Vol. 82, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1978 Pages 665-673

TRANSLATION OF MOUSE INTERFERON mRNA IN XENOPUS LAEVIS OOCYTES AND IN RABBIT RETICULOCYTE LYSATES

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Received March 8, 1978

Summary

Mouse interferon mRNA, extracted from NDV (Newcastle disease virus)-induced L-929 cells has been translated with high efficiency in Xenopus laevis oocytes and rabbit reticulocyte lysates. The translational efficiency of a crude RNA extract was 10 640 interferon units/mg RNA/hour for the Xenopus oocytes and 4 012 interferon units/mg RNA/hour for the reticulocyte lysates. The translation product fulfilled the usual criteria for mouse interferon, viz. species specificity and neutralization by specific anti-mouse interferon antiserum. Upon injection of crude interferon mRNA into Xenopus oocytes, interferon activity appeared both in the oocyte homogenates and the oocyte incubation medium. When analyzed by velocity sedimentation in formamide-sucrose, the mouse interferon mRNA showed a rather sharp peak halfway between the 4 S and 18 S RNA markers, as could be expected from a mRNA which codes for a 20,000 dalton protein.

INTRODUCTION

Interferon mRNA is one of the rare eukaryotic mRNAs a biologically active product of which can be obtained in heterologous translation systems. For human interferon mRNA (induced by $(I)_n.(C)_n$ in human fibroblast cells), efficient translation has been observed in chick embryo cells (1), hamster embryo cells (2), Xenopus laevis oocytes (3-7), rabbit reticulocyte lysates (3), mouse ascites cell extracts (3,8) and wheat germ extracts (4). For mouse interferon mRNA (induced by either NDV or $(I)_n.(C)_n$ in mouse fibroblast(oid) cells), effective translation has been obtained in a variety of heterologous recipient cells, including monkey (e.g. VERO), human (e.g. HeLa) and chick cells (9-12) and in only one cell-free (wheat germ) system (13).

We now describe the translation of mouse interferon mRNA in two

0006-291X/78/0822-0665$01.00/0

additional protein-synthesizing systems, Xenopus laevis oocytes and rabbit reticulocyte lysates. In both systems, the messenger RNA isolated from NDV-induced L-929 cells directed the synthesis of a biologically active product with the properties of mouse interferon. With mouse interferon mRNA that had been partially purified on a formamide-sucrose gradient, the Xenopus laevis translation system allowed a quantitative estimation of the amount of mRNA.

MATERIALS AND METHODS

Cells and viruses

L-929 cells, human skin fibroblasts (VGS strain) and primary rabbit kidney cells were grown in EMEM medium (Eagle's minimal essential medium) supplemented with 10 % fetal calf serum. The Kumarov strain of Newcastle disease virus (NDV) was used for interferon induction. Stock virus was prepared in the chorio-allantoic cavity of 10-day-old chick embryos. The Indiana strain of vesicular stomatitis virus (VSV) served as the challenge virus for the interferon assays. The stock virus had been propagated in BSC-1 cells.

Induction of interferon mRNA

L-929 cells were grown in 1/2 gallon roller bottles, and, when confluent, the cells were treated with 100 U/ml of mouse interferon (specific activity 10^5 to 10^6 units/mg protein) for 2 hours at 37°. After this "priming" period, the cells were inoculated with NDV at approximately 1 pfu/cell. After 1 hour incubation at 37°, the virus inoculum was removed and the cells were further incubated with EMEM for about 11 to 12 hours, at which time the cells were harvested for RNA extraction. When this work was in progress we noticed that omission of the interferon "primed" step resulted in a marked (about 10-fold) increase of the interferon mRNA activity; for this reason some experiments were performed with RNA extracted from unprimed NDV-induced L cells.

Interferon mRNA extraction

The cells were first washed with phosphate buffer saline (PBS), brought into suspension with EDTA (0.02 % in PBS) for 30 min. at 37°, washed 3 x with ice-cold PBS, and finally treated with 5 volumes of hypotonic buffer (containing HEPES 10 mM pH 7.5, 20 mM KCl, 1.5 mM $MgCl_2$ and 7 mM mercaptoethanol). After 10 min. incubation at 4°, the swollen cells were broken by 25 strokes of a tight fitting dounce homogenizer. Nuclei were removed by centrifugation for 2 min. at 2,000 g and the cytoplasm was deproteinized by a mixture of phenol, chloroform and isoamylalcohol (25 : 25 : 1, v/v, pH 8.9). The RNA was precipitated with two volumes of cold ethanol followed by 3 cycles of 2 M LiCl at 4° for 6 to 16 hours. After a last ethanol treatment, the precipitated RNA was washed 2 x with 75 % ethanol and dried under vacuum before it was finally dissolved in water at 5 - 10 mg/ml.

