The Location of the Poly(C) Tract in the RNA of Foot-and-Mouth Disease Virus

By T. J. R. HARRIS AND F. BROWN
Biochemistry Department, Animal Virus Research Institute, Pirbright, Surrey, U.K.

(Accepted 20 July 1976)

SUMMARY

Fragments of foot-and-mouth disease virus RNA of decreasing size, containing the 3' poly(A) sequence have been prepared by alkali treatment and sucrose gradient centrifugation followed by oligo(dT)-cellulose affinity chromatography. Polyacrylamide gel electrophoresis of the ribonuclease T1 resistant oligonucleotides from these polyadenylated fragments has enabled us to locate the position of some of the longer oligonucleotides on the RNA. In particular the poly(C) tract has been shown to be near the 5' end of the RNA. The possible function of the poly(C) tract is discussed in the light of these findings.

INTRODUCTION

The RNA molecules from representatives of all subgroups of the vertebrate picornaviruses, in common with other messenger RNAs, contain a heterogeneous tract of polyriboadenylic acid [poly(A); Armstrong et al. 1972; Yogo & Wimmer, 1972; Nair & Owens, 1974; Chatterjee, Bachrach & Polatnick, 1976; Frisby et al. 1976; Goldstein, Pardoe & Burness, 1976; Newman & Brown, 1976]. In poliovirus and foot-and-mouth disease virus (FMDV) RNA, the poly(A) sequence is 50 to 90 nucleotides long and is at the 3' end of the molecule (Armstrong et al. 1972; Yogo & Wimmer, 1972; Chatterjee et al. 1976; Newman & Brown, 1976). RNA from the cardioviruses, encephalomyocarditis virus (EMCV) and Mengovirus also contains poly(A) at the 3' terminus, but there is some dispute about its length, with values ranging from 15 to 70 nucleotides (Miller & Plagemann, 1972; Spector & Baltimore, 1975; Frisby et al. 1976; Goldstein et al. 1976; Newman & Brown, 1976).

In addition to poly(A) the cardioviruses and several FMDV RNAs contain a polyribocytidylic acid [poly(C)] tract which is resistant to digestion by ribonuclease T1 (Brown et al. 1974; Porter, Carey & Fellner, 1974; Frisby et al. 1976). This tract ranges in length from about 100 nucleotides in EMCV RNA to greater than 200 nucleotides in some serotypes of FMDV and is composed of 80 to 90% cytidylic acid (Brown et al. 1974).

Recent biochemical analyses of the ribonuclease T1 (RNase T1)-resistant oligonucleotides from the RNA of a virulent FMDV (serotype SAT1) by polyacrylamide gel electrophoresis have shown that the poly(C) tract is considerably longer in this RNA than the poly(C) tract in the RNA of an attenuated mutant derived from it (Harris & Brown, 1977). Since it was possible that the shortening of the poly(C) tract was in some way related to the attenuation, it was clearly important to ascertain the position of the poly(C) in the RNA as a prerequisite to any examination of its function. It has been suggested, in view of the lack of a proline-rich polypeptide in the structural proteins of EMCV, that the poly(C) region in this...
virus RNA is untranslated and therefore near either the 3' or 5' end of the RNA (Porter et al. 1974).

To find the position of the poly(C) tract in the RNA of FMDV, we have used an experimental approach based on that used to map and order the RNase T1 resistant oligonucleotides of Rous sarcoma virus (RSV) RNA (Joho, Billeter & Weissmann, 1975; Coffin & Billeter, 1976; Wang et al. 1975, 1976). In these procedures random fragments of RNA are generated by mild alkali treatment and various size classes, containing the polyadenylated 3' end, selected by sucrose gradient centrifugation and affinity chromatography. Analysis of the large RNase T1-resistant oligonucleotides produced from each size class then gives a measure of how close each oligonucleotide is to the 3' end. Thus the oligonucleotides can be ordered on the RNA relative to the 3' end.

