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Analysis of Parvovirus mRNA by Sedimentation and Electrophoresis in Aqueous and Nonaqueous Solution

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Adenovirus-associated virus (AAV)-specific RNA present in the cytoplasm of cells coinfected with a helper adenovirus was analyzed by sucrose gradient sedimentation and gel electrophoresis. In aqueous conditions both gels or gradients revealed three AAV RNA components corresponding to 30*S*, 27*S*, and 20*S* and having apparent molecular weights of 2.6×10^6, 1.75×10^6 to 1.8×10^6, and 0.9×10^6 to 1.0×10^6, respectively. In nonaqueous, denaturing solvents only the 20*S* AAV RNA species was observed. For this reason, and because they would be apparently significantly larger than a single AAV DNA strand, both the 30*S* and 27*S* species are believed to result from conformational or aggregation effects in the aqueous nondenaturing systems. It is concluded that only a single RNA molecule having a molecular weight of approximately 0.9×10^6 to 1.0×10^6 is synthesized by AAV.

This report describes a comparative analysis by sedimentation and electrophoresis in both aqueous and nonaqueous denaturing solvents of the stable cytoplasmic RNA transcribed from the genome of a defective parvovirus, adenovirus-associated virus (AAV).

Molecular weight estimations for single-stranded RNA may be complicated by conformational effects or by aggregation of molecular species. Because this is frequently an unrecognized problem, analyses should be performed in conditions which can be expected to provide data that reflects the chain length rather than conformational state of the RNA under consideration. The AAV RNA was therefore analyzed under denaturing conditions of 98% formamide or 99% dimethyl sulfoxide (Me_2SO), as well as in aqueous conditions, in an attempt to avoid these problems.

MATERIALS AND METHODS

Growth of cells and virus. KB-3 cells in a spinner culture at 37 C were coinfected with AAV type 2 (AAV-2) and adenovirus type 2 as helpers as described elsewhere (B. J. Carter and J. A. Rose, Virology, in press). RNA was labeled with [5-^{3}H]uridine (28 Ci/mmol) at a concentration of 10 μCi/ml for 4 h from 16 to 20 h after infection.

Isolation of cytoplasmic RNA. Cytoplasm from infected cells was obtained by disruption of cells in the nonionic detergent Nonidet P-40 (NP-40), and RNA was extracted by a hot phenol-detergent procedure as described elsewhere (Carter and Rose, in press).

Sedimentation analysis. Nonaqueous gradients (4.8 ml) contained 0 to 15% sucrose (Mann ultrapure) in 99% Me_2SO, 5 mM LiCl, and 0.5 mM sodium EDTA in polyallomer tubes. Samples containing less than 30 μg of RNA were diluted in 1 volume of Me_2SO and 2 volumes of dimethyl formamide in a final volume of less than 150 μliters and layered on the gradients which were centrifuged in a Spinco SW65 rotor at 60,000 rpm for 60 h at 23 to 25 C.

Aqueous sucrose gradients (11.6 ml) contained 10 to 30% sucrose in 50 mM Tris (pH 7.6), 40 mM sodium acetate, 1 mM sodium EDTA, and 0.2% SDS. RNA samples were layered in the same buffer, and the gradients were centrifuged in nitrocellulose tubes in an SW41 rotor at 40,000 rpm for 4 h at 20 C. Gradients were fractionated from the bottom, and portions of each fraction were taken for determination of total radioactivity in toluene-Triton-liquifluor scintillation fluid. The remainder of each fraction was used to detect AAV-specific RNA by hybridization (see below).

