Protein Synthesis in Cell-Free Systems: an Effect of Interferon

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Received for publication 13 October 1970

The activity of ribosome and cell-sap fractions from interferon-treated and control chick embryo fibroblasts was compared in mixed chick-mouse and purely chick cell-free systems capable of the synthesis of viral polypeptide(s) in response to viral ribonucleic acid (RNA). Interferon treatment of cells did not affect the intrinsic amino acid incorporation activity of these systems or their response to polyuridylic acid. With encephalomyocarditis (EMC) virus RNA as messenger, however, a fraction of the ribosomes from interferon-treated cells appeared less active than parallel controls. The results obtained with the corresponding cell-sap fractions were variable. Although competition between endogenous and added messengers cannot be excluded in these systems, a reduced level of translation of EMC RNA with interferon-treated cell ribosomes was also suggested by the results of analyses of tryptic digests of the products formed in response to the RNA. In addition, these analyses showed that this reduced activity must reflect a reduction in the rate or frequency of translation rather than a decrease in the length of the EMC RNA translated, for the same polypeptides were synthesized in response to the RNA with material from interferon-treated and control cells. Interferon added directly to the cell-free system was without effect. Although suggestive, these results do not provide definitive evidence for or against the hypothesis that virus protein synthesis is inhibited at the translational level in the interferon-treated cell. Possible alternative interpretations of the data are discussed.

Early work on interferon led to the suggestion that it acts by inducing the cell to synthesize a new protein which inhibits the translation of viral, but not of host-cell, messenger ribonucleic acid (mRNA) (reviewed in 19 and 20). The data obtained by Joklik and Merigan and Levy and Carter in their studies on the fate of viral mRNA in the intact cell are in accord with such a mechanism (6, 10). Further evidence in favor of this mechanism was provided by experiments with cell-free systems. Marcus and Salb concluded that ribosomes from interferon-treated cells would combine with, but not translate, viral RNA (11, 12), whereas Carter and Levy reported that such ribosomes neither bound viral RNA nor incorporated amino acids in response to it (1, 2).

Our own results using very similar systems were in marked contrast to those of Marcus and Salb (11, 12). We found no quantitative correlation between the formation and breakdown of viral RNA-ribosome complexes in the cell-free system and the messenger function of the RNA in protein synthesis (9). A reappraisal of their binding studies has led Marcus and Salb to the

same conclusion (P. I. Marcus, *personal communication*). Meanwhile animal cell-free systems capable of synthesizing encephalomyocarditis (EMC) virus-specific proteins in response to EMC RNA have been developed (7, 18). The accompanying paper (8) describes an analysis of the virus-specific polypeptide products synthesized in response to this RNA in ribosome-cell-sap systems from chick and Krebs 2 mouse ascites tumor cells.

The Krebs cell is insensitive to interferon and interferon inducers (E. M. Martin, *unpublished results*). Accordingly, chick embryo fibroblasts (CEF) were used for the experiments with interferon. In the first part of this paper, the response to EMC RNA of ribosomes from interferon-treated and control cells was compared in mixed assay systems using chick ribosomes and Krebs cell sap. A comparison was also made of the polypeptides formed in response to the RNA in these systems and of the binding of labeled RNA to the two types of ribosome. The second section of the paper deals similarly with the activity of cell-sap preparations from interferon-treated and control cells. Finally, the response to EMC RNA

of completely CEF systems from the treated and control cells is described. The results are in accord with there being an inhibition of the translation of viral RNA in the interferon-treated cell, but alternative interpretations cannot be excluded and their significance is discussed.

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 as described before (7) or in the accompanying paper (8).

Interferon. The partially-purified (100- to 1,000-fold) chick interferon, of the type described previously (4, 9) which was used for the treatment of cells, and the more highly-purified (approximately 5,000-fold) material (4), which was used for addition to the cell-free systems, were the generous gifts of Karl Fantes (Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Bucks., England). The treatment of cells with interferon, its assay, and assay of the effectiveness of the interferon treatment have already been described (9). In all cases discussed here, the interferon treatment reduced the yield of Semliki Forest virus, which was used as the challenge virus throughout these studies, by greater than 99%.

Amino acid incorporation assays and analysis of the product synthesized in the cell-free system. Ribosome and cell-sap fractions were prepared and assayed in the cell-free system, and the product synthesized was analyzed as described in the accompanying paper (8).

³H-EMC RNA. Krebs 2 mouse ascites tumor cells were harvested, washed, and suspended at 2×10^7 cells/ml in Earle's medium. The cells were infected with EMC at an added multiplicity of 5 to 10 plaque-forming units/cell. Actinomycin D (Merck & Co. Inc., Rahway, N.J.) was added to 3 μ g/ml and the culture was incubated as described previously (13). After 2 hr at 37°C, 200 μ Ci each of ³H-uridine (5 Ci/mmol) and ³H-adenosine (4.65 Ci/mmol) were added per 100 ml of culture. Virus was harvested at 16 hr and purified as described in the accompanying paper (8). The RNA was extracted as previously described (7). Typically, 100 μ g of RNA of specific activity 5,000 counts per min per μ g was obtained from 400 ml of culture. On occasion, the RNA was diluted with unlabeled RNA prior to its use in the cell-free system.

