PROTEIN SYNTHESIS DIRECTED BY ENCEPHALOMYOCARDITIS VIRUS RNA

II. THE IN VITRO SYNTHESIS OF HIGH MOLECULAR WEIGHT PROTEINS AND ELEMENTS OF THE VIRAL CAPSID

I. Boime, H. Aviv and P. Leder

Section on Molecular Genetics
Laboratory of Biomedical Sciences
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20014

Received August 30, 1971

Summary

RNA from the encephalomyocarditis virus directs the cell-free synthesis of several discrete, high molecular weight proteins. The largest of these have molecular weights of approximately 110,000, 82,000, 73,000, 61,000 and 44,000 Daltons. In addition, tryptic digestion of the in vitro products gives rise to a number of peptides corresponding to those derived from the viral capsid. The data suggest that approximately one-third of the information encoded by the EMC genome is translated in vitro as a single polypeptide chain, that this translation proceeds in an appropriate phase, and that portions of the genome corresponding to structural proteins of the virus are translated.

Despite the fact that encephalomyocarditis virus (EMC) RNA has been shown to direct protein synthesis in several cell-free systems (1-5), the products of these reactions seem to be relatively low molecular weight proteins which do not contain peptides corresponding to the mature viral capsid (4, 5). This is unexpected in that in vivo synthesis of proteins of the EMC virus is thought to proceed through high molecular weight precursors which are processed into smaller capsid and non-capsid proteins (6) (cf. polio virus [7-9]). We have recently shown that EMC RNA-directed protein synthesis in crude cell-free extracts of Krebs II ascites cells is surprisingly dependent upon the addition of exogenous tRNA (10). Moreover, this requirement can be met only by tRNA derived from rather closely related species. Since this improved system might translate the EMC genome completely, conceivably making mature viral proteins, we have begun to characterize the products of this reaction. Here

we show that EMC RNA directs the cell-free synthesis of several discrete, high molecular weight proteins which apparently include elements of the viral capsid. The largest of these products would correspond to approximately one-third of the information contained in the EMC genome.

Materials and Methods

Extracts derived from Krebs II ascites cells were prepared and pre-incubated according to Aviv, Boime and Leder (10) using a modification of the techniques described by Mathews and Korner (3) and by Kerr and Martin (5). The preparation and purification of the EMC virus, EMC RNA and Krebs II ascites tumor cell tRNA has been described (10). Radioactive virus was obtained by adding ^{35}S-methionine (specific activity, 23,000 mCi/mmole) or ^{14}C-phenylalanine (specific activity, 460 mCi/mmole) to cell suspensions four hours after infection with EMC virus and the simultaneous addition of actinomycin D (5 μg/ml). The labeled virus was purified using the techniques noted above.

Protein synthetic reaction mixtures, generally 0.1 ml, contained 30 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 120 mM KCl, 7 mM 2-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, 0.16 mg/ml creatine kinase, 40 μM 19 non-radioactive amino acids, 5 μM radioactive amino acid of high specific activity (^{35}S-methionine or ^{14}C-phenylalanine, as indicated), 0.4-1.0 A_{260} units Krebs II ascites tumor cell tRNA, pre-incubated ascites tumor cell extract containing 420 μg protein and 2 μg EMC RNA. Incubation was at 37° for 150 minutes. Reactions were stopped by adding 0.2 ml of 0.1 M KOH. Incubation was continued for 20 minutes to hydrolyze the peptidyl-tRNA, and then 1 ml of ice-cold 10% Cl_3CCOOH was added. The reactions were allowed to stand at 0° for 10 minutes and centrifuged at 3000 RPM in a Sorvall SS-34 rotor for five minutes. The precipitate was washed once with cold 5% Cl_3CCOOH and, subsequently, twice with acetone to remove residual Cl_3CCOOH.