Gel electrophoresis. Several acrylamide gel systems were employed. All gels were polymerized in glass tubes (0.6 mm by 12 cm) to a height of 11 cm, and all electrophoreses were performed at 20 C. The gels were prepared as follows. (i) Composite agarose-acrylamide-SDS gels containing 0.5% agarose and 2% acrylamide cross-linked with 0.1% bis-acrylamide were prepared as described by Dingman and Peacock (6) except that the buffer system was 50 mM Tris, 40 mM sodium acetate, and 1 mM EDTA adjusted to pH 7.6 with acetic acid. In addition, 0.2% SDS was included in the gels and buffer. (ii) Urea gels were formed exactly as for the SDS gels, but with the addition of 2 M urea to both gels and buffer. Inclusion of higher concentrations of urea prevented gelation of

composite gels, prepared as described by Dingman and Peacock (6). (iii) Formamide gels were prepared essentially as described by Staynov et al. (18). The gels contained 3.6% acrylamide and were electrophoresed in 98% formamide containing 20 mM NaCl.

After electrophoresis gels were fractionated into 1-mm slices with a Joyce-Lobel gel slicer or 1.1-mm slices with stacked razor blades, and RNA was eluted from each slice by shaking at room temperature for 16 to 20 h with 4 × SSC (0.6 M sodium chloride, 0.06 M sodium citrate) and 0.1% SDS. Recovery of total radioactive RNA and viral-specific RNA was usually 85 to 90%. Viral-specific RNA was determined by hybridization as described below.

RNA-DNA hybridization. ^{3}H-labeled, viral-specific RNA present in gradient fractions or eluted from gel slices was detected by hybridizing to purified AAV DNA immobilized on 13-mm nitrocellulose filters as described fully elsewhere (4; Carter and Rose, in press). Briefly, RNA from each gradient fraction or gel slice was incubated with one filter containing 1 μg of AAV-2 DNA and one filter containing 1 μg of *E. coli* DNA as a heterologous control for nonspecific binding. Me_2SO gradient fractions were diluted with 4 volumes of 5 × SSC to yield final concentrations of 4 × SSC and 20% Me_2SO, and annealing was performed at 58 C for 30 to 40 h. Hybridization reactions with RNA from aqueous sucrose gradients or gel slices were performed in 4 × SSC–0.1% SDS at 68 C for 30 h. Radioactivity bound to the *E. coli* DNA-containing filters (usually less than 20 counts/min) was subtracted.

RESULTS

The ^{3}H-labeled, AAV-specific RNA present in the cytoplasm of infected cells, after a 4-h labeling period, sedimented in a Me_2SO-sucrose gradient mainly as a discrete 20*S* species, although some heterogeneous material sedimented more slowly than 18*S* rRNA (Fig. 1). These data are consistent with a previous, low resolution Me_2SO gradient analysis of AAV RNA present in total cell RNA (4). The trailing heterogeneous AAV RNA represents nonpolysomal, degraded species (Carter and Rose, in press). The presence of a larger proportion of radioactivity in 18*S* rRNA relative to 28*S* rRNA does not reflect breakage of RNA but is accounted for partly by the more rapid labeling of 18*S* rRNA (9) and also by the partial shutdown of rRNA synthesis by the helper adenovirus under the conditions of these experiments (Carter and Rose, in press).

Analysis of similar RNA preparations by electrophoresis in composite acrylamide-agarose gels in a Tris-acetate-EDTA buffer containing SDS apparently revealed three species of AAV RNA corresponding approximately to 20*S*, 27*S*, and 30*S* (Fig. 2). Results similar to those in Fig. 2 were also observed when the composite gels were electrophoresed in the Tris-borate-EDTA (pH 8.5) buffer of Dingman and Peacock (6) in the absence of SDS (data not shown). Because the 27*S* and 30*S* AAV RNA species were not observed during sedimentation in Me_2SO (Fig. 1), in which conditions RNA should be fully denatured (18), they appeared to result from the effects of either conformation or aggregation upon the electrophoretic mobility of AAV RNA. In an attempt to overcome this problem, the RNA was analyzed in composite gels in 2 M urea. These conditions, although not very strongly denaturing, are sufficient to prevent anomalous electrophoretic migration of vaccinia mRNA species (A. Ball, personal communication). Prior to analysis in these gels, the RNA preparation was first incubated at 37 C with 8 M urea. However, electrophoresis in the presence of 2 M urea still showed, in addition to the 20*S* AAV RNA, a significant amount of the 27*S* species, but the 30*S* component was less

