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Characterization of the Polypeptides Formed in Response to Encephalomyocarditis Virus Ribonucleic Acid in a Cell-Free System from Mouse Ascites Tumor Cells

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The polypeptide products synthesized at different times in a cell-free system from Krebs mouse ascites tumor cells in response to the addition of encephalomyocarditis (EMC) virus ribonucleic acid (RNA) were characterized by electrophoresis on polyacrylamide gels and fingerprint analysis of their tryptic peptides. Translation of the EMC RNA genome with time occurred in a nonrandom fashion in these systems, to yield products containing sequences characteristic of both virion capsid polypeptides and EMC-specific polypeptides present only in the infected cell. The molecular weights of the products fell in a series from 20,000 to 140,000 daltons, although occasionally traces of larger polypeptides were also observed. All of the major polypeptides appeared to arise from partial or complete translation of about 60% of the EMC RNA genome. They were not formed by cleavage of a large precursor molecule. It is suggested that they are artifacts generated by premature "termination" of nascent polypeptide chains at preferred sites.

Evidence has been presented that, in the infected cell, the poliovirus ribonucleic acid (RNA) genome is translated as a single unit to yield a precursor polypeptide from which the whole spectrum of virus proteins is produced by proteolytic cleavage (5, 6, 14, 17). Similar data are available for other picornaviruses (10) including encephalomyocarditis (EMC) virus (Dobos and Martin, *in preparation*). Under normal conditions, the precursor protein is not seen in its entirety, and it is assumed that cleavage begins while it is still in the form of a nascent chain on the ribosome. Recent results with poliovirus (18, 19) and EMC virus (2) favor a sequential translation of the virus genome, with synthesis of the virion capsid polypeptides preceding that of the nonstructural virus-specific polypeptides present only in the infected cell. In the cell-free system, the stimulation of amino acid incorporation in response to EMC RNA is well documented (7, 9, 13), and there has been some progress in establishing that the products formed in response to the RNA are indeed EMC-specific polypeptides (1, 3, 8, 15). In a previous paper (3), we reported that the products formed in two mouse cell-free systems supplemented with EMC RNA were similar, and that tryptic digests of these products could be resolved to yield approximately 30 methionine-containing peptides, all but four of which were identical with peptides derived from virion coat protein polypeptides or polypeptides present only in the infected cell. From studies of this type, and a preliminary analysis of the size of the polypeptide products synthesized, we concluded that at least 30% of the EMC RNA genome was being translated in these systems to yield both virion capsid and virus-specific noncapsid polypeptides. Here, we have been concerned with a further analysis of the polypeptide products formed in response to EMC RNA in cell-free systems from Krebs mouse ascites tumor cells and an investigation of how closely events in these systems parallel those in the infected cell. We will present evidence for the sequential translation of 50% or more of the EMC RNA genome in the cell-free system to yield a series of polypeptides of up to and occasionally over 140,000 daltons in molecular weight. Although the results suggest that events in the cell-free system may well reflect those occurring in the intact cell, there are a number of uncertainities and anomalies in these systems. Their significance will be discussed.

A preliminary account of some of this work has already been presented (Kerr et al., Fed. Eur.

Biol. Soc. Meeting, Varna, 1971, Symposium vol. 22, *in press*), and Boime, Aviv, and Leder (1) and Mathews (*personal communication*) have also observed the formation of a number of polypeptides in response to EMC RNA in the Krebs cell-free system.

MATERIALS AND METHODS

The chemicals used, the Krebs 2 mouse ascites tumor cells, the EMC virus and viral RNA, the preparation of ribosome and cell sap fractions, their assay and the labeling of the polypeptide products in the cell-free system, the performate oxidation and tryptic digestion of these products, and the analysis of the tryptic digests by electrophoresis and chromatography on thin-layer silica gel plates have already been described (3, 7, 8, 12).

Analysis of the polypeptide products from the unfractionated cell-free system by electrophoresis on polyacrylamide gels after treatment with sodium dodecyl sulfate (SDS) was carried out as described previously for the 105,000 × *g* supernatant fluid (8). Here, however, initially 0.5 M and latterly 5 M urea was present in the gels, as this gave better resolution. Although gels containing 5 and 10% acrylamide were also used, all of the data presented (Fig. 1 to 3 and Table 1) were obtained with 7.5% gels. A Teflon insert was employed to allow two samples, one on either side of the insert, to be loaded onto a single "split" gel (4). Two methods were used in estimating the amount of radioactivity in different gel fractions. In the first, after autoradiography, the dried stained gel was cut into 1-mm slices. Each slice was transferred to a scintillation vial, dissolved by treatment overnight (≥6 hr) with 50 μliters of 100-volume hydrogen peroxide at 70 to 90 C, and counted in a toluene-triton scintillation fluid mix (11). In the second, the areas under the peaks obtained by tracing the autoradiographs with a Joyce Loebl densitometer were computed. In the calculation of the data presented in Fig. 2, the two methods yielded identical results.