The samples of *in vitro* product or purified EMC virus were prepared for analysis by dissolving them in 0.02 M Tris-Cl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol,

10% glycerol, 0.001% brom phenol blue and by heating to 100° for one minute (11). Discontinuous acrylamide gel electrophoresis in a linear (7-20%) acrylamide gradient containing SDS and 2-mercaptoethanol was carried out at room temperature at 200 volts for 3 hours (11, 12). The slab gel apparatus described by Reid and Bieleski (13) was used with appropriate standard marker proteins on both sides of the sample. The slabs were stained with 0.2% Coomasie blue in 50% methanol, 7% acetic acid and dried according to Maizel (12). The dried slabs could be conveniently exposed to X-ray film.

Results and Discussion

Butterworth, Stoltzfus and Rueckert (6) have shown that *in vivo* synthesis of EMC proteins proceeds through the apparent proteolytic cleavage of high molecular weight precursors. A similar mechanism appears to be involved in the production of both viral capsid and non-capsid viral proteins. In this respect, EMC virus is quite similar to polio in which precursor protein products corresponding to a large portion of information in the viral genome have been isolated *in vivo* (7-9).

Early reports of cell-free translation of EMC RNA suggested that this translation was incomplete, giving rise to low molecular weight proteins which did not contain structural elements of the viral capsid (4, 5). We have recently shown that crude extracts of Krebs II ascites tumor cells are appreciably depleted with respect to tRNA (10). The activity of this system is considerably enhanced by the addition of tRNA from an appropriate species. Thus, we felt this tRNA-supplemented system might translate more extensive regions of EMC genome. To begin to test this possibility, the radioactive products of an EMC RNA-directed reaction carried out in the presence of saturating amounts of tRNA were analyzed by SDS-acrylamide gradient gel electrophoresis (Figure 1). Five relatively discrete protein products of molecular weight greater than 41,000 Daltons were identified (Figure 1C). These are listed in Table 1. The two largest, having molecular weights of 110,000 and 82,000 Daltons, would each account for almost one-third of the information included in the EMC genome. The

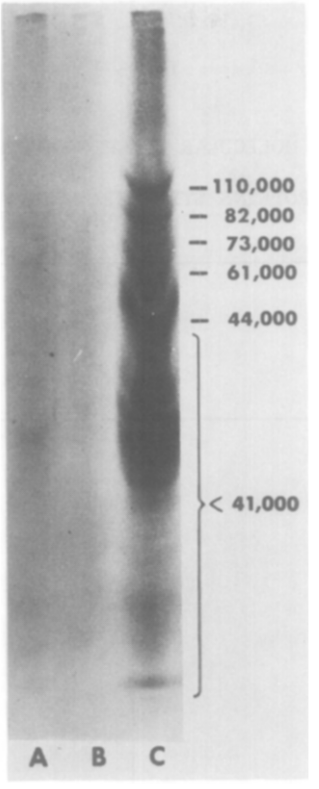

Figure 1. SDS-acrylamide gradient (7-20%) gel electrophoresis was carried out as indicated in the text. Standards, listed in the legend to Table 1, provided the calibration shown adjacent to the samples.

combined molecular weight of all five large in vitro proteins is approximately 370,000 Daltons and, if each were a discrete gene product, would account for all the information on the EMC genome [assuming a molecular weight of 2.7×10^6 Daltons for EMC RNA (14)]. However, the relation of these large in vitro products to one another and to the low molecular weight proteins also seen has not as yet been determined. Even the large proteins may bear a precursor-product relationship to one another as has been demonstrated for EMC precursor proteins in vivo (6). Control analyses carried out in the absence of added tRNA (Figure 1A) show very limited synthesis of low molecular weight material. No product is seen in the absence of EMC RNA (Figure 1B).