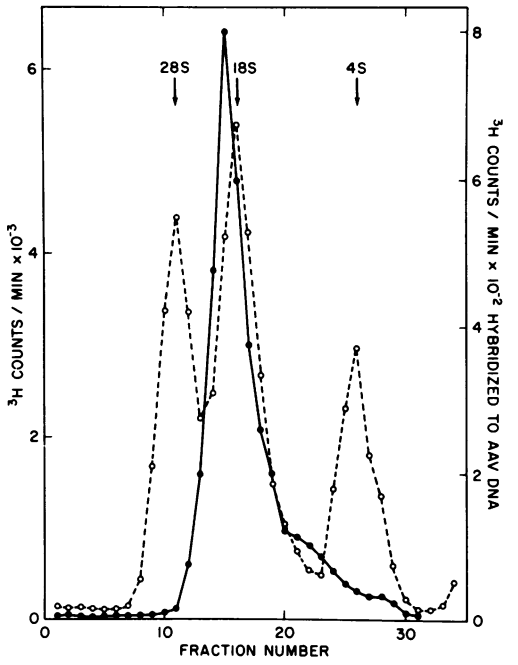

FIG. 1. *Sedimentation of ^{3}H-labeled, cytoplasmic RNA in Me_2SO-sucrose. Cytoplasmic RNA (30 μg), isolated from infected cells after a 4-h label, was sedimented in a Me_2SO-sucrose gradient. Fractions (150 μl) were collected from the bottom of the tube, and 20-μliter portions were taken for determination of total radioactivity. The remainder of each fraction was used to measure AAV-specific, ^{3}H-labeled RNA. All procedures used are described in the text. Sedimentation is from right to left.* ○, *Profile of total ^{3}H-labeled, cytoplasmic RNA;* ●, *^{3}H-labeled, AAV-specific RNA.*

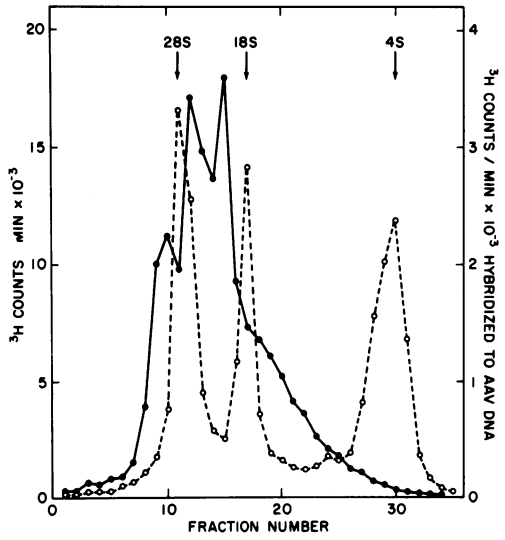

FIG. 2. *Electrophoresis of cytoplasmic RNA in a composite acrylamide-agarose-SDS gel. A sample (16 μg) of an RNA preparation similar to that in Fig. 1 was electrophoresed in 2% acrylamide gels containing 0.5% agarose in Tris-acetate-EDTA-SDS (pH 7.6) buffer for 3 h at 4.5 V/cm. The gel was cut into 1-mm slices, and RNA was eluted from each slice in 4 × SSC-0.1% SDS. Portions (20 μliters) of each eluate were taken for determination of total radioactivity, and the remaining portions were pooled in pairs and used for assay of viral-specific, ^{3}H-labeled RNA. All procedures were as described in the text. ○, Total ^{3}H-labeled RNA; ●, AAV-specific, ^{3}H-labeled RNA.*

apparent (Fig. 3). Since RNA is probably not fully denatured under the conditions of the aqueous gel analyses (Fig. 2 and 3), further electrophoresis was performed in 3.6% acrylamide gels in 98% formamide (18). In 98% formamide, as in Me_2SO, RNA should approach a fully denatured state (10). Electrophoresis in the formamide gels revealed a single major species of AAV RNA equivalent to 20*S* and a small residual 27*S* peak (Fig. 4). Analysis of RNA in lower concentration gels in formamide did not resolve any additional AAV RNA species (Carter and Rose, in press).

