JOURNAL OF VIROLOGY, August 1976, p. 325-330
Vol. 19, No. 2

Printed in U.S.A.

Encephalomyocarditis Virus RNA: Variations in Polyadenylic Acid Content and Biological Activity

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Received for publication 24 March 1976

Encephalomyocarditis (EMC) viral RNA was isolated from purified virus grown in Ehrlich ascites tumor cells. The viral RNA was found to contain polyadenylic acid [poly(A)] regions that were very heterogeneous in length. Chromatography of the EMC viral RNA on oligo(dT)-cellulose columns separated the RNA into three distinct fractions (peaks 1 to 3). Approximately 20% of the EMC viral RNA appeared as peak 1, 40% as peak 2, and 40% as peak 3. The RNA in each fraction appeared to be intact as shown by co-sedimentation with 35*S* unfractionated EMC viral RNA in SDS-sucrose density gradients. Approximately 95 to 100% of peaks 1 and 3, and 60 to 70% of peak 2, reappeared at the same elution position after rechromatography on oligo(dT)-cellulose. The RNA in peak 1 contained poly(A) with an average length of 16 nucleotides, peak 2 contained poly(A) with an average length of 26 nucleotides, and peak 3 contained an average of 74 nucleotides in its poly(A) region. The distribution in the three fractions, as well as the average length of the poly(A) moieties, was relatively unaffected by changes in the cell suspension medium used during infection. Finally, each of the three viral RNA fractions was assayed for biological activity using an infectious RNA assay on L-cell monolayers. Infectivity of the viral RNA was found to increase with poly(A) length, with peak 3 viral RNA being approximately 10 times more infectious than peak 1 viral RNA.

3'-Polyadenylic acid [poly(A)] has been detected on virus-specific mRNA of virtually all animal viruses (17) except reovirus (20). Since viruses that replicate solely in the cytoplasm of mammalian cells, for example, picornaviruses, also contain poly(A), it has been of some interest to utilize these kinds of viruses to investigate possible cytoplasmic functions for poly(A) in viral replication and protein synthesis. Data from studies on poliovirus poly(A) (18) have failed to disclose any role for poly(A) in viral protein synthesis. However, these authors do suggest that poly(A) may be important in viral RNA replication, since its removal causes a 10-fold reduction in infectivity (16).

We have investigated the structure and function of poly(A) in another picornavirus, encephalomyocarditis virus (EMC). EMC RNA was found to contain poly(A) moieties that were very heterogeneous in size, in accord with the results of Gillespie et al. (4). By using oligo(dT)-cellulose chromatography it was possible to isolate subfractions of the EMC RNA which varied considerably in both their poly(A) content and biological activity.

MATERIALS AND METHODS

Materials. Actinomycin D, pancreatic RNase A, and RNase T_1 were purchased from Sigma; Fischer medium, in the powdered form, was from GIBCO; [8-^{3}H]adenosine (21 Ci/mmol) and [5-^{3}H]uridine (28 Ci/mmol) were obtained from Schwarz/Mann; [^{32}P]orthophosphate was from Amersham-Searle; oligo(dT)-cellulose, T-3 grade, was from Collaborative Research, Inc.; and EMC virus originated from Searle.

Growth and labeling of virus. Ehrlich ascites tumor cells, which had been growing intraperitoneally for 8 days in male BD/F mice, were harvested and washed 8 to 10 times to remove contaminating erythrocytes (12). The cells were then infected by incubating 10^8 cells/ml with 8,000 hemagglutination units of EMC virus per ml for 45 min at 4°C to allow virus adsorption. This gives an input multiplicity of 20 to 30 PFU/cell. The culture was then diluted 10-fold with cold Fischer medium (supplemented with 100 U of both penicillin and streptomycin per ml) and made 5 μg/ml in actinomycin D. The resulting 5% cell suspension was transferred to a conical flask, whose volume was 10 times that of the culture volume, and rotated at 90 rpm in a 37°C water bath. At 3 h postinfection, 2 μCi of [^{3}H]adenosine or [^{3}H]uridine per ml was added. Virus replication was terminated at 9.5 h postinfection by freezing the culture at −20°C. If [^{32}P]orthophosphate was used to label the virus, the infection was carried out as described above except that Earle balanced salt solution (BSS) (phosphate free) was substituted for Fischer medium, and 10 μCi of [^{32}P]orthophosphate per ml was added at 3 h postinfection.

