Proc. Nat. Acad. Sci. USA
Vol. 72, No. 12, pp. 4881–4885, December 1975
Biochemistry

Interferon activity produced by translation of human interferon messenger RNA in cell-free ribosomal systems and in *Xenopus* oöcytes

(protein synthesis/biochemical characterization)

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Communicated by Wallace P. Rowe, September 30, 1975

ABSTRACT **Translation of messenger RNA isolated from poly(rI)·poly(rC)-induced human fibroblasts in cell-free ribosomal systems and in *Xenopus* oöcytes resulted in the production of biologically active proteins that had the properties of human fibroblast interferon. The translation in the oöcytes was much more efficient, giving approximately 500 times higher titers of interferon activity than the cell-free systems. A control messenger RNA isolated from noninduced human fibroblasts, did not code for interferon synthesis in these systems. Both messenger RNA preparations stimulated [^{3}H]amino-acid incorporation into trichloroacetic acid-insoluble material. The radioactive products and their immunoprecipitates were electrophoresed on polyacrylamide gels under denaturing conditions. The products resulting from the translation of the control (uninduced) messenger RNA in oöcytes contained a major protein of approximately 45,000 molecular weight. The messenger RNA isolated from poly(rI)·poly(rC)-induced cells stimulated the synthesis of an additional 25,000 molecular weight protein that electrophoresed in the same position as human fibroblast interferon. These results suggest that human fibroblast interferon was synthesized by the translation of its messenger RNA in *Xenopus* oöcytes and in cell-free ribosomal systems.**

Interferons are species specific extracellular glycoproteins (1) that impart an antiviral state in the recipient cells (2). Both viral infections and some polynucleotide complexes [e.g., poly(rI)·poly(rC)] can induce interferon in cells grown in tissue culture. The interferon yields can be enhanced when metabolic inhibitors are present during the induction process (3–5). This effect is called "superinduction" and suggests that, as with a number of other inducible responses, the induction of interferon is regulated posttranscriptionally (6). However, the effects of the metabolic inhibitors used in superinduction are too complex to allow an unambiguous interpretation of their mechanism of action (7). Thus studies on the molecular level are needed to determine the exact nature of the mechanism controlling interferon production.

Biochemical study of the processes involved in the regulation of interferon induction requires a sensitive method for the detection of interferon messenger RNA (mRNA). De-Maeyer-Guignard *et al.* (8) developed an assay based on the species specificity of interferon and on the ability of heterologous cells to translate interferon mRNA. They showed that when a complex of mouse interferon mRNA and diethylaminoethyldextran (DEAE-dextran) was applied to chick or monkey cells previously treated with actinomycin D, mouse interferon was produced. This system was used to characterize the interferon mRNA from mouse cells induced both by poly(rI)·poly(rC) and by Newcastle disease virus (9). Using a similar approach, we have recently shown that poly(rI)·poly(rC)-induced mRNA for human fibroblast interferon is a 10–18S, poly(A)-containing RNA molecule (10). Although this assay is very sensitive, it depends strongly on the physiological condition of chick cells; it is also limited by the difficulty in obtaining a quantitative relationship between the amount of RNA applied to the recipient cells and the amount of interferon produced, and therefore is not suitable for the quantitation of interferon mRNA.

The current study describes two additional assays for human fibroblast interferon mRNA using injection into *Xenopus* oöcytes and translation in cell-free ribosomal systems. The results presented indicate that both biologically active and highly radioactive interferon can be produced in these systems.

MATERIALS AND METHODS

Cells and Virus. Human foreskin fibroblasts (passages 5–15) were grown in roller bottles in Eagle's minimal medium supplemented with 5% fetal bovine serum and gentamicin (50 μg/ml), and maintained as described (10). Vesicular stomatitis virus (New Jersey serotype) was plaque purified on mouse L cells and propagated on L cells pretreated with DEAE-dextran (20 μg/ml). The resulting titer was 10^9 plaque-forming units/ml.

