The determination of secondary structure in the poly(C) tract of encephalomyocarditis virus RNA with sodium bisulphite

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Received 14 April 1975

ABSTRACT

The degree of secondary structure in the poly(C) tract of encephalomyocarditis virus (EMCV) RNA has been investigated using sodium bisulphite, which brings about the hydrolysis of non-base-paired cytidylic acid to uridylic acid in RNA. The percentage conversion of C to U in the poly(C) region of native EMCV RNA was similar to that found in a synthetic polynucleotide lacking secondary structure [poly(C)]. When poly(I) was annealed to either native or denatured EMCV RNA, it protected the poly(C) tract from the action of bisulphite. It is concluded that the poly(C) tract of EMCV RNA in solution is very largely single-stranded.

INTRODUCTION

Large tracts of polycytidylic acid have recently been detected within the RNA genomes of a variety of picornaviruses\textsuperscript{1,2}. In the case of encephalomyocarditis virus (EMCV), a cardiovirus, one poly(C) tract is found within each viral RNA molecule, containing 85-90C, 3A and 4U residues\textsuperscript{3}. The role, if any, of this homopolymeric region in the viral replicative cycle\textsuperscript{3} is so far unknown; however, information about both the location of the poly(C) and the extent of its participation in secondary structure would be expected to contribute to an understanding of its function.

In this paper we set out to determine whether the poly(C) region of EMCV RNA is single-stranded and exposed, or base-paired with a corresponding region of poly(G). Although no poly(G) tract of comparable length has been detected in EMCV RNA (A.G.P. and P.F., unpublished), the possibility remained that the poly(C) could be hydrogen-bonded to shorter G-rich tracts, perhaps drawn from widely separated parts of the molecule. In one case, base-pairing between distantly separated RNA segments (bacteriophage MS2 RNA) has been strongly implicated in the translational control of synthesis of the virus-induced replicase\textsuperscript{4}.
Secondary structure in tRNA has previously been studied with the use of sodium bisulphite which hydrolyses to U only those C residues not involved in base-pairing\textsuperscript{5,6}. We therefore employed this reaction as a sensitive probe for the amount of secondary structure in the poly(C) tract of EMCV RNA. Our results show that about 90 per cent of C residues in the poly(C) tract are not base-paired.

MATERIALS AND METHODS

1) Materials

Poly(C) and poly(I) were obtained from P and L Biochemicals, bacterial alkaline phosphatase and pancreatic RNase from Worthington, and T\textsubscript{1} RNase from Sankyo.

2) Methods

a) Action of bisulphite on poly(C) and poly(I) : poly(C)

Control: poly(C) (3.7 u moles) was dissolved in 0.1M NaCl (0.2 ml) and diluted to 2 ml with water. Poly(C) + bisulphite : poly(C) (3.7 u moles) was dissolved in 0.1M NaCl (0.2 ml) and diluted to 2 ml with 3M bisulphite (pH 6.0) containing 10 mM MgCl\textsubscript{2} and 5 mM hydroquinone. Poly(C) + bisulphite + urea : poly(C) (3.7 u moles) was dissolved in 6M urea (0.2 ml). After heating at 80° for 3 min, followed by rapid cooling in ice, the solution was diluted to 2 ml with 3M bisulphite (pH 6.0) containing 6M urea and 5 mM hydroquinone. Poly(I) : poly(C) + bisulphite : A mixture of poly(C) (3.7 u moles) and poly(I) (4.3 u moles) was dissolved in 0.1M NaCl (0.4 ml). After heating at 69° for 10 min and allowing to cool to room temperature over a period of one hour, the solution was diluted to 4 ml with 3M bisulphite (pH 6.0) containing 10 mM MgCl\textsubscript{2} and 5 mM hydroquinone. Poly(I) : poly(C) + bisulphite + urea : A mixture of poly(C) (3.7 u moles) and poly(I) (4.3 u moles) was dissolved in 6M urea (0.4 ml). After heating at 80° for 3 min followed by rapid cooling in ice, the solution was diluted to 4 ml with 3M bisulphite (pH 6.0) containing 6M urea and 5 mM hydroquinone.

