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Cell-free synthesis of human interferon

[protein synthesis/poly(A)-containing mRNA/human fibroblasts/poly(I)·poly(C)/oligo(dT)-cellulose]

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ABSTRACT **With mRNA prepared from induced human fibroblasts biologically active human interferon was synthesized *de novo* in a cell-free extract from mouse cells. The identity of the antiviral activity as human interferon was demonstrated by its species and antigenic specificity.**

Interferon was characterized as an antiviral entity by Isaacs *et al.* (1, 2) in 1957. Since that time a vast literature on interferon has accumulated (see refs. 3–5 for reviews). Viruses, isolated double-stranded RNA, and other compounds have been found to stimulate interferon synthesis by cells. Interferon production serves as a valuable model of induced protein synthesis in eukaryotic cells. At present, however, relatively little is known about the molecular mechanisms of interferon induction and biosynthesis. To study interferon biosynthesis and gene expression, we have partially purified interferon messenger RNA from human fibroblasts and have translated this mRNA in a cell-free system from mouse cells with the resultant production of biologically active human interferon.

MATERIALS AND METHODS

Chemicals. Poly(I)·poly(C) was supplied by the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases. Cycloheximide was obtained from the Upjohn Co.; gentamicin, from Schering Corp.; and actinomycin D, from Calbiochem.

Preparation of Human Fibroblasts Stimulated with Poly(I)·Poly(C). Each 0.5 gallon roller bottle was seeded with about 1×10^7 cells of human FS-4 fibroblasts in 150 ml of Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum and 50 μg of gentamicin per ml (6, 7). Nine days after seeding, the cultures were washed once with solution A (137 mM NaCl, 27 mM KCl, 9 mM $CaCl_2$, 5 mM $MgCl_2$, 81 mM Na_2HPO_4, and 15 mM KH_2PO_4) and incubated at 36° for 4 hr with 50 ml of serum-free MEM containing 100 μg/ml of poly(I)·poly(C) and 50 μg/ml of cycloheximide (6, 7). After this 4-hr incubation, the induced cultures were washed with ice-cold solution A and the cells were scraped off the bottles into fresh ice-cold solution A with a rubber policeman. The harvested cells were sedimented by centrifugation in the GSA rotor of the Sorvall RC-2B centrifuge at 4° and 2000 rpm for 10 min. The supernatant fraction was decanted, and the cell pellet was frozen and stored at −70° until used for the preparation of mRNA.

Preparation of mRNA from Cells. Poly(A)-containing mRNA was prepared from human fibroblasts stimulated with poly(I)·poly(C) by phenol extraction and oligo(dT)-cellulose chromatography as described (8, 9).

Cell-Free Protein Synthesis with mRNA Fractions in Mouse Ehrlich Ascites Extracts. Messenger RNA fractions were translated in a mouse Ehrlich Ascites cell-free extract as described (8, 9). Each 0.050-ml reaction mixture contained the following components: 0.15–0.23 mg of S-30 protein; 70 μg of reticulocyte polysomal salt wash fraction protein (8, 9); 0.05–0.1 A_{260} unit of mRNA; 10 mM Tris·HCl (pH 7.5) or 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.5); 120 mM KCl; 3.5 mM $MgCl_2$; 1 mM ATP; 0.1 mM GTP; 5 mM creatine phosphate; 8 μg of creatine kinase; 2 mM 2-mercaptoethanol; 0.13 μCi each of [^{14}C]leucine, [^{14}C]valine, and [^{14}C]alanine of specific activity 348, 265, and 171 mCi/mmol, respectively; and each of the unlabeled amino acids at a concentration of 40 μM. Incubations were performed for 60 min at 30°. Protein synthesis was determined by amino-acid incorporation into hot trichloroacetic acid-precipitable material. In addition, the interferon titer of the cell-free incubations stimulated by various messenger RNA fractions as well as control RNA fractions was assayed as described below.

