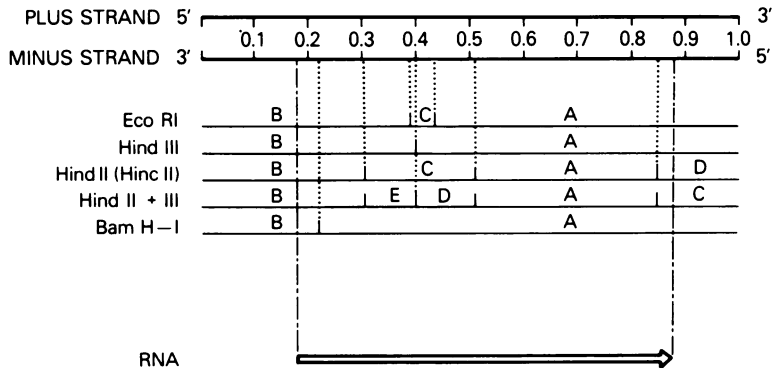

FIG. 1. *Schematic representation of RNA transcription map and restriction endonuclease fragment maps of the AAV2 genome. The top two lines represent the plus and minus strands of the AAV2 DNA genome with the strand polarity as indicated. The scale represents 1 map unit equivalent to one genome length (approximately 4,200 base pairs). The thin lines indicate the physical order and relative size of fragments produced by EcoRI, HindII or HindIII (7, 11, 12), HincII (which is an isoschizomer of HindII), or BamHI as described in this report. Enzyme cleavage sites are indicated by short vertical bars. The orientation of the restriction fragments with respect to the DNA strand polarity was determined previously (12). The large arrow indicates the direction and extent of stable AAV RNA transcription on the AAV DNA minus strand.*

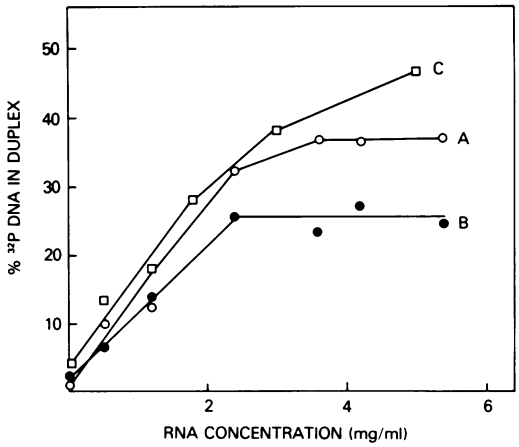

FIG. 2. *Annealing of ^{32}P-labeled AAV DNA EcoRI fragments with AAV RNA. RNA was extracted from infected cells at 20 h after infection. The DNA fragments were purified from neutral sucrose gradients (12). Reactions were carried out as described in Materials and Methods. Annealing was for 2 h at 67°C. The proportion of ^{32}P-labeled DNA in duplex was determined using S_1 nuclease. The specific activity of the DNA was 4 × 10^5 cpm/μg. Reactions contained fragment EcoRI-A at 2.5 ng/ml (○), EcoRI-B at 2.5 ng/ml (●), or EcoRI-C at 1.6 ng/ml (□).*

mate, since saturation curves were not obtained. To obtain more reliable estimates, the minus-strand components of *Eco*RI-A and *Eco*RI-B were purified from ^{32}P-labeled, bromodeoxyuridine-substituted DNA. These DNA preparations were annealed with the poly(A)-containing fraction of RNA isolated 20 h after infection. Other experiments (Carter, in press) indicate that the poly(A[+]) AAV RNA contains the same sequences as those present in either total cellular AAV RNA or in poly(A[−]) AAV RNA. The RI-A(−) and RI-B(−) strands yielded plateau levels of hybridization with AAV RNA of 79 and 56%, respectively (Fig. 3). These values are in good agreement with those obtained using the duplex fragments (Fig. 2).

