Analysis of the Secondary Structure of the Poly(C) Tract in Foot-and-Mouth Disease Virus RNAs

By E. J. C. MELLOR,† F. BROWN‡ and T. J. R. HARRIS*§

The Animal Virus Research Institute, Pirbright, Woking, Surrey GU24 0NF, U.K.

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SUMMARY

Sodium bisulphite modification of foot-and-mouth disease virus (FMDV) RNA in solution indicates that the majority of the poly(C) tract in the RNA is single-stranded in concordance with previous results with encephalomyocarditis virus RNA. The reaction kinetics are biphasic; 60% of the cytidylic acid in the poly(C) tract reacts like synthetic poly(C), and the remainder with the kinetics of the cytidylic acid in the rest of the RNA. The reactivity of the poly(C) tract with poly(I) indicates that it is looped out and exposed in the RNA. The deamination reaction has also been used to investigate the structure of the replicative form (RF) and replicative intermediate (RI) isolated from infected cells. Analysis by gel electrophoresis of the long RNase A- and T1-resistant oligonucleotides of RI suggests that it has five single-stranded poly(C) tracts to every one which is base-paired. Bisulphite reactivity of the poly(C) tract and gel electrophoresis of the ribonuclease-resistant oligonucleotides of RF indicate that the poly(C) is base-paired to a poly(G) tract in this molecule. The presence of a poly(G) tract in RF and RI provides unequivocal evidence that the poly(C) is replicated via poly(G) in the negative strand.

INTRODUCTION

The RNA genomes of viruses of the aphthovirus and cardiovirus genera of the Picornaviridae are similar to those of the entrovirus and rhinovirus genera in having a 3' poly(A) sequence, a genome-linked protein (VPg) at the 5' end and a long 5' untranslated region. They differ, however, by the presence of a poly(C) tract near the 5' end of the RNA. The length of the homogeneous poly(C) tract in these RNAs is of the order of 50 to 200 nucleotides depending on virus type and isolate (Brown et al., 1974; Harris & Brown, 1977; Black et al., 1979). The distance of the poly(C) tract from the 5' end of the RNA also varies from 150 to 500 nucleotides (Harris & Brown, 1976; Rowlands et al., 1978; Black et al., 1979). The role of the poly(C) tract in the replication cycle of the virus or in the structure of the RNA has not been determined. The poly(C) tract probably does not play a direct role in protein synthesis since in foot-and-mouth disease virus (FMDV) RNA the two initiation sites for protein synthesis have been located on the 3' side of the poly(C) tract about 1000 bp downstream from the 5' end (Sangar et al., 1980; Beck et al., 1983; Carroll et al., 1984; Forss et al., 1984). Also, removal of the poly(C) tract and all nucleotides to its 5' side by RNase H digestion in the presence of oligo(dG) (Rowlands et al., 1978) has no effect on the quality or quantity of polypeptides translated in vitro from the shortened RNA (Sangar et al., 1980).

A role for the poly(C) tract in the process of RNA replication in infected cells has not been established either. A G-rich region has been detected in double-stranded replicative form (RF) RNA (Harris & Brown, 1976) but the presence of poly(C) or poly(G) in the replicative intermediate (RI) has not been reported.

† Present address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.
‡ Present address: Wellcome Biotechnology Ltd., Ash Road, Pirbright, Woking, Surrey GU24 0NQ, U.K.
§ Present address: Celltech Ltd., 250 Bath Road, Slough, Berks. SL1 4DY, U.K.
The poly(C) tract in the RNA of encephalomyocarditis virus (EMCV) has been shown, by virtue of its reactivity towards sodium metabisulphite at pH 5, to be largely single-stranded in solution whereas, by the same criterion, the remainder of the RNA has a high degree of secondary structure (Goodchild et al., 1975). In this paper the cytidylic acid specificity of sodium metabisulphite has been used to examine the structure in solution of the poly(C) tracts of FMDV RNA and RNA species isolated from FMDV-infected cells.