If the major portion of the EMC genome is being translated in vitro, we should be able to detect tryptic peptides corresponding to those derived from the purified EMC virus. Comparisons of the two dimensional analyses of ^{14}C-phen-

TABLE 1

APPROXIMATE MOLECULAR WEIGHTS OF EMC RNA-DIRECTED PROTEINS SYNTHESIZED *IN VITRO*

Protein	Molecular weight (Daltons)
1	110,000
2	82,000
3	73,000
4	61,000
5	44,000
Total molecular weight of *in vitro* proteins	370,000

Molecular weights were calculated following SDS-acrylamide gradient (7-20%) gel electrophoresis as indicated in Figure 1. Comparison was made to purified samples of myosin (200,000), *E. coli* G factor (15) (73,000), γ-globulin [50,000 (heavy chain) and 25,000 (light chain)], myeloma protein, MOPC-41C (25,000) and lysozyme (14,300).

ylalanine and ^{35}S-methionine labeled tryptic peptides derived from purified EMC virus and the *in vitro* product are shown in Figures 2 and 3. The ^{14}C-phenylalanine labeled EMC capsid yields nineteen discrete spots; the *in vitro* product, twenty two (Figure 2). Of the twenty two spots seen *in vitro*, a minimum of ten correspond to EMC capsid peptides. Similarly, nineteen ^{35}S-methionine labeled peptides can be derived from the *in vitro* product, twenty one from the EMC-capsid (Figure 3). Of these, nine clearly correspond to one another. The fact that not all peptides derived from the EMC capsid can be identified in the *in vitro* product, despite the *in vitro* synthesis of very high molecular weight proteins, may be accounted for in several ways. It is entirely possible that that portion of the EMC