Assay for Interferon Activity. A modification of the semimicro method described by Armstrong (10) was used. Individual wells of Micro Test II tissue culture plates (Falcon Plastics) were first filled with 100 μl of MEM with 5% fetal calf serum, which had been buffered with sodium bicarbonate (0.15%), Hepes (13.2 mM), and tricine (6.6 mM) to pH 7.6 and contained 100 μg of gentamicin and 2.5 μg of amphotericin B (Fungizone) per ml. The reaction mixture for cell-free protein synthesis was diluted 4-fold with this medium and assayed directly for interferon activity. To prepare a series of 2-fold dilutions, 100 μl of the sample to be titrated was added to the first well containing the same volume of medium. Serial dilutions were prepared by transferring 100 μl of this mixture to the adjacent well after thorough mixing with an automatic micropipette. Each well was then seeded with 30,000 FS-7 human fibroblasts (obtained by trypsinization of confluent flask cultures) in 100 μl of the above medium. After 18 hr of incubation at 36° in a 5% CO_2 atmosphere, each well received 1000 plaque-forming units (PFU) of the Indiana-type vesicular stomatitis virus in 50 μl of the medium described above. Several wells on each plate served as virus controls and cell controls. The titrations were scored microscopically 48 hr after virus inoculation, when the virus controls showed complete destruction by the virus. The highest dilution of the titrated sample causing at least 50% protection of cells was considered the end point. All interferon titers are expressed in terms of reference units/ml, calibrated against the 69/19 reference standard for human interferon (6). It should be noted that the samples assayed for interferon activity were coded so that the individuals per-

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MEM, Eagle's minimal essential medium; PFU, plaque-forming units.

Table 1. Human interferon synthesis in mouse cell-free extracts stimulated by RNA fractions from induced and uninduced human fibroblasts

Oligo(dT)-cellulose RNA fraction	Cell source	Interferon activity (units/ml)
None	—	<16
Flow through	Human fibroblasts induced with poly(I)·poly(C) and cycloheximide	<16
0.1 M KCl wash	Human fibroblasts induced with poly(I)·poly(C) and cycloheximide	<16
mRNA bound	Human fibroblasts induced with poly(I)·poly(C) and cycloheximide	68
Flow through	Human fibroblasts treated with cycloheximide only	<16
0.1 M KCl wash	Human fibroblasts treated with cycloheximide only	<16
mRNA bound	Human fibroblasts treated with cycloheximide only	<16
Flow through	Human fibroblasts (uninduced)	<16
0.1 M KCl wash	Human fibroblasts (uninduced)	<16
mRNA bound	Human fibroblasts (uninduced)	<16

The RNA fractions were prepared from induced and uninduced human fibroblasts as described in *Materials and Methods*. All the RNA preparations were fractionated on oligo(dT)-cellulose into flow through, 0.1 M KCl wash, and KCl-free wash [poly(A)-containing bound mRNA] fractions as reported (8, 9). Cell-free incubations and interferon assays were performed as described in *Materials and Methods*. All assays were performed in duplicate. Assays in the absence of RNA and in the presence of RNA from induced cells were performed in quadruplicate. All samples listed as <16 were negative at the lowest dilution that could be tested.

forming the interferon assays were not aware of the composition of the cell-free reaction mixtures.

Neutralization of Interferon. Interferon, prepared in the cell-free extracts, was neutralized with the use of rabbit antiserum to human interferon as described (11, 12).

Inhibition of Interferon Activity in Cells Treated with Actinomycin D. Human fibroblasts (FS-7 cell strain) were seeded into the wells of a Micro Test II plastic tissue culture plate (30,000 cells per well). Two days after seeding, the cells in two wells were treated for 30 min with actinomycin D (2 μg/ml in 100 μl of MEM) while two wells received the same medium without actinomycin D. After 30 min the cultures were washed. One actinomycin D-treated and one control culture were incubated for 6 hr with 100 μl of the cell-free product, diluted 1/20 in MEM containing 5% fetal bovine serum (giving a final concentration of 4 reference units of interferon per ml). The two remaining cultures (one actinomycin D-treated and one control) were incubated in medium without interferon. The wells were then washed and inoculated with the Indiana type of vesicular stomatitis virus (about 3 PFU per cell). The virus was allowed to adsorb for 1 hr and the cells were then washed four times to remove unadsorbed virus. Each culture was replenished with 200 μl of MEM containing 5% fetal bovine serum. Culture fluids were collected 18 hr after virus inoculation, and the yields of virus were determined by plaque titration in cultures of mouse L cells.