**METHODS**

Radioactive RNA was prepared as described by Harris (1980) and Harris & Brown (1977). BHK cells infected with FMDV type A10 were labelled with 12.5 μCi/ml [5-3H]cytidine (TRK: 198, 25 to 30 Ci/mmol) or [3H]guanosine or 1 mCi/ml carrier-free 32P (Amersham).

**Extraction and isolation of double-stranded RNA from infected cells.** RNA was isolated from cytoplasmic extracts of BHK cells, 6 h after infection, by phenol–CHCl₃ extraction and ethanol precipitation. DNA was removed by DNase treatment (10 μg RNase-free DNase in 0.14 M-NaCl, 0.01 M-Tris-HCl pH 7.6, 0.0015 M-MgCl₂) and partially double-stranded and single-stranded RNA removed by 2 M-LiCl precipitation. The 4S RNA and double-stranded RNA in the supernatant was then precipitated with 2 vol. ethanol overnight at −20 °C. The pellet was resuspended in TNE (0.14 M-NaCl, 0.02 M-Tris–HCl pH 7.6, 0.005 M-EDTA, 0.1% SDS) containing 35% (v/v) ethanol and loaded onto a Whatman CF11 cellulose column. After washing well with TNE containing 15% (v/v) ethanol to remove single-stranded RNA, double-stranded RNA was eluted with TNE. This process was repeated two or three times until no more single-stranded RNA could be washed off the column. Double-stranded RNA was precipitated with 2 vol. ethanol overnight at −20 °C.

**Extraction and isolation of replicative intermediate from infected cells.** About 10⁸ BHK cells in monolayers were infected with FMDV type A10 at high multiplicity (100 p.f.u./cell). At 150 min after infection 1 μg/ml actinomycin D was added to the medium. Fifteen min later, 500 μCi [3H]cytidine, [3H]uridine or [3H]guanosine was added to the cells (12 μCi/ml) for 10 min and then removed. Total RNA was prepared by phenol extraction of the cell monolayers and partially double-stranded and single-stranded RNA selected by LiCl precipitation at 0 °C. The precipitate, which contained RI and single-stranded RNA, was dissolved in TNE containing 35% (v/v) absolute ethanol. The RNA was loaded onto a Whatman CF11 cellulose column and RI were prepared as described for RF.

**Bisulphite treatment of RNA.** In control reactions about 2·5 μg FMDV or EMCV RNA or FMDV RI or FMDV RF labelled with [3H]cytidine were dissolved in 50 μl 0·1 M-NaCl and diluted to 1·6 ml with water. For bisulphite treatment, RNAs were dissolved in 50 μl 0·1 M-NaCl and diluted to 1·6 ml with 3 M-sodium bisulphite buffered to pH 6·0 with sodium sulphite containing 5 mM-quinol. The RI was separated from degraded RI cores and single-stranded tails by chromatography on Whatman CF11 cellulose and differential precipitation with 2 m-LiCl.

All the above mixtures were incubated at 4 °C for up to 96 h. To complete the reaction and remove bisulphite, the mixtures were dialysed at 4 °C, three times against 0·1 M-NaCl containing 5 mM-quinol, twice against water, once against 0·5 M-disodium hydrogen phosphate (pH 9·0) and finally four times against water. After the final dialysis, each sample was made 0·3 M with respect to sodium acetate pH 5·0 containing 0·2% (w/v) SDS and the RNA was precipitated in the presence of 5 to 50 μg of yeast tRNA as carrier with 2 vol. ethanol at −20 °C.

**Isolation of polypyrimidine tracts.** Bisulphite-treated RNA was digested to completion with RNase T₁. The polypyrimidine tracts were isolated either by chromatography on Sephadex G-100 or by polycrylamide gel electrophoresis.

