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Virus Protein Synthesis in Animal Cell-Free Systems: Nature of the Products Synthesized in Response to Ribonucleic Acid of Encephalomyocarditis Virus

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Ribonucleic acid (RNA) from encephalomyocarditis (EMC) virus stimulates the incorporation of amino acids into protein in cell-free protein-synthetic systems derived from Krebs mouse ascites tumor cells and chick embryo fibroblasts; the mouse system is the more responsive to the viral RNA. The greater part of this difference in activity can be ascribed to the cell sap, but the origin of the ribosomes also has a marked effect. The nature of the polypeptides formed in these cell-free systems was investigated by electrophoresis on polyacrylamide gels and by fingerprint analysis of tryptic digests. The same product in part appears to be synthesized in response to the EMC RNA in both systems. It was not detected if the EMC RNA was partly degraded ($\leqslant 4S$) or replaced by other species of RNA, including that from influenza virus. The results suggest that EMC RNA is partially translated in these systems to yield virus-specific polypeptides.

The rapid progress made recently in the understanding of protein synthesis in bacteria has come largely from work carried out on cell-free systems. Progress in animal systems has been relatively slow, however, and it would clearly be of advantage to have an animal system analogous to the phage ribonucleic acid (RNA)-*Escherichia coli* cell-free system, in which specific viral proteins are synthesized in response to viral RNA. This is particularly true in relation to our work on interferon, for it seems likely that a detailed understanding of its mode of action will be obtained only by studies at the cell-free level on systems of this type.

Some years ago we showed that RNA from encephalomyocarditis (EMC) virus would stimulate amino acid incorporation in a ribosome-cell-sap system from Krebs 2 mouse ascites tumor cells, but definitive characterization of the product proved impossible at that time (9, 10). Nonetheless, the system has obvious advantages in the ease with which relatively large quantities of both ribosomes and viral RNA can be prepared, and it seemed likely that the use of higher specific activity isotopes might make possible the characterization of the product as virus-specific polypeptide. That this is indeed the case has been demonstrated by the recent work of Smith, Marcker, and Mathews (17). We therefore decided to reexamine this system both as a model for the study of viral protein synthesis at the cell-free level and for the study of interferon action. Unfortunately, in our hands, the Krebs ascites cell responds very poorly, if at all, to interferon or to polynucleotide inducers of interferon (*unpublished data*). Accordingly, we have also investigated the response to EMC RNA of cell-free systems from interferon-sensitive chick embryo fibroblasts (CEF) and of mixed systems using components from both chick and mouse cells.

In this paper the requirements for the stimulation of amino acid incorporation by EMC RNA in these various systems are described and the partial identity of the products synthesized in response to the RNA in the chick and mouse systems is established. The effect of interferon pretreatment of cells on the chick and mixed systems is dealt with in the accompanying paper (8).

MATERIALS AND METHODS

Materials. Chemicals for use in the cell-free system and for the isolation of cell fractions and RNA were obtained and made into solution as described previously (9, 11). ^{14}C- and ^{3}H-labeled chemicals and ^{35}S-sulfate were obtained from the Radiochemical Centre, Amersham, Bucks, England. ^{35}S-methionine was prepared from ^{35}S-sulfate by a modification of the method of Sanger, Bretscher, and Hocquard (16). It had a specific activity of 20 to 40 Ci/mmole as estimated by isotope dilution experiments in the cell-free systems described here.

Krebs 2 mouse ascites tumor cells. The origin of the cell line used and the methods for its propagation in mice have been described previously (13).

Purification of EMC virus and extraction of its RNA. EMC virus was grown in 1.5- to 2.5-liter batches of Krebs ascites cells as described previously (13). After overnight incubation the cell debris was removed by centrifugation at 10,000 × *g* for 15 min, and the crude virus was precipitated by adjusting to *p*H 5.0 with 3 N acetic acid. The precipitate was recovered by centrifuging and was suspended in an amount of PP8 buffer (0.1 M NaCl, 0.1 M sodium phosphate, and 0.2 M sodium pyrophosphate, *p*H 8.0; reference 1) equal to 2% of the original volume of crude virus. More recently, crude EMC in this form was purchased from the Microbiological Research Establishment, Porton, Wilts. The virus was further purified by a modification of the method of Burness (1). It was shaken with a mixture of one part of 2.5 M sodium phosphate (*p*H 7.5), 0.53 parts of 2-butoxyethanol, and 0.27 parts of 2-ethoxyethanol and the mixture was centrifuged to separate the phases. The disc of material at the interface, containing the virus and denatured cell proteins, was extracted three times with PP8 buffer and the extract was diluted with 0.5 volumes of water to prevent precipitation of phosphate. It was centrifuged at 85,000 × *g* for 2.5 hr, and the pelleted virus was suspended with a Dounce homogenizer in 3 ml of 0.33 M K_2HPO_4 (adjusted to *p*H 8.7). The *p*H was brought to 8.0 by addition of 0.38 ml of 0.5 M NaH_2PO_4 and the suspension was incubated for 1 hr at 37 C with 0.5 mg of trypsin per ml. Insoluble debris was centrifuged off and the pellet was washed twice with 5-ml portions of 0.02 M phosphate buffer, *p*H 6.9. The supernatants were pooled, adjusted to *p*H 6.9 by the addition of 0.79 ml of 0.5 M NaH_2PO_4, and diluted with 60 ml of water to bring the phosphate concentration to 0.025 M. This was loaded onto a column (14 by 2.4 cm) of calcium phosphate equilibrated with 0.02 M sodium phosphate buffer (*p*H 6.9). The calcium phosphate was prepared freshly each week as follows, using a method suggested by T. S. Work: Na_2HPO_4 (0.5 M, 450 ml) and calcium acetate (0.25 M, 300 ml) were separately pumped into a beaker containing 100 ml of 0.05 M Na_2HPO_4; the phosphate and calcium were added at rates of 230 and 155 ml/hr, respectively, and the reactants were vigorously stirred during the addition. The suspension of calcium phosphate was allowed to settle and was washed three times by decantation with 1-liter portions of water and then once with 500 ml of 0.02 M sodium phosphate buffer, *p*H 6.9. The calcium phosphate column was eluted with a gradient produced with a four-chamber mixer containing 75 ml each of 0.02 M, 0.2 M, 0.15 M, and 0.5 M sodium phosphate buffer (*p*H 6.9) in that order (15). Purified virus was eluted between 0.22 and 0.26 M buffer and appeared as a peak of optical density with ratios of absorbance at 260 to 280 nm of 1.63 to 1.66. The fractions containing virus were pooled and centrifuged at 150,000 × *g* for 1.5 hr. The pellet of purified virus was suspended in 0.05 M tris(hydroxymethyl) aminomethane (Tris) buffer (*p*H 7.6) containing 0.01 M 2-mercaptoethanol and stored at −70 C. The ultraviolet absorption spectrum of the virus preparations was very similar to that described for EMC virus by Burness (1).

