POLY(C)SEQUENCE IS LOCATED NEAR THE 5'-END OF ENCEPHALOMYOCARDITIS VIRUS RNA

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Received May 11,1976

Summary: The distance between the poly(A) and poly(C) tracts in the molecules of encephalomyocarditis virus RNA has been estimated by two methods. The results indicate that these tracts are situated on the opposite ends of the viral RNA molecule. Evidence is presented that the poly(A) sequence in this molecule is located at the 3'-end. It is concluded that the poly(C) tract is situated at, or near, the 5'-end of the molecule.

It has been shown that molecules of encephalomyocarditis (EMC) virus RNA contain a poly(C) sequence about 100 nucleotides long (1). Similar poly(C) tracts are present in RNAs of some other picornaviruses (2). We have recently obtained preliminary evidence indicating that the poly(C) sequence is located at, or near, the 5'-end of the EMC virus RNA molecule (3). Data strongly supporting this conclusion are reported in this communication.

Materials and Methods

Methods for preparation of ^{32}P- and ^{3}H-labeled EMC virus RNA have been described (3).

Poly(U)- and poly(I)-celluloses were prepared by a method based on the reported procedures (4,5). The RNA preparations to be chromatographed on poly(U)-cellulose were dissolved in TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 7.5, 0.01% SDS) with 0.3 M NaCl and were loaded onto a poly(U)-cellulose column at 4°C. After washing the column with the same NaCl-containing buffer, elution of the bound RNA was carried out with TE buffer at 20°C. For poly(I)-cellulose chromatography, RNA

was dissolved in TE buffer with 0.5 M NaCl and was loaded onto the column at 25°C. Under these conditions, poly(U), poly(G) and ribosomal RNA from Krebs-II cells were not retained on the column, whereas poly(A), poly(C) and EMC virus RNA were adsorbed. Then the column was washed with a buffer containing 0.0015 M Tris-HCl, 0.001 M EDTA, pH 7.5, 0.01% SDS at 25°C. This procedure resulted in the elution of poly(A). Next, the elution with the same buffer was carried out at 45°C ensuring desorption of poly(C) and EMC virus RNA (3, and manuscript in preparation). Electrophoresis of ^{32}P-labeled RNA was carried out in 1.5% agarose gels prepared in buffer E (0.02 M Tris-HCl, 0.001 M EDTA pH 7.5, and 1 ug/ml of ethidium bromide for visualization of RNA). RNA samples were mixed with melted 1.5% agarose in 0.1 x E and loaded onto gels 10 cm long. After electrophoresis for 60 min at 150 v, gels were cut into slices 2 mm thick and radioactivity in each fraction was measured by Cerenkov radiation. Then the individual slices were melted for 1 min in a boiling water bath, cooled to 41°C and each sample was supplemented with 100 ug of ribosomal RNA from Krebs-II cells and 2.5 ug of RNase T1 (Worthington). After hydrolysis for 60 min at 36°C, the samples were melted again and loaded onto 10% polyacrylamide gels prepared in 0.05 M tris-acetate, 0.001 M EDTA, pH 8.3. Electrophoretic separation of oligonucleotides was carried out for 90 min at 150 v. Gels were cut into 2 mm thick slices. Slices were dried and radioactivity of each sample was counted in a toluene-based fluor in a liquid scintillation spectrometer. For nucleotides determination (6), slices were treated with 0.3 M KOH for 18 hr at 37°C.

Oxidation of RNA by periodate treatment and reduction with ^{3}H-borohydride (320 mCi/mmole, Amersham) were performed as described (7).

Thermal fragmentation (8) of RNA was achieved by heating RNA samples in TE buffer at 100°C for 15 or 20 min.

Results

EMC virus RNA contains poly(A) (9,10, and L.I.Romanova, in preparation) as well as poly(C) (1) sequences. The distance between these polynucleotide tracts was estimated by two methods. The rationale of the first method consisted in the following. Viral RNA was subjected to a mild random fragmentation

and RNA species containing both poly(A) and poly(C) tracts were selected from the resulting mixture by consecutive affinity chromatography on poly(U)- and poly(I)-cellulose columns. The minimal size of the RNA molecules containing both homopolymeric tracts should correspond to the distance between these tracts.

^{3}H-labeled EMC virus RNA was subjected to thermal degradation for 15 min and poly(A)-containing RNA species were isolated by chromatography on poly(U)-cellulose. It was found that roughly half of the labeled fragmented material was bound to poly(U)-cellulose in contrast to entirely complete adsorption of intact EMC virus RNA molecules. When the mixture of poly(A)-containing material thus obtained was subjected to poly(I)-cellulose chromatography, some 20% of the labeled material were adsorbed under conditions characteristic of poly(C) or poly(C)-containing RNA binding. This material should represent RNA molecules having both poly(A) and poly(C) sequences.