RESULTS

Synthesis of human interferon in cell-free extracts

RNA fractions were prepared from induced and uninduced human FS-4 fibroblasts. Induced cells were stimulated with poly(I)·poly(C) and cycloheximide as described in *Materials and Methods*. Uninduced cells were treated with cycloheximide only or not treated. Fractions not containing poly(A), which passed directly through oligo(dT)-cellulose (flow through), as well as poly(A)-containing fractions, which were bound to oligo(dT)-cellulose, were translated in a cell-free ascites system (Table 1). The results indicate that interferon activity (68 units/ml) was synthesized only in the cell-free extracts directed by poly(A)-containing fractions from induced human fibroblasts. RNA fractions not bound to the oligo(dT)-cellulose from induced human fibroblasts and all fractions from uninduced human fibroblasts stimulated no detectable synthesis of human interferon activity in the cell-free extracts (Table 1). All fractions from mouse plasmacytoma cells (data not shown) stimulated no detectable synthesis of human interferon activity in the cell-free extracts.

Evidence for *de novo* synthesis of human interferon

The interferon activity formed in the cell-free incubations dependent on exogenous mRNA could represent *de novo* synthesis of interferon or, possibly, interferon or poly(I)·poly(C) carried over in the oligo(dT)-cellulose bound fraction. To differentiate between these alternatives, interferon activity was examined in cell-free reactions in the presence and absence of the 60 min incubation at 30° (Table 2). The data show that only reaction mixtures incubated at 30° for 60 min yielded interferon activity. In addition, sparsomycin, a specific inhibitor of protein synthesis (13), abolished the synthesis of human interferon in these cell-free extracts (Table 2).

Species specificity of human interferon synthesized in the cell-free system

Interferons exhibit species specificity (3–5). Thus, human interferons are devoid of antiviral activity in cultures of chick

Table 2. Synthesis of human interferon in mouse cell-free extracts: Dependence on incubation and inhibition by sparsomycin

Additions	Min at 30°	Interferon activity (units/ml)
None	60	<16
mRNA	60	40
mRNA	0	<16
mRNA + 33 μM sparsomycin	60	<16
33 μM Sparsomycin, no mRNA	60	<16

The mRNA was prepared from induced human fibroblasts and fractionated on oligo(dT)-cellulose as described in *Materials and Methods*. The mRNA fraction eluted from oligo(dT)-cellulose in the absence of KCl was used. Cell-free incubations and interferon assays were performed as described in *Materials and Methods*. All assays were performed in duplicate.

or mouse cells. The material synthesized in the cell-free mouse extracts with mRNA from induced human fibroblasts was active with human cells, but not in chick or mouse cells (Table 3). This species specificity was thus consistent with the activity synthesized being identical to human interferon.

Neutralization of human interferon with antisera against interferon

Rabbit antisera prepared against human FS-4 cell interferon were shown to neutralize the activity of homologous human interferon (11). The same antisera exerted no neutralizing activity against interferons from some heterologous human cells (12). Thus, it was determined if the activity synthesized in the cell-free extract could be neutralized with antiserum prepared against FS-4 human interferon. The antiviral activity synthesized in the cell-free extract was indeed neutralized with this antiserum (Table 4), indicating that the active product is antigenically related to human fibroblast interferon. Approximately the same dilution of antiserum was required for neutralization of human interferon produced by intact FS-4 cells as for neutralization of a comparable quantity of antiviral activity synthesized in the cell-free extract.

Prevention of cell-free interferon activity in cells treated with actinomycin D

Activity of interferon can be blocked by treating cells with actinomycin D prior to exposure to interferon (3–5). Thus, the ability of actinomycin D to prevent interferon action provides another criterion for identification of the cell-free activity. The data of Table 5 demonstrate that prior treatment of cells with actinomycin D blocked the antiviral activity synthesized in the cell-free extract.

