Interferon Messenger RNA: Translation in Heterologous Cells*

(eukaryotes/mouse/monkey/chick/VERO)

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ABSTRACT A viral inhibitor with the characteristics of mouse interferon is produced by avian and simian cells preincubated with RNA extracted from interferon-producing mouse cells. Similarly, RNA extracted from interferon-producing monkey cells induces a monkey interferon-like substance in avian cells and also in a line of simian cells, VERO, which normally lacks the capacity to produce its own interferon. In both cases, the RNA effect is inhibited by treatment of the receptor cells by cycloheximide, but not by actinomycin D. We conclude that interferon messenger RNA has been translated in the receptor cells. Thus, the production of interferon in heterologous cells can be used as a sensitive assay of interferon messenger RNA.

Study of the regulation of gene expression in eukaryotic cells requires sensitive techniques to detect messenger RNAs (mRNA) for specific proteins. Due to its properties of inducibility and species specificity, the antiviral protein, interferon (1), is easily characterized. Interferon is normally undetectable within a cell, but its synthesis can be induced by either a viral infection or double-stranded RNA (see review, ref. 2). The existence of an interferon mRNA has been implied from results showing a decreased efficiency of interferon induction in cells treated with inhibitors of RNA or protein synthesis (3). Hence it was predictable that an RNA with messenger activity could be extracted from cells induced to make interferon. Evidence for the presence of this mRNA would be given by its translation into interferon in cells incubated with the RNA extract. However, unequivocal proof of translation cannot be given in cells of the same species; in this system, it is impossible to differentiate the interferon synthesized under the direction of the exogenous mRNA from that induced in receptor cells by an inducer possibly present in the extract, such as cellular double-stranded RNA (4). We therefore decided to take advantage of the species specificity of interferon and to incubate the RNA extract with heterologous cells for translation; interferon translated from this RNA would have the properties of donor, and not of receptor, cell interferon.

We present evidence for production of mouse interferon by avian and simian cells incubated with mRNA extracted from mouse cells. Moreover, we show that mRNA extracted from simian cells can code for simian interferon in VERO cells, a clone of simian cells that have lost the capacity to synthesize their own interferon.

Abbreviations: CEF, chick embryo fibroblasts; MEF, mouse embryo fibroblasts; NDV, Newcastle disease virus; PFU, plaque-forming unit; VSV, vesicular stomatitis virus.

* Dedicated to Dr. John F. Enders on the occasion of his 75th birthday.

MATERIALS AND METHODS

Tissue Culture Medium was Eagle's minimum essential medium, supplemented with glutamine and nonessential amino acids (Gibco, Long Island, N.Y.). Unless indicated otherwise, it was enriched with 10% calf serum for growth and 3% for maintenance.

Cells. Primary chick embryo fibroblast (CEF) cultures were obtained by trypsinizing 8- or 9-day-old brown Leghorn chick embryos. The culture medium was supplemented with 15% fetal calf serum. Mouse embryo fibroblast (MEF) cultures were obtained by trypsinizing randomly bred 15- to 18-day-old Swiss mouse embryos. Secondary cultures in Roux bottles were used for interferon induction and extraction of RNA. Secondary or tertiary cultures in 35-mm plastic petri dishes (Falcon) were used for interferon determinations. VERO cells are a continuous line of African Green Monkey kidney cells that have lost the capacity to make interferon, but they are still fully susceptible to the antiviral action of interferon (5). This was confirmed in our laboratory for the particular subline received. L 809 cells are a diploid line of human embryonic lung, and ES1 cells, a stable line of *Macacrus cynomolgus* cells†. WIRA cells, a continuous line of Wistar rat embryo cells, were isolated in our laboratory in 1969.

Virus Strains. The Kumarov strain of Newcastle disease virus (NDV) was used for interferon induction. Stock virus suspensions were prepared in the chorio-allantoic cavity of 10-day-old chick embryos. Allantoic fluids were harvested 2 days later and clarified by 30-min centrifugation at 5000 rpm in a Sorvall GSA rotor. The NDV titer was 1.07×10^8 plaque-forming units (PFU) per ml, in CEF. Vesicular stomatitis virus (VSV) (Indiana strain) was used as the challenge virus in the interferon titration, unless otherwise indicated. The stock virus had been cultivated in primary cultures of CEF.

