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Adenovirus-Associated Virus Multiplication

VIII. Analysis of In Vivo Transcription Induced by Complete or Partial Helper Viruses

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We have analyzed ribonucleic acid (RNA) synthesized in KB cells co-infected with adenovirus-associated virus (AAV) type 2 and either adenovirus type 2 (Ad2) or herpes simplex virus type 1 (HSV-1). With either type of helper virus, synthesis of AAV RNA was readily detected by deoxyribonucleic acid (DNA)-RNA hybridization. As is the case for AAV RNA synthesized with helper Ad2, the AAV RNA synthesized with HSV-1 as helper annealed only to the thymidine-rich (minus) AAV DNA strand. In addition, AAV RNA synthesized with either type of helper (i) contained similar nucleotide sequences as determined by hybridization inhibition tests and (ii) had a mean molecular weight of approximately 7.5×10^5 based on sedimentation in dimethylsulfoxide-sucrose gradients. These experiments suggest that the restricted helper function of HSV-1 is not due to abnormal transcription of the AAV genome. Since the mean molecular weight of AAV RNA is equivalent to 40 to 50% of the AAV genome, as few as two or three AAV RNA species may be transcribed in vivo. In contrast to adenovirus RNA, cleavage of AAV RNA after transcription was not observed.

In contrast to the complete helper function of adenoviruses (2, 8) the herpesviruses only partially assist the multiplication of adenovirus-associated virus (AAV). Although herpesviruses promote synthesis of AAV deoxyribonucleic acid (DNA) and ribonucleic acid (RNA; 17) and antigen (1, 4), production of infectious AAV has not been observed (1, 4, 17). The specific mechanism(s) by which either type of helper acts to induce AAV macromolecular synthesis is thus far uncertain. However, the inability of herpesviruses to serve as complete helpers for AAV replication might be related to production of abnormal precursors or components of AAV, absence of a helper function, or interference at some stage in AAV synthesis or assembly.

To explore the basis of the restricted helper activity exhibited by herpesviruses, we have further analyzed AAV RNA synthesized in cells using either adenovirus type 2 (Ad2) or herpes simplex virus type 1 (HSV-1) as helper. [For simplicity when referring to AAV systems using different helper viruses, we use the following nomenclature in which the helper virus is represented in parentheses: AAV(Ad) refers to AAV-infected systems in which the helper virus is an adenovirus, and AAV(HSV) indicates that the helper virus is herpes simplex virus.] Based on DNA strand specificity, hybridization inhibition, and velocity sedimentation, the AAV RNA species transcribed in the presence of either type of helper were identical with respect to nucleotide sequence and mean molecular size. As determined by sedimentation analysis, the mean molecular weight of AAV RNA was approximately 7.5×10^5, equivalent to 40 to 50% of the AAV genome. Although cleavage of adenovirus RNA molecules after transcription did occur, no cleavage of AAV RNA molecules was observed.

MATERIALS AND METHODS

Materials. Enzymes, 5-bromodeoxyuridine (BU DR), and uridine-5-^3H (25 to 28 Ci/mmole) were obtained from sources previously given (3, 16). Dimethylsulfoxide (DMSO) was obtained from Fisher Scientific Co., Fairlawn, N.J. Chloroform was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J.

Cells and viruses. KB cells in suspension culture were simultaneously infected with AAV type 2 (AAV-2) and either Ad2 or HSV-1 as helper as described previously (3, 17). The multiplicity of infection was 10 tissue culture infective dose ($TCID_{50}$) units/cell for Ad2 and 1 to 2 plaque forming units/cell for HSV-1. The multiplicity of infection for AAV-2 was 10 $TCID_{50}$ units/cell. When HSV-1 was used as the helper, the AAV-2 innoculum was heated at 56 C for 10 min to destroy contaminating adenovirus.

Chloroform treatment of helper virus. Stocks of HSV-1 or Ad2 were centrifuged at 3,000 × *g* for 10 min to remove cell debris. Chloroform from a freshly opened bottle was then added to a final concentration of 20% (v/v) and the mixture was shaken vigorously. After 10 min at room temperature, chloroform was removed after each of two centrifugations at 3,000 × *g*. The final aqueous supernatant fluid was used as the virus inoculum. Portions of the stock to be used as controls were treated exactly as above, except that chloroform was not added.

Preparation of purified DNA. The purification of DNA from KB cells, Ad2, HSV-1, and AAV-2 and the separation of complementary strands of ^{14}C-labeled, BUDR-substituted AAV-2 DNA were carried out as described previously (14, 16, 17).