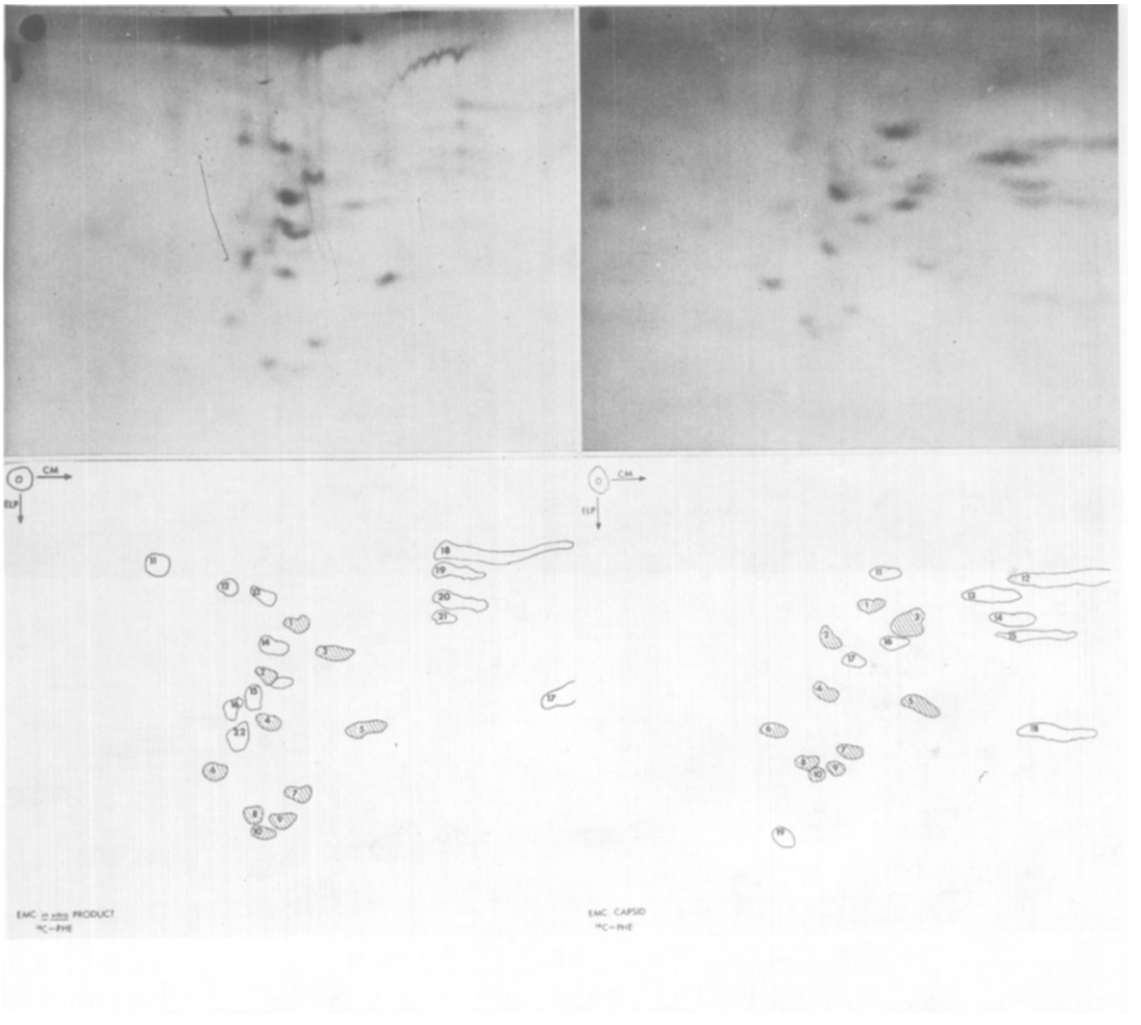

Figure 2. Radioautograms of two-dimensional tryptic peptide maps derived from EMC RNA directed in vitro products and authentic EMC capsid protein, both labeled with ^{14}C-phenylalanine. Below each radioautogram is a diagramatic tracing of the map. The cross-hatched spots correspond in each set. Analyses were carried out with appropriate dye marker standards. For tryptic digestion, samples were treated as described under Materials and Methods except that 2-mercaptoethanol and 2-hydroxy ethyl disulfide were added to give final concentrations of 2 mM and 100 mM, respectively, prior to heating. The samples were then dialyzed against 0.6% NH_4HCO_3, pH 8.0, for 16 hours, removed and incubated with a 1:1 w/w portion of trypsin for 16 hours at 37°. The digested samples were lyophilized, dissolved in a small volume of water, and applied at the corner of a 46 x 57 cm sheet of Whatman 3 MM paper together with xylene cyanole ff, acid fuchsin and orange g dye standards and developed by descending chromatography in butanol-acetic acid-water (270/80/400) for 18-20 hours. As a second dimension, the resultant chromatogram was electrophoresed at pH 3.5 at 2.0 K volts for 1.5 hours. The dried tryptic map was exposed to X-ray film for 12-30 days.

