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Mechanism of Interferon Action: Inhibition of Viral Messenger Ribonucleic Acid Translation in L-Cell Extracts

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Encephalomyocarditis (EMC) virus ribonucleic acid (RNA) stimulated the incorporation of ^{14}C-amino acids into polypeptides in cell-free systems using preincubated S10 extracts from L cells. Incorporation was linear for over 2 hr. Analysis of the tryptic peptides derived from the polypeptide products formed in response to EMC RNA showed them to be virus specific. The major product, a polypeptide of 140,000 in molecular weight, migrated on sodium dodecyl sulfate-polyacrylamide gels with one of the virus-specific polypeptides present in EMC-infected cells. A minor component of molecular weight about 230,000 may correspond to the product of complete translation of the EMC virus genome. Little or no effect of interferon or vaccinia virus infection was observed in the preincubated, cell-free system. The EMC RNA-stimulated incorporation of ^{14}C-amino acids into polypeptides was not inhibited in extracts derived from L cells early in virus infection, from interferon-treated cells, or from cells subjected to both treatments. Interferon treatment did appear to have a slight inhibitory effect on chain elongation in this system. However, treatment of cells with highly purified interferon before virus infection caused a decrease of about 80% in the capacity of non-preincubated cell extracts to translate added EMC RNA. This effect did not extend to the translation of polyuridylic acid and could be reversed by preincubation of the extracts at 37 C for 20 min. The inhibition of translation was manifest at interferon concentrations as low as 5 IU/ml, and in this respect closely paralleled the inhibition of virus growth. Inactivation of the antiviral activity of the interferon by heating or digestion with trypsin also abolished the effect on cell-free protein synthesis. The EMC-specific polypeptides formed in reduced amounts in extracts of interferon-treated vaccinia-infected cells were smaller than those formed in extracts of untreated, vaccinia-infected cells. Thus, inhibition of initiation or elongation of polypeptides, or both, can be demonstrated in cell-free systems employing non-preincubated extracts from interferon-treated, virus-infected cells. These results indicate that antiviral activity of interferon is directed against the translation of viral messenger RNA.

With the exception of some recent evidence implicating transcription of viral ribonucleic acid (RNA) as the basic defect in the virus replication mechanism in interferon-treated cells (2, 18, 23), most studies have suggested that translation of viral RNA is the primary point at which interferon action is expressed (25). Joklik and Merigan (9) demonstrated that early vaccinia virus messenger RNA (mRNA) is produced in increased amounts in interferon-treated L cells, but that this mRNA fails to enter into polyribosomes and is not translated (9). In an RNA virus infection, the translation of input viral RNA is inhibited (7). However, attempts to reproduce this inhibition in cell-free systems have either proved nonreproducible (15, 19) or have uncovered effects which have not been sufficiently clear-cut for it to be established beyond doubt that they were due to the antiviral activity of interferon (3, 12).

We present here evidence that both preincubated and non-preincubated cell-free systems from L cells can translate virus mRNA with fidelity to yield virus-specific polypeptides. Interferon pretreatment of the L cells had little effect on the ability of conventional, preincubated extracts to translate encephalomyocarditis virus

(EMC) RNA in the cell-free system, but an inhibitory effect of interferon treatment on the subsequent ability of cell extracts to translate added EMC RNA has been demonstrated. This required that the extracts be taken not simply from interferon-treated cells, but from interferon-treated, virus-infected cells. Moreover, the effect was seen in the systems from interferon-treated, vaccinia virus-infected cells only if the usual preincubation step was omitted from the preparation of the cell extracts.

MATERIALS AND METHODS

The Krebs 2 mouse ascites tumor cells (20) and the preparation of cytoplasmic extracts from them (21), the preparation of EMC virus (14) and EMC RNA (14), the labeling of polypeptide products in the cell-free system (4), 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 (4) have already been described, as has the qualitative and quantitative analysis of the polypeptide products by electrophoresis on 5 or 7.5% polyacrylamide gels (4) after treatment with sodium dodecyl sulfate (SDS). The values for the molecular weights of polypeptides synthesized in the cell-free system were obtained by comparison with marker polypeptides from purified reovirus run in parallel gels or in split gels.

Preparation of L-cell extracts: preincubated extracts. L cells were originally obtained from N. Finter (Wellcome Research Laboratories) and were grown in Eagle Spinner medium with 10% calf serum to a density of 80 to 100 $\times$ 10^4 cells/ml. Cell extracts were prepared basically by the methods of Mathews and Korner (21) originally described for Krebs mouse ascites tumor cells. Cells were washed three times by centrifugation in cold buffer (35 mM tris[hydroxymethyl]aminomethane [Tris]-hydrochloride [*p*H 7.5], and 140 mM NaCl). They were packed by sedimentation at 400 $\times$ *g* for 10 min and resuspended in 1.5 packed cell volumes of buffer (10 mM Tris-hydrochloride, *p*H 7.5, 7 mM 2-mercaptoethanol, 10 mM KCl, and 1.5 mM magnesium acetate). After 5 min they were homogenized with 20 strokes of a glass Dounce homogenizer, the homogenate was brought to 30 mM Tris-hydrochloride (*p*H 7.5), 90 mM KCl, 3.5 mM magnesium acetate, and 7 mM 2-mercaptoethanol, and centrifuged at 10,000 $\times$ *g* for 10 min. The pellet was discarded. The extract was incubated at 37 C for 40 min after the addition of adenosine triphosphate (ATP; 1 mM), guanosine triphosphate (GTP; 0.1 mM), cytidine triphosphate (CTP; 0.6 mM), creatine phosphate (10 mM), creatine kinase (0.16 mg/ml), and 40 μM each of amino acids. The extract was then passed through a fine stainless-steel mesh prior to passing through a Sephadex G-25 column which had been equilibrated with buffer containing 30 mM Tris-hydrochloride (*p*H 7.5), 120 mM KCl, 5 mM magnesium acetate, and 7 mM 2-mercaptoethanol. Samples of about 0.2 ml, containing the maximal concentration of protein and RNA, were frozen at -70 C in glass vials. Activity was stable in the frozen material for at least 2 months. These extracts contained 5 to 9 mg of protein per ml as measured by the method of Lowry et al. (17) and had an absorbancy at 260 nm (A_{260}) of 20 to 30 units/ml. Preparations with A_{260} <20 units/ml had little or no activity. All operations, unless otherwise noted, were carried out at 4 C.

Non-preincubated extracts. L cells at a concentration of 1.5 $\times$ 10^6 cells/ml were treated for 4 hr at 37 C with the indicated dose of interferon, diluted to 7.5 $\times$ 10^5 cells/ml, and incubated overnight (about 17 hr).

The cells were then infected (1) with vaccinia virus at a multiplicity of infection of 500 virus particles (about 5 plaque-forming units [PFU]) added per cell, and after 75 min were washed three times with 35 mM Tris-hydrochloride (*p*H 7.5) and 140 mM NaCl. The preparation then proceeded as described for preincubated extracts until after the 10,000 $\times$ *g* sedimentation step. At this point, samples of 0.2 ml were frozen at -70 C in glass vials. Thawed preparations were not refrozen. These extracts contained 14 to 16 mg of protein per ml as measured by the method of Lowry et al. (17) and had an A_{260} of 50 to 60 units/ml. No significant differences were noted in the protein or RNA content between extracts from interferon-treated or untreated cells.

In the case of infection with EMC, treatment was identical except that infection was at an added multiplicity of 300 PFU per cell in the presence of actinomycin D (1 μg ml). Virus was allowed to absorb over a 30-min period, and extracts were prepared after a further 2-hr period of incubation at 37 C.

