Synthesis of Capsid and Noncapsid Viral Proteins in Response to Encephalomyocarditis Virus Ribonucleic Acid in Animal Cell-Free Systems

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The polypeptide products formed in two cell-free protein-synthetic systems programmed with encephalomyocarditis (EMC) virus ribonucleic acid (RNA) have been compared with the virus-specific proteins found in EMC-infected cells and with the capsid proteins of the purified virion. Tryptic peptides of ^{35}S -methionine-labeled proteins from these three sources were compared by co-chromatography and electrophoresis and by isoelectric focussing. Fifty-two methionine-containing peptides were resolved in digests of material from infected cells, of which about one-third were also clearly present in digests of the virion capsid proteins. The product formed in response to EMC RNA in cell-free systems from Krebs mouse ascites tumor cells yielded 26 to 29 such peptides. Most of these peptides were shown to behave identically with virus-specific peptides from infected cells, whereas just under half of them appeared to be identical with peptides from the virion capsid proteins. The product formed in response to EMC RNA in the L-cell cell-free system was similar, whereas six additional EMC-specific peptides were detected in mixed Krebs L-cell systems. The results indicate that the EMC RNA genome is partially translated in the mouse cell-free systems used to yield products containing both virion capsid and virus-specific noncapsid polypeptides.

The stimulation of amino acid incorporation in animal cell-free systems in response to the ribonucleic acid (RNA) of encephalomyocarditis (EMC) virus is well documented (7, 9, 12, 15). On the basis of fingerprint analyses of ^{35}S -methionine-containing tryptic peptides, we recently reported that the same polypeptide product, in part, is synthesized in response to EMC RNA in two such systems from chick and mouse cells (8). We did not, however, present data comparing the polypeptides formed in the cell-free systems with authentic EMC-specific proteins. From such a comparison, Smith, Marcker, and Mathews found no evidence for the cell-free synthesis of virion capsid proteins, but their data did indicate some identity between the tryptic peptides derived from material synthesized in response to the viral RNA in the cell-free system and noncapsid virus protein(s) present only in infected cells (15).

Here we report the results of an analysis of the polypeptides formed in response to EMC RNA in cell-free systems from mouse Krebs 2 ascites tumor and L cells. By the use of thin-layer finger-

printing techniques, we have been able to resolve over 50 virus-specific tryptic peptides in infected cells and to show that about half of these are also present in digests of the product synthesized in the cell-free systems. In addition, we present evidence that these systems also synthesize virus capsid proteins in response to the viral RNA.

MATERIALS AND METHODS

Materials. Chemicals for use in the cell-free systems, for the isolation of cell fractions and viral RNA, and for the preparation of tryptic digests were described previously (7, 8). All radiochemicals were supplied by the Radiochemical Centre, Amersham, Buckinghamshire, England. Actinomycin D was the generous gift of H. D. Brown of Merck & Co., Inc., Rahway, N.J. The L cells were a line of interferon-sensitive cells obtained from N. B. Finter, Wellcome Research Laboratories, Beckenham, Kent, England. The origin of the Krebs 2 ascites tumor cells and the methods for their propagation were described elsewhere (11).

EMC virus and viral RNA. The growth and purification of EMC virus and the extraction of its RNA have been described (2, 7, 8). For the preparation of ^{35}S -

methionine-labeled virus, swirling suspensions of Krebs cells (11) were incubated for 3 hr with 3 μ g of actinomycin D per ml and then infected with EMC virus at an added multiplicity of 10 plaque-forming units (PFU)/cell. At 2.5 hr postinfection, 0.5 mCi of 35 S-methionine (specific activity, 30 Ci/mmol) was added, and the virus was harvested 12 to 14 hr later. It was purified as described previously (2, 8) to yield about 1.5 mg of virus having a specific activity of 2×10^6 counts per min per mg for each liter of cell suspension. The sedimentation properties ($S_{20,w} = 156$) and ultraviolet spectrum of the virus and the analysis of its constituent polypeptides on acrylamide gels were used as criteria of purity. It was subjected to performate oxidation and tryptic digestion without removal of the RNA.

