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# Encephalomyocarditis Virus RNA

## III. Presence of a Genome-Associated Protein

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A low-molecular-weight protein was found to be associated with intact 35S RNA isolated from purified encephalomyocarditis virus. This protein was positively charged at pH 3.5, sensitive to proteinase K treatment, and labeled with either $^3$H-amino acids or $^{32}P_i$.

Encephalomyocarditis (EMC) virus RNA isolated from purified virions has a molecular weight of approximately $2.7 \times 10^6$, is infectious, and serves as an efficient message in many eucaryotic cell-free protein-synthesizing systems (6). EMC virus RNA does not contain the 5'-terminal "cap" structure [$m^7G(5')ppp(5')Np$] (4) common to most other eucaryotic mRNA's (11). However, it does contain an unusual feature at or near the 5' end, namely, an untranslated polycytidylic acid sequence (1). This region has been shown to be present in the genomic RNA of EMC virus, foot-and-mouth disease virus, and several other cardioviruses (5). Its function is presently unknown. With the recent discovery that the 5' end of poliovirus virion RNA contains a covalently linked protein (3, 8, 9), it was of interest to determine if a similar genome-associated protein (GAP) was present on EMC virus RNA in addition to the polycytidylic acid sequence.

EMC virus was grown in suspensions of Ehrlich ascites tumor cells and purified as previously described (6) except that the virus was also banded in cesium chloride gradients (EMC virus banded at 1.33 g/cm$^3$) to avoid contamination by cellular debris. The viral RNA was extracted from purified virions with phenol at 45°C in the presence of 0.5% deoxycholic acid and 1 mM EDTA (pH 7.4). When the RNA purified from EMC virus grown in the presence of $^3$H-amino acids was analyzed on sodium dodecyl sulfate-sucrose gradients (Fig. 1A), it was obvious that a substantial portion of the radioactivity cosedimented with the 35S viral RNA. This radioactivity appeared to be incorporated into both protein and RNA, since incubation of the purified viral RNA with either proteinase K (Fig. 1) or a nuclease mixture (Fig. 2) resulted in approximately one-half of the $^3$H becoming associated with low-molecular-weight compounds. Treatment of the viral RNA with proteinase K had no detectable effect on the 35S viral RNA absorbancy but did reduce by 50% the $^3$H-labeled viral RNA peak, with a new peak of $^3$H radioactivity appearing at the top of the gradient (Fig. 1). This new peak of radioactivity was larger than expected from the loss of 35S material; the explanation for this increase in measured radioactivity is unknown, but the phenomenon was reproducible and may relate to a greater efficiency of counting the hydrolyzed material. When similar gradients were assayed for radioactivity by acid precipitation and membrane filtration of the gradient fractions, the radioactivity in the 35S peak again was reduced by 50% after proteinase K digestion, but no radioactivity was observed at the top of the gradient. Thus, the purified 35S viral RNA appears to contain a GAP that can be hydrolyzed to acid-soluble products by proteinase K. Attempts to label EMC viral RNA, under identical growth conditions, with either [$^{35}$S]methionine or [*methyl*-$^3$H]methionine were unsuccessful. This is in agreement with earlier attempts to label poliovirus RNA with these precursors (2).

To ascertain more exactly the nature of the $^3$H-amino acid-labeled material, EMC virus [$^3$H]RNA was completely digested with a mixture of RNase's T2, T1, and A, and the digestion products were separated by pH 3.5 paper electrophoresis. Figure 2 shows that, whereas about one-half of the radioactivity was present in the various nucleotides (predominantly cytidylic acid), the rest of the radioactivity was present in a spot that moved slightly toward the cathode during electrophoresis. About 0.14% of the $^3$H radioactivity in intact EMC virus was recoverable in this region. An identical spot is observed if one carries out the same experiment with $^{32}$P-labeled EMC virus RNA. Approximately 0.025%

