Adenovirus-Associated Virus Multiplication

VI. Base Composition of the Deoxyribonucleic Acid Strand Species and Strand-Specific In Vivo Transcription

J. A. ROSE AND F. KOCZOT

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

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The two complementary strand species of 5-bromodeoxyuridine-substituted, adenovirus-associated virus type 2 (AAV-2) deoxyribonucleic acid were preparatively separated in CsCl density gradients and further purified by sedimentation through 5 to 20% sucrose. The base composition of each strand species was determined, and it was found that the species banding at a greater density in CsCl (heavy strands) had an expected higher thymidine content (26.5%) than that (21.7%) of the less dense species (light strands). Furthermore, the base composition of in vivo-synthesized, AAV-specific ribonucleic acid was similar to that of light-strand deoxyribonucleic acid, and this ribonucleic acid apparently hybridized only with heavy strands. These observations indicate that the heavy-strand species alone serves as the transcriptional template in vivo. This study represents the first instance in which the base composition and specificity of in vivo transcription have been determined for each of the complementary strands of an animal virus deoxyribonucleic acid.

Adenovirus-associated viruses (AAV) are defective, animal viruses which were initially thought to contain double-stranded deoxyribonucleic acid (DNA; 2, 16, 18). It was subsequently discovered that AAV DNA is single-stranded in situ and that, during DNA extraction, single strands from different virions anneal to form duplex molecules (15, 20). There are at least two discrete populations of AAV particles, as two complementary strand species can be identified in neutral CsCl density gradients when 5-bromodeoxyuridine (BUDR) is substituted for thymidine in the DNA (4, 20). A similar segregation of complementary polynucleotide chains has not been found with any other virus.

The in vivo function of each AAV DNA strand species has not been determined. Some insight into the mode of AAV replication would be gained if it were known whether one or both of the viral DNA strands serves as a template for transcription in vivo. In this study the complementary DNA strands of AAV type 2(AAV-2) have been preparatively separated and their base compositions have been analyzed. An expected difference in thymidine content between strands was observed, and it was found that in vivo-synthesized AAV ribonucleic acid (RNA) apparently hybridized with only one of the DNA strand species.

MATERIALS AND METHODS

Materials. Virus stocks were assayed as reported previously (11), and KB cells were from a line originally provided by M. Green. Pancreatic ribonuclease, T1 ribonuclease, and electrophoretically purified pancreatic deoxyribonuclease I were obtained from the Worthington Biochemical Corp., Freehold, N.J., and Pronase was obtained from Calbiochem, Los Angeles, Calif. Tritiated adenosine-5'-triphosphate (4 Ci/mole), uridine-5'-triphosphate (3 Ci/mole), guanosine-5'-triphosphate (3 Ci/mole), and cytidine-5'-triphosphate (4 Ci/mole) were purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y. The sources of CsCl, BUDR, thymidine-*methyl*-³H, ³²P, 5-fluorodeoxyuridine (FUDR), Sarkosyl, papain, and trypsin have been given (4).

Virus production and purification. Adenovirus type 2 and AAV-2 (grown with adenovirus type 2 as a helper) were produced in KB cells in suspension culture (4). AAV containing BUDR-substituted, ³²P-labeled DNA, or ³H-labeled DNA were prepared as before (4). AAV containing BUDR-substituted, ³H-labeled DNA was also obtained as described previously (4) except that DNA was labeled with ³H-thymidine (0.5 μ Ci/ml) instead of ³H-BUDR. The Freon-extraction step was eliminated from the adenovirus type 2 purification procedure (9, 18). The AAV purification technique (4) was also modified. After treatment with deoxycholate and trypsin, the virus preparation was again sonically treated, and debris was removed by centrifugation at $10,000 \times g$ for 5 min.

The supernatant fluid was then adjusted to a density of 1.40 g/ml with solid CsCl, and the AAV was banded three times as described previously (18).

DNA extraction and analysis. Adenovirus DNA and the double-stranded form of AAV DNA were extracted and purified by methods detailed previously (18, 20). The complementary strand species of BUDR-substituted AAV DNA were separated by isopycnic CsCl centrifugation and further purified by sedimentation through 5 to 20% sucrose gradients (4). Base analyses of enzymatic hydrolysates of ^{32}P -labeled viral DNA were performed as reported previously (18).

