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Protein-Synthetic Activity of Ribosomes from Interferon-Treated Cells

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Cell-free protein-synthetic systems from normal and interferon-treated chick cells were compared. No difference was found in the amino acid incorporation activities of such ribosome-cell sap systems or in their response to polyuridylic acid. Throughout a variety of experiments we failed to detect the formation of a discrete peak of virus-specific polysomes, when ribosome monomers and subunits (from interferon-treated or control cells) were incubated with labeled Sindbis or Semliki Forest virus ribonucleic acid (RNA). Some binding of viral RNA did occur, but the complexes formed were evident in sucrose gradients as a broad, rapidly sedimenting shoulder on the ribosome monomer peak. Interferon pretreatment of cells did not affect the formation of these complexes in vitro, nor did it alter their rate of breakdown on incubation under amino acid incorporation conditions. Experiments with inhibitors of protein synthesis showed that such "breakdown" was not dependent upon amino acid incorporation and was not an index of translation. In these respects, our results are in marked contrast to those of Marcus and Salb. These results, together with our failure to detect any significant change in the protein composition of ribosomes from interferon-treated cells, suggest that such treatment does not result in a modification of the ribosome per se. They do not, however, rule out the involvement of a factor(s) required for ribosomes and viral RNA to function in viral protein synthesis. Indeed, it remains likely that interferon acts through such a mechanism, although the precise level at which the inhibition occurs remains to be elucidated.

The inhibition of viral growth by interferon is well documented (7, 33), yet its mode of action is still not known. Early investigations by Taylor (31, 32), Friedman and Sonnabend (10, 11), Lockart (21), and Levy and Merigan (20) suggested that exposure of cells to interferon induces the synthesis of a new protein which inhibits the synthesis of viral macromolecules without affecting the normal synthetic activity of host cells. It appears to be the synthesis of viral protein rather than nucleic acid which is inhibited (16, 27, 30). This inhibition could theoretically occur at any stage in the processing of the viral messenger ribonucleic acid (mRNA) prior to or during its combination with the ribosome or at any point in its subsequent translation. Strong support for the latter mechanism, i.e., an inhibition of translation, was provided by Marcus and Salb (23), who compared the interaction of labeled viral and host cell RNA species with ribosomes from interferon-treated and control chick embryo fibroblasts (CEF). They concluded that ribosomes from interferon-treated cells could combine with, but not translate, viral RNA, although they retained the ability to translate host cell mRNA isolated from polysomes. It was later shown that mild trypsin treatment of these ribosomes restored their ability to translate a viral RNA (24). They thus provided strong evidence not only for the altered ribosome hypothesis but also for the involvement in this alteration of protein(s) synthesized in response to interferon. Some reduction in the apparent affinity of interferon-treated cell ribosomes for viral RNA was also noted by these workers (23, 24), an effect which was even more pronounced in the results of similar studies reported by Carter and Levy (3). Moreover, both groups observed that cell-free systems from treated cells incorporated amino acids less well in response to the addition of viral RNA than did suitable control preparations (4, 23).

Our own approach was twofold. Firstly, we sought evidence for changes in the protein composition of the host cell in response to interferon, particularly in the proteins of the ribosome fraction, which might reflect the synthesis of the hypothetical inhibitory protein. However, an

extensive comparison of ^{14}C-labeled ribosomal proteins from interferon-treated cells with ^{3}H-labeled proteins from control ribosomes detected no significant change. This work has been summarized (29) and will be reported in detail elsewhere. Secondly, at the functional level, we initiated studies to compare the protein-synthetic activities of cell-free systems from interferon-treated and control cells. The contrast between our failure to detect differences in ribosomal proteins and the results of Marcus and Salb (23, 24) and Carter and Levy (3, 4), however, prompted us to a detailed examination of the interaction of viral RNA with ribosomes under the conditions described by Marcus and Salb (23). It is this aspect of our work which is reported here. The first half of the paper is concerned with the characterization of the cell-free systems used and with a comparison of ribosomes from interferon-treated and control cells, with respect to both their intrinsic amino acid incorporation activities and their response to a synthetic mRNA (polyuridylic acid). In agreement with both Marcus and Salb (23) and Carter and Levy (4), we found no difference between the two types of ribosome in these respects. In the second half of the paper the interaction of viral RNA with these ribosomes is described. The results, in marked contrast to those of Marcus and Salb, do not support the altered ribosome hypothesis. Our recent findings concerning the response of cell-free systems to viral RNA do, however, favor an inhibition of the function of viral RNA in protein synthesis in the interferon-treated cell, but the level at which this occurs remains to be elucidated.

MATERIALS AND METHODS

Materials. Solutions of pyruvate kinase, adenosine triphosphate (ATP), guanosine triphosphate (GTP), phosphoenolpyruvate (C. F. Boehringer und Soehne, Mannheim, Germany), and ethylenediaminetetraacetic acid (EDTA; BDH Chemicals Ltd., Poole, England) were adjusted where necessary to *p*H 6.5 to 7.0 with 1 N KOH and buffered with 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.6). β-Mercaptoethanol (Koch-Light Laboratories, Colnbrook, Bucks, England) was redistilled and made up as a 1 M solution buffered with 20 mM Tris-hydrochloride (*p*H 7.6). Sodium dodecyl sulfate (SDS; BDH Chemicals Ltd.) was recrystallized from ethyl alcohol prior to use. Bentonite (Fisher Laboratory Chemicals, Fairlawn, N.J.) was washed as described by Fraenkel-Conrat, Singer, and Tsugita (9) and then with distilled water, freeze-dried, and stored in suspension (50 mg/ml) in 50 mM EDTA (*p*H 7.0). All of these reagents were stored at −20 C. Polyuridylic acid (potassium salt) was from Miles Chemical Corp., Elkhart, Ind. All radioactive chemicals were obtained from the Radiochemical Centre, Amersham, Bucks, England.

Interferon. Partially purified chick interferon was the generous gift of Karl Fantes (Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Bucks, England). It mostly consisted of material equivalent to that purified on diethylaminoethyl (DEAE) cellulose as described by Fantes (5). It was used as a solution of between 5,000 and 50,000 units/ml in 0.1 M sodium phosphate buffer (*p*H 6.5). Assayed as described previously (25), it had a specific activity of from 10^4 to 10^5 units/mg of protein, compared with 10^2 to 5×10^2 units/mg of protein for crude interferon.

Cells. Monolayer cultures of fibroblasts from 10-day-old chick embryos were routinely prepared as described by Taylor (32). In experiments requiring large numbers of cells (over 10^{10}), these were occasionally obtained in suspension (approximately 7.5×10^7 cells/ml) in Parker's 199 medium containing 10% calf serum from the Microbiological Research Establishment, Porton, Wilts, England, and were prepared as described by Zwartouw and Algar (34). The cells were diluted to 5×10^6 cells/ml in Gey's salt solution containing 5% calf serum, 0.25% lactalbumin hydrolysate, and 0.1% protease peptone (Difco) and transferred to 20-oz (600 ml) flat bottles (2×10^8 to 3×10^8 cells/bottle) yielding monolayers of 10^8 to 1.5×10^8 cells/bottle. For interferon treatment of the cells or virus growth, Gey's salts solution supplemented with 2.5% calf serum, 0.25% lactalbumin hydrolysate or 0.1% protease peptone, or both, 7.5 mM Tris-hydrochloride (*p*H 7.6), and 0.025% $NaHCO_3$ was routinely used.

