The Size and Location of the Poly(A) Tract in EMC Virus RNA

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SUMMARY

Encephalomyocarditis (EMC) virus RNA, selected by its affinity for oligo(dT)-cellulose, contains poly(A) of size: (i) about 14 nucleotide residues long, based on the percentage of radioactivity in the RNA resistant to digestion by a mixture of pancreatic and T1 RNases; (ii) about 15 residues long, as measured by the ratio of the amount of terminal adenosine to internal adenylic acid in isolated poly(A); and (iii) in the range 12 to 45 residues, the majority of tracts being about 16 to 18 residues long, based upon electrophoretic mobility on polyacrylamide gels using poly(A) molecules of known size as mol. wt. markers.

The poly(A) appears to be located at the 3'-terminus of the virus genome since the tract, liberated by digestion with a mixture of pancreatic and T1 RNases, was shown by compositional analysis to contain a non-phosphorylated 3'-terminus and only adenine residues. The size heterogeneity in the poly(A) tracts revealed by gel electrophoresis is also consistent with a terminal location.

Comparison of our data for EMC virus with published data for other picornaviruses suggests that the sizes of poly(A) tracts in polio- and Mengovirus RNA have been overestimated; poly(A) tracts in cardioviruses appear to be smaller than those in poliovirus; the minimum size of poly(A) required for full infectivity of picornavirus RNA has also been overestimated; a tract of at least 13 adenine residues long is required for full infectivity of EMC virus RNA.

INTRODUCTION

Adenylic acid-rich [poly(A)] segments are present in most eukaryotic cell messenger RNA (mRNA) molecules (Brawerman, 1974) and in the genomes of those RNA viruses in which the virus mRNA has the same polarity as the virion RNA (Shatkin, 1974).

The presence of poly(A) in the genome of poliovirus has been clearly demonstrated (Armstrong et al. 1972; Yogo & Wimmer, 1972; Spector & Baltimore, 1974). Its presence in the RNA of other picornaviruses has been less firmly established. For instance, preparations of encephalomyocarditis (EMC) virus were claimed to contain both long and heterogeneous short poly(A) (Gillespie et al. 1973), molar amounts (Frisby et al. 1976), or tracts with about 20 adenosine residues (Goldstein, Pardoe & Burness, 1976). Closely related Mengovirus RNA has been variously reported to contain a poly(A) segment of about 16 (Miller & Plagemann, 1972), 50 (Sullivan & Roberts, 1973), or 50 to 70 (Spector & Baltimore, 1975b) nucleotides. Columbia SK virus RNA was said to contain poly(A) similar in

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size to that found in Sindbis virus (not a picornavirus but a Togavirus; Melnick, 1975) when extracted at neutral pH (Johnston & Bose, 1972). Subsequently it was shown that the bulk of Sindbis virus RNA is released into the aqueous layer by extraction at neutral pH and that this RNA fraction contained a poly(A) tract claimed to be 60 to 80 residues long (Eaton & Faulkner, 1972). The poly(A) content of foot-and-mouth disease virus RNA was found to be about the same as that in poliovirus RNA (Newman & Brown, 1976), while in rhinovirus RNA the poly(A) was estimated to be about 90 nucleotides long (MacNaughton & Dimmock, 1975), or about 150 residues long if calculated from the data given (Nair & Owens, 1974).

The poly(A) tract of poliovirus RNA (Spector & Baltimore, 1974) and EMC virus RNA (Goldstein et al. 1976) have been reported to be required for infectivity of the RNA. Spector & Baltimore (1975b) have since stated that ‘it is likely that a minimum of 40 to 50 nucleotides of poly(A) is necessary for replication of picornaviruses’. Some of the values given above for the size of poly(A) tracts in picornaviruses, including that which we reported (Goldstein et al. 1976), are below this size. In this communication we describe further studies on the size of the poly(A) tract in EMC virus RNA. At the same time we have identified pitfalls which may be responsible for some of the previously reported size discrepancies for picornavirus poly(A) in general.

**METHODS**

**Materials.** Oligo(dT)-cellulose T2 from Collaborative Research, Inc., Massachusetts, U.S.A. contained chains of up to 10 nucleotides long and had a capacity of 1 g dry powder to 33-5 \( E_{260} \) units of poly(rA) of mol. wt. greater than 100,000. DEAE-Sephadex (A25) was obtained from Pharmacia Fine Chemicals, Inc., New Jersey, U.S.A. Pancreatic RNase A (EC. 2.7.7.16) and snake venom phosphodiesterase (EC. 3.1.4.1) were from Worthington Biochemical Corp., New Jersey, U.S.A. Ribonuclease T1 (EC. 2.7.7.26) was from Sigma Chemical Company, Missouri, U.S.A. RNase T2 (EC. 2.7.7.17), nucleotides, nucleosides, purine and pyrimidine bases and poly(C) were from Calbiochem, La Jolla, California, U.S.A. \(^3\)H-poly(A) and unlabelled poly(A) preparations, estimated by intrinsic viscosity measurements by the manufacturer (Janik & Sommer, 1972) to contain on average 25, 45 and 90 nucleotide residues, were purchased from Miles Laboratories, Inc., Indiana, U.S.A. Oligoriboadenylate preparations containing 9, 10 and 15 residues were prepared by high resolution DEAE-cellulose chromatography in the presence of 7 M-urea and identified from compositional analysis by the supplier, Collaborative Research, Inc., Massachusetts, U.S.A. Carrier-free \(^32\)P-orthophosphoric acid and 2,8-\(^3\)H-adenosine (30 Ci/mmol) were from New England Nuclear, Massachusetts, U.S.A. Bio-Solv BBS-3 and 2,5-diphenyloxazole were from Beckman Instruments, Inc., California, U.S.A. Urea (ultra pure) and RNase-free sucrose were from Schwarz/Mann, New York, U.S.A., and all other materials and chemicals from Fisher Scientific Co., New Jersey, U.S.A.