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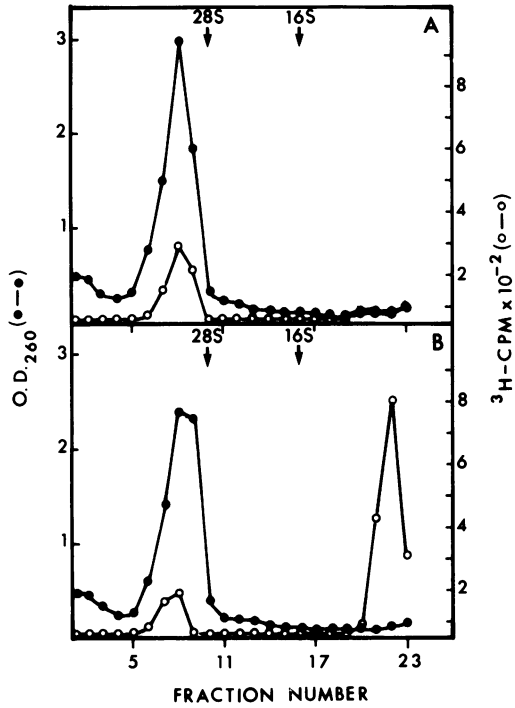


FIG. 1. *Sedimentation analysis of $^3$H-amino acid-labeled EMC virus RNA. EMC virus was grown in the presence of a 5-μCi/ml $^3$H-amino acid mixture (Schwarz/Mann, algal profile) plus 5 μg of actinomycin D per ml (Sigma). The viral RNA was subsequently extracted with phenol at 45°C and was ethanol precipitated (6). Two equal portions of the viral RNA were removed from the ethanol by centrifugation and suspended in 0.2 ml sodium dodecyl sulfate-gradient buffer and incubated at 37°C for 1 h. (A) Control, no additions; (B) proteinase K treated, 200 μg/ml (3). The RNA was then layered over an 11.5-ml, 5 to 20% sodium dodecyl sulfate-sucrose gradient and centrifuged at 35,000 rpm at 24°C for 4.75 h in an SW41 rotor (6). Fractions (0.5 ml) were collected from the bottom and analyzed for optical density at 260 nm ($OD_{260}$). Radioactivity was determined by mixing each fraction with 5 ml of Bray solution and counting it in a liquid scintillation counter.*

of the total $^{32}$P radioactivity recovered was present in this spot, corresponding to a yield of two phosphates per 35S RNA molecule. This calculation assumes complete recovery of the radioactive material and uniform $^{32}$P labeling of the four nucleotides, assumptions that may not be correct (3). Pretreatment of the nuclease digests of either $^3$H- or $^{32}$P-labeled viral RNA with proteinase K before electrophoresis resulted in a 70 to 80% reduction in the radioactivity migrating toward the cathode. Therefore, based on its migration towards the cathode and sensitivity to proteinase K, it appears that this material is a basic protein (GAP).

Figure 3 shows the analysis of the nuclease digestion products of EMC virus [$^{32}$P]RNA on a sodium dodecyl sulfate-polyacrylamide gel. A GAP was present that migrated between capsid protein δ and the bromophenol blue marker. This GAP was sensitive to proteinase K, as evidenced by a reduction in band intensity after exposure to this enzyme. Although this particular gel system cannot be used to accurately measure the size of the GAP, it would appear that the GAP is relatively small, with a molecular weight of less than 10,000.

Taken together, the data presented here suggest that, like poliovirus, EMC virus contains a GAP. This protein remains associated with the viral RNA through phenol treatments at 45°C and sodium dodecyl sulfate-sucrose gradient centrifugations, but we have not yet carried out experiments to prove its covalent attachment to the RNA. EMC virus GAP appears to be a small basic protein that can be detected in nuclease digests of intact EMC virus RNA by using either $^{32}$H-amino acids or $^{32}P_i$ as a source of label. Thus, the properties of GAP from EMC virus are sim-