METHODS

Viruses and 32P labelling. FMDV, serotype SAT1, was a clone from the 82nd passage in BHK cells of a virus isolated in Haci Pasa in Turkey in 1962 (Mowat, Barr & Bennett, 1969; Harris & Brown, 1976). The virus was grown in monolayers of 10^8 BHK cells. Labelling of the RNA with 32P was accomplished by growing the virus in 20 ml of phosphate-free Earle's saline containing 200 to 500 μCi/ml of carrier-free 32P-orthophosphate (Radiochemical Centre, Amersham).

Preparation of 32P-virus RNA. Virus was purified from the medium essentially as described by Brown & Cartwright (1963), with the modifications outlined by Harris & Brown (1976). RNA was isolated from purified virus after dilution in buffer (0.15 M-NaCl, 0.005 M-EDTA, 0.05 M-tris-HCl, pH 7.6) containing 0.1 % SDS, by two extractions with phenol-chloroform (1:1), followed by precipitation with two vol. ethanol. The RNA was reprecipitated once with ethanol before use.

Preparation and separation of RNA fragments. The RNA was dissolved in 0.1 ml of 0.025 M-Na2CO3 and incubated at 37 °C for 25 min to introduce random breaks (Rabbitts & Milstein, 1975). The solution was then diluted with 0.4 ml of 0.1 M-sodium acetate (pH 5.0) containing 0.1 % SDS, loaded on to a 14 ml 5 to 25 % sucrose gradient (in 0.1 M-acetate, 0.1 % SDS, pH 5.0) and the gradient centrifuged at 60000 to 65000 g for 16 h at 20 °C (20000 rev/min, in an MSE rotor no. 59108). The gradient was collected in 0.4 ml fractions and the radioactivity in 5 μl of each fraction assessed by liquid scintillation counting. Appropriate fractions were then pooled (see Fig. 1), 200 μg of E. coli tRNA (BDH) added as carrier, and the RNA precipitated by addition of two vol. ethanol.

Isolation of polyadenylated fragments by affinity chromatography. Polyadenylated fragments were isolated from the pooled RNA fractions by oligo(dT)-cellulose chromatography (Aviv & Leder, 1972). This was done on 25 mg columns of oligo(dT)-cellulose (type 7, P-L Biochemicals) in Pasteur pipettes, run and monitored as described in detail by Harris & Wildy (1975), but omitting the KOH wash and the SDS from the elution buffer. Using these conditions, the unbound RNA was clearly separated from the bound RNA, greater than 95 % of the unbound RNA being eluted in the first high salt fraction and the remainder by the second wash. The bound RNA, eluted with 0.01 M-tris-HCl, pH 7.6, was precipitated by ethanol after addition of 100 μg of E. coli tRNA carrier.

RNase T1 digestion and oligonucleotide separation. The bound RNA precipitates were dissolved in 10 μl of 0.01 M-tris-HCl, 0.001 M-EDTA, pH 7.4 containing RNase T1 (Sankyo, Japan) at an enzyme-substrate ratio of 1:20, and incubated for 1 h at 37 °C. The oligonucleotides resistant to the enzyme were separated by one-dimensional electrophoresis on
Location of poly(C) in FMDV-RNA

Fig. 1. Sucrose gradient sedimentation of alkali-treated FMDV RNA. The letters A to F refer to those fractions pooled and subjected to oligo(dT)-cellulose chromatography. The positions of the 28S and 18S rRNA markers were determined from a parallel gradient.

Table 1. Oligo(dT)-cellulose chromatography of alkali-fragmented FMDV RNA

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Approximate S values*</th>
<th>Unbound (ct/min × 10⁻³)</th>
<th>Bound (ct/min × 10⁻³)</th>
<th>Bound (ct/min bound × 100)</th>
<th>Recovery† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>34–38</td>
<td>39.8</td>
<td>35.2</td>
<td>46.6</td>
<td>71.5</td>
</tr>
<tr>
<td>B</td>
<td>29–33</td>
<td>90.1</td>
<td>37.7</td>
<td>29.3</td>
<td>78.0</td>
</tr>
<tr>
<td>C</td>
<td>24–28</td>
<td>155.8</td>
<td>52.5</td>
<td>25.2</td>
<td>84.0</td>
</tr>
<tr>
<td>D</td>
<td>19–23</td>
<td>195.3</td>
<td>61.2</td>
<td>23.6</td>
<td>80.5</td>
</tr>
<tr>
<td>E</td>
<td>14–18</td>
<td>172.8</td>
<td>42.4</td>
<td>19.5</td>
<td>74.0</td>
</tr>
<tr>
<td>F</td>
<td>9–13</td>
<td>114.6</td>
<td>24.0</td>
<td>17.2</td>
<td>78.1</td>
</tr>
</tbody>
</table>