Annealing of AAV RNA with R·*Hind* fragments. The five fragments of AAV2 DNA produced by cleavage with endonuclease R·*Hind*II+III were purified from 1.4% agarose gels. Individual fragments were denatured and annealed with RNA from infected cells. The concentration of each fragment was adjusted in proportion to its genetic complexity (i.e., molecular weight) so that all DNA fragments were incubated at equimolar concentrations. Under these conditions, if the AAV RNA transcripts contain equimolar amounts of all parts of the transcribed region, then plateau levels of hybridization for any fragment should be reached at the same RNA concentration. The actual plateau level reached may, of course, vary depending upon the proportion of the fragment that is transcribed. This expectation is apparently realized, as shown in Fig. 4. Incubation of the *Hind*II+III fragments A, D, and E with AAV RNA yielded in each case a maximum level of hybridization that approached 50%. The *Hind*II+III fragments B and C, containing, respectively, the left and right molecular ends, each gave hybridization plateaus at about 20%, which indicated that each contained a large part of the nontran-

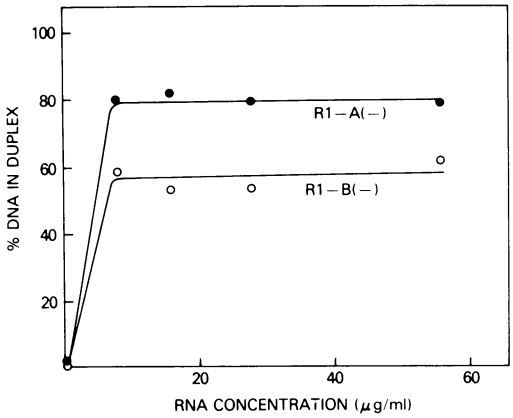

FIG. 3. *Annealing of minus-strand components of AAV DNA EcoRI-A and EcoRI-B fragments with AAV RNA. The EcoRI-A(−) and EcoRI-B(−) strands were obtained from ^{32}P-labeled bromodeoxyuridine-AAV2 DNA (specific activity, 4 × 10^5 cpm/μg) as described previously (12). Reactions contained ^{32}P-labeled DNA (2 ng/ml), 0.5 M NaCl, 0.01 M Tris (pH 8.0) and were incubated for 22 h at 67°C. Under these conditions, there was less than 1% annealing of the DNA alone. Symbols (●), EcoRI-A(−); (○), EcoRI-B(−) DNA.*

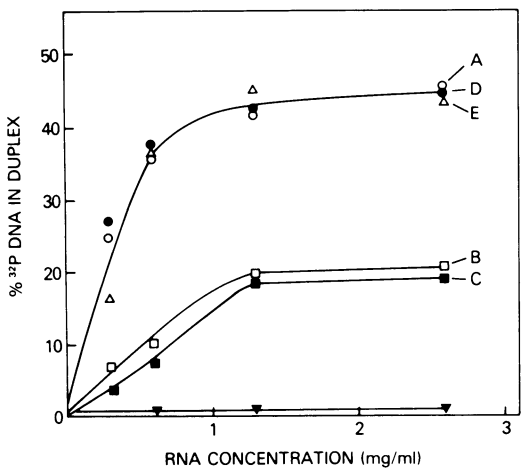

FIG. 4. *Annealing of ^{32}P-labeled AAV DNA HindII+III fragments with AAV RNA. The RNA was isolated from KB cells at 20 h after infection with AAV2 and Ad2. Denatured, ^{32}P-labeled DNA fragments purified from 1.4% agarose gels were incubated for 2 h at 68°C in reaction mixtures containing 0.21 M NaCl, 0.01 M Tris-hydrochloride (pH 8.0) and the indicated amount of RNA. The specific activity of the ^{32}P-labeled DNA fragments was 10^6 cpm/μg. Reactions contained specific DNA fragments as follows: (○) fragment A, 2.5 ng/ml; (□) fragment B, 2.0 ng/ml; (■) fragment C, 1.2 ng/ml; (●) fragment D, 0.7 ng/ml; (△) fragment E, 0.5 ng/ml. A control reaction (▼) contained fragment A, 2.5 ng/ml, and RNA from uninfected KB cells.*

scribed region. As a control, the *Hind*II+III fragment A was annealed with RNA isolated from uninfected KB cells and no annealing to DNA was observed. The results obtained here are in good agreement with those obtained using *Eco*RI fragments (see below).