All the above reaction mixtures were incubated at 4° for 4 days. They were dialyzed at 4° three times against 0.1M NaCl containing 5 mM hydroquinone, twice against water, once against 0.05M sodium phosphate (pH 9.0) and finally four times against water.

b) Determination of C to U conversion

The polynucleotides were precipitated in 0.2M sodium acetate with 3 volumes ethanol and collected by centrifugation. They were dissolved in 1.0 ml aliquots of 0.3M NaOH and kept at 37° for 16 hr. The solutions
were neutralized with Dowex 50 (pyridinium form) and taken to dryness. After evaporation of several volumes of 1N NH₄OH to remove pyridine, the products were incubated with 0.1 units of bacterial alkaline phosphatase in 0.6M Tris-HCl pH 8.2 (150 ul) at 37° for 16 hr.

The nucleotides were separated on cellulose TLC plates in isopropyl alcohol : conc HCl : H₂O (65 : 16.7 : 18.3), eluted with 0.01N HCl and their UV spectra measured.

c) Action of bisulphite on EMCV RNA

Control : EMCV [³²P] RNA (2-3 ug, 10⁷ c.p.m.) was dissolved in 0.1M NaCl (0.2 ml) and diluted to 2 ml with water. EMCV RNA + bisulphite : EMCV [³²P] RNA (2-3 ug, 10⁷ c.p.m.) was dissolved in 0.1M NaCl (0.2 ml) and diluted to 2 ml with 3M bisulphite (pH 6.0) containing 10 mM MgCl₂ and 5 mM hydroquinone. EMCV RNA + bisulphite + urea : EMCV [³²P] RNA (2-3 ug, 10⁷ c.p.m.) was dissolved in 6M urea (0.2 ml). After heating at 80° for 3 min followed by rapid cooling in ice, the solution was diluted to 2 ml with 3M bisulphite (pH 6.0) containing 6M urea and 5 mM hydroquinone. Denatured EMCV RNA + poly(I) + bisulphite : EMCV [³²P] RNA (2-3 ug, 10⁷ c.p.m.) was dissolved in 25 ul water and 1 A₂₆₈₄ unit poly(I) added (12 ul). After heating at 80° for 3 min, the mixture was allowed to cool slowly to room temperature, left at 0° for 1.2 hr, and then diluted to 2 ml with 3M bisulphite containing 10 mM MgCl₂ and 5 mM hydroquinone. Native EMCV RNA + poly(I) + bisulphite : As above except that heating to 80° was omitted.

The reactions were conducted as for poly(I) : poly(C). After the final dialysis the RNA in each sample was ethanol precipitated in the presence of 100 ug non-radioactive carrier RNA.

d) Determination of C to U conversion in EMCV RNA

To measure the overall C to U conversion in bisulphite-treated EMCV [³²P] RNA, an aliquot (5 per cent) of each sample of RNA (see previous section) was incubated in 20 ul 0.3N NaOH for 16 hr at 37°. The resulting mononucleotides were resolved by electrophoresis (50V/cm; 4 hr) on Whatman No. 52 paper at pH 3.5 (5% acetic acid; 0.5% pyridine v/v) and their molarities determined by scintillation counting.

e) Determination of C to U conversion in the poly(C) tract of EMCV RNA

Bisulphite-treated EMCV [³²P] RNA (see above) was digested to completion with T₁ RNase (0.5 hr at 37°) in 40 ul of 0.02M Tris-HCl pH 7.5; 0.002M EDTA, at an enzyme : substrate (w/w) ratio of 1:20. To isolate the polypyrimidine segment, the T₁ RNase oligonucleotides in each digest were fractionated by electrophoresis in 20 x 40 cm polyacrylamide (10 per cent)
The poly(C) or poly(U) fragments were isolated from each gel slice, completely digested with pancreatic RNase and the products resolved by paper electrophoresis, identified and quantitated by counting (for full details see Figs. 2 and 3 of Ref. 1).