Assay of the interaction of ³H-EMC RNA with ribosomes in the cell-free system. The interaction of ³H-EMC RNA with ribosomes was studied with cell-free systems under exactly the conditions used for the routine assay of amino acid incorporation in response to the RNA (8), scaled down to a total volume of 0.05 ml. Typically, in the full system 2.0 μ g of ³H-EMC RNA (4,000 counts per min per μ g) was added to 25 μ g of "74S" CEF ribosomes in the presence of 200 μ g of cell-sap protein and 0.31 μ Ci of a mixture of ¹⁴C-L-amino acids (Radiochemical Centre, Amersham, Bucks., England, catalogue no. CFB104). Analysis of the ³H-EMC RNA bound to ribosomes and of the fate of the ¹⁴C-amino acids in-

corporated into protein was on 30-ml 7.5 to 45% (w/v) sucrose gradients in 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6), 10 mM KCl, 1.5 mM MgCl₂. Centrifugation was in the 30 rotor of the Spinco model L centrifuge for 90 min at 65,000 $\times$ g unless otherwise stated. Fractions (1.5 ml) were collected by insertion of a tube to the bottom of the gradients which were then pumped out using a Varioperpex peristaltic pump (LKB-Produkter, Bromma 1, Sweden). Fractions from a marker gradient containing only "74S" ribosomes were monitored for material absorbing light at 260 nm. RNA and protein were precipitated by the addition of 0.3 N trichloroacetic acid at 0°C, collected on Oxoid membrane filters (Grade 0.45; Oxo Ltd., London, England), washed three times with 5.0-ml batches of 0.3 N trichloroacetic acid at 0°C, once each with ethyl alcohol, ethyl alcohol-ether [1:1 (v/v)], and ether, and dried. After addition of scintillator [4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(4-methyl-5'-phenyloxazolyl) benzene per liter of toluene], the membranes were assayed for ³H and ¹⁴C in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Control experiments with unlabeled EMC RNA, in which the recovery of ¹⁴C radioactivity using this procedure was compared with that involving a prior digestion of the cell-free system with alkali, indicated that less than 5% of the ¹⁴C-amino acids recovered here could be bound to tRNA rather than incorporated into protein.

RESULTS

The response of ribosomes from interferon-treated and control CEF to EMC RNA. The characteristics of the chick and mixed chick-Krebs cell-free systems and their stimulation by EMC RNA are described in the accompanying paper (8). The CEF ribosomes routinely used throughout these studies were a "74S" fraction (including ribosome subunits and monomers and a few residual dimers) isolated from the total microsome fraction without deoxycholate (DOC) treatment. Treatment with DOC in the presence or absence of cell sap, although it had no effect on intrinsic activity or the ability of the ribosomes to respond to polyuridylic acid, markedly reduced their response to EMC RNA [Fig. 2 accompanying paper (8)].

The response to EMC RNA of such ribosomes from interferon-treated and control cells, assayed in the presence of Krebs cell sap, is shown in Fig. 1. The total amino acid incorporation in response to EMC RNA with ribosomes from cells exposed to 35 or 140 units of interferon per ml was 60% and 30%, respectively, of the corresponding controls. Rates of incorporation were also measured, from which it can be seen that EMC RNA both stimulates the initial rate of incorporation and prolongs the period over

FIG. 1. Response to encephalomyocarditis (EMC) RNA of ribosomes from interferon-treated and control cells. Stimulation of amino acid incorporation by EMC RNA with untreated "74S" ribosomes from control chick embryo fibroblasts (CEF, ■) and CEF which had been exposed to 35 (●) and 140 (▲) units of interferon per ml was assayed in the presence of Krebs cell sap and a mixture of ^{14}C -L-amino acids. Incubations were for 40 min at 37°C as described under Methods in the accompanying paper (8).

which it occurs (Fig. 2). The rate of incorporation in response to EMC RNA, however, is clearly slower with ribosomes from interferon-treated cells.