Table 4. Neutralization of antiviral activity synthesized in cell-free extracts with antiserum against human fibroblast interferon

Interferon source	Neutralization titer
Cell-free product	1:800
Standard interferon preparation	1:1500

The neutralization titer is expressed as the highest dilution of antiserum prepared against human fibroblast interferon neutralizing 10 reference units of interferon activity per ml. The cell-free product was prepared with the use of mRNA prepared from human fibroblasts treated with poly (I)·poly(C). The standard interferon preparation was produced by intact human FS-4 cells stimulated with poly(I)·poly(C) as described (6).

DISCUSSION

The results demonstrate that mRNA for human interferon can be isolated from human fibroblasts stimulated by poly(I)·poly(C). This mRNA was used in a cell-free mouse extract to produce biologically active human interferon.

The following evidence indicates we have synthesized human interferon *de novo:* synthesis of interferon was dependent on addition of mRNA from induced human fibroblasts, and synthesis required incubation at 30°. In addition, a specific inhibitor of protein synthesis, sparsomycin, abolished synthesis of interferon.

The antiviral activity synthesized had the species specificity characteristic of interferon. It was active on human cells, but not on chick or mouse cells. In addition, specific antiserum against human fibroblast interferon inactivated the activity synthesized in the mouse cell-free extract. Furthermore, as with interferon, the antiviral activity of the cell-free synthesized material was not expressed in cells treated with actinomycin D. The results, therefore, are consistent with the *de novo* synthesis of human interferon in a cell-free extract from mouse cells. We have used a tracer amount of radioactivity in these experiments (see Table 3 legend). With the use of a greater number of labeled amino acids of high specific activity, highly labeled interferon could be synthesized.

While this work was in progress, Reynolds, Zicha, and Pitha (14) also reported the cell-free synthesis of human in-

Table 3. Species specificity of active cell-free product

mRNA from induced human fibroblasts	Interferon activity (units/ml)		
	Human cells	Chick cells	Mouse cells
Exp. 1			
None	<20	<20	—
mRNA	80	<20	—
Exp. 2			
None	<20	—	<20
mRNA	40	—	<20

The mRNA prepared from human fibroblasts stimulated with poly(I)·poly(C) was translated in mouse ascites S-30 and assayed for interferon activity as described in *Materials and Methods* and the legend of Table 1. In the absence of mRNA, 27 and 24 pmol of [^{14}C]aminoacids were incorporated into protein in Exps. 1 and 2, respectively. In the presence of induced mRNA, 74 and 56 pmol of [^{14}C]aminoacids, respectively, were incorporated.

Table 5. Effect of actinomycin D on antiviral activity produced in cell-free extracts

Interferon treatment of cells	Virus yield (PFU/ml × 10^{-5})	
	No actinomycin D	Plus actinomycin D
Exp. 1		
None	30	32
Cell-free product	0.9	21
Exp. 2		
None	46	32
Human fibroblast interferon	2.6	34

The ability of actinomycin D to prevent the action of interferon was determined as described under *Materials and Methods*. The cell-free product was identical to the preparation used in Table 4. The translational product in the ascites cell-free extract was diluted 1/20 directly into MEM containing 5% fetal bovine serum.

terferon. Previously, RNA isolated from cells induced to produce interferon was used to produce interferon in heterologous intact cells (15–17). It was concluded in those studies that mRNA for interferon was translated in the heterologous intact cells.

The results demonstrate that biologically active human interferon can be synthesized in cell-free extracts. Biochemical and physical characterization of the cell-free product will serve to answer whether it is synthesized as a precursor and whether carbohydrate substitutions are required for full biological activity as was suggested previously (11). We do not know whether the active cell-free product was glycosylated. Study of interferon mRNA in stimulated and unstimulated fibroblasts should provide some insight into the control and mechanism of interferon biosynthesis. In general, the ability to isolate and translate interferon mRNA in a cell-free system will permit us to investigate the biosynthesis of interferon and its gene expression.