Virus stocks and assay of viruses, viral RNA, and interferon. Preparation of high-titer Semliki Forest virus (SFV) and Sindbis virus stocks, which were free from interferon, and the assay of viruses, infectious RNA, and interferon were as described for the SFV-CEF system by Mécs et al. (25). Virus stocks routinely contained 0.5 μg/ml of actinomycin D (a gift from H. D. Brown of Merck & Co., Inc., Rahway, N.J.).

Assay of ^{3}H-RNA. For the assay of RNA labeled with ^{3}H-uridine, whether free as ribonucleoprotein or in intact virus, 5 ml of 0.3 N trichloroacetic acid was added to samples (up to 1.0 ml) of the RNA solution at 0 C. The precipitated RNA was collected on Oxoid membrane filters (grade 0.45; Oxo Ltd., London, England), washed three times with 5-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 radioactivity in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) with an efficiency of 22% for ^{3}H.

Preparation of radioactive SFV RNA from infected cells. At 1 or 2 days after seeding, monolayer cultures of CEF in 6-cm petri plates (2.5×10^7 cells/plate) were infected with 0.5 ml of SFV at an added multiplicity of 10 to 50 plaque-forming units (PFU)/cell. After 45 min at 37 C, the infecting medium was replaced with 4 ml per plate of fresh medium containing 2 μg/ml of actinomycin D, and the incubation was

continued at 37 C. At 1 hr later ^{3}H-uridine (20 μc in 0.1 ml of medium, 500 to 3,000 c/mole) was added to each plate. At 6 hr the medium was removed, the cells were chilled to 0 C, and the RNA was extracted as described by Mécs et al. (25), except that the SDS-phenol extraction was carried out at 60 C. Bentonite was added to the aqueous layer from the phenol extraction to a final concentration of 0.4 mg/ml, and the mixture was stored at −70 C. Subsequently 4 M NaCl was added to 0.4 M, and the RNA was precipitated by the addition of 2 volumes of ethyl alcohol. After standing overnight at −20 C, the RNA was pelleted and dissolved in 10 mM Tris-hydrochloride (*p*H 7.6), 0.1 M KCl, and 1 mM EDTA (TKE buffer). Typically, about 10^6 counts/min of ^{3}H-RNA was obtained from 10^8 cells. The RNA was layered on a 15 to 30% (w/v) sucrose gradient (25 ml) in TKE buffer and centrifuged at 45,000 × *g* for 17 to 18 hr at 0 to 4 C in the swing-out rotor (3 by 25 ml) of the MSE 50 ultracentrifuge (Measuring & Scientific Equipment, Ltd., London, England). Gradient fractions were collected and monitored for material absorbing light at 260 nm and for radioactivity; Fig. 1A shows a typical preparation. Peak fractions were pooled to yield 45*S* and 26*S* + 45*S* SFV RNA preparations. To each pool, bentonite and 2 M potassium acetate were added to final concentrations of 0.1 mg/ml and 0.2 M, respectively. The RNA was precipitated by the addition of 2 volumes of ethyl alcohol and stored at −20 C. Small samples of each of these preparations were analyzed (in parallel to their use in the cell-free system) on 5-ml 5 to 20% (w/v) sucrose gradients in TKE buffer with mouse ribosomal RNA as a sedimentation marker. Centrifugation was at 4 C for 110 min at 130,000 × *g* in the swing-out rotor (3 by 5 ml) of the MSE 50 ultracentrifuge. Typical results are shown in Fig. 1B and C.

Preparation of radioactive RNA from purified Sindbis virus. Monolayer cultures of CEF in 20-oz (600 ml) flat bottles (10^8 cells/bottle) were infected with 4 ml of stock Sindbis virus (10 to 50 PFU/cell). After 40 min at 37 C, the infecting medium was replaced with 14 ml of fresh medium containing 0.5 μg/ml actinomycin D. After 1.0 hr at 37 C, 50 μc of ^{3}H-uridine (2,300 c/mole) in 1.0 ml of medium was added to each bottle. At 15 hr the culture fluid was harvested and centrifuged at 800 × *g* for 30 min at 4 C, and the supernatant fluid was stored at −70 C. Concentration and purification of the virus were carried out by zonal centrifugation by using a slight modification of the method described by Fox et al. (8). The infected culture fluid from 15 × 10^8 cells (225 ml containing 3 × 10^6 counts/min of ^{3}H-RNA) was formed into a 15 to 30% (w/w) sucrose gradient over 60 ml of 40% and 120 ml of 60% (w/w) sucrose in an MSE aluminum BXIV zonal rotor. Centrifugation was for 17 hr at 43,000 × *g* at 4 C. More than 90% of the virus was recovered in a volume of 45 ml at an average sucrose concentration of 43% (w/w). The peak fractions were pooled and diluted to 90 ml. Methanol was added to a final concentration of 25% (v/v), and the material was stored at −20 C overnight prior to centrifugation at 80,000 × *g* for 3.5 hr at 4 C in the no. 30 rotor of the Spinco model L ultracentrifuge.

For extraction of the RNA, the virus was resuspended in 2 ml of 0.5% SDS, 50 mM Tris-hydrochloride (*p*H 7.6), and 0.2 mg/ml of bentonite and mixed for 10 min at 37 C with an equal volume of freshly distilled phenol, which had been equilibrated with 50 mM Tris-hydrochloride (*p*H 7.6). Potassium acetate was added to a final concentration of 0.2 M, and the mixture was held at 0 C for 10 min before centrifugation at 10,000 × *g* for 10 min at 4 C. Bentonite was added to the aqueous layer to a final concentration of 0.1 mg/ml, and the RNA was precipitated with ethyl alcohol in the presence of potassium acetate as described above. The RNA was subsequently fractionated on a 25-ml 15 to 30% (w/v) sucrose gradient in TKE buffer. Centrifugation was for 16 hr at 35,000 × *g* at 4 C in the SW25 rotor of the Spinco model L ultracentrifuge. Fractions containing the peak of ^{3}H-RNA sedimenting at 40 to 45*S* were pooled and precipitated with ethyl alcohol as above. Samples of this RNA were analyzed on sucrose gradients, as described for ^{3}H-SFV RNA, in the presence of mouse ribosomal RNA as sedimentation marker (Fig. 2). The total ^{3}H-RNA recovered by using this procedure was 90 to 100% of that present in the purified virus.

RNA preparations were routinely stored either as the alcohol precipitate at −20 C or in solution in TKE buffer at −70 C.

Interferon treatment. CEF monolayers in 20-oz (600 ml) flat bottles (10^8 to 1.5 by 10^8 cells/bottle) were used throughout. At 1, or occasionally 2, days after seeding, the monolayers were exposed to fresh medium (20 to 30 ml/bottle) containing either 30 to 150 units per ml of interferon or, for control preparations, an amount of potassium phosphate buffer (*p*H 6.9) equivalent to that present in the interferon preparation. Incubation was continued for 17 to 24 hr before the cells were harvested. The efficacy of the interferon treatment was always checked by assay of virus growth in sample monolayers of the interferon-treated and control cells infected with 10 to 50 PFU/cell of SFV. In all cases interferon treatment reduced the virus yield by greater than 99%. In some experiments cells were exposed after harvesting to puromycin (Lederle Laboratories, Pearl River, N.Y.) at a concentration of 0.33 mM for 30 min at 37 C with the object of obtaining an increased yield of monomeric ribosomes (15, 18, 23). In the experiments involving comparison of ribosome and cell-sap fractions from interferon-treated infected, infected, and control cells, interferon treatment was for 14 hr at a concentration of 55 units/ml and infection with SFV was at an added multiplicity of 20 PFU/cell. After infection, incubation was continued for 5.5 hr in the presence of 55 units /ml of interferon, in the case of the interferon-treated cells, and 0.25 μg/ml of actinomycin D, in the case of the infected cells not treated with interferon. The control cells were incubated for similar periods with medium changes paralleling those required during interferon treatment and infection. Sample cultures of the interferon-treated-infected and infected cells were incubated for 9 hr postinfection, and the yields of virus were assayed to check virus growth and the effectiveness of the interferon treatment.

