Aminoacylation of Encephalomyocarditis Virus RNA

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SUMMARY

RNA extracted from purified encephalomyocarditis (EMC) virus (EMC-RNA) can be aminoacylated with synthetase preparations from *Escherichia coli*, beef and rabbit liver. The extent of aminoacylation is between 0-024 and 0-080 moles per mole EMC-RNA and occurs only with serine. Neither removal of possible low mol. wt. contaminants with 3 M-sodium acetate nor periodate oxidation of the virus RNA affects its aminoacylation capacity.

The genome of RNA viruses must possess structural features that are recognized by the cellular systems concerned with replication of the virus. The RNA of picornaviruses acts as messenger RNA and also as template for the polymerase that replicates the virus RNA. While some proteins required for virus replication may be entirely coded for by the virus genome it seems likely that many enzymes necessary for virus replication may, in part at least, be made up from host proteins, as is known to be the case for the polymerase that replicates Qβ RNA (Blumenthal, Landers & Weber, 1972). Investigation of the ability of the RNA extracted from purified particles of a picornavirus to interact with non-virus proteins may reveal cellular proteins necessary for virus replication.

A transfer RNA (tRNA)-like structure has been demonstrated in several groups of plant viruses (see Chen & Hall, 1973) and in a picornavirus, namely Mengo virus (Salomon & Littauer, 1974). The frequent discovery of tRNA-like structures in plant viruses strongly suggests some role for this structure in virus replication but the role is not apparent. In tobacco mosaic virus (TMV) and turnip yellow mosaic virus (TYMV) the amino acid appears to be added directly to the 3'-terminus of the virus RNA or after the addition of a single nucleotide (Yot et al. 1970; Guilley, Jonard & Hirth, 1975). Functional significance has been attributed to such aminoacylation (Litvak et al. 1973). However, charging may not occur in vivo and factors that recognize a tRNA-like structure other than aminoacyl tRNA synthetases may be functionally important during virus replication. The ability of virus RNA to be charged with an amino acid may therefore be inconsequential although at RNA-like structure may play an essential role in virus replication.

We report here the ability of aminoacyl tRNA synthetases to charge EMC-RNA specifically with serine. We have demonstrated that tRNA preparations of prokaryotic origin are protective against infection of mice with EMC virus and that this appears to be a feature of some but not all species of tRNA (Stebbing et al. 1976). We suggest that the tRNA-like structure in EMC-RNA plays a role of some functional importance in replication of the virus and that this function is suppressed by treatment of mice with tRNA.

All radioactive amino acids were obtained from the Radiochemical Centre, Amersham, Bucks. The sp. act. (Ci/mmol) of individual ¹H-amino acids were: alanine 42, arginine HCl 16, aspartic acid 0-178, glutamic acid 3-6, glycine 2-8, histidine 55, isoleucine 12, leucine 54, lysine HCl 19, phenylalanine 11, serine 15, tyrosine 53, and valine 17-7. A mixture of 15 tritiated amino acids including those just cited plus proline and threonine (Radiochemical Centre mixture TRK 440) and ³⁵S-methionine with a sp. act. of 38 Ci/mmol was also used.
EMC virus, obtained from Dr I. M. Kerr, was grown by passage in Krebs II ascites tumour cells in serum-free medium and purified by precipitation with polyethylene glycol 6000 (Kerr & Martin, 1972). EMC-RNA was produced from purified virus by phenol-chloroform extraction in the presence of 1% SDS as described by Porter, Carey & Fellner (1974) and stored at -20 °C in 20 mM-HEPES. Each preparation was checked for integrity and homogeneity by treatment with 100% formamide for 5 min at 60 °C followed by electrophoresis on 1.9% polyacrylamide gels prepared as described by Loening (1969).

A crude mixture of aminoacyl tRNA synthetases (E.C. 2.3.2.) was obtained from fresh rabbit livers by homogenization for 4 min in 0.05 M-KCl, 0.1 M-tris HCl, pH 7.6, containing 30 μg/ml of streptomycin sulphate, followed by sedimentation at 140000 g for 60 min. After 95% ammonium sulphate treatment, the supernatant was centrifuged at 40000 g for 30 min, the pellet raised in 0.002 M-tris HCl and repeatedly dialysed against the same buffer. The resultant enzyme preparation was stored at -20 °C. Purified aminoacyl tRNA synthetase mixtures from beef liver and E. coli were obtained from Biogenics Research Corporation Chagrin Falls, Ohio, U.S.A. A crude synthetase preparation from E. coli, strain K12 CA265, was supplied by Dr Atkinson of MRE, Porton, U.K. This enzyme preparation was found to contain only low levels of nucleases while the other synthetase preparations were heavily contaminated with nucleases. All four synthetase preparations gave essentially similar incorporation of labelled amino acids with a standard tRNA preparation using the mixture of labelled amino acids. All these synthetase preparations were also found to contain tRNA nucleotidyl transferase activity (EC. 2.7.7.25).