Preparation of ribosome and cell-sap fractions. Components for the cell-free system were prepared from Krebs cells as described previously: the endogenous protein synthetic activity was reduced by preincubation of the postmitochondrial supernatant fluid (7, 8). Ribosomes were prepared from L cells as follows: cells (about 10^8 to 2×10^8), grown to confluence in rolling cultures, were trypsinized and suspended in Eagle's Spinner medium containing 10% calf serum at a concentration of 5×10^6 cells/ml. After incubation in Spinner culture for 24 hr, the cells were washed and disrupted, and the postmitochondrial supernatant fluid was prepared as for the Krebs cell system (7). It was supplemented with adenosine triphosphate (ATP), guanosine triphosphate (GTP), phosphoenolpyruvate, and pyruvate kinase at the concentrations used in the cell-free system assay, incubated for 30 min at 37 C, and centrifuged at $105,000 \times g$ for 1.5 hr. The ribosome pellet was suspended in a small volume of 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6), 10 mM KCl, and 0.1 mM magnesium acetate and layered over a solution of 0.5 M sucrose containing these components. It was centrifuged in a swinging-bucket rotor at $120,000 \times g$ for 2 hr, and the pellet of washed ribosomes was suspended in 0.25 M sucrose and 20 mM Tris-hydrochloride (pH 7.6) at a concentration of 10 mg of ribosomes per ml. The ribosomes were stored at -70 C and used only once after thawing. Cell sap was prepared from disrupted L cells as for the Krebs cell system (7).

Assay of protein-synthetic activity and labeling of polypeptides in the cell-free system. To assay the response of both Krebs and L-cell ribosomes to EMC RNA, the 30- μ l assay method described previously was used (8). For the preparation of material for the analysis of tryptic digests, the assay was scaled up 10- to 20-fold, and the 14 C-amino acid mixture was replaced by 35 S-methionine (specific activity, 15 to 45 Ci/mmol, 0.3 to 0.6 mCi/ml of assay) plus the "other 19" unlabeled amino acids. The reaction mixture was supplemented with 30 to 60 μ g of EMC RNA per ml and incubated for 40 min at 37 C; ribonuclease (25 μ g/ml) and ethylenediaminetetraacetic acid (EDTA) (50 mM) were added and the incubation was continued for an additional 15 min. The reaction product was precipitated, washed, treated with performic acid, and digested with trypsin (8).

Labeling of viral proteins in infected cells. Virus-specific proteins were labeled in the infected cell by a modification of the fluoride-reversal method of Smith et al. (15). Krebs cells (25 ml of a suspension of 2×10^7 cells/ml in Earle's medium in a 250-ml conical flask) were incubated at 37 C for 3 hr with actinomycin D (3 μ g/ml) and then infected with EMC virus at an added multiplicity of 10 to 15 PFU/cell. A small portion of the cells (e.g., 4 ml in a 25-ml flask) was incubated for 30 min before the main culture to monitor total protein synthesis. Samples of these cells (0.1 ml) were added to 2 ml of Earle's medium containing 0.2 μ Ci of 14 C-leucine (specific activity, approximately 300 Ci/mmol) in a 25-ml flask which had been gassed with 5% CO_2 in air and equilibrated at 37 C. After 10 min, 8 ml of ice-cold phosphate-buffered saline, containing 0.5% (w/v) unlabeled L-leucine, was added and the cells were immediately filtered through a 3-cm "Oxoid" membrane (45 μ m pore size). The cells were washed five times with 5% (w/v) trichloroacetic acid and once each with 90% (w/v) ethanol, ethanol-ether (50/50), and ether. The membrane was dried, covered with scintillation fluid (0.4%, w/v, 2,5-diphenyl-oxazole in a mixture of two parts xylene and one part of "Triton X-100"), and counted in a Beckman model LS150 liquid scintillation counter. In this way, a result was obtained less than 25 min after sampling, and it was possible to determine precisely when viral protein synthesis would be maximal in the main culture (Fig. 1). At this time (usually 3.5 hr postinfection), 1 M sodium fluoride was added to the main bulk of the cells to a final concentration of 20 mM, and incubation was continued for 20 min. The cells were washed five times with Earle's medium at room temperature by centrifugation for 5 min at $100 \times g$ and resuspended in 25 ml of warm (37 C) Earle's medium. 35 S-methionine (0.1 to 0.2 mCi; specific activity, 30 Ci/mmol) was added, and the incubation was continued for the appropriate time (usually 15 min). The suspension was cooled and centrifuged (5 min at $100 \times g$ at 4 C), and the cells were washed with phosphate-buffered saline containing 0.1% (w/v) of unlabeled L-methionine. The cells were further washed with Tris-buffered saline, suspended in 6 mM magnesium acetate, and disrupted by Dounce homogenization (7). A volume of solution A (210 mM Tris-hydrochloride, pH 7.6; 150 mM KCl; 60 mM mercaptoethanol; 6 mM magnesium acetate, 6 mM EDTA; 1.5 M sucrose) equivalent to three-fifths of the packed cell volume was added to the homogenate, and the nuclei and debris were removed by centrifugation at $700 \times g$ for 10 min (7). The nuclear pellet was washed once, and the washings were returned to the postnuclear supernatant fluid. This was then centrifuged at $13,000 \times g$ for 15 min to yield the large-particle ("mitochondrial") pellet which includes, in EMC-infected as with the majority of small RNA virus-infected cells, the membrane-associated complexes in which most of the virus protein and RNA synthesis occurs (9; Dobos and Martin, *in preparation*). The supernatant fluid was discarded as it contained only small amounts ($\leq 10\%$) of the radioactivity in the newly synthesized virus-specific proteins. The "mitochondrial" pellet was suspended in 1 to 2 ml of distilled water and either treated with