Interferon Assay. Interferon was assayed with vesicular stomatitis virus, either by the colorimetric method of Finter (11) or, when more accurate estimates of titers were required, by reduction in yield (12). One unit of interferon is defined as the concentration giving 50% reduction in virus yield. One unit of international reference standard titrated as 1 unit in our assay.

Induction and Preparation of mRNA. Human fibroblasts were induced with 100 μg/ml of poly(rI)·poly(rC) (P. L. Biochemicals), in phosphate-buffered saline (0.1 M NaCl and 0.02 M sodium phosphate buffer, pH 7.0) in the presence of cycloheximide (50 μg/ml) (5, 10). After 1 hr of incubation at 37°, poly(rI)·poly(rC) was removed and cells were washed and overlayed with Eagle's minimal medium with 2% fetal calf serum containing 50 μg/ml of cycloheximide; 5 hr later, medium was removed and cells were washed with phosphate-buffered saline, scraped, centrifuged, and stored at −70°. The control fibroblasts were treated with cycloheximide, but did not receive poly(rI)·poly(rC).

Total cellular mRNA was prepared as follows. Cell pellets from 30 roller bottles were homogenized in 50 ml of cold 0.2 M Tris·HCl buffer, pH 9.0, 0.05 M NaCl, 0.01 M Na_2EDTA, and 0.5% sodium dodecyl sulfate. The homogenate was extracted five times with an equal volume of phenol–chloroform mixture (1:1, vol/vol) saturated with the same buffer (13). The aqueous phase was made 0.2 M in sodium acetate

Abbreviation: Con A, concanavalin A.

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and precipitated with 2.5 volumes of cold ethanol. To remove any contaminating double-stranded RNA which could interfere with the protein synthesis (14), the precipitate was redissolved in 0.2 M sodium acetate and adjusted to 2 M in LiCl; the single-stranded RNA was pelleted by centrifugation and redissolved in 0.5 M NaCl buffered with 10 mM Tris·HCl, pH 7.5. Under these conditions, radioactive poly(rI)·poly(rC) did not copurify with the mRNA preparation. Poly(A)-containing RNA was prepared by chromatography on oligo(dT)-cellulose (Collaborative Research, Inc.); the fraction retained in 0.5 M NaCl and eluted with 10 mM Tris·HCl, pH 7.5, was precipitated with ethanol, redissolved, and used as mRNA (15).

Oöcyte Assay. Oöcytes were obtained by dissecting an adult female *Xenopus laevis*, and the injections were performed as described (16). Ten micrograms of mRNA preparation was dissolved in 50 μl of 66% L-15 medium and injected into 50 oöcytes. For radiolabeling, 500 μCi of [^{3}H]leucine (New England Nuclear) was dissolved in 50 μl of 66% L-15 medium (minus leucine) and injected with the RNA preparation. Injected oöcytes were incubated at 25° in medium for 24 hr; incorporation of [^{3}H]leucine stimulated by injected mRNA under these conditions was 15-fold higher than in the endogenous reaction.

Cell-Free Protein Synthesis. A preincubated cell-free ribosomal system was prepared from Krebs-II cells (17) and enriched with a preparation of partially purified rabbit reticulocyte initiation factors (18); 5 μg of mRNA gave, under our conditions, optimal stimulation of amino-acid incorporation (50-fold above the endogenous reaction). Incubation was at 37° for 1 hr. A cell-free protein synthesis system was also prepared from rabbit reticulocytes (19), and the reaction mixtures were incubated at 30° for 45 min.

Biochemical Characterization of Products from Oöcytes Injected with mRNA and from Cell-Free Ribosomal Systems. Immunoprecipitations were performed as described (17). Since unpurified human fibroblast interferon (purified human fibroblast interferon is not available as yet) was used for immunization, the rabbit interferon antiserum was partially purified by affinity chromatography on an agarose column (Affi-Gel-10) with covalently attached preparation of cell contaminating proteins (isolated by ammonium sulfate precipitation from tissue culture medium after overnight incubation with human fibroblasts).