On occasion a nonspecific reduction in the intrinsic amino acid incorporation activity of ribosomes in the absence of added EMC RNA was observed with material from cells exposed to some preparations of partially-purified interferon. These proved cytopathic on prolonged exposure of cells to high concentrations (500 units/ml for 48 hr at 37°C). The interferon used here, however, was not cytopathic under these latter conditions and the intrinsic activities of the "74S" ribosome, total microsome, total ribosome, and polysome fractions from the interferon-treated cells were each identical with corresponding controls. Moreover, treatment of Krebs cells for 24 hr at 37°C with 50 to 100 units of the same chick interferon per ml (specific activity 10,000 units/mg) or of chick cells with the same interferon after heat inactivation had no effect on the ability of ribosomes subsequently isolated from these cells to respond to EMC RNA. Besides, the effect of interferon treatment of the

chick cells on the ribosomes was the same at the same interferon concentration for interferon preparations of 10-fold different purity (specific activity 10,000 to 100,000 units/mg). Finally, in contrast to the results with EMC RNA, there was no difference in the response of the "74S" ribosomes from interferon-treated and control cells to polyuridylic acid (Table 1). Thus, there was no evidence for a nonspecific effect of the interferon, and the difference in activity of the ribosomes appeared to relate only to their ability to

FIG. 2. Kinetics of amino acid incorporation in response to encephalomyocarditis (EMC) RNA. Untreated "74S" ribosomes from chick embryo fibroblasts were assayed in the presence of Krebs cell sap and a mixture of ^{14}C -L-amino acids (8). (A) Control ribosomes in the presence (●) and absence (○) of EMC RNA (5 μg). (B) Time course of net EMC RNA-stimulated incorporation. Figures shown in this graph were obtained from the data presented in (A) by subtracting the value for amino acid incorporation in the absence from that in the presence of EMC RNA for each time point. Control ribosomes (●); interferon ribosomes (■).

TABLE 1. *Response to polyuridylic acid (poly U) of ribosomes from interferon-treated and control chick embryo fibroblasts*

Poly U (μ g)	¹⁴ C-phenylalanine incorporation ^a by ribosomes ^b from	
	Control cells	Interferon-treated cells
0	280	210
5	1,010	970
7.5	2,420	1,470
10	3,870	3,330
12.5	5,100	5,400
15	6,780	6,760

^a Expressed as counts per minute per 100 μ g of ribosomes. The assays were scaled up to 0.2 ml in the presence of 100 μ g of ribosomes, 1.0 mg of Krebs cell-sap protein, 15 mM MgCl₂, and 0.33 μ Ci of ¹⁴C-L-phenylalanine (495 Ci/mole). The other 19 amino acids were omitted.

^b Untreated "74S" ribosomes from control cells and cells which had been exposed to 35 units/ml of interferon for 17 to 24 hr at 37 C.

translate EMC RNA. The products synthesized in response to this RNA with the two types of ribosome were, therefore, compared.

Nature of the product(s) formed in response to EMC RNA with ribosomes from interferon-treated and control cells. The "74S" ribosomes from interferon-treated and control CEF were assayed with cell sap from Krebs cells in the presence and absence of EMC RNA. Autoradiographs of the peptide maps of tryptic digests of the ³⁵S-methionine labeled products formed are shown in Fig. 3. It is clear that EMC RNA stimulates amino acid incorporation into peptides (Fig. 3B), which are not detectable in the minus RNA controls (Fig. 3A). The distribution of these EMC RNA-stimulated peptides was qualitatively identical for systems with ribosomes from interferon-treated or control cells (Fig. 3B and C). In fact, the total radioactivity in the EMC-stimulated peptides appeared surprisingly similar for the two systems, despite the lower total stimulation with ribosomes from interferon-treated cells. The question arose, therefore, as to whether the absolute amounts of EMC RNA-stimulated incorporation were in fact different in the two systems or whether the same response to RNA was being observed against differentially reduced levels of intrinsic incorporation, the reduction presumably resulting from a competition between EMC RNA and endogenous mRNA.

To try to answer this question tryptic digests from interferon-treated and control ribosome systems were electrophoresed on paper in a single dimension at pH 6.5. The paper was dried, cut

into sections (1 by 2 cm) and assayed for radioactivity in a scintillation counter. The distribution of radioactivity (corrected for the total radioactivity recovered) for a control system and for interferon and control systems in the presence of EMC RNA is shown in Fig. 4. In the absence of EMC RNA only the neutral and basic peptides were sufficiently radioactive to be detected, whereas a considerable proportion of the label from the EMC RNA-stimulated systems was in acidic peptides. Accordingly, the difference in incorporation into this acidic, EMC-specific, peptide region for the interferon and control ribosome systems (Fig. 4) would suggest that one is not in fact observing the same response to EMC RNA against a differentially reduced background, but a truly reduced level of translation of the viral RNA in the interferon ribosome system. This is not, of course, to rule out the possibility that competition between messengers is also occurring. Good quantitative evidence for such competition has been provided by similar studies of the response to EMC RNA of cell-free systems using ribosome and cell-sap fractions both derived from CEF (Kerr, *unpublished results*).