*Preparation of virus RNA.* Growth of the K2 strain of EMC virus in Krebs ascites tumour cells (Sanders, Huppert & Hoskins, 1958), virus purification, RNA extraction using a phenol: CHCl₃ procedure (Perry et al. 1972), and oligo(dT)-cellulose chromatography (Aviv & Leder, 1972) to recover virus RNA containing poly(A) tracts have been described previously (Goldstein et al. 1976).

*Ribosomal RNA* (rRNA) and a mixture of 4S and 5S RNAs were obtained from Krebs ascites tumour cells as follows: cells at a concentration of \( 5 \times 10^7/m\)l in phosphate buffered saline (PBS) were extracted with an equal vol. of water-saturated phenol, and the aqueous layer, recovered by centrifuging at about 3000 \( g \) for 5 min, was re-extracted twice more.
with phenol before extracting with ether to remove the phenol. Sodium acetate was then added to 3 M to precipitate rRNA at −20°C overnight, followed (after removal of the rRNA) by ethanol to precipitate the 4S and 5S RNAs. Physarum 4S RNA was a gift from Dr P. W. Melera and was prepared as previously described (Melera & Rusch, 1973).

DEAE-Sephadex chromatography was performed in a similar manner to that described for DEAE-cellulose by Tomlinson & Tener (1963). Samples were mixed with either 5 E100 units of Krebs cell 4S and 5S RNA and/or a pancreatic RNase A digest of Krebs cell rRNA, and the solution adjusted to contain 0.1 M-NaCl and 7 M-urea. The mixture was chromatographed on a 0.9 × 2 cm column of DEAE-Sephadex using a linear 0.1 to 0.6 M-NaCl gradient containing 7 M-urea and 0.02 M-tris-HCl, pH 7.6 throughout. The gradient was generated by an Ultrograd (LKB Instruments, Inc., Maryland, U.S.A.) set for 16 h for the complete gradient with a flow rate of about 8 ml/h. Extinction at 260 nm and radioactivity were measured for each 1 ml sample and conductivity on every fifth sample with a YSI model 31 conductivity bridge, fitted with a YSI model 3417 conductivity cell (Yellow Springs Instrument Co., Ohio, U.S.A.) and converted to NaCl molarity using standards of known concentration.

Electrophoresis was performed in 0.6 × 10 cm 12% polyacrylamide gels as described by Peacock & Dingman (1967). The running time was 2 h at 4 mA/gel. Gels were scanned in an Acta C III spectrophotometer (Beckman Instruments) at 260 nm, or at 600 nm after staining with 0.2% (w/v) methylene blue in 0.4 M-acetic acid and destaining in water. Radioactive bands were located by pulverizing the gels with a Gilson gel fractionator, adding 1 ml water to each fraction and, after soaking overnight, measuring E260 and radioactivity in cocktail T (5 g diphenyloxazole in 11 toluene and 200 ml Bio-Solv BBS-3).

Conditions for enzyme digestion. RNA was digested by incubation at 37°C for 30 min with either pancreatic RNase A alone (RNase A: substrate ratio 1:25, w/w) or together with RNase T1 (T1: substrate ratio 1:40, w/w) in 0.01 M-tris-HCl, pH 7.6 containing 0.3 M-NaCl in which poly(A) resists digestion, at least by pancreatic RNase A (Beers, 1960). Adenylic acid-rich material resistant to digestion with either pancreatic RNase A alone, or combined with RNase T1, and subsequently selected by binding to oligo(dT)-cellulose is described, for simplicity, as [RNase A]poly(A) or [RNase A + T1]poly(A), respectively, following the convention used by Yogo & Wimmer (1972).

In some experiments [RNase A]poly(A) was further incubated at 37°C for 1 h with RNase T1 (enzyme: substrate ratio 1:40) in 0.02 M-tris-HCl, pH 7.6. The elution profile of the digest during DEAE-Sephadex chromatography suggested the presence of cyclic phosphates at the 3'-terminus of some of the oligonucleotides (I. Pardoe & A. T. H. Burness, unpublished results). To open these cyclic phosphates, the solution was adjusted to pH 2 with 1 M-HCl immediately after T1 digestion and incubation at 37°C continued for a further 2 h before adjusting the solution to a final pH 7.6 with 1 M-NaOH.