Electrophoresis was performed in 10% polyacrylamide gels (20). Mobilities were calculated as described (21). Measurements of radioactivity and interferon activity of gel fractions were performed as described (21).

Affinity chromatography on concanavalin A-agarose (Con A-agarose, glycosyl × A) (Miles-Yeda) was used to purify the radioactive material produced both in oöcytes and in cell-free ribosomal systems and was performed as described (22).

Chromatography on columns of rabbit antibody against interferon (which was partially purified as described above) covalently linked to agarose, was done as described (22, 23).

RESULTS

Antiviral Activity Produced in Oöcytes Injected with mRNA and in Cell-Free Ribosomal Systems. Table 1 shows the antiviral activity produced by human mRNA in *Xenopus* oöcytes and cell-free incubation mixtures. The mRNA prepared from human fibroblasts induced by poly(rI)·poly(rC) stimulated the production of measurable quantities of antiviral activity in the Krebs-II and reticulocyte cell-free ribosomal systems; however, approximately 500-fold higher titers of antiviral activity were obtained when this mRNA was injected in *Xenopus* oöcytes. The antiviral substances produced in these systems were species specific; only human fibroblasts, but not mouse L cells, were protected against replication of vesicular stomatitis virus. The antiviral activity of the product was stable to ribonuclease treatment; trypsin treatment (1 unit/ml) reduced the titers of the material produced in oöcytes from 64,000 units/ml to levels lower than 100 units/ml, and completely destroyed the antiviral activity produced in the cell-free system. The same decrease in antiviral activity was achieved by freezing and thawing of the oöcyte homogenate. This may be due to the high proteolytic activity of oöcyte homogenate (Premkumar, unpublished).

The ability of the mRNA preparation to stimulate the synthesis of a biologically active product deteriorated upon prolonged storage and repeated freezing and thawing; treatment of the RNA preparation with ribonuclease completely destroyed the template activity of this RNA to code for the synthesis of human interferon in cell-free systems (Krebs-II ascites). The preparation of mRNA from induced cells, when used in the same concentration as used in cell-free systems (100 μg/ml, with or without ribonuclease treatment), did not induce the antiviral state in human fibroblast cells and thus was free of any contamination by human fibroblast interferon (Table 1). Thus, these results indicate that the interferon was synthesized in the oöcytes and in the cell-free ribosomal system, and its synthesis was an RNA-mediated response.

Biochemical Characterization of Radioactive Products from mRNA-Injected Oöcytes. Electrophoresis of radiolabeled products present in homogenates from *Xenopus* oöcytes injected with mRNA isolated from poly(rI)·poly(rC)-induced human fibroblast cells and with mRNA from the control cells is shown in Fig. 1. Both homogenates contained a major radioactive peak which corresponded to molecular weight of 45,000; however, electrophoretic analyses of the homogenate from oöcytes injected with mRNA from induced cells showed a diffused spread of radioactivity in the region between 18,000 and 40,000 daltons. With the same amount of injected mRNA, the incorporation of [^{3}H]leucine into the acid-precipitable products in oöcytes was significantly higher (2–3 times) with mRNA from induced cells than with mRNA from the control cells.

When the nonradioactive homogenate from the oöcytes injected with induced mRNA was analyzed by gel electrophoresis, antiviral activity (5000 units/ml) was recovered (10% recovery) only from the 18,000–25,000 dalton region. No biological activity (less than 10 units/ml) was recovered from the gel of homogenate from oöcytes injected with control mRNA. We have previously shown (21) that human fibroblast interferon, when analyzed under identical conditions, gives only a single 25,000 dalton peak of antiviral activity. Thus, these results indicate that the product of translation of mRNA isolated from poly(rI)·poly(rC)-induced human fibroblast cells in oöcytes has a similar electrophoretic mobility as human fibroblast interferon induced by poly(rI)·poly(rC) in these cells in tissue culture.