Binding of EMC RNA to ribosomes from interferon-treated and control cells. Assuming that EMC RNA is functioning as an mRNA for viral protein synthesis in these systems, one of the simplest explanations for the quantitative difference in the response to the RNA would be a corresponding quantitative difference in the amount of the RNA bound to the ribosomes. Both Marcus and Salb and Carter and Levy (1, 11, 12) have reported such differences. Accordingly, the formation and breakdown of ³H-EMC RNA-ribosome complexes were examined in these systems. Sufficient radioactive RNA was used for stimulation of ¹⁴C-amino acid incorporation to be studied in parallel in the same systems. As an additional control the formation and breakdown of DOC-treated ribosome-³H-EMC RNA complexes were studied. These ribosomes retain their intrinsic activity and ability to respond to polyuridylic acid (9) but show a much reduced response to EMC RNA [Fig. 2 of accompanying paper (8)]. The results of these various studies in which at the end of incubation the cell-free systems were analyzed on sucrose gradients are presented in Fig. 5 to 7.

On incubation at 0 C in the cell-free system, ³H-EMC RNA does not bind to "74S" CEF ribosomes in the absence of cell sap but does bind to cell-sap protein in the absence of ribosomes, yielding a rather heterogeneous pattern on fractionation on a sucrose gradient (Fig. 5A). This is quite distinct, however, from the pattern of com-

FIG. 3. Comparison of tryptic digests of the product formed in response to encephalomyocarditis (EMC) RNA by ribosomes from interferon-treated and control cells. Untreated "74S" ribosomes from control chick embryo fibroblasts (CEF) and from CEF which had been treated with 35 units of interferon per ml were incubated for 40 min at 37 C with ^{35}S -methionine and Krebs cell sap in the presence or absence of EMC

FIG. 4. A quantitative comparison of tryptic digests of the products formed in response to encephalomyocarditis (EMC) RNA by ribosomes from interferon-treated and control cells. ^{35}S -methionine-labeled tryptic peptides from digests prepared as for Fig. 3 were electrophoresed on paper at pH 6.5 for 27 min at 4 kv and the distribution of radioactivity estimated as described in the text. Digests were of control ribosome systems with (Δ) and without ($\bullet$) added EMC RNA and of an interferon ribosome system with EMC RNA ($\circ$). Results obtained with ribosomes from interferon-treated cells assayed without EMC RNA were very similar to those for control ribosomes without EMC RNA. The same total radioactivity (3,000 counts/min) was loaded in each case. The results are corrected for recoveries of 70 to 100% during electrophoresis and to total values in proportion to those obtained for incorporation in the original cell-free systems, i.e., 3,000 counts/min in the absence of EMC RNA and of 4,350 and 5,550 counts/min, respectively, for the EMC RNA-stimulated interferon and control ribosome systems.

plexes formed on incubation of the RNA at 0 C in the full cell-free system, when the majority of the RNA appears to bind to the 74S monomers, irrespective of the source of the ribosomes or cell sap or whether or not the ribosomes have been

RNA (8). Tryptic peptides were prepared and analyzed by two-dimensional chromatography and electrophoresis as detailed in the accompanying paper (8). The load was 12,000 counts/min in each case, and the autoradiographs were developed after 180 days. A, control ribosomes; B, control ribosomes plus EMC RNA; C, interferon ribosomes plus EMC RNA. As presented, chromatography was from an origin near the bottom center of each fingerprint and electrophoresis was in the plane from left to right with the anode to the left. The position to which a phenol red marker migrated during the electrophoresis is indicated with the letters PR.

FIG. 5. Binding of ^3H -encephalomyocarditis (EMC) RNA to ribosome and cell-sap fractions in the cell-free system. ^3H -EMC RNA was incubated at 0 C in chick embryo fibroblast (CEF) ribosome-Krebs cell-sap systems and subsequently analyzed on sucrose density gradients centrifuged for 180 min (A) and 90 min (B) as described under Methods. Sedimentation was from right to left. Separate gradients were run in parallel with 74S ribosomes (80 μg) as an optical density (O.D.) marker (.....). (A) ^3H -EMC RNA (Δ); ^3H -EMC RNA in the full cell-free system minus cell sap ($\circ$) or minus ribosomes ($\times$). (B) ^3H -EMC RNA in the full cell-free system with untreated "74S" ribosomes from control (Δ) and interferon-treated ($\circ$) CEF and with deoxycholate-treated ($\square$) "74S" ribosomes from control CEF. Radioactivity in the pellets from these gradients (^3H) and those in Fig. 6, 7, and 9 (^3H or ^{14}C) was always less than 10% of the total radioactivity (^3H or ^{14}C) recovered from the gradients.

treated with DOC (Fig. 5B and 9A). On incubation at 37 C there was a rapid release or breakdown, or both, of the majority of the RNA bound to ribosomes in these systems. The results obtained with untreated ribosomes from interferon-treated and control cells assayed with cell sap from Krebs cells are shown in Fig. 6A and 7A. There was no significant difference between these two systems in the breakdown of the ribosome-RNA complexes on incubation for either 7.5 or

15 min at 37 C. Despite the rapid release and breakdown of a large portion of the RNA, amino acid incorporation was stimulated in response to it. This can be seen from Fig. 6B and 7B which show the distribution of the ^{14}C -amino acids incorporated into protein in the same assays. Clearly, in the presence of ^3H -EMC RNA there was an increased incorporation of ^{14}C -amino acids in both the 74S monomer (nascent polypeptide) and soluble cell-sap regions of the gradient at

