Inhibition of Protein Synthesis in Cell-Free Systems from Interferon-Treated, Infected Cells: Further Characterization and Effect of Formylmethionyl-tRNA_F

IAN M. KERR, R. M. FRIEDMAN, R. E. BROWN, L. A. BALL, AND J. C. BROWN

National Institute for Medical Research, Mill Hill, London NW7 1AA, and Department of Microbiology, University of Virginia, Charlottesville, Virginia, 22901

Received for publication 3 August 1973

The translation of encephalomyocarditis virus (EMC) RNA is markedly inhibited in cell-free systems from interferon-treated, vaccinia virus-infected L-cells (10, 11). The polypeptide products synthesized in response to EMC RNA in cell-free systems from these and untreated infected cells have been analyzed by electrophoresis on polyacrylamide gels. Qualitatively, the same EMC-specific polypeptides were synthesized throughout. In experiments using preincubated microsomes from normal Krebs cells to assay cell sap from L-cells which had been exposed to interferon prior to infection, only the amount of the EMC-specific polypeptide products was reduced. This result suggests that there is an inhibition very early in translation in interferon-treated, infected cells. Initiation seems a priori the more attractive site for this inhibition, but an effect shortly after initiation cannot be excluded. With unfractionated cell-free systems from interferon-treated infected L-cells, however, there appeared to be an additional minor inhibitory effect on polypeptide chain elongation, in that the EMC-specific polypeptides synthesized showed not only a reduction in amount but also a bias towards lower molecular weight. The formylated methionyl initiator tRNA (Fmet-tRNA_F) was used as a further probe into the apparent effect on initiation. With this reagent we have confirmed that there is one major initiation site for the translation of EMC RNA in these cell-free systems. In addition, the results have shown that EMC-specific polypeptide chains initiated with Fmet escape the major interferon-mediated inhibition at or shortly after initiation.

In the interferon-treated, vaccinia virus-infected L-cell, vaccinia virus mRNA is synthesized but not translated (16, 21). Recently, we have shown that the translation of encephalomyocarditis virus (EMC) RNA is inhibited in cell-free systems from such cells (10, 11). It is now well established from our own work (6, 11, 18) and that of others (1, 2, 8, 23, 27) that the EMC RNA genome is translated with fidelity in cell-free systems from mouse L- and Krebs 2 ascites tumour cells. The products are a series of high-molecular-weight EMC-specific polypeptides. The majority of these arise as a result of premature termination at preferred sites during the translation of this large mRNA (molecular weight 2.7×10^6) (2, 18). Here we have made use of our knowledge of these polypeptide products to analyze the nature of the inhibitory event(s) in the translation of EMC RNA in cell-free systems from interferon-treated, vaccinia virus-infected L-cells. In addition, we have used the formylated methionyl initiator

tRNA (Fmet-tRNA_F) (14, 28) to confirm (23, 27) that initiation of translation of EMC RNA in the cell-free system is at a unique site as in the intact cell. With this established for the cell-free systems with which we are working, we have used the Fmet-tRNA_F as a probe in our investigation of the translation of EMC RNA in these systems. The results of the electrophoretic analysis of the products suggest that the major inhibition in the translation of EMC RNA in cell-free systems from interferon-treated, infected cells is at or shortly after initiation, with a minor secondary effect on chain elongation. With EMC RNA as messenger, however, polypeptide chains initiated with Fmet from Fmet-tRNA_F appear to escape the major inhibition occurring early in translation.

MATERIALS AND METHODS

Materials. Chemicals for use in the cell-free system, for the isolation of cell fractions and viral RNA and for the preparation of tryptic digests were as

described previously (6, 19). All radiochemicals were supplied by the Radiochemical Centre, Amersham, Buckinghamshire, England. The calcium leucovorin was from Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y. Some of the EMC virus was obtained from Products for Research, G.D. Searle and Co. Ltd., High Wycombe, Buckinghamshire, England.

Methods. The Krebs 2 mouse ascites tumour cells and the preparation of cytoplasmic extracts from them (6, 19), the preparation and purification of EMC virus (20) and the extraction of EMC RNA (19), the labeling of 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 (6) have already been described. Qualitative and quantitative analysis of the polypeptide products by electrophoresis on 5 or 7.5% polyacrylamide gels after treatment with sodium dodecyl sulphate (SDS) was as described previously (18). Further digestion of the tryptic digests with Pronase was overnight at 37°C in 0.2 M ammonium bicarbonate, 10 mM CaCl₂.

Preparation of L-cell extracts. Nonpreincubated post-mitochondrial supernatant—S10—fractions were prepared as before (11) from interferon-treated (50 to 100 reference units/ml) and control, vaccinia virus-infected L-cells. Centrifugation of this material at 100,000 × *g* for 90 min yielded the supernatant cell sap fractions used in the mixed system assays with Krebs cell microsomes.

Amino acid incorporation assays. For the Krebs and L-cell systems the assays of amino acid incorporation in response to saturating amounts of EMC RNA (20 to 40 µg/ml) were carried out as previously (6, 11). Assays in the mixed system in which L-cell sap was assayed with preincubated microsomes from normal Krebs cells were incubated at 30°C under the same conditions as for the L-cell system (11) but using 200 µg of Krebs microsomes and 10 µliters of L-cell sap (approximately 150 µg of protein) per 50-µliter assay. Assays involving the use of ³⁵S-Fmet- or met-tRNA_F and/or AUG(U)₃₅ were routinely carried out in a final volume of 25 µliters. To saturate these systems 2 pmol (35,000 to 250,000 counts/min per pmol for methionine at a specific activity of 20 to 150 Ci/mmol at 80% efficiency of counting) of ³⁵S-met- or Fmet-tRNA_F were added per 25-µliter sample. Unlabeled methionine (50 µM) was included in the amino acid mixture used in all such assays. AUG(U)₃₅ was added at a saturating concentration of 80 µg/ml (1.6 OD₂₆₀ units/ml) and was assayed at the same magnesium ion concentration (4.35 to 4.85 mM) as for EMC RNA in the L-cell system. It was prepared according to the method of Sundararajan and Thach (30).

Interferon preparation and assay. Mouse L-cell interferon (>10⁶ reference units per milligram of protein) was purified by the method of Paucker (25) and assayed as previously described (11).