RNA was extracted from the purified virus as described previously (9). These preparations routinely contained more than 80% of intact 37*S* EMC RNA. Occasionally, degraded RNA was obtained from virus which had been frozen and thawed several times; such preparations were used in the studies with partly degraded EMC RNA. The RNA could be stored for many months at −20 C, without any apparent degradation, as a precipitate in 2:1 (v/v) ethanol–saline. Before use in the cell-free system it was centrifuged, the tube drained and wiped thoroughly to remove traces of ethanol, and the pellet dissolved in sterile distilled water at a concentration of 1 to 2 mg/ml. These solutions were stored in small samples or as the lyophilized powder at −70 C.

Influenza RNA. Allantoic fluid from eggs infected with the X-31 hybrid strain of influenza virus was purchased from Evans Medical, Speke, Liverpool, U.K. It was concentrated by continuous flow zonal ultracentrifugation basically as described by Gerin and Anderson (5) but on a smaller scale. It was further purified by differential centrifugation on sucrose and potassium tartrate gradients by the method of J. J. Skehel and G. C. Schild (*in press*). The RNA was extracted from the purified virus as described for Sindbis virus (11) and stored as for EMC RNA. In agreement with the findings of others (4), the RNA was heterogeneous with the major components sedimenting between 9 and 18*S*. Six discrete high-molecular-weight components were detected by electrophoresis on acrylamide gels (J. J. Skehel, *unpublished results*).

Preparation of ribosome and cell-sap fractions from Krebs cells. Cells were harvested, cooled to 0 C, washed, and homogenized, and a post mitochondrial supernatant was prepared (9). For preincubated ribosomes 10-fold concentrated solutions of adenosine triphosphate (ATP), guanosine triphosphate (GTP), phosphoenolpyruvate, and pyruvate kinase were added to the postmitochondrial supernatant to the same final concentrations as used in the cell-free system assays and the mixture was incubated for 30 min at 37 C. Preincubated and untreated ribosomes were isolated from the appropriate postmitochondrial supernatants by centrifugation at 105,000 × *g* for 90 min at 4 C. The ribosome pellets were resuspended by gentle hand homogenization in a loosely fitting glass Dounce homogenizer in 0.25 M sucrose, 20 mM Tris-hydrochloride (*p*H 7.6) at a concentration of 10 mg/ml and centrifuged at 12,000 × *g* for 10 min at 4 C to remove insoluble material. Cell sap was isolated and concentrated by pressure dialysis as described previously (9). Both ribosome and cell-sap fractions were stored in small samples at −70 C. Once thawed they were not refrozen and reused.

Preparation of ribosome and cell-sap fractions from CEF. Batches of 2 to 5 × 10^{10} CEF in suspension prepared as described by Zwartouw and Algar (18) were obtained from the Microbiological Research Establishment, Porton, Wilts., England. Monolayer cultures were prepared from these as described pre-

viously (11) except that the cells were incubated in 2.5-liter screw-capped bottles (yielding approximately 10^9 cells per bottle) on a roller drum. The medium was routinely changed after 24 hr as described previously (11), and the cells were used 20 to 24 hr later. "74S" ribosome and polysome fractions which had been treated with sodium deoxycholate (DOC) and cell sap were prepared as before (11). Untreated "74S" ribosomes were prepared from the total microsome fraction in the same way (11) but omitting the treatment with DOC. This omission reduced the yield of "74S" ribosomes to about one third. Cell-sap and ribosome fractions were stored as for the Krebs cell system.

Preparation of ribosomes and cell sap from chick embryos. Chick embryos (11 day) were decapitated, minced, and homogenized at 0 C in three volumes of 0.3 M sucrose, 10 mM Tris-hydrochloride (*p*H 7.6) by eight strokes at 1,500 rev/min of a Potter homogenizer. The homogenate was centrifuged at 4 C for 30 min at 10,000 × *g* to yield the postmitochondrial supernatant. The cell sap was prepared from the untreated postmitochondrial supernatant as described previously (9). Preincubated ribosomes were prepared as for the Krebs cell system, but, after preincubation and centrifugation of the postmitochondrial supernatant at 105,000 × *g* for 90 min, the microsome pellets were resuspended in 5% sucrose, 50 mM Tris-hydrochloride (*p*H 7.6), 25 mM KCl, 5 mM $MgCl_2$, 20 mM β-mercaptoethanol layered on 5 ml of 7.5% sucrose in 10 mM Tris-hydrochloride (*p*H 7.6), 10 mM KCl, and 1.5 mM $MgCl_2$ and centrifuged at 105,000 × *g* for 90 min at 4 C. The pellets were resuspended, centrifuged to remove insoluble material, and stored as above.