RNA preparation and analysis. KB cells in suspension culture (3×10^6 cells/ml) were simultaneously infected with AAV-2 and adenovirus type 2 [10 tissue culture infective dose (TCID)₅₀ units of each per cell] and RNA synthesized in infected cells was labeled with ^{32}P (4 $\mu\text{Ci}/\text{ml}$) after cells were washed with phosphate-free medium and suspended in medium containing 10^{-6} M phosphate and 5% dialyzed horse serum. RNA was extracted by a hot phenol procedure (23), incubated with electrophoretically purified deoxyribonuclease (50 $\mu\text{g}/\text{ml}$), and additionally purified by chromatography on Sephadex G-25 (3). AAV-specific RNA, selected by hybridization with AAV DNA (see below), was used for base composition determinations and hybridization experiments. Base analyses of alkaline hydrolysates of ^{32}P -labeled viral RNA were performed by the method of Sebring and Salzman (24). The preparation of in vitro-synthesized viral RNA (17) was slightly modified. *Escherichia coli* B RNA polymerase (containing sigma factor) prepared by the method of Zillig et al. (30) was obtained from P. Qasba. In addition, the incubation time for RNA synthesis was extended to 40 min, and the subsequent incubation with deoxyribonuclease was increased to 30 min. As before, extracted (double-stranded) AAV DNA was used as a template for AAV RNA synthesis (19). The synthetic AAV RNA product had an average sedimentation coefficient of 10 when sedimented through a linear 5 to 20% sucrose gradient under conditions described in Fig. 2.

Nucleic acid hybridization. DNA-RNA hybrids were formed on nitrocellulose membrane filters (8) or in free solution. Hybridization reactions on filters (13 mm in diameter) were carried out in 0.3 ml of $4\times$ SSC (0.60 M NaCl plus 0.06 M sodium citrate) at 67 C for 30 hr. Filters were then washed on each side with 50 ml of $4\times$ SSC, soaked in 20 ml of $2\times$ SSC at 70 C for 15 min, incubated with 1 ml of $2\times$ SSC containing 20 μg of pancreatic ribonuclease at 37 C for 30 min, and rewashed on each side with 100 ml of $4\times$ SSC. After drying under a heat lamp, filters were assayed for radioactivity in Liquifluor-toluene (Nuclear-Chicago Corp., Des Plaines, Ill.). With this procedure, filters without DNA (blank filters) retained 0.002 to 0.005% of added ^{32}P -RNA counts. Hybridization-selected RNA was eluted from filters by boiling in a dilute salt solution (1). Filters from which RNA was to be eluted were dried overnight in vacuo at room temperature before counting. The counted filters were then washed with toluene, boiled for 5 min in 3 ml of $0.01\times$ SSC, and fast-cooled. Between 90 and 95% of ^{32}P -RNA was eluted from

filters by boiling. The RNA solution was adjusted to 0.15 M NaCl, 0.005 M MgCl_2 , and 0.05 M tris-(hydroxymethyl)aminomethane (Tris) pH 7.5, and incubated with electrophoretically purified deoxyribonuclease (50 $\mu\text{g}/\text{ml}$) for 30 min at 37 C. Sodium dodecyl sulfate (SDS) was then added to a final concentration of 0.3%, and the mixture was incubated with Pronase (400 $\mu\text{g}/\text{ml}$) for another 30 min at 37 C. After addition of 2 mg of yeast RNA as carrier, the mixture was extracted three times with hot phenol, and RNA was precipitated from alcohol. RNA precipitates were collected by centrifugation at $10,000 \times g$ for 30 min; the final recovery of hybridized ^{32}P -RNA was 80%. For hybridization in free solution, sucrose gradient-purified preparations of ^3H -BUDR heavy and light strands were first self-annealed in SSC at 70 C for 1 hr and then incubated with in vivo- or in vitro-synthesized AAV RNA in 0.37 ml of $2\times$ SSC for 5 hr at 67 C. Samples were then mixed with an equal volume of a ribonuclease solution [150 μg of heated pancreatic ribonuclease per ml (14) and 500 units of T1 ribonuclease per ml in 0.1 M Tris, pH 7.5] and incubated for 30 min at 37 C. A 0.025-ml addition of 30% Sarkosyl was made, and each sample was brought to a final volume of 3.5 ml with 0.01 M Tris, pH 7.5. Samples were adjusted to 1.780 g/ml with solid CsCl and centrifuged at 40,000 rev/min for 48 hr at 25 C in a Spinco model SW50 rotor. Gradients were fractionated by collecting drops through a puncture in the bottom of the centrifuge tube, and the density and acid-insoluble radioactivity of gradient fractions were determined as described previously (18). Conditions for the formation and detection of RNA-RNA hybrids were based on methods described by Colvill et al. (5). Double-stranded, ^3H -labeled reovirus RNA was obtained from A. J. Shatkin.