RESULTS

Characterization of the polypeptides synthesized in the cell-free system in response to EMC RNA with time. The polypeptide products labeled with ^{35}S-methionine produced at different times in the cell-free system in response to EMC RNA were fractionated according to their molecular weights by electrophoresis on polyacrylamide gels in the presence of SDS (Fig. 1). In the absence of added EMC RNA, there was a low level of incorporation of ^{35}S-methionine into Krebs cell proteins, which appeared spread throughout the gel. This presumably reflected the completion of residual nascent polypeptide chains, and the distribution of label in the species of different molecular weights did not change with time throughout the incubation. In the presence of EMC RNA, however, it is clear that a number of polypeptides were synthesized, the maximal molecular weight of which increased steadily with time (Fig. 1 and 3). At 2 and 3 min (not shown), the EMC RNA-stimulated product was very heterogeneous, reaching a molecular weight of not more than 20,000 daltons. By 5 min, the majority of it appeared to co-migrate in the gels with trypsinogen (23,500 daltons; Fig. 1). Thereafter, the number and molecular weights of the polypeptide products increased until about 40 min. Beyond 40 min, there appeared to be an increase in the amount of material of higher molecular weight, but little increase, if any, in maximal molecular weight (cf. Fig. 3).

Without wishing to imply that they represent unique homogeneous molecular species, the major polypeptides observed at 40 min have been arbitrarily designated A to G (Fig. 1). This pattern has been observed on a variety of occasions, with a number of different cell-free systems and EMC RNA preparations analyzed in 5, 7.5, and 10% gels in the presence of 0.5 or 5 M urea. In addition, a number of these products prepared on different occasions have been subjected to electrophoresis together on split gels without any difference in the molecular weights of the polypeptide species being detected. There has, however, been some variation in the degree of resolution observed on different occasions and in different gels. For example, polypeptide A has been resolved into two or three components, and polypeptide D, in particular, usually appeared to be rather heterogeneous (Fig. 1 and 3). The overall impression, therefore, has been one of minor variation upon an underlying regularity in pattern.

Direct comparison of EMC virion polypeptides and polypeptides from the EMC-stimulated cell-free system by electrophoresis on split gels showed no obvious correspondence. It is impossible, however, in view of the complexity of the pattern of polypeptides obtained in these systems to exclude some identity with virion coat polypeptides on this basis alone.

Estimation of the molecular weights of the polypeptides synthesized in response to EMC RNA. Estimates of the molecular weights of the major polypeptide species synthesized in response to EMC RNA in the cell-free system are listed in Table 1. The values given were obtained by comparison with marker polypeptides of known molecular weight, notably those from purified reovirus (the kind gift of J. J. Skehel), run in parallel gels, in the same gels, and in split gels. The largest polypeptide (G) routinely formed in response to EMC RNA has an apparent molecular weight of approximately 140,000 daltons. Polypeptides of higher molecular weight