Charging and formylation of Krebs tRNA_F^{Met}. The enzyme fraction used for the charging and formylation of the tRNA was prepared from *Escherichia coli* (MRE 600) according to Muench and Berg (22). The dialyzed and concentrated DEAE-cellulose

fraction in 50% glycerol was employed throughout. The activity of the enzymes was monitored by using pure *E. coli* tRNA_F^{Met} (the generous gift of Kellmers and Novelli, Oak Ridge National Laboratory). 100% charging and >80% formylation of this tRNA was routinely observed. *E. coli* Fmet-tRNA_F has been reported (14) to be active at a low level in the rabbit reticulocyte system, but with the Krebs and L-cell systems employed here, although there was some incorporation of label from the *E. coli* ³⁵S-Fmet-tRNA_F in response to EMC RNA, analysis of the Fmet tryptic peptides showed it to be nonspecific. Accordingly, the *E. coli* enzymes were used to charge and formylate Krebs cell tRNA_F^{Met}. The Krebs cell tRNA was obtained by phenol extraction of whole cells followed by salt fractionation (31) and chromatography on DEAE-cellulose (32). Initially, it was fractionated on BD-cellulose to yield the tRNA_F^{Met} and tRNA_F^{Met} species (27). Having confirmed (12) that the *E. coli* enzyme preparation was inactive with the mammalian noninitiating tRNA_M^{Met} species, however, unfractionated tRNA was used in all of the work described here. Charging and formylation were carried out simultaneously in a reaction mixture containing the following components: 50 mM HEPES buffer, pH 7.5; 50 mM KCl; 5 mM Mg acetate; 2 mM dithiothreitol, 4 mM ATP, 1 mM CTP; 10 µM Ca leucovorin; 200 µg of Krebs cell tRNA per ml (approximately 7,000 pmol of total tRNA equivalent to 140 pmol of tRNA_F^{Met} at 2%) and 1 µM L-³⁵S-methionine (40 to 150 Ci/mmol). Incubation was for 15 min at 30°C. For the preparation of nonformylated met-tRNA_F the formyl donor Ca leucovorin was omitted. The Ca leucovorin was made up and stored in 0.25 N HCl. On addition to the cell-free system, the change in pH converts it to the unstable active form (26). The amount of *E. coli* enzyme required for optimum charging and formylation varied considerably from preparation to preparation and with storage and was determined on a small scale prior to preparative use. The charged and formylated tRNA was extracted with phenol before purification at pH 4.5 on a small DEAE-cellulose column (32). After ethanol precipitation the RNA was dissolved in water at 2 × 10⁵ counts per min per µliter. To assay the extent of formylation a sample of the ³⁵S-Fmet-tRNA_F was precipitated with 5% trichloroacetic acid at 0°C, washed with cold acid on a cellulose nitrate filter (13-mm diameter, Sartorius Membranfilter GMBH, Gottingen, Germany), rinsed with ethanol, and the amino acyl-tRNA was discharged by incubation of the filter for 30 min at 37°C in 0.4 N NH₄OH. The eluted Fmet and met were fractionated by electrophoresis for 1 h at 400 V on Whatman 3 MM paper in pyridine acetate buffer, pH 6.5. The amounts of Fmet and met were determined by using a strip scanner or by cutting the dried paper into 0.5-cm strips and counting these in a scintillation counter. Charging was to a level of 2 to 2.4 pmol of Met-tRNA_F per 100 pmol of total tRNA. Only 70 to 80% formylation of the Krebs met-tRNA_F was usually obtained. However, the residual (20 to 30%) nonformylated methionine in these preparations was not incorporated into acid-insoluble products in response to EMC RNA. Any nonformylated N-terminal methionine is rapidly cleaved (14, 15) from the nascent

polypeptide chain. In addition, although when working with completely nonformylated initiator met-tRNA_F there is a significant incorporation of methionine into internal sites in the polypeptide chain (7, 13, and our own unpublished results), no significant incorporation of this type occurred from the nonformylated fraction (20 to 30%) of the Fmet-tRNA_F preparation used here. For example, complete digestion of the EMC RNA-stimulated polypeptide products with trypsin and Pronase consistently yielded $\geq 95\%$ Fmet and $\leq 5\%$ methionine. It could be, therefore, that the 20 to 30% of apparently nonformylatable met-tRNA_F was artefactual or represented damaged nonfunctional RNA.

RESULTS

Throughout the experiments to be described, the L-cell extracts employed in the cell-free systems were neither preincubated nor subjected to Sephadex chromatography. With such nonpreincubated material, amino acid incorporation in response to EMC RNA in the cell-free system is not reduced by prior infection of the cells and interferon treatment alone has only a small inhibitory effect (11). A clear-cut 50 to 90% inhibition of the translation of the added EMC RNA is seen, however, in systems employing material of this type from L-cells exposed to both interferon treatment and infection (10, 11, I. M. Kerr et al., *Advances in the Biosciences*, vol. 11, in press). It is with the further characterization of this enhanced inhibition of translation triggered by infection that we will be concerned here.

Characterization of the polypeptide products synthesized in response to EMC RNA in cell-free systems from interferon-treated, infected cells. An analysis by electrophoresis on SDS polyacrylamide gels of the EMC-specific polypeptide products synthesized in response to EMC RNA in cell-free systems from vaccinia-virus-infected and interferon-treated, vaccinia-infected cells is shown in Fig. 1. A qualitative autoradiographic analysis of the products synthesized after incubation for 30, 60, 120, and 240 min in the presence of EMC RNA yielded the results shown in Fig. 1A, whereas a quantitative analysis of the distribution of the products at 120 min is shown in Fig. 1B. It should be emphasized that in this experiment pretreatment of the cells with interferon prior to infection resulted in a 70% inhibition of incorporation in response to EMC RNA in the cell-free system (Fig. 1C). To compensate for this the autoradiographs of gels 6 to 9 (Fig. 1A) (interferon treated) were exposed for longer than the corresponding controls (gels 1 to 4, Fig. 1A). Similarly, for ease of comparison, the data in Fig. 1B are expressed as percentage of recovered radioactivity rather than in terms of absolute