RESULTS

Base composition of complementary AAV DNA strands. A preparative purification of each complementary strand species of AAV DNA was based on techniques reported previously (4). The complementary strands separate when denatured, BUDR-substituted DNA is centrifuged to equilibrium in a neutral CsCl density gradient (Fig. 1), indicating a differential incorporation of density label between the strands. Further purification is achieved by sucrose sedimentation of self-annealed heavy- and light-strand components from the CsCl gradient (Fig. 2). It is estimated that sucrose-fractionated, heavy-strand pools are less than 5% contaminated with light strands, whereas the light-strand pools are 5 to 10% contaminated with heavy strands (Fig. 2). The greater contamination of light strands is probably due to their close association with duplex molecules formed during CsCl centrifugation and partially included with the light-strand fraction (Fig. 1). In the light-strand sucrose gradient (Fig. 2B) a slow-sedimenting, double-stranded component (15S) and a larger, fast-sedimenting, single-stranded compo-

FIG. 1. Isopycnic CsCl centrifugation at 40,000 rev/min for 48 hr at 25 C in a Spinco model 65 rotor of 30 μg of ^3H -BUDR AAV-2 DNA. DNA (specific activity, 10,500 counts per min per μg) was alkali-denatured and then neutralized. Fractions (0.125 ml) were collected and 5- μliter samples were counted. Heavy-strand (H) and light-strand (L) pools are indicated by bracketed lines.

FIG. 2. Sucrose sedimentation of the DNA strand pools fractionated from the CsCl density gradient shown in Fig. 1. Gradients of 5 to 20% sucrose containing 1 M NaCl, 0.15% Sarkosyl, 0.02 M (ethylenedinitrilo)-tetraacetic acid and 0.05 M Tris(hydroxymethyl)-aminomethane (pH 8.0) were overlaid with DNA in a 0.3-ml volume and centrifuged at 40,000 rev/min for 5 hr at 20 C in a Spinco model 40.1 rotor. Fifty-eight 0.2-ml fractions were collected from each gradient. (A) Heavy-strand pool, 5- μliter samples counted; (B) Light-strand pool, 10- μliter samples counted. Heavy (H)- and light (L)-strand preparations fractionated from these gradients are indicated by bracketed lines. The positions of double- and single-stranded DNA in the gradients are indicated (15 and 24S, respectively). Sedimentation is from right to left.

ment (24S) can be seen along with heterogeneously dispersed DNA not observed in the heavy-strand gradient (Fig. 2A). This heterogeneous DNA probably represents aggregated duplex molecules. The base composition of each complementary strand species, labeled with ^{32}P and similarly purified, is shown in Table 1. The expectation that strands which contain more BUDR (heavy strands) would have a higher thymidine content is confirmed. The thymidine content of heavy strands is 22% greater than that of the light strands. Complementarity between the strand species is reflected by equivalence of the paired bases. The predicted base composition of double-stranded molecules (heavy plus light strands) is the same as that found for extracted duplex DNA. The presence of BUDR in extracted DNA did not affect base composition determinations because bromodeoxyuridylic and thymidylic acids were electrophoretically indistinguishable. It can be seen that the base composition of extracted DNA was the same whether BUDR was present (DS BUDR) or absent (DS). Double-stranded helper virus DNA (adenovirus type 2 DNA) base composition was also analyzed as a control, and the values found are in close agreement with other published data (9). Although the base composition of AAV-2 DS DNA and adenovirus type 2 DNA are nearly the

TABLE 1. Base composition of adenovirus-associated virus type 2 (AAV-2) DNA and RNA

DNA or RNA	Nucleotides (moles/100 moles) ^f				
	A	T(U)	G	C	% G + C
AAV-2 DNA					
Heavy strands	20.5	26.5	26.7	26.3	53.0
Light strands	25.2	21.7	26.6	26.5	53.1
Heavy plus light strands ^a	22.9	24.1	26.6	26.4	53.0
DS BUDR ^b	22.3	24.4	26.9	26.4	53.3
DS ^c	22.5	24.1	26.8	26.6	53.4
Adenovirus type 2 DNA	22.8	22.3	27.7	27.2	54.9
AAV-2 RNA					
DS-selected ^d	26.2	20.7	26.4	26.7	53.1
Heavy strand-selected ^e	26.6	21.5	25.1	26.8	51.9

^a Predicted base composition of double-stranded DNA obtained by averaging observed values of each nucleotide component of the separated heavy and light strands.