Incubation mixtures of 50 μl contained 10 μl of synthetase, 10 μCi of amino acids and 16 μg of EMC-RNA in 10 mM-ATP, 10 mM-MgCl₂, 3 mM-dithiothreitol, 20 mM-tris HCl, pH 7.4. After 15 min at 37 °C, 2 ml of 10% ice-cold trichloroacetic acid (TCA) was added, and after 30 min the precipitate was collected on Whatman 2 cm diam. GF/C paper discs and washed three times with 10 ml of 10% TCA and once with ethanol/ether (3:1). The discs were dried in hot air and counted in 10 ml of toluene-PPO-POPOP in a Nuclear Chicago Mk. II liquid scintillation spectrometer at an efficiency of 28%. Yeast tRNA, prepared as described previously (Stebbing et al. 1976), was used as a control in amino acid acceptor assays.

The crude rabbit liver synthetase preparation was found to cause rapid degradation of EMC-RNA by loss of TCA precipitable counts from a reaction mixture containing radiolabelled EMC-RNA. An incubation time of 15 min was chosen since this time interval achieved over 85% incorporation of labelled amino acid into yeast tRNA and did not cause complete degradation of EMC-RNA. The size classes of EMC-RNA fragments remaining after a 15 min incubation with the rabbit liver synthetase were examined by electrophoresis on polyacrylamide gels. Polyacrylamide gels (2-2%) run with buffer containing SDS at pH 5.7 showed no intact EMC-RNA, but only a heterogeneous population of fragments around the position of a tRNA marker. On a 10% polyacrylamide slab gel run at 4 °C in the presence of 6 M-urea, 22 major bands of RNA were found between 5 and 200 bases in length, and 17 of these bands were over 40 bases in length.

Three tritium-labelled amino acid mixtures including a total of 13 amino acids were used with the crude rabbit liver synthetase to test for EMC-RNA-stimulated incorporation of amino acids into TCA precipitable material. Each amino acid mixture was tested with EMC-RNA and yeast tRNA as a control and the results, shown in Table 1, include the background counts from parallel assays to which no RNA had been added. From these results it is clear that only the first amino acid mixture gave significant incorporation with EMC-RNA over the appropriate background level, and from other assays containing only
Table 1. Charging of EMC-RNA with different amino acids by rabbit liver synthetase

<table>
<thead>
<tr>
<th>Labelled amino acids</th>
<th>tRNA</th>
<th>EMC-RNA</th>
<th>Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>ser, ileu, phe</td>
<td>2122</td>
<td>3312</td>
<td>1429</td>
</tr>
<tr>
<td>leu, asp, tyr, gly, ala</td>
<td>11765</td>
<td>1243</td>
<td>1025</td>
</tr>
<tr>
<td>val, glu, lys, arg, his</td>
<td>20527</td>
<td>806</td>
<td>824</td>
</tr>
<tr>
<td>ser</td>
<td>1983</td>
<td>2944</td>
<td>1260</td>
</tr>
<tr>
<td>ileu</td>
<td>349</td>
<td>389</td>
<td>332</td>
</tr>
<tr>
<td>phe</td>
<td>788</td>
<td>599</td>
<td>535</td>
</tr>
<tr>
<td>his</td>
<td>12037</td>
<td>1602</td>
<td>1509</td>
</tr>
<tr>
<td>met</td>
<td>2464</td>
<td>2046</td>
<td>2280</td>
</tr>
</tbody>
</table>

one labelled amino acid, also shown in Table 1, this appears to be due entirely to incorporation of serine. We attribute the low incorporation of labelled ileu and phe into the yeast tRNA to low levels of the appropriate synthetase, since other synthetase preparations gave significant incorporation with these amino acids.