To purify the radioactive material produced in oöcytes, the oöcyte homogenates were precipitated with partially purified rabbit antiserum against interferon and then analyzed by gel electrophoresis (Fig. 1). Two radioactive peaks at 45,000 and 25,000 daltons were detected by this procedure in homogenate from oöcytes injected with induced mRNA; the 45,000 dalton peak contained approximately five times

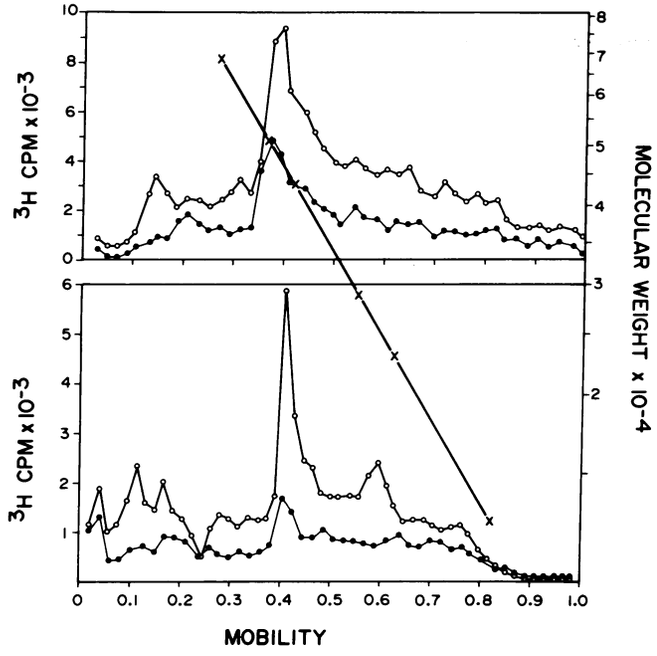

FIG. 1. Electrophoretic analysis of the [^{3}H]leucine-labeled proteins produced in oöcytes injected with mRNA. The upper panel shows samples (50 μl) of the homogenate from oöcytes that were injected with induced mRNA (5 μg) (○) and uninduced mRNA (5 μg) (●). Electrophoresis of the immunoprecipitates of homogenates with antiserum against interferon is shown in the lower panel; oöcytes injected with induced mRNA (○) and with uninduced mRNA (●). Molecular weight standards (×) are bovine serum albumin (68,000), heavy chain of IgG (50,000), ovalbumin (43,000), carbonic anhydrase (29,000), light chain of IgG (23,500), and cytochrome *c* (12,170).

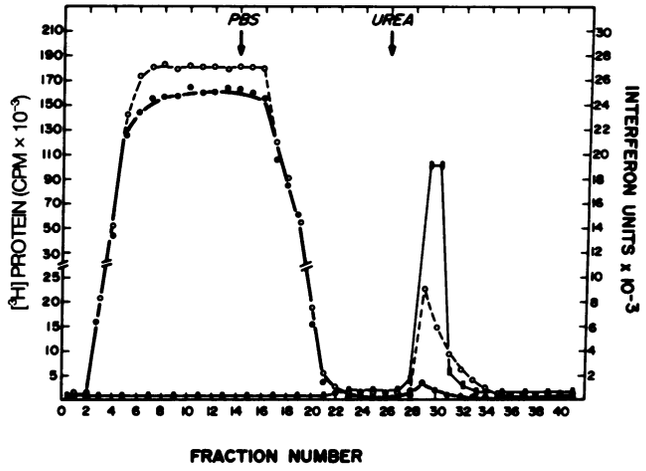

FIG. 2. Chromatography of homogenates from oöcytes injected with mRNA on Con A-agarose column. Homogenates from oöcytes were diluted to 10 ml in phosphate-buffered saline (pH 7.0) (PBS) and applied to the column. The column was then washed with 10 ml of phosphate-buffered saline and eluted with urea buffer (6 M urea, 0.1% sodium dodecyl sulfate, in 0.02 M sodium phosphate buffer, pH 7.0). Interferon activity was regenerated as described (21). Human fibroblast interferon (10 ml, 5000 units/ml) was assayed by antiviral activity (□); [^{3}H]leucine-labeled product from oöcytes that were injected with the uninduced mRNA (●); oöcytes injected with the induced mRNA (○).

more radioactive material than the 25,000 dalton peak. Immunoprecipitation appears to result in a more distinct peak at 25,000 daltons, which is not present in the control.

