Physical Map and Strand Polarity of Specific Fragments of
Adenovirus-Associated Virus DNA Produced by Endonuclease
\textit{R·EcoRI}

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Cleavage of adenovirus-associated virus type 2 (AAV2) DNA linear duplex monomers with the restriction endonuclease \textit{R·EcoRI} yielded three fragments, A, B, and C, having approximate mol wt of $1.6 \times 10^4$, $1.1 \times 10^4$, and $1.3 \times 10^4$, respectively. Radioactive labeling the 5' termini of AAV DNA before cleavage with \textit{R·EcoRI} showed that A and B were terminal fragments and C was internal. Separation of the complementary strands of fragments A and B showed that A contained the 5' terminus of the minus strand and the 3' terminus of the plus strand, and conversely for fragment B. The physical map of the AAV \textit{R·EcoRI} fragments can thus be unambiguously determined and is drawn with B at the left-hand and A at the right-hand end. On this map, transcription of stable AAV mRNA from the minus strand proceeds from left to right, beginning in fragment B and terminating in fragment A. The asymmetry in distribution of thymidine between the AAV DNA plus and minus strands is preferentially located in fragment A, which represents the right-hand half of the duplex molecule. These experiments enable preparative separation of all four single-strand termini of AAV DNA and provide a basis for orientation of fragment maps derived by cleavage with other restriction enzymes.

Individual particles of adenovirus-associated virus (AAV) contain either a plus or minus linear single strand of DNA and, upon isolation, these strands reassociate to yield duplex molecules (14, 17). As a consequence of a limited permutation in the nucleotide sequence of AAV DNA strands, some of the duplex molecules contain cohesive ends that cause formation of circular monomers and linear and circular oligomers (4, 9, 13). From an analysis of in vivo transcription of AAV DNA, we have suggested that this genome may contain a single gene (5, 7, 8) and is thus a potentially useful probe for studying certain aspects of genetic expression in eukaryotic systems. To provide specific DNA fragments for use in further analysis of AAV functions, we have begun construction of a restriction enzyme cleavage map of the AAV genome. In a previous report (4), we showed that the restriction endonuclease \textit{R·EcoRI} makes two site-specific cleavages in AAV DNA duplexes to yield three fragments, A, B, and C. Cleavage of the AAV duplex oligomers with \textit{R·EcoRI} yielded two of the fragments (A and B) held together by cohesive ends, and it was therefore possible to deduce a tentative physical order for the three fragments (4).

In this report we describe experiments designed to determine unambiguously the physical order and strand polarity of the fragments derived from adenovirus-associated virus type 2 (AAV2) DNA duplexes by cleavage with \textit{R·EcoRI}. These experiments enable the preparative separation of all four single-strand termini. In addition, the data obtained indicate that the asymmetric distribution of thymidine between the plus and minus strands of AAV DNA is preferentially located in fragment A. The physical map obtained with the \textit{R·EcoRI} enzyme allows a more accurate determination of the region of the AAV genome that is stably transcribed and provides a basis for orientation of further physical maps derived with other restriction endonucleases. A preliminary account of these data has been given (B. Carter, D. Denhardt, and G. Khoury, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S248, p. 255).

**MATERIALS AND METHODS**

**Viruses and cells.** The growth of AAV2 in KB-3 cells in spinner culture with adenovirus type 2 as a helper has been described (6, 8).
Preparation and purification of viral DNA. The preparation of viral DNA uniformly labeled with $^{3}H$ or $^{32}P$ and substituted with 5-bromodeoxyuridine (BUDR) when desired has been fully described (4). In the BUDR-substituted DNA, the $^{3}H$ label is present in thymidine.

Enzymes. Restriction endonuclease R-EcoRI purified from Escherichia coli RY13 according to Mulder and Delius (15) was generously provided by G. C. Fareed. Bacterial alkaline phosphatase (EC 3.1.3.1, Worthington, BAPC) was further purified on DEAE-cellulose (19). T4 polynucleotide kinase was purified according to Richardson (16) and was generously provided by S. Eisenberg. S1 nuclease was purified according to Ando (1).