RNase T2 was used for compositional analysis of RNA, oligonucleotides and poly(A) by incubating, under typical conditions, 10 μl substrate (about 1 μg) plus 1 μl 1 M-ammonium acetate, pH 4.5, plus 1 μl RNase T2 (1 unit). Under these conditions, digestion was complete within about 1 h at 37°C but incubation was continued for 3 h or, to prove the specificity of the enzyme, for 6 h (see Table 1). The digestion products were separated, as described below, by compositional analysis.

Snake venom phosphodiesterase (SVD) purified by the method of Sulkowski & Laskowski (1971) was used for compositional analysis by incubating 50 μl substrate (2 μg) in 10 mM-MgCl2 and 10 mM-tris-HCl, pH 8.5, with 5 μl (10 μg) enzyme for 30 min at 37°C. Digestion products were separated by compositional analysis.
Table 2. Composition of 3H-adenosine-labelled EMC virus RNA

<table>
<thead>
<tr>
<th>Method of hydrolysis</th>
<th>Nucleotide*</th>
<th>Nucleoside* or base*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>HCl</td>
<td>0</td>
<td>ND†</td>
</tr>
<tr>
<td>KOH</td>
<td>34</td>
<td>2294</td>
</tr>
<tr>
<td>T2 RNase</td>
<td>0</td>
<td>4980</td>
</tr>
</tbody>
</table>

* HCl hydrolysis (1 h at 100 °C) produces 3'-pyrimidine nucleotides (Cp and Up) and purine bases (A and G); KOH (18 h at 37 °C) or T2 RNase hydrolysis (6 h at 37 °C, in this experiment) produces 3'-nucleotides (Cp, Ap, Gp and Up).
† ND, Not determined.

Compositional analysis. RNA, oligonucleotides or poly(A) were hydrolysed with RNase T2, SVD (as described above), or with 0.3 M-KOH at 37 °C for 18 h, after which the excess KOH was removed by precipitation with HClO₄. Appropriate nucleotides and nucleosides were added, as required, to serve as markers and to measure recoveries, and the enzyme or KOH digest subjected to paper electrophoresis in 0.05 M-ammonium formate, pH 3.5, as previously described (Burness, Pardoe & Fox, 1973). Some RNA preparations were hydrolysed in 2 M-HCl at 200 °C for 2 h, after addition of purine bases and pyrimidine nucleotides to serve as markers, the hydrolysates were paper chromatographed with iso-propanol-HCl as developing solvent as previously described (Burness et al. 1973).

RESULTS

Preparation of RNA

EMC virus used in these studies was prepared by a method which gives a highly purified product (Burness, 1969). Included in the procedure is a pancreatic RNase step which would be expected to eliminate non-encapsidated RNA, as has been shown (Burness, 1969). However, any trace of residual RNase was eliminated during extraction of the virus RNA since 3H-adenosine-labelled RNA retained its infectivity and high sedimentation rate even after 6 months or more of handling.

The purified virus sedimented on sucrose gradients as a single, apparently homogeneous peak at 160S (Burness & Clothier, 1970). More than 90% of the RNA extracted from this virus sedimented on sucrose gradients at 37S (Burness, Vizoso & Clothier, 1963). Fractions comprising the RNA peak were combined and, following ethanol precipitation, chromatographed on oligo(dT)-cellulose. As described previously (Goldstein et al. 1976), about 20% of the RNA was not retained probably because it contained a poly(A) tract below a critical size for retention on oligo(dT)-cellulose; the remaining 80% of the RNA was retained, but eluted under conditions expected to elute poly(A)-containing molecules. This RNA, which will be referred to as poly(A)+RNA, was used for most of the experiments reported here although compositional analyses were performed on total RNA before separation by oligo(dT)-cellulose chromatography.

Compositional analysis of RNA

3H-Adenosine-labelled virus RNA was hydrolysed with 1 M-HCl, 0.3 M-KOH or T2 RNase, and the products separated either by paper chromatography or by paper
Table 2. Composition of poly(A) from EMC virus RNA

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Cp</th>
<th>Ap</th>
<th>Gp</th>
<th>Up</th>
<th>Adenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>32P-labelled [RNase A]poly(A)</td>
<td>55</td>
<td>291</td>
<td>427</td>
<td>49</td>
<td>ND*</td>
</tr>
<tr>
<td>32P-labelled [RNase A + T1]poly(A)</td>
<td>39</td>
<td>384</td>
<td>27</td>
<td>9</td>
<td>ND</td>
</tr>
<tr>
<td>3H-adenosine-labelled [RNase A + T1]poly(A)</td>
<td>38</td>
<td>3005</td>
<td>20</td>
<td>0</td>
<td>216</td>
</tr>
</tbody>
</table>

* All samples were hydrolysed with T2 RNase.
† ND, not determined.

electrophoresis. All procedures gave essentially the same result of 86 to 90% of the activity in adenine with the remainder in guanine and background levels only in the pyrimidine nucleotides (Table 1).