* Estimated from the 28S and 18S rRNA markers (Fig. 1).
† Recovery of RNA expressed as Total ct/min from oligo(dT)-cellulose chromatography steps x 100.

The values therefore represent the recovery of both the ethanol precipitation and the oligo(dT)-cellulose chromatography steps.

a 10 % polyacrylamide gel at pH 8.3 in 6 M-urea (Porter et al. 1974), or by two-dimensional polyacrylamide gel electrophoresis (De Wachter & Fiers, 1972; Frisby et al. 1976). The dimensions of the gels and the running conditions were the same as those used by Harris & Brown (1976). Autoradiography of the gels was done as described by Frisby et al. (1976).
RESULTS

Location of poly(C) in FMDV RNA

FMDV (SAT1-82) was labelled with $^{32}$P and the RNA extracted and treated with alkali. The randomly fragmented RNA molecules were separated by sucrose gradient centrifugation and different size classes pooled into six fractions. The distribution of radioactivity in the gradient and the fractions which were pooled are shown in Fig. 1. Each fraction was then subjected to oligo(dT)-cellulose chromatography to select those molecules containing the 3' poly(A) sequence. Isolation of polyadenylated RNA fragments after size separation (rather than before) has the advantage that the oligo(dT)-cellulose binding can be monitored accurately (cf. Wang et al. 1975; Coffin & Billeter, 1976). Table I shows that, as expected, the percentage of the RNA binding to oligo(dT)-cellulose was gradually reduced in the shorter molecules. About 50% of fraction A which contains RNA of full length (34 to 38S, Table I) was bound to the column. This percentage is similar to that found by others using undegraded FMDV RNA (Chatterjee et al. 1976).

The oligo(dT)-cellulose bound RNA from each of the six fractions was then digested with RNase T1 and the resistant oligonucleotides separated, according to size, by one dimensional polyacrylamide gel electrophoresis in 6.0 M-urea at pH 8.3 (Fig. 2). The oligonucleotides obtained from the RNA in fraction A (34 to 38S, Table I) were similar to those obtained from SAT1-82 virus RNA which had not been subjected to oligo(dT)-cellulose chromatography (Harris & Brown, 1976). The RNase T1-resistant oligonucleotide moving more slowly than the xylene cyanol dye marker (Fig. 2A) is the poly(C) tract and is about 100 nucleotides long (Harris & Brown, 1976). Fig. 2 shows that this poly(C) tract is present in molar amounts only in the RNA from fraction 1; the poly(C) tract is clearly sub-molar in the RNA from other fractions. Since nearly twice as much RNA was used for analysis of fractions C and D compared with fraction A (see Table I), the best comparison is between fractions A and B which contained approximately the same amount of radioactivity. This comparison shows that the poly(C) is virtually absent from RNA molecules of 29 to 33S which contain the 3' end indicating that the poly(C) is near the 5' end of the RNA. The small amount of the poly(C) in fractions B to F (Fig. 2) is probably due to some non-specific retention of non-polyadenylated RNA fragments by the oligo(dT)-cellulose (Coffin & Billeter, 1976). Fig. 2 also shows that the background haze, running slightly faster than the poly(C) tract (just ahead of the xylene cyanol dye marker), gradually increases from A to F. This is probably due to oligonucleotides containing the heterogeneous poly(A) at their 3' end, as these will increase proportionately in the shorter polyadenylated RNA fragments (see next section and Fig. 3). Moreover, the heterogeneity of the poly(A) tract and its apparent absence from full length RNA (Fig. 2A) explains why it was not detected previously in RNase T1 digests of various picornavirus RNAs analysed by one-dimensional gel electrophoresis (Porter et al. 1974; Brown et al. 1974). The prominent band appearing in all fractions running more slowly than the bromophenol blue marker (Fig. 2) is probably the long RNase T1 oligonucleotide numbered 4 in Fig. 3 as this is located towards the 3' end of the RNA (see next section).