Assay of amino acid incorporation in the cell-free system. The basic system for the assay of amino acid incorporation contained the following components at the final concentrations indicated in a volume of 0.1 ml: 100 mM Tris-hydrochloride (*p*H 7.6); 50 mM KCl; 5 mM $MgCl_2$ (15 mM where polyuridylic acid was added); 20 mM β-mercaptoethanol; 1 mM ATP; 0.25 mM GTP; 5 mM phosphoenolpyruvate; pyruvate kinase, 40 μg/ml; the "other" amino acids, each 0.05 mM. In addition, the assay contained 0.625 μCi of a mixture of ^{14}C-L-amino acids (Radiochemical Centre, catalogue number CFB104, 54 Ci/gatom of carbon), 50 μg of ribosomes and cell sap containing 500 μg of protein. This was the mixture used for the assay of intrinsic incorporation (i.e., that due to endogenous host messenger RNA) for both chick and mouse systems and for viral RNA-stimulated activity in the chick system. For the assay of viral RNA-stimulated activity in the Krebs cell system the final assay volume was reduced to 0.03 ml, and it contained 100 μg of ribosomes and 125 to 225 μg of cell-sap protein. Unless otherwise stated incubations were for 40 min at 37 C.

The number of amino acids incorporated per ribosome was calculated from the results of isotope dilution experiments in which the specific activities of the precursor pools of the individual amino acids (^{14}C-valine, ^{14}C-leucine, or ^{35}S-methionine) were determined in the different cell-free systems. For analysis of the product reaction mixtures were scaled proportionally. As much as 1.0 mg of ribosomes and a maximum of 100 μCi of ^{35}S-methionine or 3 μCi of the ^{14}C-L-amino acid mixture were used.

Electrophoresis on acrylamide gels. After incubation the cell-free system was centrifuged at 105,000 × *g* for 90 min at 4 C to remove ribosomes. In a typical experiment 0.1 ml (500 μg of protein) of the supernatant from this step was treated with ribonuclease (10 μg/ml for 15 min at 37 C) and diluted to 0.4 ml with distilled water, and 10% sodium dodecyl sulfate (SDS) and 1.0 M β-mercaptoethanol were added to final concentration of 2% and 0.1 M, respectively. The mixture was heated to 90 C for 5 min and dialyzed at room temperature for 17 to 24 hr against 3 liters of 10 mM sodium phosphate buffer (*p*H 7.3) containing 0.1% SDS. The dialyzed protein (0.5 ml) was analyzed by electrophoresis using 10 by 0.8 cm, 7% acrylamide gels; the buffer used was 0.1 M sodium phosphate (*p*H 7.3) containing 0.1% SDS, and electrophoresis was for 16 hr at 4 mamps per gel. The gels were stained with 0.1% amido black, destained with acetic acid–methanol–water [5:40:55 (v/v)], and cut into four longitudinal slices. The middle two slices were dried on to cellophane and autoradiographed using "Ilfex" X-ray film.

Fingerprint analysis of tryptic peptides. After incubation of the cell-free system, unlabeled cell-sap protein was added where necessary to bring the amount of protein up to 2 mg. Ribonuclease (British Drug Houses, Ltd., Poole, U.K.) and 0.2 M ethylenediaminetetraacetic acid (EDTA) were added to 25 μg/ml and 50 mM, respectively, and the mixture was incubated at 37 C for a further 15 min. A 5.0-ml amount of 0.3 N trichloroacetic acid was added to precipitate the protein, which was then washed with 0.3 N trichloroacetic acid, ether–ethyl alcohol [3:2 (v/v)] and ether and dissolved in formic acid–methanol [5:1 (v/v)] for performate acid oxidation as described by Hirs (7). After lyophilization the performate-oxidized protein (1 to 2.0 mg) was resuspended in 0.8 ml of 0.2 M ammonium bicarbonate (*p*H 8.5). A solution of trypsin in 1 mM HCl, equivalent to a total of 2.5% by weight of the protein to be digested, was added in two equal portions at zero time and after 2 hr. Incubation was for 4 hr at 37 C with vigorous shaking. The tryptic digest was lyophilized, 1.0 ml of distilled water was added, and the solution was relyophilized to remove residual ammonium bicarbonate. The tryptic digest was dissolved in a small volume (<0.1 ml) of 0.3% (w/v) ammonium hydroxide. Up to 10% of the radioactivity frequently proved insoluble at this stage. Samples (10 to 20 μliters), including any insoluble material as a suspension, were applied to Whatman no. 1 paper (W. & R. Balston Ltd., England) for descending chromatography in *n*-butanol–acetic acid–pyridine–water [90:18:60:72 (v/v)] for 17 hr at room temperature. For each sample the paper strip containing the chromatographed material was cut out and sewn into a second sheet of Whatman no. 1 paper. Electrophoresis at right angles to the direction of chromatography was performed in a pyridine-acetic acid buffer [pyridine–acetic acid–water, 25:1:474 (v/v),

*p*H 6.5] at 4 kv for the period necessary (usually 50 min) for a phenol red marker to travel a distance of 10 to 12 cm. Electrophoresis in a single dimension at *p*H 6.5 or 3.5 was carried out similarly: the *p*H 3.5 buffer consisted of pyridine–acetic acid–water [1:10:289 (v/v)]. The papers were set up for autoradiography with "Ilfex" X-ray film (Ilford Ltd., Essex, U.K.) and exposed for 20 to 180 days.

Protein and ribosome concentrations. Protein was estimated by the method of Lowry et al. (12) with bovine serum albumin as standard. The concentration of ribosomes [milligrams (wet weight)/milliliter] was calculated from the absorbance at 260 nm assuming the $E^{260}_{1mg/ml}$ to be 12.