Hydrolysis of 32P-labelled EMC virus RNA with SVD to 5'-nucleotides gave the composition pC = 21.2%, pA = 29.3%, pG = 19.6% and pU = 29.9%. Hydrolysis of a similar preparation of RNA with T2 RNase which yields 3'-nucleotides gave the composition Cp = 27.3%, Ap = 26.1%, Gp = 20.0% and Up = 26.7%.

Since 32PO4 enters RNA in the 5' position of the nucleotide precursor, the radioactivity in any one species of 5'-nucleotide in an RNA molecule is related both to the specific radioactivity and to the number of copies of that nucleotide present, i.e. base composition. It is generally accepted that the 32P-radioactivity in any one species of 3'-nucleotide in RNA is related only to the number of copies of that nucleotide present; i.e. base composition on the assumption that the 32P of 5' origin is randomly distributed throughout the RNA. If this assumption is correct, it follows that the ratio of the 32P-radioactivity in the 5'-nucleotide to that in the 3'-nucleotide is a reflection of the specific radioactivity of the 5'-nucleotide.

The assumption that the 32P-radioactivity in 3'-nucleotides is representative of the number of each species of nucleotide present appears justified since the result given above for T2 RNase hydrolysates of EMC virus RNA is similar to the base composition obtained by direct determination of the E260 of the separated components (Burness, 1970). However, due to the non-random nature of a homopolymer tract, the same assumption may not hold unless the specific radioactivity of the 5'-nucleotide comprising the tract is about the same as the average for all nucleotides in the RNA. The ratio of 32P-radioactivity in 5'- to 3'-nucleotides for EMC virus RNA was 1.12 for adenylic and uridylic acids, 0.78 for cytidylic acid, and 0.98 for guanylic acid. Nevertheless, although in no case was the difference from unity in specific radioactivity sufficient to cause serious errors in estimating the number of nucleotides present in a homopolymer tract, differences were taken into account in calculating the length of the poly(A) tract in EMC virus RNA described in the following section.

Poly(A) size

A characteristic of poly(A) is its resistance to digestion with either pancreatic or T1 RNase, the specificities of which result in hydrolysis at pyrimidine residues and at guanine residues, respectively (Brawerman, 1974). This property of poly(A) permits its recovery for the further investigation of its size by a variety of procedures, some of which are now described.
Table 3. Composition of oligonucleotides from DEAE-Sephadex*

<table>
<thead>
<tr>
<th>Tentative identification</th>
<th>Ct/min</th>
<th>Ap</th>
<th>Gp</th>
<th>Up</th>
<th>Ap + Gp</th>
<th>Chain length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mononucleotides</td>
<td>1598</td>
<td>164</td>
<td>153</td>
<td>1690</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>Dinucleotides</td>
<td>280</td>
<td>349</td>
<td>440</td>
<td>354</td>
<td>1.2</td>
<td>2</td>
</tr>
<tr>
<td>Trinucleotides</td>
<td>592</td>
<td>1562</td>
<td>1501</td>
<td>856</td>
<td>2.1</td>
<td>3</td>
</tr>
<tr>
<td>Tetranucleotides</td>
<td>456</td>
<td>1489</td>
<td>1099</td>
<td>419</td>
<td>3.0</td>
<td>4</td>
</tr>
<tr>
<td>Pentanucleotides</td>
<td>152</td>
<td>182</td>
<td>529</td>
<td>194</td>
<td>3.6</td>
<td>5</td>
</tr>
<tr>
<td>Hexanucleotides</td>
<td>231</td>
<td>1196</td>
<td>921</td>
<td>212</td>
<td>4.8</td>
<td>6</td>
</tr>
</tbody>
</table>

* Oligonucleotides, isolated from pancreatic RNase digests of 32P-labelled EMC virus RNA by chromatography on DEAE-Sephadex, were subjected to compositional analysis by paper electrophoresis following hydrolysis with T2 RNase.

**Poly(A) size from percentage of RNA resisting enzymic digestion**

Poly(A)+RNA labelled with 3H-adenosine was digested with pancreatic RNase A and the [RNase A]poly(A) fraction, recovered by oligo(dT)-cellulose chromatography, was found to contain about 1% of the total radioactivity. EMC virus RNA contains about 8000 nucleotides (Duffy, Burness & Sanders, manuscript in preparation), of which about 26% are present as adenine residues (Burness, 1970; also see 'Compositional analysis of RNA' section above). The 1% radioactivity in [RNase A]poly(A) is thus equivalent to a poly(A) tract containing 20 adenine residues, assuming the radioactivity was present as adenine residues only. However, as described above, about 10% of the radioactivity in 3H-adenosine-labelled [RNase A]poly(A) was present in guanine residues and, thus, a size of 20 residues for the poly(A) tract was regarded as tentative only.