FIG. 6. Fate of ^3H -encephalomyocarditis (EMC) RNA and of the ^{14}C -amino acids incorporated in cell-free systems with ribosomes from control cells. ^3H -EMC RNA was incubated with untreated "74S" ribosomes from control chick embryo fibroblasts in the presence of Krebs cell sap and a mixture of ^{14}C -amino acids (Methods). After incubation the systems were analyzed on sucrose gradients (Methods). Sedimentation was from right to left. A separate gradient was run in parallel with 74S ribosomes (80 μg) as a sedimentation marker. (A) ^3H -EMC RNA from systems incubated for 15 min at 0 C ($\circ$) and 7.5 ($\square$) and 15 (Δ) min at 37 C. (B) ^{14}C -amino acid incorporation after 7.5 (Δ) and 15 ($\circ$) min at 37 C in the presence of ^3H -EMC RNA and after 7.5 ($\blacktriangle$) and 15 ($\bullet$) min at 37 C in its absence.

FIG. 7. Fate of ^3H -encephalomyocarditis (EMC) RNA and of the ^{14}C -amino acids incorporated in cell-free systems with ribosomes from interferon-treated cells. Procedure was as for Fig. 6 except that the ribosomes were from chick embryo fibroblasts which had been exposed to 35 units of interferon per ml. (A) ^3H -EMC RNA from systems incubated for 15 min at 0°C (●) and 7.5 (■) and 15 (▲) min at 37°C. (B) ^{14}C -amino acid incorporation after 7.5 (▲) and 15 min (■) at 37°C in the presence of ^3H -EMC RNA.

both time points. In fact, rather oddly, the ratio of counts incorporated in the ribosome and soluble fraction remained constant at 70% soluble to 30% monomer-associated at 7.5, 15, and 40 min in both systems plus or minus added RNA.

Putting the results for the fate of the ^3H -EMC RNA and those for the incorporation of ^{14}C -amino acid together, and assuming that EMC RNA is indeed functioning as a mRNA in these systems, it can be concluded that only a small fraction (<15%) of the RNA originally bound to ribosomes at 0°C is functioning in this way at any given time. For example, only about 10% of the total RNA recovered from the systems after incubation at 0°C still sediments at 74S or greater after 7.5 min at 37°C and only 2 to 5% after 15

min. In the absence of any real indication as to how much of the "release" of the radioactive RNA at 37°C represents dissociation of molecules from the ribosome and how much nucleolytic cleavage, however, it would seem premature to attempt any further analysis of the size and behavior of this fraction in these systems. It is none the less clear that there is no straight-forward quantitative correlation either between the amount of RNA bound to ribosomes in these systems at 0°C or the breakdown of the RNA-ribosome complexes on incubation at 37°C and the functioning of the RNA in protein synthesis. It is also clear that the difference in the response to EMC RNA of ribosomes from interferon-treated and control cells does not result from a gross change in the binding of the viral RNA to the ribosomes as measured at 0°C in these systems.

The activity of cell-sap fractions from interferon-treated and control cells. Cell-sap fractions from interferon-treated and control CEF are equally capable of supporting endogenous or polyuridylic acid-stimulated incorporation by ribosomes from interferon-treated or control cells (9). It was of interest to see if there is any difference in their ability to support EMC RNA-stimulated incorporation. When assayed with preincubated Krebs cell ribosomes, control CEF cell sap supported a consistent two- to threefold stimulation of incorporation by EMC RNA (Fig. 2, accompanying paper (8)). The results obtained with cell-sap preparations from three different batches of interferon-treated cells, however, were variable. Typical results are presented in experiments 1 and 2 of Table 2. In the first experiment the response to EMC RNA with cell sap from cells exposed to 35 or 140 units of interferon per ml was the same as that observed with control cell sap. The alternative type of result, with interferon cell sap supporting about 60% of the response of corresponding controls, is presented in experiment 2 (Table 2). No similar variation in the results obtained from assay to assay, or even within the same set of assays for a given cell fraction, has been obtained with any of the other Krebs or chick cell fractions. The reason for this unusual variability is not known.

Nature of the products formed in response to EMC RNA in the presence of cell sap from interferon-treated and control cells. Tryptic digests of the ^{35}S -methionine-labeled polypeptide products formed by Krebs cell ribosomes in response to EMC RNA in the presence of cell sap from interferon-treated and control cells are compared in Fig. 8. There was no obvious qualitative difference in the nature of the products synthesized in the two systems in response to the RNA. In the absence of added RNA, as with the minus EMC

TABLE 2. Ability of cell sap from interferon-treated and control cells to support EMC RNA-stimulated incorporation in the cell-free system

Experiment ^a	EMC RNA (μ g)	¹⁴ C-amino acid incorporation ^b with	
		Control cell sap	Interferon cell sap ^c
1	0	5,250	5,200 (4,000)
	1	7,100	8,100 (7,650)
	2	9,300	8,650 (9,200)
	4	9,300	9,950 (9,000)
2	0	5,250	5,500
	0.5	8,200	7,700
	1	10,400	8,700
	2	8,600	7,820
	4	9,000	7,600

^a Cell-sap preparations used in these two experiments were prepared on separate occasions from different batches of chick embryo fibroblasts. The interferon and control preparations were from interferon-treated and untreated portions of the same batch of cells.