counts. From this data it can be seen that the same series (A to G, Fig. 1A) of high-molecular-weight EMC-specific polypeptides was synthesized in both systems. These are apparently identical in molecular weight to those synthesized in cell-free systems from uninfected Krebs and L-cells. The nomenclature A to G is that which was used to identify the EMC-specific polypeptides in our previous work (Fig. 3, reference 18). In addition, the results for the earlier 30-min (gels 1 and 6) and 60-min (gels 2 and 7) time points indicate that there is no striking difference in the time at which the polypeptide chains first reach a size of 130,000 in molecular weight, (polypeptide G), for example. This in turn suggests that there is no gross overall reduction in the rate of polypeptide chain elongation in the inhibited systems. Nevertheless, there does appear to be a slight inhibitory effect on chain growth in that the amount of the major polypeptide G accumulating at later time points (gels 3 and 4) is significantly reduced (gels 8 and 9). This is shown quantitatively for the 120-min time point in Fig. 1B, in which the bias in the distribution of the polypeptide products towards lower molecular weight is obvious in the inhibited system. The nature of this relatively small effect is uncertain, but it is clearly insufficient to account for the 70% inhibition of total incorporation (Fig. 1C). Accordingly, the results show that the major inhibition must occur prior to the products reaching a size detectable on these gels, i.e., most probably at, or possibly shortly after, initiation.

Characterization of the polypeptide products synthesized in response to EMC RNA in mixed cell-free systems. In general, on fractionation of the L-cell extracts, the amount of the interferon-mediated inhibitory effect recovered with the cell sap (S100) and microsome fractions has proved variable. It may be that this variability is correlated, for example, with the degree of polysome disaggregation or ribosome dissociation in the system. The results obtained in preliminary assay of the crude microsome fraction with normal Krebs cell sap were qualitatively similar to those for the unfractionated system (Fig. 1). It should be emphasized that this may merely reflect the crudeness of the fractionation procedure. Different results were obtained, however, on assay of cell sap from interferon-treated, vaccinia virus-infected cells with preincubated microsomes from normal Krebs cells. With this system only the major inhibition at or shortly after initiation was observed, with no secondary effect on elongation. This is shown in Fig. 2. In this experiment interferon treatment prior to infec-

FIG. 1. Characterization of the polypeptide products synthesized in response to EMC RNA with time in cell-free systems from interferon-treated, vaccinia virus-infected cells. Unfractionated postmitochondrial supernatant (S10) fractions were used throughout. Incubation in the cell-free system in the presence of ^{35}S -methionine (100 $\mu\text{Ci}/\text{ml}$, 100 Ci/mmol) and EMC RNA (40 $\mu\text{g}/\text{ml}$) was as already described (Materials and Methods). A, Analysis of the polypeptide products by electrophoresis in SDS-polyacrylamide gels. Gels 6 to 9 were with interferon-treated, vaccinia-infected cell extracts; Gels 1 to 4 were with corresponding extracts from infected cells without interferon pretreatment. After incubation of the cell-free systems for 30 min (gels 1 and 6), 60 min (gels 2 and 7), 120 min (gels 3 and 8) and 240 min (gels 4 and 9), 50 μl samples were treated with SDS and subjected to electrophoresis on SDS-polyacrylamide gels. The figure shows autoradiographs of the dried gels and, as an index of molecular weight, a photograph of a stained gel on which the polypeptides of purified reovirus were electrophoresed in parallel. The figures to the right represent the molecular weight in thousands of the major reovirus polypeptides $\lambda 1$, $\lambda 2$, $\mu 2$, and $\sigma 3$ (29). The autoradiographs of gels 1 to 4 were exposed for 4 days; those for gels 6 to 9 for 20 days. B, Quantitative analysis of the molecular weight distribution of the products at 120 min. Gels corresponding to 3 and 8 (Fig. 1A) were fixed, sliced, prepared for radioactive counting, and counted as previously (18). The abscissa shows the distance migrated from the top of the gel (1-mm slices) with the origin to the left; the major peak (fractions 8 to 10) corresponds to G in the gels 3 and 8. The ordinate gives the radioactivity incorporated into polypeptide recovered in each fraction as a percentage of the total recovered from the complete gel. Symbols: $\bullet$, no interferon pretreatment of the cells; $\circ$, interferon pretreated. The total recoveries from the gels (100% values) were 53,000 counts/min without and 15,000 counts/min with interferon pretreatment. C, Direct quantitative assay of ^{35}S -methionine incorporation in response to EMC RNA with time in the cell-free systems used in 1A and 1B above. After incubation for the indicated time 5- μl samples were assayed for incorporation of radioactivity into protein in the usual way. Symbols: $\bullet$, vaccinia-infected cell extracts; $\circ$, interferon-treated, vaccinia-infected cell extracts.

FIG. 2. Characterization of the polypeptide products synthesized in response to EMC RNA with time in the cell-free systems in the presence of cell sap fractions from vaccinia virus-infected and interferon-treated vaccinia virus-infected cells. The cell sap fractions were assayed with preincubated microsomes from Krebs ascites tumour cells. Incubation in the cell-free system in the presence of ^{35}S -methionine (100 $\mu\text{Ci}/\text{ml}$, 100 Ci/mmol) and EMC RNA (40 $\mu\text{g}/\text{ml}$) was as already described. A, Analysis of the polypeptide products by electrophoresis in SDS-polyacrylamide gels. Gels 1 to 3 employed cell sap from vaccinia virus-infected cells. Gels 4 to 6 were with cell sap from interferon-treated, vaccinia virus-infected cells. After incubation of the cell-free systems for 30 min (gels 1 and 4), 60 min (gels 2 and 5), and 120 min (gels 3 and 6), 40- μl samples were treated with SDS and subjected to electrophoresis on SDS-polyacrylamide gels in parallel with reovirus polypeptides as described in Fig. 1. The figures to the right represent the molecular weights in thousands of the major reovirus polypeptides (Fig. 1). The autoradiographs were exposed for 4 days. B, Quantitative analysis of the molecular weight distribution of the products at 120 min. This was carried out with gels corresponding to 3 and 6 in A above as described in Fig. 1B. The data are expressed as in Fig. 1B. Symbols: $\bullet$, No interferon; $\circ$, interferon pretreated. The total recoveries from the gels (100% values) were 75,000 counts/min without and 21,000 counts/min with interferon pretreatment. C, A direct quantitative assay of ^{35}S -methionine incorporation in response to EMC RNA with time in the cell-free systems used in A and B above was carried out as in Fig. 1C. Symbols: $\bullet$, vaccinia virus-infected cell extracts; $\circ$, interferon treated, vaccinia virus-infected cell extracts.

tion reduced the level of translation of EMC RNA to 50% of the control value (Fig. 2C) without apparently affecting the nature of the EMC-specific polypeptides formed (Fig. 2A and B). In the inhibited system identical products

were synthesized (Fig. 2B) at apparently the same rate of polypeptide chain growth (gels 1 and 4, Fig. 2A) but in reduced amounts (Fig. 2C).