Although it cannot be rigorously excluded, it seems unlikely that the absence of the 27*S* and 30*S* components in formamide or Me_2SO was caused by physical or chemical breakage or nuclease degradation of AAV RNA because no significant increase in the proportion that was smaller than 18*S* rRNA was observed in these solvents when compared to the aqueous analyses. Also, any breakage occurring would be expected to produce random-sized fragments rather than a discrete 20*S* species. That the presence of the 27*S* and 30*S* AAV RNA species was a function of the ionic conditions and solvent employed and did not result from some intrinsic difference in the characteristics of gels compared to gradients is shown by the experiment described in Fig. 5. Cytoplasmic RNA from infected cells was analyzed in aqueous sucrose gradients under the same ionic conditions as the composite SDS gel analysis detailed in Fig. 2. In the aqueous sucrose gradients (Fig. 5), significant amounts of AAV RNA sedimented at 27*S* and 30*S*, in addition to the 20*S* peak. This result was similar to that obtained in the electrophoresis analysis in the presence of SDS (Fig. 2).

From the several sedimentation and electrophoretic analyses performed (Fig. 1 through 5), the apparent molecular weights of each AAV RNA species were calculated (Table 1). All measurements in both aqueous and nonaqueous conditions yielded an estimated molecular weight for the 20*S* species of 0.9×10^6 to 1.0×10^6. The apparent molecular weights of the 27*S* and 30*S* species observed in the aqueous analy-

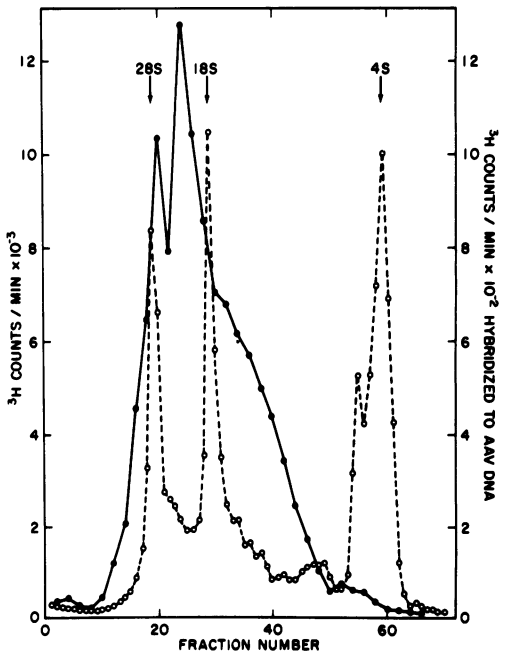

FIG. 3. *Electrophoresis of infected-cell cytoplasmic RNA in a composite gel in the presence of 2 M urea. All conditions were as described in Fig. 2 except that the gels and buffer contained 2 M urea. A 13-μg sample of RNA was adjusted to 8 M urea with solid urea and was incubated at 37 C for 3 min prior to layering on the gel and electrophoresing for 2.5 h at 7.5 V/cm. Gels were cut into 1-mm slices and analyzed as described in the legend to Fig. 2. ○, ^{3}H-labeled, total cytoplasmic RNA; ●, ^{3}H-labeled, AAV-specific RNA.*

ses were estimated to be approximately 1.75×10^6 to 1.8×10^6 and 2.6×10^6, respectively. It is interesting that these values appear to be multiples of the estimated size of the 20S RNA.

DISCUSSION

The experiments reported here suggest that a single, stable 20S RNA species is transcribed from AAV DNA, since the 27S and 30S species were not observed in conditions known to favor RNA denaturation. The presence of 27S and 30S components of AAV RNA in aqueous conditions is therefore taken to reflect conformational differences or more probably aggregation of RNA rather than the presence of AAV RNA with strands which were longer than that of the 20S species. Because it is difficult to be certain that absolutely all formal secondary structure is removed from RNA even at high concentrations of formamide or Me_2SO, the residual 27S AAV RNA component observed in the formamide gel analysis probably represents incomplete denaturation of some AAV RNA molecules.