In the chromatographic method, the RNase T₁ oligonucleotides were diluted to 0·5 ml with TNE and the polypyrimidine tracts isolated from the void volume of a Sephadex G-100 column (7 × 0·4 cm) equilibrated in TNE. The fraction containing the largest oligonucleotides was precipitated with ethanol in the presence of 5 μg of carrier yeast tRNA. For isolation by electrophoresis, an equal volume of marker dye mixture (0·1% xylene cyanol, 0·1% bromophenol blue in 20% glycerol) was added to the RNase T₁ digest which was then fractionated on a 10% polyacrylamide gel buffered to pH 8·3 with Tris–borate. Gels were analysed by autoradiography. Samples labelled with [14C]nucleotides were detected by fluorography (Bonner & Laskey, 1974). Oligonucleotides labelled with [3H]nucleotides were detected by fluorography (Bonner & Laskey, 1974). Oligonucleotides labelled with [3P]P were eluted from the gel using a crush and soak method (Frisby et al., 1976).

RNase T₁ and RNase A oligonucleotides from RI and RF were fractionated on 10% Tris–borate gels in tubes (10 × 0·4 cm) at 100 V for 4 h. The gels were cut into 1·5 mm slices and each slice was solubilized in 0·5 ml of a 1:6 mixture of water: NCS (Beckman) at 37 °C for 16 h, 5 ml of toluene scintillant was added and the distribution of radioactivity throughout the gel determined. Poly(I) was removed from FMDV RNA and synthetic poly(C) and the RI and RF preparations were denatured by boiling them in 0·01 M-Tris–HCl pH 7·6 and 0·001 M-EDTA (TE) buffer for 2 min, followed by rapid cooling in a methanol solid CO₂ bath before digestion with RNase T₁.

**Determination of C to U conversion in FMDV and EMCV RNA, isolated polypyrimidine tracts and synthetic poly(C).** RNA or isolated polypyrimidine tracts were digested with RNase T₁ in TE buffer at 37 °C for a minimum of 2 h. The mononucleotides were separated on small (10 × 6·5 cm) polyethyleneimine (PEI) cellulose plates (Volckaert...
Table 1. Percentage C to U conversion for FMDV and EMCV RNA after reaction with 3 m-sodium metabisulphite for 96 h

<table>
<thead>
<tr>
<th>Sample</th>
<th>Non-denatured RNA</th>
<th>RNA denatured in 10 mM-CH₃HgOH*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(C) tract from FMDV RNA</td>
<td>81</td>
<td>80</td>
</tr>
<tr>
<td>Synthetic poly(C)</td>
<td>82</td>
<td>81</td>
</tr>
<tr>
<td>Poly(C) tract from poly(I)-protected FMDV RNA</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Poly(I)-protected synthetic poly(C)</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>FMDV RNA</td>
<td>38</td>
<td>60</td>
</tr>
<tr>
<td>EMCV RNA</td>
<td>36</td>
<td>64</td>
</tr>
<tr>
<td>Poly(C) tract from EMCV RNA</td>
<td>78</td>
<td>-</td>
</tr>
<tr>
<td>Poly(I)-protected FMDV RNA</td>
<td>32</td>
<td>-</td>
</tr>
</tbody>
</table>

* The presence of 10 mM-CH₃HgOH was shown not to interfere with the reaction by deaminating single-stranded synthetic poly(C) in the presence or absence of denaturant.

& Fiers, 1977). Two μl of each digest, containing a maximum of 5 μg of RNA, was applied to the plates and the mononucleotides were visualized under shortwave u.v. light. The radioactive spots were revealed by autoradiography, cut out of the plate and their radioactive contents measured. When 3H-labelled material was separated, the plates were impregnated with 30% PPO in acetone and fluorographed before the molarities were determined by scintillation counting. No account was taken of the potential small error arising from 3H isotope exchange during deamination of the 5,6-dihydro-6-sulphonate intermediate (Hayatsu, 1976), since similar results were obtained with RNA labelled with [3H]cytidine or 32P.