Sedimentation analysis. Neutral sucrose gradients (4.9 or 11.9 ml) contained a 5 to 20% sucrose gradient in 1 M NaCl, 50 mM Tris-hydrochloride (pH 8.0), 1 mM Na$_2$ EDTA, and 0.15% Sarkosyl in nitrocellulose tubes. Alkaline sucrose gradients (11.9) contained a 5 to 20% sucrose gradient in 0.3 M NaOH, 0.7 M NaCl, and 0.15% Sarkosyl. The gradients were centrifuged in the SW50L or SW41 rotor of a Beckman L265B centrifuge at 20 C and fractionated by collecting drops from the bottom of the tube.

Gel electrophoresis of viral DNA. Analysis of viral DNA in composite gels containing 2.0% acrylamide and 0.5% agarose in a buffer containing 50 mM Tris-acetate, 40 mM sodium acetate, 1 mM Na$_2$ EDTA (pH 7.6), and 0.1% sodium dodecyl sulfate was performed as described before (4).

Cleavage of DNA with restriction endonuclease. AAV2 DNA was cleaved with R-EcoRI as described before (4), and products of the reaction were analyzed on neutral sucrose gradients or acrylamide-agarose gels.

End labeling of DNA. To label $^{3}H$-labeled BUDR-AAV2 DNA duplexes with 5'-terminal $^{32}P$, the DNA was first incubated with bacterial alkaline phosphatase (19) at 65 C for 25 min. Phosphatase was added to a concentration of 2 U/ml at 0 and 10 min. The reaction was stopped by addition of ethylene glycol-β-aminoethyl ether)N,N'-tetraacetic acid to 10 mM and incubating for an additional 30 min (11). The 5'-termini were then labeled with $^{32}P$, using T4 polynucleotide kinase and γ-$^{32}P$]ATP according to Richardson (16), except that 2 mM phosphate was included in the reaction to ensure inhibition of the phosphatase. γ-$^{32}P$]ATP was prepared and purified by the procedure of Glynn and Chappel (10). Reactions with kinase were incubated for 3 h at 36 C and then stopped by addition of EDTA to 40 mM. The 5'-terminal-labeled DNA was then purified by sucrose gradient centrifugation (see Fig. 6).

Separation of plus and minus strands of AAV DNA. $^{3}H$-labeled, BUDR-substituted AAV2 DNA was denatured by alkali, renatured, and rapidly adjusted to a density of 1.815 g/ml by addition of solid CsCl. The mixture was then centrifuged in the Beckman 65 rotor at 20 C and 35,000 rpm for 45 to 60 h. The gradients were fractionated from the bottom of the tube, and the density of appropriate fractions was immediately determined by measuring the refractive index, using an Abbe refractometer (Bausch and Lomb) at room temperature. Densities were corrected to 25 C according to Vinograd et al. (18).

DNA-RNA hybridization. The preparation of unlabeled total cell RNA from KB cells 24 h after infection with AAV2 and adenovirus type 2 has been described (5, 7). Purified single-strand components of restriction fragments were boiled for 10 min in 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl and chilled in ice before use in annealing reactions. The denatured DNA was then incubated for appropriate periods in the absence or presence of unlabeled, infected cell RNA at 68 C as described previously (5, 12). The proportion of labeled DNA in hybrid molecules was assayed by single-strand-specific S1 nuclease (1, 5).

RESULTS

Preparative separation of complementary strands of AAV R-EcoRI fragments. Because the intact strands of AAV DNA contain an asymmetric distribution of thymidine, substitution of BUDR for thymidine allows preparative separation of the strands in a CsCl buoyant density gradient (3). We determined whether the complementary strands of the AAV2 DNA R-EcoRI fragments could be separated in a similar way.