Cleavage of AAV DNA with endo R·*Bam*HI. To enable more accurate mapping of the location of the AAV RNA on AAV DNA, we searched for other restriction nucleases that would make useful cleavages in AAV DNA. Digestion of AAV DNA with endo R·*Bam*HI produced two fragments (denoted *Bam* A and *Bam* B), as shown by sedimentation in neutral sucrose gradients (Fig. 5) or by electrophoresis in 1.4% agarose gels (Fig. 6). Cleavage of AAV DNA with R·*Hind*II+III followed by cleavage with R·*Bam*HI resulted in the specific loss of the *Hind*II+III B fragment and the production of one fragment that comigrated with the *Bam*HI B fragment and one fragment that was smaller than the *Hind*II+III E fragment (Fig.

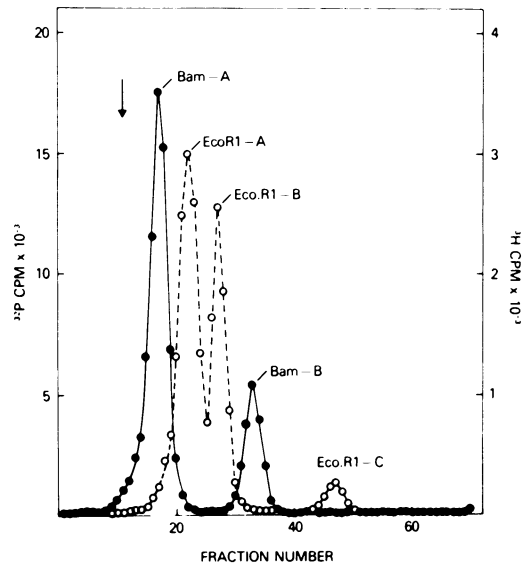

FIG. 5. *Cleavage of AAV2 DNA with R·BamHI. Linear duplexes of ^{32}P-labeled and ^{3}H-labeled AAV2 DNA were separately cleaved with R·BamHI or R·EcoRI, respectively. The two reactions were then mixed together and centrifuged in a neutral sucrose gradient for 16 h at 35,000 rpm at 20°C. Gradients were fractionated from the bottom and counted in toluene-Triton scintillation cocktail. The data was corrected for approximately 2% ^{32}P cross-talk into the ^{3}H counting channel. Sedimentation is from right to left. The arrow indicates the position of linear, duplex AAV2 DNA centrifuged in a parallel gradient. Symbols: (●) ^{32}P-labeled AAV2 DNA cleaved by R·BamHI; (○) ^{3}H-labeled AAV2 DNA cleaved by R·EcoRI.*