Labeling and isolation of RNA from infected cells. RNA synthesized in infected KB cells was labeled in the presence of uridine-*5-^{3}H* (5 to 10 μCi/ml) for intervals indicated in individual experiments. RNA was extracted by a hot phenol procedure and further purified by treatment with electrophoretically pure pancreatic deoxyribonuclease (16). When the RNA was to be used for molecular-weight determinations in DMSO-sucrose gradients, incubation with deoxyribonuclease was omitted to avoid possible degradation of the RNA. In these RNA preparations, less than 0.02% of the total acid-insoluble isotope was present in DNA as determined by resistance to alkaline hydrolysis (0.5 N KOH for 18 hr at 37 C).

Sedimentation analysis of RNA. RNA was analyzed in DMSO-sucrose gradients according to the method of Strauss et al. (21). The RNA samples (up to 50 μliters containing less than 40 μg of RNA) were mixed with equal volumes of 99% DMSO and layered on 0 to 15% sucrose gradients (in 99% DMSO, 10 mM LiCl, and 1 mM ethylenedinitrilotetraacetic acid in polyallomer tubes). Gradients were centrifuged in the SW 65 Spinco rotor in a Beckman L2 65B centrifuge for 10 hr at 60,000 rev/min and 25 C. Fractions of 0.15 ml were collected from each gradient through a hole punctured in the bottom of the tube. Samples (10 μliters) were assayed for acid-insoluble radioactivity (5) and (as noted) for alkali-resistant, acid-insoluble radioactivity. Appropriate fractions were then pooled, and the RNA was recovered by precipitation with 99% ethanol in the presence of 0.1 M NaCl and unlabeled yeast RNA (0.2 mg) as carrier. For direct use in hybridization reactions, the precipitated RNA was dissolved in 4 × SSC (0.6 M NaCl, 0.06 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS). For deoxyribonuclease treatment after RNA precipitation, RNA was dissolved in a solution (2 ml) containing 0.1 M NaCl, 5 mM $MgCl_2$, and 0.05 M tris(hydroxymethyl)aminomethane (*p*H 7.0), and was incubated with pancreatic deoxyribonuclease (50 μg/ml) for 1 hr at room temperature. Pronase (previously self-digested for 2 hr at 37 C) was then added to a final concentration of 50 μg/ml, and the incubation at room temperature was continued for 1 additional hr. After three extractions with phenol, RNA was again precipitated with ethanol and redissolved in 4 × SSC–0.1% SDS as above. Approximately 75 to 80% of acid-insoluble radioactivity in gradient fractions was recovered in the ethanol-precipitated RNA.

Determination of strand specificity of AAV(HSV) RNA. The strand specificity of in vivo-synthesized AAV(HSV) RNA was determined exactly as described previously for AAV(Ad) RNA (16). Briefly, hybridization-selected, ^{3}H-AAV(HSV)-specific RNA was annealed in free solution with nonlimiting amounts of ^{14}C-labeled, BUDR-containing plus or minus AAV DNA strands. (The plus and minus strand preparations were first self-annealed to eliminate any reactions which could result from cross-contamination of the strand species.) After hybridization, the mixtures were treated with pancreatic and T1 ribonucleases, and were banded in CsCl density gradients; the acid-insoluble radioactivity and density of gradient fractions were then determined.

DNA-RNA hybridization and hybridization inhibition. DNA-RNA hybridization reactions were carried out on nitrocellulose filters (13 mm) in 4 × SSC–0.1% SDS as described previously (16). Blank filters containing either no DNA or heterologous *Escherichia coli* DNA retained less than 0.005% of the added radioactivity. DNA-RNA hybridization inhibition tests were performed by a two-step procedure. In the first step, AAV-2 DNA (0.05 or 0.1 μg) immobilized on nitrocellulose filters was incubated without RNA or with increasing amounts of unlabeled, competing RNA in 0.6 ml of 4 × SSC-0.1% SDS at 67 C for 24 hr. In the second step, the RNA solution was removed, and the filters were rinsed twice with 4 × SSC and then incubated with saturating amounts of ^{3}H-labeled RNA in 0.6 ml of 4 × SSC-0.1% SDS for an additional 24 hr at 67 C. Blank filters containing no DNA or *E. coli* DNA were included in each vial. Hybridization data represent averages of duplicate reactions except for DMSO-sucrose gradients, with which only single reactions were performed with each fraction.

RESULTS

Under the described conditions of infection, maximal yields of labeled AAV RNA (i.e., up to 1 to 1.5% of the total incorporated isotope was present in AAV RNA) were obtained between 17 and 20 hr after infection with Ad2 as helper or between 13 and 16 hr with HSV-1 as helper (17; B. J. Carter, *unpublished data*). With either type of helper virus, little AAV RNA can be detected prior to 10 hr after infection (17). When HSV-1 is the helper, there is no increase in the AAV infectivity titer by 20 hr whereas with helper Ad2 up to a 1,000-fold increase in AAV titer is observed after the same interval (17).