Translation of interferon mRNA in Xenopus laevis oocytes

Xenopus laevis oocytes were microinjected with the mRNA preparations as described previously (14). Two hundred and fifty ng of RNA dissolved in 50 nl water was injected into each oocyte (10 oocytes per group). The oocytes were incubated for 16 hours at 19° in 0.1 ml of Barth's incubation medium (14) in (1.5 ml) Eppendorf plastic tubes. The incubation medium was

collected and the oocytes were homogenized in 100 µl of fresh Barth's medium. The homogenates were centrifuged at 10,000 g for 2 min. in an Eppendorf microcentrifuge. The supernatant was carefully removed and stored at -70° until it was assayed for interferon activity.

Translation of interferon mRNA in rabbit reticulocyte lysates

The reticulocyte lysates were prepared as described previously (15). The lysates were depleted from their endogenous mRNA activity by treatment with micrococcal nuclease (10 µg/ml) in the presence of 1 mM $CaCl_2$. After 15 min. incubation at 20°, 2 mM EGTA was added to terminate the nuclease reaction (16). To monitor protein synthesis, the nuclease-pretreated lysates were incubated for 45 min. at 31° in the presence of 150 µg/ml of calf liver tRNA (Boehringer, Mannheim).

Interferon assay

Mouse interferon was assayed by inhibition of VSV-induced cytopathogenicity in L-929 cells. A standard calibrated against the National Institutes of Health mouse interferon reference standard G 002-904-511 was included in each assay.

RESULTS AND DISCUSSION

Synthesis of mouse interferon in Xenopus laevis oocytes

Total cytoplasmic RNA extracted from NDV-induced L cells directed in Xenopus laevis oocytes the synthesis of a product which had the properties of mouse interferon (Table I) : it was active in inhibiting virus replication in mouse L cells but not in human or rabbit cells. Furthermore, its antiviral activity was completely neutralized by antiserum to mouse interferon (Table I), and, when chromatographed on a CPG column (Fig. 2), the oocyte translation product behaved as authentic mouse interferon.

Translation within the oocytes was required since the interferon mRNA did not show antiviral activity by itself when directly transferred to L cells (Table I). In contrast with the RNA from NDV-induced L cells, RNA extracted from uninduced L cells did not stimulate the synthesis of an antiviral product in oocytes (Table I). The presence of adventitious protein or double-stranded RNA contaminants in the RNA extracts was avoided by several cycles of deproteinization followed by three cycles of precipitation with 2 M LiCl (or further digestion with proteinase K).

As could be expected for an mRNA that codes for a protein made for export from the cell, interferon mRNA activity was also detected in the RNA extracted from membrane-bound polyribosomes (Table I).

If one assumes that mRNA represents 2 % of cytoplasmic RNA and that interferon mRNA itself represents 1 % of the total mRNA population (which is probably overrated), each oocyte should have been injected with 0.05 ng of interferon mRNA at the most. The sensitivity of the translation assay,

Table I

Antiviral activity of oocyte translation product

RNA injected	Cells used for interferon assay	Interferon titer (units/ml)
- no	L cells	≤ 10
- total cytoplasmic RNA from uninduced L cells	L cells	≤ 10
- total cytoplasmic RNA from NDV-induced L cells	L cells	1,500
- id. but no incubation after injection in oocytes	L cells	≤ 10
- total cytoplasmic RNA from NDV-induced L cells	Human skin fibroblasts (VGS cells)	≤ 10
- id.	Primary rabbit kidney cells	≤ 10
- total RNA from membrane-bound polyribosomes	L cells	800
- total cytoplasmic RNA from NDV-induced L cells	L cells, after neutralization of oocyte homogenate with mouse interferon antiserum	≤ 10

combined with the extremely high biological activity of interferon ($\sim 10^{-17}$ mole/unit) and the selectivity of the interferon assay thus allows to detect the activity of a minor mRNA species within a complex mixture of different RNA species.

The time-course of interferon synthesis in Xenopus laevis oocytes after injection of cytoplasmic RNA from NDV-induced L cells is shown in Fig. 1. Interferon was already detectable in the oocyte homogenate within 3 hours of mRNA injection. While maximal interferon yields in the oocyte homogenates were obtained after 12 hours of incubation, interferon activity appeared in the medium surrounding the oocytes and continued to increase, when the interferon activity within the oocytes declined (after 20 hours of incubation) (Fig. 1).