Taken together these results are consistent

with a major block at or shortly after initiation, with a minor inhibition of polypeptide chain elongation, the latter being seen only in the unfractionated system.

Incorporation of Fmet from Fmet-tRNA_F in response to EMC RNA. The initiation of polypeptide chain synthesis in eukaryotes is thought to occur exclusively with methionine from one of the methionine tRNAs: the initiator tRNA_F^{Met}. Normally, this N-terminal methionine is cleaved from the nascent polypeptide chain when it is still quite short (14, 15). Thus, ³⁵S-methionine from labeled initiator ³⁵S-met-tRNA_F is removed from the N-terminus of the nascent polypeptide chains almost immediately after it is incorporated and is not normally detected. If, however, the methionine on the initiator tRNA is formylated, the Fmet once incorporated N-terminally on the nascent chain is not cleaved and is readily detected (14, 27). Here, therefore, we have used Krebs cell initiator tRNA_F^{Met} loaded and formylated with enzymes from *E. coli* as a probe in our studies on initiation. The *E. coli* enzymes do not recognize the noninitiator mammalian methionine tRNA species (tRNA_M^{Met}) responsible for the donation of methionine into internal sites (12, and Materials and Methods). Moreover, the presence of the formyl group both prevents the proteolytic cleavage of the N-terminal (formyl) methionine and removes any possibility of incorporation of the methionine into internal sites in the polypeptide chain. Incubation of ³⁵S-methionine-labeled Fmet-tRNA_F with EMC RNA in the Krebs cell-free system provides a product which on digestion with trypsin yields a single major EMC-specific Fmet-peptide (23, 27). Further analysis of this peptide by Smith has shown it to be identical (with the addition of the formyl group) to the unique methionine initiation peptide formed in the translation of the entire EMC RNA genome in the intact EMC-infected cell (27). Thus, initiation of protein synthesis with Fmet-tRNA_F in response to EMC RNA in the Krebs cell-free system occurs at the same site as does initiation of EMC polypeptide synthesis in the intact cell.

N-terminal incorporation of ³⁵S-methionine labeled Fmet from Fmet-tRNA_F, as a measure of initiation, and a mixture of free ¹⁴C-amino acids or ³H-leucine, as an index of total synthesis, were compared for the systems under study here. With systems using material from interferon-treated, infected cells there was the usual inhibition of ¹⁴C-amino acid or ³H-leucine incorporation in response to EMC RNA, but no such inhibition in the incorporation of ³⁵S-Fmet was observed (Fig. 3). On occasion, with unfrac-

tionated systems showing a profound effect some inhibition (<50%) of Fmet incorporation did occur, but this was always much less than that observed with the free amino acids (≥80%).

Accepting that EMC-specific polypeptide chains initiated with Fmet-tRNA_F do escape the major inhibition one might, a priori, have expected to see a decrease in the inhibition of ³H-leucine incorporation in those systems receiving Fmet-tRNA_F as in the experiments shown in Fig. 3B. However, the added Fmet tRNA_F is in competition with endogenous met tRNA_F and only 0.01 to 0.05 pmol of Fmet are incorporated per 0.34 pmol of added EMC RNA. No accurate figure for the total number of EMC-specific polypeptide chains initiated in response to the added RNA is yet available (not all EMC RNA molecules need be active in this respect), but it is clearly possible that Fmet from the added Fmet-tRNA_F is only used in a small fraction of the initiation events (our calculations suggest a figure of no more than 15%). Accepting this, no significant decrease in the inhibition of ³H-leucine incorporation would be expected in the presence of Fmet-tRNA_F.

There are a number of possible explanations why incorporation of Fmet from Fmet-tRNA_F might escape inhibition. It could be, for example, that the major inhibition operates at the level of charging or breakdown of the initiator tRNA_F^{Met}. Thus, by adding ³⁵S-Fmet-tRNA_F one might already be beyond the site of inhibition in the sequence of events involved in initiation. Indeed, we have some preliminary evidence for a reduced ability to charge the methionine tRNAs in these systems, but on fractionation this does not appear to be responsible for the major inhibition observed (L. A. Ball, unpublished data). Nevertheless a reduced charging activity could result in a reduced pool of endogenous met-tRNA_F in the cell-free system. This, in turn, would result in an increase in the effective specific activity of the added ³⁵S-Fmet-tRNA_F in the inhibited system and a spuriously increased level of incorporation. The results obtained with AUG(U)₃₅ as message, however, argue against any such explanation.

Incorporation from Fmet-tRNA_F and met-tRNA_F in response to AUG(U)₃₅. When assayed with AUG(U)₃₅ as mRNA, incorporation of label from initiator tRNA_F^{Met} was inhibited under conditions where incorporation of Fmet in response to EMC RNA escaped inhibition (Table 1). This was true with AUG(U)₃₅ as message both for the formylated Fmet-tRNA_F and the nonformylated met-tRNA_F species, although in some experiments the inhibition with

A **B**

FIG. 3. Incorporation of ^{35}S -Fmet from ^{35}S -Fmet-tRNA_f in the cell-free system: absence of inhibition in extracts from interferon-treated, vaccinia virus-infected cells. A, Unfractionated extracts. The graph on the left shows the incorporation of a mixture of ^{14}C -amino acids, that on the right the incorporation in parallel assays of ^{35}S -Fmet from ^{35}S -Fmet tRNA_f with time at 30 C in the cell-free system. Interferon-treated, vaccinia virus-infected cell extracts plus $\bigcirc$ — $\bigcirc$ and minus $\bullet$ — $\bullet$ EMC RNA; vaccinia virus-infected cell extracts plus $\bigcirc$ — $\bigcirc$ and minus $\bullet$ — $\bullet$ EMC RNA. The symbols are the same for both graphs. B, Assays of L-cell sap with preincubated microsomes from normal Krebs cells. Here the incorporation in response to EMC RNA of free ^3H -leucine incorporation with vaccinia virus-infected cell extracts ($\bullet$) and interferon-treated, vaccinia virus-infected cell extracts (Δ). ^{35}S -Fmet incorporation with vaccinia virus-infected cell extracts ($\bigcirc$) and with interferon-treated, vaccinia virus-infected cell extracts (Δ). The assays were carried out as described in Materials and Methods with 0.25 μCi of ^{14}C -amino acid mix (50 mCi/mAtom of carbon) or 5 μCi of ^3H -leucine (52 Ci/mmol) plus ^{35}S -Fmet tRNA_f equivalent to 3×10^6 to 4×10^6 counts per min (4 pmol at 45 to 50 Ci/mmol).