Preparation of ribosome and cell-sap fractions. Ribosome and cell sap fractions were prepared by a

method based on that described by Kerr et al. (17) for Krebs 2 mouse ascites-tumor cells. All procedures were carried out as rapidly as possible at 0 to 4 C. The medium was decanted, and the cell monolayers were chilled on ice, washed twice with saline, and once with 50 mM Tris-hydrochloride (*p*H 7.6), 25 mM KCl, 5 mM $MgCl_2$, 20 mM β-mercaptoethanol. The cells were scraped from the glass and homogenized in the above buffer (10^8 cells/ml) by 25 strokes in a glass Dounce homogenizer; 2 M sucrose was added to a final concentration of 0.25 M. Under these conditions, greater than 95% cell disruption was achieved without obvious nuclear damage. The homogenate was centrifuged for 30 min at 10,000 × *g*, and the supernatant solution was recentrifuged for 90 min at 150,000 × *g* in the MSE 50 ultracentrifuge. The fat layer was discarded, and the remainder of the supernatant solution was concentrated to 5 to 10 mg of protein per ml by pressure dialysis at 8 to 12 lb/in² overnight at 4 C against 40 to 200 volumes of 20 mM Tris-hydrochloride (*p*H 7.6), 5 mM mercaptoethanol, and 1 mM EDTA. After centrifuging at 12,000 × *g* for 10 min at 4 C, this dialyzed supernatant solution ("cell sap") was stored in suitable small batches at −70 C. The microsome pellets from the high-speed centrifugation could be stored at −70 C for at least a month without loss of activity. To prepare ribosomes, the microsomes from, for example, 5 × 10^9 cells were resuspended, with the aid of gentle mixing with a Potter or loose-fitting Dounce homogenizer, in 2 ml of 250 mM sucrose, 50 mM Tris-hydrochloride (*p*H 7.6), 25 mM KCl, 5 mM $MgCl_2$, and 20 mM β-mercaptoethanol. Sodium deoxycholate (DOC; 5%) was added with mixing to a final concentration of 0.5% and either total ribosomes or "74*S*" ribosome and polysome fractions were isolated as rapidly as possible. In some of the later experiments, homologous cell sap was added (1 to 2 mg of protein/ml) to the microsome suspension prior to DOC treatment. Inclusion of cell sap at this stage yielded ribosome preparations which supported an up to twofold greater level of amino acid incorporation in the cell-free system and which contained fewer monomer ribosomes (approximately 20% instead of 30 to 40%). For total ribosomes, the DOC-treated microsomes were layered on 5 ml of 7.5% (w/v) sucrose in 10 mM Tris-hydrochloride (*p*H 7.6), 10 mM KCl, and 1.5 mM $MgCl_2$ (TKM buffer) and centrifuged for 3 hr at 150,000 × *g* in the MSE 50 ultracentrifuge. For "74*S*" ribosome and polysome fractions, the DOC-treated microsomes were layered as rapidly as possible on 30-ml 7.5 to 45% (w/v) sucrose gradients in TKM buffer and centrifuged for 100 min at 80,000 × *g* in the no. 30 rotor of the Spinco model L ultracentrifuge. Appropriate fractions were pooled to yield the "74*S*" and polysome preparations which were diluted twofold with TKM buffer and centrifuged for 3 hr at 150,000 × *g* in the MSE 50 ultracentrifuge. The ribosome pellets were immediately resuspended in 0.25 M sucrose and 20 mM Tris-hydrochloride (*p*H 7.6) with the aid of gentle mixing in a loose-fitting Dounce homogenizer. The resuspended pellets were centrifuged at 10,000 × *g* for 15 min at 4 C to remove "insoluble" material, and the supernatant ribosome fraction was divided into small batches for storage at −70 C. Ribosome and cell-sap preparations could be stored for approximately 6 months without loss of activity in the amino acid incorporation assay, but deteriorated gradually thereafter, or on repeated freezing and thawing. Sucrose-gradient analysis of typical total and "74*S*" ribosome preparations is shown in Fig. 3. In view of the possible involvement of ribosomal subunits in the initiation of protein synthesis, these were deliberately included in the "74*S*" ribosome preparations, which also usually contained a few residual dimers. With these methods, yields of 125 to 250 μg of total or 40 to 80 μg of "74*S*" ribosomes per 10^8 cells were obtained. About 25% of the ribosomes were discarded with the mitochondrial fraction with this procedure, but inclusion of the latter resulted in preparations which were less active in the cell-free system.

Assay of amino acid incorporation in the cell-free system. Two assays were routinely used. For those in which incorporation of a single radioactive amino acid was studied, the systems contained the following (final concentrations) in a volume of 0.5 ml: 100 mM Tris-hydrochloride (*p*H 7.6); 50 mM KCl; 5 mM $MgCl_2$ (15 mM where polyuridylic acid was added); 20 mM β-mercaptoethanol; 1 mM adenosine triphosphate (ATP); 0.25 mM guanosine triphosphate (GTP); 5 mM phosphoenolpyruvate; pyruvate kinase, 40 μg/ml; the "other" 19 amino acids, each 0.05 mM; ^{14}C-L-leucine (0.2 μc per assay, 165 c/mole) or ^{14}C-L-phenylalanine (0.2 μc per assay, 495 c/mole); and 0.25 to 0.5 mg of ribosomes and saturating amounts of cell sap. For those in which incorporation of a mixture of ^{14}C-amino acids was studied, the assay was identical except for the adjustments necessary to the use of 40 to 100 μg of ribosomes and 0.625 μc of a mixture of ^{14}C-L-amino acids (54 c/gatom of carbon) in a total volume of 0.1 ml. Incubations, unless otherwise stated, were for 45 min at 37 C. At the end of the incubation, an excess of cold amino acid(s) in 1 N NaOH was added, and the mixture was incubated for 5 to 10 min at 37 C before precipitation of the protein in 0.3 N trichloroacetic acid. The precipitated protein was collected on Oxoid membrane filters, and the membranes were processed and counted as for the estimation of ^{3}H-RNA.