^b Double-stranded, 5-bromodeoxyuridine (BUDR)-substituted AAV DNA.

^c Double-stranded, unsubstituted AAV DNA.

^d RNA selected by hybridization with double-stranded (unfractionated) AAV DNA.

^e RNA selected by hybridization with the heavy strand component of AAV DNA.

^f Abbreviations: A, deoxyadenylic or adenylic acid; T, thymidylic acid; U, uridylic acid; G, deoxyguanylic or guanylic acid; C, deoxycytidylic or cytidylic acid.

same, AAV-2 is efficiently helped by other adenoviruses which have substantially different DNA base compositions (11, 19).

Base composition of in vivo-synthesized AAV RNA. Because of strand differences in adenine and thymidine content, the base composition of AAV-specific RNA from infected cells should provide a clue as to whether one or both DNA strands is transcribed in vivo. AAV RNA can first be detected 10 to 12 hr after simultaneous infection of KB cells with AAV-2 and adenovirus type 2, and AAV infectivity titers are observed to increase by 17 to 20 hr (J. A. Rose et al., *in preparation*). It would thus be expected that most, if not all, species of AAV RNA should be labeled during a 12- to 20-hr pulse with ^{32}P . Figure 3 shows the hybridization saturation of 0.2 μg of double-stranded AAV DNA with 12- to 20-hr ^{32}P -labeled RNA. To obtain a proportionate representation of transcribed DNA sequences, RNA was recovered only from those filters where DNA was saturated. The template specificity of eluted RNA was tested by rehybridization with AAV, adenovirus type 2, and KB DNA (Table 2). As shown in Table 2, this RNA was highly specific for AAV DNA which bound 62 to 67% of added RNA, whereas less than 1% of the RNA hybridized with DNA from helper adenovirus type 2 and KB cells. The base composition of the AAV-specific RNA (DS-selected) is shown in Table 1. It is similar to that of the light DNA strands and suggests that the heavy-strand species is the transcriptional template in vivo.

Self-annealing of AAV RNA. An attempt was made to detect complementarity among species in the hybridization-selected AAV RNA. The ribonuclease resistance of several viral RNA

FIG. 3. Saturation of AAV DNA with ^{32}P -RNA labeled 12 to 20 hr after AAV infection. Filters contained 0.2 μg of double-stranded AAV DNA. Points represent averages of duplicate reactions, and blank filter values determined for each level of added RNA have been subtracted.

preparations after they were subjected to annealing conditions is shown in Table 3. Reovirus RNA serves as a double-stranded RNA control which is largely resistant to ribonuclease treatment until

TABLE 2. Specificity of hybridization-selected adenovirus-associated virus type 2 (AAV-2) RNA

Source of DNA	Amt of DNA on filter (μg)	^{32}P -RNA bound (counts/min)	Added ^{32}P -RNA bound ^b (%)
AAV(BU) ^a	0.04	758	16.6
	0.2	1,679	36.9
	0.5	2,433	53.5
	1.0	2,814	61.8
	2.0	2,763	60.8
AAV ^c	0.15	1,453	31.9
	0.2	2,305	50.7
	0.5	2,762	60.8
	1.0	3,050	67.0
	2.0	3,034	66.7
Adenovirus type 2	4.0	42	0.9
KB	4.0	8	0.2

^a Double-stranded form of DNA approximately 70% substituted with 5-bromodeoxyuridine.

^b Each reaction contained 4,550 counts/min of hybridization-selected ^{32}P -AAV RNA.

^c Unsubstituted, double-stranded form of DNA.

TABLE 3. Ribonuclease resistance of viral RNA

RNA ^a	Treatment	% Ribonuclease resistant ^c
Reovirus (DS)	Ribonuclease ^b	81
	Boil + ribonuclease ^d	6
Adenovirus type 2 (syn)	Ribonuclease	7
	Boil + ribonuclease	4
AAV-2 (syn)	Ribonuclease	36
	Boil + ribonuclease	8
AAV-2 (in vivo)	Ribonuclease	10
	Boil + ribonuclease	7

^a Samples of double-stranded (DS) and synthetic (syn) ^{32}P -RNA and hybridization-selected, in vivo-synthesized ^{32}P -RNA were incubated in 0.60 M NaCl plus 0.06 M sodium citrate (4X SSC) at 67°C for 4 hr. After incubation, acid-insoluble counts/min were: 1,100, reo (DS); 60,979, adenovirus type 2 (syn); 47,875, adenovirus-associated virus type 2 (AAV-2) (syn); and 1,212, AAV-2 (in vivo).