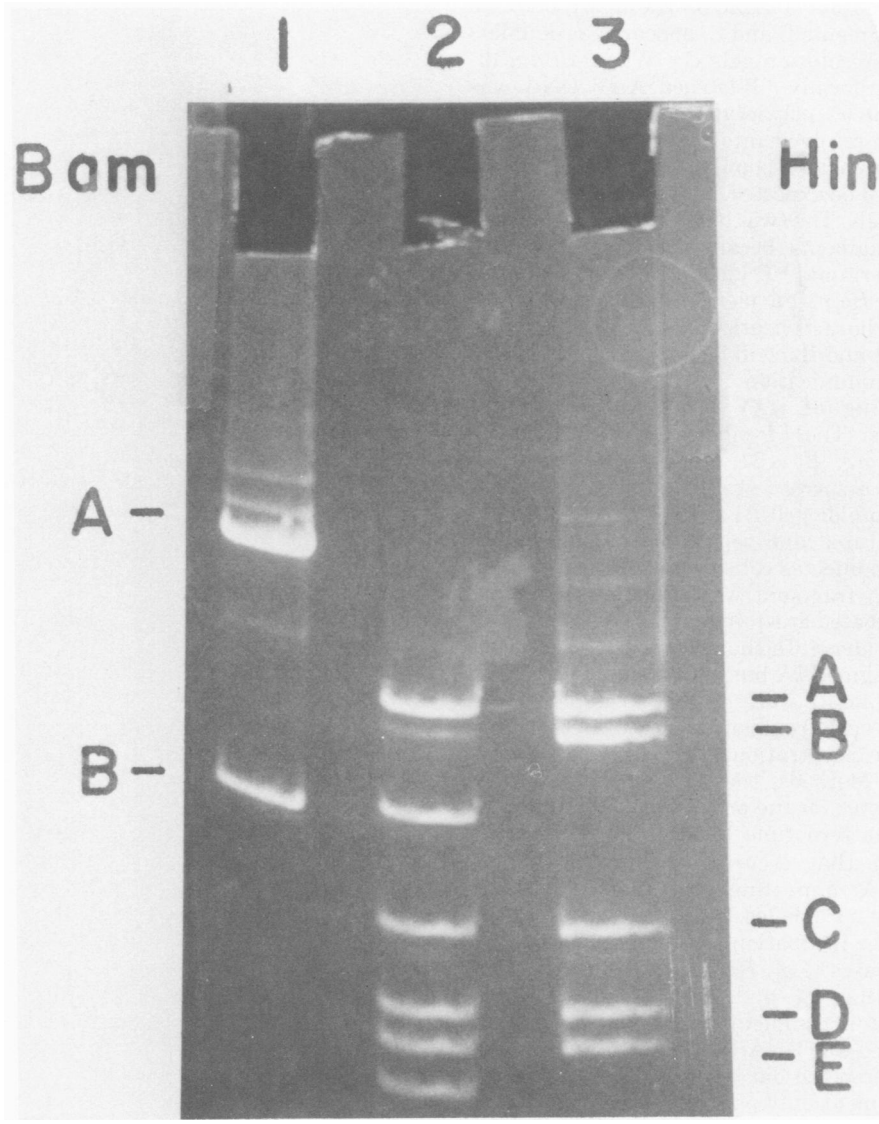

FIG. 6. *Digest of duplex AAV DNA with endo R·BamHI (lane 1), R·BamHI followed by R·HindII+III (lane 2), and R·HindII+III (lane 3) run on a 1.4% agarose gel. The fragments have been labeled in order of increasing electrophoretic mobility.*

6). Molecular weights of the new fragments were estimated by comparing their electrophoretic mobilities to those of the *Hind*II+III fragments. This yielded estimates of 6.1×10^5 and 2.4×10^5 for the large (*Bam* B) and small new fragments, respectively. The sum of these values is in good agreement with the molecular weight of 8.6×10^5 previously determined for *Hind*II+III B (7). The molecular weights of the two *Bam* fragments were also estimated from the distribution of the uniform ^{32}P label in neutral sucrose gradients (Fig. 5). This showed that the *Bam* A fragment contained 78% and the *Bam* B fragment 22% of the total ^{32}P label, which corresponded to molecular weights of 2.2×10^6 and 6.2×10^5, respectively. Similar values are also obtained (Fig. 5) based upon the rate of sedimentation and using the *Eco*RI fragments for comparison. Thus, endo R·*Bam*HI makes a single cleavage in AAV2 DNA at 0.22 map units from the left molecular end (Fig. 1), producing fragments with molecular weights of approximately 6.1×10^5 and 2.2×10^6.

It was shown previously that the terminal

fragments from a *Hind*II+III digest of AAV DNA (fragments B and C) appeared as doublets in higher-resolution gels (7). When a *Bam* digest of uniformly ^{32}P-labeled AAV DNA was analyzed in 6% polyacrylamide gels, the B fragment was resolved into two components (Fig. 7). Although two populations of fragment A should also be expected, they were not resolved in these gels. The two components of B are each *Bam* B fragments, because AAV DNA containing a 5'-terminal ^{32}P label yielded, upon digestion with *Bam*, an identical pattern (Fig. 7). Further characterization of the *Bam* B fragments (B1 and B2) will be presented in a subsequent communication.