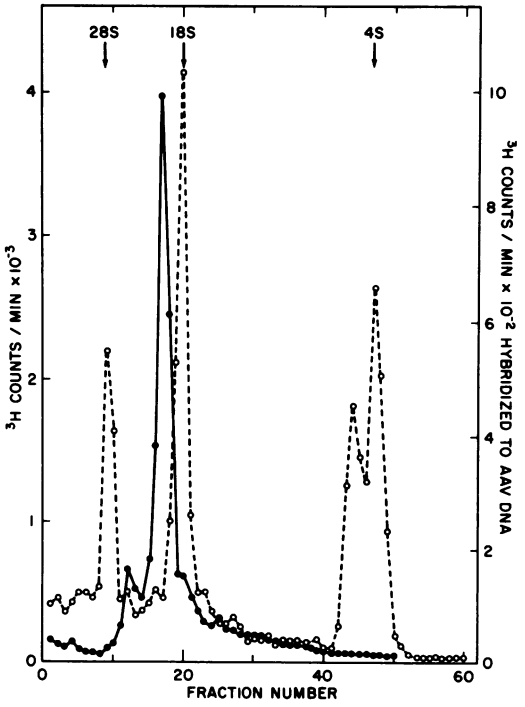

FIG. 4. *Analysis of infected-cell cytoplasmic RNA in a formamide gel. A 20-μg sample of an RNA preparation similar to that in Fig. 1 was electrophoresed in a 3.6% acrylamide gel in 98% formamaide for 5 h at 9.1 V/cm as described in the text. The gel was sliced into 1.1-mm slices at 4 C with a stack of razor blades, and RNA was eluted and analyzed as in Fig. 2 and 3.* O, *^{3}H-labeled, total cytoplasmic RNA;* ●, *^{3}H-labeled, AAV-specific RNA.*

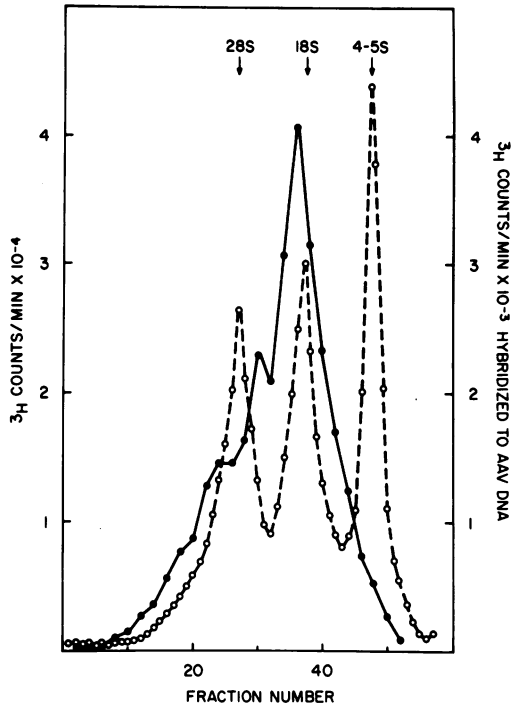

FIG. 5. *Sedimentation of ^{3}H-labeled cytoplasmic RNA in an aqueous sucrose gradient. An RNA preparation (30 μg) similar to that in Fig. 1 was sedimented in a 10 to 30% sucrose gradient in Tris-acetate-EDTA-SDS (pH 7.6) buffer. 20-μliter portions of each fraction (240 μliter) were taken for determination of total radioactivity. Remaining portions of each fraction were pooled in pairs and used for measurement of AAV-specific radioactivity.* O, *Total ^{3}H-labeled RNA;* ●, *AAV-specific, ^{3}H-labeled RNA.*

The above conclusions are consistent with other studies on AAV. First, the apparent molecular weights of the 30S and 27S species (Table 1) are significantly larger than that of an entire single strand of AAV DNA of which the molecular weight is 1.35×10^6 to 1.4×10^6 (8, 11). Secondly, we have demonstrated, from studies of AAV DNA-RNA hybrids formed in solution, that only 70 to 75% of the AAV DNA minus strand and none of the plus strand is represented in stable RNA transcripts (3; B. J. Carter and G. Khoury, manuscript in preparation). This places a maximal value on the expected size of AAV RNA of approximately 0.95×10^6 to 1.0×10^6, which is in good agreement with the estimated size of the 20S AAV RNA.