^b Incubated samples adjusted to SSC were treated with pancreatic ribonuclease (75 $\mu\text{g}/\text{ml}$) and T1 ribonuclease (250 units/ml) for 30 min at 37°C.

^c Percentage of acid-insoluble counts/min remaining after ribonuclease treatment.

^d Unincubated samples in 0.1X SSC were boiled for 5 min, fast-cooled, adjusted to 1X SSC, and treated with ribonuclease as described in footnote b.

FIG. 4. Isopycnic CsCl centrifugation of AAV DNA-RNA hybrids formed in free solution. (A) In vivo-synthesized ^{32}P -AAV RNA (27,000 counts/min) hybridized with 0.8 μg of ^3H -BUDR heavy-strand DNA fraction-

ated in a sucrose gradient (Fig. 2A). (B) Same amount of in vivo-synthesized ^{32}P -AAV RNA hybridized with 0.8 μg of sucrose gradient-fractionated ^3H -BUDR light-strand DNA (Fig. 2B). (C) In vitro-synthesized ^3H -AAV RNA (28,000 counts/min) hybridized with 0.8 μg of the heavy-strand preparation used in A. (D) Same amount of in vitro-synthesized ^3H -AAV RNA hybridized with 0.8 μg of the light-strand preparation used in B. The recoveries of DNA in gradients A and B were 93 and 83%, respectively.

it is denatured by boiling. The in vivo-synthesized AAV RNA was not significantly resistant to ribonuclease, suggesting that symmetrical transcription of the complementary strand species does not occur in infected cells. Of interest is the finding that 36% of the AAV RNA synthesized with *E. coli* polymerase was resistant to ribonuclease digestion, whereas similarly prepared adenovirus type 2 synthetic RNA was only 7% resistant, the level observed with denatured samples. These data indicate that the double-stranded form of AAV DNA is symmetrically transcribed in vitro.

DNA strand-specificity of AAV RNA. Pools of ^3H -labeled, heavy- and light-strand fractions were prepared (Fig. 1 and 2), and the ability of each strand species to hybridize with in vivo- and in vitro-synthesized AAV RNA was determined. The presence of BUDR in AAV DNA produced no gross alteration in its affinity for complementary RNA (Table 2). Before hybridization tests with RNA, each strand pool was again subjected to annealing conditions to reduce reactions which could result from cross-contamination of the strand species. The DNA-RNA hybridization reactions were carried out in free solution in the presence of nonlimiting amounts of DNA. Hybrids were subsequently detected in CsCl density gradients so that hybridized RNA could be located with respect to specific DNA components (Fig. 4). Hybridization-selected AAV RNA (labeled 12 to 20 hr after virus infection) hybridized well with the heavy-strand species (Fig. 4A). Approximately 30% of added RNA was bound. The preparation of light strands, however, was associated with only 1.5% of added ^{32}P -RNA (Fig. 4B); furthermore, these RNA counts did not form a discrete peak coincident with the main component of the DNA preparation. This component banded at the density of light strands (Fig. 4B), whereas its less dense shoulder is probably double-stranded DNA (Fig. 1), expected as discussed above. A comparable shoulder is not seen with the heavy-strand preparation (Fig. 4A). A similar selective hybridization was also observed between heavy strands and AAV RNA labeled during the first 4 hr of detectable AAV RNA synthesis (10 to 14 hr after infection).

Synthetic AAV RNA was also hybridized with

ated in a sucrose gradient (Fig. 2A). (B) Same amount of in vivo-synthesized ^{32}P -AAV RNA hybridized with 0.8 μg of sucrose gradient-fractionated ^3H -BUDR light-strand DNA (Fig. 2B). (C) In vitro-synthesized ^3H -AAV RNA (28,000 counts/min) hybridized with 0.8 μg of the heavy-strand preparation used in A. (D) Same amount of in vitro-synthesized ^3H -AAV RNA hybridized with 0.8 μg of the light-strand preparation used in B. The recoveries of DNA in gradients A and B were 93 and 83%, respectively.

the same amounts of the heavy- and light-strand DNA preparations that were used for the *in vivo* RNA tests. Because both synthetic RNA and AAV DNA were labeled with tritium, RNA hybridization would be evident only if radioactivity exceeded that resulting from the DNA alone (Fig. 4A and B). Figure 4C and D clearly shows that synthetic RNA hybridized well with heavy- and light-strand species. About 30% of added RNA hybridized with each strand preparation. This finding was expected from ribonuclease resistance studies (Table 3), and demonstrates, additionally, that light-strand DNA is capable of hybridizing with RNA. Again, a double-stranded DNA shoulder is seen only with the light-strand preparation (Fig. 4C and D).