^b Expressed as counts per minute per 100 μ g of ribosomes. The cell-sap preparations were assayed with preincubated Krebs cell ribosomes in the routine system described in the accompanying paper (8).

^c Interferon cell sap was from cells which had been exposed to 35 units/ml or, in the case of the figures in brackets, to 140 units of interferon per ml for 17 to 24 hr at 37 C.

RNA control shown in Fig. 3A, incorporation was into a large number of different peptides and none was present in sufficient quantity to give rise to a discrete spot on autoradiography. It should be emphasized that in this experiment there was a clear quantitative difference in the response to EMC RNA observed with the two different cell-sap preparations. Incorporation of ³⁵S-methionine in the presence of control cell sap plus and minus EMC RNA was 74,000 and 28,000 counts per min, whereas with cell sap from interferon-treated cells the corresponding figures were 54,000 and 28,000, respectively. In order to compensate for this quantitative difference and underline the similarity of the product, in Fig. 8 the autoradiogram for the system in which cell sap from interferon-treated cells was used was exposed for a longer period of time than the control. With fingerprints exposed for the same period of time, the EMC RNA-stimulated spots in this system were fainter than the corresponding controls. This would indicate that there was a reduced level of translation of the EMC RNA in this system, irrespective of whether or not competition occurred between the endogenous messenger and added viral RNA.

Response to EMC RNA of cell-free systems derived entirely from CEF and the nature of the products formed. So far the response to EMC RNA of mixed systems only has been described.

FIG. 8. Comparison of the product(s) formed in the cell-free system in response to encephalomyocarditis (EMC) RNA in the presence of cell sap from interferon-treated and control cells. Preincubated Krebs cell ribosomes were assayed with ³⁵S-methionine and EMC RNA in the presence of cell sap from control chick embryo fibroblasts (CEF; A) and CEF exposed to 35 units of interferon per ml (B). Tryptic peptides were prepared and analyzed by two-dimensional chromatography and electrophoresis (8). Loads were 48,000 and 38,000 counts/min, and exposure was for 26 and 97 days, respectively, for the control and interferon cell-sap systems. (Allowing for the half-life of ³⁵S-methionine, the different extent of stimulation by EMC RNA and the different exposure times, it can be calculated that the total exposure to net EMC RNA-stimulated radioactivity in the control system was 70% of that in the system using cell sap from interferon-treated cells). Chromatography and electrophoresis were as in Fig. 3. The position to which a phenol red marker migrated during electrophoresis is indicated with the letters PR.

With cell-free systems derived entirely from CEF, the stimulation of amino acid incorporation in response to EMC RNA is much smaller [Fig. 2, accompanying paper (8)]. Nevertheless, it seems likely that translation of EMC RNA, in competition with that of endogenous messenger, is occurring in these chick systems (8). Accordingly, the response to EMC RNA of systems from interferon-treated and control cells was compared and typical results are presented in Table 3. Control experiments indicated that there was no difference in the size of the cold amino acid pools in the two cell-sap preparations. In the two experiments shown (Table 3), the intrinsic levels of incorporation in the absence of added RNA were 10,000 and 10,200 counts/min for the control systems and 8,700 and 11,300 for the systems from interferon-treated cells. The values given in Table 3, therefore, represent relatively small differences between large numbers which may account for their variability. Despite this variability, the response to EMC RNA of the systems

from interferon-treated cells never exceeded that of the control systems. This is typical and, in general, it can be said that the response to EMC RNA of such systems is never more and usually less than that of corresponding controls.

It seemed possible that the relatively poor response of chick systems to EMC RNA might reflect a more rapid destruction of the RNA by nuclease. The results of an experiment in which the fate of ^3H -EMC RNA was followed on incubation in the completely CEF cell-free system would argue against this, however (Fig. 9). After incubation at 0°C, on sucrose density gradient analysis the majority of the ^3H -EMC RNA sedimented with the 74S ribosome monomers. As with the mixed systems (Fig. 6 and 7), on

TABLE 3. Stimulation of amino acid incorporation by EMC RNA in cell-free systems from interferon-treated and control chick embryo fibroblasts

Experiment ^a	EMC RNA (μg)	Net EMC RNA-stimulated incorporation ^b in cell-free systems from	
		Control cells	Interferon-treated cells
1	2	1,725	1,280
	4	2,040	2,070
	6	3,450	1,600
2	2	530	550
	4	3,200	2,000
	6	2,100	300

^a Ribosome and cell-sap fractions used in these two experiments were prepared on separate occasions from two different batches of cells. The interferon and control systems were from interferon-treated (35 units/ml for 17 to 24 hr at 37°C) and untreated portions of the same batch of cells.