RESULTS AND DISCUSSION

Denatured and native EMCV [\(^{32}\)P] RNA, and poly(I)-protected EMCV [\(^{32}\)P] RNA were incubated at 0\(^{0}\) for 4 days in 3M bisulphite in the presence of hydroquinone. This was followed by successive dialyses (NaCl, H\(_2\)O, phosphate buffer pH 9.0, H\(_2\)O) to complete the conversion of single-stranded C to U, and remove unreacted bisulphite. These conditions were similar to those found satisfactory for tRNA\(^{5,6}\), but we found it necessary to carry out the reaction at 4\(^{0}\) instead of room temperature to avoid scission of the viral RNA (see Methods for details).

In order that any conclusions drawn from the conversion of C to U in the poly(C) region of EMCV RNA might be relevant to the normal conformation of the viral RNA in solution, it was necessary to perform control bisulphite reactions that would demonstrate that the following conditions were met:

1. The poly(C) region would undergo complete reaction if it were not base-paired.
2. The poly(C) region would not undergo reaction if it were base-paired.
3. The secondary structure of EMCV RNA is not destroyed under the conditions of the reaction.

To demonstrate that the first condition was met, a control reaction was carried out in which the percentage conversion of C to U was determined for the poly(C) region in denatured EMCV RNA. The RNA, in 6M urea, was heated at 80\(^{0}\) and cooled rapidly in ice. The bisulphite solution, also in 6M urea, was then added and the reaction performed as described in the Methods section. The polypyrimidine segment was isolated from the viral RNA by T\(_1\) RNase digestion (Fig. 1) and the nucleotide composition was determined by identifying and quantitating the products of subsequent pancreatic RNase digestion. In two experiments, 80 per cent of the C residues in the tract were converted to U (Table 1). The presence of urea was shown not to interfere with the bisulphite reaction by performing the reaction on synthetic poly(C) in both the presence and absence of 6M urea. The percentage conversion of C to U was practically the same in each case (Table 1), and similar to the 80 per cent conversion in the poly(C) region of denatured EMCV RNA. To demonstrate that the procedure for denaturation was effec-
Fig. 1. Gel electrophoresis of T\textsubscript{1} RNase digestion products of bisulphite-treated \textsuperscript{32}P labelled EMCV RNA. The gels were prepared and electrophoresis carried out as described in Methods and in Ref. 1, and autoradiographed to visualize the radioactive bands. The positions of the large T\textsubscript{1} RNase fragments consisting largely of C or U are indicated. Tract 1, native EMCV RNA (no bisulphite); tract 2, bisulphite-treated denatured EMCV RNA; tract 3, bisulphite-treated native EMCV RNA; tract 4, poly(I) annealed to denatured EMCV RNA, then bisulphite treated.
Table 1. Reaction of encephalomyocarditis virus RNA and its Poly(C) tract with sodium bisulphite.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C→U conversion by action of bisulphite (%)</th>
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<tbody>
<tr>
<td></td>
<td>Nondenatured RNA</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>1. EMCV RNA</td>
<td>36</td>
</tr>
<tr>
<td>2. Poly(C) tract in EMCV RNA</td>
<td>74</td>
</tr>
<tr>
<td>3. Poly(C) tract in denatured EMCV RNA annealed to poly(I)</td>
<td>20</td>
</tr>
<tr>
<td>4. Poly(C) tract in native EMCV RNA annealed to poly(I)</td>
<td>18</td>
</tr>
<tr>
<td>5. Poly(I) : poly(C)</td>
<td>16</td>
</tr>
<tr>
<td>6. Poly(C)</td>
<td>84</td>
</tr>
</tbody>
</table>

The C to U conversion resulting from the action of bisulphite is calculated from the alkali digestion products of whole EMCV RNA (row 1), the pancreatic RNase digestion products of the Poly(C) tracts (rows 2, 3 and 4) and the nucleosides derived from phosphatase treatment of the alkali digestion products of synthetic Poly(C) and Poly(I) : Poly(C) (rows 5 and 6).

tive, at least for the complex poly(I) : poly(C) (see * below), the complex was treated with bisulphite in the presence and absence of urea. In the absence of urea, 16 per cent of C residues reacted. In the presence of 6M urea, 80 per cent reacted, the same as for poly(C) alone. Taken together, these results satisfy the first condition, that the poly(C) region within the viral RNA would undergo maximum reaction if it were not base-paired.