Preparations were assayed as described below with an Mg^{2+} concentration of 4.5 mM except in the case of the polyuridylic acid experiments where the Mg^{2+} concentration was 10 mM and the incubation time was 1 hr at 37 C.

Assay for amino acid incorporation. Each 50-μliter reaction mixture contained the following constituents in the given concentration: 110 mM KCl, 30 mM Tris-hydrochloride (*p*H 7.5), 7 mM 2-mercaptoethanol, magnesium acetate as indicated (usually 4.5 mM), 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, 0.16 mg of creatine kinase per ml, 5 μCi of ^{14}C-amino acid mixture per ml (The Radiochemical Centre, Amersham, Bucks, England), 40 μM each of amino acids not present in the ^{14}C-amino acid mixture (methionine, histidine, glutamine, asparagine, cysteine, and tryptophan), and 10 to 15 μliters of L-cell extract. In the case of labeling with ^{35}S-methionine, 100 μCi of ^{35}S-methionine per ml and 40 μM of cold amino acids (minus methionine) were added per 50-μliter incubation mixture. Each L-cell preparation was tested to determine which volume of the preparation yielded optimal incorporation. Incubation was usually for 120 min at 30 C. The reaction was halted, and the mixtures were analyzed for acid-precipitable radioactivity as previously described (4).

Interferon preparation and assay. Mouse interferon was prepared by the method of Paucker (24). The preparations used contained at least 1.5 $\times$ 10^7 IU of mouse interferon activity per mg of protein. Interferon was assayed by its inhibitory effect on the growth

of EMC virus in L cells. Virus yield was estimated by hemagglutinin production (20). In the case of vaccinia virus-infected cells, the inhibition of vaccinia virus protein synthesis and its effect on the polyribosome pattern were also used to monitor interferon action (9). The mouse interferon used in this study was prepared by K. Paucker and purchased through special funds from the National Cancer Institute, Bethesda, Md.

Chick interferon was prepared by the method of Fantes (6). The preparation used was donated by K. H. Fantes and contained 13,000 international chick units per mg of protein.

RESULTS

Characteristics of the translation of EMC RNA by L-cell extracts. When we tested extracts of Krebs ascites cells prepared by previously described methods, we confirmed that incorporation of ^{14}C-amino acids was stimulated by the addition of EMC RNA (Table 1). We prepared, in a similar manner, extracts of L cells and tested these at 37 C and 30 C. With L-cell preparations at 37 C, we found eightfold stimulation, but a 60-fold stimulation was seen when these extracts were assayed at 30 C (Table 1). In L-cell extracts, the stimulation by EMC RNA was optimal when the Mg^{2+} concentration was about 4.5 mM. The optimal K^+ concentration was 110 mM. An additional 10 to 20% incorporation of amino acid was occasionally observed by including transfer RNA in the L-cell incubation mixtures.

We used similarly prepared L-cell extracts to test whether mouse globin mRNA (27) or vaccinia mRNA (F. Fournier, D. Tovell, and D. Metz, *manuscript in preparation*) (11) produced by vaccinia virus cores would also stimulate amino acid incorporation. Both did to some degree (about five- to sixfold over endogenous incorporation) but to a much lesser extent than did EMC RNA. The concentrations of Mg^{2+} and K^+ which were optimal for stimulation of amino acid incorporation by each of the mRNAs used were somewhat different although each mRNA used was more effective at 30 C than at 37 C in the L-cell systems.

TABLE 1. *EMC RNA-directed protein synthesis in Krebs ascites or L-cell extracts*

Origin of preparation	Assay temp (C)	Counts/min/50-μliter incubation mixture	
		−EMC RNA	+EMC RNA
Krebs cells[a]	37	1,400	18,200
L cells	37	970	7,600
L cells	30	1,000	62,100

[a] Krebs ascites cell or non-preincubated L-cell extracts were prepared and assayed as described in Materials and Methods. Each 50-μliters of assay mixture contained 10-μliters of extract.

The stimulation of amino acid incorporation by EMC RNA in L-cell extracts was linear for 120 min. Thereafter until 300 min, the rate of the reaction decreased (Fig. 1). Part of the explanation for the prolonged ability of EMC RNA to stimulate amino acid incorporation in L-cell extracts at 30 C was due to the marked stability of EMC RNA under these conditions. After 1 hr at 30 C, 80% of this RNA ran with mobility of EMC RNA on electrophoresis in polyacrylamide gels.

The effect of the concentration of viral mRNAs on stimulation of ^{14}C-amino acid incorporation by L cells was also studied. Increasing concentrations of EMC RNA, up to 2 or 3 μg per 50-μliter incubation mixture, stimulated proportional increases in incorporation of ^{14}C-amino acids by L-cell extracts. Higher concentrations were inhibitory.

Nature of the product. The polypeptides formed in response to EMC RNA in the cell-free systems

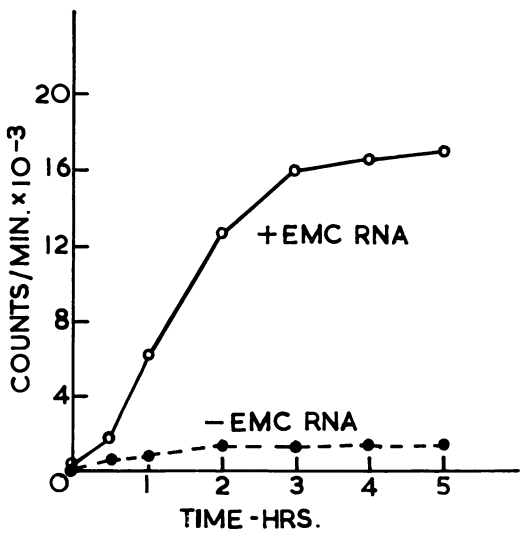

FIG. 1. *Stimulation of amino acid incorporation by EMC RNA in L-cell extracts: time course. Two 500-μliter incubation mixtures were prepared containing the concentrations of constituents listed in Materials and Methods, 150 μliters of an L-cell preparation, 50 μliters of ^{14}C-amino acids, and 0 or 20 μg of EMC RNA. At each indicated time point, a 25-μliter sample was removed and assayed for ^{14}C-amino acid incorporation. ○——○, 0.04 mg of EMC RNA per ml present; ●-----●, no EMC RNA added.*

were analyzed by polyacrylamide gel electrophoresis and by two-dimensional peptide mapping on thin-layer plates. In both L-cell and Krebs ascites cell extracts, product size was found to increase with increasing time of incubation at 30 C. The identification of virus-specific peptides was facilitated by the polydisperse nature of the product and the very low incorporation by the extracts in the absence of EMC RNA (Fig. 2). The general patterns seen on SDS-polyacrylamide gels were similar to those previously reported for Krebs ascites cells with the exception that, by 120 min, the largest prominent peptide found (molecular weight about 140,000) was more conscpicuous in L-cell than Krebs ascites cell preparations (13). The nature and origin of these polypeptides have been extensively discussed in previous communications from this laboratory (4, 13). It is sufficient to note here that the formation of very large polypeptide products was stimulated by EMC RNA in L-cell extracts and that these products closely resembled in size those previously identified as being characteristic of polypeptides stimulated by EMC RNA in Krebs ascites cell extracts.