TABLE 1. *Incorporation of ^{35}S -methionine from the initiator $\text{tRNA}_F^{\text{Met}}$ species in response to $\text{AUG}(\text{U})_{35}$ in cell-free systems from interferon-treated, infected cells^a*

Cell-free systems from	Incorporation in response to $\text{AUG}(\text{U})_{35}$ with		Incorporation in response to EMC RNA with	
	^{35}S -met- tRNA_F (counts/min)	^{35}S -Fmet- tRNA_F (counts/min)	^{35}S -Fmet- tRNA_F (counts/min)	^3H -phenylalanine (counts/min)
Vaccinia virus-infected cells	15,600	6,500	2,500	11,500
Interferon-treated, vaccinia virus-infected cells	4,400 (28%) ^b	2,200 (30%) ^b	2,000 (80%) ^b	2,050 (18%) ^b

^a Unfractionated postmitochondrial supernatant (S10) fractions were used throughout. Met-tRNA and Fmet-tRNA were from the same preparation and had essentially the same specific activity. Two μl of one or other of the ^{35}S -met-labeled initiator $\text{tRNA}_F^{\text{Met}}$ species (4×10^5 counts/min, 2.2 pmol) were added to each assay (25 μl of cells). All assays were carried out in duplicate under the conditions already described (Materials and Methods). Incorporation from the abnormal Fmet-tRNA was apparently less efficient than from the normal met-tRNA_F in accord with the results of Brown and Smith (3).

^b % of incorporation in the vaccinia-infected system.

the formylated species was marginally less. (With $\text{AUG}(\text{U})_{35}$ as message, nonformylated met-tRNA_F can be used since the N-terminal methionine is not cleaved from the relatively short nascent met(phe)_n oligopeptides [3].) Initiation with saturating amounts of $\text{AUG}(\text{U})_{35}$ is over more rapidly (5 to 10 min) in these systems than with EMC RNA (20 to 40 min, Fig. 3). However, control experiments involving the addition of these messengers progressively with time have shown no difference in the stability of functional Fmet and met-tRNA_F species in these systems. It seems unlikely, therefore, that the apparent side-stepping by Fmet of the inhibitory effect with EMC RNA (Fig. 3) simply reflects a reduction in the size of the endogenous met-tRNA_F pool (Table 1), or any difference in the relative stabilities of the normal endogenous (or added) initiator met-tRNA_F as compared with the formylated Fmet-tRNA_F species.

Characterization of the Fmet-labeled polypeptide products synthesized in response to EMC RNA in cell-free systems from interferon-treated, infected cells. An obvious possible alternative explanation for the escape of the Fmet incorporation from the major inhibition would be provided if, in these particular systems, initiation with Fmet occurred at an abnormal site on the EMC RNA genome. The Fmet-labeled polypeptide products synthesized in response to EMC RNA were, therefore, analyzed by electrophoresis on SDS polyacrylamide gels (Fig. 4). In addition, the major EMC-specific Fmet-labeled initiation peptide released on tryptic digestion of the polypeptide products was compared (Fig. 5) with that formed in response to EMC RNA in the Krebs cell-free system. This latter has been shown by Smith to be identical (plus the formyl group) to

that normally involved in the initiation of EMC polypeptide synthesis in the intact infected cell (27). From a comparison of the autoradiographs shown in Fig. 4 with those in Fig. 1 and 2 and previously (18), it is clear that ^{35}S -Fmet from Fmet-tRNA_F is incorporated predominantly into the same series of high-molecular-weight EMC-specific polypeptides as is free methionine. Double labeling experiments with ^{35}S -Fmet-tRNA_F and ^3H -leucine have confirmed this and similar results have been reported by Oberg and Shatkin for the Krebs cell-free system (23). The relatively greater amounts of radioactivity in the lower molecular weight material with ^{35}S -Fmet as label (compare Fig. 4 with Fig. 1 and 2) is consistent with incorporation of Fmet exclusively at the N-terminus, for over the molecular weight range of 25,000 to 130,000 observed here, the ratio of N-terminal Fmet per molecule to randomly distributed methionine or leucine would be expected to decrease by a factor of 5. Accurate quantitation in these systems, which seem to show less premature termination of translation of EMC RNA than is observed in the Krebs system, is complicated by the fact that some cleavage of nascent high-molecular-weight EMC precursor polypeptide may be occurring (R. M. Esteban and I. M. Kerr, manuscript in preparation). There is, in addition, a significant (e.g., Fig. 3A) background of endogenous incorporation in these nonpreincubated systems. Nevertheless, on complete digestion of these Fmet-labeled products with Pronase, $\geq 95\%$ of the label co-electrophoresed with Fmet and $< 5\%$ with methionine, confirming that at least 95% of the label incorporated is N-terminal. On tryptic digestion the same EMC-specific Fmet initiation peptide (A, Fig. 5A) was obtained with all of these systems