$^{3}H$-labeled BUDR-AAV2 DNA was incubated with the R-EcoRI restriction endonuclease and sedimented through a neutral sucrose gradient. The sedimentation profile (Fig. 1a) that was obtained indicated at least four major peaks; the three slowest peaks contained fragments A, B, and C with mol wt of 1.6 x 10$^6$, 1.1 x 10$^6$, and 1.3 x 10$^6$, respectively (4). The components AB' and AB arise from cleavage of oligomeric forms of AAV DNA and consist, at least in part, of fragments A and B held together by cohesive end joining (4). Fragments A and B, purified from the neutral sucrose gradient (Fig. 1a), were analyzed in alkaline sucrose (Fig. 1b) and by electrophoresis in composite gels (Fig. 1c), which showed that each fragment contained very few single-strand nicks and was at least 90% pure. These fragment preparations were then used for strand separation in CsCl (Fig. 2). Fragment C is not discussed since it was too small to allow strand separation in CsCl.

The preparative separation in a CsCl equilibrium density gradient of the complementary strands of intact $^{3}H$-labeled BUDR-AAV2 DNA is shown in Fig. 2a. Under these conditions, the duplex $^{3}H$-labeled BUDR-AAV DNA banded at a density of approximately 1.81 g/ml (not shown), which is equivalent to approximately 90% substitution of thymidine by BUDR. The minus strands (Fig. 2a) banded at a higher density than the plus strands because of their higher content of BUDR. The shoulder on the less dense side of the plus strand peak was due
Fig. 1. Preparation of EcoRI cleavage products of AAV2 DNA. (a) 3H-labeled BUdR-AAV2 DNA was cleaved with the restriction endonuclease EcoRI and sedimented in a neutral sucrose gradient in the SW41 rotor of a Beckman centrifuge for 12 h at 35,000 rpm. A 5-μl portion of each fraction was taken to determine the radioactivity profile. Sedimentation in this and succeeding figures is from right to left. Fractions containing fragments RI-A and RI-B were pooled as indicated and dialyzed extensively against 0.01 M Tris-hydrochloride (pH 8.0)−0.01 M NaCl. A portion of each pool was taken to assess the purity of the preparations, and the remainder of each pool was used for the experiment described in Fig. 2. (b) Aliquots of the RI-A and RI-B pools were sedimented in separate alkaline sucrose gradients for 12 h at 35,000 rpm in the SW41 rotor at 20 C. The sedimentation profiles of RI-A (solid line) and RI-B (broken line) are plotted in the same panel. (c) Portions of the RI-A and RI-B pools were electrophoresed in composite acrylamide-agarose gels as described in Materials and Methods. Gels, 12 cm long and 6 mm in diameter, were electrophoresed for 14 h at 4 mA/gel and then cut into 1.25-mm slices, solubilized in H2O2, and counted in toluene-Triton scintillation cocktail. RI-A and RI-B were electrophoresed on parallel gels, and the results are plotted in a single panel. Migration is from left to right.

to the presence of reannealed duplex material.

The preparative strand separation of the RI-A and RI-B fragments is shown in Fig. 2b and c. In both cases, the light (plus strand) component banded at a density similar to that of intact plus strands (Fig. 2a). However, for fragment A (Fig. 2b), the heavy (minus strand) component banded at a higher density than intact minus strands (Fig. 2a). Conversely, for fragment B (Fig. 2c), the minus-strand component banded at a lower density than intact minus strands. We infer from these data (assuming uniform substitution of thymidine by BUdR) that the asymmetry in distribution of thymidine between the plus and minus strands is restricted predominantly to the Eco-RI-A fragment.

From the CsCl gradients shown in Fig. 2b and c, the minus- and plus-strand components of fragments A and B (designated A(−), A(+), B(−), and B(+), respectively) were further purified. The DNA from the pooled regions (Fig. 2) was dialyzed to remove CsCl and then
AAV DNA species in CsCl buoyant density gradients. A fragment obtained from the experiment described in Fig. 1a. The fractions containing the separated DNA preparations were denatured with alkali, renatured, and centrifuged to equilibrium in CsCl density gradients as shown in Fig. 3. The cross-contamination should result in formation of duplexes of 13.0S and 11.5S for fragments A and B, respectively (4). The A(−) strand preparation (Fig. 3a) had only a very low degree of contaminating duplex (11%), whereas the A(+) strand preparation (Fig. 3b) had a significant proportion of 13.0S duplex material (39%). The A(−) single strands (21.5S) sedimented slightly faster than the A(+) single strands (19.5S), presumably because of the significantly different amounts of BUdR in the two strands. For the B(−) and B(+) strand preparations (Fig. 3c and d), the extent of cross-contamination as evidenced by the large proportion of 11.5S duplex material was 63 and 50%, respectively. These values were significantly higher than those observed with the A strands, consistent with the poorer resolution of the B strands in CsCl (Fig. 2). Also, both pure B(−) and B(+) strands had similar sedimentation constants of approximately 16.5S, consistent with the lower degree of asymmetry in the distribution of thymidine (and consequently BUdR) between the B strands. From the neutral sucrose gradients shown in Fig. 3, the purified A(−), A(+), B(−), and B(+) strands were obtained by pooling peak fractions of the 21.5S, 19.5S, 16.5S, and 16.5S components, respectively. Concentration and extensive reassociation of these strand preparations to Ct values of $10^{-2}$ converted not more than 5% of each preparation to duplex DNA as assayed by the single-strand-specific S1 nuclease (see below). Thus each of the complementary strands of fragments A and B was present in relatively pure form.