Virus purification and RNA extraction. EMC vi-

rus was purified according to the polyethylene glycol precipitation method of Kerr and Martin (6), except that the CsCl step was omitted in most virus preparations. The viral RNA was extracted from the purified virions with 45°C phenol in the presence of 0.5% deoxycholate and 1 mM EDTA (5). The RNA was then precipitated twice with ethanol and stored as a precipitate in ethanol at −20°C until use, as it was observed that EMC RNA was somewhat unstable in aqueous solution.

SDS-sucrose sedimentation analysis. A small sample of the radioactive viral RNA suspension was centrifuged at 12,000 × *g*, and the RNA pellet was suspended in 0.5 ml of SDS-gradient buffer (50 mM NaCl, 1 mM EDTA, 10 mM Tris-hydrochloride [pH 7.4], and 0.2% SDS), which contained 120 μg of cytoplasmic RNA isolated from Ehrlich ascites cells (12). The RNA was then layered over a 12-ml, 5 to 20% linear sucrose gradient in SDS-gradient buffer. Centrifugation was performed using an SW41 rotor at 35,000 rpm at 24°C for 4.75 h in a Beckman L-265B centrifuge. After centrifugation, 0.5-ml fractions were collected from the bottom. Absorbancy at 260 nm was measured, and then each sample was made 5% in trichloroacetic acid and chilled. The resulting precipitates were collected on glass-fiber filters (Whatman GF/A) and counted in 10 ml of a toluene-based scintillation fluid using a scintillation counter (Beckman LS-330).

Oligo(dT)-cellulose chromatography. Fractionation of viral RNA on oligo(dT)-cellulose was carried out essentially according to the methods of Aviv and Leder (1). A column of oligo(dT)-cellulose (0.7 by 2.5 cm) was poured and prepared as detailed. The column was first washed with 5 ml of wash solution (0.1 N NaOH and 0.5 M NaCl). The column was then equilibrated with binding buffer (0.5 M NaCl, 1 mM EDTA, 10 mM Tris-hydrochloride, pH 7.5, and 0.2% SDS), and 120 μg of Ehrlich ascites cytoplasmic RNA was passed onto it. This was eluted with 5 ml of elution buffer II (1 mM EDTA, 10 mM Tris-hydrochloride, pH 7.5, and 0.2% SDS), and the column was reequilibrated with binding buffer. This series of steps was repeated each time before the fractionation of viral RNA, to presaturate nonspecific binding sites and improve reproducibility of results (J. A. Bantle, I. H. Maxwell, and W. E. Hahn, submitted for publication). The viral RNA, in 1.0 ml of binding buffer, was then applied to the column. This was followed by seven 1.0-ml portions of binding buffer, eight 1.0-ml portions of elution buffer I (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-hydrochloride, pH 7.5, and 0.2% SDS), and four 1.0-ml portions of elution buffer II. One-milliliter fractions were collected and analyzed for either absorbancy at 260 nm or radioactivity. The material from the peak fractions was pooled, and the SDS was removed by chilling the sample to 0°C followed by centrifugation. The RNA was subsequently isolated by ethanol precipitation in the presence of 0.15 M potassium acetate (pH 5.3).

RNase degradation. Viral RNA samples, labeled with [^{3}H]adenosine, were removed from the ethanol by centrifugation and dissolved in 1.8 ml of distilled water, which was subsequently divided into two 0.9-ml portions. A 10× mixture of RNase T_1 (500 U/ml) and RNase A (100 μg/ml) in 0.1 ml of 2 M NaCl-100 mM Tris-hydrochloride (pH 7.5) was added to the experimental tube, and 0.1 ml of buffer was added to the control tube (11). The reactions were run at 37°C for 30 min, after which 1.5 ml of ice-cold 10% trichloroacetic acid and 0.5 ml of carrier RNA (200 μg of yeast RNA per ml) were added. Precipitates were collected on glass-fiber filters and counted. [^{3}H]poly(A) (Miles Laboratories) and [^{3}H]-tRNA were run as controls with each series of reactions.

Infectivity and hemagglutination assays. The hemagglutination titer of virus preparations was determined by microtiter analysis according to Martin et al. (9). To determine the PFU titer, confluent monolayers of L-929 cells were prepared in tissue culture dishes (60 by 15 mm; Falcon Plastics). The nutrient medium, Earle minimum essential medium (MEM) supplemented with 10% calf serum, was removed and the monolayers were washed twice with warm phosphate-buffered saline. A 0.2-ml portion of an appropriate dilution of viral RNA was added, and the monolayers were incubated at 37°C for 30 min. The inoculum was then removed and the cells were covered with 2.5 ml of 45°C 1% agar (Difco certified) in MEM. After the agar had hardened, 2.5 ml of MEM was added and the plates were incubated at 37°C for 48 to 72 h in the presence of 5% CO_2. Monolayers were then stained for 2 h with 0.01% neutral red, and the plaques were counted.