RESULTS

Characteristics of the cell-free systems. The basic Krebs and chick cell-free systems used were essentially scaled-down versions of those already described (9, 11). They showed typical dependence on the ribosome and cell-sap concentrations, an ATP-generating system, *p*H and Mg^{2+} optima, and the usual sensitivity to ribonuclease and inhibitors of protein synthesis (9, 11). Most of the amino acid incorporation occurred during the first 20 min of incubation at 37 C and was complete by 45 min (9, 11). In either system using total untreated ribosomes, the activity observed corresponded to approximately 20 amino acids incorporated per ribosome. Assayed under the same conditions, cell-free systems from rat liver and rabbit reticulocyte were of similar and roughly twofold greater activity, respectively.

Response of cell-free systems from Krebs cells to viral RNA. Various methods for preparing ribosomes were investigated to obtain systems which showed a low intrinsic amino acid incorporation activity and yet responded well to EMC RNA. Preparations with a relatively low activity (5 to 25% of controls) could be obtained from cells preincubated under a variety of conditions (9), by fractionation of the ribosomes to exclude existing polysomes or by preincubation of the postmitochondrial supernatant prior to the isolation of the ribosomes (14). The last was by far the most convenient method and was routinely used.

The response of untreated and of preincubated ribosomes from Krebs cells to EMC and influenza virus RNA is shown in Fig. 1. With EMC RNA the stimulation of amino acid incorporation was proportional to the amount of RNA added with as little as 0.1 μg producing a detectable response. Under these conditions high-molecular-weight influenza virus RNA was apparently inactive. Degraded EMC RNA ($\leq$4S), ribosomal RNA, and Krebs cell tRNA (in addition to that present in the cell-sap preparations)

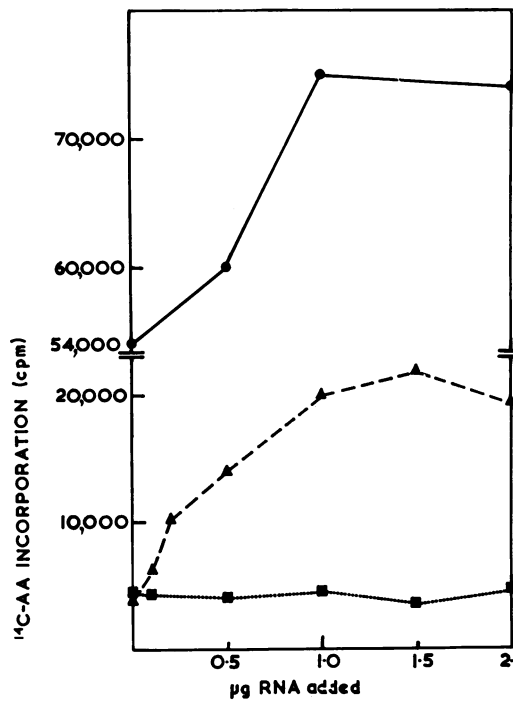

FIG. 1. *Response of Krebs cell systems to encephalomyocarditis (EMC) and influenza virus RNA. Preincubated and control ribosomes from Krebs cells were assayed in the cell-free system with Krebs cell sap and a mixture of ^{14}C-L-amino acids in the presence and absence of viral RNA under the conditions described under Methods. Incubation of the cell-free systems was for 40 min at 37 C. Symbols: ●, untreated ribosomes plus EMC RNA; ▲, preincubated ribosomes plus EMC RNA: ■, preincubated ribosomes plus influenza virus RNA.*

were inactive as reported previously (9). Clearly, therefore, high-molecular-weight EMC RNA is required for stimulation. As with ribosomes from preincubated cells (9), the optimum Mg^{2+} concentration was 5 to 7 mM both for intrinsic incorporation and incorporation in response to EMC RNA. The response to EMC RNA was dependent upon both the cell-sap protein and the ribosome concentrations. These were 3.75 to 7.0 mg/ml and 3.3 mg/ml, respectively. A fourfold reduction in the concentration of either or both abolished the response to RNA. This was rather surprising in that with the above concentration of cell-sap protein optimum intrinsic incorporation (amino acids incorporated per ribosome) was achieved at the lower ribosome concentration of 0.5 mg/ml. The reason EMC RNA does not stimulate amino acid incorporation at this lower ribosome concentration is not clear, especially as a good response to the RNA was

routinely obtained with CEF ribosomes assayed under these conditions with the same Krebs cell sap. Indeed, this was the concentration at which the response of chick ribosomes to EMC RNA was normally assayed.

The response to viral RNA of chick and mixed chick Krebs cell systems. The CEF ribosomes routinely used were a "74*S*" fraction (including ribosome monomers, subunits, and few residual dimers) isolated from the total microsome fraction by separation on sucrose gradients in the absence of any prior treatment with DOC. Treatment with 0.5% DOC, in the presence or absence of cell sap, markedly reduced the response of the ribosomes to EMC RNA (Fig. 2). In the absence of added RNA these "74*S*" ribosomes showed about one third of the activity (amino acids incorporated per ribosome) of unfractionated ribosomes and one quarter the activity of polysomes assayed under the same conditions in the cell-free system. Occasionally, total microsomes from whole chick embryos, preincubated as for the Krebs cell system, were used but these tended to respond less well to the addition of EMC RNA.