In addition, human interferon may be the first biologically active eukaryotic protein synthesized in a cell-free system dependent on exogenous mRNA. The synthesis of a biologically active protein indicates that the cell-free system not only is capable of synthesizing the primary amino acid sequence, but also has the capacity for performing those post-translational processes, if any, required for producing a biologically functional molecule. In the cell-free system all these processes can be delineated.

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1. Isaacs, A. & Lindenmann, J. (1957) "Virus Interference. I. The interferon," *Proc. R. Soc. London Ser. B* **147**, 258–267.
2. Isaacs, A., Lindenmann, J. & Valentine, R. C. (1957) "Virus interference. II. Some properties of interferon," *Proc. R. Soc. London Ser. B* **147**, 268–273.
3. Ng, M. H. & Vilček, J. (1972) "Interferons: Physicochemical properties and control of cellular synthesis," *Adv. Protein Chem.* **26**, 173–241.
4. Colby, C. & Morgan, M. J. (1971) "Interferon induction and action," *Annu. Rev. Microbiol.* **25**, 333–360.
5. DeClercq, E. & Merigan, T. C. (1970) "Current concepts of interferon and interferon induction," *Annu. Rev. Med.* **21**, 17–46.
6. Havell, E. A. & Vilček, J. (1972) "Production of high-titered interferon in cultures of human diploid cells," *Antimicro. Ag. Chemother.* **2**, 476–484.
7. Vilček, J. & Havell, E. A. (1973) "Stabilization of interferon messenger RNA activity by treatment of cells with metabolic inhibitors and lowering of the incubation temperature," *Proc. Nat. Acad. Sci. USA* **70**, 3909–3913.
8. Green, M., Graves, P. N., Zehavi-Willner, T., McInnes, J. & Pestka, S. (1975) "Cell-free translation of immunoglobulin messenger RNA from MOPC-315 plasmacytoma and MOPC-315 NR, a variant synthesizing only light chain," *Proc. Nat. Acad. Sci. USA* **72**, 224–228.
9. Green, M., Zehavi-Willner, T., Graves, P. N., McInnes, J. & Pestka, S. (1975) "Isolation and cell-free translation of immunoglobulin messenger RNA," *Arch. Biochem. Biophys.* **170**, in press.
10. Armstrong, J. A. (1971) "Semi-micro, dye-binding assay for rabbit interferon," *Appl. Microbiol.* **21**, 723–725.
11. Havell, E. A., Vilček, J., Falcoff, E. & Berman, B. (1975) "Suppression of human interferon production by inhibitors of glycosylation," *Virology* **63**, 475–483.
12. Havell, E. A., Berman, B., Ogburn, C. A., Berg, K., Paucker, K. & Vilček, J. (1975) "Two antigenically distinct species of human interferon," *Proc. Nat. Acad. Sci. USA* **72**, 2185–2187.
13. Pestka, S. (1971) "Inhibitors of ribosome functions," *Annu. Rev. Micro.* **25**, 487–562.
14. Reynolds, F. H., Zicha, M. & Pitha, P. M. (1975) "Biochemical characterization of human interferon activity produced in a cell-free protein synthesis system," *Abstracts of the Annual Meeting of the American Society for Microbiology* (S339), 270.
15. De Maeyer-Guignard, J., DeMaeyer, E. & Montagnier, L. (1972) "Interferon messenger RNA: Translation in heterologous cells," *Proc. Nat. Acad. Sci. USA* **69**, 1203–1207.
16. Montagnier, L., Collandre, H., DeMaeyer-Guignard, J. & De Maeyer, E. (1974) "Two forms of mouse interferon messenger RNA," *Biochem. Biophys. Res. Commun.* **59**, 1031–1038.
17. Reynolds, F. H., Jr. & Pitha, P. M. (1974) "The induction of interferon and its messenger RNA in human fibroblasts," *Biochem. Biophys. Res. Commun.* **59**, 1023–1030.