**Fig. 2. Separation of complementary strands of AAV DNA species in CsCl buoyant density gradients.** DNA preparations were denatured with alkali, renaturated, and centrifuged to equilibrium in CsCl density gradients as described in Materials and Methods. Portions (10 μl) of each gradient fraction were taken to determine the radioactivity profile. The density of appropriate fractions was also measured. (a) Separation of the complementary strands of intact 3H-labeled BUdR-AAV2 DNA. (b) Separation of the complementary strands of the RI-A fragment obtained from the experiment described in Fig. 1a. (c) Separation of the complementary strands of the RI-B fragment obtained from the experiment described in Fig. 1a. The fractions containing the separated strands of fragments RI-A and RI-B were pooled as indicated in panels (b) and (c) and subjected to further purification (see Fig. 3).

**Annealing of viral RNA with separated strands of restriction fragments.** In the above experiments, the designation of the complementary components of fragments A and B as minus or plus was made assuming that in each case the heavy component arose from the original intact minus strand. To verify these assignments of minus and plus sense, the separated strands of fragments A and B obtained from the experiment described in Fig. 3 were annealed with concentrated, adjusted to 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate), and annealed at 68°C to a Ct value in excess of $10^{-2}$, which is more than 100-fold above the Ct value required for complete reassociation of AAV DNA strands (5). Thus virtually all cross-contamination in the single-strand preparations should be removed by formation of duplex molecules. The remaining single-strand DNA was then purified from the contaminating duplexes by sedimentation in neutral sucrose gradients as shown in Fig. 3. The cross-contamination should result in formation of duplexes of 13.0S and 11.5S for fragments A and B, respectively (4). The A(−) strand preparation (Fig. 3a) had only a very low degree of contaminating duplex (11%), whereas the A(+) strand preparation (Fig. 3b) had a significant proportion of 13.0S duplex material (39%). The A(−) single strands (21.5S) sedimented slightly faster than the A(+) single strands (19.5S), presumably because of the significantly different amounts of BUdR in the two strands. For the B(−) and B(+) strand preparations (Fig. 3c and d), the extent of cross-contamination as evidenced by the large proportion of 11.5S duplex material was 63 and 50%, respectively. These values were significantly higher than those observed with the A strands, consistent with the poorer resolution of the B strands in CsCl (Fig. 2). Also, both pure B(−) and B(+) strands had similar sedimentation constants of approximately 16.5S, consistent with the lower degree of asymmetry in the distribution of thymidine (and consequently BUdR) between the B strands. From the neutral sucrose gradients shown in Fig. 3, the purified A(−), A(+), B(−), and B(+) strands were obtained by pooling peak fractions of the 21.5S, 19.5S, 16.5S, and 16.5S components, respectively. Concentration and extensive reassociation of these strand preparations to Ct values of $10^{-2}$ converted not more than 5% of each preparation to duplex DNA as assayed by the single-strand-specific S1 nuclease (see below). Thus each of the complementary strands of fragments A and B was present in relatively pure form.