RESULTS

EMC RNA purification. EMC virus was grown in 5% suspensions of Ehrlich ascites tumor cells in Fischer medium for leukemic mouse cells. This particular medium was used because it affords a far greater in vitro lifetime for Ehrlich ascites cells, even in the absence of serum, than do more traditional cell suspension media (2). The yield of virus grown in this manner was 2 to 3 times greater than that using other media.

The virus was purified, and the RNA was subsequently extracted according to the methods of Kerr and Martin (6). A sedimentation profile of the purified EMC RNA is presented in Fig. 1. A single peak of viral RNA sedimented at about 35*S*. There was little evidence of lower-molecular-weight RNA species that might correspond to either viral RNA breakdown products or contaminating host cell species. An identical viral RNA sedimentation pattern was obtained by monitoring absorbancy at 260 nm, in the absence of marker RNA species.

EMC RNA fractionation according to poly(A) content. Most polyadenylated RNA species contain poly(A) that is highly heterogeneous in size. Therefore, by using conventional RNA fractionation techniques, it should be possible to select for differentially adenylated pop-

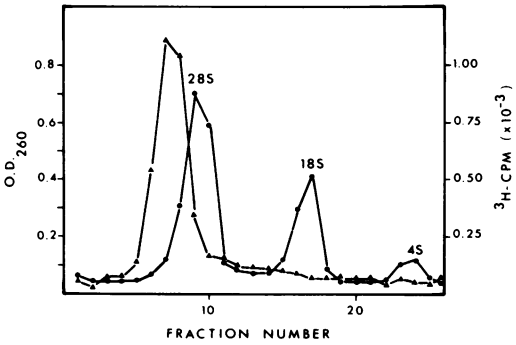

FIG. 1. *Sedimentation analysis of EMC RNA. ^{3}H-labeled EMC RNA was co-sedimented with unlabeled Ehrlich ascites cell cytoplasmic RNA as described in Materials and Methods. Absorbancy at 260 nm (●); trichloroacetic acid-precipitable counts per minute (▲).*

ulations of EMC RNA. Several different methods were examined for their ability to fractionate EMC RNA, including benzoylated cellulose (13), nitrocellulose filters (7), and poly(U)-glass-fiber filters (14). Although all of these procedures separated the bulk EMC RNA into a bound and unbound fraction, they all exhibited one or more of the following problems: low recovery, breakdown of EMC RNA, and lack of specificity.

The method we have found to be most satisfactory is oligo(dT)-cellulose chromatography. Before fractionation of EMC RNA, the columns were always presaturated with ribosomal RNA to reduce nonspecific binding to lignins in the cellulose and improve recovery. Figure 2 demonstrates that EMC RNA can be fractionated into three fractions on oligo(dT)-cellulose: peak 1, 2, and peak 3. The specificity of binding was relatively good, in that 95 to 100% of peaks 1 and 3 was found to rechromatograph in the same position. Only 60 to 70% of peak 2 RNA rechromatographed as peak 2, so it was always repassed at least once prior to further analysis to reduce the level of contaminating species. The absolute proportion of RNA in each of the three peaks varied slightly between different preparations, but the usual RNA distribution was approximately 20% in peak 1, 40% in peak 2, and 40% in peak 3.

Since EMC RNA appeared to be somewhat unstable in solution, it was important to determine the amount of degradation caused by oligo(dT)-cellulose chromatography. When each of the three peaks was centrifuged after fractionation, they still sedimented with a velocity of 35S, indicating there was little if any degradation (Fig. 3). EMC RNA breakdown was accelerated if the SDS was omitted from the buffers used during chromatography.

Poly(A) content of EMC RNA. The usual method for measuring the poly(A) content of an mRNA is to determine the percentage of the adenylic acid residues in the RNA that are resistant to degradation by a mixture of RNase A and T_1. To utilize this procedure, it is necessary to know the number of adenylic acid residues present in the RNA molecule to calculate percentage of resistance. Therefore, ^{32}P-labeled EMC RNA was hydrolyzed with KOH, and the resulting nucleotides were separated by pH 3.5 paper electrophoresis (12). By this method the nucleotide composition was determined to be: UMP, 24.5%; GMP, 24.4%; AMP, 26.9%; and CMP, 24.2% (data not shown). This agrees well with previously published values (3). If we assume that EMC RNA (molecular weight, 2.7×10^6) contains about 8,000 nucleotides, this yields an estimate of 2,160 adenylic acid residues.