When being synthesized in the oocyte, interferon can be labelled with (^{35}S)methionine. This interferon has been further characterized by affinity chromatography on Controlled Pore Glass (CPG) beads, a procedure which has proven useful for the purification of both human and murine fibroblast interferon (17). Figure 2 shows the results of such an experiment. While only 1 % of the total (^{35}S)methionine counts were eluted from the CPG column with 0.01 M glycine-HCl (pH 2.5), almost all interferon activity present in the oocyte (homogenate) sample applied to the CPG column could be recovered with

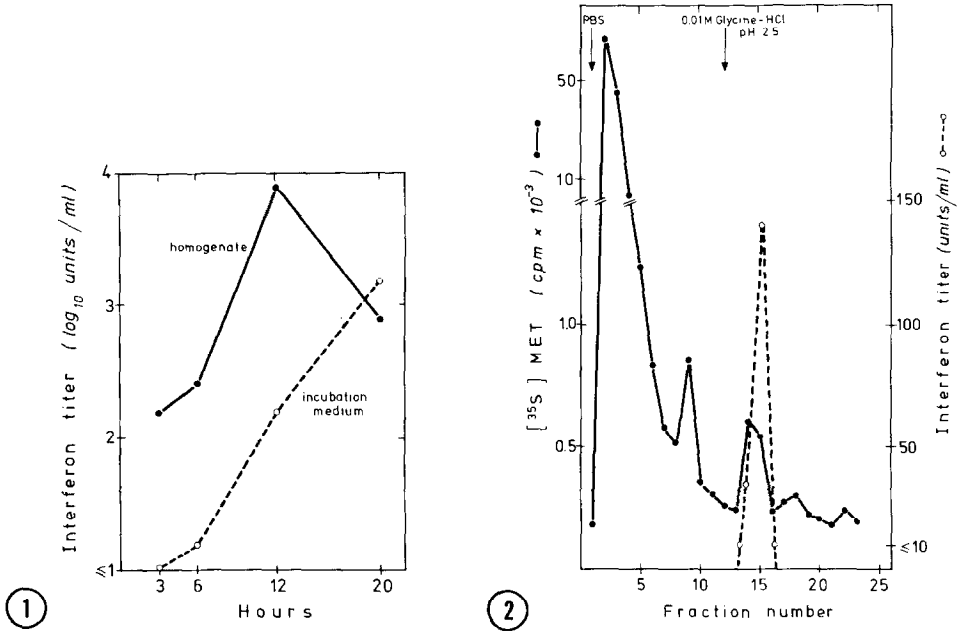


Figure 1. Kinetics of interferon synthesis in Xenopus laevis oocytes. Groups of 10 oocytes were injected with 250 ng of cytoplasmic RNA extracted from NDV-induced L cells; each group was incubated at 19° for different periods of time, as indicated in the abscissa. Both the oocytes homogenates (●——●) and incubation medium (o---o) were assayed for interferon content.

Figure 2. Controlled Pore Glass (CPG) chromatography of interferon translated in Xenopus laevis oocytes. Fifty oocytes were injected with 250 ng of cytoplasmic RNA from NDV-induced L cells and incubated at 19° in the presence of 180 µCi/ml (^{35}S)methionine (specific activity : 600 Ci/mmole). After 20 hours the oocytes were homogenized in Barth's medium and the homogenates were chromatographed on CPG, as described (17). One ml fractions were collected. Twenty-five µl aliquots of these fractions were assayed for interferon activity (o---o) (whereas 100 µl aliquots of the fractions were assayed for TCA-precipitable radioactivity (on 3 MM Whatman paper disks) (●——●).

glycine-HCl as a single peak (fractions 14-15 : Fig. 2), reproducing exactly the chromatographic pattern that one would obtain with an authentic mouse fibroblast interferon (17).

Synthesis of mouse interferon in rabbit reticulocyte lysates

Cytoplasmic RNA extracted from NDV-induced L cells could also be translated in a micrococcal nuclease preincubated rabbit reticulocyte cell-free system. This system allows the translation of extremely low concentrations of mRNA (1 - 5 µg/ml) and responds quite well in the presence of a large excess of ribosomal RNA (16).