sodium dodecyl sulfate (SDS) for acrylamide gel analysis (8) or oxidized with performic acid and digested with trypsin for fingerprinting (8). Occasionally, the sodium fluoride treatment was omitted, but the results of the tryptic peptide analyses were less satisfactory (Fig. 5).

Examination of tryptic peptides. The separation of the radioactive peptides obtained after trypsin hydrolysis was achieved by electrophoresis and chromatography on thin-layer sheets (20 by 10 cm) of silica gel (reference 14; Eastman "Chromatogram," catalogue no. 6061). The sheets were lightly sprayed with electrophoresis buffer, and 1- to 4- μ l samples of digest (10 to 100 μ g of peptides and 10^4 to 2×10^4 counts/min) were applied. Electrophoresis was for 2.5 hr at 400 v, with buffer of pH 6.5 or 3.5 (acetic acid-pyridine-water, 1:25:474 or 1:10:289, respectively). After drying, chromatography was in *n*-butanol, acetic acid, water (3:1:1) at room temperature until the solvent front had reached the top of the sheet (1.5 to 2 hr). The dried sheets were autoradiographed for 10 to 30 days with "Ilflex" (Ilford) or "Osray M" (Agfa-Gevaert) X-ray film. The autoradiogram was used to locate particular peptides on the original sheet. For further characterization, these peptides were scraped off, the silica was moistened with 5 to 10 μ l of water, and a paste of the gel was spread as a thin 1.5-cm line on a second sheet of moistened silica gel, which was then subjected to electrophoresis at a pH different from that used in the original fingerprinting. Alternatively, the peptide was extracted three times with 50- μ l portions of water and subjected to isoelectric focussing on plates (20 by 15 cm) of 5% acrylamide gel containing 2% (w/v) "Ampholine" (pH 3 to 10; references 1, 13). Isofocussing was for 18 hr at 500 v, after which the plates were dried immediately with a hair drier (without acid treatment) for autoradiography (20 to 40 days; Fig. 4).

RESULTS

Characterization of the polypeptides found in EMC-infected Krebs cells. When EMC virus infected Krebs cells, there was a progressive inhibition of host protein synthesis which was enhanced by incubation of the cells with actinomycin for several hours before infection. Under these conditions, the rate of total protein synthesis fell by 2 hr after infection to about 25% of the uninfected controls; thereafter it increased, reaching a peak at 3.5 hr, before falling to zero at about 6 hr (Fig. 1B). We have analyzed the proteins synthesized in the infected cell during this sequence of events. The results of SDS-acrylamide gel electrophoresis are shown in Fig. 1A. In the control cells (I), there was an appreciable synthesis of very high-molecular-weight polypeptides; however, by 3.5 hr postinfection (II) synthesis of these polypeptides had ceased. Meanwhile, during the period of increased protein synthesis from 2.5 to 3.5 hr, a pattern of 12 to 14 polypeptides characteristic of the infected