Hybridization with *in vivo* and *in vitro* AAV RNA produced little shift in the densities of the DNA strand species (Fig. 4). This could be explained by the presence of excess DNA in hybridization reactions with relatively small RNA molecules. Figure 5 shows that the hybridization-selected RNA was of small size, predominantly 4 to 5S. The synthetic AAV RNA (10S) was also relatively small.

On the basis of the data shown in Fig. 4, RNA hybridizing with heavy strands should have a base composition resembling that of light strands and DS-selected RNA (Table 1). The base composition of 12- to 20-hr ^{32}P -RNA hybridizing with heavy-strand DNA immobilized on nitrocellulose filters (heavy strand-selected) is also given in Table 1. As expected, the values agree with those for light-strand and DS-selected RNA.

DISCUSSION

The preparative separation of each of the two complementary strand species of AAV DNA has

FIG. 5. Sedimentation of 12- to 20-hr labeled ^{32}P -AAV RNA through a 0 to 15% sucrose gradient in 99% dimethyl sulfoxide (27). Centrifugation was for 11 hr at 60,000 rev/min at 25°C in a Spinco model SW65 rotor. KB cell RNA labeled with ^3H -uridine was used as a marker (17). Sedimentation is from right to left.

permitted their characterization with respect to base composition and transcriptional function. It was observed previously that more BUDR was incorporated into one of the strand species, a bias that allows separation of the complementary strands in a CsCl density gradient (4). From the densities of the heavy- and light-strand components in CsCl, it was estimated that heavy strands should contain 26% thymidine, whereas light strands should have a thymidine content of 18% (4). The direct determination of base composition for each strand species (Table 1) gave values for thymidine content which are reasonably close to those predicted from density differences. The base composition of *in vivo*-synthesized AAV RNA resembles that of the light strands (Table 1), and this RNA appears to hybridize only with heavy strands (Fig. 4A and B). It is therefore concluded that during infection the heavy strands alone serve as a template for viral RNA synthesis. This finding permits a designation of "minus" strands for the heavy-strand species and "plus" strands for the RNA-like light-strand species.

It is interesting that while AAV transcription *in vivo* was confined to minus strands, RNA was synthesized from both plus and minus strands by *E. coli* RNA polymerase *in vitro* (Fig. 4C and D). This loss of specific transcriptional control *in vitro* is also indicated by the detection of symmetrically transcribed RNA molecules (Table 3). In contrast, *in vitro*-synthesized adenovirus RNA was asymmetrically transcribed. Other studies of adenovirus DNA transcription by *E. coli* RNA polymerase (13) have shown that *in vitro* RNA is asymmetric and that all sequences transcribed *in vitro* were also present in RNA from virus-infected cells. The reduced fidelity of AAV transcription *in vitro* is possibly due to the occurrence of discontinuities in both strands of template molecules (29) generated when plus and minus strands are hybridized during extraction of DNA from virions (20).

There is little known, at present, concerning the means by which the two complementary progeny species of AAV DNA are synthesized. The simplest mechanism for AAV replication would seem to require conversion of the single-stranded genome into a double-stranded template which could then mediate either transcription or synthesis of the plus and minus strands. This mechanism is analogous to that described for bacteriophage ϕX174 (10, 26), a virus containing only plus DNA strands (10) with a molecular weight (25) near that of single-stranded AAV DNA (20). However, whereas AAV transcriptional functions are restricted to one strand, as is the case for ϕX174 (10), the replication of progeny DNA strands would proceed nonselectively, unlike that ob-

served for ϕ X174. The apparent equivalence of plus and minus strands in AAV particles (18) suggests a random synthesis of both species in infected cells.

In contrast with ϕ X174, covalently closed-circular molecules have not been identified in DNA extracted from AAV (16, 28) or other parvoviruses [Kilham rat virus (22) or minute virus of mice (7)]. This absence of circular genomes is not strictly associated with the occurrence of plus and minus virions (as found with AAV), as only one-strand species can be extracted from Kilham rat virus (21) or minute virus of mice (6) particles. It is possible, however, that for AAV and other parvoviruses a circular DNA species is involved during the replication process in infected cells.

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