The sedimentation profiles of the initial fragmented RNA preparation, the poly(A)-containing fraction thereof, and the fraction possessing both poly(A) and poly(C) tracts are shown in Fig. 1. It is seen that while the two former preparations are represented by heterogenous sets of RNA species, the latter

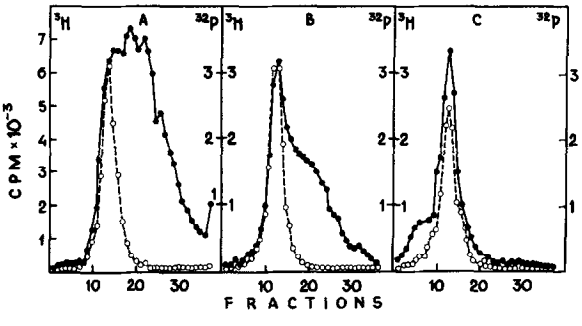

Fig. 1. Sedimentation profiles of an unfractionated fragmented tritium-labeled EMC virus RNA preparation (a), a poly-(U)-bound fraction (b), and a fraction isolated by consecutive binding to poly(U)-and poly(I)-celluloses (c) (●-----●). The conditions for fragmentation and chromatography are described in the text. Each sample contained intact phosphorus-labeled EMC virus RNA as the internal marker (o- - o). Sedimentation was performed in 5-20% sucrose concentration gradients prepared in TE buffer with 0.1 M NaCl in a Beckman SW65 rotor at 60,000 rpm for 75 min at 20°C.

fraction is fairly homogenous and its sedimentation coefficient coincides with that of the marker virion RNA. This result indicates that poly(A) and poly(C) sequences are located on the opposite ends of EMC virus RNA molecule.

It seemed necessary, however, to prove that the sequences in viral RNA responsible for binding it to poly(I)-cellulose are indeed identical to poly(C) tracts found in the T1 RNase hydrolysates of this RNA (1). To this end, the following approach was adopted. First, a method for the estimation of the content of poly(C) tracts in EMC virus RNA or its fragments was devised. ^{32}P-labeled viral RNA was hydrolysed by T1 RNase and electrophoresed in 10% polyacrylamide gel. Fig. 2 shows the result of such an experiment. A prominent peak larger than the tRNA marker is clearly discerned. The nucleotide composition analysis of this material revealed that it contained almost exclusively cytidylic acid residues. No such peak could be revealed after RNA hydrolysis with a mixture of pancreatic RNase and T1 RNase. Thus, the material in this peak seems to be identical to the poly(C) tract described by Porter et al. (1). The radioactivity in this peak appears to comprise 1.7-2.1% of the viral RNA radioactivity before the treatment with

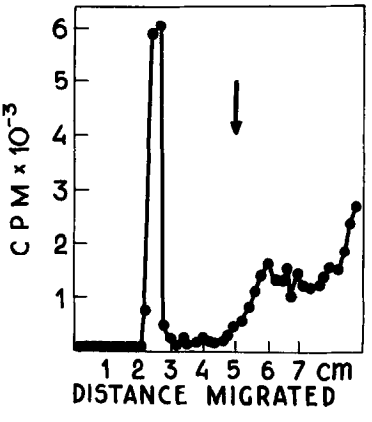

Fig. 2. Electrophoretic pattern of oligonucleotides present in T1 RNase hydrolysate of EMC virus RNA. Phosphorus-labeled viral RNA was treated with T1 RNase in E buffer without ethidium bromide and electrophoresed in 10% polyacrylamide gel as described in the text. The position of Krebs-II cells tRNA visualized by toluidine methylene blue staining (16) is indicated by the arrow.