FIG. 1. Effect of virus infection on protein synthesis by Krebs ascites tumor cells. Cells were incubated with 3 μ g of actinomycin per μ l for 3 hr before infection at time 0 with 10 PFU of EMC virus per cell. (A) At zero time (I) and 3.5 hr postinfection (II), cells were incubated for 15 min with ³⁵S-methionine; the "mitochondrial" fractions were isolated, treated with SDS, and subjected to acrylamide gel electrophoresis; and the dried gels were autoradiographed. The figure presents densitometer tracings of the autoradiograms, with the origin to the right. The arrows VP1 to 4 mark the positions of viral capsid proteins identified by the inclusion of these as markers (Dobos and Martin, in preparation); their molecular weights are approximately 38,000, 31,000, 24,000, and 10,000, respectively. (B) The rate of total protein synthesis (monitored by 10-min incubations with ¹⁴C-leucine) during the course of preincubation and infection. I and II indicate the times of sampling for the acrylamide gel analyses shown in A.

cell developed. These newly synthesized proteins did not obviously correspond to any which were synthesized before infection, suggesting that a switch from the synthesis of host to virus-specific protein had occurred. Evidence that this is so and that there is a complete change in the nature of the proteins synthesized on infection was provided by a comparison of tryptic digests of infected- and control cell proteins (see, for example,

Fig. 5a, e, and f), whereas a comparison of fingerprints of tryptic peptides from infected cells with those from purified virus (Fig. 2C and 3B) established that by 3.5 hr postinfection a major portion of the newly synthesized protein in the infected cell is indeed virion capsid protein. Thus, it would seem reasonable to conclude that by 3.5 hr postinfection virtually all protein synthesis in the infected cell is virus-specific. The details of the work establishing this more rigorously will be

published elsewhere (Dobos and Martin, *in preparation*).

About 52 methionine-containing peptides can be resolved by electrophoresis and chromatography of tryptic digests of the virus-specific proteins derived from the infected cell (Fig. 2C). This is about the number one would expect assuming the complete translation of a genome of the size of EMC RNA (i.e., 2.7×10^6 daltons; reference 3) into protein containing about 2% of

FIG. 2. Fingerprint analysis of tryptic digests of ^{35}S -methionine-labeled proteins from infected cells and cell-free systems. (A) A map of the peptides present in digests of protein from EMC-infected Krebs cells traced from fingerprint C; the numbering is arbitrary. (B) A mixture of equal amounts (on a radioactivity basis) of the digests used in C (infected-cell protein) and in Fig. 3C (from the EMC RNA-stimulated Krebs cell-free system). (C) Digest of protein from EMC-infected Krebs cells labeled with ^{35}S -methionine at 3.5 hr postinfection after treatment with sodium fluoride. (D) L-cell cell-free system supplemented with EMC RNA. The digests were placed at the bottom center of the silica-gel sheets (arrow) and subjected to electrophoresis at pH 6.5; the anode is to the left in all cases. Chromatography was towards the top of the sheet. Autoradiography was for 10 to 30 days. As an aid to the comparison of the different fingerprints, where there is sufficient resolution of the spots for this to be done, those peptides thought to correspond to peptides appearing in the digests of the EMC RNA-stimulated Krebs cell-free system have been numbered accordingly (cf. Fig. 3C and Table 1). The absolute distance moved by a particular peptide in each run varies possibly as a result of unavoidable small differences in temperature and degree of wetting of the silica gel in different experiments. This is true even with duplicate experiments with the same digest. Although, therefore, exact correspondence cannot be demonstrated, the relative distance moved by the different peptides one to another remains reasonably constant. The coordinates have been drawn to demonstrate this. In each case, the scales of the coordinates are related to the distance peptide 41 has migrated from the origin. It has been arbitrarily assigned the average coordinates B (basic), 2.75; C (chromatography), 3. With this reference it is clear, for example, that in Fig. 2B and C and in Fig. 3B, C, and D, peptide 2 has the average coordinates A (acidic), 4.75; C, 2; peptides 11 and 12 (unresolved in this system), A, 2.75; C, 2.2; peptide 15, A, 1.9; C, 2.4; and peptide 19, A, 1.8; C, 3.15. The correspondence in Fig. 2D (the L cell-free system) is less convincing as the fingerprint has skewed rather badly, possibly through uneven wetting during electrophoresis. Nevertheless, the same general pattern can be seen.

randomly distributed methionine. A map of these peptides, with the numbers we have arbitrarily assigned to them, is shown in Fig. 2A; some of the spots seen in a typical fingerprint (Fig. 2C) have been resolved into two to three peptides by further analysis, and this information is included in the map. A further basic peptide (no. 52) moved too far to be detected in the autoradiogram shown in Fig. 2C. Methionine sulfoxide, which would result from performic oxidation of free ^{35}S -methionine, moved to the position of spot 25, which may have arisen in this way. Similar fingerprints of digests from proteins labeled with ^{35}S -methionine in control cells resolved large numbers of peptides. With the possible exception of spot 25, none of these, however, obviously coincided in position with the peptides obtained from the infected cell. An analysis of this type of material by electrophoresis in a single dimension at pH 6.5 is shown in Fig. 5f.