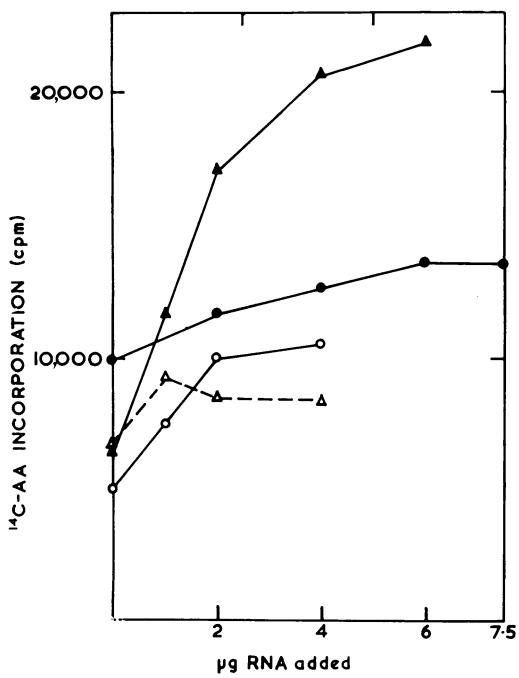

FIG. 2. *Response of chick and mixed chick-Krebs cell-free systems to encephalomyocarditis (EMC) RNA. Incorporation of a mixture of* ^{14}*C-amino acids was assayed in the following cell-free systems as described under Methods:* ●, *"74S" chick embryo fibroblast (CEF) ribosomes and CEF cell sap;* ▲, *"74S" CEF ribosomes with Krebs cell sap;* △, *deoxycholate-treated "74S" CEF ribosomes with Krebs cell sap;* ○, *preincubated Krebs cell ribosomes with CEF cell sap. Incubations were for 40 min at 37 C.*

Mixed Krebs ribosome–CEF cell sap or CEF ribosome-Krebs cell-sap systems both responded well to EMC RNA (Fig. 2). The response to EMC RNA of completely chick cell-free systems, however, was relatively poor (Fig. 2), at best resulting in only a 40% stimulation of incorporation. This response, although small, was reproducible, for ribosome and cell-sap preparations from four different batches of CEF assayed on seven different occasions all gave similar results. Under these assay conditions the response to influenza virus RNA was small and variable; stimulation when observed never exceeded 15% in any of these systems.

As discussed above the ribosome concentration used for the assay of stimulation of incorporation by EMC RNA was different for chick and Krebs cell ribosomes. In all other respects the characteristics and requirements for optimum stimulation of these chick and mixed systems were the same as for those for the completely Krebs system. For example, because larger assay volumes were used, it was necessary to add a higher absolute amount of EMC RNA to elicit the optimum response in the chick and mixed systems (Fig. 1 and 2); however, the concentration of the RNA required for this response was approximately the same (50 μg/ml) in the different systems. Like poliovirus RNA (6), EMC RNA appeared to bind cell-sap proteins (cf. Fig. 5, ref. 8); despite this, variation in the order of addition of the ribosomes, cell sap, and RNA to any of the Krebs, chick, or mixed assay systems affected incorporation only marginally (±10%). Similarly, preincubation of the RNA with cell sap for 1 min at 37 C in the cell-free system prior to the addition of the ribosomes had no effect.

Nature of the product formed in response to EMC RNA in the cell-free system. In many of the following studies ^{35}S-methionine was used as the radioactive marker. Although our preparations were contaminated with ^{35}S-methionine sulfoxide and sulfone, competition experiments showed that these were not incorporated into protein in the cell-free system. Furthermore, chromatographic analyses of exhaustive Pronase digests of the performate-oxidized radioactive protein from these systems indicated that methionine was the only radioactive substance incorporated.

When the cell-free systems were incubated for from 7.5 to 40 min at 37 C and centrifuged at 105,000 × *g* for 90 min, it was found that from 30 to 75% of the radioactivity incorporated

into polypeptides in response to EMC RNA was released into the soluble cell-sap fraction. With Krebs cell systems using ribosomes from preincubated cells, it was shown previously that at least 60% of this released material sedimented in a sucrose density gradient with a velocity of 2 to 8*S* with a broad peak at 2.6*S* (9). In the present experiments, acrylamide gel analysis of the radioactive protein present in the 105,000 × *g* supernatant from the Krebs cell-free system (Fig. 3) showed that the product formed in the presence of EMC RNA was apparently a mixture of polypeptides with molecular weights ranging from 20,000 to 40,000; no proteins of this size were detected with these preincubated ribosomes in the absence of viral RNA. EMC virus capsid proteins labeled during infection with ^{35}S-methionine showed three major protein bands (Fig. 3), with molecular weights of 21,500, 29,000, and 31,000 (the last two were not resolved by the 7% gel used in this experiment); a much fainter fourth protein band of molecular weight 10,000 can usually be just discerned. These results are included to show that these cell-free systems are capable of the synthesis of high-molecular-weight polypeptides in response to EMC RNA. It is not intended that they should be taken as evidence either for or against the possible identity of the product with the virus capsid proteins.

Fingerprint analysis of tryptic peptides from the product formed in response to EMC RNA. Autoradiographs of the tryptic peptide fingerprints obtained with the products formed in the Krebs and chick systems are shown in Fig. 4. In the absence of added viral RNA, incorporation was into a large number of different peptides and few were present in sufficient quantities to give discrete spots on the autoradiograph. This is particularly so with ribosomes of low intrinsic activity, where only a faint streak of neutral peptides was obtained with the amounts of radioisotope routinely used (*see* Fig. 3, ref. 8). However, host-specific peptides can be detected in the products obtained by incubating Krebs microsomes of high intrinsic activity in the absence of viral RNA, and this is illustrated in Fig. 4A (Fig. 4B shows a corresponding fingerprint from the same system to which EMC RNA was added). A pattern similar to that of Fig. 4A can also be obtained with low-activity ribosomes if the fingerprints are autoradiographed for a prolonged period (e.g., 20,000 counts/min for 180 days). The important feature of these control autoradiographs is that no discrete acidic peptides were detected when viral RNA was omitted from the incubation.