^{35}S-methionine-labeled EMC polypeptides from L-cell extracts programmed with EMC RNA were also subjected to electrophoresis on polyacrylamide gels with the EMC-specific polypeptides labeled by ^{3}H-methionine in virus-infected HeLa cells (Fig. 3). The electropherogram shows that the polypeptide with a molecular weight of 140,000 which is formed in the in vitro L-cell system is the same size as one of the large polypeptides formed in virus-infected cells. Similar results have recently been reported (5). The in vitro product may therefore be related to one of the large polypeptides formed in virus-infected cells.

We have analyzed the tryptic peptide maps derived from EMC RNA-directed products labeled with ^{35}S-methionine (Fig. 4). The L-cell and the Krebs ascites extract products were compared. The numbers assigned to the different spots seen in the autoradiograms of the thin-layer plates designate peptides which have been previously shown by detailed analysis of peptide maps to be present in EMC virus-infected Krebs cells and, in the case of some of these, also in purified EMC virus (4). These tryptic peptides are definitely specified by the EMC virus genome. There is a close correspondence in Fig. 4 between the peptides formed by the two systems, and it can be concluded that L-cell extracts are capable of producing a number of EMC-specific polypeptides.

Lack of effect of vaccinia virus infection or interferon treatment on translation of EMC virus RNA in preincubated extracts. In cell-free systems derived from L cells treated with 50 IU of mouse interferon per ml, which was sufficient to inhibit virus production by more than 99%, no effect on

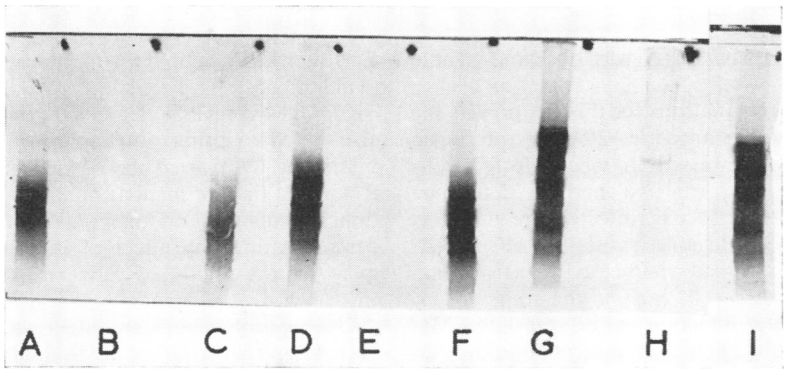

FIG. 2. *Electrophoretic analysis of products of L cell and Krebs cell-free system on polyacrylamide gels. A 500-μliter incubation mixture contained ^{35}S-methionine, 100 μliters of L cell or Krebs cell extract, and 0 or 20 μg of EMC RNA. After incubation at 30 C, samples were removed and prepared for polyacrylamide gel electrophoresis, and samples containing 50,000 counts per min were applied to each gel. The 5% gels were subjected to electrophoresis overnight, fixed, stained, sliced, dried, and autoradiographed as previously described (4). The autoradiographs were developed after 1 week. The molecular weights of the products were estimated from those of reovirus proteins which were subjected to electrophoresis with the samples. A, D, and G; L-cell extracts with EMC RNA, 30, 60, and 120 min of incubation, respectively. B, E, and H; L-cell extracts; no EMC RNA added; incubations were for 30, 60, and 120 min. C, F and I; Krebs ascites cell extracts with EMC RNA; 30, 60 and 120 min of incubation.*

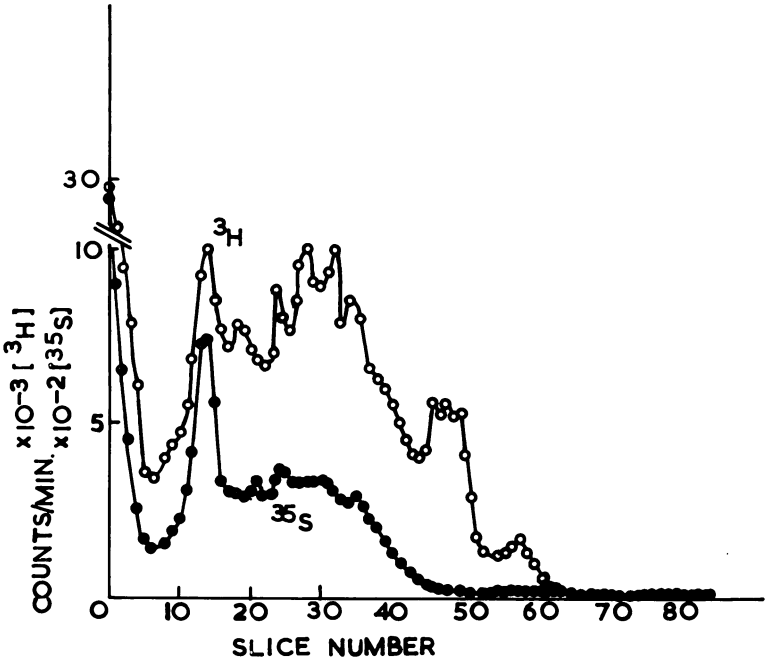

FIG. 3. *Co-electrophoresis of EMC RNA-directed products from L cell-free system and EMC virus-infected HeLa cells. The ^{35}S-methionine-labeled cell-free product (●) was the EMC-directed 120 min L-cell specimen from Fig. 2G. ^3H-methionine-labeled EMC-directed product from HeLa cells (○) was produced by infecting actinomycin D (2 μg/ml)-treated HeLa cell monolayers with EMC virus at a multiplicity of 100. After 3 hr when, under these conditions, the protein synthesis in these cells is almost entirely virus-directed (J. J. Skehel, personal communication), the monolayers were washed five times with balanced salt solution and incubated for 15 min with methionine-free medium to which 25 μCi of ^3H-methionine had been added. The cells were washed five times, and an extract was prepared for gel electrophoresis, mixed with the ^{35}S-labeled in vitro product, the mixture was subjected to electrophoresis and the gels were sliced and counted as previously described (16).*

translation of EMC RNA was observed (Table 2A).

Among the explanations for this failure was the possibility that the antiviral effect of interferon might only be expressed in virus-infected cells (25). To test this, we first had to determine whether extracts of cells infected by a heterologous virus would still translate EMC RNA. We therefore examined whether extracts from vaccinia virus-infected cells early in the course of infection (1 hr after infection) could translate EMC RNA. The results (Table 2B) indicate that they do so at least as efficiently as extracts from control cells. By 1 hr after infection, vaccinia virus markedly inhibited cell-directed protein synthesis and vaccinia virus proteins dominated the pattern of protein being produced; however, no cytopathic effect of the virus was observed at this time (22).

It was thus possible to test whether interferon-treated, vaccinia virus-infected cells would translate EMC RNA. Indeed, cell-free systems from these cells were just as capable of translating EMC RNA as those from vaccinia virus-infected cells which had not been treated with interferon (Table 2C).

Changes in several elements in the assay system also failed to bring out any effect of interferon treatment. We varied the concentration of EMC RNA, the time of incubation, and the concentrations of magnesium or potassium ion without demonstrating any effect of interferon pretreatment on the ability of cell extracts to translate EMC RNA.