Assay of the interaction of radioactive viral RNA with ribosomes in the cell-free system. Unless otherwise stated, studies concerning the interaction of radioactive viral RNA with ribosomes and cell sap were carried out under the conditions described by Marcus and Salb (23). The incubations were carried out in two stages. First, 85 to 250 μg of "74*S*" ribosomes was incubated with ^{3}H-RNA in the presence of 50 mM Tris-hydrochloride (*p*H 7.6), 100 mM NH_4Cl, 7.5 mM $MgCl_2$, and 5 mM β-mercaptoethanol in a final volume of 0.25 ml for 30 min at 0 C. Further additions were then made to give final concentrations of 50 mM Tris-hydrochloride (*p*H 7.6); 150 mM NH_4Cl; 7.5 mM $MgCl_2$; 5 mM β-mercaptoethanol; 1 mM ATP; 0.25 mM GTP; 5 mM phosphoenolpyruvate; and 40 μg/ml of pyruvate kinase in a total volume of 0.5 ml. Incubation was continued as indicated in the individual experiments. In experiments not involving the assay of amino acid incorporation, all 20 unlabeled amino

acids were included at a final concentration of 0.05 mM. In those in which amino acid incorporation was measured, 500 μg of ribosomes was used, the assay was scaled-up to a final volume of 1 ml, and cold leucine was replaced with 0.2 μc of ^{14}C-L-leucine (165 c/mole). Cell sap was included in amounts sufficient to saturate the amino acid incorporation activity of the ribosomes. Estimation of ^{14}C-leucine incorporated was as described above. Analysis of ^{3}H-RNA bound to ribosomes was on 30-ml, 7.5 to 45% (w/v) sucrose gradients in TKM buffer. Centrifugation was in the Spinco SW 25 or, for analysis of more than three samples in parallel, in the 30 rotor under the conditions detailed in the individual experiments. Identical results were obtained in trial experiments with the two rotors. Gradients were routinely monitored for material absorbing light at 260 nm by using the Isco density gradient fractionator (Instrumentation Specialties Co., Inc., Lincoln, Neb.). Fractions (1 ml) were collected and assayed as above for ^{3}H-RNA.

Assay of ribonuclease activity in the ribosome and cell-sap fractions. Incubations were carried out under conditions identical to those described for the second stage of the assay of the interaction of ^{3}H-viral RNA with ribosomes in the cell-free system, using ^{3}H-uridine-labeled SFV RNA as substrate. Incubation was for 30 min at 37 C and the remaining acid-insoluble ^{3}H-RNA was estimated as already described.

Protein. Protein was estimated by the method of Lowry et al. (22), with bovine serum albumin as standard.

RESULTS

Characteristics of the cell-free system. The characteristics of the amino acid-incorporation activity of a typical cell-free system from CEF are given in Table 1. In a system using total unfractionated CEF ribosomes, the incorporation routinely observed corresponds to approximately 20 amino acids incorporated per ribosome, or 5 nmoles of amino acid per mg (0.25 nmoles) of ribosomes. Assayed under the same conditions, cell-free systems from Krebs mouse ascites-tumor cells and rat liver were of similar activity whereas the rabbit reticulocyte system was two to three times more active. Amino acid incorporation in the chick system showed a typical dependence on ribosomes, cell sap, an energy (ATP)-generating system, and the Mg^{2+} concentration. It was inhibited by cycloheximide and puromycin (Table 1). Most of the incorporation occurred during the first 20 min of incubation at 37 C and was complete by 45 min (*see* Fig. 7). Analysis of the product both by electrophoresis on acrylamide gels and by fingerprinting tryptic peptides showed that incorporation was into a number of proteins.

Cell-free systems from interferon-treated and control cells. In experiments involving comparison of interferon-treated and control cell material, ribosome and cell-sap preparations were always isolated in parallel from the same batch of cells, a portion of which had been exposed to interferon. There was no difference in the yield, polysome pattern or ultraviolet light absorption spectra of ribosomes from these two types of cell. In a typical experiment the yields from 5×10^9 cells of total and "74S" ribosomes were 6.4 and 2.1 mg, respectively, for control, and 6.2 and 1.7 mg for interferon-treated cells. The ratios of optical density at 260 to optical density at 280 nm of the control and interferon-treated cell ribosomes were 1.76 and 1.79. Assays in which the concentration of the radioactive amino acid was varied indicated that the pools of unlabeled amino acids in the systems from interferon and control cells were small and of similar size, and a direct comparison of their activity was, therefore, possible.

The intrinsic amino acid incorporation activities of such systems, prepared on four separate occasions, are given in Table 2. The assays were in the presence of limiting amounts of ribosomes. No differences in ribosomal activity were detected; nor could any differences be detected in the activities of the cell-sap preparations, when assayed either at rate-limiting concentrations or with highly active rabbit reticulocyte ribosomes. The responses to polyuridylic acid of "74S" ribosome preparations from interferon-treated and control

TABLE 1. *Characteristics of the cell-free system*

System	Incorporation[a]	
	^{14}C-Amino acid mixture	^{14}C-Leucine
Complete[b]	8,765	
Minus ribosomes	500	
25 μg of ribosomes	4,260	
75 μg of ribosomes	11,690	
Minus cell sap	800	
35 μg of cell sap	5,900	
70 μg of cell sap	8,500	
200 μg of cell sap	8,000	
Complete[c]		8,000
2.5 mM Mg^{2+}		1,350
7.5 mM Mg^{2+}		8,000
10 mM Mg^{2+}		7,800
15 mM Mg^{2+}		5,000
Minus ATP, GTP, phosphoenolpyruvate and pyruvate kinase		1,285
Plus 3.6 mM cycloheximide		1,950
Plus 1 mM puromycin		1,640

[a] Expressed in counts per minute per assay.
[b] Complete system contained 50 μg of ribosomes and 150 μg of cell sap protein.
[c] Complete system contained 0.5 mg of ribosomes, 0.75 mg of cell-sap protein, and 5 mM Mg^{2+}.

TABLE 2. *Amino acid incorporation by ribosome-cell sap systems from interferon-treated and control cells*

Ribosomes	Cell sap	Incorporation of ^{14}C-amino acid mixture[a]			
		1[b]	2	3	4
Control	Control	5,740	6,100	3,500	6,200
Interferon[c]	Control	6,200	5,900	4,750	6,400
Interferon[d]	Control				6,200
Control	Interferon[c]		5,950		6,650
Control	Interferon[d]				7,300
Interferon[c]	Interferon[c]		6,300		6,200
Interferon[d]	Interferon[d]				6,300
Rabbit reticulocyte[e]	Control		10,000		
Rabbit reticulocyte[e]	Interferon[c]		11,000		

[a] Expressed as counts per minute per 50 μg of ribosomes.

[b] Experiment number. The results presented as experiments 1 to 4 represent data obtained with total ribosome and cell sap preparations from four different batches of cells; each value represents the average of at least two assays.

[c] Interferon treatment of cells was at concentrations of from 35 to 50 units/ml.

[d] Interferon treatment of cells was at a concentration of 140 units/ml.

[e] The rabbit reticulocyte ribosomes were the kind gift of R. A. Cox and were prepared as was the "light" ribosome fraction described by Arnstein and Cox (1).

TABLE 3. *Response to polyuridylic acid (poly U) of cell-free systems from interferon-treated and control cells*[a]

Ribosomes	Cell sap	Poly U[b]	^{14}C-Phenylalanine incorporation[c]	
			No poly U	+ Poly U
Control	Control	12.5	1,100	10,650
		20		9,900
Interferon	Control	12.5	1,000	9,900
		20		9,360
Rabbit reticulocyte	Control	13	280	8,500
		20		14,000
		33		24,000
Rabbit reticulocyte	Interferon	13	0	7,000[d]
		20		13,500[d]
		33		24,000[d]

[a] Assays were with saturating amounts of cell sap in the complete cell-free system with 15 mM Mg^{2+}. "74S" ribosomes from interferon-treated and control cells were used. The rabbit reticulocyte ribosomes were the kind gift of R. A. Cox and were prepared as was the "light" ribosome fraction described by Arnstein and Cox (1).

[b] Expressed in micrograms per 0.5 mg of ribosomes.

[c] Expressed in counts per minute per 0.5 mg of ribosomes.