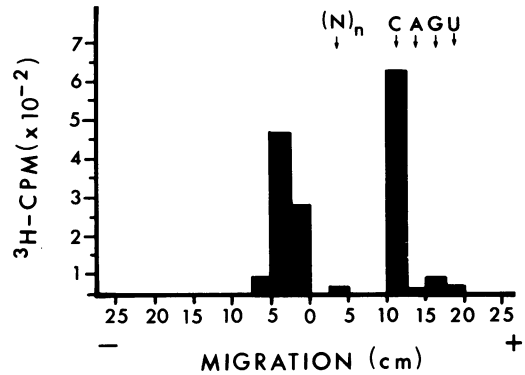


FIG. 2. *Ionoelectrophoresis of a nuclease digest of $^3$H-amino acid-labeled EMC virus RNA. A portion of the $^3$H-amino acid-labeled EMC virus RNA was centrifuged from the ethanol, suspended in 100 μl of ribonuclease mixture (RNase T2 [5 μg/ml]-RNase A [10μg/ml]-RNase T1 [50 U/ml] in 0.5 M ammonium acetate, pH 5.0) in the presence of 40 μg of bovine serum albumin, and incubated at 37°C for 1 h (3). After digestion, the products were spotted in the middle of a Whatman 3MM filter paper strip and subjected to electrophoresis at 900 V for 5 h in 0.05 M ammonium formate (pH 3.5) (10). After electrophoresis, the positions of the nucleotides were visualized under UV light. The filter strip was then cut into 2.5-cm strips and counted by liquid scintillation. All reactions and transfers were carried out with polypropylene test tubes and plastic pipette tips. C, A, G, and U represent the positions of the four nucleotides; $(N)_n$ represents a small amount of nondigested RNA that, upon extraction and redigestion, is broken down to nucleotides.*

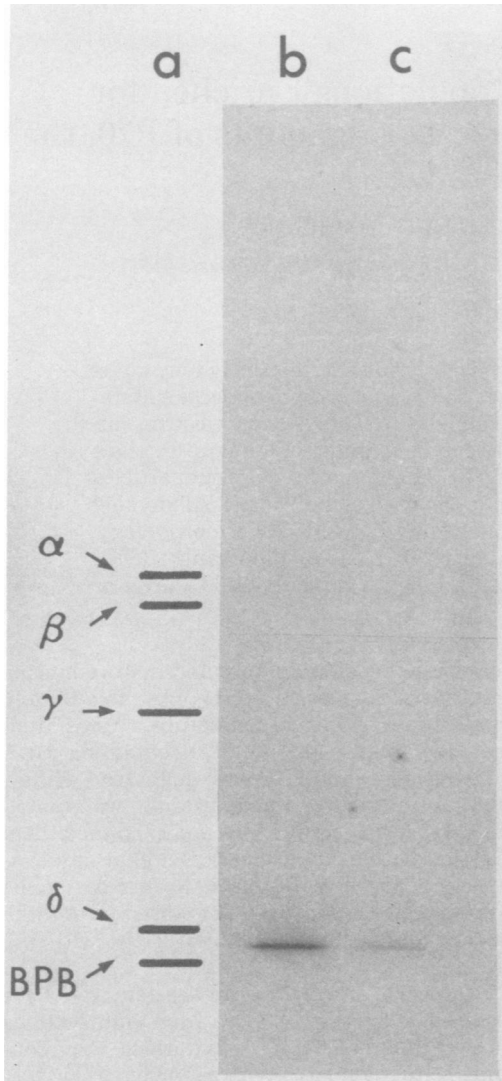


FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a nuclease digest of $^{32}$P-labeled EMC virus RNA. Two identical portions of EMC virus [$^{32}$P]RNA (2.5 × 10$^6$ cpm) were subjected to nuclease digestion as described in the legend of Fig. 2. One tube was then made 200 μg/ml in proteinase K, and both tubes were incubated at 37°C for an additional hour. The digestion products were acetone precipitated and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7) followed by autoradiography. (a) EMC virus capsid proteins (molecular weights: α, 34,000; β, 30,000; γ, 23,000; δ, 9,000) were located by staining with Coomassie brilliant blue (BPB is a bromophenol blue dye marker); (b) nuclease digest of EMC virus [$^{32}$P] RNA; (c) proteinase K-treated nuclease digest of EMC virus [$^{32}$P]RNA.*

ilar to the reported characteristics of poliovirus VPg (9) by all the criteria tested here. The presence of GAP may be common to all picornaviruses. The function of GAP is, as yet, unknown, but its study may yield some insight into the viral replication process itself.

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