FIG. 4. Characterization of the ^{35}S -Fmet-labeled polypeptide products synthesized in response to EMC RNA with time in the cell-free system. A, Unfractionated extracts from vaccinia virus-infected (gels 1 to 4) and interferon-treated, vaccinia virus-infected (gels 5 to 8) cells were incubated in the presence of 8×10^6 counts per min of ^{35}S -Fmet-tRNA_F (equivalent to 100 pmol of ^{35}S -met at 45 Ci/mmol) and 20 μg of EMC RNA (7.4 pmol) per 0.5-ml assay. At 30 min (gels 1 and 5), 60 min (gels 2 and 6), 120 min (gels 3 and 7), and 240 min (gels 4 and 8) 0.1-ml samples were treated with SDS and subjected to electrophoresis on SDS-polyacrylamide gels in the presence of reovirus polypeptides as molecular weight markers. The figures to the right represent the molecular weights in thousands of the major reovirus polypeptides detailed in the legend to Fig. 1. Autoradiographs of the dried gels are shown. Incorporation of Fmet in the interferon-treated, infected cell system (gels 5 to 8) was inhibited to some extent (25 to 40%) in this experiment, but much less so than was the incorporation of ^3H -leucine and free ^{35}S -methionine (70 to 80%) assayed in parallel. B, L-cell sap from vaccinia-infected (gels 1 to 3) and interferon-treated, vaccinia-infected (gels 4 to 6) cells were incubated with preincubated microsomes from normal Krebs cells in the presence of 4×10^6 counts per min of ^{35}S -Fmet tRNA_F (equivalent to 50 pmol of ^{35}S -met at 45 Ci/mmol) and 20 μg of EMC RNA (7.4 pmoles) per 0.5-ml assay. At 30 min (gels 1 and 4), 60 min (gels 2 and 5), and 120 min (gels 3 and 6) 0.125-ml samples were treated with SDS and subjected to electrophoresis as in A. There was no significant inhibition (<10%) of incorporation of Fmet in this experiment whereas there was a 50% inhibition of incorporation of ^3H -leucine and of free ^{35}S -methionine assayed in parallel. In both A (240 min) and B (120 min) samples were taken and completely digested with trypsin then with Pronase for further electrophoretic analysis which showed that essentially all ($\geq 95\%$) of the radioactivity incorporated into polypeptides was recoverable as Fmet.

FIG. 5. *Fmet*-tryptic peptides formed in response to EMC RNA in Krebs and L-cell-free systems. The polypeptide products from the various cell-free systems labeled with ^{35}S -*Fmet*-tRNA_f or, in the case of band 3 in A, free ^{35}S -methionine, were digested with trypsin. A, Electrophoresis on thin-layer plates at pH 6.5. Samples (2 to 5 μl) were placed at 0 with the anode to the top of the figure. Krebs cell-free system plus (1) and minus (2) EMC RNA. (3) Krebs cell-free system plus EMC RNA with free ^{35}S -methionine. Vaccinia-infected L-cell system plus (4) and minus (5) EMC RNA. Interferon-treated, vaccinia-infected system plus (6) and minus (7) EMC RNA. The remaining assays were with preincubated microsomes from normal Krebs cells and vaccinia virus-infected cell sap plus (8) and minus (9) EMC RNA and interferon-treated, vaccinia virus-infected cell sap plus (10) and minus (11) EMC RNA. (B) Two-dimensional chromatography and electrophoresis on thin layer plates (6). The digests were placed at the bottom center of the sheets (arrow) and subjected to electrophoresis at pH 6.5 with the anode to the left. P.R. indicates the position to which a phenol red marker migrated during the electrophoresis. Chromatography was towards the top of the sheet. The results with the *Fmet*-tryptic peptides from all of the systems in Fig. 5A were essentially identical to the example from the Krebs cell system shown here. The same major *Fmet* peptide was detected on analysis of the product after 5, 10, and 120 min of incubation in the Krebs cell-free system. Experiments in which the digests labeled with *Fmet* were run separately and mixed with digests labeled with free methionine showed that the major *Fmet* peptide migrated slightly faster in both dimensions than the major peptide X (sample 3 in Fig. 5A) labeled with free methionine. The assays carried out as in Materials and Methods were scaled up to 0.3 ml for the Krebs cell system and 0.5 ml for the L- and Krebs microsome L-cell sap systems. ^{35}S -*Fmet*-tRNA_f equivalent to 4×10^6 counts per min (22.6 pmol of methionine at a specific activity of 100 Ci/mmol) was added to each assay. Incorporation in response to EMC RNA varied from 20,000 counts/min (0.11 pmol) for the Krebs system to 100,000 counts/min (0.55 pmol) for the mixed system.

(inhibited and control, unfractionated and fractionated cell sap with Krebs microsomes) as with the Krebs cell system (Fig. 5A). The minor Fmet-peptide (B, Fig. 5A) (usually <10% of the total radioactivity) is thought to be a further breakdown product of the major (23, 27). It seems reasonable to conclude that initiation with Fmet occurs at the normal site on EMC RNA and that the Fmet is incorporated into the normal spectrum of EMC-specific polypeptides in these systems. Thus, the ability of Fmet incorporation to escape the major inhibition is not the result of its incorporation at an abnormal site.

Comparing the results for the analyses of the Fmet N-terminally labeled polypeptide products synthesized at different times in the cell-free system using unfractionated (S10) material from interferon-treated and untreated, infected cells (Fig. 4A), the same bias away from the higher molecular weight products is seen in the interferon case as was noted for the incorporation of free amino acids (Fig. 1). Again, in agreement with the results in Fig. 2, there is no such bias in the systems in which corresponding cell sap fractions were assayed with Krebs microsomes (Fig. 4B). Thus, even though polypeptide chains initiated with Fmet escape the major inhibition early in translation, they are still subject to the minor inhibition affecting the accumulation of high molecular weight material in the unfractionated system.

DISCUSSION

It is impossible as yet to tell how the inhibition characterized here for systems from interferon-treated, vaccinia virus-infected cells, which apparently affects the translation of EMC RNA, AUG(U)₂₅ and mouse globin mRNA (I. M. Kerr et al., in press; Friedman unpublished data) but not of poly U (11), correlates with the effect we have seen in systems from interferon-treated, EMC-infected L-cells, which appears to show some specificity for different mRNAs (I. M. Kerr et al., in press; R. M. Esteban, D. R. Tovel, and I. M. Kerr, manuscript in preparation). Nor is it clear how this inhibition correlates with the much smaller effect of interferon treatment in the absence of infection that we have reported for these (11) and other systems (17), or with the large effect reported in the absence of infection by Falcoff et al. (9). The possibility exists that the inhibition of protein synthesis seen in the interferon-treated, vaccinia-virus-infected cells, is a secondary effect of the abortive replication of virus in these systems. Alternatively, it could repre-