[d] These values were corrected by a factor of 1.08 for the difference in the size of the pool of unlabeled phenylalanine in the cell sap preparations from interferon-treated and control cells.

cells were identical (Table 3). Similarly, the cell-sap preparations from these cells were equally capable of supporting polyuridylic acid-stimulated incorporation, even when assayed with the highly active reticulocyte ribosomes. Incubation of the assays was for 45 min at 37 C; accordingly, all of the figures quoted are for extents of incorporation. Rates of incorporation were also measured and were identical. Clearly, therefore, interferon treatment of cells had no detectable effect on either the intrinsic activity of these systems or on their response to polyuridylic acid. In one series of experiments with a particular batch of partially purified interferon, ribosome and cell-sap preparations from interferon-treated cells were 50% less active than corresponding control preparations. This batch of interferon, however, unlike those used in the remainder of these studies, was cytopathic when used at high concentrations (150 to 450 units/ml). As this cytopathic effect was not obvious at the concentrations routinely used (35 to 50 units/ml), these results emphasize the caution necessary in interpreting any differences observed on treatment of cells with only partially purified interferon.

It seemed possible a priori that the antiviral state might only be fully manifest in the interferon-treated cell after infection. Accordingly, the activities of ribosome and cell-sap preparations from control, 5.5 hr-infected, and interferon-treated infected cells were compared. There was no significant difference in the activities of the three cell sap fractions, and, although ribosomes from interferon-treated cells were less active than controls, they were more active than those from infected cells (Table 4). This would argue against any hypothesis involving the triggering by infection of a generalized inhibition of protein synthesis in the interferon-treated cell. Nor was there any evidence for an inhibitory effect of material from interferon-treated cells on the activity of ribosomes from infected cells. These latter were equally active when assayed with cell sap from control and interferon-treated infected cells (Table 4), and, in a separate experiment, the addition of ribosomes from interferon-treated

TABLE 4. *Amino acid incorporation by cell-free systems from interferon-treated infected, infected, and control cells*[a]

Ribosomes	Cell sap	Incorporation ^{14}C-amino acid mixture[b]
Control	Control	13,000
	Interferon-treated infected	11,900
	Infected	10,550
Interferon-treated infected	Control	6,400
	Interferon-treated infected	8,500
	Infected	7,610
Infected	Control	3,200
	Interferon-treated infected	3,500
	Infected	4,300

[a] Details of the interferon treatment and infection of the cells and of the isolation and assay of the ribosome and cell sap fractions are given under Methods. Each result represents the average of at least two assays.

[b] Expressed in counts per minute per 50 μg of ribosomes.

TABLE 5. *Amino acid incorporation by ribosomes from Semliki Forest virus (SFV)-infected cells in the cell-free system in the presence and absence of ribosomes from interferon-treated cells*

Ribosome preparations		Incorporation of ^{14}C-amino acid mixture[a]	
Infected[b]	Interferon[c]	Assayed separately: sum of separate assays	Mixed assay
μg/assay	*μg/assay*		
90		5,380	
90 + 45		8,050	8,900
90 + 90		10,710	10,000
90	33	7,880	8,340
90	66	8,660	9,600
90	99	9,420	9,700

[a] Expressed as counts per minute per assay. The separate and mixed assays were in final volumes of 0.15 and 0.3 ml, respectively. Assays were in the presence of saturating amounts of cell sap from interferon-treated cells (135 μg of cell-sap protein per 0.15-ml assay) under the conditions described under Methods.

[b] Total ribosomes from SFV-infected cells.

[c] "74*S*" ribosomes from interferon-treated cells.

cells had no effect on the activity of ribosomes from infected cells (Table 5). Unfortunately, however, whereas the detection of such an inhibitory effect might have indicated the presence of the hypothetical translation-inhibitory protein or functionally similar substance(s), no firm conclusion is possible in the absence of such an effect.

Formation, breakdown and significance of viral RNA-ribosome complexes in the cell-free system. The results of Marcus and Salb (23) indicated that ribosomes from CEF combined with ^{3}H-labeled Sindbis RNA at 0 C in the cell-free system to form complexes which sedimented on sucrose gradients as a prominent discrete peak in the 250*S* region, where virus-specific polysomes would be expected to appear. On incubation at 37 C, these complexes broke down. This "breakdown" paralleled amino acid incorporation and was interpreted as representing translation of the viral RNA message. With ribosomes from interferon-treated cells, similar complexes were formed (although with reduced efficiency) at 0 C. On incubation at 37 C, however, "breakdown" did not occur, and this was interpreted to result from an inhibition of translation (23). In similar experiments to be described here, ^{3}H-labeled RNA from purified Sindbis virus (Fig. 2) and both ^{3}H-labeled 45*S* SFV RNA (Fig. 1C) and a mixture of 26*S* and 45*S* SFV RNA (Fig. 1B) from CEF infected in the presence of actinomycin D were used, together with "74*S*" ribosome preparations from interferon-treated and control cells (Fig. 3). Incubation of the ^{3}H-labeled RNA with ribosomes in the cell-free system was under the conditions described by Marcus and Salb (23). The RNA bound to the ribosomes was estimated by analysis on sucrose gradients. The values given (Table 6) are for the amount of RNA which formed complexes sedimenting more rapidly than 74*S*. No significant difference in the ability to bind viral RNA under these conditions was observed for ribosomes from control and interferon-treated cells regardless of the source of the RNA (Table 6).

The nature of the viral RNA-ribosome complexes formed was investigated by analysis on sucrose gradients. In a typical experiment (Fig. 4), control "74*S*" ribosomes were incubated at 0 C with 26*S* plus 45*S* ^{3}H-SFV RNA under the conditions of Marcus and Salb (23) prior to analysis. Encephalomyocarditis (EMC) virus was included as a sedimentation marker (156*S*) (6). As always, a diffuse spread of radioactive RNA bound to ribosomes was seen in the polysome region (*see* Fig. 5 to 7). No consistent difference was observed in the gradient patterns obtained with systems from interferon-treated and control

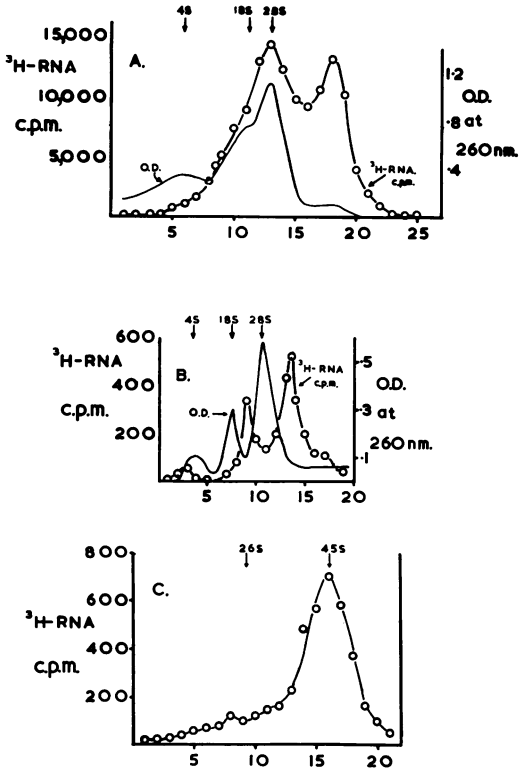

FIG. 1. *Sucrose-gradient analysis of ^{3}H-Semliki Forest virus (SFV) RNA from infected cells. (A) Analysis of total ^{3}H-RNA from the cytoplasm of CEF infected with SFV in the presence of actinomycin D and labeled with ^{3}H-uridine from 1 to 6 hr postinfection. The optical density trace shows 4S sRNA and 18S and 28S ribosomal RNA. (B) Analysis of 26S + 45S ^{3}H-SFV RNA. Analysis of a sample of the RNA present in pooled fractions 13 to 17 from the gradient shown in Fig. 1A. The optical density trace represents 4S, 18S, and 28S mouse ribosomal RNA species included as markers. (C) Analysis of 45S ^{3}H-SFV RNA. Analysis of a sample of the RNA present in pooled fractions 18 to 20 from the gradient shown in Fig. 1A.*