Effect of chloroform on helper virus activity. Adenoviruses and AAV are insensitive to chloroform, which completely abolishes the infectivity of the herpesviruses (4). To eliminate the possibility that AAV RNA synthesis was induced by a contaminating adenovirus in the HSV-1 stock, we tested the sensitivity of helper function to

pretreatment with chloroform. Synthesis of AAV RNA between 12 and 20 hr after infection with either Ad2 or HSV-1 as helper is shown in Table 1. In the absence of chloroform pretreatment, the level of AAV RNA synthesized with each helper was similar (approximately 0.3% of the labeled RNA was AAV-specific). After chloroform extraction, however, the HSV-1 inoculum lost its ability to promote AAV RNA synthesis, whereas the same treatment had little effect on the helper function of Ad2. We therefore conclude that an adenovirus contaminant is not responsible for the observed helper activity of the HSV-1 stock.

Determination of strand specificity of AAV(HSV) RNA. Previous work in this laboratory demonstrated that in AAV(Ad)-infected cells AAV RNA is transcribed only from the thymidine-rich (minus) strand of AAV DNA (16). The inability of HSV-1 to act as a complete helper might be due to faulty transcription of the AAV genome. To investigate this possibility, hybridization-selected ^{3}H-AAV RNA from AAV(HSV)-infected cells labeled with ^{3}H-uridine at 13 to 16 hr was annealed with ^{14}C-labeled, BUDR-containing minus or plus strands of AAV DNA, and the resulting DNA-RNA hybrids were banded in CsCl density gradients (Fig. 1). Minus strands (Fig. 1A) and plus strands (Fig. 1B) banded at densities of 1.826 and 1.800 g/cc, respectively. The AAV RNA hybridized extensively with the minus strands, 37% of the added ^{3}H-RNA being recovered in the hybrid (Fig. 1A), whereas less than 1% of the added ^{3}H-RNA was associated with the plus strands (Fig. 1B). That the density of the minus strands was not appreciably altered by annealing with RNA can be accounted for by the presence of nonlimiting amounts of DNA in the annealing reactions and the low molecular weight (approximately 4*S*) of the hybridization-selected AAV RNA, equivalent to less than 5% of a single strand of AAV DNA. It was shown previously that the presence of BUDR in AAV DNA does not significantly affect formation of DNA-RNA hybrids (16). We conclude that the strand specificity of AAV RNA synthesized with helper HSV-1 is identical to that synthesized with helper Ad2 (16).

Comparison of AAV(HSV) and AAV(Ad) RNA by hydridization inhibition. Alternatively, the partial helper function of HSV-1 might be due to a failure to promote transcription of all of the AAV RNA species synthesized when Ad2 is the helper. Thus, some sequences of the AAV genome represented in AAV(Ad) RNA might be absent, or present in greatly reduced amount, in AAV(HSV) RNA. This possibility was tested in hybridization inhibition experiments. The amount of ^{3}H-AAV(Ad) RNA (labeled 15 to 24 hr after infection) required to saturate 0.1 μg of AAV-2 DNA was first determined (Fig. 2). DNA was saturated with an input of approximately 175 μg of ^{3}H-RNA (1.5×10^{6} counts/min). A two-step hybridization inhibition experiment was then performed, with the use of this amount of the same RNA. In the first step, filters containing 0.1 μg of AAV-2 DNA were incubated with unlabeled AAV(Ad) RNA or AAV(HSV) RNA, isolated 20 and 19 hr after infection, respectively. In the second step, the filters were incubated with the ^{3}H-AAV(Ad) RNA. The results (Fig. 3) demonstrate that ^{3}H-RNA binding was inhibited to the same extent by either AAV(Ad) RNA or AAV(HSV) RNA. In each case, inhibition

TABLE 1. *Effect of chloroform on HSV-1 helper function*

Helper virus[a]	Chloroform treatment of helper virus[b]	^{3}H-labeled RNA (counts/min)[c]		Input radioactivity bound to filter (%)
		Input[d]	Bound to filter[e]	
HSV-1	No	82,000	286	0.35
		331,000	850	0.26
HSV-1	Yes	216,000	2	0.001
		865,500	15	0.002
Ad2	No	73,900	140	0.19
		295,600	847	0.29
Ad2	Yes	218,400	773	0.35
		873,000	1,713	0.20

[a] Cultures (100 ml) of KB cells were infected with AAV-2 (heated at 56 C for 10 min) and the indicated helper virus.
[b] Helper viruses were treated with or without chloroform as described in Materials and Methods.
[c] RNA was isolated from cultures that were labeled with ^{3}H-uridine from 12 to 20 hr after infection.
[d] ^{3}H-RNA (12.0 or 50.0 μg) was incubated with filters containing 1 μg of AAV-2 DNA for 24 hr.
[e] Values for blank filters (0.001 to 0.002% of input radioactivity) have been subtracted.

was at least 90%. In control reactions, KB cell RNA produced little, if any, nonspecific inhibition.