Isolation of negative strand from RI and RF. This method was based on an observation that addition of low concentrations of LiCl to solutions of double-stranded EMCV RNA in 75% DMSO at low concentration resulted in a significant decrease in the melting temperature of the RNA (Chumakov, 1979; Cumakov et al., 1979). One μg of 3H-labelled RNA was dissolved in 25 μl of denaturation buffer (100 mM-LiCl, 10 mM-HEPES pH 7.5, 1 mM-EDTA) and 75 μl DMSO was added. The solution was mixed well, heated to 32 °C for 3 min, cooled rapidly in a methanol/solid CO₂ bath to 0 °C and then supplemented with 25 μl 2 M-LiCl containing a fivefold molar excess (2.5 μg) of 32P-5' end-labelled fragmented virus RNA. The RNA was allowed to anneal at 30 °C for 30 min and then diluted with 2 ml TNES. Absolute ethanol was added to the mixture to a final concentration of 35% (v/v). The double-stranded negative strand/positive strand fragments and undenatured RF were separated from the full-length positive strands and excess fragments of virus RNA on Whatman CF11 cellulose columns. The double-stranded molecules were eluted from the column, precipitated with ethanol, dissolved in 50 μl of denaturation buffer and heated at 56 °C for 15 min. DMSO (50 μl) and dimethylformamide (100 μl) were added and the samples loaded onto a 5 to 25% sucrose gradient in DMSO and centrifuged at 50000 r.p.m. for 18 h at 20 °C in a Beckman SW50 rotor (Bishop et al., 1969). The 32P-5' end-labelled fragments were separated from the full-length RNA species (which now contained an excess of negative strand) and the non-denatured RF. The full-length RNA, whose position was estimated from a marker run on a parallel gradient, was precipitated from the gradient with ethanol.

RESULTS

Examination of the secondary structure of the poly(C) tract in virus RNA

Bisulphite modification has been used in functional and conformational studies of a variety of RNA and DNA molecules (Goddard & Maden, 1976; Braverman et al., 1975; Sklyadneva et al., 1979; Domday et al., 1978). Addition of bisulphite to the 5:6 double bond of cytidylic acid at slightly acid pH causes a slow deamination to a uridylic acid analogue. The adduct may be converted by treatment at higher pH to uridylic acid (Hayatsu, 1976). This modification depends on the secondary structure of the RNA; cytidylic acid is fully reactive in single-stranded regions but resistant to modification in base-paired regions (Goddard & Schulman, 1972).

To examine the amount of secondary structure in the poly(C) tract of FMDV RNA in solution, it was important to determine the extent of deamination in fully denatured and fully base-paired RNA, and also to show that the secondary structure of the RNA is not destroyed under the reaction conditions.

[5-3H]Cytidine-labelled or 32P-labelled FMDV RNA denatured with 10 mM-CH₃HgOH was reacted with 3 M-bisulphite for 96 h, the polypyrimidine tract isolated by RNase T₁ digestion and the conversion of C to U determined by PEI plate chromatography. Table 1 shows that 80%
of the cytidylic acid (C) is converted to uridylic acid (U) in the poly(C) tract in denatured FMDV RNA. The amount of reactivity expected if the poly(C) tract were fully base-paired was determined by converting it into a double-stranded complex by annealing synthetic poly(I) to the RNA at 0 °C. After reaction with bisulphite, the polypyrimidine tract was isolated and the nucleotide composition showed that 9.5% of the cytidylic acid had been converted to uridylic acid. This result is similar to the conversion seen in a native synthetic poly(I):poly(C) complex (Table 1).

Although these results indicate that the bisulphite reaction is not completely single-strand-specific under these conditions, they do provide an estimate of the C to U conversion expected if the poly(C) tract were single-stranded (80%) or fully base-paired (10%) in the virus RNA.