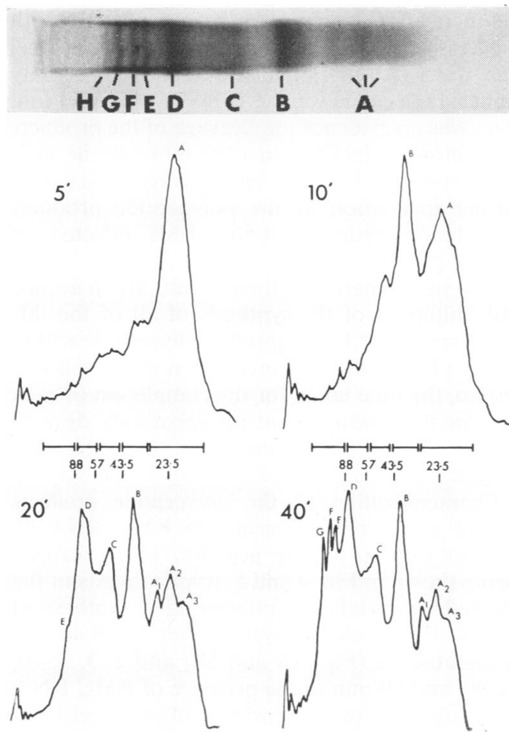

FIG. 1. *Synthesis of EMC-specific polypeptides with time in the cell-free system. Cell-free systems were incubated at 37 C in the presence of 50 μg of EMC RNA per ml and 167 μCi of ^{35}S-methionine per ml (≥20 Ci/mmole) for 5, 10, 20, and 40 min as shown. After incubation, for each time point, samples equivalent to 50,000 counts per min of acid-insoluble polypeptide were prepared and subjected to electrophoresis on SDS-polyacrylamide gels. The figure shows tracings of autoradiographs of the dried stained gels and, for comparison, a photograph of the autoradiograph from which the tracing for the 40-min sample was made. As presented, electrophoresis was from left to right. Standard marker proteins underwent electrophoresis in parallel with the cell-free system products in separate and in split gels. The markers used here were transferrin, ovalbumin, and trypsinogen of molecular weights 88,000, 43,500, and 23,500 daltons, respectively. In the figure, the positions to which they migrated are indicated by the values for their molecular weights in thousands. In addition, the pyruvate kinase (57,000 daltons) present as part of the adenosine triphosphate-generating system in the cell-free system provided a useful internal marker. The horizontal bars in the center of the figure represent the fractions pooled in the quantitation of these and similar results for the calculation of data of the type presented in Fig. 2.*

(155,000 to 180,000 daltons, Table 1) were only occasionally observed (Fig. 1 and 3).

The presence of high-molecular-weight precursor viral polypeptides in the intact cell was demonstrated by the use of inhibitors of the

TABLE 1. *Apparent molecular weights of the polypeptide species synthesized in the cell-free system in response to encephalomyocarditis virus RNA*

Species	Apparent molecular wt (daltons)[a]
J	180,000
I	165,000
H	155,000
G	140,000
F	120,000
E	89,000
E1[b]	(92,000)
E2	(86,000)
D	64,000
D1[b]	(69,000)
D2	(54,000)
C	48,000
B	37,000
A	26,000
A1[b]	(28,000)
A2	(26,000)
A3	(22,500)

[a] These values are based on comparisons of distance migrated on electrophoresis in SDS-acrylamide gels with standard proteins. Three sets of marker proteins were employed in the split gel technique: those used in Fig. 1, those of partially oxidized bovine serum albumin (4), and the polypeptides of purified reovirus (Fig. 3). The values for polypeptides A to G are all averages of 10 or more determinations; those for H to J are each for 1 or 2 determinations.

[b] Peptides A, D, and E were, on occasion, resolved into two or three components with the apparent weights shown in parentheses.

proteases involved in their cleavage (5). Of those used, phenyl methyl sulfonyl fluoride was the most effective in the EMC-infected Krebs cell system, but amino acid analogues (5), tosyl phenylalanyl chloromethyl ketone, and tosyl lysyl chloromethyl ketone were also active to some extent in inhibiting cleavage (Dobos and Martin, *in preparation*). There was, however, no change in the distribution of the polypeptide species of different molecular weights in the cell-free system in the presence and absence of these inhibitors at the same concentrations (1 to 3 mM) as were effective in the infected cell (concentrations at which amino acid incorporation in these systems in response to EMC RNA was not greatly reduced). Moreover, the kinetics of formation of the different species of polypeptide were not in accord with a high-to-low molecular-weight precursor-to-product relationship.

Kinetics of formation of the polypeptides synthesized in response to EMC RNA. As shown in Fig. 2, the ^{35}S-methionine accumulated first in the