^b ^{14}C -amino acid incorporation expressed as counts per minute per 50 μg of ribosomes. Values for incorporation in the absence of added EMC RNA of 10,000 and 10,200 counts/min for the control systems and 8,700 and 11,300 for the interferon systems, in experiments 1 and 2, respectively, have been subtracted from the figures for total incorporation to obtain the data given here. The assays were with 50 μg of untreated "74S" ribosomes and 500 μg of cell-sap protein in the routine 0.1-ml assay system described in the accompanying paper (8).

FIG. 9. Fate of ^3H -encephalomyocarditis (EMC) RNA and of the ^{14}C -amino acids incorporated into cell-free systems derived entirely from chick embryo fibroblasts (CEF). ^3H -EMC RNA was incubated in the cell-free system with cell sap and untreated "74S" ribosomes from control CEF in the presence of ^{14}C -amino acids (Methods). After incubation the systems were analyzed on sucrose gradients (Methods). Sedimentation was from right to left. A separate gradient was run in parallel with 74S ribosomes (80 μg) as an optical density (O.D.) marker (.....). (A) ^3H -EMC RNA after 7.5 min at 0°C (○) or 37°C (▲). (B) ^{14}C -amino acid incorporation after 7.5 min at 37°C in the presence of ^3H -EMC RNA (●).

incubation at 37°C most of this RNA was released from the ribosomes. In marked contrast to the results with the mixed systems, however, the released RNA remained relatively intact (Fig. 9A). Apart from this, the results obtained with the completely chick systems were very similar to those for the mixed systems (Fig. 6 and 7). For example, on incubation at 37°C, only a small amount of EMC RNA remained apparently associated with the 74S ribosomes on which the majority of the ^{14}C -amino acid incorporation occurred (Fig. 9B). Clearly, therefore, as with the mixed systems, there was no quantitative correlation between the amount of the RNA bound to ribosomes at 0°C in these systems and its messenger function.

An analysis has also been made of tryptic digests of the products synthesized in these systems. Patterns similar to that shown in Fig. 4F of the accompanying paper (8), for the completely chick cell-free system plus EMC RNA, were obtained for systems from both interferon-treated and control cells in the presence of EMC RNA.

The addition of highly purified chick interferon to a final concentration of 80 units/ml in the assay had no consistent effect on the small stimulation of amino acid incorporation observed in response to EMC RNA in these systems from interferon-treated and control CEF (Table 3), nor did it have any effect on the nature of the products formed as determined by fingerprint analysis of their tryptic digests. It was similarly without effect on the stimulation of amino acid incorporation by EMC RNA observed with the mixed chick-mouse systems.

DISCUSSION

It is clear that EMC RNA codes for the synthesis of virus specific polypeptide(s) in cell-free systems employing material from mouse and chick cells (8 and 18). It can be concluded, therefore, that the difference in response to EMC RNA observed here for ribosomes from interferon-treated and control cells reflects a difference in protein synthetic activity *in vivo*. The results are in accord with this being an interferon-mediated rather than a nonspecific effect. Although not large, it was proportional to the interferon concentration over a 10-fold range of purity of interferon preparations, was not observed on treatment of mouse (Krebs) cells with chick interferon or of chick cells with heat-inactivated interferon, and appeared specific for viral RNA. It should be noted, however, that these results were obtained with only the "74S" fraction of the microsomes (the total microsomes, ribosomes, and polysomes not being amenable

to testing in this way), that the effect of a contaminant in the partially purified interferon cannot be entirely excluded, and that a test of the activity of the ribosomes with an added nonviral mRNA other than polyuridylic acid has not yet been possible.

It might have been expected in view of the reports of others (1, 11, 12) that the reduced level of translation of viral RNA in these systems would have been reflected in a reduction of the binding of viral RNA to the ribosomes. No such effect was observed. The experiments described here following the fate of ^3H -EMC RNA in the cell-free system differed from our previous experiments with ^3H -Semliki Forest virus (SFV) RNA (9) in the use (i) of ribosomes which had not been exposed to DOC and (ii) of sufficient ^3H -EMC RNA to follow ^{14}C -amino acid incorporation in response to it in the system under test. Despite this, they led to the same conclusion that there is no quantitative correlation between the formation and breakdown of viral RNA-ribosome complexes in the cell-free system and the messenger function of the viral RNA.

The difference in response to EMC RNA observed with ribosomes from interferon-treated and control cells is clear and reproducible. This was not the case with the cell-sap fractions. It would be premature, however, as is discussed below, to exclude a role for cell-sap factors in the interferon response.