Characterization of the virus capsid polypeptides. Fingerprints of tryptic digests of the total protein from purified virus (Fig. 3B) usually

showed 12 to 16 clearly defined and several faint spots. The most easily distinguished of the capsid-specific peptides were the basic peptides 40, 41, and 42 which are clearly resolved in all of the fingerprints in which they appear (Fig. 2B, C, D and 3B, C, D). By repeated comparison of fingerprints of capsid proteins with those from the infected cell (cf. Fig. 3B and 2C) and further comparison of eluted spots by one-dimensional electrophoresis and isofocussing, it has been possible to correlate 18 of the capsid peptides with peptides 2, 9, 11, 12, 13, 15, 19, 26, 27, 29, 30, 32, 34, 40, 41, 42, 46, and 51 (Fig. 2A) from digests of infected-cell material. The remaining minor peptides could not be unambiguously identified and their significance is uncertain; they could, for example, merely represent partial or over-digestion products, or they could be derived from the protein (VP 1) which is only present in small amounts in the virion capsid. For this reason, it is not yet possible to give a precise number to the methionine-containing peptides derived from the virion. Nevertheless, the results are in reason-

FIG. 3. Fingerprint analysis of tryptic digests of the ^{35}S -methionine-labeled products from the Krebs cell-free system and from purified EMC virus. (A) Digest from a Krebs cell-free system incubated without EMC RNA. (B) Digest of purified EMC virus. (C) Digest of a Krebs cell-free system supplemented with EMC RNA. (D) A mixture of equal amounts (on a radioactivity basis) of the digests shown in B (EMC virus) and C (cell-free system plus EMC RNA). Other details, including numbering of peptides and coordinates, as for Fig. 2.

able agreement with the figure of about 20, which one might expect for a virus containing 2.3% methionine (5) randomly distributed in capsid proteins of 10^5 daltons total molecular mass (Dobos and Martin, *in preparation*).

Characterization of the products from the Krebs cell-free system. Fingerprints of the digests of the polypeptides formed in the Krebs cell-free system are shown in Fig. 3. In the absence of viral RNA (Fig. 3A), the pattern is characterized by a smear of neutral peptides, with few if any other peptides being resolved; when the system was supplemented with EMC RNA (Fig. 3C), a pattern of about 26 discrete spots representing at least 29 peptides (Table 1) was obtained. Comparison of this pattern with those obtained from purified virus (Fig. 3B) and from infected cells (Fig. 2C) suggested that many of these reaction product peptides could be identical with peptides found in the digests of infected-cell material and that about half of them were probably derived from capsid proteins. To confirm this apparent identity, digests of the cell-free system product were mixed with digests from the purified virus or from infected cells, and the mixtures were subjected to fingerprint analysis (Fig. 2B and 3D); peptides which are identical should appear as a single spot on the mixed fingerprints. This proved satisfactory for those peptides which were clearly separated but was not particularly useful for the poorly resolved neutral peptides. Further evidence for the identity of these peptides (and the better resolved peptides) was provided by elution and additional analysis by isofocussing (Fig. 4) or electrophoresis at pH 3.5 (the original electrophoresis was at pH 6.5). Because of the difficulty of eluting the relatively small amounts of radioactive peptide in a sufficiently small volume to load conveniently ($<5 \mu\text{liters}$), electrophoresis was less satisfactory than the isofocussing technique with which 100- μliter load samples were used. Nonetheless, the results obtained by electrophoresis agreed with the assignment of identity based on other criteria. With respect to the isofocussing, in the particular example shown (Fig. 4), peptides 19, 25, 32, and 34 from the poorly resolved slightly acidic, neutral, and slightly basic areas of fingerprints of the EMC RNA-stimulated Krebs cell-free system, the infected cell and EMC virus (cf. Fig. 2 and 3) were eluted and analyzed together with several others which either contained too little radioactivity for convincing identification or are of little interest. The significant point here is that peptides 32 and 34 from the cell-free system (p, Fig. 4), the infected cell (i), and the purified virion (e) clearly correspond, whereas the same is true for material from the