Digests of material from systems incubated in the presence of EMC RNA and ^{35}S-methionine exhibited a number of characteristic peptides; the most readily distinguishable of these were the acidic peptides in the areas labeled 1, 2, and 3 (Fig. 4B to E). These peptides were present in digests of EMC RNA-stimulated Krebs

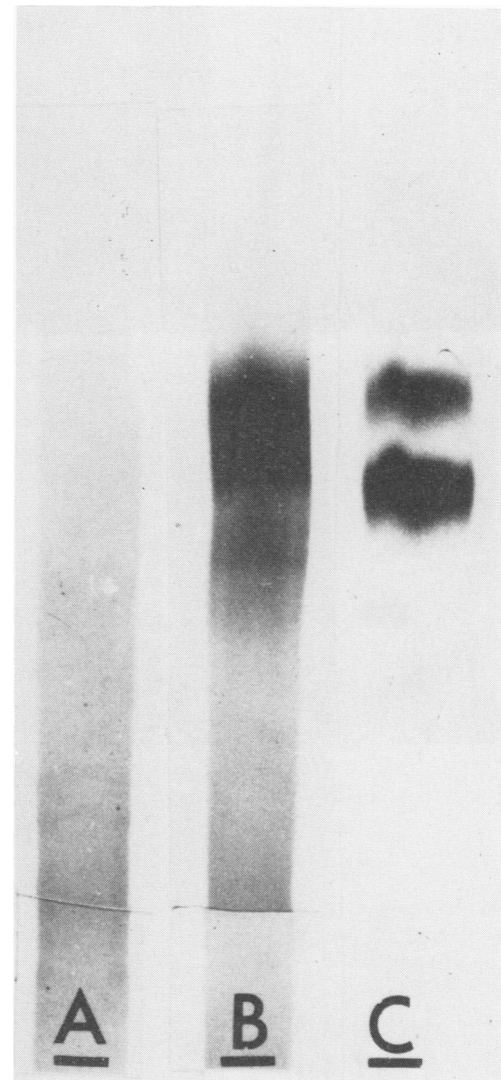

FIG. 3. *Analyses of the product synthesized in the cell-free system. After incubation of cell sap and preincubated ribosomes from Krebs cells with ^{35}S-methionine in the absence (A) or presence (B) of encephalomyocarditis (EMC) RNA, the cell-sap fractions were analyzed by electrophoresis on sodium dodecyl sulfate-acrylamide gels as described under Methods. Radioactive EMC virus proteins were electrophoresed in parallel on a separate gel (C) as molecular weight markers. Autoradiographs of the dried, sliced gels are shown, with the origins at the foot of the figure.*