The above experiment did not exclude the possibility that AAV(HSV) RNA contained additional AAV sequences not present in the AAV(Ad) RNA. To test this possibility, the hybridization inhibition experiment was repeated, but this time ^{3}H-AAV(HSV) RNA (labeled from 13 to 16 hr after infection) was used. Saturation of 0.05 μg of AAV-2 DNA (Fig. 4) was reached with about 140 μg of ^{3}H-AAV(HSV) RNA (3×10^6 counts/min). The hybridization inhibition experiment (Fig. 5) shows that annealing of the ^{3}H-AAV(HSV) RNA was also inhibited by at least 90 to 95% with either unlabeled AAV(HSV) RNA or AAV(Ad) RNA. These experiments indicate that the same AAV sequences are transcribed whether Ad2 or HSV-1 is used as the helper.

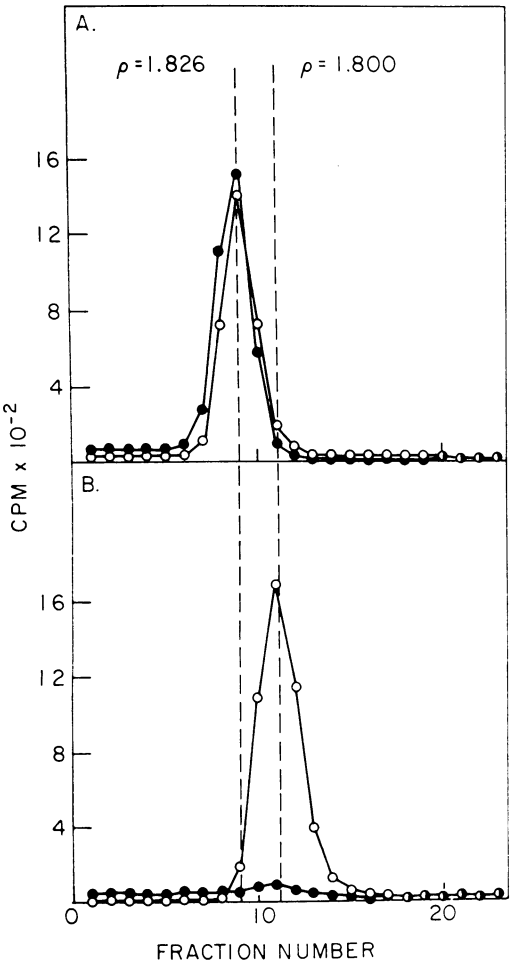

FIG. 1. *DNA strand specificity of hybridization-selected AAV(HSV) RNA. (A) ^{3}H-AAV(HSV) RNA (11,000 counts/min) incubated with 0.8 μg of ^{14}C-labeled minus strands. (B) ^{3}H-AAV(HSV) RNA incubated with 1.0 μg of ^{14}C-labeled plus strands. Specific activity of ^{14}C-DNA was 4,170 counts per min per μg (minus strands) and 4,900 counts per min per μg (plus strands). The specific activity of viral RNA was in excess of 40,000 counts per min per μg, since this is the specific activity of the total cell RNA preparation from which the AAV-RNA was selected. Recovery of the isotope in the gradients was: (A) ^{14}C, 98%; ^{3}H, 37%. (B) ^{14}C, 96%; ^{3}H, less than 1%. (○) Counts/minute of ^{14}C-DNA; (●) counts/minute of ^{3}H-RNA.*

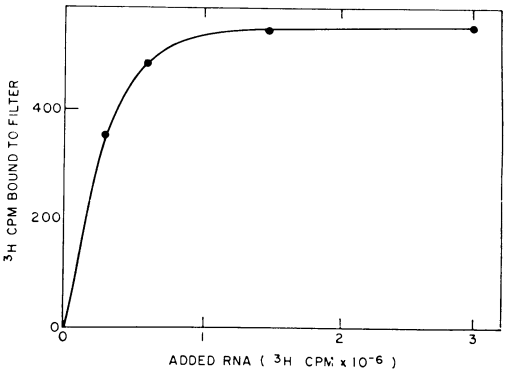

FIG. 2. *Saturation of AAV DNA with ^{3}H-AAV(Ad) RNA. Increasing amounts of ^{3}H-AAV(Ad) RNA were incubated with filters containing 0.1 μg of AAV-2 DNA for 24 hr at 67 C. Blank filter values have been subtracted.*