To confirm that the secondary structure of the RNA was not destroyed by the reaction, the overall C to U conversion was determined by quantification of the products of alkaline or RNase T2 digestion of the RNA. Table 1 shows that 38% of the C residues reacted in native FMDV RNA and 36% in native EMCV RNA used as a control. The value for EMC virus RNA is in exact agreement with the 36% C to U conversion obtained during bisulphite deamination by Goodchild et al. (1975). These results indicate that the secondary structure of FMDV RNA in solution is probably conserved during the reaction. The overall extent of deamination of cytidylic acid in denatured FMDV and EMCV RNA was also determined. The 60 and 64% C to U conversion obtained (Table 1) is rather low by comparison with that observed with synthetic denatured poly(C), probably indicating that 10 mM-CH3HgOH does not fully denature all G:C pairs under these conditions.

To determine the extent of C to U conversion in the poly(C) tract of native FMDV and EMCV RNAs, a total RNase T1 digest of bisulphite-treated [3H]cytidine-labelled RNA was filtered through a Sephadex G-100 column and fractions containing the large polypyrimidine tracts were digested to constituent mononucleotides with RNase T2; nucleotides were separated on PEI-cellulose plates. Analysis showed 81% C to U conversion in the poly(C) tract from the native FMDV RNA and 78% for the poly(C) from EMCV RNA (Table 1). The C to U conversion was that expected for a single-stranded poly(C) tract and shows that the amount of secondary structure in the poly(C) tract of FMDV RNA in solution is similar to that in the poly(C) tract from EMCV RNA (Goodchild et al., 1975).

The effect of bisulphite treatment on the poly(C) tract from FMDV RNA was also examined by electrophoresis in polyacrylamide gels. Fig. 1 shows that the polypyrimidine tracts in native and denatured RNA treated with bisulphite migrate more slowly than the poly(C) tracts from unreacted RNA, and that the decreased mobility increases with reaction time. The polypyrimidine tracts were eluted from the gel and the C to U conversion was determined by RNase T1 digestion and separation on PEI-cellulose plates. The overall C to U conversion for full-length FMDV RNA for each time of reaction with bisulphite was determined in a similar way and the results were combined to produce Fig. 2, showing the kinetics of the reactivity of FMDV RNA and the pol(y)tract towards bisulphite. Both curves show an initial high reactivity, followed by a slower linear increase up to 96 h. The initial rapid increase in the U content of the poly(C) tract isolated from native FMDV RNA after reaction with bisulphite occurs at a similar rate to that found with a synthetic pol(y)C.

The rate of deamination of the poly(C) tract from 0 to 6 h can be represented in terms of bases modified per hour. The half-life for this period of deamination in the poly(C) tract is 5.5 h, which is similar to the value of 5 h determined for a synthetic poly(C) reacted under similar conditions (Goddard & Schulman, 1972). This means that 60% of the reactive residues in the poly(C) tract react at a similar rate, and so presumably have the same conformation in solution, as synthetic poly(C). The remaining reactive bases in the poly(C) tract react at a rate similar to the majority of the reactive cytidylic acid residues in the full-length RNA. Further evidence for a biphasic reaction can be seen when Fig. 1 is examined more closely. In the presence of 10 mM-CH3HgOH, the deamination of the poly(C) in denatured RNA was complete (81% C to U conversion) after 24 h (Fig. 1, lane 2) whereas in native RNA only about 60% of the poly(C) tract was deaminated (Fig. 1, lane 3). After 96 h, however, both the poly(C) tracts from native and denatured RNA migrated more slowly, presumably because both now have a U content of 81%
Poly(C) tract in FMDV RNA

Fig. 1. Effect of bisulphite modification on the migration of polypyrimidine tracts from [³H]cytidine-labelled FMDV RNA on polyacrylamide gels. Lanes 1, 4 and 7, poly(C) tracts from RNA incubated with H₂O for 96 h at 4 °C. The RNA was treated for 24 h (lanes 2 and 3), 48 h (lanes 5 and 6) or 96 h (lanes 8 and 9) in the presence (lanes 2, 5 and 8) or absence (lanes 3, 6 and 9) of 10 mM CH₃HgOH. O, Origin.