The idea that interferon itself is the antiviral agent has recently been promoted by the work of Sheaff and Stewart (17), but interferon *per se* had no effect on the EMC RNA-stimulated systems described here. Until more extensive studies under a variety of conditions are performed, however, this possibility should not be excluded.

The same product is synthesized in response to EMC RNA with ribosomes and cell sap from interferon-treated and control cells (Fig. 3 and 8). The decreased response to EMC RNA observed with material from interferon-treated cells must, therefore, reflect a reduction not in what is translated but in the rate or frequency of its translation. Unfortunately, the data do not indicate at which level in translation (initiation, peptide bond formation, or even release) the limitation lies. As competition may be occurring between viral and endogenous mRNA, we cannot even be certain that the limitation operates only upon the processing of the viral RNA. There appears to be a number of protein factors of the F3(B) type in *Escherichia coli* responsible for selection between mRNA at the translational level (3, 15, 16). Given an analogous situation in animal cells, limitation of one or a few such factors might

affect the translation of viral RNA and a fraction of host mRNA, without total host protein synthesis being noticeably affected.

In short, by using cell-free systems capable of the synthesis of virus polypeptide in response to virus RNA, we have obtained results which are consistent with there being an inhibition of translation of viral RNA in cells previously exposed to interferon. Although these results would be in accord with a translational site for interferon action, they nevertheless remain open to a variety of interpretations. In the continued absence of pure preparations of interferon, an effect of a contaminant in even the relatively highly-purified material used here cannot be entirely excluded. Similarly, despite the above comments, the possibility of competition between endogenous and added viral RNA complicates any interpretation of the results. Even accepting that the observed effect is real and mediated by interferon, the nature of the alteration in translation remains to be established. In addition, it must be asked whether this inhibition of translation can account for the greater than 99% inhibition of virus growth observed in the interferon-treated cell. Is the effect primary to interferon action or a mere secondary consequence of it?

With respect to the nature of the change the simplest interpretation of the data might seem to be that, in agreement with the altered ribosome hypothesis (1, 2, 11, 12), it lies entirely in the ribosome fraction. In general, however, experiments with different cell-sap preparations have indicated that cell-sap factors (or factors variably distributed between the cell sap and ribosomes) are involved in the translation of EMC RNA. Indeed the variability of the results obtained with cell sap from interferon-treated cells would suggest this. In addition, here we are working largely with mixed systems and it is possible that the detection and particularly the quantitation of any change in the fractions from interferon-treated chick cells could be complicated by the presence of an excess or compensating factor in the Krebs cell component of the system. The important requirement, therefore, would seem to be to define the number and nature of the factors controlling the translation of the viral RNA, irrespective of whether they are isolated with the ribosome, or cell-sap fractions or both. Until this has been done no precise understanding of the nature of the effect will be possible. It would certainly be premature on the basis of this data alone to ascribe any effect which interferon treatment of cells may have on translation simply to an altered ribosome.

The question remains whether the 30 to 70% inhibition of translation of EMC RNA observed here can account for the greater than 99% in-

hibition of virus growth observed in the interferon-treated cell. In fact, a direct comparison of these figures may be misleading. We are observing the translation of EMC RNA in the cell-free system but measuring the effectiveness of interferon action by its ability to inhibit the replication of SFV in the intact cell. Apart from any compensatory effect of the Krebs cell component, in these mixed systems probably only a portion of the RNA genome is being translated and it is conceivable that initiation of translation might not be at the site at which it normally occurs *in vivo* (8). This could have a profound effect upon the extent to which an inhibition of translation in the interferon-treated cell would be manifest in these cell-free systems. Early events in virus replication are less inhibited in the interferon-treated cell than the final yield of virus (14). It is not impossible, therefore, that a partial inhibition of virus protein synthesis might cumulatively result in a marked reduction in virus yield. This apart, recent work on the translation of phage mRNA in cell-free systems from normal and T4-infected *E. coli* has shown that selection between mRNA can occur at the translational level (3, 5, 15, 16). It would hardly be surprising, therefore, if the results reported here reflected the operation of a similar mechanism in the virus-infected and interferon-treated animal cell.

This work has indicated some of the limitations and pitfalls intrinsic to the cell-free systems employed. Nevertheless, the results do suggest that such systems, perhaps from cells susceptible to the more highly purified mouse interferons now available, should be capable of providing definitive answers to many of the remaining questions and hence lead to a more detailed understanding of the factors controlling virus protein synthesis in the infected cell.

ACKNOWLEDGMENTS

The excellent technical assistance of R. E. Brown is most gratefully acknowledged. I am heavily indebted to K. H. Fantes and E. M. Martin for their generous gifts of interferon and purified encephalomyocarditis virus, to D. H. Metz for the interferon assays, and to Martin for stimulating discussions during the preparation of this paper.

This investigation was much facilitated by the purchase of chick cells from the Microbiological Research Establishment, Porton, Wilts., England. The help of K. Sargeant, P. Buck, and F. C. Belton in this connection is greatly appreciated.

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