Analysis of polyadenylated fragments of FMDV RNA by two-dimensional gel electrophoresis

An analogous experiment was done to confirm that the sucrose gradients were fractionating the alkali-treated RNA according to size, and to show that the oligo(dT)-cellulose was selecting predominantly polyadenylated molecules. A sucrose gradient of alkali-
Fig. 2. Separation of the RNase T₁ resistant oligonucleotides of FMDV RNA fragments of different size classes, containing the 3' poly(A) sequence, on a 10 % polyacrylamide gel at pH 8.3 in 6.0 M-urea for 16 h at 4 °C. Direction of electrophoresis is from top to bottom. X and B denote respectively the position of the xylene cyanol and bromophenol blue dye markers.
Fig. 3. Two-dimensional gel electrophoresis of the RNase T₁ resistant oligonucleotides of FMDV RNA fragments of different size classes containing the 3' end. The arrows indicate the direction of electrophoresis. The numbers refer to those large oligonucleotides analysed by RNase A digestion (Harris & Brown, 1976). Oligonucleotide number 1 is the poly(C) tract and oligonucleotide number 2 is the heterogeneous poly(A). The letter (O) denotes the origin of the gels.
Location of poly(C) in FMDV-RNA

treated SAT$_1$-82 RNA was pooled into five fractions and the polyadenylated molecules in each size class isolated by oligo(dT)-cellulose chromatography. The amount of RNA from each size class binding to oligo(dT)-cellulose followed a reduction similar to that found earlier (Table 1) and a similar amount of RNA was bound and recovered. This bound RNA was digested with RNase T$_1$ and the resistant oligonucleotides separated by two-dimensional gel electrophoresis (Fig. 3). The pattern obtained for fraction A (Fig. 3a) which again contained full length RNA, was the same as that obtained for SAT$_1$-82 virus RNA not selected by oligo(dT)-cellulose chromatography (Harris & Brown, 1977). However, as slower sedimenting RNA molecules were analysed (Fig. 3b to e) there was a gradual loss of the long RNase T$_1$-resistant oligonucleotides, and an overall reduction in the complexity of the maps. This result suggested that the sucrose gradient was separating the RNA molecules according to their size and since there was also a proportional increase in the streak containing the poly(A) (Fig. 3, spot 2; see also Frisby et al. 1976) it demonstrated that the oligo(dT)-cellulose was binding predominantly polyadenylated molecules. Two-dimensional gel analysis of fragmented RNA also allows the characteristic T$_1$ oligonucleotides to be ordered on the molecule relative to the poly(A) at the 3' end. The poly(C) tract (spot 1 in Fig. 3 and see Harris & Brown, 1977) was again detected only in fraction 1 showing that the poly(C) was near to the 5' end of the molecule. The long oligonucleotides 3, 5 and 11 are also near the 5' end whereas spots 4 and 16 are near the 3' end (see also Fig. 2).

DISCUSSION

Using a modification of the experimental approach adopted by Duesberg & Billeter and their colleagues to map the oligonucleotides of RSV RNA we have shown that the poly(C) tract is near the 5' end of the RNA of FMDV although its exact location has not been ascertained. In FMDV RNA the poly(C) tract is not present in polyadenylated RNA fragments of 29 to 33 S (Fig. 1b and 2b). These S values correspond to mol. wt. of approx. 1.7 to $2.1 \times 10^6$, i.e. molecules of 61 to 75% of the full length RNA. We have recently increased the resolution of the experimental procedure by taking eight smaller fractions from a sucrose gradient of alkali-treated FMDV RNA. Analysis of the RNase T$_1$ oligonucleotides of the polyadenylated RNA in each fragment by one-dimensional gel electrophoresis (as in Fig. 2) again showed that the poly(C) tract was only present in full length RNA. Molecules of less than 84% of full length did not contain the sequence. We can conclude therefore, assuming FMDV RNA contains 8000 to 8500 nucleotides, that the poly(C) tract is located within the 1200 nucleotides at the 5' end.