Table II

Mouse interferon mRNA translation in rabbit reticulocyte lysates

mRNA added	Interferon titer (units/ml)	
	before incubation	after 45 min. incubation at 31°
no RNA	⩽ 10	⩽ 10
RNA from uninduced L cells (200 µg/ml)	⩽ 10	⩽ 10
RNA from NDV-induced L cells (160 µg/ml)	⩽ 10	500

Translation efficiency of interferon mRNA
(1) in rabbit reticulocyte lysates :
500 units/ml/45 min. = 666 units/ml/hour (with 160 µg/ml of mRNA) = 4012 units/mg RNA/hour.
(2) in Xenopus laevis oocytes :
800 units/ml/6 hours = 133 units/ml/hour (with 50 oocytes and 250 ng mRNA per oocyte) = 10640 units/mg RNA/hour.

Crude RNA extracted from NDV-induced cells stimulated the production of an antiviral product in the reticulocyte lysate cell-free system (Table II). This antiviral activity strictly depended on the appropriate incubation period, indicating that the observed activity was not due to contamination of the interferon mRNA by interferon.

The ability of the reticulocyte lysate to translate mouse interferon mRNA was not affected by the addition of spermidine (100 - 400 µM) (data not shown). Yet, Thang et al. (13) and Raj and Pitha (4) reported that in the wheat germ cell-free system the presence of spermine is essential for efficient translation of both mouse and human interferon mRNA.

We have recently noted that there exist remarkable differences in the capacity of double-stranded (ds)RNAs to inhibit protein synthesis in rabbit reticulocyte lysates (15) : natural dsRNAs such as bacteriophage f2 dsRNA and reovirus dsRNA effectively inhibited protein synthesis at a concentration of 10 to 100 ng/ml whereas synthetic homopolymer pairs such as $(I)_n.(br^5C)_n$ and $(I)_n.(s^2C)_n$ failed to do so. Table III clearly indicates that these findings can be extended to interferon synthesis in reticulocyte lysates. At 10 - 100 ng/ml the natural double-stranded RNAs f2 dsRNA and reovirus dsRNA reduced the synthesis of interferon by about 70 % whereas the synthetic homopolymer pairs $(I)_n.(br^5C)_n$ and $(I)_n.(s^2C)_n$ did not cause any inhibition at all (Table III).

The translation efficiency of interferon mRNA in the rabbit reticulocyte lysate system, expressed in interferon units/mg RNA/hour, amounted to

Table III

Effect of various double stranded RNAs on the translation of mouse interferon mRNA in rabbit reticulocyte lysates

mRNA added	double-stranded (ds)RNA added	(^{35}S)methionine net cpm per 50 µl reaction	Interferon titer (units/ml) after 45 min. incubation at 31°
no	no	-	< 30
200 µg/ml	no	122.120	500
200 µg/ml	10 ng/ml f2 dsRNA	18.780	150
200 µg/ml	100 ng/ml f2 dsRNA	39.330	150
200 µg/ml	10 ng/ml reovirus dsRNA	30.850	150
200 µg/ml	100 ng/ml reovirus dsRNA	33.630	150
200 µg/ml	10 ng/ml $(I)_n.(s^2C)_n$	123.630	500
200 µg/ml	100 ng/ml $(I)_n.(s^2C)_n$	125.860	500
200 µg/ml	10 ng/ml $(I)_n.(br^5C)_n$	111.610	500
200 µg/ml	100 ng/ml $(I)_n.(br^5C)_n$	123.380	500

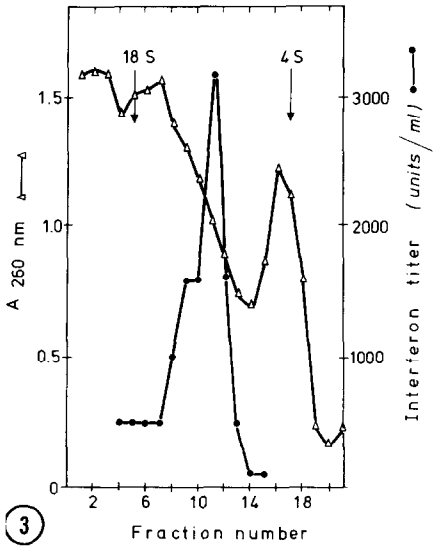
The origin of the dsRNAs and the methodology employed for measuring protein synthesis in rabbit reticulocyte lysates have been described previously (15).

about 40 % of the interferon mRNA translation efficiency obtained by Xenopus laevis oocytes (Table II). Although the Xenopus oocytes certainly represent the most sensitive assay system to detect low quantities of interferon mRNA, the contention that Xenopus oocytes would give 500 times higher interferon titers (calculated per µg RNA used) than the cell-free systems (3) does apparently not hold for the nuclease-preincubated rabbit reticulocyte cell-free system.