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RNA from AAV-infected cells. Since the stable AAV transcripts present in infected cells anneal only to the intact minus strand (5), only the minus-strand components of the restriction fragments should form duplexes with AAV RNA. The results of the experiment are listed in Table 1. In the absence of RNA, little reassociation of any of the DNA preparation was observed even at C\textsubscript{0}t\textsubscript{4} values 20- to 25-fold higher than the C\textsubscript{0}t\textsubscript{4} values for RI-A and RI-B duplexes (4). In the presence of AAV RNA, only the A(−) and B(−) strands show extensive formation of DNA-RNA duplex, thus verifying their minus-strand origin. In a prior calibration experiment (not shown) under conditions similar to those described in Table 1, a concentration 0.2 mg/ml of the same RNA preparation was sufficient to yield a saturation reaction with 5 ng of intact AAV minus-strand DNA (5). The concentration of 1.8 mg/ml (Table 1) should be sufficient to completely saturate 5 ng of A(−) or B(−) strand, and therefore the data obtained afford a measure of the proportion of these strands that are stably transcribed.

**Physical order of AAV2 DNA RI fragments.** To determine the physical order in the AAV genome of RI fragments A, B, and C, the 5' termini of \textsuperscript{3}H-labeled BUdR-AAV DNA were labeled with \textsuperscript{32}P before EcoRI cleavage. Two preparations of \textsuperscript{3}H-labeled BUdR-AAV2 DNA were used. One preparation consisted of unfractionated duplex DNA formed during ex-

<table>
<thead>
<tr>
<th>DNA strand</th>
<th>DNA in duplex* (%)</th>
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<tbody>
<tr>
<td>RI-A(−)</td>
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</tr>
<tr>
<td>RI-A(+)</td>
<td>4.5</td>
</tr>
<tr>
<td>RI-B(−)</td>
<td>5.0</td>
</tr>
<tr>
<td>RI-B(+)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

\* Reaction mixtures (0.6 ml) containing \textsuperscript{3}H-labeled DNA (approximately 5 ng with a specific activity of \textsuperscript{10} counts/min per ng), 0.5 M NaCl, and 5 mM Tris-hydrochloride buffer (pH 8.0) were incubated at 68 C for 20 h and then chilled in ice.

\* Preparations of purified DNA strands were obtained from the experiment shown in Fig. 3.

\* Proportion of DNA duplex was measured by using the single-strand-specific S\textsubscript{1} nuclease as described previously (1, 5).

\* Annealing reactions were performed in the absence of any added RNA.

\* Annealing reactions contained RNA (to a final concentration of 1.8 mg/ml) isolated from KB cells 20 h after infection with AAV2 and adenovirus type 2.

**Fig. 3. Purification of separated strands of the RI-A and RI-B fragments.** The preparations of individual minus and plus strands of fragments A and B obtained from the experiment shown in Fig. 2 were separately concentrated and reassociated as described in the text and then sedimented through neutral sucrose gradients in the SW41 rotor for 7.5 h at 35,000 rpm and 20 C. (a) A(−) strand from Fig. 2b. (b) A(+) strand from Fig. 2b. (c) \textsuperscript{3}H-labeled BUdR-AAV2 DNA. (f) \textsuperscript{32}P-labeled AAV2 DNA. All gradients were run in parallel. Peak fractions of the A(−) strand (21.5S), the A(+) strand (19.5S), and the B(−) and B(+) strands (16.5S) were pooled and dialyzed to remove sucrose and then used in the experiment detailed in Table 1. S values for intact AAV DNA (panels e and f) and duplex fragments A (panel b) and B (panels c and d) were determined previously, using Simian virus 4D DNA as a standard (4). The reported values are the means of seven determinations. Approximate S values of the single-strand components of fragments A and B were determined directly from the data shown.
traction from virions and thus included the circular and linear oligomer molecules. The second DNA preparation contained only 15.5S linear monomer molecules that had been purified from the neutral sucrose gradient shown in Fig. 4. Both DNA preparations contained more than 90% intact strands as judged by sedimentation in alkaline sucrose. The 5' termini of both DNA preparations were labeled with 32P by successive incubations with bacterial alkaline phosphatase and then T4 polynucleotide kinase and γ-[32P]ATP. Under the conditions used, the 5' termini of internal nicks such as those involved in cohesive end joining in component AB' would also be labeled with 32P (19).