Annealing of AAV RNA with R·*Bam*HI fragments. The ^{32}P-labeled AAV DNA fragments *Bam*A, *Bam*B1, and *Bam*B2 were purified from a sucrose gradient (*Bam*A) or a 6% polyacrylamide gel (B1 and B2). Each fragment was denatured and separately annealed with RNA from infected cells (Fig. 8). The amount of each DNA fragment was adjusted so that all were incubated at equimolar concentrations, as discussed above. In the presence of RNA, 40 to 42% of fragment A but only about 13% of fragments B1 or B2 were present in duplex molecules, but each fragment was saturated at similar RNA concentrations. As described in Materials and Methods, the data plotted in Fig. 8 was corrected for the proportion of DNA duplex present at zero time but not for DNA-DNA annealing that occurred during incubation. DNA-DNA annealing is reflected by the amount of ^{32}P-labeled DNA resistant to S_1 nuclease after incubation in the absence of RNA. In most cases (e.g., *Bam*A, Fig. 8), this value was less than 1%, and, in any case, for a fragment that is completely or nearly completely transcribed this DNA-DNA annealing would be largely competed out by AAV RNA. However, for a fragment only partially transcribed (e.g., *Bam*B1 and -B2) DNA-DNA annealing of only a few percent may be significant because most of this could not be competed by AAV RNA. For instance, the annealing of *Bam*B1 and -B2 was 3.4 and 2.5%, respectively, in the absence of RNA and 13 to 14% with RNA at saturation. Since about 80% of the *Bam*B fragments are not transcribed, most of the DNA-DNA annealing would not be competed out by RNA and, therefore, probably should be subtracted. This yields a value of 11.0 and 11.6% for annealing of *Bam*B1 and *Bam*B2, respectively, with AAV RNA (Table 1). The control reaction in Fig. 8 shows no detectable annealing of the *Bam*A fragment in the presence of RNA from cells infected only with Ad2 RNA.

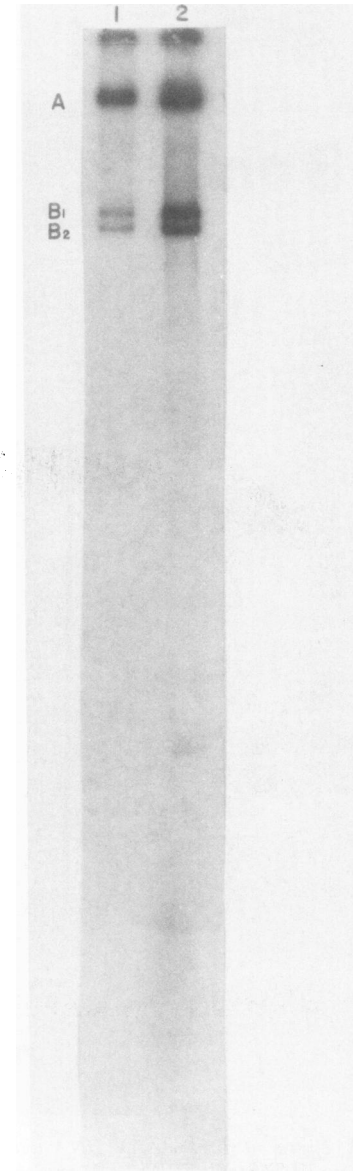

FIG. 7. *Autoradiographs of 6% polyacrylamide slab gels of digested AAV DNA. Endo R·BamHI digests of duplex AAV DNA either uniformly labeled with ^{32}P (lane 1) or labeled only at the 5' termini with ^{32}P (lane 2) showing the resolution of the two B fragments (denoted B1 and B2).*

Location of the transcribed region of AAV DNA. All mature AAV mRNA sequences are contained in a single molecule of molecular weight 0.9×10^6 to 10^6 (10, 13, 16). The data reported in this paper are in accord with the suggestion of a single region of transcription on the minus strand. Using the data derived from