Interferon Induction by NDV. Confluent MEF in 600-ml Roux bottles were incubated for 45 min at 37° with 10 ml of a 1:5 dilution of the NDV stock virus in maintenance medium. The inoculum was removed, the cells were washed once with warm medium, and 60 ml of fresh medium was added for further incubation at 37°. At time selected for the experiment, the culture medium was removed, cells were washed twice

† L 809 and ES1 cells were received from Dr. A. Boué, Centre International de l'Enfance, Paris.

TABLE 1. *Effect of RNase on the ability of mouse RNA to direct interferon synthesis in chick cells*

RNA donor cells	RNA fraction	Treatment of RNA	
		None	RNase*
MEF, poly(I·C) (12 hr)	A	10†	0
MEF, poly(I·C) (15 hr)	A	6	0
	A	3	0
	I	30	0
	I	15	0
MEF, poly(I·C) (24 hr)	A	3	0
MEF, NDV (12 hr)	A	15	3

Chick cells treated with actinomycin D were incubated with the indicated RNA fraction extracted from mouse embryo fibroblasts (MEF), induced by poly(I·C) or Newcastle disease virus (NDV), 12, 15, or 24 hr after the beginning of induction.

* With 10 µg/ml RNase (Worthington Biochemical Co., Freehold, N.J.) prepared in standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate) for 15 min at 37°.

† Each value is given in units of mouse interferon per 1.5 ml.

with cold fresh medium and immediately stored at -70°, without medium, until RNA could be extracted.

Interferon Induction by Poly(I·C). MEF were incubated for various times with maintenance medium containing 10 µg/ml of DEAE-Dextran and 10 µg/ml of poly(I·C) (P. L. Biochemicals, Milwaukee, Wisc.). The medium was then removed, and the cells were washed twice with Eagle's minimal medium, and frozen at -70° either immediately or after further incubation with medium for 12 hr.

RNA Extraction. The initial steps are similar to those already described for extraction of double-stranded RNA (6). Cell monolayers stored at -70° were rapidly thawed and scraped with a rubber policeman in a mixture of 0.04 M ethylene diamine tetraacetic acid (EDTA), 1% sodium dodecyl sulfate, 0.1% bentonite, 0.1% sodium deoxycholate, and an equal volume of phenol which was directly added to each cold bottle. The homogenate was shaken for 15 min at room temperature. After centrifugation, the aqueous phase (fraction A) was reextracted twice with phenol and precipitated twice with ethanol. It was then incubated for 15 min at 37° with 10 µg/ml of RNase-free DNase (Worthington) in 2 ml of 0.1 M Tris·HCl (pH 7.8)-8 mM MgCl₂-2 mM CaCl₂, reextracted twice with phenol, precipitated with ethanol, and redissolved in 0.1 M Tris·HCl (pH 7.8)-0.1 M NaCl-1 mM EDTA. Traces of phenol were removed by washing with ether, and the ether was removed by nitrogen bubbling. The interface of the first phenol extraction (fraction I) was washed twice with ethanol and incubated with 1 mg/ml of Pronase for 18 hr at 37°. The solution was then treated with phenol and processed as for fraction A.

Fraction A contains most of the cytoplasmic RNA and some of the nucleolar precursors of ribosomal RNA. Fraction I contains membrane-bound RNA and the heterogeneous nuclear RNA in an aggregate form (Montagnier, L. & Colandrea, H., in preparation).

Estimation of RNA was made from the absorbance at 260 nm, taking concentration in µg/ml = (absorbance × 1000)/25.

Incubation of Recipient Cells with RNA. Cells in 35-mm plastic petri dishes were pretreated with actinomycin D (General Biochemicals, 0.1 µg/ml for chick cells and 1 µg/ml for VERO cells) for 6 hr before the RNA was added (early experiments, without using actinomycin D, had given irregular results). Thereafter, the culture medium was removed, and the cells were washed with 2 ml warm phosphate buffered saline; 0.5 ml of phosphate buffered saline containing 75 µg of DEAE-Dextran (Pharmacia, Uppsala, Sweden) per ml was then added to each petri dish, followed by 0.25 ml of a suitable dilution of the RNA preparation in phosphate buffered saline. The RNA-Dextran mixture was left on the cells for 45 min at 37°, and then aspirated. 2 ml of culture medium were added to each plate, and the cells were incubated at 37° in a humidified atmosphere of 4% CO₂ in air. Supernatants were harvested 15 hr later, unless indicated otherwise, and tested for interferon content.