The terminally labeled DNA was then purified by sedimentation in neutral sucrose gradients, and the appropriate regions were pooled as indicated in Fig. 5a and b. For the unfractionated DNA (Fig. 5a), the region pooled excluded the largest oligomers; this had the effect of decreasing the yield of component AB upon subsequent EcoRI cleavage. The 5'-terminal-32P-labeled, 3H-labeled BUdR-AAV2 DNA was then incubated with the R-EcoRI enzyme and sedimented in neutral sucrose gradients. In Fig. 6a and b are shown the sedimentation profiles of the DNA before incubation with the enzyme. The unfractionated DNA contained a proportion of circular monomers (fraction 12 of gradient in Fig. 6a). After incubation with the R-EcoRI enzyme, the 5'-terminal 32P label was equally distributed between fragments A and B and there was no detectable peak of 32P radioactivity in fragment C (Fig. 6c and d). Cleavage of the unfractonated DNA (Fig. 6c) also produced some component AB' that contained a peak of 32P label. The data from Fig. 6c and d are summarized quantitatively in Table 2, in which the observed ratio of 32P/3H in intact DNA or the R-RI fragments is compared with the 32P/3H ratio expected for any individual species, assuming it contained only one 5' terminus of the original DNA (fragment A, B, or C) or two 5' termini (intact DNA or component AB'). These data clearly show that fragments A and B each contained one 5' terminus of AAV DNA and C did not. Also, component AB' contained the amount of 32P label expected if it consisted of fragments A and B held by cohesive ends.

**Strand polarity of the R-EcoRI fragments.** The preceding experiments showed that RI fragments A and B each contained one end of the original intact duplex AAV genome. In addition, the complementary strands of each

![Fig. 4. Purification of linear duplex monomer molecules of AAV2 DNA.](4)

![Fig. 5. Purification of 3H-labeled BUdR-AAV2 DNA after addition of a 5'-terminal 32P label.](5)

'the horizontal bar which contained linear monomer duplex molecules was taken and used in subsequent experiments (Fig. 5–7). The arrow indicates the position of circular, hydrogen-bonded, monomer duplexes sedimenting 1.13 times more rapidly than linear duplex monomers.
fragment could be separated in CsCl. These results provided the basis for determining the strand orientation of each fragment. RI fragments A and B obtained by cleavage of $^3$H-labeled BUdR-AAV2 DNA containing a 5'-terminal $^32$P label were denatured, and the complementary strands were separated in CsCl equilibrium buoyant density gradients (Fig. 7). At least 80% of the 5'-terminal $^32$P in fragment A was contained in the heavy minus-strand component (Fig. 7a). The small proportion of $^32$P banding in the position of the light plus-strand component could be accounted for by reannealed duplex fragments, by some $^32$P labeling of internal nicks, and by a low degree of cross-contamination of fragment A with fragment B. As noted before in CsCl gradients, the complementary strands of the RI-B fragment did not separate as well as those of RI-A. However, Fig. 7b clearly shows that the majority of the terminal $^32$P label in RI-B banded at the density of the light plus-strand components.

These data provide evidence that the RI-A fragment contains the 5' terminus of the AAV DNA minus strand and the 3' terminus of the plus strand. Conversely, RI-B contains the 5' terminus of the plus strand and the 3' terminus of the minus strand.

**DISCUSSION**

The experiments described here allow an arbitrary but unambiguous definition of left
and right ends of the AAV2 DNA duplex with respect to the polarity of the plus and minus strands. We have defined the AAV linear duplex genome map as shown in Fig. 8 so that the left molecular end is in RI-B and contains 3' terminus of the minus strand and the 5' terminus of the plus strand. The right molecular end is therefore in RI-A and contains the 5' terminus of the minus strand and the 3' terminus of the plus strand.