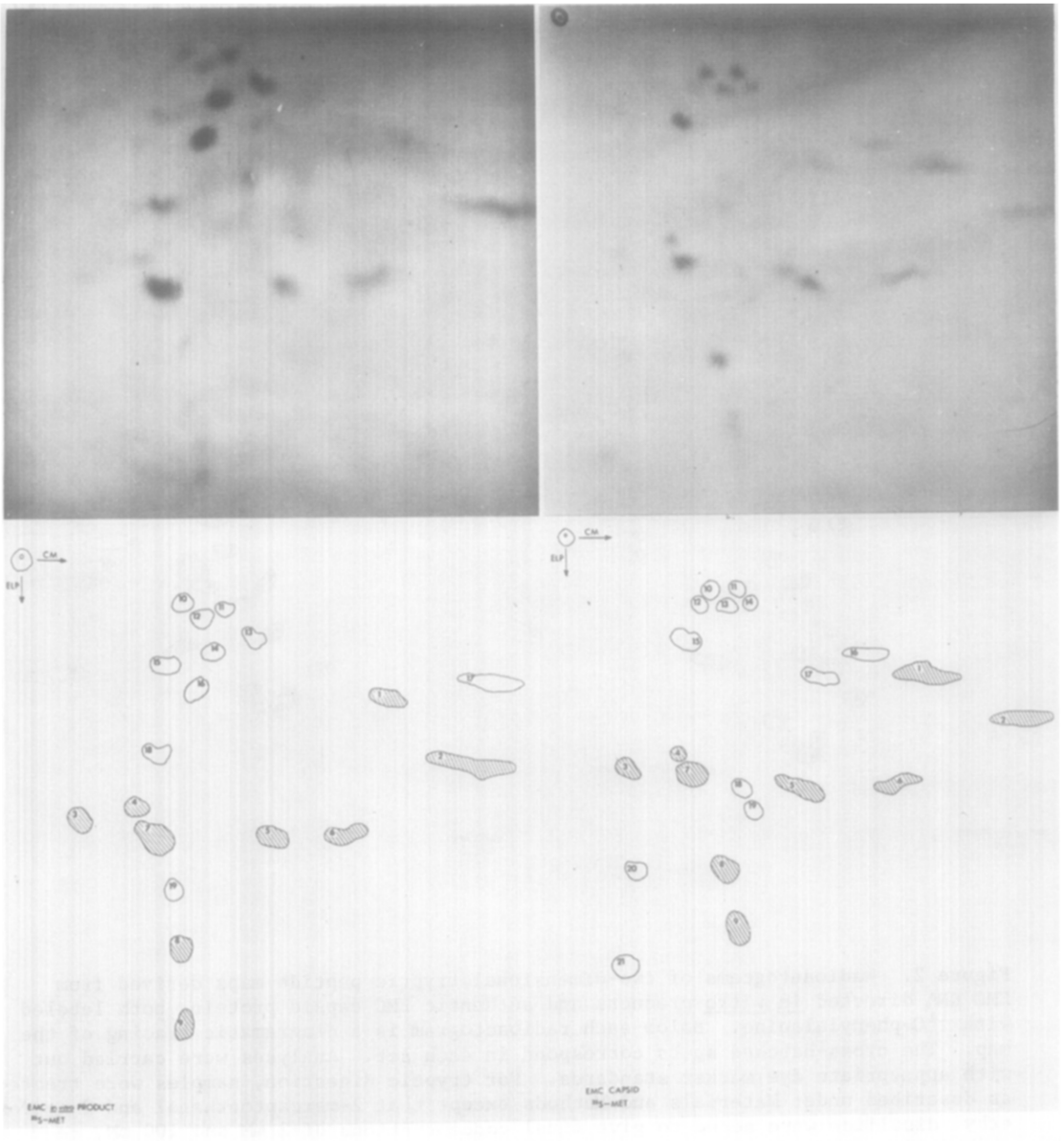

Figure 3. Radioautograms of two-dimensional tryptic peptide maps derived from EMC RNA directed *in vitro* products and authentic EMC capsid protein, both labeled with ^{35}S-methionine. Below each radioautogram is a diagramatic tracing of the map. The cross-hatched spots correspond in each set. Analyses were carried out with appropriate dye marker standards as indicated under Figure 2.

genome corresponding to the capsid is only partially translated. However, the number of peptides which clearly correspond to one another has been conservatively

estimated, and further identities may be brought out by comparison of spots in a third dimension (cf. ^{35}S-capsid peptides, 10-15, ^{14}C-capsid peptides, 12-15). It is also possible that certain of the capsid peptides appear only after *in vivo* cleavage of procapsid molecules and cannot be derived directly from the tryptic digestion of the unprocessed procapsid. Further, the mature virion may be enriched with respect to certain proteins which are represented to a lesser extent among the *in vitro* products.

While these results are preliminary, they do indicate that the tRNA supplemented cell-free system is able to translate long stretches of the EMC genome in appropriate phase giving rise to discrete protein products. It is also clear that elements of the viral capsid are among the products synthesized. Recent studies by Kerr and his associates (personal communication) also suggest that elements of the viral capsid are synthesized *in vitro*. It will be interesting to see which, if any, of these high molecular weight proteins give rise to the viral capsid proteins and to correlate these with specific regions of the viral genome.

Acknowledgment

We are grateful to Dr. J. V. Maizel for his advice regarding acrylamide gel electrophoresis, to Drs. R. Adelstein and E. Appella for their gifts of purified myosin and myeloma protein, respectively, and to Dr. O. Smithies for providing us with 2-hydroxyethyl disulfide.

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