sent the sum of a virus-mediated inhibition of host protein synthesis and an interferon-mediated inhibition of virus, rather than a blanket interferon-mediated inhibition of both. On the other hand, our preliminary analyses of these different systems have indicated points in common as well as apparent differences, suggesting that a common underlying mechanism may be involved. Moreover, the idea that infection, or other insult to the cell, may be required to trigger the full development of the interferon-mediated inhibition has its attractions. According to one variation on this hypothesis, there could be a switch-off of protein synthesis on infection of the interferon-treated cell during which time either the events leading to cell death are initiated, i.e., the cell dies, thus preventing the spread of infection, or the input viral nucleic acid is inactivated so that, after a period, normal host metabolism can resume. In many cases interferon pretreatment does not prevent cell death after infection, it merely reduces the virus yield and this hypothesis could provide the basis for further work should it turn out that there is no significant selection between host and viral mRNAs in the effect upon translation. This, however, is only one possibility; the results in the interferon-treated SV40-infected cell systems (24) strongly suggest a specific inhibition of viral functions, and the absence of selectivity for host and viral mRNA in the cell-free system has by no means been established. Globin mRNA, particularly in the form in which it is presented to the cell-free system may, for example, be atypical of host message in the intact cell. A much more detailed analysis of these cell-free systems and of the possible importance of the different methods used in their preparation and assay will be required before the relationships between them and their significance to interferon action can be resolved.

It is, however, with the further characterization of the enhanced inhibition of translation, triggered by infection in the interferon-treated, vaccinia-virus-infected cell system that we have been concerned here. Even if this inhibition is secondary to the primary event of interferon action (an event perhaps capable of affecting transcription and translation in different cell-virus systems in different ways), the absence of an effect of the translation of poly U (11) and on the incorporation of Fmet from Fmet-tRNA_F (Fig. 3) clearly indicates that a highly specific mechanism is involved which must be of interest in the control of protein synthesis. The results of the analysis of the polypeptide products synthesized in response to EMC RNA in cell-free systems of this type are consistent with

a major interferon-mediated inhibition of translation at, or shortly after initiation, with a minor secondary inhibition of chain elongation (Fig. 1 and 2). On the basis of this data alone it is possible that the major inhibition occurs shortly after initiation when the product is too small to be seen on acrylamide gels. It would be premature to exclude such a mechanism, but for it to be unique to an early event in chain elongation it seems likely that it would be closely linked to initiation, as indeed is suggested by the results obtained with the initiator Fmet-tRNA_F.

Fmet from Fmet-tRNA_F is incorporated N-terminally into the normal spectrum of high molecular weight polypeptides synthesized in response to EMC RNA in these systems (Fig. 4), but is present as a common N-terminal peptide (Fig. 5). This, therefore, provides further evidence in favour of the model that we (18) and others (2) have previously proposed for the translation of EMC RNA in the cell-free system. According to this model, initiation in the cell-free system occurs at a unique site on the EMC RNA as in the intact infected cell, the majority of the high molecular weight polypeptides being formed by premature termination of translation at different preferred sites (possibly of nucleolytic cleavage) on the large EMC RNA message.

The interesting aspect of the results obtained with Fmet-tRNA_F here, however, is that the incorporation of Fmet from it escapes the inhibition of translation seen in the interferon-treated, infected-cell system. The results with AUG(U)₃₅ as message (Table 1) and those concerning the stability of the charged initiator tRNAs argue against any simple explanation for this escape, such as would be involved if the inhibitory event affected the charging or breakdown of the normal initiator met-tRNA_F so that the inhibition preceded the reactions after the addition of the precharged Fmet-tRNA_F. Nor does the side-stepping of the inhibition by the Fmet-tRNA_F reflect initiation at an abnormal site (Fig. 5). The most likely explanation seems to be that the abnormal formyl group on the Fmet-tRNA is not recognized by the inhibitor. But if this were the whole answer one would not have expected the incorporation of Fmet from Fmet-tRNA_F to be inhibited with AUG(U)₃₅ as message (Table 1). There is evidence from work with the reticulocyte system, for example, that both with Fmet-tRNA_F (5) and with synthetic messages (4), the factor requirements for initiation are different from those observed with the nonformylated initiator met-tRNA_F or natural messages. This emphasizes, incidentally, the

fact that the inhibition of incorporation in response to AUG(U)₃₅ in these systems should not be taken as proof that all messages will be affected: if there are additional factors specifically required for the translation of host messages it is possible that they could provide the specificity not apparent here. Returning to the effect of Fmet-tRNA_F, however, there are, in fact, a number of possible explanations for this intriguing phenomenon which are currently under investigation. Meanwhile, taking these results together, they suggest that the presence of the abnormal formyl group on the initiator Fmet-tRNA_F allows a factor(s) involved in the initiation of translation of EMC RNA, at least, to escape recognition at the site of the major interferon-mediated inhibition of translation.

ACKNOWLEDGMENTS

We thank D. Risby, A. Douglas, and Susan O'Connor for technical assistance and J. A. Sonnabend for much useful discussion. R.M.F., National Cancer Institute staff member at the National Institutes of Health, was a visiting scientist. The interferon was obtained from The Microbiological Research Establishment, Porton, Wiltshire, England (C. J. Bradish) and from K. Paucker, and purified reovirus was the generous gift of J. J. Skehel.