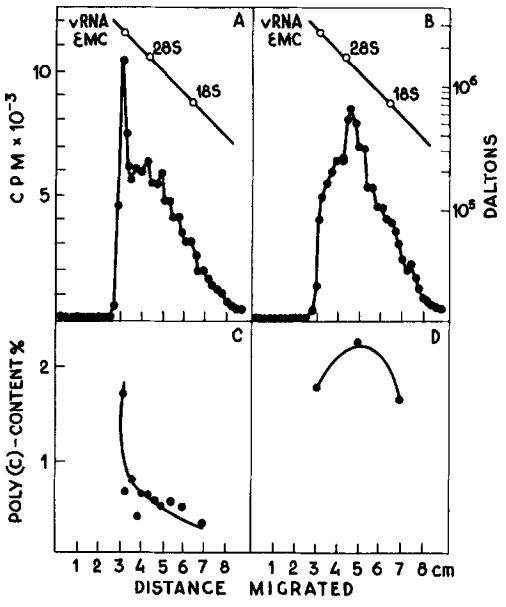

Fig. 3. Poly(C) content in different fractions of EMC virus RNA fragments. Phosphorus-labeled EMC virus RNA was subjected to thermal degradation (15 min) and fractionated on poly(U)-cellulose. Bound (a) and unbound (b) fractions were electrophoresed in 1.5% agarose gels. Intact EMC virus RNA, 28S rRNA, and 18S rRNA from Krebs-II cells were run as markers in a separate gel. Several zones of the gels a and b were treated with T1 RNase and analyzed in 10% polyacrylamide gels as described in the text. The radioactivity found in poly(C) tracts in per cent of the total radioactivity of the given fractions is plotted against the distance migrated by this fraction in the agarose gel. Poly(U)-bound (c) and -unbound (d) RNA species.

T1 RNase. Then the following experiment was performed. ^{32}P-labeled viral RNA was fragmented by heating and separated into a poly(A)-containing and a poly(A)-lacking fractions by chromatography on poly(U)-cellulose. These fractions were electrophoresed in agarose gels (Fig. 3a,b). RNA in several zones of these gels was treated with T1 RNase and the resulting material was analysed by electrophoresis in polyacrylamide gels. The ratio of radioactivity in the poly(C) peaks to the total radioactivity in the given zone of agarose gels was calculated (Fig. 3c,d). It is seen that in the poly(A)-containing fraction, the content of poly(C) dropped abruptly with an even small decrease in the molecular weight of fragments. This result indicates that only

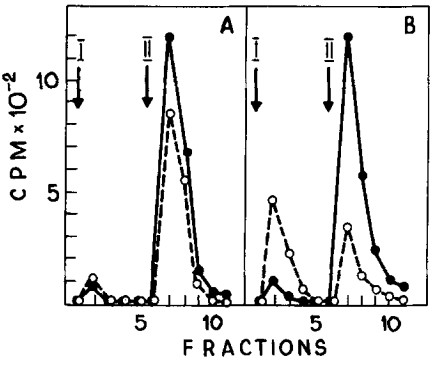

Fig. 4. Chromatographic profiles on poly(U)-cellulose of intact (a) and fragmented (b) EMC virus RNA labeled either uniformly with phosphorus (o - - o) or at the 3'-end with tritium (• ----- •). Samples containing both labeled RNAs in TE buffer with 0.3 M NaCl were loaded onto poly(U)-cellulose columns at 4°C (I), washed under the same conditions and eluted with TE buffer at 20°C (II).

longest poly(A)-containing molecules possess the poly(C) sequence. No such drop in poly(C) content was observed in different zones of the poly(A)-lacking fragments studied as a control. Thus, these data strongly support the conclusion that poly(A) and poly(C) stretches are located on the opposite ends of the EMC virus RNA molecule.

In eukaryotic cells (11) and viral (12) mRNAs poly(A) sequence is located at the 3'-end of the molecule. We felt that it was desirable to obtain direct evidence for the 3'-terminal localization of the poly(A) tract in EMC virus RNA. A preparation of viral RNA labeled with ^{3}H at the 3'-end by periodate-borohydride treatment was mixed with uniformly ^{32}P-labeled viral RNA and was fractionated on poly(U)-cellulose column before and after a 20 min thermal degradation. It was found that fragmentation resulted in the displacement of a large part of ^{32}P label to the fraction which was not retained on poly(U)-cellulose while the chromatographic behaviour of 3'-terminal ^{3}H label changed insignificantly, if at all. This should mean that the poly(A) tract is located near the 3'-end of the molecule; the poly(C) tract therefore is situated at, or near, the 5'-end of EMC virus RNA.

Discussion

The results presented in this paper establish the 5'-terminal localization of poly(C) tract in the EMC virus RNA molecule. The role played by this sequence in viral multiplication is unknown. Poly(C) may be located to the right or to the left of the single initiation site of translation (14) of this RNA. The former hypothesis seems to be less plausible because no polyproline sequences have been reported to be present in EMC virus-specific proteins. If poly(C) is located before the initiation site of translation it may participate in the interaction with ribosomes and/or with some regulatory proteins. On the other hand, poly(C) tract in the virion RNA or the 3'-terminal poly(G) tract in the complementary RNA may be involved in the RNA replication process. Some of these possibilities are under study in this laboratory.

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