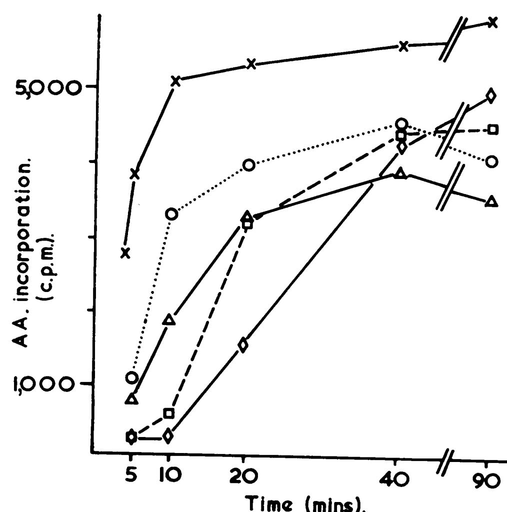

FIG. 2. *Synthesis of individual EMC-specific polypeptides at different times in the cell-free system. Cell-free systems were incubated at 37 C for the times indicated in the presence of EMC RNA and ^{35}S-methionine, as in Fig. 1. After electrophoresis in acrylamide gels, the radioactivity in the individual peaks corresponding to the different polypeptides (Fig. 1) was estimated, and the percentage of the total radioactivity in each of the polypeptides at each of the time points was calculated. From this and the known values for the total radioactivity incorporated at each time (in this experiment, 5,500, 10,700, 15,250, 22,100, and 22,500 counts per min per 5-μliter sample at 5, 10, 20, 40, and 90 min, respectively), the absolute amounts of radioactivity in the different components present at each time were calculated and plotted. The data are for the polypeptide(s) in peak A (26,000), ×; peak B (37,000),○; peak C (48,000), △; peak D (64,000), □; and peaks E, F, and G combined (≥ 89,000), ◇. The figures above in parentheses are for the apparent average molecular weights of the polypeptide(s) in each peak, in daltons.*

lower-molecular-weight polypeptides and only later in higher-molecular-weight material. There was virtually no increase in the absolute amounts of the lower-molecular-weight polypeptides A (26,000 daltons) after 5 min and B (37,000 daltons) after 10 min, and the higher-molecular-weight components E, F, and G (≥ 90,000 daltons) were not detected until relatively late. In accord with this, addition of ^{35}S-methionine to the cell-free system at 10, 20, 30, and 40 min after initiation of an incubation lasting 90 min yielded the results shown in Fig. 3. The usual pattern of labeling, comparable to that shown for the 40-min time point in Fig. 1, was obtained on addition of the label at zero time. Had proteolytic cleavage been occurring, even on nascent polypeptide chains of intermediate size, the formation of some low-molecular-weight polypeptide late in the incubation would have been expected. With progressively later addition, however, the label was restricted to polypeptides of progressively higher molecular weight (Fig. 3). In addition, there was no evidence for cleavage of the products on prolonged incubation (210 min) of the cell-free system at 37 C. Nor was cleavage detected on further incubation of the polypeptide products with homogenates of 4.5-hr EMC-infected or control cells.

In general, therefore, these results are in accord with initiation of the synthesis of all of the different species of EMC-specific polypeptide within the first few minutes of incubation in the cell-free system, the time taken for the completion of each and for its appearance as an apparently discrete species being proportional to its molecular weight.

Characterization of the polypeptide products formed with time in response to EMC RNA by analysis of their tryptic peptides. In the experiments shown in Fig. 4 and 5, tryptic digests of the ^{35}S-methionine-labeled polypeptides synthesized at 37 C in the cell-free system after 2 and 40 min in the absence (Fig. 4A and 5E) and 2, 3, 5, 10, 20, 40, and 90 min in the presence of EMC RNA were subjected to fingerprint analysis by electrophoresis and chromatography on thin-layer plates. Fingerprints of digests of the virus-specific polypeptides from EMC-infected cells and from purified virus are included for ease of comparison only (Fig. 5C and D). The evidence establishing the identity of the different tryptic peptides from the cell-free system with those from virion capsid proteins or EMC-specific polypeptides present only in the infected cell has already been presented (3) and will not be repeated here. The numbering system for the individual peptides is based on the 52 peptides present in digests from the infected cell (Fig. 5D and F). It is thought that these represent all of the methionine-containing peptides coded by the EMC RNA genome (3).

It is clear that the number of EMC-specific peptides increased from 2 to over 20 during 2 to 40 min of incubation in the cell-free system (Fig. 4 and 5). Although the data do not allow a complete definition of the origin and exact order of appearance of all of the different peptides, the following observations can be made. Virion coat protein peptides 2 and 11 + 12 (not resolved here) appeared to be just detectable at 5 min and were clearly present at 10 min, by which time peptides 15 and possibly 26 and 34 were also apparent. All of these plus peptides 40, 41, and 42 were obvious by 40 to 90 min, and traces of peptide 51 were also seen by the latter time. Thus, it can be stated with a fair degree of certainty