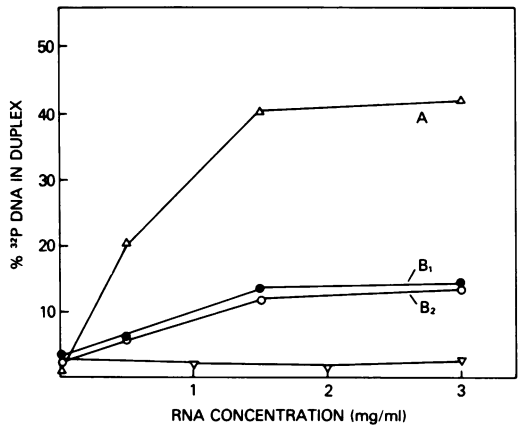

FIG. 8. *Annealing of ^{32}P-labeled AAV DNA BamHI fragments with AAV RNA. Reactions contained denatured ^{32}P-labeled AAV DNA fragment, 0.21 M NaCl, 0.01 M Tris-hydrochloride (pH 8.0), and the indicated amount of RNA isolated 20 h after infection of cells with AAV2 and Ad2 together. Annealing was for 2 h at 68°C. The proportion of ^{32}P-labeled in duplex molecules was estimated using nuclease S_1. The specific activity of the ^{32}P-labeled DNA fragments was 1.3 × 10^6 cpm/μg. Reactions contained: (△) BamA fragment, 2 ng/ml; (○) BamB1 fragment, 0.5 ng/ml; (○) BamB2 fragment, 0.5 ng/ml. A control reaction (▽) contained BamA fragment (2 ng/ml) and RNA isolated at 20 h after infection of cells with Ad2 alone.*

the experiments described in Fig. 2, 3, 4, and 8, the proportion of each AAV DNA restriction fragment that was transcribed was calculated (Table 1). From these data were calculated the map locations of the 5′ and 3′ ends of the AAV RNA, as listed in Table 1 and diagrammatically represented in Fig. 1. Each set of fragments yielded an independent estimate of the location of the RNA termini. The data have been corrected where necessary for DNA-DNA annealing as discussed above. The values obtained for the location of the 5′ end of the RNA are all in good agreement with a range of 0.170 to 0.187 map units and an average value of 0.176 map units from the left molecular end. Similarly, all the estimates for the location of the 3′ end of the AAV RNA are in reasonably good agreement and range from 0.87 to 0.90, with an average of 0.882. The data from each set of fragments are internally consistent and each yields an estimate of 69 to 73% for the proportion of the minus strand, which is stably transcribed. This is in good agreement with estimates of 70 to 75% obtained from annealing of nuclear, cytoplasmic, polysomal, or poly(A[+]) or poly(A[−]) RNA fractions of AAV-infected cells with whole AAV DNA minus strands (Carter, in press).

DISCUSSION

The minimum and maximum extents of the transcribed region of AAV DNA reported here are equivalent to 68 and 73%, respectively, of the minus-strand length. Previous studies showed that AAV RNA consisted of a major polyadenylated 20S species present in both nucleus and polysomes and the only apparent message species (10, 16; Carter, in press). A second population of non-polyadenylated AAV RNA, ranging in size from 18S to 4S, is present in the nucleus and cytoplasm supernatant (16). This second population may be degradation products of the 20S RNA or incomplete transcripts. However, both populations apparently contain the same nucleotide sequences, which anneal to 70 to 75% of the DNA minus strand (13; Carter, in press). The 20S AAV RNA (having a molecular weight of approximately 10^6) is equivalent to 70% of the length of the AAV DNA strand, which indicates that a single continuous transcript is synthesized from this region. The present mapping data are therefore in excellent accord with this. Also, the observation that all regions of the AAV transcript are present at equal concentration is consistent with a single transcript.

Assuming that a single continuous region of AAV DNA is transcribed and that the direction of transcription on the DNA minus strand was as determined previously (Fig. 1), the data from each set of restriction fragments showed that a single region of about 70% of the DNA minus strand was transcribed. Transcription began between 0.170 and 0.187 map units and proceeded rightwards, terminating between 0.871 and 0.899 units. Thus, both the 5′ and 3′ ends of the RNA transcript could be located within specific regions of the AAV genome of about 100 nucleotides in length. The estimates obtained by S_1 nuclease might be slightly low due to "end nibbling" by the enzyme, but this is reported to be not more than 30 to 40 bases (33), which is not more than 0.01 map units for AAV DNA. In addition, other experiments using both S_1 or hydroxyapatite chromatography (which might give slightly high estimates) for analysis of AAV DNA-RNA hybrids gave very similar results with either method.

Previous experiments provided no evidence for any larger precursor of the 20S AAV RNA even though precursors to Ad mRNA were readily detected in the same experiments (13, 16). However, the experimental procedures used, including those in the present report, may have detected only relatively stable AAV

RNA. We cannot eliminate the possibility that larger transcripts of AAV RNA might be synthesized but degraded very rapidly or that a precursor only slightly larger might exist. Very recently it has been reported that the initial steps in post-transcriptional cleavage of Ad RNA occur extremely rapidly (3). At a minimum, the data presented here provide an accurate map of the stable, and therefore presumably informational, AAV RNA. Since there is apparently only one AAV RNA transcript, it is reasonable to suppose that there may be only one promoter in the AAV genome. The location of this promoter is not immediately obvious except that it must be located at, or upstream (i.e., leftwards) from, 0.18 map units from the left-hand end of the AAV DNA.