The stable AAV RNA synthesized in infected cells, in the presence of either adenovirus type 2 or simple virus type I as the helper, hybridized only to the AAV DNA minus strand (5). Therefore, transcription of stable AAV RNA on the minus strand would proceed from left to right. Since the stable AAV RNA transcript was equivalent in size to approximately 70% of the DNA strand length (7, 8) and was complementary to approximately 70% of the minus strand (5), it would represent a single contiguous region of this strand. In addition, the inverted, terminally repeated nucleotide sequences that comprise 1.5 to 5% of the DNA strand length at each terminus do not appear to be represented in the stable AAV RNA (2, 5, 13; manuscript in preparation). Furthermore, RI-C is completely within the transcribed region (manuscript in preparation). The most extreme possible leftward or rightward locations of the stably transcribed region of the minus strand consistent with the above observations are indicated in Fig. 8. Based upon the data in Table 1 and the proportion of the genome contained in RI-A, RI-B, and RI-C (4), stable transcription would be expected to begin between 0.1 and 0.15 map units in RI-B and to terminate between 0.8 and

Table 2. Distribution of 5'-terminal \(^{32}P\) in R-EcoRI fragments of \(^{3}H\)-labeled BUDR-AAV DNA

<table>
<thead>
<tr>
<th>AAV2 DNA</th>
<th>Mol wt</th>
<th>Relative ratio (^{32}P/^{3}H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>2.8 x 10^5</td>
<td>1.0</td>
</tr>
<tr>
<td>RI-A</td>
<td>1.6 x 10^5</td>
<td>0.88</td>
</tr>
<tr>
<td>RI-B</td>
<td>1.1 x 10^5</td>
<td>1.27</td>
</tr>
<tr>
<td>RI-C</td>
<td>0.13 x 10^5</td>
<td>10.88</td>
</tr>
<tr>
<td>RI-AB'</td>
<td>2.7 x 10^5</td>
<td>1.03</td>
</tr>
</tbody>
</table>

\(^{3}H\)-labeled BUDR-AAV2 DNA was terminally labeled with 5' \(^{32}P\) and then incubated with R-EcoRI and analyzed in neutral sucrose gradients as described in Fig. 6.

\(^{3}P/^{3}H\) was calculated directly from sucrose gradients such as those shown in Fig. 6. The results from two experiments are shown. All values were normalized by taking the expected ratio for intact DNA as 1.0.

It was determined previously (4) that the expected ratio of \(^{32}P/^{3}H\) for RI fragment A, B, or C was taken as 1/molecular weight, assuming that each fragment contained one 5' \(^{32}P\) terminus of the original DNA. For intact DNA or AB', the expected ratio of \(^{3}P/^{3}H\) was calculated as 2/molecular weight, assuming two 5'-terminal phosphates were present in each molecule. All values were normalized by taking the expected ratio for intact DNA as 1.0.

The values for the molecular weights of the EcoRI cleavage products were determined previously (4).

The complementary strands of these fragments were then separated in CsCl buoyant density gradients under conditions similar to those described in Fig. 2. (a) Fragment RI-A; (b) fragment RI-B; (C) \(^{3}H\) general label; (D) \(^{32}P\) 5'-terminal label; (E) density of CsCl.
Fig. 8. Physical map and strand polarity of AAV2 DNA EcoRI fragments and direction of transcription. The minus and plus strands of AAV2 DNA are represented by the heavy horizontal lines with the strand polarity as indicated. The location of various features of the genome are noted according to the scale at the top of the diagram in which 1 map unit is equivalent to the length of the AAV DNA strand. B, C, and A indicate the order and position of the R-EcoRI fragments. C is equivalent to 4.5% of the genome length. Eco1 and Eco2 indicate the approximate positions of the two R-EcoRI cleavage sites. The direction of transcription on the DNA minus strand is indicated by the horizontal stipled arrows (see Discussion). The sequences depicted at the ends of the DNA strands are shown merely to indicate the presence of the inverted repetitious sequences (see Discussion) and are not intended to be representative of the actual terminal sequence. A natural terminal repetition is also present (9).

0.85 units in RI-A. A more extensive analysis of the stably transcribed region will be reported elsewhere.

The physical map of AAV2 DNA detailed here relates the EcoRI fragment map to the direction of RNA transcription and provides a basis for the orientation of further specific fragments derived with other restriction enzyme. The fragment map produced should also be useful in the further analysis of AAV2 functions such as determining the origin and direction of DNA replication.

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LITERATURE CITED