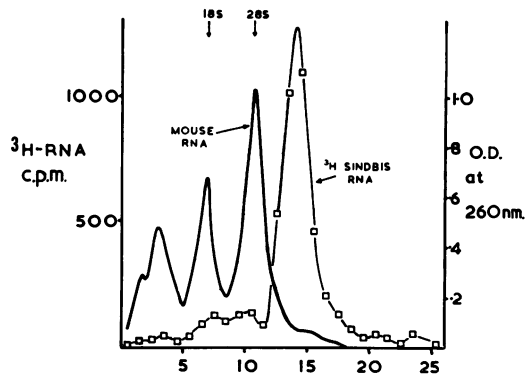

FIG. 2. *Sucrose-gradient analysis of ^{3}H-Sindbis RNA from purified virus. Mouse soluble and ribosomal RNA species were included as sedimentation markers.*

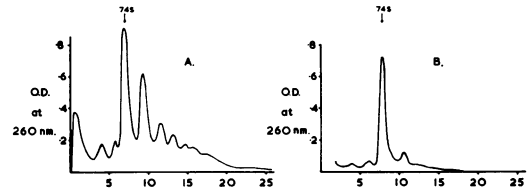

FIG. 3. *Sucrose gradient analysis of total (A) and "74S" (B) ribosome preparations from CEF. Centrifugation was at 4 C on 30 ml of 7.5 to 45% sucrose gradients in TKM buffer for 3.25 hr at 65,000 × g in an SW 25 rotor of a modified MSE 50 ultracentrifuge. Sedimentation was from left to right.*

cells (*see* Fig. 6). Many attempts were made with the different viral RNA and ribosome preparations to detect a discrete peak of virus-specific polysomes of the type described by Marcus and Salb (23), without success. The very small peak of radioactivity observed towards the bottom of the gradient in Fig. 7 is an artifact of the collection of the gradient consistently observed with the Isco recording apparatus. No such peak was ever observed with alternative methods of collection. Among the variations tried were the use of fresh and frozen cell saps prepared by the method of Marcus and Salb (23), the use of unfrozen viral RNA, an increase in Mg^{2+} concentration to 15 mM and, finally, incubation under the conditions routinely used by us in the amino acid incorporation studies described above [these differed slightly from those described by Marcus and Salb (23) and gave somewhat higher (20 to 40%) incorporation activities]. Nor did such a discrete peak of polysomes form on incubation of these systems for brief periods (2 to 10 min) at 37 C.

When viral RNA, ribosomes, and cell sap were mixed at 0 C and incubated at 37 C, there was a gradual breakdown of the viral RNA-ribosome complexes (Fig. 5), irrespective of whether the ribosomes were from interferon-treated or control cells (Fig. 6). The results in Fig. 6 were obtained with ribosomes from each of these sources incubated in the cell-free system with unfrozen ^{3}H-Sindbis RNA and freshly prepared undialyzed cell sap of the type described by Marcus and Salb. Similar results were obtained with both types (Fig. 1B and C) of ^{3}H-SFV RNA in the routine system with dialyzed and concentrated cell sap from either inferferon-treated or control cells.

To determine whether such "breakdown" paralleled protein synthesis in these systems, the fate of the viral RNA-ribosome complexes was followed under conditions inhibitory to amino acid incorporation (Fig. 7). ^{3}H-SFV RNA bound to ribosomes in a cell-free system held at 0 C was

compared, by analysis on sucrose gradients, with that from three other systems each incubated 25 min at 37 C—one in the complete system, one in the absence of the ATP-generating system, and

TABLE 6. *Binding of ^{3}H-labeled viral RNA to ribosomes in the cell-free system*[a]

RNA preparation	Ribosomes	RNA bound to ribosomes	
		Counts/min	Fraction of added RNA
			%
SFV RNA (26 + 45S)	Control	14,300	58
	Interferon	19,070	81
SFV RNA (45S)	Control	750	48
	Interferon	562	36
Sindbis virus RNA	Control	1,580	76
	Interferon	1,540	72
Sindbis virus RNA[b]	Control[c]	555	61
	Interferon[c]	694	77
Sindbis virus RNA	Control[d]	2,361	77
	Interferon[d]	2,395	78

[a] Details of the assay conditions are given under Methods. "74S" ribosomes and control-cell cell sap were used throughout. Incubations (second stage of the Marcus and Salb type assay) were for 45 min at 0 C. The "RNA bound to ribosomes" was that fraction of the ^{3}H-RNA sedimenting at, or more rapidly than, 74S in a 7.5 to 45% sucrose gradient of the type shown in Fig. 5 and 7.

[b] Fresh unfrozen Sindbis virus RNA.

[c] Assayed in the presence of unfrozen cell sap freshly prepared according to the method of Marcus and Salb (23).

[d] Ribosomes from cells exposed to a pulse of puromycin.

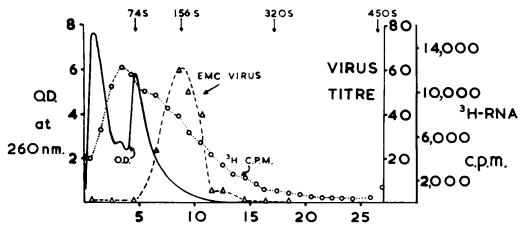

FIG. 4. *Sucrose-gradient analysis of the complexes formed on incubation of 26S plus 45S ^{3}H-SFV RNA with "74S" control-cell ribosomes at 0 C in the cell-free system. Centrifugation was at 4 C for 90 min at 65,000 × g in the SW25 rotor of a modified MSE 50 ultracentrifuge. The encephalomyocarditis (EMC) virus was included as a sedimentation marker; its titer is in hemagglutination units (6). The higher S values given here and in Fig. 5, 6, and 7 were calculated by extrapolation from the distances moved by the 74S ribosome and 156S virus markers.*

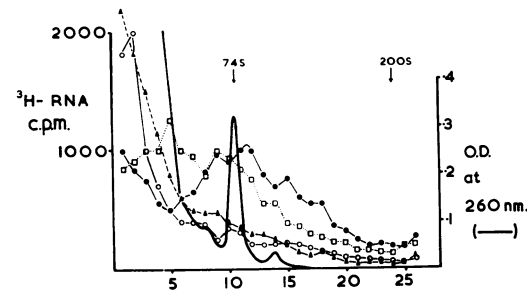

FIG. 5. *Sucrose-gradient analyses showing breakdown of the ^{3}H-viral RNA-ribosome complexes on incubation at 37 C in the cell-free system. Control-cell "74S" ribosomes were mixed with 26S plus 45S ^{3}H-SFV RNA and incubated in the complete cell-free system with control-cell cell sap. Incubation was for 45 min at 0 C (●) and for 5 (□), 25 (▲), and 45 (○) min at 37 C. Centrifugation was for 90 min at 80,000 × g in the no. 30 rotor of the Spinco model L ultracentrifuge.*