In preliminary experiments we have also found that the poly(C) tract is near the 5' end of Mengovirus RNA. This suggests that the poly(C) tract is in the same position in the RNA of the cardioviruses as it is in the RNA of the FMD viruses.

The first 40% of the RNA of picornaviruses starting from the 5' end codes for the virus structural proteins. In FMDV neither these polypeptides nor the non-structural polypeptides synthesized in infected cells are unduly rich in proline (D. V. Sangar, personal communication). This observation suggests therefore, in view of the nature of picornavirus protein synthesis, that the poly(C) tract is in an untranslated part of the 5' region, preceding the initiation site of protein synthesis. As far as some of the other long RNase T$_1$ resistant oligonucleotides are concerned, it is only possible at this stage to say that they are near the 5' or 3' ends. It is not yet possible to relate them to any biological function.

The 5' ends of poliovirus, EMC virus and FMDV RNA molecules are probably not 'capped' with a m$^3$G in 5'–5' pyrophosphate linkage (m$^3$G(5')ppp(5')Xp) (Hewlett, Rose...
& Baltimore, 1976; Nomoto, Lee & Wimmer, 1976; P. Fellner, personal communication). It is possible therefore that the poly(C) region is 5' terminal in the RNA of FMDV and the cardioviruses. This suggestion is supported by the apparent variation in the length of the poly(C) tract in different FMDV serotypes and even in very closely related viruses of the same serotype (Brown et al. 1974; Harris & Brown, 1976).

The poly(C) tract may play a role in protein synthesis. However, as this sequence is absent from enterovirus RNA, the role must be specific for the FMD viruses and the cardioviruses (Brown et al. 1974; Frisby et al. 1976). This difference between these groups and the enteroviruses must be taken into account in any speculation concerning the role of the poly(C) tract in picornavirus replication. Alternatively the poly(C) tract may function in RNA replication, possibly via a poly(G) tract in the negative strand, which would in turn initiate new strands of RNA of virus polarity. Preliminary results have shown that a long oligonucleotide of a size similar to the poly(C) tract is found in ribonuclease A digests of denatured double stranded RF RNA, isolated from BHK cells infected with FMDV. A similar long oligonucleotide was not found in digests of poliovirus RF RNA (T. J. R. Harris, unpublished data). This observation suggests that the poly(C) tract is transcribed. Work is currently in progress to investigate this point more fully and to examine further the 5' end of FMDV RNA.

During the preparation of this paper for publication the work of Chumakov & Agol (1976) was brought to our attention. Using a similar approach they have located the poly(C) tract in EMC virus RNA near the 5' end of the molecule.

We thank Dr P. Fellner, Searle Research Laboratories, for useful discussions, and Mr P. Wallbridge of this Institute for the photographs.

REFERENCES


CHUMAKOV, K. M. & AGOL, V. I. (1976). Poly(C) sequence is located near the 5' end of encephalomyocarditis virus RNA. Biochemical and Biophysical Research Communications 71, 551–557.


Location of poly(C) in FMDV-RNA


NOMOTO, A., LEE, Y. F. & WIMMER, E. (1976). The 5' end of poliovirus mRNA is not capped with m7G(5')ppp(5')Nm. Proceedings of the National Academy of Sciences of the United States of America 73, 375-380.


WANG, L. H., DUESBERG, P., BEEMON, K. & VOGT, P. K. (1975). Mapping RNase T1-resistant oligonucleotides of avian tumor virus RNAs: sarcoma-specific oligonucleotides are near the poly(A) end and oligonucleotides common to sarcoma and transformation defective viruses are at the poly(A) end. Journal of Virology 16, 1051-1070.


(Received 9 July 1976)