We also charged EMC-RNA with the mixture of tritiated amino acids including serine and compared the incorporation of amino acids into TCA precipitable material with a similar reaction mixture containing excess unlabelled serine. The results in Table 2 show that incorporation of the labelled amino acid into EMC-RNA is severely depressed in the presence of excess unlabelled serine. Moreover, when excess unlabelled amino acids (excluding serine) were added to a reaction mixture with serine as the only labelled amino acid, there was no depression in incorporation of label, as shown by the 3rd and 4th reactions in Table 2. We conclude that there is a population of EMC-RNA molecules that can be aminoacylated only with serine. However, we have not excluded the possibility of another population of EMC-RNA molecules that are chargeable with one of the five amino acids not tested directly in labelled form (i.e. cysteine, cystine, glutamine, asparagine or tryptophan).

Evidence that serine is esterified to EMC-RNA in the manner usual for a tRNA was provided by the observation that treatment of the reaction mixture for 15 min in pH 9 buffer after the incubation with synthetase and tritiated serine, completely reverses the appearance of label in TCA precipitable material as shown by reaction 5, Table 2.

The two E. coli synthetase preparations and the beef liver enzyme preparation also caused aminoacylation of EMC-RNA, although the enzyme from E. coli K12 CA265 was about 50% less efficient than the other enzyme preparations. Periodate oxidation of EMC-RNA and tRNA, using the conditions of Hunt (1965), completely eliminated aminoacylation of tRNA but had no effect on aminoacylation of EMC-RNA. With a counting efficiency of 28%, serine with a sp. act. of 15 Ci/mmol gives $9.4 \times 10^{12}$ ct/min/mmol. Taking the mol. wt. of EMC-RNA to be $2.7 \times 10^6$ (Burness & Clothier, 1970) we estimate that the efficiency of aminoacylation of EMC-RNA with serine, using the data shown in Table 1, is $1.974 \times (5.5 \times 10^4)^{-1} = 0.036$ mol serine/mol EMC-RNA. Different assays gave aminoacylation efficiencies between 0.024 and 0.080 mol serine/mol EMC-RNA.

Although analysis of formamide treated EMC-RNA on polyacrylamide gels did not reveal any low mol. wt. RNA in the 4S position (see Emtage, Carey & Stebbing, 1976), we examined RNA, extracted with 3 M-sodium acetate to eliminate low mol. wt. contaminants (method of Palmiter, 1973), for its ability to be aminoacylated. EMC-RNA, 50 μg, was
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Table 2. Competitive effect of various amino acids on charging of EMC-RNA with serine

<table>
<thead>
<tr>
<th>Reaction number</th>
<th>Treatment</th>
<th>&quot;H-labelled amino acids</th>
<th>ct/min/50 µl incubation (background subtracted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>&quot;Mixture&quot;*</td>
<td>1874</td>
</tr>
<tr>
<td>2</td>
<td>Excess serine</td>
<td>&quot;Mixture&quot;</td>
<td>357</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>Serine</td>
<td>1462</td>
</tr>
<tr>
<td>4</td>
<td>Excess amino acids except serine†</td>
<td>Serine</td>
<td>2694</td>
</tr>
<tr>
<td>5</td>
<td>pH 9‡</td>
<td>Serine</td>
<td>0</td>
</tr>
</tbody>
</table>

* The "H-labelled amino acid 'mixture' contained 15 protein amino acids including serine. Reaction 2 contained 570 µg/ml serine.
† Reaction 4 contained 715 µg/ml all protein amino acids except serine.
‡ Reaction mixture 5 was adjusted to pH 9 for 15 min, after charging with serine, before filtration.

heated at 65 °C for 5 min in 40 µl de-ionized formamide and then cooled rapidly. After ethanol precipitation and drying, the RNA was washed with 3 M-sodium acetate, centrifuged, and the pellet washed with ethanol and dried. This pelleted material could still be aminoacylated using the beef liver synthetase preparation.