One possibly significant effect was found, however. In extracts from both vaccinia virus-infected and uninfected cells, the products formed were analyzed on polyacrylamide gels. The proportion of counts in the highest-molecular-weight EMC-specific polypeptide obtained (about 140,000) was larger in extracts from controls than from interferon-treated cells (Fig. 5). This was suggested in the results seen after 120 min of incubation but was even more evident in the 240-min specimens. This can be seen at a quantitative level in the results of the experiment shown in Fig. 6 which illustrates an additional point of interest. With

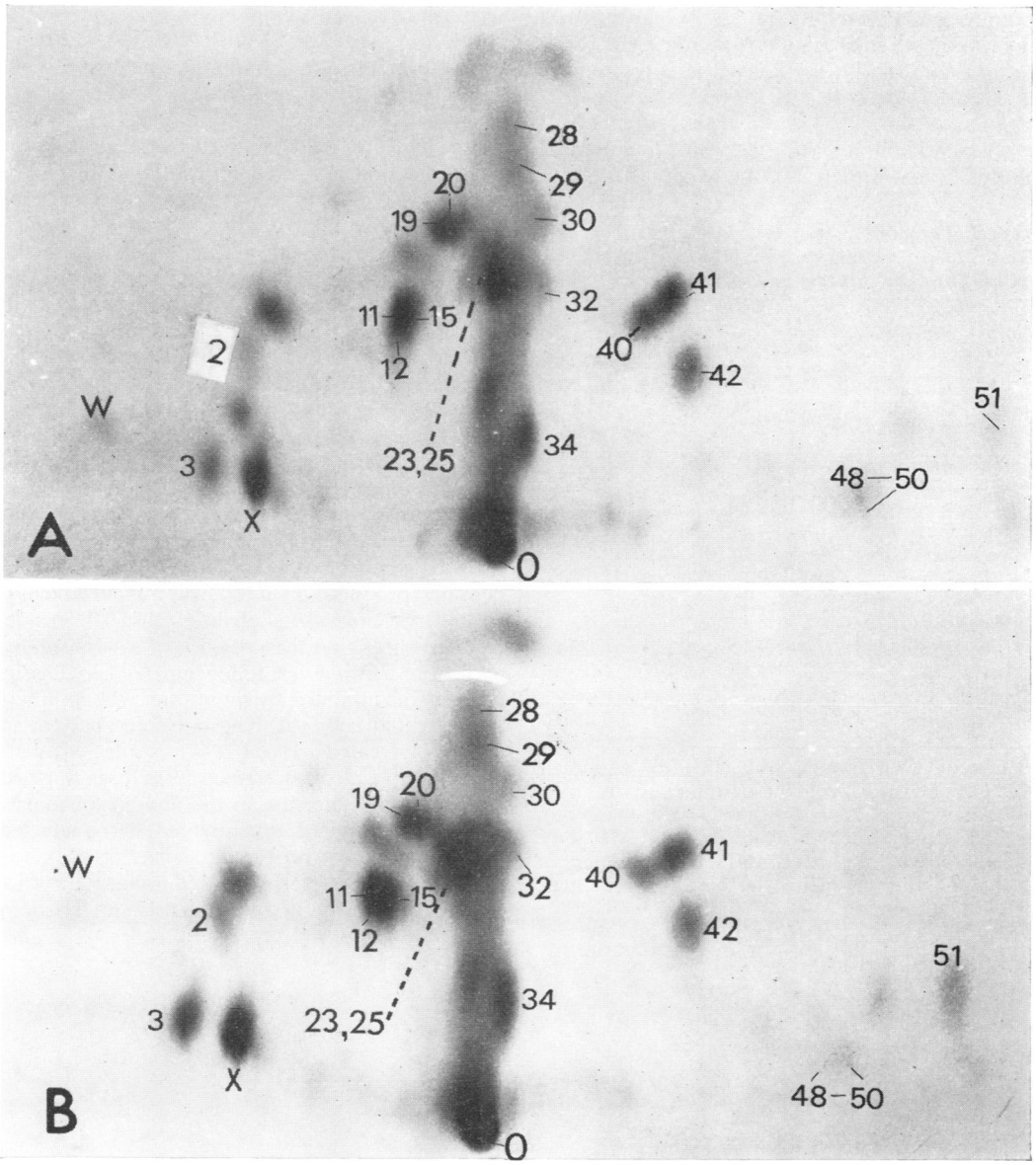

FIG. 4. *Radioautograms of two-dimensional tryptic peptide maps from EMC-directed products formed in L-cell and Krebs cell extracts. The ^{35}S-methionine-labeled products were the 120-min L-cell and Krebs cell specimens incubated with EMC RNA in Fig. 2 (G and I). Performate oxidation, trypsin treatment, electrophoresis, and chromatography on thin-layer plates, and autoradiography were carried out as previously described (4). The numbers are those previously assigned in this laboratory to EMC-specific peptides (4). Products formed in (A) L-cell extracts and (B) Krebs cell extracts.*

long exposures of the autoradiograms, small amounts of a polypeptide of at least 230,000 in molecular weight have been consistently observed among the products formed by extracts from control cells (Fig. 6, arrow). This polypeptide is sufficiently large for it to correspond to the complete translation product of the EMC RNA genome. It is not seen in extracts from interferon-treated cells (Fig. 6).

Inhibition of EMC RNA-directed polypeptide synthesis in non-preincubated extracts from interferon-treated L cells. L-cell extracts which had not

been preincubated for 40 min at 37 C during their preparation are capable of translating EMC RNA (Table 3A). As with the conventionally preincubated extracts described in Table 2, however, pretreatment of the cells with sufficient (50 units/ml) interferon to inhibit EMC virus growth in parallel cultures by more than 99% had only a small inhibitory effect on chain elongation during the translation of added EMC RNA in this system. In addition (Table 3B), non-preincubated, cell-free systems, which were derived from L cells infected with vaccinia virus, translated EMC RNA. Accordingly, as neither interferon treatment nor virus infection appeared individually to have a significant effect on these systems, it was possible to test their combined effect. When this experiment was performed, a marked inhibition in the translation of EMC RNA was observed with non-preincubated extracts from interferon-treated, vaccinia virus-infected cells (Table 4).

TABLE 2. *Lack of effect of interferon treatment and vaccinia virus infection on EMC RNA-directed protein synthesis in L-cell extracts*

Treatment of cells[a]	Preincubation of extract[b]	Counts/min/50-μliter incubation mixture	
		−EMC RNA	+EMC RNA
A. None	+	400	15,400
Interferon	+	800	3,700
B. None	+	400	7,400
Vaccinia virus infection	+	440	12,000
C. Vaccinia virus infection	+	640	6,200
Interferon + vaccinia virus infection	+	670	7,200

[a] L cells were treated with 50 units of mouse interferon per ml for 14 hr or infected with vaccinia virus, or both, and extracts were made and assayed as described in Materials and Methods. Each 50-μliter mixture contained 0 or 2 μg of EMC RNA and was incubated for 120 min at 30 C.
[b] 37 C, 40 min.