32P-Labelled poly(A)+RNA was digested with pancreatic RNase, and the radioactivity resistant to digestion was isolated by binding to oligo(dT)-cellulose. About 1.2 to 1.5% of the total 8000 nucleotides was bound, this being equivalent to a chain of approx. 96 to 120 residues in contrast to the 20 residue tract estimated above for 3H adenosine-labelled [RNase A]poly(A). Taking into account the higher average specific activity for adenine residues, calculated as described above, a poly(A) tract containing 1.2 to 1.5% of the radioactivity is equivalent to a chain of approx. 86 to 107 residues for 32P-labelled [RNase A]poly(A), which is still considerably different from the 20 residue tract estimated for 3H-adenosine-labelled [RNase A]poly(A).

Compositional analysis of 32P-labelled [RNase A]poly(A) following digestion with T2 RNase gave values of Cp = 7%, Ap = 34%, Gp = 52% and Up = 7% (Table 2). This finding showed the 32P-labelled [RNase A]poly(A) was far from pure, and the apparent long chain length resulted from contamination with non-adenine-rich oligonucleotides. These observations illustrate the dangers of estimating poly(A) size using either digestion with a single enzyme and/or RNA preparations, all labelled with the same isotope, even when digestion is followed by oligo(dT)-cellulose chromatography.

To reduce this oligonucleotide contamination, another poly(A)+RNA labelled with 3H-adenosine was incubated with a mixture of pancreatic and T1 RNases and the [RNase A + T1]poly(A) was recovered by oligo(dT)-cellulose chromatography. Of the radioactivity in the approx. 2000 adenine residues, about 0.7% was present in this poly(A) fraction, which is equivalent to a total chain length of about 14 adenine residues per RNA molecule.

A similar experiment was performed with 32P-labelled poly(A)+RNA except that the RNA was first digested with pancreatic RNase A alone and the [RNase A]poly(A), isolated
by oligo(dT)-cellulose chromatography, was further digested with T1 RNase. The $^{32}$P-labelled [RNase A + T1]poly(A) was then recovered by DEAE-Sephadex chromatography and shown by compositional analysis to contain Cp = 1.0%, Ap = 98.1%, Gp = 0.7% and Up = 0.2% (Table 2), demonstrating that the material was now virtually pure poly(A). The product accounted for about 0.2% of the total radioactivity in the $^{32}$P-labelled RNA or the equivalent of a tract containing about 14 adenine residues per RNA molecule, allowing for the slightly higher specific radioactivity of adenine residues.

**Poly(A) size by DEAE-Sephadex chromatography**

Oligonucleotides present in pancreatic RNase digests of EMC virus RNA were eluted in sharp, well-separated peaks when chromatographed on DEAE-Sephadex despite the relatively small bed volume of resin we used (Fig. 1a). We confirmed that the oligonucleotides were eluted in order of increasing chain length by measuring the ratios of internal purine to terminal pyrimidine residues in fractions derived from pancreatic RNase A digests (Table 3). A plot of the logarithm of chain length in digests of virus RNA or Krebs rRNA against molarity of NaCl for elution was linear in the range of dinucleotides to heptanucleotides, the longest oligonucleotide detected under the conditions used; mononucleotides, although not emerging in the void volume, were eluted by a NaCl concentration close to that of starting buffer and did not fall on the same straight line (Fig. 2).

EMC virus [RNase A]poly(A) was eluted after Physarum 4S RNA as a sharp peak from...
DEAE-Sephadex by about 0.44 M-NaCl containing 7 M-urea (Fig. 1b). EMC virus [RNase A + T1]poly(A) was also eluted from DEAE-Sephadex by about the same salt concentration (result not shown). This NaCl concentration was estimated by extrapolation (Fig. 2) to elute oligonucleotides of about 13 residues long. However, we do not regard this as a reliable estimate of chain length under the conditions we used because Physarum 4S RNA, which presumably contains about 80 nucleotides (Dayhoff & McLaughlin, 1969) was eluted from DEAE-Sephadex by 0.42 M-NaCl (Fig. 1b), a concentration expected to elute oligonucleotides containing about 11 residues only (Fig. 2). Thus, extrapolation of molarities for elution of oligonucleotides containing 7 residues or less to larger molecules is invalid under the conditions of chromatography we (and others) have used and is imprudent under other conditions without adequate controls. This problem cannot be circumvented by calibrating the DEAE-Sephadex columns with homopolymers since we have already shown that poly(A) molecules of average chain lengths 25, 45 and 90 are all eluted by about 0.44 M-NaCl (Burness, Pardoe & Goldstein, 1975), the same molarity required to elute EMC virus [RNase A]- and [RNase A + T1]poly(A).

**Poly(A) size from compositional analysis**

Although DEAE-Sephadex chromatography had little value for estimating polynucleotide size, it was invaluable for removing small, contaminating oligonucleotides, the presence of which would lead to serious errors in determining poly(A) size by compositional analysis. Therefore, EMC virus poly(A) for such studies and for electrophoretic analysis (described below) was a product of both pancreatic and T1 RNase digestion and was purified by chromatography both on oligo(dT)-cellulose and on DEAE-Sephadex.