Partial purification of mouse interferon mRNA

The release of interferon activity from the Xenopus laevis oocytes into the surrounding medium (Fig. 1) may be related to the presence of extraneous mRNA (i.e. NDV mRNA) in the crude interferon mRNA preparations which were used to inject the oocytes. If the RNA preparation was first purified on a formamide-sucrose gradient (Fig. 3) and then injected into the Xenopus oocytes, no more release of interferon activity in the supernatant fluid of the oocytes could be witnessed (data not shown).

Fractionation of the cytoplasmic RNA (from NDV-induced L cells) by velocity ultracentrifugation in formamide-sucrose revealed a characteris-



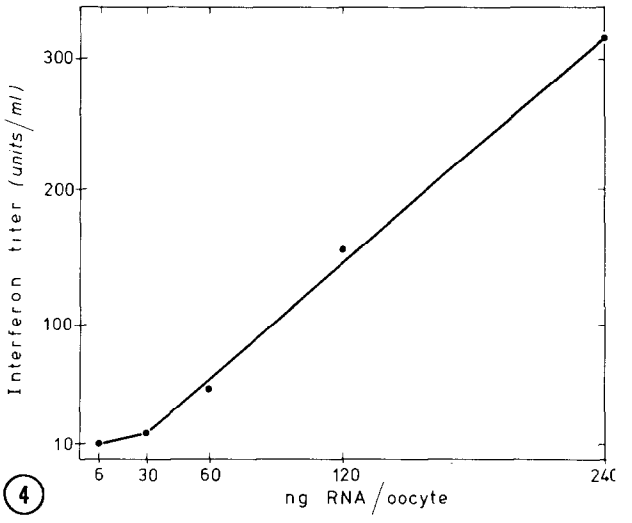


Figure 3. Sucrose gradient analysis of interferon mRNA activity. Samples of 300 µg of cytoplasmic RNA from NDV-induced L cells were first incubated for 2 min. at 37° in 50 % formamide and then layered onto a linear 5 - 20 % w/v sucrose gradient in 1 mM EDTA, 0.1 M Tris-HCl pH 7.5 and 1 % SDS. The gradients were centrifuged at 20° for 16 to 17 hours at 40,000 rpm in a Beckman SW 60 Ti rotor. [^{3}H]uridine-labelled cytoplasmic mouse cell RNA was run in a parallel gradient. The 18 S and 4 S RNA sedimented at the positions indicated by the arrows. The absorbancy at 260 nm was recorded for all fractions (Δ——Δ). For most fractions, RNA was precipitated with ethanol redissolved in water at 2 mg/ml and microinjected into Xenopus laevis oocytes. Finally, the oocyte translation products were assayed for interferon activity in L cells (●——●).

Figure 4. Interferon synthesis in Xenopus laevis oocytes as a function of the quantity of RNA injected per oocyte. Varying amounts of partially purified cytoplasmic RNA (corresponding to the peak activity obtained in sucrose gradients : see Fig. 3) were injected into Xenopus laevis oocytes. The RNA had been dissolved in water and was injected at the indicated amounts in a total volume of 50 nl per oocyte. Groups of 10 oocytes were used and the interferon synthesized in the oocytes was assayed on L cells.

tic sedimentation pattern as exemplified in Fig. 3. The interferon mRNA activity sedimented as a rather narrow band at approximately 11 S, as may be expected from a mRNA which codes for a protein of circa 20,000 daltons. A slightly lower sedimentation value (about 8 to 9 S) has been suggested previously for the mouse and human interferon mRNAs induced by $(I)_n.(C)_n$ (1, 18).

The dependence of interferon synthesis in oocytes on the amount of mRNA injected was determined with the peak interferon mRNA fraction obtained in the formamide-sucrose gradient (Fig. 3). As shown in Fig. 4, there

was a nearly linear increase in interferon yield with increasing amounts of RNA injected (in the range of 30 to 240 ng of RNA per oocyte) (Fig. 4). A similar quantitative relationship between the amounts of mRNA injected and the amounts of interferon produced has previously been established with human interferon mRNA (extracted from $(I)_n.(C)_n$-induced FS-4 fibroblast cells) (5,6).

Acknowledgements. These investigations were supported by grants from the Belgian F.N.R.S. (Fonds National de la Recherche Scientifique), F.G.W.O. (Fonds voor Geneeskundig Wetenschappelijk Onderzoek) and the Fonds Derde Cyclus (Katholieke Universiteit Leuven). I.A.B. is the recipient of a predoctoral fellowship of the Fonds Derde Cyclus (Katholieke Universiteit Leuven).

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