That an antiviral state had been established by the interferon treatment employed was checked by (i) testing the ability of samples of cells (not in this case infected with vaccinia virus) to support EMC virus growth, (ii) checking the pattern of polyribosomes in the cytoplasm of the interferon-treated, vaccinia virus-infected cells against that of untreated, vaccinia virus-infected cells, and (iii) analyzing on polyacrylamide gels the polypeptides produced in interferon-treated and untreated, vaccinia virus-infected cells. The results of these checks on the presence of a viral inhibitory state showed the following. (i) EMC virus growth was inhibited by more than 99% in interferon-treated cells. (ii) The polysome pattern in interferon-treated, vaccinia virus-infected cells was, as previously described (9), disaggregated with a marked increase in the number of monosomes as compared to untreated, virus-infected cells. (iii) Almost no protein (virus or cell) was produced in interferon-treated, vaccinia virus infected cells, whereas a pattern characteristic o

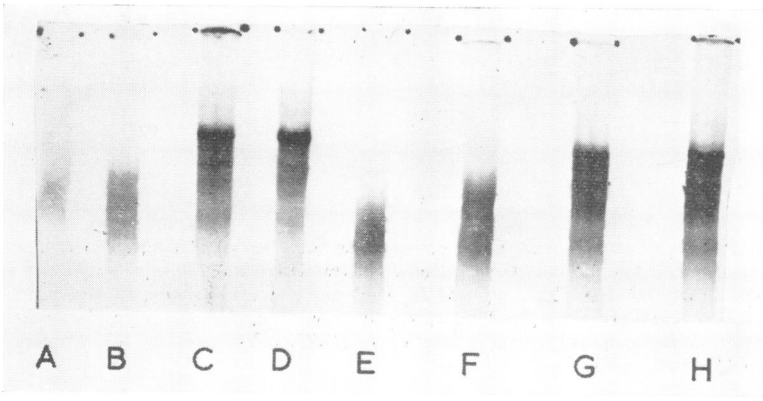

FIG. 5. *Analysis of products of cell-free systems by electrophoresis on polyacrylamide gels: effect of interferon treatment and vaccinia virus infection. Extracts from vaccinia virus-infected cells were treated for 14 hr with 50 units of purified mouse interferon per ml prior to infection, and virus-infected controls were prepared and analyzed as described in the legend to Fig. 2. In this experiment, however, samples were taken after 30, 60, 120, and 240 min of incubation with EMC RNA. A, B, C, and D: extracts from control cells at 30, 60, 120, and 240 min, respectively. E, F, G, and H: extracts from interferon-treated cells at 30, 60, 120, and 240 min.*

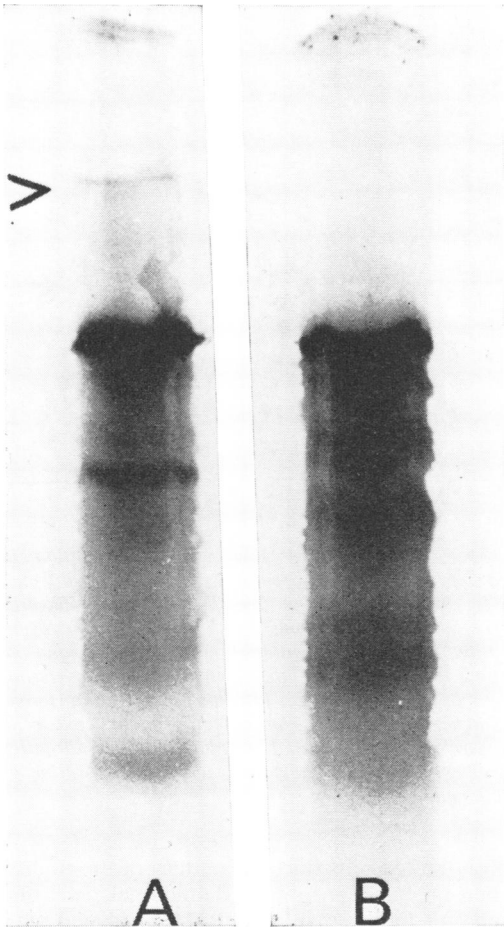

FIG. 6. *Analysis of products of cell-free systems by electrophoresis on polyacrylamide gels: possible effect of interferon treatment and vaccinia virus infection. Samples were prepared in a manner identical to those in Fig. 5. However, films were exposed to the dehydrated gels for 14 days instead of 5. A, Extract from vaccinia virus-infected cells; B, extract from interferon-treated, vaccinia virus-infected cells.*

vaccinia virus infection was already established in untreated cells by 1 hr after infection (22).

One additional point should be made concerning the results in Table 4. At 1 hr after infection, almost all of the protein being produced in cells infected with vaccinia virus is vaccinia virus directed (22). Therefore, the endogenous incorporation observed in the absence of added EMC RNA largely represents vaccinia virus-directed protein synthesis. It might be expected that interferon treatment would have some effect on translation of endogenous vaccinia virus mRNA and indeed

TABLE 3. *Translation of EMC RNA by L-cell extracts*

Treatment of cells	Treatment of extract	Counts/min/50-μliter incubation mixture	
		−EMC RNA	+EMC RNA
A. None	None	3,830	8,700
Interferon[a]	None	3,970	8,370
B. Vaccinia infection	Present[b]	580	7,300
Vaccinia infection	None	1,330	8,310

[a] Cells were treated with 50 units of interferon per ml, and non-preincubated or preincubated extracts were prepared and assayed as described in Materials and Methods. Each 50-μliter incubation mixture contained 15 μliters of the L-cell preparation and 0 or 2 μg of EMC RNA.

[b] After 1 hr of infection with vaccinia virus, extracts were preincubated for 40 min at 37 C and then filtered through Sephadex G25.

TABLE 4. *Inhibition of EMC RNA translation in non-preincubated extracts of interferon-treated, vaccinia virus-infected L cells*

Treatment of cells[a]		Volume of L-cell extract per 50-μliter incubation mixture (μliters)	Counts/min/50-μliter incubation mixture	
Interferon (50 units/ml)	Vaccinia virus infection		−EMC RNA	+EMC RNA
−	+	10	1,750	6,930
		15	1,560	8,320
		20	1,230	5,840
+	+	10	291	738
		15	323	597
		20	337	631

[a] L cells were treated with interferon (50 units/ml) and infected with vaccinia virus as described. Each 50-μliter incubation mixture contained the indicated volume of the non-preincubated L-cell preparations employed.

a three- to fourfold inhibition of ^{14}C amino acid incorporation was observed (Table 4).

Taken together, these results suggest that the antiviral effect of interferon is directed against the translation of viral mRNA and that operation of this inhibition is only fully manifest in extracts from virus-infected cells. As shown below, this effect of interferon is reversible. Previous attempts to demonstrate this effect in conventionally prepared extracts (S-10 preparations) failed, probably because some step in the preparative procedure inactivated a factor(s) responsible.

Characteristics of the interferon effect in extracts of interferon-treated, vaccinia virus-infected L cells. At all concentrations of EMC RNA tested, extracts from interferon-treated, vaccinia virus-infected cells showed less stimulation of amino acid incorporation than did those from untreated, vaccinia virus-infected cells (Fig. 7). The optimal concentration of EMC RNA with both extracts was 40 μg/ml. With extracts from both interferon-treated, virus-infected and untreated, virus-infected cells, incorporation was linear for about 60 min (Fig. 8). At all times studied, preparations from interferon-treated, virus-infected cells incorporated amino acids less well than did preparations from untreated, virus-infected cells. The difference noted was not due to more rapid breakdown of EMC RNA by extracts from the interferon-treated cells since the pattern of the counts, when analyzed on polyacrylamide gels, was the same for radioactive EMC RNA incubated with either cell extract.