Interferon Titrations. Samples to be examined for mouse interferon were assayed by a plaque reduction method in MEF with VSV as challenge virus. Monolayers were incubated overnight with appropriate dilutions of the samples. The medium was then removed, cell sheets were washed with warm Eagle's minimal medium, and about 50 PFU of VSV in 0.5 ml were added. After 1 hr, the cells were covered with 2 ml of nutrient starch overlay and further incubated. Plaques were counted 48 hr later. Interferon titers are expressed as units, one unit corresponding to the amount of inhibitor necessary to reduce the number of challenge plaques by 50%. Some results are not represented as interferon units, but as percent inhibition of challenge virus.

RESULTS

A preliminary study of kinetics of interferon induction in MEF with NDV and poly(I·C) indicated that maximal production occurred about 12 hr after contact with the inducer. Therefore, an RNA extract was made from induced MEF at that time, and added to CEF as described in *Methods*. The

TABLE 2. *Effect of cycloheximide treatment of CEF on synthesis of mouse interferon*

Origin of RNA	Units of mouse interferon made by CEF when incubated with RNA			
	RNA fraction	No cycloheximide	Cycloheximide 1 hr before RNA	Cycloheximide 1 hr before and during incubation with RNA
MEF, poly(I·C) (15 hr)	A	6	7	0
	A	3	4	0
	I	15	ND†	5
MEF, poly(I·C) (24 hr)	A	3	3	0

Chick embryo fibroblasts (CEF) treated with actinomycin D were incubated with the indicated RNA fraction extracted from poly(I·C)-induced MEF, 15 or 24 hr after the beginning of induction.

* Each value is given in units of mouse interferon per 1.5 ml.

† ND = not done.

culture medium was removed 15 hr later and assayed for antiviral activity both in mouse and chick cells. A clearcut inhibition of VSV plaque formation in mouse cells, but not in chicken cells, was observed; the inhibition was proportional to the amount of RNA added to the recipient chick cells. However, concentrations of fraction A RNA over 50 $\mu\text{g}/\text{ml}$ were not as efficient as lower concentrations; therefore, 50 $\mu\text{g}/\text{ml}$ was chosen as the concentration of fraction A RNA to be routinely tested. A similar phenomenon of decreased efficiency at higher concentrations was not observed with fraction I, but in view of the limited amounts of RNA in this fraction, all tests were routinely carried out at concentrations of 25 $\mu\text{g}/\text{ml}$.

Further analysis showed that:

(i) Single-stranded RNA is involved. As shown in Table 1, production of the antiviral factor in CEF was prevented by pretreatment with RNase of MEF RNA. Control experiments (not shown), also indicated that the DNase treatment used routinely in the RNA extraction procedure (see *Methods*) had no effect. Treatment of fraction A RNA with Pronase similarly did not change its activity.

(ii) The action of the RNA depends only on a translation process. Treatment of the recipient cells with actinomycin D (see *Methods*) does not decrease, but on the contrary increases, the RNA-directed synthesis of the viral inhibitor. Therefore, a transcription process is unlikely to be involved. In contrast, a significant inhibition was observed with cycloheximide, a reversible inhibitor of protein synthesis at the translational level (Table 2). CEF were treated with 10 $\mu\text{g}/\text{ml}$ of cycloheximide starting 1 hr before RNA addition and continuing during the 45-min incubation with RNA. It can be seen from Table 2 that the appearance of the viral inhibitor was either completely inhibited or much reduced under this treatment. On the other hand, when the cycloheximide treatment was interrupted before the RNA was added, no inhibition occurred. This strongly suggested that protein synthesis took place very soon after the RNA was added. A kinetic experiment was done that confirmed the rapidity of the production of viral inhibitor (Table 3). Already by 2 hr after uptake of the RNA a significant proportion of inhibitor was released into the culture fluid, and at 4 hr, there was as much activity as after 15 hr. The rapidity of synthesis of the inhibitor is

TABLE 3. *Kinetics of appearance of viral inhibitor in RNA-treated CEF*

Origin of inhibitor			Viral inhibitory activity obtained after*						
RNA donor cells	Fraction	Recipient cells	0 hr	1 hr	2 hr	3 hr	4 hr	15 hr	
MEF, NDV (12 hr)	I	CEF	0	9	12	13	17	12	
MEF, NDV (24 hr)	I	CEF	0	—	3	—	4	2	

Chick cells treated with actinomycin D were incubated under the usual conditions with fraction I RNA extracted from NDV-induced MEF, 12 or 24 hr after the beginning of induction. Culture media were harvested at time indicated. Time 0 refers to the time at which the RNA was removed from the cultures.

* Expressed in interferon units per 1.5 ml.