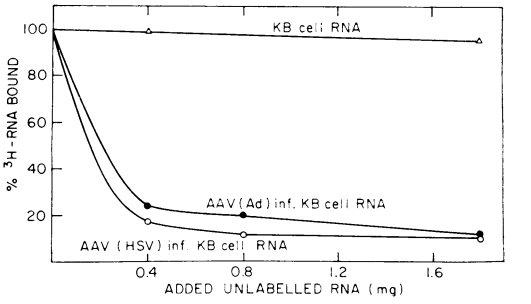

FIG. 3. *Inhibition of ^{3}H-AAV(Ad) hybridization by AAV(Ad) RNA and AAV(HSV) RNA. In the first step, the filters (containing 0.1 μg of AAV-2 DNA) were incubated with increasing amounts of unlabeled RNA from uninfected KB cells (△), AAV(Ad)-infected cells (●) or AAV(HSV)-infected cells (○). In the second step, the filters were incubated with nonlimiting amounts of ^{3}H-AAV(Ad) RNA. Per cent ^{3}H-RNA bound refers to the fraction of RNA bound relative to binding in the absence of any unlabeled RNA; i.e., the amount of radioactive RNA bound in the absence of unlabeled RNA is taken as 100%. Blank filter values have been subtracted.*

Molecular size of AAV-specific RNA. Velocity sedimentation in DMSO-sucrose gradients was used to measure the size of virus-specific RNA synthesized in AAV(Ad)- and AAV(HSV)-infected cells. The positions of viral RNA species in the gradients were determined by DNA-RNA hybridization.

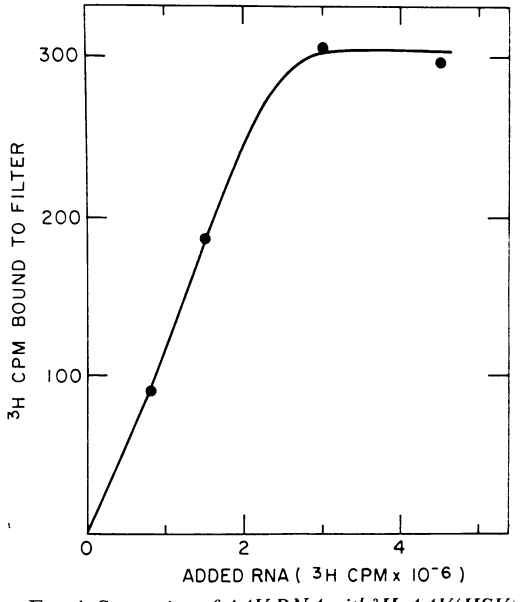

FIG. 4. *Saturation of AAV DNA with ^{3}H-AAV(HSV) RNA. Increasing amounts of ^{3}H-AAV(HSV) RNA were incubated with filters containing 0.05 μg of AAV-2 DNA for 24 hr at 67 C. Blank filter values have been subtracted.*

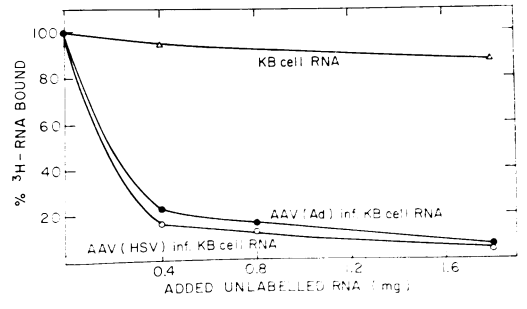

FIG. 5. *Inhibition of ^{3}H-AAV(HSV) RNA hybridization by AAV(Ad) and AAV(HSV) RNA. In the first step, the filters (containing 0.05 μg of AAV-2 DNA) were incubated with increasing amounts of unlabeled RNA from uninfected KB cells (△), AAV(Ad)-infected cells (●), or AAV(HSV)-infected cells (○). In the second step, the filters were incubated with non-limiting amounts of ^{3}H-AAV(HSV) RNA. The values for blank filters were subtracted. The amount of radioactivity bound to the filters was normalized as described in the legend to Fig. 3.*

Initially, portions of an AAV(Ad)-infected culture were labeled with ^{3}H-uridine from 14 to 17, 17 to 20, or 20 to 23 hr after infection. Equal amounts of RNA isolated at the end of each labeling period were mixed and sedimented in a DMSO-sucrose gradient. The overall sedimentation profile of the ^{3}H-labeled RNA is shown in Fig. 6A. The profiles of AAV and Ad2 RNA as determined by DNA-RNA hybridization are shown in Fig. 6B. The AAV RNA sedimented in a single peak at a slightly faster rate than 18S ribosomal RNA. [In these gradients, the ribosomal and transfer RNA species (28S, 18S, 5S, and 4S) are designated by sedimentation coefficients determined in the absence of DMSO.] Ad2 RNA was heterogeneous and mostly sedimented at rates equal to, and faster than, 28S ribosomal RNA. There was a small component of