Fig. 2. Kinetics of the reaction of FMDV RNA (●) and the isolated poly(C) tract (■) with bisulphite.

(Fig. 1, lanes 8 and 9). If all the nucleotides in the poly(C) tract had the same conformation as synthetic poly(C), a similar pattern of migration would be expected in native and denatured RNA after 24 h of reaction.

Examination of the amount of secondary structure in the poly(C) tracts of RI from FMDV-infected cells

The RI can be isolated from FMDV-infected cells about 2-25 h after a high multiplicity infection. When RNA synthesis is at a maximum in picornavirus-infected cells, about 80 to 90% of the RI species contain a full-length negative-sense strand synthesizing positive-sense mRNA,
Fig. 3. Gel electrophoresis of polypyrrimidine tracts isolated from (a) single-stranded virus RNA, (b) unfractionated RI, (c) RI cores or (d) sheared RI tails. The panels on the left represent control unreacted samples and those on the right samples reacted with 3 M-bisulphite for 96 h at 4 °C. The position of the poly(C) and poly(U) tracts are shown. The samples were run on 10 cm polyacrylamide gels in tubes. After electrophoresis, the gels were sliced into 1 mm slices. Only the top portions of the gels are shown. The RI was labelled with [³H]cytidine.

and the remainder contain a full-length positive-sense strand synthesizing negative-sense RNA (Bishop et al., 1969). The amount of secondary structure in the poly(C) tracts of FMDV RI was examined by treating RI with 3 M-bisulphite for 96 h at pH 6·0. It was essential to ensure that degraded RI molecules were removed from the reaction mixture before analysis of the reactivity of the poly(C) tracts. RI molecules and RI cores (containing a double-stranded backbone but no single-stranded tails) were separated from sheared tails by chromatography on Whatman CF11 cellulose (Mellor, 1982). The double-stranded cores and RI molecules were separated by differential precipitation in 2 M-LiCl. All three species, sheared tails, RI cores and the RI were denatured and digested to completion with RNase T₁ and the polypyrrimidine tracts were isolated by polyacrylamide gel electrophoresis (Fig. 3). In a control experiment with single-stranded virus RNA, all the poly(C) tracts were deaminated to poly(U) (Fig. 3a), as were the poly(C) tracts from the sheared RI tails (Fig. 3d). None of the poly(C) tracts from the RI cores were deaminated to poly(U) (Fig. 3c), suggesting that the poly(C) tract is base-paired with poly(G) in the negative strand.
RI molecules reacted with bisulphite produced a mixture of poly(U) and poly(C) tracts (Fig. 3b). The distribution of radioactivity in the gel suggests that the RI preparation contains five poly(C) tracts which are single-stranded and can be deaminated to poly(U) to every one which is unreactive, and presumably base-paired. If 10 to 20% of this RI preparation contains RI which is synthesizing negative strands, containing one poly(C) tract in the double-stranded backbone and presumably unreactive, about 10 to 20% of the counts remaining as unreactive poly(C) would be accounted for by these molecules. Thus, it is likely that, when RNA synthesis is maximal, among the major RI species in the cells there are five or six single-stranded poly(C) tracts to every one which is unreactive.
Structure of double-stranded RNA isolated from FMDV-infected cells