[RNase A + T1]poly(A) produced in this way from ³H-adenosine-labelled EMC virus RNA was hydrolysed with T2 RNase and the digest subjected to paper electrophoresis under conditions which separate adenosine from adenylic acid. From the ratio of radioactivity in these two adenine derivatives (Table 2), an average chain length of 15 adenine residues was calculated for EMC virus [RNase A + T1]poly(A).

In this kind of experiment, it is essential to check that digestion is complete and that none of the products are preferentially lost, for instance, by the handling procedures or by
Poly(A) in EMC virus

Fig. 3. Electrophoresis on separate 12% polyacrylamide gels of poly(A) preparations containing on average 25, 45 and 90 nucleotides, oligoriboadenylates containing 9, 10 and 15 nucleotides and [RNase A + T1]poly(A) from 3H-adenosine-labelled EMC virus RNA. The position of the 4S and 5S RNAs run at the same time and used to align parallel gels are indicated by the arrows. ---, Cl/min; ----, recorder deflection, E600.

 inadvertent dephosphorylation by a contaminating enzyme. In preliminary experiments, we found that under the conditions of T2 RNase incubation we used, digestion was complete within 1 h; but, nevertheless, for compositional analysis, incubation was extended to 3 h. For both time-points, only background counts were detected at the origin of the paper electropherograms. In pilot studies the handling procedures were shown to yield from 87 to 100% recovery of the added optical density markers. To test for the possible presence of a contaminating nuclease, 3H-adenosine-labelled EMC virus RNA was incubated with T2 RNase for 6 h, i.e. twice as long as used for compositional analysis of poly(A). The presence of a phosphatase would have been revealed by a substantial amount of radioactivity in adenosine. Most of the radioactivity was in Ap with about 14% in Gp and background counts only were associated with the added adenosine, Cp and Up markers (Table 1).

Poly(A) size from mobility on polyacrylamide gels

[RNase A + T1]poly(A), released from 3H-adenosine-labelled virus RNA by enzyme digestion and recovered by oligo(dT)-cellulose and DEAE-Sephadex chromatography, was co-electrophoresed on 12% polyacrylamide gels with a mixture of Krebs cell 4S and 5S RNA. After electrophoresis, the gel was crushed in 1 mm segments and the position of 4S and 5S RNAs and of EMC virus poly(A) was estimated by measuring E260 and radioactivity respectively. To estimate the size of the 3H-adenosine-labelled [RNase A + T1]poly(A) tract, unlabelled oligoriboadenylates containing 9, 10 and 15 residues, poly(A) preparations containing on average 25, 45 and 90 adenine residues and a mixture of 4S and 5S RNA were also run on separate parallel gels. The positions of the oligo(A) and poly(A) molecules and the 4S and 5S RNA were revealed by staining. The 4S and 5S RNA markers on the crushed gels containing [RNase A + T1]poly(A) and on the stained gels were used to align the two kinds of gel.
[RNase A+T1]poly(A) appeared heterogeneous with a size range of about 14 to 45 residues long (Fig. 3). However, most tracts were in the size range of 16 to 18 residues long based on the mobility of the poly(A) and oligoriboadenylate standards. As we reported elsewhere (Burness et al. 1975), the 4S and 5S RNA molecules had the electrophoretic mobilities expected of poly(A) molecules containing about 25 and 52 residues, respectively.

Location of the poly(A) tract in EMC virus RNA

Heterogeneity in poly(A) size

The nucleotide sequence in a given species of RNA molecules would be expected to be identical. However, variability might be tolerated at the extreme ends of the RNA. The size heterogeneity in EMC virus poly(A) (Fig. 3) is consistent, therefore, with the tract being located either at the 3'- or 5'-terminus rather than in an internal position.

Evidence from compositional analysis for non-phosphorylated 3'-terminus in poly(A)

Most poly(A) tracts studied so far appear to be located at the 3'-terminus of the RNA and, therefore, themselves are terminated by a nucleoside residue unphosphorylated in the 3'-position of the ribose (Brawerman, 1974).

If this were so for EMC virus poly(A), digestion of such a tract with T2 RNase should release internal residues as nucleotides and the 3'-terminal residue as a nucleoside. As described above [poly(A) size from compositional analysis], T2 RNase digestion of 3H-adenosine-labelled [RNase A+T1]poly(A) liberated one adenosine for every 14 adenylic acid residues (Table 2), which demonstrates that the poly(A) tract had no 3'-terminal phosphate and thus must have been located at the 3'-terminus of the RNA.