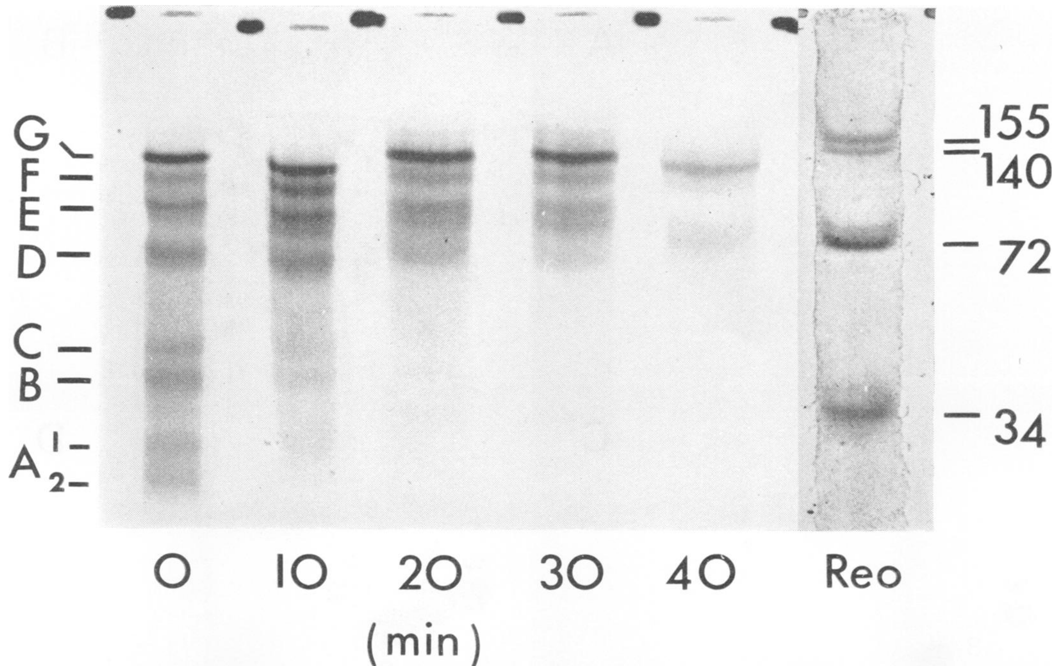

FIG. 3. *Synthesis of EMC-specific polypeptides with time in the cell-free system as shown by the addition of ³⁵S-methionine after different periods of incubation. A cell-free system was incubated at 37 C in the presence of EMC RNA as in Fig. 1. To portions of it, ³⁵S-methionine (≥ 20 Ci/mmole) was added to 167 μCi/ml at zero time and after 10, 20, 30, and 40 min. In all cases, incubation at 37 C was continued to 90 min. Individual samples were analyzed by electrophoresis on SDS-polyacrylamide gels in the presence of 5 M urea. Autoradiographs of the dried stained gels are presented together with a photograph of a similarly prepared gel on which the polypeptides of purified reovirus had undergone electrophoresis in parallel, to provide molecular weight markers. The figures to the right represent the molecular weights, in thousands, of the major reovirus polypeptides λ1, λ2, μ2, σ3 (16). The minor virion polypeptides μ1, γ1, and γ2 (16) were not resolved here. The individual polypeptides in the cell-free systems are identified by the letters A to G.*

that translation of the coat protein cistrons had started by 5 min, and by 40 to 90 min all 15 of the readily resolved coat protein peptides could be detected in these digests. This would suggest, by comparison with the results in Fig. 1 to 3, that translation of the coat protein cistrons starts *within* 25,000 daltons (or 250 amino acids) of the N-terminus of the cell-free system product and is complete before, or by the time that, the product has reached a molecular weight of 140,000 daltons.

Turning now to the noncoat protein peptides, it is of interest that, of those present early, W and Y are peptides which in our previous study (3) were only occasionally seen in digests of infected-cell proteins and could not be unambiguously identified as EMC-specific, whereas X was a peptide present in high proportion in all digests of EMC RNA-stimulated cell-free system products, but was absent from the infected cell. We previously suggested that X may represent the peptide translated first in the cell-free system where it might retain the methionine cleaved in the intact cell (3). Of the identifiable nonstructural virus-specific peptides, however, peptide 3 appeared to be present from the earliest times (2 and 3 min). By 5 min, and certainly by 10 min, peptides 8 and possibly 10 were present, and there clearly was more material in the area of peptides 19, 20, and 23 than could readily be accounted for by the coat protein peptide 19. In fact, all of these noncoat protein peptides could have been present at 5 min, the time at which the first well-resolved coat protein peptides appeared. It was not until after 40 min that traces of further readily identifiable nonvirion peptides were detected. For example, although not obvious from the photograph presented here (Fig. 5B), traces of material were occasionally observed at later time points in the areas of peptides 36 to 38, 48 to 50, and 52.