TABLE 4. *Mouse interferon produced by chick cells as a function of time of RNA extraction from mouse cells induced by poly(I·C) or NDV*

Time of RNA extraction after onset of induction	Inducer: RNA fraction:	Poly(I·C)		NDV	
		A	I	A	I
5 min		0	0	ND*	ND
6 hr		3†	3	ND	ND
		10	6		
12 hr		10	10	15	12
				2	12
15 hr		6	30	ND	ND
		3	15		
24 hr		3	0	3	5
				3	2
Uninduced controls		0	0	0	0

* ND = not done.

† Antiviral activity against VSV in MEF expressed as interferon units per 1.5 ml. When two values are given, results were obtained independently in two different experiments.

clearly in accord with the hypothesis of a translation of mRNA.

(iii) Only cells induced to make interferon yield active RNA. As shown in Table 4, RNA extracted from MEF not induced to make interferon, or at a very early time of induction, is not active in CEF.

(iv) The antiviral product obtained fulfills the criteria that characterize interferon.

- It is destroyed by proteolytic enzymes and not by RNase. The viral inhibitory activity appearing in the medium of CEF after treatment with RNA extracted from induced mouse cells was completely destroyed after incubation with 100 $\mu\text{g}/\text{ml}$ trypsin (Crystalline, A grade, Calbiochem) for 45 min at 37°. The activity was destroyed after treatment with 100 $\mu\text{g}/\text{ml}$ Pronase (Pronase, B grade, nuclease free, Calbiochem) for 45 min at 37°, and it was not affected by incubation with 10 $\mu\text{g}/\text{ml}$ RNase for 45 min at 37°.
- It is active against different types of viruses. Several samples of inhibitor obtained from CEF were tested for antiviral activity in MEF against herpes simplex virus and Semliki forest virus. An inhibition of plaque formation comparable to that obtained with a corresponding amount of mouse interferon units, was observed.
- It affects a single growth cycle of VSV in MEF. Therefore the decreased plaque number observed in MEF is due to an effect on viral replication and not on plating efficiency (Table 5).
- Its action is inhibited by actinomycin D. Simultaneous overnight incubation of MEF with the viral inhibitor and 0.12 or 0.25 $\mu\text{g}/\text{ml}$ actinomycin D completely abolished the antiviral activity against VSV in a single growth cycle.
- Its molecular weight, as determined by gel filtration on Dextran gel (Sephadex G-75) and subsequent titration of the eluted fractions in L cells, was found to be 32,000 (8).
- This is mouse interferon.
- It is species specific, an important characteristic of interferon. The antiviral activity of the product obtained in

TABLE 5. *Effect on virus yield in a single growth cycle of VSV in MEF*

Origin of viral inhibitor		Recipient cells	VSV yield (PFU/ml)
RNA donor cells	RNA fraction		
MEF, poly(I·C) (12 hr)	A	CEF	3.3×10^4
MEF, NDV (12 hr)	I	CEF	8.5×10^4
Controls			3.0×10^6 2.7×10^6

MEF were incubated overnight with a 1:2 dilution of each sample or with control fluid. The media were then removed, the plates were washed once with warm Eagle's minimal medium, and the cells were infected with VSV at an input multiplicity of 20 PFU/cell. After 1 hr, the cells were washed three times and growth medium was added. 10 hr later, cells were freeze-thawed twice, and the combined virus content of media and cells was determined.

chick cells after incubation with mouse RNA, was examined not only in mouse cells, but also in chick, human, primate, and rat cells. It can be seen from Table 6 that a significant reduction of VSV plaque number was observed only when the inhibitor was titrated in mouse cells; even in chick cells, in which the inhibitor had been produced, no antiviral activity was observed.

- (b) It is inactivated by sheep serum prepared against mouse interferon. Table 7 summarizes the effect of the anti-mouse interferon serum on six different preparations of the viral inhibitor. The antiviral activity of all six preparations were significantly inhibited by the antiserum,

TABLE 6. *Species specificity of viral inhibitor made by CEF or VERO cells incubated with mouse RNA*

Origin of inhibitor		% Inhibition of challenge VSV plaques when tested in					
Donor cells	RNA fraction	Recipient cells	MEF	CEF	REF*	Mon-key (ES1)	Hu-man (809)
MEF, poly-(I·C) (12 hr)	A	CEF	100	0	11	0	0
	I	CEF	100	0	13	0	0
MEF, NDV (12 hr)	I	CEF	91	0	ND†	0	ND
MEF, poly-(I·C) (15 hr)	A	CEF	53	0	21	ND	ND
	A	VERO	65	0	0	ND	ND

The antiviral substance examined in these experiments had been produced by chick or VERO cells, after incubation with fraction A and I extracted from MEF induced with poly(I·C) or NDV. The basic procedure was the same for all cells tested: after overnight incubation with the samples at a 1:3 dilution, the plates were washed with warm Eagle's minimal medium, and a plaque assay against VSV under starch overlay was done. As much as possible, two plates per sample and per cell type were used, but sometimes it was necessary to use only one plate because of the limited amounts of material available.