Utilizing this information, analytical RNase degradation (11) was carried out on an oligo(dT)-cellulose-fractionated EMC RNA preparation that was labeled with [^{3}H]adenosine. The three EMC RNA fractions were found to vary markedly in their poly(A) contents, with the RNAs in peaks 1, 2, and 3 containing poly(A) stretches with average lengths of 16, 26, and 74 nucleotides, respectively (Table 1). The average poly(A) length of 61 nucleotides for unfractionated EMC RNA is roughly equivalent to that reported for polioviral poly(A) isolated from purified virions (22). In contrast, the EMC RNA poly(A) regions are of different size than those reported for mengovirus (10) and rhinovirus (11). Also note that this method, oligo(dT)-cellulose chromatography, yields a poly(A)-de-

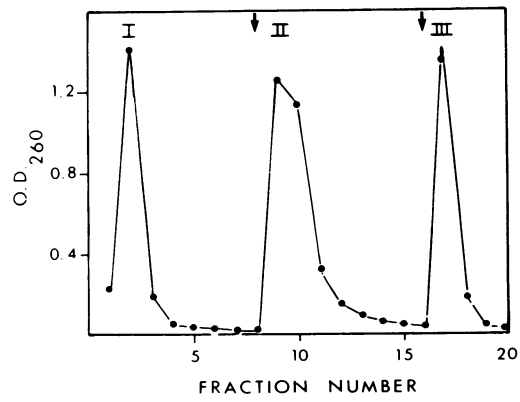

FIG. 2. *Oligo(dT)-cellulose fractionation of EMC RNA. EMC virus was purified, the RNA was extracted, and an RNA sample was chromatographed on an oligo(dT)-cellulose column as described in Materials and Methods. Arrows denote buffer changes.*

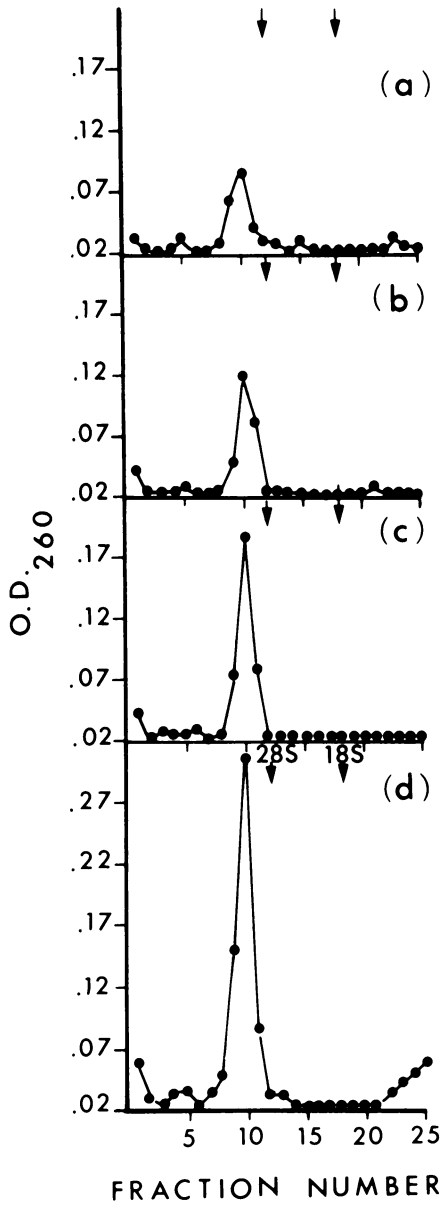

FIG. 3. *Sedimentation profiles of oligo(dT)-cellulose-fractionated EMC RNA. Unlabeled EMC RNA was fractionated on oligo(dT)-cellulose. The RNA from each peak was ethanol precipitated. A portion was centrifuged out of the ethanol and suspended in 0.5 ml of SDS-gradient buffer. Sucrose density gradient centrifugation was performed. An additional gradient containing Ehrlich ascites cell cytoplasmic RNA was run as a reference. (a) Peak 1 EMC RNA; (b) peak 2 EMC RNA; (c) peak 3 EMC RNA; (d) unfractionated EMC RNA.*

ficient fraction (peak 1) comparable to that produced by enzymatic treatment (16), i.e., containing residual poly(A) of an average length of 10 to 15 nucleotides.