It was shown that interferons can be purified by affinity chromatography with interferon antibodies (23–25). To examine the affinity of the radioactive product formed in oöcytes to these antibodies, the [^{3}H]leucine-labeled material produced in oöcytes injected with mRNA from induced cells was absorbed on a column of agarose to which rabbit antibodies against interferon had been covalently linked. Five percent of the input radioactivity was eluted with pH 2.2 buffer (which elutes human fibroblast interferon from these columns) while with the control material, 2% of the input radioactivity was eluted (data not shown).

The [^{3}H]leucine-labeled material produced in oöcytes was further purified by affinity chromatography on columns of Con A covalently attached to agarose (22), and bound radioactivity was eluted with a urea-containing buffer (Fig. 2). The urea buffer eluted a large peak of [^{3}H]leucine-labeled material from the homogenate prepared from oöcytes injected with induced mRNA; a much smaller peak of radioactivity was eluted from the control experiment. Electrophoretic analysis of the radioactive material eluted from a Con A column with urea is shown in Fig. 3. The Con A purified material from oöcytes injected with induced mRNA contained approximately the same amount of radioactivity in both 45,000 and 25,000 molecular weight peaks. The material

Table 1. Antiviral activity of translation products

Translation system	mRNA	Interferon titer in units: Human fibroblasts Exp. 1	Human fibroblasts Exp. 2	Mouse L cells Exp. 1
Krebs-II ascites	Control	<6	<6	<6
	Induced	96	96	<6
Rabbit reticulocyte	Control	<6	<6	<6
	Induced	96	96	<6
Xenopus oöcytes	Control	<10	<10	<10
	Induced	128,000	64,000	<10
—	Induced*	<1	—	—

The titers reported are for 0.1 ml of cell-free ribosomal system or for 50 oöcytes homogenized in 1 ml of phosphate-buffered saline (pH 7.2). All interferon assays were done in Eagle's minimal medium supplemented with 15% fetal calf serum. Exps. 1 and 2 were done with two different preparations of mRNA.

* mRNA preparation (100 μg/ml) in Eagle's minimal medium supplemented with 15% fetal calf serum, was applied directly on the cells; after overnight incubation, cells were washed and assayed for the presence of antiviral state by reduction in yield of vesicular stomatitis virus.