Lack of Gp, Cp or Up in poly(A)

All oligonucleotides produced by digestion of RNA with a combination of pancreatic and T1 RNases will be terminated with Cp, Up or Gp residues except for the oligonucleotide located at the 3'-terminus, unless one of these three residues was the naturally-occurring 3'-terminus. As described above [poly(A) size from % RNA resisting enzyme digestion], 32P-labelled [RNase A+T1]poly(A) was found, using T2 RNase digestion, to contain 98 % Ap, 1.0 % Cp, 0.7 % Gp, and 0.2 % Up (Table 2). Since we have shown above that most poly(A) tracts in EMC virus RNA comprise about 14 to 18 residues, there are 0.14, 0.10 and 0.03 residues of Cp, Gp and Up, respectively, per tract. This suggests that Cp, Gp and Up are not present in EMC virus poly(A) from which we conclude that the poly(A) tract must have been located at the 3'-end of the RNA.

DISCUSSION

Spector & Baltimore (1975b) suggested that a poly(A) tract of minimum size 40 to 50 nucleotides long in picornavirus genomes is necessary for replication in these viruses. This suggestion resulted from their conclusions that polio- and Mengovirus RNA contained poly(A) tracts of 50 to 125 and 50 to 70 nucleotides long, respectively, and in the case of poliovirus, enzymatic reduction of the tract to a size they estimated to be less than 40 residues long caused a marked decrease in infectivity of the RNA (Spector & Baltimore, 1974). However, the results we have presented here clearly show that most EMC virus RNA poly(A) tracts are less than 20 nucleotides long, yet such EMC virus RNA preparations are infectious (Goldstein et al. 1976). We believe this uncertainty concerning minimum size for
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infectivity arises from the fact that Spector & Baltimore and others have overestimated the size of the RNA poly(A) tract in picornaviruses for the reasons now discussed.

A wide range of sizes for the poly(A) tract in picornavirus RNA has been reported (see Introduction for sizes and references). Techniques used to measure size include: (i) % RNA resistant to digestion by pancreatic and T1 RNases (Armstrong et al. 1972; Miller & Plagemann, 1972; Nair & Owens, 1974; MacNaughton & Dimmock, 1975; Spector & Baltimore, 1975a, b; Newman & Brown, 1976); (ii) DEAE-Sephadex chromatography (Yogo & Wimmer, 1972, 1973, 1975); (iii) sedimentation rate or electrophoretic mobility on polyacrylamide gels (Armstrong et al. 1972; Johnston & Bose, 1972; Miller & Plagemann, 1972; Yogo & Wimmer, 1972; Porter, Carey & Fellner, 1974; Spector & Baltimore, 1975a, b); (iv) hybridization to (3H)poly(U) followed by enzyme digestion (Gillespie, Marshall & Gallo, 1972; Gillespie et al. 1973; Sullivan & Roberts, 1973); and (v) compositional analysis (Armstrong et al. 1972; Yogo & Wimmer, 1972; Spector & Baltimore, 1975).

There are many potential pitfalls in using these methods. For instance, method (i), % RNA resistant to digestion, contains difficulties in that: (a) the resistant fraction comprises only a few percent of the total and is, therefore, difficult to measure with accuracy; (b) contamination with what would normally be considered trace amounts of labelled material can cause serious errors; the method assumes (c) that all the nucleotide residues have the same specific radioactivities; and (d) that 100% recovery of poly(A) is achieved. In this report we have illustrated the difficulties in trying to estimate 32P-labelled EMC virus (RNase A)poly(A) size by this method unless contamination is rigorously excluded. Because of such difficulties, we suggest that this method is of limited use and gives only a rough guide to poly(A) size.

Using method (ii), it has been shown that poliovirus RNA poly(A) (Yogo & Wimmer, 1972) and poly(U) from poliovirus replicative intermediate RNA (Yogo & Wimmer, 1973, 1975) were eluted from DEAE-Sephadex at higher salt concentrations than those required to elute tRNA, evidence thought to indicate that the homopolymers were larger than tRNA (Yogo & Wimmer, 1973, 1975). However, we have shown elsewhere (Burness et al. 1975) that tRNA, containing about 80 nucleotides (Dayhoff & McLaughlin, 1969), was eluted at lower salt concentrations than poly(A) preparations containing on average 25, 45 and 90 residues, and possibly even fewer, as we have shown here for poly(A) from EMC virus RNA (Fig. 1 b). Thus, although DEAE-Sephadex gives excellent resolution of small oligonucleotides (see Fig. 1 a) and is useful for removing small contaminating oligonucleotides from poly(A), the technique is of little value in estimating poly(A) size under the conditions which have been used for this purpose.

Factors contributing to difficulties in interpreting DEAE-Sephadex chromatography data are the differences in physical properties between homo- and heteropolymers. These differences are further emphasized by the electrophoretic properties of these molecules which in turn have led to possible errors when method (iii) is used to determine poly(A) size. We have shown elsewhere (Burness et al. 1975) and in this report (Fig. 3) that 4S RNA (of about 80 residues long; Dayhoff & McLaughlin, 1969) and 5S RNA (about 120 residues long; Forget & Weissman, 1967) have mobilities expected of homopolymers of chain lengths 25 and 52 residues long, respectively, or of about 29 and 57 residues long if run under the denaturing conditions induced by formaldehyde (Burness et al. 1975). Morrison, Merkel & Lingrel (1973) have also commented upon the apparent anomalous electrophoretic mobilities of homopolymers.