It was previously suggested (13), and is supported by this work, that the terminal regions of AAV DNA containing the regular and inverted repetitious nucleotide sequences were not stably transcribed. It should be noted, though, that this has not been tested rigorously by annealing AAV RNA to purified terminal fragments. Attempts to do this are in progress. That the terminal sequences may not be transcribed would be consistent with the probable role of these sequences in DNA replication rather than genetic coding (4, 24).

Cleavage of AAV DNA with *Bam*HI resulted in a single cleavage at 0.22 map units, which is about 160 bases to the right ("downstream") from the location of the 5′ end of AAV RNA. The smaller fragment, *Bam*B, was resolved on the basis of electrophoretic mobility in acrylamide gels into two components, B_1 and B_2. This phenomenon was previously observed with the terminal *Hind* fragments of AAV2 DNA and was ascribed to differences in size resulting from a limited permutation in the DNA nucleotide sequence (7, 21). We report elsewhere (20) that *Hae*III cleavage produces two main terminal fragments (denoted as α and γ, respectively) and variable amounts of a minor component (denoted as β) from either end of the AAV DNA duplex and also from the duplex panhandle region of purified single strands. Further characterization of the *Bam*B1 and -B2 fragments will be reported in a subsequent communication.

Assuming that the regions of the AAV genome from 0 to 0.18 and from 0.88 to 1.0 map units are not stably transcribed, the nucleotide sequences of these relatively short regions become of interest because they presumably contain a number of potential recognition sites, including those of the origin of DNA synthesis and initiation and termination of transcription. Experiments to determine the nucleotide sequences of these regions of the genome are in progress.

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LITERATURE CITED

1. **Ando, T.** 1966. A nuclease specific for heat-denatured DNA isolated from a product of *Aspergillus oryzae*. Biochim. Biophys. Acta 114:158–168.
2. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligo thymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. 69:1408–1412.
3. **Bachenheimer, S., and J. Darnell.** 1975. Adenovirus-2 mRNA is transcribed as part of a high-molecular-weight precursor RNA. Proc. Natl. Acad. Sci. U.S.A. 72:4445–4449.
4. **Berns, K. I.** 1974. Molecular biology of the adeno-associated viruses, p. 1–20. *In* W. Arber et al. (ed.), Current topics in microbiology and immunology, vol. 65. Springer-Verlag, New York.
5. **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.
6. **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.
7. **Berns, K. I., J. Kort, K. H. Fife, W. Grogan, and I. Spear.** 1975. Study of the fine structure of adeno-associated virus DNA with bacterial restriction endonucleases. J. Virol. 16:712–719.
8. **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.
9. **Blin, N., A. V. Gavain, and H. Bujard.** 1975. Isolation of large molecular weight DNA from agarose gels for further digestion by restriction enzymes. FEBS Lett. 53:84–86.
10. **Carter, B. J.** 1974. Analysis of parvovirus mRNA by sedimentation and electrophoresis in aqueous and nonaqueous solution. J. Virol. 14:834–839.
11. **Carter, B. J., and G. Khoury.** 1975. Specific cleavage of adenovirus-associated virus DNA by a restriction endonuclease R·*Eco*R1 – characterization of cleavage products. Virology 63:523–538.
12. **Carter, B. J., G. Khoury, and D. T. Denhardt.** 1975. Physical map and strand polarity of specific fragments of adenovirus-associated virus DNA produced by endonuclease R·*Eco*RI. J. Virol. 16:559–568.
13. **Carter, B. J., G. Khoury, and J. A. Rose.** 1972. Adenovirus-associated virus multiplication. IX. Extent of transcription of the viral genome in vivo. J. Virol. 10:1118–1125.
14. **Carter, B. J., F. J. Koczot, J. Garrison, J. A. Rose, and R. Dolin.** 1973. Separate function provided by adenovirus for adeno virus-associated virus multiplication. Nature (London) New Biol. 244:71–73.
15. **Carter, B. J., and J. A. Rose.** 1972. Adenovirus-associ-