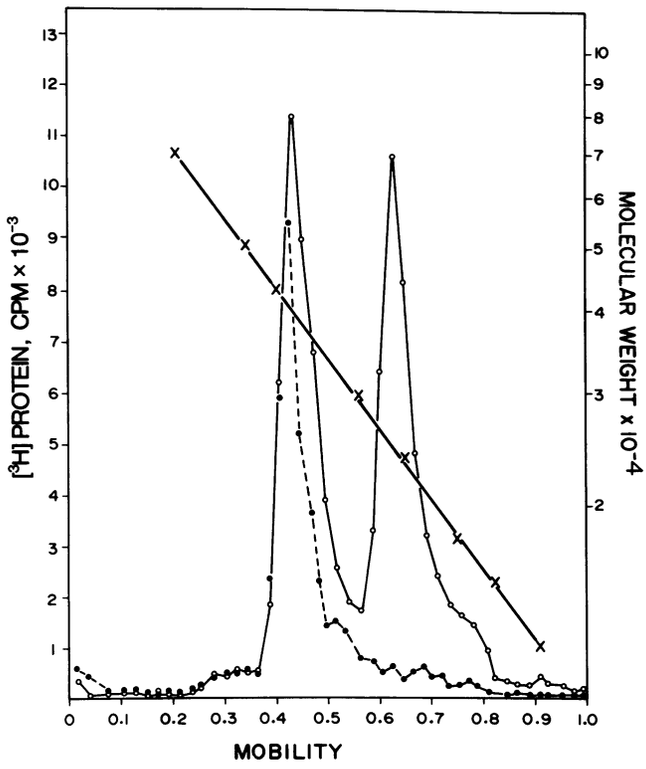

FIG. 3. Electrophoretic analysis of [^{3}H]leucine-labeled products from oöcyte homogenate purified by Con A-agarose chromatography. The fractions (28–34) from the Con A columns (shown in Fig. 2) were pooled, dialyzed for 6 hr against deionized water, lyophilized, dissolved in buffer (10 mM sodium phosphate, pH 7.0, 1% sodium dodecyl sulfate, 0.1 M 2-mercaptoethanol, and 8 M urea), and electrophoresed. Material from oöcytes injected with uninduced mRNA (●); from oöcytes injected with induced mRNA (○). Molecular weight standards (×) are bovine serum albumin (68,000), heavy chain of IgG (50,000), ovalbumin (43,000), carbonic anhydrase (29,000), light chain of IgG (23,500), myoglobin (17,200), hemoglobin (15,500), and cytochrome *c* (12,170).

from oöcytes injected with uninduced mRNA contained only one major peak of radioactivity, of 45,000 molecular weight.

Thus the affinity chromatography procedure on a Con A-agarose column, as well as on columns (23, 24) of antibodies against interferon coupled to agarose (Reynolds *et al.*, unpublished), did not separate the biologically active (25,000 dalton material) and inactive (45,000 dalton) products synthesized in the oöcyte system.

Biochemical Characterization of Radioactive Products from Krebs-II Cell-Free Ribosomal System. The levels of biologically active interferon produced in cell-free ribosomal systems were much lower than those synthesized in oöcytes. This could be caused not only by the lower rate of interferon synthesis, but also by a difference in biological activity or stability of the interferon synthesized either in cell-free systems or oöcytes. The [^{3}H]proteins synthesized in the Krebs-II cell-free ribosomal system were therefore analyzed by gel electrophoresis. Electrophoresis of the postribosomal supernatant from cell-free incubation mixtures stimulated by mRNA isolated from induced cells revealed, among many other protein fractions, the existence of a 25,000 molecular weight radioactive peak. Under identical conditions, human fibroblast interferon contained only a single 25,000 dalton peak of antiviral activity (21). Immunoprecipitation of the postribosomal supernatant with antiserum against interferon prior to electrophoresis did not preferentially enrich the preparation for the 25,000 molecular weight species (data not shown). These data indicate that the products from the cell-free ribosomal system synthesized as a response to the mRNA from induced cells were more heterogeneous than those synthesized in oöcytes. Thus, both biochemical characterization of the radioactive products and low biological activity indicate that the interferon synthesis in the cell-free system used was much less effective than in oöcytes.

DISCUSSION

The present data suggest that human interferon activity was produced in *Xenopus* oöcytes and in cell-free ribosomal systems as a response to mRNA isolated from poly(rI)·poly(rC)-induced human fibroblast cells. The translation in the oöcyte system was much more efficient, giving approximately 500 times higher titers of interferon activity (calculated per μg of mRNA used) than the cell-free ribosomal systems. The interferon activity resulted only from the injection of mRNA from poly(rI)·poly(rC)-induced cells into *Xenopus* oöcytes, and not from the injection of mRNA from the uninduced cells. Also, the appearance in the immunoprecipitate of a 25,000 molecular weight species that had the same electrophoretic mobility as the interferon activity produced in oöcytes and human fibroblast interferon was seen only when oöcytes were injected with mRNA from induced cells. The sensitivity to trypsin treatment and the insensitivity to ribonuclease indicate that a protein is responsible for the observed biological effect. This, plus the species specificity of the antiviral activity produced, suggests that interferon was synthesized in *Xenopus* oöcytes by translation of its mRNA.