DISCUSSION

Previously (4), we presented evidence that EMC RNA is translated in cell-free systems from mouse Krebs ascites tumor and L cells to yield

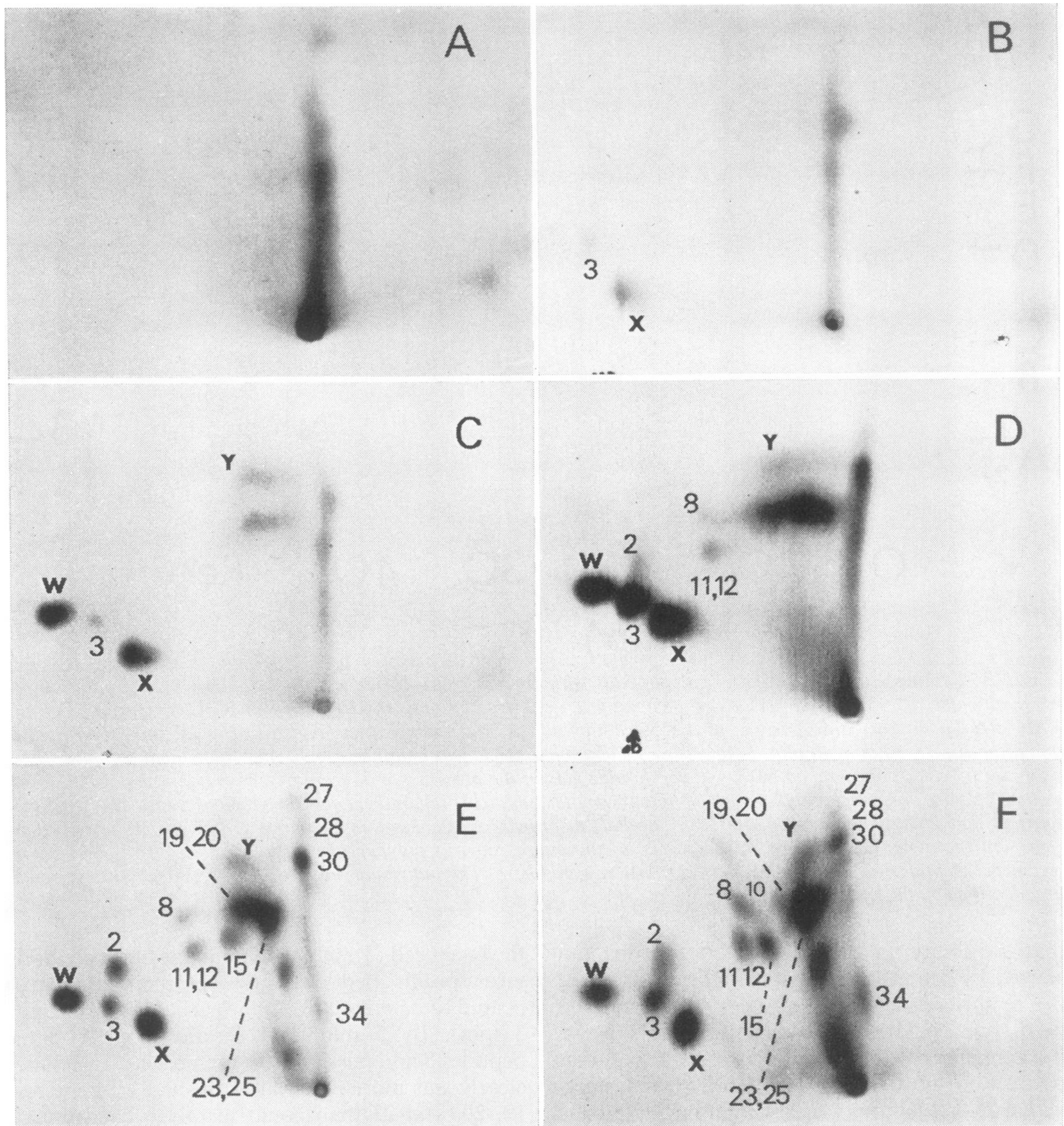

FIG. 4. *Fingerprint analysis of tryptic digests of the* ^{35}*S-methionine-labeled polypeptide products from cell-free systems incubated at 37 C for different times in the presence and absence of EMC RNA: (A) 2 min without EMC RNA; (B, C, D, E, and F) 2, 3, 5, 10, and 20 min, respectively, with EMC RNA. The 2-, 3-, 5-, 10-, and 20-min digests were from the same incubation mixtures as the samples analyzed in Fig. 1. The digests were placed at the bottom center of the silica gel sheets and subjected to electrophoresis at pH 6.5; the anode was to the left in all cases. Chromatography was toward the top of the sheet. Autoradiographs of the dried fingerprints are shown. A detailed account of the procedure is given in reference 3. As an aid to the comparison of the different fingerprints, where there is sufficient resolution of the spots for this to be done, peptides have been numbered as previously (3), according to a system based on the fingerprint of the EMC-specific peptides present in digests of protein from EMC-infected Krebs cells (cf. Fig. 5D). A map of the latter is shown (Fig. 5F).*

polypeptides which were shown, by analysis of their tryptic peptides, to contain sequences of amino acids identical to those in virion coat protein polypeptides and polypeptides present only in the infected cell. Translation could, however, have been initiated and terminated virtually at random over about half of the EMC RNA genome. Here, we have shown that translation is