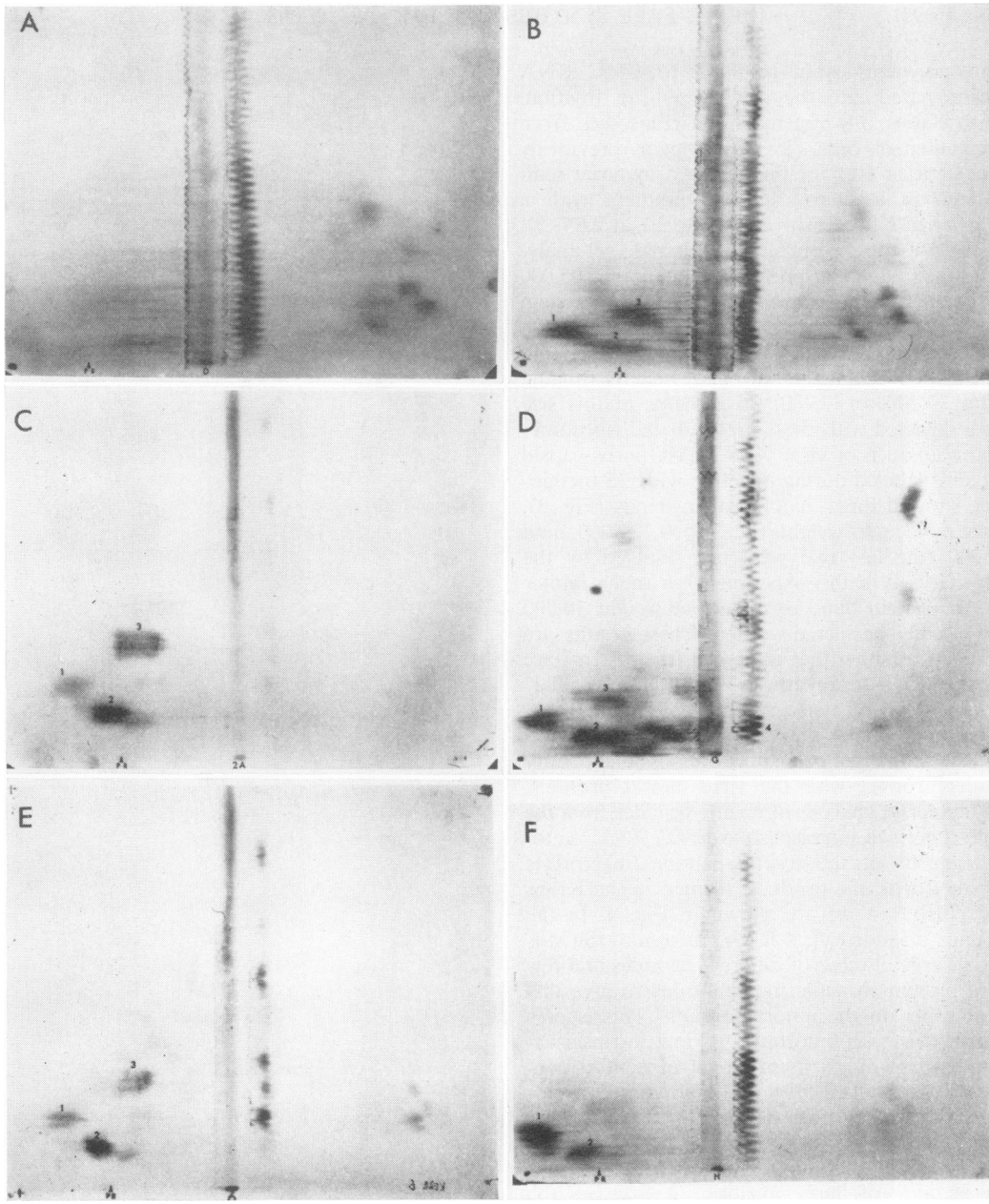

FIG. 4. *Comparison of tryptic digests of the products synthesized in response to encephalomyocarditis (EMC) RNA in mouse and chick cell-free systems. Incubations in the cell-free systems, preparation of the tryptic peptides, and their analysis by two-dimensional chromatography and electrophoresis were as described under Methods. The autoradiographs shown are from analyses of the following systems: A and B, untreated Krebs cell microsomes assayed with Krebs cell sap and ^{35}S-methionine in the absence (A) and presence (B) of EMC RNA (load 100,000 counts per min, autoradiography 20 days); C, preincubated Krebs cell ribosomes assayed with Krebs cell sap and EMC RNA in the presence of ^{35}S-methionine (load 20,000 counts per min, autoradiography 87 days); D, as for C except that the isotope used was a mixture of ^{14}C-amino acids (load 50,000 counts per min, autoradiography 47 days); E, "74S" chick embryo fibroblast (CEF) ribosomes assayed with Krebs cell sap, EMC RNA and ^{35}S-methionine (load 12,000 counts per min, autoradiography 180 days); F, preincubated chick embryo microsomes assayed with CEF cell sap, EMC RNA, and ^{35}S-methionine (load 50,000 counts per min, autoradiography 47 days). Chromatography was from the origin marked with a letter or figure near the bottom center of each fingerprint, and electrophoresis at pH 6.5 was in the plane from left to right with the anode to the left. Arrow and lettering PR indicate the position to which a phenol red marker migrated during the electrophoresis.*

systems in which either untreated or preincubated microsomes were used (Fig. 4B and C). They were also present in digests of EMC RNA-stimulated mixed systems [Fig. 4E and Fig. 8 of the accompanying paper (8)] and in EMC RNA-stimulated systems in which a mixture of ^{14}C-amino acids replaced the ^{35}S-methionine as label (Fig. 4D). On a statistical basis one would expect that acidic peptides must be present in digests of control (minus EMC RNA) systems which had been incubated in the presence of a mixture of ^{14}C-amino acids, but again such peptides were never present in sufficient quantities for them to be detected in these experiments. The minor differences in the positions of areas 1, 2, and 3 in the different fingerprints can be accounted for by minor variations in the chromatographic separation, for not all of these digests were chromatographed in parallel. The electrophoresis step was more reproducible and there is good correspondence between the fingerprints in this respect, as can be seen from a comparison with the position of the phenol red marker which gives some direct measure of relative mobility. The use of cell sap from EMC-infected Krebs cells in place of normal cell sap had no effect on the product synthesized in response to EMC RNA in these systems.

Acidic peptides were again detected in areas 1 and 2 when the products from purely chick cell systems stimulated with EMC RNA were analyzed. Similar patterns were obtained when either preincubated microsomes from whole chick embryos (Fig. 4F) or "74S" ribosomes from CEF were assayed with ^{35}S-methionine, EMC RNA, and CEF cell sap. When eluted and reelectrophoresed at a different *p*H (*p*H 3.5) these peptides comigrated with the ^{35}S-methionine- and ^{14}C-amino acid-labeled peptides from corresponding areas of the fingerprint from the RNA-stimulated Krebs systems (Fig. 5). In the absence of amino acid sequence data, the identity of these peptides from the chick and mouse systems is not beyond doubt; however, it is likely that they do correspond and, therefore, that the EMC RNA codes for their synthesis in both systems.

With the preincubated microsomes from whole chick embryos used in the experiment shown in Fig. 4F, little (<10%) or no stimulation of incorporation was observed in response to EMC RNA when they were assayed with CEF cell sap. However, a specific product appeared to be synthesized in response to the RNA (Fig. 4F). This implies that a lack of stimulation in the cell-free assay does not necessarily indicate failure to translate the RNA. It was of interest, therefore, to see if this were true for the influenza virus RNA. Analysis of digests of both Krebs and chick systems which had been incubated with influenza RNA indicated that this was not the case as these were indistingushable from corresponding controls incubated without viral RNA.

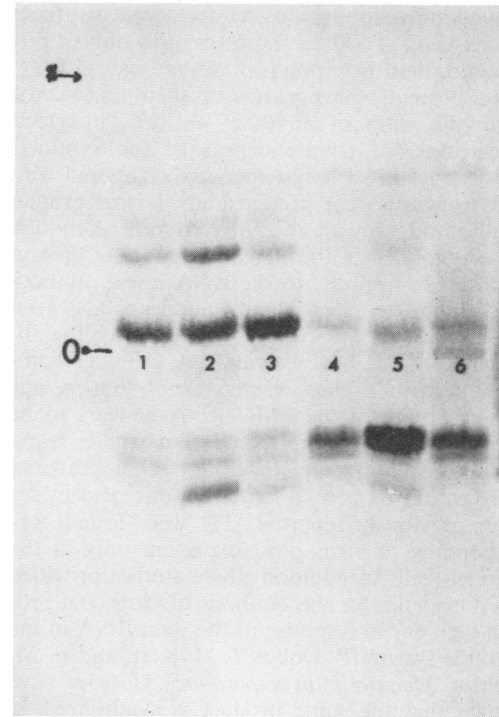

FIG. 5. *Identity of the ^{14}C-amino acid and ^{35}S-methionine labeled products synthesized in response to encephalomyocarditis (EMC) RNA in cell-free systems from mouse and chick cells. Radioactive peptides from areas 1 and 2 of selected fingerprints were eluted and reelectrophoresed at pH 3.5 (Methods). Origin is marked 0 and the anode is at the top of the figure. Numbers 1 and 4 were the ^{35}S-peptides eluted from areas 1 and 2, respectively, for the chick system plus EMC RNA (Fig. 4E); numbers 2 and 5 were the corresponding ^{35}S-peptides from the Krebs system plus EMC RNA (Fig. 4C); numbers 3 and 6 were the corresponding peptides from areas 1 and 2 for the Krebs system plus EMC RNA assayed in the presence of the ^{14}C-amino acid mixture (Fig. 4C). The arrow indicates the position of a phenol red marker. The loads were not quantitatively identical but were approximately 2,000 to 3,000 counts per min in each case. Autoradiograph was developed after exposure for 75 days.*