The RF accumulates late in the infectious cycle of FMDV-infected cells. It is a double-stranded structure containing one positive strand and one negative strand. RF was isolated from FMDV-infected cells and reacted with 3 M-bisulphite for 96 h at 4 °C. As the RF is considered to be fully double-stranded, the same amount of reactivity with bisulphite as seen with double-stranded synthetic poly(I):poly(C) (about 10%) was expected (see Table 1). However, over 20% of the cytidylic acid residues in RF were deaminated to uridylic acid. Ten mM-CH₃HgOH did not denature the RF fully under these conditions. The reason for the unexpected reactivity towards bisulphite was discovered when RF was electrophoresed in 1% agarose gels. RF was resolved into two major bands, RF1 and RF2 (Fig. 4a); there was twice as much RF2 as RF1. The ribonuclease sensitivities of these two species showed that RF1 was ribonuclease-insensitive whereas most of the RF2 was sensitive (Fig. 4b). The products of RNase treatment, however, were 90% TCA-precipitable and migrated as large fragments on agarose gels (Fig. 4b). RF1 was resistant to RNase over a wide range of concentrations. However, 25% of the material running as RF2 was found in a resistant form, even at high RNase concentrations (Fig. 4b). This result would suggest that there are three species of double-stranded RNA in FMDV-infected cells, two of which are RNase-resistant, characteristic of RF and one which has the characteristics of RI cores. Bisulphite treatment of RF1, isolated after electrophoresis on agarose gels, resulted in 5% of the C being deaminated to U. Presumably, the remaining C to U conversion in unfraccionated RF is due to contaminating RI in the double-stranded RNA preparation.
Isolation of the poly(G) tract from the negative strand of RI and RF

Treatment of \(^{3}H\)cytidine- and \(^{3}H\)guanosine-labelled FMDV RF with RNase A after heating to 110 °C resulted in the formation of a large oligonucleotide fragment, about the same size as the poly(C) tract. Base analysis of the fragment showed that it consisted of equal amounts of the nucleotides C and G, suggesting that the RF was not fully denatured around the base-paired poly(C):poly(G) tracts by the heating step. Similar RNase-resistant fragments and partially denatured structures containing both single-stranded and double-stranded regions have been found in EMCV RF (Chumakov, 1979). The difficulty in melting the RF is probably due to the presence of a very stable hydrogen-bonded poly(G):poly(C) duplex. Addition of LiCl to EMCV RF in DMSO has given a significant reduction in the melting temperature of the RNA (Chumakov et al., 1979). This observation was carried further when Chumakov (1979) used this method to study the melting and reassociation of EMCV RF. This method has been extended to provide a gentle method for the isolation of negative strands from FMDV RF and RI. A poly(G) tract running more slowly than the poly(C) tract could be isolated from the negative strand of RF by RNase A digestion. This G-rich tract was not only resistant to RNase A but sensitive to RNase T1 (Fig. 5a to d). Approximately equimolar amounts of poly(G) and poly(C) were recovered from the RF when the ratio of incorporation of \(^{3}H\)cytidine and guanosine was taken into consideration.

As predicted by sodium bisulphite modification, the presence of a complementary poly(G) tract in the full-length negative strand of RI was confirmed when a large RNase A-resistant oligonucleotide was isolated on a polyacrylamide gel. The positive strands which did not bind to the CF11 cellulose column (see Methods) were collected, digested with RNase T1, and the oligonucleotides separated by polyacrylamide gel electrophoresis (Fig. 5e,f). The number of \(^{3}H\) d.p.m. in the poly(C) tract was measured and compared to the number of counts in the RNase A-resistant band. This gave an estimate of six poly(C) tracts to one complementary poly(G) tract in the RI when the ratio of incorporation of \(^{3}H\)cytidine and guanosine was taken into consideration.

DISCUSSION

The amount of secondary structure in the poly(C) tracts in the RNAs from FMDV particles and FMDV-infected cells has been studied by chemical modification of the RNA. Sodium metabisulphite will deaminate cytidylic acid residues to uridylic acid in single-stranded regions of RNA. Since G:C base pairs are more stable than A:U pairs and regions with secondary structure are likely to be enriched for G:C pairs (Cox et al., 1973), the amount of cytidylic acid deaminated in this reaction will give a good indication of the number of residues involved in base pairing and thus give a reasonable estimate of the overall amount of secondary structure in the RNA in solution. The value of 38% C to U conversion in FMDV RNA in solution gives an estimate of 62% of the cytidylic acid being base-paired and hence unreactive. This value is in good agreement with an estimate, obtained by Bachrach (1964) using circular dichroism, of 64% of the RNA in solution being in secondary structure conformation; 36% of the cytidylic acid was deaminated to uridylic acid in the EMCV RNA in solution used as a control. This value is in exact agreement with the value obtained by Goodchild et al. (1975) using similar conditions. Again the estimates obtained using chemical modification agree well with estimates for the amount of secondary structure of this RNA using physical methods (Frisby et al., 1977).