* REF = rat embryo fibroblasts.

† ND = not done.

which furthermore was shown to have no effect on either chick or monkey interferon and some effect on rat interferon; the latter is not surprising since it is known that mouse and rat interferons have a certain degree of cross reactivity.

Similar experiments were performed with RNA extracted from monkey cells (ES1 line) induced by NDV and with CEF and VERO cells as receptor cells. In this case, an antiviral substance showing the characteristics of monkey interferon was obtained (Table 8).

DISCUSSION

The *in vivo* biological activity of eukaryotic RNAs has been claimed several times in the past (see review, ref. 8). (The term *in vivo* refers to intact cells, either cultured or in living organisms.) Recently, rabbit globin mRNA from reticulocytes has been translated in amphibian oocytes (9). We now

TABLE 7. *Effect of anti-mouse interferon serum on the antiviral activity of inhibitor made in chick cell*

Origin of inhibitor				% Inhibition of challenge when inhibitor is pre-incubated with	
Donor cells	RNA fraction	Recipient cells	Antiviral activity tested in	Anti-mouse interferon serum	Control serum
MEF, poly(I·C) (12 hr)	A	CEF	MEF	18	66
	A	CEF	MEF	17	68
	I	CEF	MEF	34	84
	I	CEF	MEF	8	70
MEF, NDV (12 hr)	I	CEF	MEF	5	42
MEF, poly(I·C) (12 hr)	A	VERO	MEF	0	13
Mouse reference interferon					
1st assay			MEF	18	78
2nd assay			MEF	0	38
Chick interferon			CEF	87	89
Monkey interferon			ES1	93	94
Rat interferon			WIRA	47	69

Anti-mouse-interferon sheep serum was obtained through the courtesy of Dr. B. Fauconnier (Faculté de Médecine, Rennes, France). It had been prepared by repeated injections into sheep of a semipurified and concentrated mouse interferon prepared in L cells with NDV as inducer. Serum of a normal unimmunized sheep was used as control. Sera were inactivated at 56° for 25 min before use. All preparations were mixed with antiserum at the minimal concentration necessary to inhibit about 5 units of mouse interferon. Control serum was used at a corresponding dilution. The mixtures were put in a water bath at 37° for 45 min, and then added to the culture plates in which the interferon assay was to be done. Care was taken to work with concentrations of interferon or inhibitor that did not completely inhibit the number of challenge plaques as compared to the controls, in order to make the assay as sensitive as possible. After overnight incubation at 37°, the cultures were further treated as for an interferon titration. The results are given as the % inhibition of challenge virus; each value is the average of two or three plates.

TABLE 8. *Production of viral inhibitor by CEF or VERO cells treated with RNA from induced ES1 cells*

Origin of inhibitor			Antiviral activity when tested in	
RNA donor cells	RNA fraction	Recipient cells	VERO	MEF
ES1, NDV _{UV} (17 hr)	I	CEF	8*	0
	I	VERO	10	0

Before interferon induction in ES1 cells, NDV was UV-irradiated at a dose of 750 ergs/mm².

* Expressed as interferon units per 1.5 ml.

describe a reproducible *in vivo* assay for the mRNA of an inducible protein. Our results show that, among many species of RNA, interferon mRNA can be translated in heterologous cells. The yield of interferon obtained from this translation was low, but this was not unexpected in view of the poor uptake of extraneous RNA by cells (8). A limiting factor may also be other RNAs contained in the extract that conceivably may compete with interferon mRNA for ribosome sites of recipient cells. The increased efficiency of mRNA translation in cells treated with actinomycin D may similarly be explained by the increased availability of their ribosomes, freed from endogenous mRNAs.

An obvious pitfall in our studies was the possibility that our RNA extract was acting as an inducer and not as a messenger. This possibility was ruled out by the fact that the interferon produced by chick cells was mouse interferon and

not chick interferon, as would have been the result of a direct induction. Further evidence was provided by the results obtained in VERO cells: in spite of their inability to produce interferon upon induction, these cells were able to make mouse and monkey interferon when incubated with the corresponding mRNA.

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