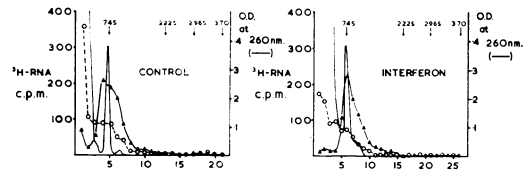

FIG. 6. *Sucrose-gradient analyses comparing the breakdown in the cell-free system of the complexes formed by ^{3}H-Sindbis virus RNA with ribosomes from interferon-treated and control cells. "74S" ribosomes from interferon-treated and control cells were mixed with ^{3}H-Sindbis RNA from purified virus (Fig. 2) and incubated in the complete cell-free system in the presence of fresh, unfrozen cell sap prepared from control cells, as described by Marcus and Salb (23). Incubations were for 25 min at 0 C (▲) and 25 min at 37 C (○). Centrifugation on sucrose gradients was for 45 min at 80,000 × g in the no. 30 rotor of the Spinco model L ultracentrifuge.*

the third in the presence of cycloheximide. The inhibitory effect of these latter two conditions on endogenous amino acid incorporation is shown in the lower graph (Fig. 7B). Despite this inhibition, breakdown of the RNA-ribosome complexes occurred as rapidly in these systems as in the control (Fig. 7A).

As there appeared to be no correlation between "breakdown" and amino acid incorporation, nucleolytic degradation of the viral RNA in these complexes seemed a likely alternative explanation. Indeed, the various preparations used were found to have significant nuclease activity, although no more than other typical cell-free systems (Table 7). Despite this, much of the viral

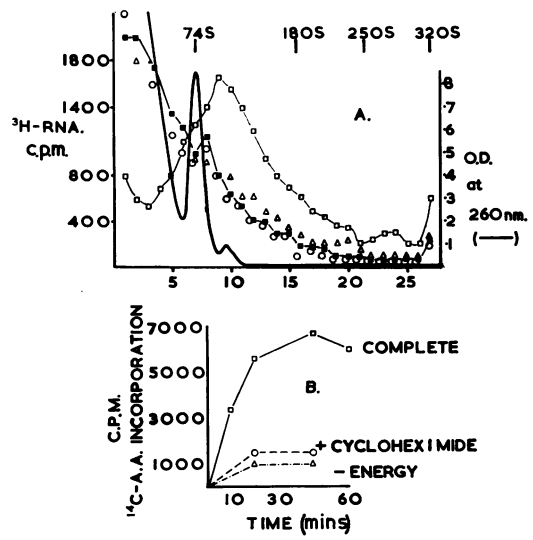

FIG. 7. *Breakdown of ^{3}H-viral RNA-ribosome complexes in the cell-free system under conditions inhibitory to amino acid incorporation. (A) "74S" ribosomes from interferon-treated cells were mixed with ^{3}H-SFV RNA and incubated for 25 min with control-cell cell sap in the complete cell-free system (□) at 0 C; (■) at 37 C; (○) at 37 C in the presence of 3.6 mM cycloheximide; and (Δ) at 37 C in the absence of ATP, GTP, and phosphoenolpyruvate (energy). Centrifugation on sucrose gradients was for 50 min at 80,000 × g in the no. 30 rotor of the Spinco model L ultracentrifuge. (B) ^{14}C-leucine incorporation in the cell-free system under the conditions of incubation used in A. The amino acid incorporation activity of total ribosomes from control cells was assayed under conditions identical to those used above: the assays were carried out in parallel to those in A by using the same solutions. Addition of ^{3}H-SFV RNA, at the concentrations used in A, to the complete cell-free system had no effect on incorporation. The abscissa represents the time of incubation of the assays at 37 C.*

RNA survived for a sufficient time in the cell-free system for it, in theory, to function as messenger. Samples of ^{3}H-labeled 45S SFV RNA were incubated in the cell-free system for 60 min at 0 C and for 10, 20, and 45 min at 37 C, extracted with phenol-SDS at 37 C, and fractionated on sucrose gradients with marker ^{14}C- 26S plus 45S SFV RNA (Fig. 8). The incubation at 0 C had little effect on the RNA. More than 50% of it apparently remained intact after 10 min at 37 C, and 30% survived the first 20 min of incubation (Fig. 8). Clearly, this method of analysis would not detect "hidden breaks" in the RNA, but similar studies concerning the survival of infectivity of EMC RNA in the cell-free system (I. M. Kerr, *unpublished results*) provided further evidence for an unexpected degree of resistance of viral RNA to nucleolytic degradation in these systems. An attempt to saturate the contaminating nuclease by the addition of excess unlabeled ribosomal RNA had no effect on the formation or breakdown of the viral RNA-ribosome complexes.

Having established that "breakdown" did not necessarily parallel amino acid incorporation and could not be taken as an index of it, the question arose as to whether the binding observed in the formation of the viral RNA-ribosome complexes was of the type involved in the functional binding of mRNA. No conclusive answer to this question

TABLE 7. *Nucleolytic degradation of ^{3}H-Semliki Forest virus (SFV) RNA on incubation in the cell-free system*

Ribosomes	Cell sap	Time at 37 C (min)	Acid-insoluble RNA recovered after incubation	
			Counts/min	Fraction of added RNA
				%
No additions[a]	No additions[a]	0	1,880	100
		30	1,860	99
	Chick liver (60 μg)	0	1,737	92
	Chick liver (60 μg)	25	1,430	76
	Control (57 μg)	25	1,524	81
	Interferon (52 μg)	25	1,650	88
	Rabbit reticulocyte[b] (50 μg)	25	1,359	72
	Control (114 μg)	30	1,520	81
	Control[c] (130 μg)	30	1,560	83
Control (20 μg)	Chick liver (60 μg)	25	1,305	69
Control (20 μg)	Control (60 μg)	25	1,347	71
Control (20 μg)	Rabbit reticulocyte[b] (50 μg)	25	1,274	68

[a] Complete system as for the second stage of the assay of the interaction of ^{3}H-viral RNA with ribosomes, but omitting ribosomes and cell sap.

[b] The rabbit reticulocyte (*p*H 5) enzyme fraction was the kind gift of A. R. Williamson. In all assays involving its use, transfer RNA from rabbit reticulocytes was included in sufficient quantity to support optimum amino acid incorporation.

[c] Unfrozen cell sap was freshly prepared by the method of Marcus and Salb (23).