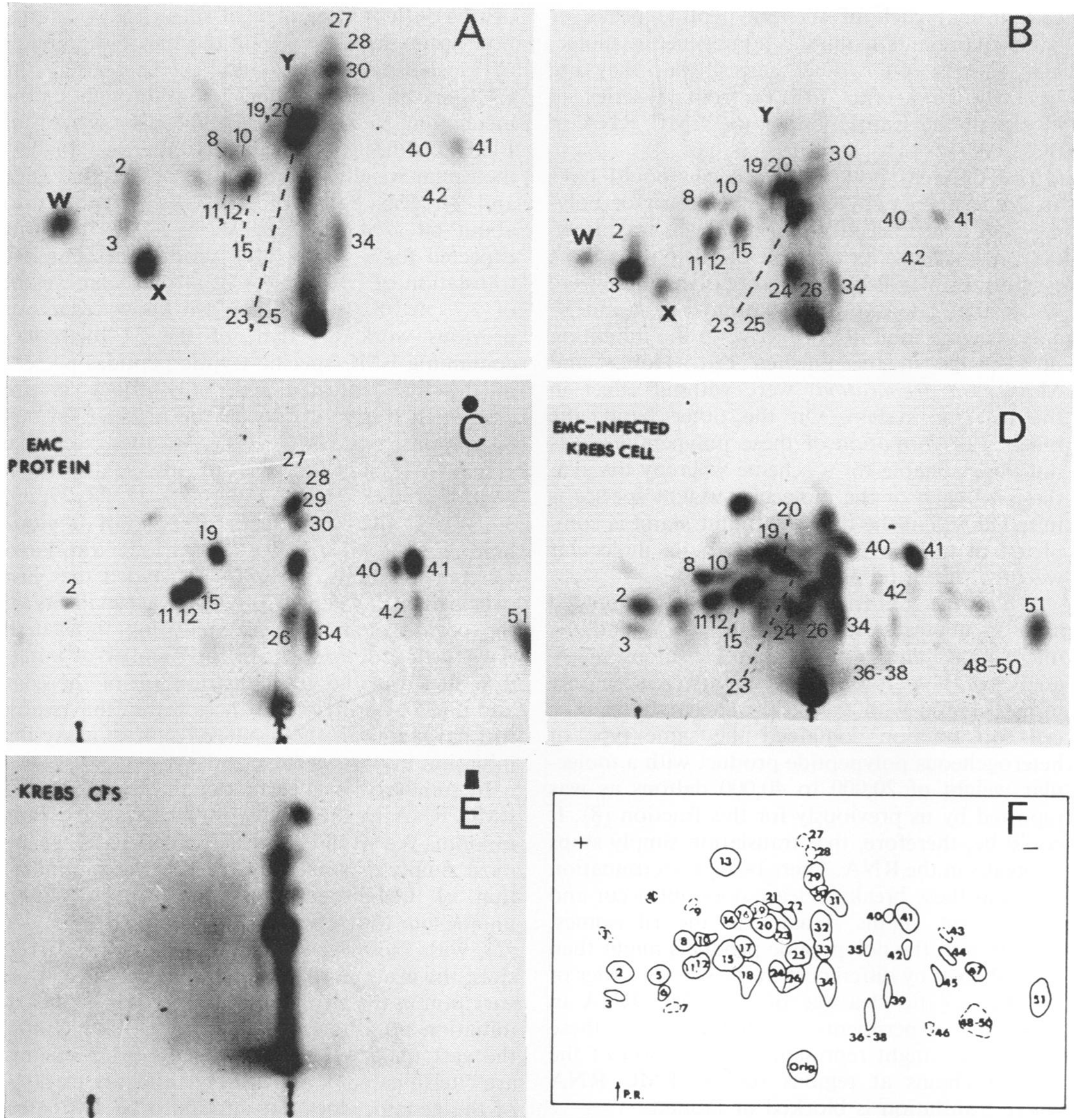

FIG. 5. Fingerprint analysis of tryptic digests of ^{35}*S-methionine-labeled polypeptides from cell-free systems, purified EMC virus, and EMC-infected Krebs cells. (A and B) Digests from cell-free systems incubated for 40 and 90 min, respectively, at 37 C in the presence of EMC RNA. The 40-min digest was from the same incubation mixture as was used in Fig. 1. (E) Digest from a cell-free system incubated for 40 min in the absence of added RNA. Fingerprints of digests of (C)* ^{35}*S-methionine-labeled EMC virus and of (D) the polypeptides from EMC-infected Krebs cells (3) are included for comparison. A map of the latter upon which the numbering system for the peptides used here and in Fig. 4 is based is also shown (F). Other details as for Fig. 4.*

far from random in the Krebs cell-free system, and the results of preliminary studies carried out in collaboration with R. M. Friedman would suggest that this is equally true for the L-cell system.