TABLE 1. *Characterization of the methionine-containing tryptic peptides from the Krebs cell-free system programmed with EMC RNA*

Peptide ^a no.	Polarity at pH 6.5	Comparison by co-finger printing ^b		Comparison by isofocussing ^c	
		With infected cell protein	With virus coat protein	With infected cell protein	With virus coat protein
W	Acidic	OP ^d	OP		
2	Acidic	+	+		
3	Acidic	+			
X	Acidic				
8	Acidic	+			
10	Acidic	+			
11	Acidic	+	+	+	+
12	Acidic	+	+	+	+
15	Acidic	+	+	+	+
19	Acidic	+	+	+	+
20	Acidic	+			
Y	Acidic				
23	Acidic	+			
Z	Slightly acidic				
24	Slightly acidic	+		+	
25 ^e	Neutral	+		+	
26	Neutral	+	+	+	+
27	Neutral	+	+		+
28	Slightly basic	+			
30	Slightly basic	+	+		
32	Slightly basic	+	+	+	+
34	Slightly basic	+	+	+	+
36-38	Basic	Too faint			
39	Basic	Too faint			
40	Basic	+	+	— ^f	
41	Basic	+	+	— ^f	
42	Basic	+	+	— ^f	

^a Numbering of these peptides synthesized in the cell-free system in response to EMC RNA is based on that of the 52 tryptic peptides identified in fingerprints of proteins labeled with ³⁵S-methionine in the infected cell (Fig. 2A and C). The letters W to Z refer to peptides found only, or mainly, in digests of the cell-free system product (Fig. 3C). The numbers in boldface refer to peptides synthesized in the cell-free system in response to EMC RNA which are also found in digests of capsid protein from purified virus.

^b Plus sign indicates that, on analysis of mixtures of digests (e.g., from the cell-free system and virus capsid proteins) by two-dimensional chromatography and electrophoresis, the peptide from the EMC RNA-stimulated cell-free system moves identically with that from the infected cell or virion capsid protein (Fig. 2B and 3D).

^c Peptides were eluted from fingerprints and subjected to isoelectric focussing (e.g., Fig. 4). Peptides from the cell-free system which moved to an identical position forming a continuous line with those from infected cells or virus capsid protein are indicated by a plus sign. A blank indicates that a comparison was not made.

^d Only occasionally present.

^e Possibly methionine sulfoxide.

^f That these peptides from the cell-free system were identical with those from virion capsid proteins was confirmed by electrophoresis of the original digests of the cell-free system product at two pH values in parallel with the virion peptides.

area of peptide 19 for the first two of these sources. The identity of these latter peptides with peptide 19 from the purified virion (not included in Fig. 4) was established in a separate experiment. Peptide 25 may be methionine sulfoxide, and material from this area of the fingerprints of the infected cell and the cell-free system migrated in parallel on isofocussing as would be expected (Fig. 4). Thus, the data in Fig. 4 establish the identity of peptides 32 and 34 from the three different sources and the possible identity of peptide 19 from two of them. Identity between the additional peptides indicated in Table 1 was established in a similar fashion in analyses not shown here.

One-dimensional electrophoresis at pH 6.5 was useful for the rapid examination of products from different systems. The patterns obtained were characteristic (Fig. 5) and clearly showed, for example, the presence of at least one of the basic capsid-specific peptides (number 41) in digests of the cell-free system product. Apparent identity in this case was confirmed by elution and electrophoresis at pH 3.5.

Table 1 summarized most of the results based on analyses of the above types. At least 29 EMC RNA-stimulated peptides were resolved, and all

FIG. 4. Further analysis of fingerprinted peptides by isoelectric focussing in acrylamide gels. Peptides 19, 25, 32, and 34 from the fingerprints of the EMC RNA-stimulated Krebs cell-free system (Fig. 3C), the infected cell (Fig. 2C), and EMC virus (Fig. 3B), indicated here by the letters p, i, and e, respectively, were eluted and transferred to filter paper strips placed in the positions indicated at the bottom of the figure on an acrylamide gel plate containing "Ampholine" (pH 3 to 10). Electrophoresis was for 18 hr at 500 v with the anode to the top of the figure, after which the gel was dried and autoradiographed.