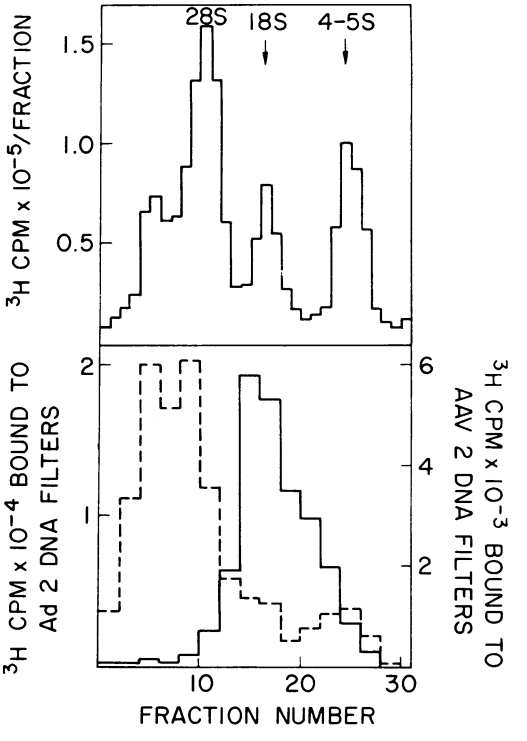

FIG. 6. *Sedimentation analysis of viral RNA synthesized in AAV(Ad)-infected cells. (A) Sedimentation profile of the ^{3}H-labeled RNA. The positions of 28S, 18S, and 4 to 5S RNA (indicated by arrows) were determined from sedimentation of RNA from uninfected cells in parallel gradients. (B) RNA recovered from pools of paired fractions from A was hybridized in vials with three filters containing 4 μg of AAV-2 DNA (solid line), 4 μg of Ad2 DNA (dashed line), or no DNA. Blank filter values have been subtracted. Sedimentation in this and succeeding figures is from right to left.*

Ad2 RNA sedimenting at about 5*S*, which may be 5*S* AAV RNA (13, 19).

In a similar experiment, equal amounts of RNA isolated from portions of an AAV(HSV)-infected culture labeled at 10 to 13, 13 to 16 and 16 to 19 hr were pooled and sedimented in DMSO-sucrose. The sedimentation profile of ^{3}H-labeled RNA (Fig. 7A) reflects the more rapid and extensive shutdown of ribosomal RNA synthesis in HSV-1-infected cells (7, 22) in comparison with that found in Ad2-infected cells (12). Also, induction of a 5*S* RNA component was not observed in HSV-1-infected KB cells (B. J. Carter and J. A. Rose, *unpublished data*). The mean size of AAV RNA synthesized in AAV(HSV)-infected cells (Fig. 7B) was similar to that synthesized in AAV(Ad)-infected cells (Fig. 6B). The HSV-1 RNA (Fig. 7B) showed a heterogeneous sedimentation profile consistent with observations by others (7, 22). No Ad2 RNA synthesis was detected (Fig. 7B), indicating that Ad2 in the AAV inoculum had been inactivated by heating (see Materials and Methods).

Experiments in which RNA was labeled for longer periods were also performed to determine whether AAV RNA is cleaved after transcription, a process known to occur with adenovirus RNA (10, 11). RNA was isolated from AAV(Ad)-infected cells labeled for 9 hr (from 14 to 23 hr after infection) and sedimented in DMSO-sucrose as shown in Fig. 8A. AAV RNA was again observed in a single peak sedimenting slightly