To check both the specificity of the RNase degradation reaction and the base composition of the EMC RNA poly(A), the following experiment was performed. Unfractionated ^{32}P-labeled EMC RNA was subjected to RNase degradation, and then the resistant polynucleotide stretches were isolated on benzoylated cellulose under ionic conditions that will bind poly(A)-containing RNA (13). The bound RNA was then eluted and hydrolyzed with 0.3 N KOH, and its base composition was determined. Over 99% of the recovered radioactive nucleotides co-electrophoresed with AMP (Table 2), indicating that the EMC RNA contains few if any other nucleotides interspersed in its poly(A) region.

Media effects on EMC RNA polyadenylation. There has been some question as to whether the cell suspension medium in which a virus is grown can modulate the extent of polyadenylation. We have examined this possibility because the methods used here not only allow an estimation of poly(A) length but also give the distribution of the various poly(A) size

TABLE 1. *RNase degradation analysis of oligo(dT)-cellulose-fractionated EMC virus RNA*[a]

Oligo(dT)-cellulose fraction	% RNase resistance	Avg poly(A) length (nucleotides)
Unfractionated	2.41	60.7
Peak 1	0.73	15.8
Peak 2	1.19	25.7
Peak 3	3.44	74.4

[a] [^{3}H]adenosine-labeled EMC RNA, which had been extracted from virions grown in the presence of Fischer medium, was fractionated on oligo(dT)-cellulose, and the RNA in each fraction was subjected to RNase degradation (see Materials and Methods). Approximately 5×10^4 cpm of viral RNA was used per reaction and all reactions were run in duplicate. Average poly(A) length was calculated by multiplying the percentage of RNase resistance times the number of adenosine residues present in the EMC genome (2,160).

TABLE 2. *Base composition of EMC RNA poly(A)*[a]

Base	^{32}P cpm	% of total cpm
UMP	0	0.00
GMP	1	0.21
AMP	463	99.36
CMP	2	0.43

[a] ^{32}P-labeled EMC RNA was subjected to RNase degradation. The reaction mixture was chromatographed on benzoylated cellulose (13). The bound polynucleotides were eluted and subjected to KOH hydrolysis followed by pH 3.5 paper electrophoresis (12). The displayed counts equal the counts in nucleotide spot minus counts in an identically sized spot from a parallel position on the paper.

TABLE 3. *Analysis of media effects on EMC RNA polyadenylation*[a]

Growth medium	Oligo(dT)-cellulose binding		RNase degradation of unfractionated EMC RNA	
	Fraction	%	% RNase-resistant cpm	Average poly(A) length (nucleotides)
Fischer	Peak 1	18.0		
	Peak 2	49.5	3.07	66.3
	Peak 3	32.5		
Fischer (+ 1 mM adenine)	Peak 1	21.2		
	Peak 2	31.7	2.86	61.8
	Peak 3	47.1		
Earle BSS ($-PO_4$)	Peak 1	18.3		
	Peak 2	39.6	2.75	59.4
	Peak 3	42.1		
Earle BSS ($+PO_4$)	Peak 1	18.9		
	Peak 2	38.2	3.08	66.5
	Peak 3	42.9		

[a] EMC virus was grown in 5% suspensions of Ehrlich ascites tumor cells suspended in one of the four growth media, in the presence of 2 μCi of [^{3}H]adenosine per ml. The virus was harvested and purified at 9.5 h after the start of infection. The RNA was extracted from virus with phenol and purified by two cycles of ethnol precipitation. Each RNA was then chromatographed on oligo(dT)-cellulose columns. Also, a sample of the unfractionated EMC RNA was used to determine poly(A) content by its resistance to RNase degradation.

classes. The media that were examined were Fischer medium, Fischer medium plus 1 mM adenine (15), and Earle medium BSS plus or minus phosphate. Phosphate has been shown to alter polyadenylation in *E. coli* (19). The data in Table 3 demonstrate that the cell suspension medium probably plays no role in determining either the length or distribution of poly(A) moieties in EMC RNA. Regardless of the cell suspension medium used, the average length of poly(A) in unfractionated EMC RNA was in the range of 59 to 67 nucleotides.