FIG. 5. Analysis of virus-specific tryptic peptides by electrophoresis at pH 6.5 on thin-layer silica gels. Samples of each digest were placed at 0, with the anode to the top of the figure. (a) Protein from 3.5-hr EMC-infected Krebs cells labeled with ^{35}S -methionine, without prior treatment with sodium fluoride; (b) and (e) protein from EMC-infected cells which were pretreated with sodium fluoride; (c) product from the Krebs cell-free system supplemented with EMC RNA; (d) ^{35}S -methionine-labeled EMC virus; (f) protein from control cells labeled with ^{35}S -methionine.

except 4 (W, X, Y, and Z, Table 1 and Fig. 3C) could be tentatively identified on the basis of the original fingerprints with peptides present in digests from infected cells. The identity of 13 of them [numbers 11, 12, 15, 19, 24, 25 (possibly methionine sulfoxide), 26, 27, 32, 34, 40, 41, and 42, Table 1] was confirmed by at least two further criteria and another eight (numbers 2, 3, 8, 10, 20, 23, 28, and 30) by one additional test; the remaining peptides were obtained in insufficient amounts for further identification. In addition, eight of the peptides synthesized in the cell-free system (numbers 11, 12, 15, 19, 26, 27, 32, and 34) were found to be identical with viral capsid protein peptides by three or more criteria (Table 1). Five others (peptides 2, 30, 40, 41, and 42) appeared to correspond to capsid peptides on the basis of the fingerprint data alone (Fig. 3), and identity was confirmed for peptides 40, 41, and 42 by electrophoretic analysis of the original digests at two pH values.

Two of the four peptide spots which could not be unambiguously identified (marked W and Y, Fig. 3C) were occasionally seen in digests of infected cell proteins. They may represent smaller peptides released by over-hydrolysis with trypsin. The area Z, which varied in different preparations,

probably contains partial digestion products. The spot marked X, which was present in high proportion in all digests of the EMC RNA-stimulated cell-free systems, may contain the peptide which is translated first and which, in the cell-free system, might still retain the initial methionine residue that is cleaved in the intact cell.

Characterization of the products from the L-cell cell-free system. The L-cell system is less responsive to EMC RNA than is the Krebs cell system. For example, on addition of optimum amounts of EMC RNA, a 5- to 10-fold increase in amino acid incorporation occurred in the Krebs system, whereas with the L-cell system only a 2- to 3-fold increase over a similar intrinsic level was observed. Most of this discrepancy can be ascribed to differences in the cell sap fractions, as L-cell ribosomes incubated with Krebs cell sap responded to viral RNA almost as well as the completely Krebs system, whereas Krebs ribosomes incubated with L-cell cell sap behaved like the totally L-cell system.

When the tryptic digests of the L-cell system reaction product were analyzed, the fingerprints were, in general, similar to those obtained with the Krebs system, but there were small reproducible differences (cf. Fig. 2D and 3C). A peptide(s) in the area of peptide Y was greatly increased, and we are not yet certain of the identity of the peptides tentatively identified as W, 2, 3, and X in Fig. 2D. However, the most significant difference is in the number and intensity of the basic peptides. With the Krebs system, only three basic peptides (number 40, 41, and 42) have been consistently observed and these only in small amounts. In contrast, these capsid peptides are prominent in fingerprints of the L-cell product (Fig. 2D), whereas traces of other basic peptides were also detected. Finally, the maximum translation of the EMC RNA genome that we have thus far observed in the cell-free system appears to occur when Krebs cell ribosomes are incubated with EMC RNA in the presence of L-cell cell sap. Analysis of the product yielded six basic peptides in addition to those observed in digests of the completely Krebs system, making a total of at least 35 EMC RNA-stimulated peptides in all. These additional peptides have been tentatively identified with peptides 45, 48, 49, 50, 51, and 52 in digests from infected cells. Thus, although the L-cell sap seems to be less efficient than Krebs cell sap in supporting amino acid incorporation in response to the viral RNA, in its presence more of the viral genome appears to be translated.

DISCUSSION

In a previous paper we reported that the same product, in part, was synthesized in response to

EMC RNA in cell-free systems derived from chick fibroblasts and Krebs mouse ascites tumor cells (8). In this study, we showed that the products formed in two mouse systems supplemented with EMC RNA are similar and that the tryptic digests of these products can be resolved into 29 to 35 methionine-containing peptides, all but 4 of which appear to be identical with virus-specific peptides derived from the infected cell. Of the four unidentified peptides, three may represent over- or partial-digestion products, whereas the fourth may be an initiation peptide from which methionine is cleaved in the intact cell. In addition, we find that at least 11 of the peptides specified by EMC RNA in the cell-free system are identical with virus coat protein peptides. We conclude that each of the cell-free systems we have thus far examined, Krebs cell, L cell, and chick fibroblast (8), are capable of translating EMC RNA to yield virus-specific polypeptides.