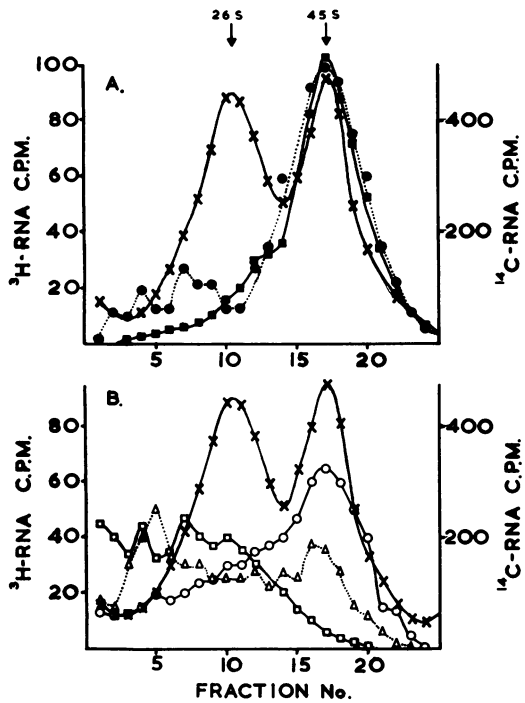

FIG. 8. *Sucrose-gradient analysis of ^{3}H-Semliki Forest virus (SFV) RNA reextracted after various periods of incubation in the cell-free system. 45S ^{3}H-SFV RNA was incubated with 74S ribosomes from control cells in the complete cell-free system in the presence of control-cell cell sap, extracted with phenol-SDS at 37 C, ethyl alcohol-precipitated, stored, and analyzed on 5 ml of 5 to 20% sucrose in TKE buffer gradients. Centrifugation was at 4 C for 2.5 hr at 130,000 × g in the SW39 rotor of the MSE 50 ultracentrifuge. 26S + 45S ^{14}C-SFV RNA was included as a sedimentation marker (×). (A) Untreated 45S ^{3}H-SFV RNA (■) and ^{3}H-SFV RNA incubated for 60 min at 0 C (●). (B) 45S ^{3}H-SFV RNA incubated for 10 min (○), 20 min (△), and 45 min (□) at 37 C in the cell-free system. The total recovery of acid-insoluble ^{3}H-SFV RNA after incubation for 60 min at 0 C was 95% and after 45 min at 37 C was 70% of that initially added to each system.*

was obtained, but several experiments which bear upon it were carried out. Cell sap was essential for the binding of both types of ^{3}H-SFV RNA to ribosomes, but, strangely, there was no such requirement in the case of Sindbis RNA. Sucrose gradient analyses produced no evidence for the formation of a viral RNA-cell sap protein complex sedimenting at >70S, such as was described by Girard and Baltimore (13) for poliovirus RNA. The results of competition studies were conflicting, for, although the addition of excess ribosomal RNA had no effect on the binding of viral RNA, the latter was equally unaffected (at 7.5 or 15 mM Mg^{2+}) by the addition of saturating amounts of polyuridylic acid, irrespective of whether this was added before or after the viral RNA.

Finally, Darnell's group has used as a criterion for the true polysome nature of ribonucleoprotein-RNA complexes sedimenting in the polysome region of sucrose gradients the fact that, in HeLa cells at least, polysomes break down very rapidly (within 2 min at 37 C) on exposure to puromycin at concentrations inhibitory to protein synthesis (J. E. Darnell, *personal communication*; *see* also references 15, 18). In cell-free systems from CEF, however, although puromycin at 0.4 mM inhibited amino acid incorporation by 65%, the effect of its presence from 2 to 10 min of incubation at 37 C on naturally occurring polysomes was slight and could only be detected with certainty by an increase in the relative size of the peak of monomeric ribosomes. A similar effect of the addition of puromycin was observed on occasion, with the viral RNA-ribosome complexes formed with ribosomes from both interferon-treated and control cells, but the results were variable.

Interestingly, with ribosomes from cells which had been treated with puromycin, the ribosome-viral RNA complexes formed in the cell-free system both sedimented more rapidly (although still as a broad shoulder) on sucrose gradients and appeared to be more stable to incubation at 37 C than did those from corresponding preparations from untreated cells. The reason for this is not known.

Putting these observations together, they do perhaps suggest some specificity in the binding of viral RNA with respect to messenger function, but the several conflicting results make any firm conclusion impossible.

DISCUSSION

Throughout these studies, in agreement with Marcus and Salb (23, 24) and Carter and Levy (4), we found no difference in the cell-free system between the intrinsic amino acid incorporation activities of ribosome and cell-sap preparations from interferon-treated and from control cells, or in their response to polyuridylic acid. Moreover, although it seemed possible a priori that interferon could act through a generalized inhibition of protein synthesis which is not expressed until triggered by infection, our results with material from interferon-treated infected cells give no support to this hypothesis.

Despite many experiments under a variety of conditions, we were unable to detect the formation of a discrete peak of virus-specific polysomes,

when "74S" ribosomes, regardless of source, were incubated with labeled Sindbis or SFV RNA. Some attachment of viral RNA to ribosomes occurred, but on analysis on sucrose gradients this was evident as a broad shoulder extending from the 74S peak to approximately 300S. No difference in the efficiency of formation of such complexes was observed for ribosomes from interferon-treated and control cells, nor was any difference seen in the rate of breakdown of the complexes at 37 C. Experiments with inhibitors of protein synthesis indicated that this "breakdown" was not related to amino acid incorporation, and hence could not be taken as a reliable index of translation. In all of these respects our results are in marked contrast to those of Marcus and Salb (23).

Obviously, in such complex systems, minor differences in methodology could be crucial. For example, the conditions of growth and treatment with interferon were different in our experiments from those for the specially aged cells used by Marcus and Salb (J. Salb, *personal communication*). Although, using 7-day-old cells Toy and Lockart obtained results identical to our own (R. Z. Lockart, *personal communication*), it is still impossible to exclude an effect of other, as yet unrecognized, differences in procedure. Recently, however, Marcus and Engelhardt (P. I. Marcus, *personal communication*) have found that "breakdown" of the virus-specific polysomes formed in vitro in their systems does not, as first reported by Marcus and Salb (23), correlate with amino acid incorporation. They, too, would now conclude, therefore, that such "breakdown" does not necessarily involve translation of the viral RNA. Accordingly, whereas it would still be intriguing to know the reason for these conflicting results, it seems likely that the differences observed have no functional significance.

In summary, as described elsewhere, we have found no consistent difference in the protein composition of ribosomes from interferon-treated and control cells (29), and, in the experiments reported here, we observed no differences in the activity of such preparations in the cell-free system. It is of interest, therefore, that Guggenheim et al. (14) have concluded, from their studies concerning the response of human-chick cell heterokaryons to human and chick interferons, that it is unlikely that interferon induces a modification in the ribosomes. Although our results clearly do not disprove the hypothesis (23) that interferon acts through an inhibition of translation at the ribosomal level, they have served to focus our attention on possible alternatives. As early as 1966, Joklik and Merigan followed the fate of vaccinia virus mRNA in interferon-treated cells and concluded that it did not combine with ribosomes to form virus-specific polysomes (16), a result which the authors emphasized could be in accord with alterations at a number of levels besides the ribosome, as indeed could much of the work reported by Carter and Levy (3, 19). From studies with cell-free systems, these latter authors have presented evidence that ribosomes from interferon-treated cells showed a reduced ability to bind viral RNA (3) and a negligible incorporation of amino acids in response to it (4), whereas their studies on the fate of ^{3}H-labeled parental viral RNA in cells in tissue culture showed a reduction in the ability of such virus RNA to combine with what was taken to be the 40S ribosomal subunit of interferon-treated cells (19).

It would seem to us, therefore, that the apparently conflicting results in this field could be resolved if the alteration in the interferon-treated cell were to involve a factor(s) required for virus protein synthesis which is not an integral part of the ribosome, but which could be associated with it under certain isolation conditions. There are numerous reports in the literature suggesting the possible involvement of factors of this type, for example, in the initiation of protein synthesis (2, 12, 26, 28).

Recently, we have returned to EMC virus as a convenient source of viral RNA active in the cell-free system (17). Preliminary analysis of the peptides produced by tryptic digestion of the product synthesized would indicate that incorporation in response to this RNA in the cell-free system is into a specific viral protein(s), and initial studies with it have provided results which would be in accord with the scheme outlined above. Nonetheless, only when the nature of the product formed in such systems has been confirmed and the factors required for the initiation and maintenance of protein synthesis have been elucidated, will the resolution of these many conflicting observations be possible, and with this, perhaps, an understanding of the mode of action of interferon will be achieved.

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