LITERATURE CITED

1. Boime, I., H. Aviv, and P. Leder. 1971. Protein synthesis directed by encephalomyocarditis virus RNA. II. The *in vitro* synthesis of high-molecular-weight proteins and elements of the viral capsid. *Biochem. Biophys. Res. Commun.* **45**:788-795.
2. Boime, I., and P. Leder. 1972. Protein synthesis directed by encephalomyocarditis virus mRNA. III. Discrete polypeptides translated from a monocistronic messenger *in vitro*. *Arch. Biochem. Biophys.* **153**:706-713.
3. Brown, J. C., and A. E. Smith. 1970. Initiator codons in eukaryotes. *Nature (London)* **226**:610-612.
4. Crystal, R. G., and W. F. Anderson. 1972. Initiation of haemoglobin synthesis. Comparison of model reactions that use artificial templates with those using natural messenger RNA. *Proc. Nat. Acad. Sci. U.S.A.* **69**:706-711.
5. Crystal, R. G., A. W. Nienhuis, P. M. Prichard, D. Picciano, N. A. Elson, W. C. Merrick, H. Graf, D. A. Shafritz, D. G. Laycock, J. A. Last, and W. F. Anderson. 1972. Initiation of globin synthesis. *FEBS Lett.* **24**:310-314.
6. Dobos, P., I. M. Kerr, and E. M. Martin. 1971. The synthesis of capsid and non-capsid viral proteins in response to encephalomyocarditis virus ribonucleic acid in animal cell-free systems. *J. Virol.* **8**:491-499.
7. Drews, J., G. Högenauer, F. Unger, and R. Weil. 1971. Incorporation of methionine from met-tRNA_F^{Met} into internal positions of polypeptides by mouse liver polyosomes. *Biochem. Biophys. Res. Commun.* **43**:905-912.
8. Eggen, K. L., and A. J. Shatkin. 1972. *In vitro* translation of cardiomyovirus ribonucleic acid by mammalian cell-free extracts. *J. Virol.* **9**:636-645.
9. Falcoff, E., R. Falcoff, B. Lebleu, and M. Revel. 1972. Interferon treatment inhibits mengo RNA and haemoglobin mRNA translation in cell-free extracts of L cells. *Nature. N. Biol. (London)* **240**:145-147.

10. Friedman, R. M., R. M. Esteban, D. H. Metz, D. R. Tovell, I. M. Kerr, and R. Williamson. 1972. Translation of RNA by L cell extracts: effect of interferon. *FEBS Lett.* **24**:273-277.
11. Friedman, R. M., D. H. Metz, R. M. Esteban, D. R. Tovell, L. A. Ball, and I. M. Kerr. 1972. Mechanism of interferon action: inhibition of viral messenger ribonucleic acid translation in L-cell extracts. *J. Virol.* **10**:1184-1198.
12. Gupta, N. K., N. K. Chatterjee, K. K. Bose, S. Bhaduri, and A. Chung. 1970. Roles of methionine transfer RNAs in protein synthesis in rabbit reticulocytes. *J. Mol. Biol.* **54**:145-154.
13. Gupta, N. K., N. K. Chatterjee, C. L. Woodley, and K. K. Bose. 1971. Protein synthesis in rabbit reticulocytes: factors controlling internal and terminal methionine codon recognition by the methionyl tRNA species. *J. Biol. Chem.* **246**:7460-7469.
14. Housman, D., M. Jacobs-Lorena, U. L. Rajbhandary, and H. F. Lodish. 1970. Initiation of haemoglobin synthesis by methionyl-tRNA. *Nature (London)* **227**:913-918.
15. Hunter, A. R., and R. J. Jackson. 1971. The origin and nature of the methionine residue initiating the synthesis of haemoglobin *in vivo* and *in vitro*. *Eur. J. Biochem.* **19**:316-322.
16. Joklik, W. K., and T. C. Merigan. 1966. Concerning the mechanism of action of interferon. *Proc. Nat. Acad. Sci. U.S.A.* **56**:558-565.
17. Kerr, I. M. 1971. Protein synthesis in cell-free systems; an effect of interferon. *J. Virol.* **7**:448-459.
18. Kerr, I. M., R. E. Brown, and D. R. Tovell. 1972. Characterization of the polypeptides formed in response to encephalomyocarditis virus ribonucleic acid and in a cell-free system from mouse ascites tumor cells. *J. Virol.* **10**:73-81.
19. Kerr, I. M., N. Cohen, and T. S. Work. 1966. Factors controlling amino acid incorporation by ribosomes from Krebs 2 mouse ascites tumour cells. *Biochem. J.* **98**:826-835.
20. Kerr, I. M., and E. M. Martin. 1972. Simple method for the isolation of encephalomyocarditis virus ribonucleic acid. *J. Virol.* **9**:559-561.
21. Metz, D. H., and M. Esteban. 1972. Interferon inhibits viral protein synthesis in L-cells infected with vaccinia virus. *Nature (London)* **238**:385-388.
22. Muench, K., and P. Berg. 1966. Preparation of aminoacyl ribonucleic acid synthetases from *Escherichia coli*, p. 375-383. In G. L. Cantoni and D. R. Davies (ed.), *Procedures in nucleic acid research*. Harper and Row, London.
23. Öberg, B. O., and A. J. Shatkin. 1972. Initiation of picornavirus protein synthesis in ascites cell extracts. *Proc. Nat. Acad. Sci. U.S.A.* **69**:3589-3593.
24. Oxman, M. N. 1973. Interferon, tumors and tumor viruses, p. 391-479. In N. B. Finter (ed.), *Interferon and interferon inducers*, 2nd ed. North Holland Publishing Company, Amsterdam.
25. Paucker, K., B. J. Berman, R. R. Golgher, and D. Stancek. 1970. Purification, characterization and attempts at isotopic labeling of mouse interferon. *J. Virol.* **5**:145-152.
26. Rabinowitz, J. C. 1963. Folic acid p. 185-252. In P. D. Boyer, K. Lardy, and K. Myrback (ed.), *The enzymes* 2nd ed. 2. Academic Press Inc., London.
27. Smith, A. E. 1973. The initiation of protein synthesis directed by the RNA from encephalomyocarditis virus. *Eur. J. Biochem.* **33**:301-313.
28. Smith, A. E., and K. A. Marker. 1970. Cytoplasmic methionine transfer RNAs from eukaryotes. *Nature (London)* **226**:607-610.
29. Smith, R. E., H. J. Zweerink, and W. K. Joklik. 1969. Polypeptide components of virions, top component and cores of reovirus type 3. *Virology* **39**:791-810.
30. Sundararajan, T. A., and R. E. Thach. 1966. Role of the formylmethionine codon AUG in phasing translation of synthetic messenger RNA. *J. Mol. Biol.* **19**:74-90.
31. Von Ehrenstein, G. 1967. Isolation of sRNA from intact *E. coli* cells. p. 588-592. In L. Grossman and K. Moldave (ed.), *Methods in enzymology*, vol. 12, part A. Academic Press Inc., London.
32. Waters, L. C., and G. D. Novelli. 1971. Analytical reversed-phase chromatography of *Escherichia coli* aminoacyl-tRNAs, p. 39-41. In K. Moldave and L. Grossman (ed.), *Methods in enzymology*, vol. 20, part C. Academic Press Inc., London.