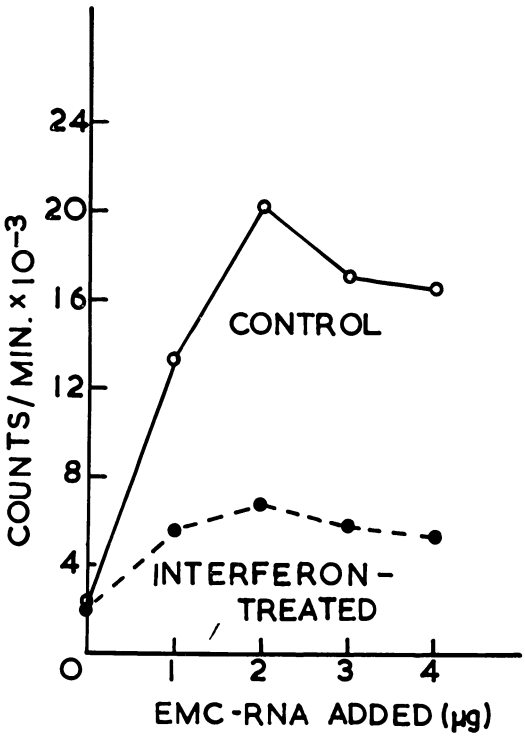

FIG. 7. *Inhibition of EMC RNA translation in extracts of interferon-treated, vaccinia virus-infected cells: effect of EMC RNA concentration. Cells were treated with 50 units of interferon per ml and infected with vaccinia virus. Controls were only virus infected. Extracts were prepared from these cells. Each 50-μliter incubation mixture contained the indicated amount of EMC RNA and 15 μliters of non-preincubated extract. Incubation was at 30 C for 2 hr.* ○——○, *Extracts from untreated, vaccinia virus-infected cells (control);* ●-----●, *extracts from interferon-treated, vaccinia virus-infected cells (interferon-treated).*

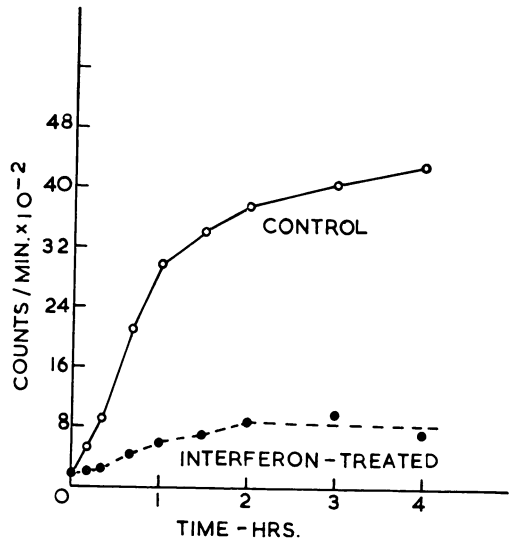

FIG. 8. *Inhibition of EMC RNA translation in extracts of interferon-treated, vaccinia virus-infected cells: effect of incubation time. Two 250-μliter mixtures were prepared containing constituents in the concentration indicated in Materials and Methods and 75 μliters of non-preincubated extract from interferon-treated, vaccinia virus-infected cells (interferon-treated;* ●-----●) *or untreated, virus-infected cells (control;* ○——○). *At the indicated times, 20-μliter samples were removed and assayed for incorporation of* ^{14}C*-amino acids.*

In addition, extracts from both interferon-treated, virus-infected cells and from untreated, virus-infected cells incorporated amino acids optimally at 4.5 mM Mg^{2+}; however, the extracts from interferon-treated, virus-infected cells had much lower levels of activity at all Mg^{2+} and K^+ concentrations where significant stimulation of amino acid incorporation was seen in untreated, virus-infected controls.

The polypeptide products synthesized in response to EMC RNA in the different cell-free systems used were analyzed by electrophoresis on SDS-polyacrylamide gels (Fig. 9B, D, and F). Parallel analyses of the products of systems in the absence of EMC RNA are shown in Fig. 9A, C, and E. With material from untreated L cells, whether the cell-free systems were preincubated (Fig. 9B) or not (not shown), a characteristic (Fig. 2) series of polypeptides was synthesized in response to the added virus RNA, the predominant product after 2 hr of incubation at 30 C

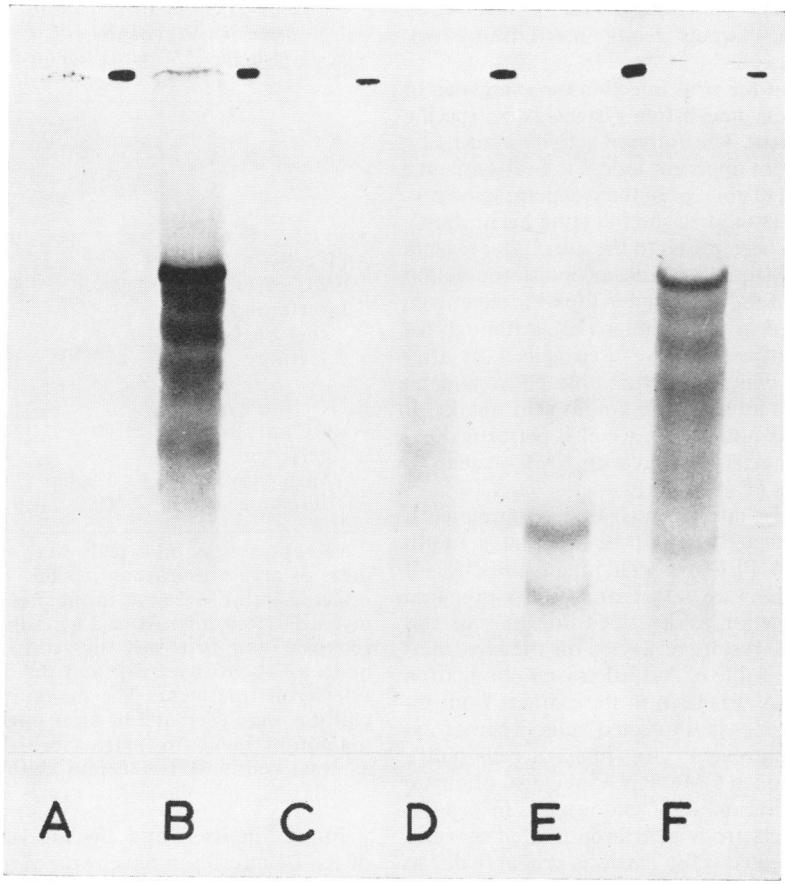

FIG. 9. *Inhibition of EMC RNA translation in extracts of interferon-treated, vaccinia virus-infected cells: electrophoretic analysis of polypeptides formed in the cell-free system.* ^{35}*S-labeled products were prepared for analysis on 5% SDS-polyacrylamide gels as previously described. About 50,000 counts per min were loaded on each gel. The gels were prepared for radioautography, and the film was exposed for 7 days. Preincubated extracts from uninfected L cells in the absence (A) and presence (B) of EMC RNA. Non-preincubated extracts from interferon-treated, vaccinia virus-infected cells in the absence (C) and presence (D) of EMC RNA. Non-preincubated extract from untreated, vaccinia virus-infected cells in the absence (E) and presence (F) of EMC RNA.*

being a large polypeptide of approximately 140,000 in molecular weight (Fig. 9B). Vaccinia virus infection did not alter the nature of the polypeptides synthesized in response to EMC RNA in these systems. This is shown for a non-preincubated system in Fig. 9F. Interferon treatment alone had, at most, a marginal effect on the relative amounts of the higher-molecular-weight products synthesized (Fig. 5 and 6). In contrast, with non-preincubated systems from cells subjected to both interferon treatment and infection, only small amounts of low-molecular-weight polypeptides were produced in response to the added EMC RNA (Fig. 9D). These results indicate that not only is there less product made in response to the added RNA, but also the product size is altered. This would argue against an effect exclusively on initiation of translation of the viral RNA.