To satisfy the second condition that, if the poly(C) tract in EMCV RNA were base-paired, it would not react with bisulphite, the tract was conver-
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ted into a double-stranded complex by annealing poly(I) to EMCV RNA. This was done in two ways. In one experiment, EMCV RNA was denatured by heating and then allowed to cool to room temperature in the presence of poly(I).

In a second experiment, poly(I) was annealed to native EMCV RNA (see Methods section). After reacting both mixtures with bisulphite, the percentage C to U conversion was determined as before by isolating the tract (Fig. 1) and determining its nucleotide composition (Table 1). In both experiments, only 18-20 per cent of C residues in the poly(I)-protected poly(C) tracts reacted, practically the same as for native poly(I): poly(C), and about one quarter of the reaction of the poly(C) region of unprotected, native EMCV RNA (Table 1). Although these results indicate the bisulphite reaction is not completely specific for single-stranded C under these conditions, they do provide an estimate of the C to U conversion to be expected if the poly(C) tract is base-paired in the native viral RNA.

Evidence that the third condition was satisfied was obtained by taking aliquots of the bisulphite-reacted native and denatured EMCV RNA, and determining the percentage conversion of C to U in whole EMCV RNA. This was done by quantitating the products of total alkali digestion of the viral RNA. 36 per cent and 65 per cent of C residues reacted in native and denatured EMCV RNA, respectively. The 65 per cent conversion in denatured EMCV RNA is low by comparison with the percentage conversion of C to U in poly(C) or denatured poly(I): poly(C), and indicates that not all G:C pairs in the viral RNA are melted, even at 80° in 6M urea*. If 18 per cent and 80 per cent C to U conversion represent the minimum and maximum reaction respectively (Table 1), an estimate of the percentage of C residues in EMCV RNA that are not base-paired is given by:

\[
\frac{36-18}{80-18} \times 100 \% = 29\%
\]

This assumes a linear relation between the extent of reaction and number of exposed C residues in the range 18-80 per cent conversion of C to U. Thus about 71 per cent of all C residues are base-paired in EMCV RNA.

Thus is in reasonable agreement with an estimate of 60 per cent secondary structure for EMCV RNA in free solution (D. Frisby and R. Cotter, unpublished and ref. 9), particularly since the base-paired regions might be enriched for G:C pairs, as is the case for other large RNA molecules10. These results indicate that the secondary structure of 'native' viral RNA is conserved during the reaction.
To determine the extent of C to U conversion in the poly(C) tract of native EMCV RNA, a total T, RNase digest of bisulphite-treated [32P] labelled RNA was electrophoresed in polyacrylamide gel. Fig. 1 shows that the poly pyrimidine tracts of native and denatured EMCV RNA moved appreciably slower than the poly(C) tracts of both unreacted EMCV RNA and poly(I)-protected, bisulphite-treated EMCV RNA. Analyses showed that the less mobile polypyrimidine tracts had undergone extensive C to U conversion (74 per cent and 80 per cent conversion in native and denatured EMCV RNA, respectively), whereas those from poly(I)-protected EMCV RNA underwent only 18-20 per cent conversion (Fig. 1 and Table 1). Presumably fragments with a high proportion of U migrate more slowly in polyacrylamide than those rich in C because of decreased base stacking, and consequently an increased Stokes' radius causing their greater sieving during electrophoresis 11, 12. Again taking 18 per cent and 80 per cent as minimum and maximum reaction, the percentage of non-base-paired C residues in the poly(C) tract of EMCV RNA is given by:

\[
\frac{(74-18)}{(80-18)} \times 100 \% = 90.3\%
\]

Thus the poly(C) tract of EMCV RNA in solution is largely single-stranded. Furthermore, since the poly(C) tract in native EMCV RNA is accessible to large polynucleotides such as poly(I), it cannot be buried within the viral RNA. An exposed single-stranded poly(C) region could specifically interact with macromolecules during the course of viral replication or protein synthesis, or perhaps with capsid proteins either during virion assembly or within the virus particle itself.

ACKNOWLEDGEMENTS

We wish to thank Drs. N.H. Carey, D. Frisby, R. Cotter and M. Eaton for valuable discussion, Mr. C. Newton for help in some of the experiments and Dr. A.J. Hale for the provision of excellent research facilities.

REFERENCES


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