ated virus multiplication. VIII. Analysis of in vivo transcription induced by complete or partial helper viruses. J. Virol. 10:9-16.
16. **Carter, B. J., and J. A. Rose.** 1974. Transcription in vivo of a defective parvovirus: sedimentation and electrophoretic analysis of RNA synthesized by adeno-associated virus and its helper adenovirus. Virology 61:182-199.
17. **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.
18. **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.
19. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids, p. 413-428. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
20. **Denhardt, D. T., S. Eisenberg, K. Bartok, and B. J. Carter.** 1976. Multiple structures of adeno-associated virus DNA: analysis of terminally labeled molecules with endonuclease R·*Hae*III. J. Virol. 18:672-684.
21. **Gerry, H. W., T. J. Kelly, and K. I. Berns.** 1973. The arrangement of nucleotide sequences in adeno-associated virus DNA. J. Mol. Biol. 79:207-226.
22. **Glynn, I. M., and J. B. Chappell.** 1964. A simple method for the preparation of ^{32}P-labeled adenosine triphosphate of high specific activity. Biochem. J. 90:147-149.
23. **Hayward, G. S., and M. G. Smith.** 1972. The chromosome of bacteriophage T5. I. Analysis of the single-stranded DNA fragments by agarose gel electrophoresis. J. Mol. Biol. 63:383-395.
24. **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 adeno-associated virus DNA. Proc. Natl. Acad. Sci. U.S.A. 70:215-219.
25. **Lillehaug, J. R., and K. Kleppe.** 1975. Kinetics and specificity of T4 polynucleotide kinase. Biochemistry 14:1221-1225.
26. **Mayor, H. 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.
27. **Middleton, J. H., M. H. Edgell, and C. A. Hutchison III.** 1972. Specific fragments of ϕX174 DNA produced by a restriction enzyme from *Hemophilus aegyptius*, endonuclease Z. J. Virol. 10:42-50.
28. **Richardson, C. C.** 1965. Phosphorylation of nucleic acid by an enzyme from T4 bacteriophage-infected *E. coli*. Proc. Natl. Acad. Sci. U.S.A. 54:158-165.
29. **Roberts, R. J., J. B. Breitmeyer, N. F. Tabachnik, and P. A. Myers.** 1975. A second specific endonuclease from *Hemophilus aegyptius*. J. Mol. Biol. 91:121-123.
30. **Rose, J. A., K. I. Berns, M. D. Hoggan, and F. Koczot.** 1969. Evidence for a single-stranded adeno-associated virus genome: formation of a DNA density hybrid on release of viral DNA. Proc. Natl. Acad. Sci. U.S.A. 64:863-869.
31. **Rose, J. A., and F. J. Koczot.** 1971. Adenovirus-associated virus multiplication. VI. Base composition of the deoxyribonucleic acid strand species and strand-specific in vivo transcription. J. Virol. 8:771-777.
32. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry 12:3055-3063.
33. **Shenk, T. E., C. Rhodes, P. W. J. Rigby, and P. Berg.** 1975. Biochemical method for mapping mutational alterations in DNA with S1 nuclease: the location of deletions and temperature-sensitive mutations in Simian virus 40. Proc. Natl. Acad. Sci. U.S.A. 72:989-993.
34. **Smith, H. O., and D. Nathans.** 1973. A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. J. Mol. Biol. 81:419-423.
35. **Sutton, W. D.** 1971. A crude nuclease preparation suitable for use in DNA reassociation experiments. Biochim. Biophys. Acta 240:522-531.
36. **Weiss, B., T. R. Live, and C. C. Richardson.** 1968. Enzymatic breakage and rejoining of deoxyribonucleic acid. V. End group labeling and analysis of deoxyribonucleic acid containing single strand breaks. J. Biol. Chem. 243:4530-4542.
37. **Wilson, G. A., and F. E. Young.** 1975. Isolation of a sequence-specific endonuclease (*Bam*I) from *Bacillus amyloliquefacians* H. J. Mol. Biol. 97:123-125.