Infectious RNA assay. Spector and Baltimore (16) have shown that enzymatic removal of poly(A) from poliovirus RNA with RNase H lowers its infectivity. We have carried out similar analyses using peak 1 to 3 EMC RNA to infect L-cell monolayers. The results are given in Table 4. The infectivity of the EMC RNA appears to increase with increasing poly(A) length. Peak 3 EMC RNA was 4 times more infectious than peak 2 RNA and 10 times more infectious than peak 1 RNA. The explanation for the lower infectivity of the under-adenylated EMC RNA is unknown at this time. To investigate whether this might be at the level of RNA adsorption to the cell surface, infectivity assays were carried out in the presence of DEAE-dextran, which facilitates the cell-viral RNA interaction (21). This treatment increased the specific infectivity of all three RNA preparations about 10-fold, but did not increase the relative infectivity of the under-adenylated EMC RNA (Table 4).

DISCUSSION

The data presented here have demonstrated that within a purified population of EMC RNA molecules, there exists a wide variety of poly(A) lengths, ranging from less than 15 nucleotides to greater than 75 nucleotides in size (Table 1). This heterogeneity can be used to fractionate the EMC RNA on oligo(dT)-cellulose to yield three differentially polyadenylated subpopulations, peaks 1 to 3 (Fig. 2). This separation appears to be specific, quite reproducible, and does not degrade the viral RNA (Fig. 3). Both the distribution and absolute size of EMC RNA poly(A) appear to be insensitive to changes in ionic and nutrient concentrations in the cell suspension media (Table 3).

The viral RNA in peaks 1 to 3 also exhibited differences in ability to initiate infection. When assayed on L cells, it appeared that the presence of poly(A) was required for full infectivity of the EMC RNA (Table 4). This requirement could not be overcome by use of DEAE-dextran to facilitate RNA adsorption to the L cells. The

TABLE 4. *Infectious RNA assay of oligo(dT)-cellulose-fractionated EMC RNA*[a]

RNA	PBS		PBS + 100 μg of DEAE-dextran per ml	
	PFU/μg of RNA ($\times 10^{-2}$)	Relative %	PFU/μg of RNA ($\times 10^{-2}$)	Relative %
Peak 3	5.19	100	55.6	100
Peak 2[b]	1.27	24.5	11.5	20.7
Peak 1	0.54	10.4	8.4	15.1

[a] Unlabeled EMC RNA which had been fractionated on oligo(dT)-cellulose was spun down out of ethanol and suspended in a small amount of phosphate-buffered saline (PBS), and its absorbancy at 260 nm was read. Then, using either PBS or PBS plus 100 μg of DEAE-dextran per ml, the EMC RNA was diluted from 10^{-1} to 10^{-5}. These dilutions were used to carry out an infectious RNA assay as described in Materials and Methods. PFU per microgram of viral RNA was calculated assuming 1 absorbancy (260 nm) unit of RNA equals 40 μg.

[b] Twice passed.

nature of the poly(A) requirement in infectivity is unclear. This sort of effect has also been observed for polioviral RNA (16). It is possible that under-adenylated viral RNA is more susceptible to RNase degradation (8), and hence is degraded before it has a chance to interact with the translational apparatus of the host cell and initiate infection. Or, it is also possible, as suggested by others (18), that poly(A) is central to picornaviral RNA replication.

Oligo(dT)-cellulose column chromatography should be a generally useful procedure for analyzing the size distribution of poly(A) moieties in RNA populations, as well as for isolating under-adenylated RNA for comparative functional studies. For the study of EMC RNA, this procedure has the advantage of permitting the isolation of viral RNAs with polyadenylated regions as short as those obtained by enzymatic deadenylation (16) while reducing the possibility of RNA degradation by avoiding an enzyme incubation step. Also, it has provided an independent method for corroborating the effect of deadenylation on infectivity; the under-adenylated viral RNA prepared enzymatically or by oligo(dT)-cellulose fractionation both showed a similar 10-fold decrease in specific infectivity (Table 4 and reference 16). Currently, oligo(dT)-cellulose-fractionated EMC RNA is being utilized in this laboratory as a probe into poly(A) function(s) in protein synthesis and viral replication.

ACKNOWLEDGMENTS

We thank T. Sakai for valuable discussions on this project. We thank R. W. Bolin and O. K. Sharma for helpful comments on the manuscript. This work was supported by Public Health Service training grant 5-TO1-GM02219 from the National Institute of General Medical Sciences and by American Cancer Society grant no. NP-200.

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