Despite the differences in electrophoretic behaviour between hetero- and homopolymers, method (iii) can be used to estimate the size of poly(A) tracts with 4S and 5S RNA as size
markers provided appropriate corrections are made. For instance, most poly(A) tracts from EMC virus have mobilities, compared with 4S and 5S RNA, of heteropolymers containing 50 to 60 nucleotides (Fig. 3). However, if the mobilities of 4S and 5S RNA are regarded as equivalent to homopolymers containing 25 and 52 residues, then the mobilities of most EMC virus poly(A) tracts are consistent with homopolymers containing less than 20 nucleotides, in reasonable agreement with results obtained using poly(A) of known size as electrophoretic markers (Fig. 3) and with our chemical data.

Similar corrections are possible for the reported data of others. Poly(A) from poliovirus RNA was found to have an electrophoretic mobility slightly less than that of tRNA, whether the latter was denatured with formaldehyde or not, and a size for poliovirus poly(A) of slightly longer than 80 nucleotides was proposed (Yogo & Wimmer, 1972). However, since native and formaldehyde-denatured tRNA behave like homopolymers containing 25 and 29 nucleotides, respectively (Burness et al. 1975), the results are consistent with a size for poliovirus poly(A) of only slightly longer than 25 to 29 nucleotides rather than the 80 nucleotides proposed. In other studies, most poly(A) tracts from poliovirus were found to have mobilities between formamide-denatured 4S and 5S RNA and a size of 50 to 120 nucleotides was reported (Spector & Baltimore, 1975a, b), but we suggest these mobilities are those expected for homopolymers in the range 15 to 50 nucleotides long. Similar corrections suggest that most poly(A) tracts from Mengovirus RNA are about 16 to 25 nucleotides long, and thus about the same size as EMC virus poly(A), rather than the 50 to 70 nucleotides proposed (Spector & Baltimore, 1975b).

We have no experience with method (iv), hybridization to (3H)poly(U); however, this method suffers from some of the difficulties referred to in discussing method (i) and, where gel electrophoresis is used to analyse the product, method (iii).

The main difficulties in determining poly(A) size by compositional analysis, method (v), are that the samples have to be rigorously purified, and that the hydrolysis conditions and handling procedures must not lead either to the creation of artifacts or to differential loss of products. For instance, alkaline hydrolysis of poly(A) results in conversion of about 1% of the adenylic acid to a nucleotide behaving like cytidylic acid (Molloy & Darnell, 1973). If the size of poly(A) in picornavirus RNA has been overestimated previously, then the minimum size of poly(A) required for full RNA infectivity has also been overestimated. Spector & Baltimore (1974) found that removal of 60% or 82% of the poly(A) reduced the infectivity of poliovirus RNA to 16.5% or 6.7%, respectively, of normal controls. The percentage of RNase resistant RNA in these preparations was 0.42% and 0.9% (Table 2 in Spector & Baltimore, 1974), which we calculate is equivalent to average poly(A) chain lengths of 9 and 20 nucleotides long, respectively. Moreover, our recalculation of their electrophoresis data (Fig. 2 in Spector & Baltimore, 1974) suggests that these preparations contained poly(A) segments close to 10 and 14 residues long, respectively, taking into consideration the apparently anomalous behaviour of 4S and 5S RNA markers.

Re-interpretation of the data in this way gives results entirely consistent with our observations made on EMC virus. Electropherograms of 3H-adenosine-labelled (RNase A+T1)-poly(A) from EMC virus poly(A)+ RNA, previously adsorbed to oligo(dT)-cellulose, reveals almost no poly(A) segments containing less than about 13 nucleotides (Fig. 3). This probably means that oligo(dT)-cellulose cannot retain molecules which contain poly(A) segments of 13 residues or less. We have shown elsewhere (Goldstein et al. 1976) that RNA not bound by oligo(dT)-cellulose is much less infectious than bound RNA.

We conclude, therefore, that if there is a minimum poly(A) size for full infectivity of picornavirus RNA, then this minimum for EMC virus is about 13 nucleotides in length.
We suggest that re-interpretation of already published data for poliovirus leads to similar conclusions. However, the possibility exists that, since typical animal virus preparations contain 1 p.f.u. per 10 to 1000 physical particles, only those picornavirus particles containing genomes with long poly(A) tracts are infectious.

Why a poly(A) tract is required for infectivity of some, if not all, picornaviruses is intriguing and under investigation, particularly because genomes of other viruses such as tobacco mosaic virus or Nodamura virus lack poly(A) but nevertheless contain infectious RNA (Newman & Brown, 1976). It would be interesting to learn whether the messenger RNA of these two viruses contains a poly(A) segment. By such comparative studies with viruses we may begin to learn the function of poly(A) segments which has proved an enigma since their discovery.

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