Clearly, the translation of added viral RNA (EMC RNA) is inhibited in cell-free systems from interferon-treated, vaccinia virus-infected cells. The results of the analysis of these systems in the absence of EMC RNA are in accord with a corresponding inhibition of the translation of endogenous vaccinia virus mRNA in the interferon-treated, virus-infected cell system. Although the polypeptides detectable in the non-preincubated system from vaccinia virus-infected cells (Fig. 9E) have not been fully characterized, they are almost certainly vaccinia virus-specific (22), and they are absent in systems from cells subjected to interferon treatment prior to infection (Fig. 9C). These results indicate that added EMC RNA and endog-

enous vaccinia virus mRNA are not efficiently translated in extracts from interferon-treated, virus-infected cells.

Requirement for virus infection for expression of antiviral activity in cell-free systems is not specific for vaccinia virus. The antiviral activity induced by interferon is not apparent unless the cells are also virus-infected (Table 3). In these experiments, vaccinia virus was used as the infecting agent. Some objection has been raised to the use of the vaccinia virus-L cell system in studies of interferon action on the basis of the profound cytopathic effect seen in cells treated in this manner (10). Although for preparation of extracts we used cells 1 hr after infection, in which no cytopathic effect and no effect of virus infection on amino acid uptake or pool sizes was noted (22), we also performed experiments on cells infected with EMC instead of vaccinia virus (Table 5, A).

In this study, interferon-treated or untreated L cells were infected with EMC virus at a multiplicity of 300 PFU per cell in the presence of actinomycin D (1 μg/ml). Extracts were prepared 2 hr after infection as described in Materials and Methods. The results of assays on these extracts are shown in Table 5, A. Little or no stimulation by EMC RNA was seen in the extracts from interferon-treated, virus-infected cells, whereas extracts from untreated, virus-infected cells had a response to added EMC RNA. We also observed a marked inhibition in the endogenous incorporation of extracts from interferon-treated as compared to untreated cells. This was probably due to the fact that, by 2 hr after infection, most of the polyribosomes in cells not treated with interferon were engaged in making EMC proteins, whereas in interferon-treated cells neither cell nor viral products were being produced, since in the presence of actinomycin D, a rapid inhibition of L-cell protein synthesis takes place on infection with EMC virus (4).

Additional studies have shown that multiplicities of vaccinia or EMC virus of 1 or 25 PFU, respectively, are sufficient to establish antiviral activity in cell-free systems from interferon-treated cells. In the case of vaccinia virus, the antiviral state is well established by 15 min after infection in interferon-pretreated L cells.

Inhibition of translation is interferon mediated. Krebs ascites cells were treated with interferon (50 units/ml), infected with EMC virus at a multiplicity of 300 PFU per cell, and non-preincubated extracts were prepared as described for L cells in Materials and Methods. Interferon treatment was ineffective in inducing an inhibition of translation in extracts of Krebs ascites cells which are resistant to induction of antiviral activity by interferon (Table 5B).

TABLE 5. *EMC RNA translation in non-preincubated extracts of interferon-treated EMC virus-infected L cells or Krebs ascites cells*

Treatment of cells[a]	EMC virus growth hemagglutin in (units/ml)	Counts/min/50-μliter incubation mixture	
		−EMC RNA	+EMC RNA
A. L cells			
EMC infection.	1,280	4,300	9,900
Interferon, then EMC infection.....	<20	1,530	1,840
B. Krebs cells			
EMC infection.	2,560	5,620	24,200
Interferon, then EMC infection....	2,560	9,180	28,300

[a] Krebs ascites or L cells were treated with 50 units of mouse interferon per ml. They were then infected with EMC virus in the presence of actinomycin D (1 μg/ml). After 2 hr, 5-ml samples were removed, and virus was allowed to replicate for 9 hr to assay virus growth and the effectiveness of interferon treatment. The major portions of the cultures were used at 2 hr after infection for making non-preincubated extracts which were assayed as described in Materials and Methods.

Interferon itself and the antiviral activity induced by interferon have some very typical properties (25). The antiviral activity and the inhibition of EMC RNA translation in cell-free systems, which was induced by the interferon preparation we used, were both stable to pretreatment of the interferon at *p*H 2. Also, neither activity was reversed by washing the cells which had been treated with the interferon preparation. The antiviral activity induced by interferon and the in vitro inhibition of EMC RNA translation were stable at 37 C for 30 min but were destroyed by 80 C or trypsin treatment for 30 min (Table 6). Partially purified chick interferon, in titer sufficiently high (80 units/ml) to inhibit Semliki Forest virus growth by more than 99% in chick cells, had no effect on L cells, and an interferon preparation (kindly donated by R. Z. Lockart), purified by a different method (26) than Paucker's (24), was as effective as the standard preparation which we used in most of our studies.

In addition, Table 7 shows that both the antiviral activity induced and the inhibition of EMC RNA translation in cell-free systems varied directly with the concentration of interferon which had been used to treat L cells. Maximum activity of both was reached between 5 and 15 units/ml. It

TABLE 6. *Effect of inactivation of interferon on the inhibition of EMC RNA translation in extracts of interferon-treated, vaccinia virus-infected L cells*

Treatment of interferon[a]	Antiviral activity[b]	Counts/min/50-μliter incubation mixture	
		−EMC RNA	+EMC RNA
Control, no interferon	−	1,280	4,140
None	+	530	970
Trypsin[c]	−	1,090	4,450
37 C[d]	+	706	1,120
80 C[e]	−	1,890	7,300

[a] In all cases 50 units of interferon per ml or its equivalent prior to inactivation was employed to treat cells.

[b] Antiviral activity was assayed by inhibition of EMC virus hemagglutinin production, inhibition of vaccinia virus-directed protein synthesis, and effect on polyribosome pattern in vaccinia virus-infected cells.

[c] Trypsin (100 μg/ml) was added to a concentrated interferon preparation (1,500 units/ml) for 30 min at 37 C. At the end of this time, soya bean trypsin inhibitor (200 μg/ml) was added and the preparation was diluted appropriately.

[d] Treated exactly as footnote *c* except that trypsin was omitted.

[e] Interferon (1,500 units/ml) was heated to 80 C for 30 min, and diluted.

is also interesting to note (Table 7) that, in cell-free systems, the level of endogenous incorporation, which is almost certainly directed by vaccinia virus mRNA, also varied with the interferon dose. This agreed quite well with the degree of cell polysome disaggregation (9) noted in cells treated with different interferon doses. In cells treated with 5 units of interferon per ml, some viral polysomes could be found, but with higher concentrations a clear-cut disaggregation of polysomes occurred.

We also tested the ability of these extracts to direct the incorporation of ^{14}C-phenylalanine in the presence of polyuridylic acid [poly(U)]. At the lowest Mg^{2+} concentration where effective phenylalanine incorporation was observed (10 mM) and at higher Mg^{2+} concentrations, no difference was seen in the incorporation of ^{14}C-phenylalanine between the extracts from interferon-treated, virus-infected cells and untreated, virus-infected cells (Fig. 10). This result indicates that the cytoplasmic elements, such as ribosomes and various factors necessary for the functioning of the poly(U) system, are present in equal amounts in preparations from both interferon-treated, virus-infected cells and untreated, virus-infected controls.

Reversal of the antiviral activity in cell-free systems induced by interferon treatment of cells. It has been observed that there is no difference in the ability to translate EMC RNA by extracts from

TABLE 7. *Effect of the interferon concentration used in the treatment of cells on the inhibition of EMC RNA translation in extracts of interferon-treated, vaccinia virus-infected L cells*

Interferon concn (units/ml)	EMC virus growth (hemagglutinin units produced)[a]	Counts/min/50-μliter incubation mixture	
		−EMC RNA	+EMC RNA
0[b]	1,280	2,080	18,450
5	30	1,180	6,940
15	<20	540	2,820
50	<20	630	2,630

[a] Before vaccinia virus infection, L-cell samples were removed, washed, and infected with EMC for 8 hr, and yields of EMC hemagglutinin were assayed to estimate the effectiveness of the interferon treatment.