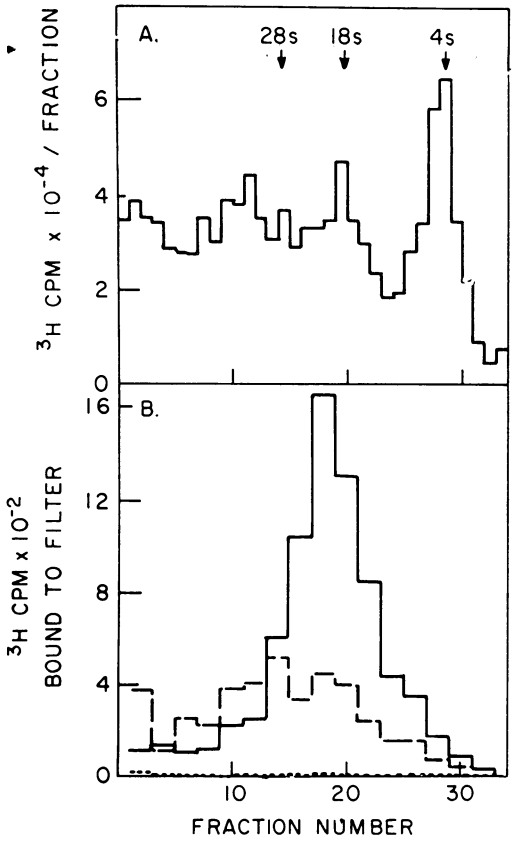

FIG. 7. *DMSO-sucrose sedimentation analysis of ^{3}H-RNA from AAV(HSV)-infected cells. (A) Sedimentation profile of the ^{3}H-labeled RNA. Arrows indicated the expected positions of 4S, 18S, and 28S RNA species. (B) RNA from pools of paired fractions was annealed in vials with three filters containing, respectively, 2 μg of AAV-2 DNA (solid line), 1.5 μg of HSV-1 DNA (dashed line), or 2 μg of Ad2 DNA (dotted line).*

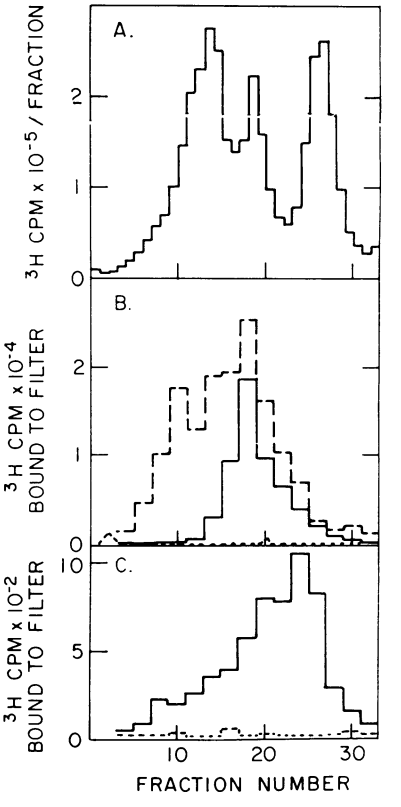

FIG. 8. *DMSO-sucrose sedimentation analysis of ^{3}H-RNA isolated from AAV(Ad)-infected cells. (A) Sedimentation profile of the ^{3}H-labeled RNA. Pairs of fractions were pooled, and the RNA was collected and annealed in vials with four filters containing, respectively, AAV-2 DNA (4 μg), Ad2 DNA (4 μg), KB cell DNA (8 μg) or E. coli DNA (4 μg). (B) Radioactivity annealing to filters containing AAV-2 DNA (solid line) or Ad2 DNA (dashed line). The level of acid-insoluble, alkali-resistant radioactivity in the gradient fractions from A is also shown (dotted line). (C) Radioactivity annealing to filters containing KB cell DNA (solid line) or E. coli DNA (dashed line).*

faster than 18S ribosomal RNA (Fig. 8B). Ad2 sedimented in a bimodal distribution with a major peak close to 18S and a smaller peak ahead of 28S ribosomal RNA (Fig. 8B). The bulk of RNA that hybridized with KB cell DNA was considerably smaller than 18S ribosomal RNA, whereas binding to a heterologous DNA (*E. coli* DNA) was not a significant (Fig. 8C).

Since RNA preparations analyzed in DMSO-sucrose gradients were not treated with deoxyribonuclease, it was necessary to be certain that radioactivity bound to the filters was not contained in DNA. Firstly, less than 0.02% of the acid-insoluble isotope in the RNA preparations was resistant to alkali (see Materials and Methods). Furthermore, this alkali-resistant, acid-insoluble radioactivity was not significant when compared with the amounts of radioactivity which hybridized with viral DNA (Fig. 8B). As a further check RNA isolated from AAV(Ad)-infected cells (labeled from 14 to 23 hr after infection) was sedimented in DMSO-sucrose (Fig. 9A) and, prior to hybridization, gradient fractions were treated with pancreatic deoxyribonuclease. The sedimentation profile of the AAV RNA (Fig. 9B) was comparable to that seen with the same RNA preparation in the absence of deoxyribonuclease treatment (Fig. 8B).

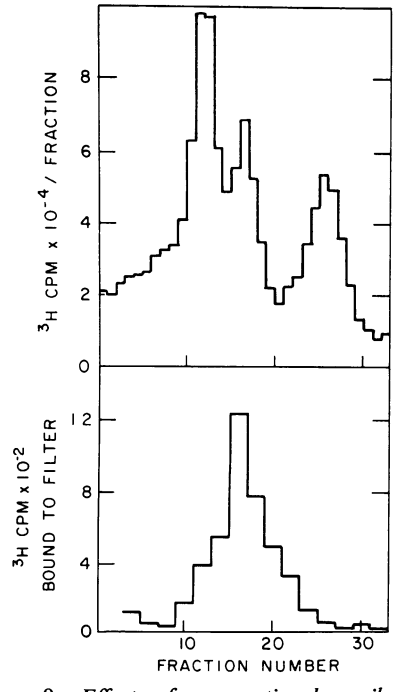

FIG. 9. *Effect of pancreatic deoxyribonuclease treatment on the DMSO-sucrose sedimentation profile of AAV RNA. (A) Sedimentation profile of ^{3}H-RNA isolated from AAV(Ad)-infected cells. RNA collected from paired fractions was treated with pancreatic deoxyribonuclease prior to incubating with filters containing AAV-2 DNA (4 μg). (B) Radioactivity annealing to the AAV-2 DNA. Nonspecific binding to filters containing 4 μg of E. coli DNA (included with each reaction) has been subtracted.*