Whether the observed conformational effects reflect interstrand aggregation of RNA mole-

TABLE 1. *Apparent molecular weights of cytoplasmic AAV-2 RNA species determined by sedimentation or electrophoresis*[a]

Apparent S value	Mol wt				
	Sedimentation[b]		Electrophoresis[c]		
	SDS-sucrose	Me_2SO-sucrose	SDS-agarose-acrylamide	Urea-agarose acrylamide	Formamide-acrylamide
30S	2.5×10^6		2.6×10^6		
27S	1.75×10^6		1.8×10^6	1.75×10^6	
20S	0.9×10^6	0.9×10^6	0.95×10^6	1.0×10^6	0.95×10^6

[a] Molecular weights were calculated from the data shown in Fig. 1 through 5. The following molecular weights were taken as standards: 28S rRNA, 1.9×10^6; 18S rRNA, 0.71×10^6 (13); and 4S RNA, 2.6×10^4 (2).

[b] For the aqueous sucrose gradient (SDS-sucrose), the apparent molecular weights were computed using the cell 28S and 18S rRNA as standards. For the Me_2SO-sucrose gradients, molecular weights were computed by the method of Strauss et al. (19).

[c] For all electrophoretic analyses, the apparent molecular weights were determined assuming, for all RNA species, a linear relationship between electrophoretic mobility (distance migrated) and the logarithm of the molecular weight (2, 6, 18). In all the gel systems employed, a linear relationship was obtained for the following molecular weight standards: 28S rRNA, 18S, rRNA, 4S RNA, and adenovirus VA RNA (Carter and Rose, in press).

cules or intrastrand base-pairing or base-stacking is not clear. The 27S and 30S components might result from aggregation with ribosomal RNA, but similar results were obtained in an analysis of immunoglobulin light-chain mRNA purified by specific immunoprecipitation and oligo(dT) cellulose chromatography to remove rRNA (16). It is possible that some of the conformational effects in aqueous solution might be related to the presence of polyadenylate sequences in AAV RNA (Carter, manuscript in preparation), although phage ϕx174 mRNA, which has no polyadenylate sequence, also aggregates in aqueous solution (17). Studies on both bacteriophage mRNA (17) and globin mRNA (13) have indicated that the use of phenol in RNA purification may promote aggregation. Several other groups also have recently analyzed the effects of RNA secondary structure on molecular weight estimates obtained in aqueous and nonaqueous solutions (1, 7, 12, 15). Kolakofsky et al. (12) suggested that analysis of Sendai virus RNA in 99% Me_2SO may yield aberrantly low molecular weight estimates due to different degrees of denaturation of the Sendai and marker RNAs used. This argument seems less likely to apply to the analysis of AAV RNA since the size of the AAV RNA observed in nonaqueous conditions is in excellent agreement with the proportion of the AAV DNA which is stably transcribed (see above).

The experiments reported here emphasize the importance of analyzing RNA in fully denaturing conditions to avoid incorrect molecular weight estimates due to the conformational state of the molecules. In addition, this work, together with other analyses (3; Carter and Khoury, manuscript in preparation), illustrates the value of obtaining size estimations of RNA employing procedures, such as molecular hybridization, which are less influenced by RNA conformation than are procedures involving sedimentation or electrophoresis. Thus, hybridization of an mRNA species to a DNA fragment of defined size and which contains the gene for the given RNA may be a useful general procedure for obtaining estimates of RNA size. For DNA viral genomes, fragments of defined size can be readily obtained by cleavage with restriction endonucleases (5).

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