The rather poor efficiency of aminoacylation of EMC-RNA with various synthetase preparations and the necessity for nuclease contamination of the enzyme preparations is now apparent. Although the location of the tRNA-like structure in EMC-RNA is not known, it cannot be at the 3'-terminus at which there is an oligo A tract (see Emtage et al. 1976). The tRNA-like structure in EMC-RNA must therefore be cleaved out prior to aminoacylation. Successful aminoacylation of Mengo virus RNA with histidine was also found to be associated with fragmentation of the virus RNA (Salomon & Littauer, 1974). Poliovirus RNA and foot-and-mouth disease virus (FMDV) RNA have not been found to be aminoacylatable (Oberg & Philipson, 1972; Chatterjee, Bachrach & Polatnick, 1976). However, Chatterjee et al. (1976) used a synthetase preparation that was virtually free of nucleases. Detection, by the aminoacylation reaction, of tRNA-like structures which are not at the 3'-end of a virus RNA probably requires the presence of tRNA nucleotidyl transferase and phosphatases in order to generate a 3'-terminal -CCA sequence necessary for esterification of an amino acid. Our enzyme preparations all contained nucleotidyl transferase activity but those of Oberg & Philipson (1972) and Chatterjee et al. (1976) may not have.

Aminoacylation of EMC-RNA occurred to approximately the same extent with both the eukaryotic and prokaryotic synthetase preparations. Similar results have been obtained with some, but not all, plant virus RNAs (Yot et al. 1970; Oberg & Philipson, 1972; Litvak et al. 1973). It is also of interest to note that while different members of the same taxonomic group of plant viruses can all be aminoacylated with the same amino acid, this would not appear to be the case for animal viruses since EMC virus and Mengo virus are closely related picornaviruses within the cardiovirus sub-group. There can be no doubt, from the results in Table 1, that our EMC-RNA preparations lack the ability to be aminoacylated with histidine. Since the tRNA-like structures in animal virus RNAs are not 3'-terminal it is possible that the particular amino acid with which they can be artificially charged is unimportant but that the situation is different for plant viruses which have 3'-terminal tRNA-like structures.

The possibility of a contaminating tRNA not covalently linked to EMC-RNA as the basis of our observations is discounted by several observations. Firstly, removal of low mol.
wt. polynucleotides by 3 M-sodium acetate after denaturation in formamide has no effect on aminoacylation of 37S EMC-RNA. Secondly, periodate oxidation of EMC-RNA preparations would be expected to prevent aminoacylation of a contaminating tRNA as well as a tRNA-like structure that was situated at the 3'-terminus of EMC-RNA. Thirdly, a contaminating tRNA that was hydrogen-bonded to EMC-RNA might not be affected by periodate but would then be expected to act as a primer for reverse transcriptase as occurs in oncornaviruses (Faras et al. 1974). However, no complementary DNA can be made from EMC-RNA without addition of synthetic primers (Emtage et al. 1976). Finally, the possibility of base sequences related to tRNAs close to the 3'-terminal oligo A tract or the poly C tract in EMC-RNA (Porter et al. 1974; Emtage et al. 1976) was previously investigated by looking for S1-nuclease resistant fragments in DNA primed with oligo dT or oligo dG on EMC-RNA using reverse transcriptase, after annealing the DNA to E. coli tRNA (Emtage et al. 1976). No such protected fragments were found. However, the similarity in terms of base sequences of tRNAs and the tRNA-like region in virus RNA may be small, as is now known to be the case for the histidine tRNA-like structure in TMV-RNA (Guilley et al. 1975). Moreover, other factors, already discussed, could have prevented detection of a tRNA-like structure by this procedure (Emtage et al. 1976).

The role of a tRNA-like structure in virus RNAs is not apparent but different functions have been proposed for these structures in the aminoacylated and non-aminoacylated states. Litvak et al. (1973) suggest that aminoacylation may aid differentiation of plus and minus strand replication or promote preferential translation of virus messenger RNA by forming a complex with elongation factors that have high affinity for ribosomes. A direct role in protein synthesis seems unlikely since in all cases examined the amino acid donor activity of virus RNAs is poor (Chen & Hall, 1973; Haenni et al. 1973). We suspect that aminoacylation of the plant virus RNAs may not occur naturally and that the presence of a tRNA-like structure serves primarily for achieving a specific interaction with a protein that forms part of a functional enzyme such as the elongation factor Tu forms part of the Qβ replicase (Blumenthal, Landers & Weber, 1972). While elongation factors seem to interact only with aminoacylated tRNAs it is possible that a tRNA-like structure not at the 3'-terminus of a virus RNA may be recognized as if it were aminoacylated. Moreover, the inability of a tRNA-like structure to be aminoacylated is not an indication that the structure is not recognized by tRNA binding proteins: direct binding assays show that aminoacylated and non-aminoacylated tRNAs are recognized equally well by tRNA synthetases (Yarus & Berg, 1967).

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