Taking the molecular weight of 18S ribosomal RNA as 7.0×10^5 (20), the mean molecular weight of the AAV RNA is calculated (21) to be approximately 7.5×10^5. It seems likely that the observed peak of AAV RNA may contain more than one species which are not resolved in these experiments. This is suggested by comparison of the peaks of 18S ribosomal RNA (Fig. 6A) and AAV RNA (Fig. 6B). The AAV RNA is clearly more heterogeneous than 18S ribosomal RNA.

DISCUSSION

These studies indicate that the AAV RNA species synthesized with either adenovirus or herpesvirus as a helper were similar when compared by several criteria. Firstly, AAV RNA synthesized in AAV(HSV)-infected cells was transcribed only from the thymidine-rich strand of AAV DNA. This result is identical to that reported previously for AAV RNA synthesized in AAV(Ad)-infected cells (16). Secondly, hybridization inhibition experiments revealed no significant differences between the nucleotide sequences contained in AAV RNA synthesized in either AAV(Ad)- or AAV(HSV)-infected cells. These experiments were performed with RNA during periods when both AAV DNA and RNA synthesis were occurring at maximal rates and when infectious AAV particles were appearing in AAV(Ad)-infected cells. Finally, the sedimentation profiles of AAV RNA isolated from either AAV(Ad)- or AAV(HSV)-infected cells were similar. In both cases, AAV RNA had a mean molecular weight of approximately 7.5×10^5.

The basis for the difference between the complete and partial helper functions of adenovirus and herpesvirus, respectively, remains to be determined. Evidence reported here indicates that HSV-1 promotes normal AAV transcription, though subtle differences may not have been detected. Since AAV antigen is synthesized in HSV-1-helped cells (1, 4), it would also seem that HSV-1 does not block transport of AAV RNA into the cytoplasm. In addition, the level of AAV DNA synthesis with HSV-1 as helper is roughly comparable to that observed with Ad2 as helper (17). Others have reported production of infectious AAV DNA in AAV-1(HSV)-infected BSC-1 cells (6).

Although the above data do not rigorously exclude abnormalities in AAV DNA synthesis or RNA transcription when HSV-1 is the helper, other possible causes of the restricted helper

function of HSV-1 should be considered. For instance, in the presence of HSV-1, abnormal translation of AAV RNA may occur. Alternatively, assembly of AAV particles may fail to occur either because of a direct inhibitory effect by HSV-1 or, perhaps, because HSV-1 lacks a specific helper function required at this level.

Since the molecular weight of a single strand of the AAV DNA is 1.5×10^6 to 1.8×10^6 (9, 15), an estimated mean molecular size of 7.5×10^5 for AAV RNA is equivalent to 40 to 50% of the AAV genome. Taking this together with the apparent heterogeneity of AAV RNA when compared to 18S ribosomal RNA (Fig. 6), and further assuming that 100% of the minus strand is transcribed, we would estimate that as few as two or three AAV RNA species may be synthesized in infected cells. Recent studies indicate that at least two species can be resolved (B. J. Carter and J. A. Rose, *in preparation*). It is interesting that the AAV particle contains three proteins: a major component of molecular weight 62,000 and two minor components of molecular weights 87,000 and 73,000 (18). Thus, the AAV RNA observed in our experiments is large enough to specify any of these three proteins. It should be noted, as suggested previously, that all three proteins may not be specified by three individual AAV genes, since the total coding capacity required appears to exceed that of the AAV genome (18).

It is considered unlikely that the observed size of the AAV RNA results from degradation of a larger molecule during isolation. This conclusion is supported by the fact that adenovirus RNA isolated in the same preparations was several times larger than the AAV RNA. However, the immediate product of transcription from AAV DNA might be a larger molecule which is subsequently cleaved as, for instance, occurs with adenovirus RNA (10, 11). But comparison of the viral RNA species synthesized in AAV(Ad)-infected cells during 3- or 9-hr labeling periods indicated that, although adenovirus RNA was cleaved, no alteration in the size of AAV RNA was observed. This result suggests that either AAV RNA is not cleaved after transcription, or it is cleaved more rapidly than adenovirus RNA.

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