In contrast to the high overall secondary structure in the RNA, the poly(C) tracts in FMDV and EMCV RNA are largely single-stranded. Their reactivity with poly(I) suggests that they are looped out and exposed on the surface of the RNA. The 80% C to U conversion in the poly(C) tracts of EMCV and FMDV RNA after 96 h of reaction would at first sight indicate that the poly(C) tracts were fully single-stranded as 80% C to U conversion is the maximum observed in these reactions. Indeed, based on the 74% C to U conversion in the poly(C) tract of EMCV RNA after 96 h of reaction in their experiments, Goodchild et al. (1975) calculated that 90% of the poly(C) tract is single-stranded. They calculated this value by assuming minimum and maximum C to U conversions from the amount of reaction in double-stranded poly(I):poly(C) and denatured poly(C) preparations and by assuming that there was a linear relationship...
between the extent of reaction and the number of exposed cytidylic acid residues within this range. However, this assumption is not valid because the kinetics of the reaction between the poly(C) tract and bisulphite are not linear, but biphasic (Fig. 2). Comparison of the C to U conversion in the poly(C) tract in native and denatured RNA leads to the conclusion that only 60% of the poly(C) tract in solution reacts in a way similar to that observed in a single-stranded synthetic poly(C). The remainder of the poly(C) tract reacts at a rate similar to that observed in the majority of the single-stranded cytidylic acid in the RNA and this is much slower than in the synthetic single-stranded molecules.

The deamination reaction has also been used to examine the structure of the RNAs isolated from infected cells. The presence of more than one species of double-stranded RNA in FMDV-infected cells was discovered when this RNA was reacted with bisulphite. The C to U conversion in the RF was higher than expected; the reason for this is probably the presence of RNase-sensitive material in the double-stranded RNA preparations. Two ribonuclease-resistant double-stranded RNA molecules were resolved in agarose gels. RF1 is probably a linear RF; the nature of RF2 is uncertain. It is possibly a circular form of RF; these have been found in preparations of cardiovirus RF examined under the electron microscope (Agol et al., 1970, 1972; Robberson et al., 1982).

A gentle denaturation method has been used to isolate negative strands from the RF molecules and ribonuclease digestions have demonstrated the presence of a poly(G) tract in these negative strands. The majority of the RI isolated from infected cells at the peak of RNA production has a full-length negative strand from which positive strands are synthesized. Examination of the amount of secondary structure in the poly(C) tracts in FMDV RI indicates that five or six poly(C) tracts are deaminated to poly(U) for every one which is not, suggesting that each RI making positive strands contains five or six newly synthesized molecules. The same numbers are derived if the radioactivity recovered as RNase A-resistant poly(G) is compared to that in RNase T₁-resistant poly(C) from RI denatured as for RF (Fig. 5e,f), taking into account the ratio of [³H]cytidine to [³H]guanosine found in virus RNA, the recoveries of poly(G) and poly(C) from RF (Fig. 5a to d) and the fact that about 10 to 20% of an RI preparation consists of molecules synthesizing negative strands. These observations on RI and RF provide unequivocal evidence that the poly(C) tract is replicated via a complementary poly(G) in the negative strand.

Apart from a role in RNA replication or protein synthesis it is possible that the poly(C) tract is important in the structure of the virus particle, by mediating RNA-protein interaction. If the poly(C) tract were single-stranded in RNA inside the virus particle it would be available for extensive interaction with capsid proteins possibly as a nucleation site during particle assembly. Results of u.v. and chemical cross-linking of virus particle proteins to RNA, however, have not provided good evidence that the poly(C) tract acts in this way (Mellor, 1982).

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REFERENCES

Poly(C) tract in FMDV RNA


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