[b] Cells were treated with the indicated concentration of interferon and then infected with vaccinia virus as described in Materials and Methods.

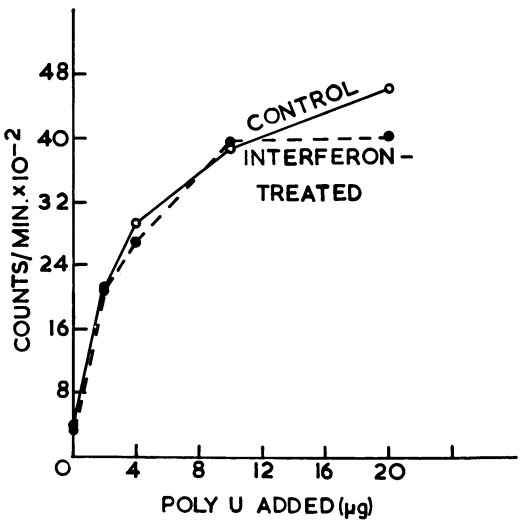

FIG. 10. *Lack of effect on poly(U) translation in extracts of interferon-treated, vaccinia virus-infected cells. Extracts were prepared as described in Materials and Methods. Concentrations of the constituents of the reaction mixture were as described except that Mg^{2+} was 10 mM and the incubation was for 1 hr at 37 C. The amount of poly(U) indicated is per 50-μliter reaction mixture.* ○——○, *Non-preincubated extracts from untreated, vaccinia virus infected cells (control);* ●-----●, *non-preincubated extracts from interferon-treated, virus-infected cells (interferon-treated).*

interferon-treated, virus-infected cells or untreated, virus-infected cells when they are preincubated for 40 min at 37 C during their preparation (Table 2). The conventional preincubation procedure used also involves filtration of extracts through a Sephadex G25 column, but this alone did not reverse the inhibition of the translation of EMC RNA observed with extracts from interferon-treated, vaccinia virus-infected cells. When all of the steps in this procedure were employed, however, the antiviral activity of the extract was destroyed (Table 8). When only incubation at 37 C for 40 min was carried out, the inhibition of EMC RNA translation was also lost. Indeed, preincubation of extracts from such cells at 37 C for as little as 20 min in the presence or absence of amino acids, ATP, and an ATP generating system was sufficient to reverse the inhibitory effect of interferon treatment on EMC RNA translation. This was true despite the fact that, under these latter conditions, amino acid incorporation during the preincubation was inhibited by more than 85%. On this preliminary basis, therefore, it seems unlikely that protein synthesis is required during the preincubation for the reversal of the interferon-mediated inhibition to occur.

DISCUSSION

In this study we have discussed two basic systems. The first involved preincubated extracts from interferon-treated but uninfected cells which, with the possible exception of an inhibition of chain elongation (Fig. 5 and 6), translated EMC RNA as well as controls from cells which had not been treated with interferon (Table 2). Infection with vaccinia virus did not impair the ability of extracts from interferon-treated or untreated cells to translate EMC RNA (Table 3). The second cell-free system consisted of non-preincubated extracts from interferon-treated, virus-infected cells. The results from this system clearly show marked inhibition of translation of EMC RNA. Both interferon treatment and virus infection were required for the demonstration of this inhibitory state.

TABLE 8. *Effect of preincubation of extracts of interferon-treated and virus-infected L cells on their ability to translate EMC RNA*

Treatment of cells	Treatment of extract	Counts/min/50-μliter incubation mixture	
		−EMC RNA	+EMC RNA
None	None	2,740	5,470
Interferon	None	944	1,450
None	37 C, Sephadex G25[a]	635	6,480
Interferon	37 C, Sephadex G25	528	4,510
None	37 C only[b]	764	5,020
Interferon	37 C only	786	4,360

[a] Extracts were treated at 37 C for 40 min and were then passed through a Sephadex G25 column (5).

[b] Extracts were treated as in footnote *a* except that amino acids were omitted from the preincubation mixture and no gel filtration was carried out.

Taken together, these results and previously published work suggest the following mechanism of action for interferon. In interferon-treated cells, a potential antiviral state is induced. This process seems to require cellular RNA and protein synthesis and may manifest itself by a slight inhibition of cell growth (24) and of translation of viral RNA (3, 8). Such a potential state may account for the inability to demonstrate any profound effect of interferon treatment on the processes of uninfected cells (25). When interferon-treated cells are infected with virus, the antiviral state is fully realized, and the translation of viral RNA in cell-free systems derived from such cells is markedly inhibited. The factor(s) responsible for this inhibition in cell-free systems from interferon-treated, vaccinia virus-infected cells is heat labile. Current studies on such extracts indicate that the factor(s) is present in both microsomal and cell sap fractions. Interestingly, the addition of cell sap from vaccinia virus-infected, interferon-treated L cells to ribosomes from interferon-insensitive Krebs cells also results in an inhibition of the translation of EMC RNA.

An intriguing aspect of these systems is that current results obtained on infection of interferon-treated cells with EMC do differ in some details from those obtained with the interferon-treated, vaccinia virus-infected cell systems. With systems from the interferon-treated, EMC-infected cells, the inhibitory effect is confined to the cell sap fraction (D. Tovell et al., *manuscript in preparation*). Moreover, the results of two experiments would suggest that the translation of both mouse globin mRNA and EMC RNA is reduced in cell-free systems from vaccinia virus-infected, interferon-treated cells. This reflects the complete inhibition of host and viral protein synthesis in these cells (9).

Although it is possible that the results obtained were due to a nonspecific effect of interferon

treatment and virus infection, we feel this is unlikely for several reasons. The mouse interferon preparations employed were highly purified and, aside from inhibiting virus growth, had no marked effect on the cells. The activity in the interferon preparation which induced the antiviral state in both cells and cell-free systems had the chemical and physical characteristics of an interferon. Two different viruses could be used to activate the antiviral state and, finally, in extracts from interferon-treated, vaccinia virus-infected cells, the inhibition of viral RNA translation was reversed by preincubation at 37 C for 40 min.

Viral RNA was not degraded more quickly in extracts from interferon-treated, virus-infected cells than in extracts from untreated, virus-infected cells (L. A. Ball, *unpublished observation*). Our results suggest that the interferon-induced inhibition we have observed may be due to an effect on the elongation and possibly also on the initiation step of virus polypeptide synthesis. Total incorporation of amino acids into viral products was markedly inhibited, and those polypeptides which were produced were of a small size compared to the EMC RNA-specific polypeptides normally produced in cell-free systems.

Two recent studies have suggested that interferon pretreatment inhibits the synthesis of viral mRNAs mediated in the infected cell by the virion polymerases of parental vesicular stomatitis (18) and vaccinia (2) viruses. The question arises whether this effect or the inhibition of translation reported here is the basis of the antiviral activity of interferon. The crucial points in this respect are (i) how general is the effect on transcription, (ii) can the apparent inhibition of the virion polymerases in the intact cell be seen with low doses of highly purified interferon preparations, and (iii) can the effect be shown to be directly on the viral polymerases by studies in the cell-free system? However, the inhibition of translation reported in this study parallels a similar effect in the intact cell (22, 25). Translation inhibition is seen with low doses of the two most highly purified interferon preparations available and is quantitatively sufficient to account for the inhibitory effect of interferon treatment on virus replication.

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