A series of polypeptides of increasing molecular weight up to at least 140,000 daltons was synthesized in the Krebs cell-free system in response to EMC RNA (Fig. 1 and 3). Differences have been observed, however, both in the number of these peptides resolved by electrophoresis and in their relative amounts and it is by no means

certain that each of the polypeptide peaks or bands represents a unique homogeneous molecular species. We would suggest that they are basically an artifact arising from a series of blocks in the translation of the EMC RNA in these systems.

The different polypeptide species could have arisen, in theory, by cleavage of precursor polypeptides as in the infected cell. There was, however, no evidence for cleavage of this type in these systems. Low-molecular-weight polypeptides were not formed late in the incubation (Fig. 2 and 3), and protease inhibitors effective in the inhibition of cleavage in the infected cell (Dobos and Martin, *in preparation*) were without effect in the cell-free system. On the other hand, the kinetics of formation of these polypeptides was not unreasonable for a scheme whereby the synthesis of each of the molecular-weight species is initiated within the first few minutes and is completed at a time proportional to its molecular weight (Fig. 2 and 3).

When the cell-free system was fractionated after incubation with the EMC RNA, and the microsome and supernatant fractions were analyzed, the polypeptides A to G were seen only in association with the particulate material. The cell sap fraction contained the same type of heterogeneous polypeptide product with a molecular weight of 20,000 to 40,000 daltons as was reported by us previously for this fraction (8). It could be, therefore, that translation simply stops at breaks in the RNA. There being no termination triplet at these breaks, release does not occur and the nascent chains remain on the ribosomes. Variation in the polypeptide products might then be explained by differences in the sites or order of endonucleolytic cleavage of the EMC RNA in different experiments. Alternatively these "products" might represent accumulation of the nascent chains at regions on the EMC RNA where translation is blocked or limited.

How then do these results fit in detail with what is known of events in the intact cell? Recent experiments have indicated that the EMC RNA genome is translated as a single unit in a fashion similar to that of poliovirus (Dobos and Martin, *in preparation*). Moreover, translation appears to be initiated with the coat protein cistrons clustered at or near the 5'-terminus of the genome (2). Crudely, therefore, in vivo translation is from coat to noncoat. In detail, however, the position of some of the final cleavage products, particularly those of less than 20,000 daltons in size, in relation to the genetic map remains rather speculative (2). In the cell-free system EMC-specific polypeptide synthesis is initiated at one or a very limited number of sites (Fig. 4), initiation occurring only during the first few minutes of incubation (Fig. 2 and 3). Thereafter, the EMC-specific polypeptides grow throughout the incubation to a maximal molecular weight of 140,000 although occasionally traces of higher-molecular-weight material were observed (Fig. 1 and 3). This figure of 140,000 corresponds to about 60% of the 250,000 to 270,000 daltons expected for a polypeptide product of complete translation of EMC RNA of a molecular weight of 2.7×10^6. In addition, we know from our previous work (3) that, of the 52 methione-containing EMC-specific tryptic peptides present in the EMC-infected cell, only about 30 are present in tryptic digests of the product formed in response to EMC RNA in these cell-free systems (cf. Fig. 5). All of the coat protein peptides [no. 2, 11, 12, 15, 19, 26, 27, 28, 29, 30, 34, 40, 41, 42, and traces of 51 (not obvious here), Fig. 5] do, however, appear to be translated (3). On this basis, it can be concluded that just over half of the genome and the vast majority of the coat protein cistrons are being translated. Any further decision as to the number of initiation sites and the exact distribution of the coat and noncoat protein sequences within this region will have to await the results of a direct assay for initiation in our systems.

In summary, it is clear that the translation of EMC RNA in the Krebs cell-free system is not random. We would suggest that the data can be most simply explained on the basis of the initiation of EMC-specific protein synthesis at a unique site towards the 5'-terminus of the RNA (2), with "termination" at a series of preferred sites, the exact position of which may depend on variation in the structure of the RNA. Secondary initiation sites, however, cannot yet be ruled out; the vast majority of nascent polypeptide chains are "terminated" prematurely, and about 40% of the genome does not appear to be translated to any significant extent. Clearly, there is much that remains to be done.

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