From these results, it would appear that a maximum of about half of the viral RNA genome is translated in the Krebs system and up to 60% of it, if L-cell cell sap, is used. Variation in the extent to which the cell saps are contaminated with ribonuclease could account for the difference, but we have no direct evidence for this. We have evidence that a viral precursor protein having a molecular weight of 240,000 daltons accumulates under appropriate conditions in samples of the type used here for the tryptic digests of infected cell material (Dobos and Martin, *in preparation*). Accordingly, it seems likely that in the intact cell the EMC RNA genome like that of other picornaviruses (6, 10) is translated as a single unit to yield a single precursor polypeptide from which the whole spectrum of viral proteins is produced by subsequent cleavage. Furthermore, although we have presented data relating only to the virus-specific peptides in the large-particle fraction (*see above*), which quantitatively contains 90% of the newly synthesized virus-specific protein, the remaining 10% of this material in the other cell fractions behaves essentially identically with the majority, for example, on electrophoresis in acrylamide gels (Dobos and Martin, *in preparation*). Therefore, despite the fact that we have presented data relating only to the virus-specific peptides present in a particular fraction of the infected cell at a particular time, it seems likely that the methionine in the 52 peptides resolved here represents the vast majority, if not all, of the methionine coded by the virus genome. It must be stressed, however, that there are other uncertainties in estimating the extent of translation; for example, partial cleavage products of a high-molecular-weight precursor(s) of viral proteins

present in the infected cell (6, 10; Dobos and Martin, *in preparation*) could yield, on tryptic digestion, peptides which might or might not be expected to appear in the cell-free system. If this were the case, an even larger portion of the genome may be translated than the 50 to 60% suggested above. On the other hand, this figure is based on the assumption of a random distribution of methionine throughout the polypeptide coded by the viral genome. If a large section towards the COOH-terminal half of the molecule (presumably untranslated in the cell-free system) were deficient in methionine, it would mean that this figure would be correspondingly too high. The value of 50 to 60% should, therefore, on balance, be considered a maximum estimate. A minimum of about 30% is set by the size of the EMC RNA-stimulated polypeptide products synthesized in these cell-free systems which reach a maximum of 85,000 to 100,000 daltons (Kerr, *in preparation*). It is not yet known whether this limit is determined by cleavage of the nascent polypeptide chains as in the intact cell or of the EMC RNA or simply by our failure to keep these systems functioning for more prolonged periods.

Our main concern here, however, is not to identify every peptide detected or exactly how much of the viral genome is translated but to establish that a significant portion of the EMC RNA genome is in fact being translated in these systems, and this clearly is the case. In addition, preliminary studies concerning the kinetics of synthesis of capsid-specific and noncapsid EMC-specific peptides have shown a sequential appearance of different peptides, the major capsid-specific peptides appearing relatively late. This suggests that viral protein synthesis is initiated with noncapsid proteins and that the capsid protein cistrons are either distributed throughout the RNA molecule or are grouped near the middle. It will be of interest to compare more detailed results of this type with the genetic data of Cooper and his co-workers, who have found that the coat protein cistrons of poliovirus are grouped at one end of the genome (4). It is, of course, possible that translation in these cell-free systems may be initiated at the wrong, or more than one, site on the RNA molecule; neither we nor A. E. Smith (*personal communication*), however, have any evidence to suggest this.

The data presented here relate only to the identity of the peptide sequences of the proteins synthesized in the cell-free systems in response to EMC RNA; it tells us nothing directly concerning the proteins themselves. Electrophoretic analysis on acrylamide gels has rather surprisingly, however, shown that a number of discrete proteins having molecular weights of as high as

85,000 to 100,000 daltons are formed in the Krebs system in response to the viral RNA. The relationship of these proteins to those found in the infected cell is being investigated.

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ADDENDUM IN PROOF

A similar correspondence to that described here between the product synthesized in the Krebs cell-free system and EMC capsid protein from purified virus has been found by H. Aviv, I. Boime, and P. Leder (*personal communication*).

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