DISCUSSION

It is clear from the studies reported here and elsewhere (9, 14, 17) that EMC RNA will stimulate the synthesis of specific high-molecular-

weight polypeptides in cell-free systems from Krebs cells. To date, however, the only direct evidence that these polypeptides are indeed EMC virus proteins (or portions of them) has come from the work of Smith et al. (17), in which fingerprints of tryptic digests of the products synthesized in such systems were compared with those of virus coat protein and of virus protein synthesized only in the infected cell. Although unable to detect the synthesis of coat protein peptides in response to the RNA, these authors were able to show correspondence between peptides from the cell-free system and the infected cell. We have carried out a similar comparison with similar results except that in our systems more of the EMC RNA appears to be translated. For example, in recent studies using higher resolution fingerprinting techniques than were employed here, a larger number of peptides than previously reported (17) were found corresponding to virus protein present only in the infected cell. In addition, these studies provided good evidence for the synthesis of virus coat protein peptides in response to the viral RNA in the cell-free system (P. Dobos, I. M. Kerr, and E. M. Martin, *Manuscript in preparation*). Here we have shown that the same product is synthesized in part in response to EMC RNA in two totally different systems, those from mouse and chick cells (Fig. 4 and 5). Accordingly, it can be concluded that EMC RNA is indeed acting as a messenger RNA for the synthesis of virus-specific polypeptide in these systems.

Accepting this it is of interest to ask how much of the RNA is translated and how frequently. EMC RNA has a molecular weight of 2.7×10^6 (2, 3) and is theoretically capable of coding for approximately 2,700 amino acids. We know that, although apparently larger polypeptides have been observed on occasion, much of the EMC RNA-stimulated incorporation is into heterogeneous polypeptides of molecular weight 20,000 to 40,000 (Fig. 3), corresponding to translation of 7 to 15% of the RNA. This provides only a minimum estimate, however, for we do not know whether these polypeptides arise from translation of the same or different sections of the RNA. A more realistic estimate may be provided by the number of methionine-containing peptides present in tryptic digests of the EMC RNA-stimulated product. As would be suggested by the results presented in Fig. 5, further detailed analyses at higher resolution have indicated that such digests contain more peptides than is obvious from the original fingerprints (Fig. 4). Recently we have resolved 25 such peptides from the cell-free system and by comparison about 50 virus-specific peptides from parallel digests of material from methionine-labeled infected cells (P. Dobos, I. M. Kerr, and E. M. Martin, *manuscript in preparation*). This latter is approximately the value to be expected for complete translation of the viral genome assuming the viral proteins to contain 2% methionine. These results would, therefore, suggest the translation in the cell-free system of about 50% of the EMC genome yielding protein(s) up to a total of 135,000 in molecular weight. How frequently a given RNA molecule is translated we do not know. Although on a purely quantitative basis several hundred amino acids are incorporated per molecule of EMC RNA, we do not know how many of the molecules added actually function as messengers. Nor do we know whether individual RNA molecules function continuously or different ones sequentially.

A curious feature of the fingerprint is the preponderance of acidic peptides in digests of the viral RNA-stimulated product (Fig. 4C to F), in contrast with the more usual distribution seen with control preparations (Fig. 4A). It is particularly striking in the digest of the product labeled with the mixture of ^{14}C-amino acids shown in Fig. 4D. The significance of this is not known. The presence of peptides in acidic areas 1 and 2 of all of the fingerprints shown in Fig. 4C to F, however, suggests that these may be at or near the N-terminus of the EMC RNA-stimulated polypeptides synthesized in these systems. If so, it appears that initiation of translation is consistent in the chick and mouse systems, although not necessarily identical with that occurring in vivo (it is possible that translation in the cell-free system could begin at a point closer to the 3′-end of the viral RNA).

RNA from influenza virus does not appear to act as messenger in either the chick or mouse systems. It is possible that this RNA requires additional factors for its translation which are absent from these systems as currently prepared, or quite simply that the correct assay conditions for this RNA have not yet been found. It is also conceivable that the virion RNA molecules do not function as messengers but represent complementary strands which have to be transcribed to produce functional messengers.

Although both the chick and mouse systems used in these studies appear to translate EMC RNA, there are important differences between them. The chick system is less active quantitatively in translating the RNA (Fig. 1 and 2) and appears to translate less of the genome (Fig. 4C and F). This latter is more obvious from recent experiments in which more of the RNA genome appears to have been translated in the Krebs system than in the experiments shown in Fig. 4. The

reduced translation in the chick system cannot simply be attributed to increased nucleolytic degradation of the viral RNA (cf. Fig. 9 and 6 of ref. 8). From the results with mixed systems, it is clear that part of the difference in activity can be ascribed to the effect of the cell sap. For example, EMC RNA stimulates both chick and Krebs ribosomes to a greater extent in the presence of Krebs cell sap (Fig. 1 and 2), and more of the RNA genome appears to be translated when chick ribosomes, in particular, are incubated with Krebs cell sap (Fig. 4E and F). The quantitative difference may be due to the presence of more or other initiation factors in the Krebs cell sap, whereas the change in the amount of the genome translated may reflect a difference in the distribution of specific tRNA. On the other hand, in the chick system at least, an additional factor(s) associated with the ribosomes may be required for the translation of EMC RNA. This is suggested, for example, by the effect of DOC treatment (Fig. 2) and of interferon pretreatment of cells (8) on the response of chick ribosomes to viral RNA. The nature of these ribosomal and cell-sap factors, which apparently govern the extent of translation of EMC RNA, and their significance in the initiation and control of virus protein synthesis are presently under investigation.

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