It is unlikely that the preparation of interferon mRNA was contaminated with interferon protein. The level of interferon present in the induced human cells at the time when mRNA was extracted was very low (10), and the titers of interferon resulting from the injection of this mRNA preparation into oöcytes were 2-fold higher than those produced by induction of human fibroblasts under optimal conditions. Also, the preparation of mRNA from induced cells, when tested directly on human fibroblast cells, was not able to induce an antiviral state in these cells. Thus, these results indicate that the low levels of interferon activity obtained in cell-free systems are not due to contamination of the mRNA preparation with human fibroblast interferon. Ribonuclease treatment destroyed the ability of the induced mRNA to stimulate interferon synthesis in the Krebs-II ascites system. We have previously shown with the identical mRNA preparation that treatment with ribonuclease or sodium hydroxide completely destroyed the ability of the RNA to code for human interferon in chick cells, but treatment with deoxyribonuclease or Pronase was without any effect (10).

There are two main reasons why the oöcyte system seems to be superior to the previously described assay in chick cells. It is much more efficient, giving 50-fold higher levels of interferon than the chick cell system (10), and, thus, is probably more sensitive for detection of low levels of interferon mRNA. Furthermore, in the oöcyte system, highly radioactive human interferon was synthesized, while the labeling of human interferon synthesized in the chick cells was very low (Reynolds and Pitha, unpublished). This difference probably reflects the lower internal amino-acid pool in the oöcytes. Whether this system is suitable for the quantitation of interferon mRNA needs further evaluation. For studies of events regulating the synthesis of interferon, however, the

cell-free system is most appropriate. Although the activity resulting from translation of the mRNA from induced cells in the cell-free ribosomal systems used was rather low, the fact that biologically active protein was obtained by translation of its mRNA in the *in vitro* system is encouraging; it may be the first evidence that a biologically active protein can be synthesized in cell-free systems as a response to stimulation by eukaryotic mRNA.

While the antiviral activity was produced in *Xenopus* oöcytes only as a result of stimulation by mRNA isolated from poly(rI)·poly(rC)-induced cells, and not by mRNA from uninduced control cells, both preparations of mRNA served as effective templates for the synthesis of radioactive proteins in this system. This reflects the fact that preparation of a total poly(A)-rich RNA was used for injection and no attempts were made in the present study to purify the interferon mRNA. One major radioactive protein was identified from the oöcyte homogenate by gel electrophoresis. This 45,000 molecular weight species was synthesized in response to both induced and uninduced mRNA. The amount of this protein produced in oöcytes injected with mRNA from induced cells was higher than in oöcytes injected with mRNA from the uninduced control. This may indicate that stimulation of human fibroblasts with poly(rI)·poly(rC) not only induces transcription of interferon mRNA, but may also enhance the transcription of mRNA for proteins other than interferon that are constitutively produced by these cells. A difference in stability between the induced and control mRNA preparations, however, could also account for these results.

It has been suggested that human fibroblast interferon, like other species interferons, is a glycoprotein. It is not completely clear as yet whether glycosylation of the interferon molecule is essential for its antiviral activity, or a prerequisite for the secretion of interferon by the cell. The question, therefore, arises whether glycosylation of the interferon molecule occurred also in the oöcytes. Although the binding to immobilized Con A may be an indication of the presence of a single or multiple carbohydrate moieties on the interferon molecule, it is not an unambiguous proof, since the possibility of hydrophobic interaction between Con A and human fibroblast interferon has also been suggested (22).

In summary, the production by *Xenopus* oöcytes of a 25,000 dalton protein with the properties of interferon, and its partial purification, suggests that this system may be suitable for preparation of radiolabeled human interferon, suitable for physicochemical studies. The synthesis of biologically active interferon in cell-free systems should permit study of the regulation of interferon synthesis on the molecular level. Further purification of interferon mRNA from the majority of other cellular mRNAs should increase both the efficiency of translation and the purity of the synthesized interferon.

We thank Dr. A. H. Owens, Jr. for continuous interest and encouragement, Dr. K. Paucker for the gift of antisera, and Drs. S. P. Staal and T. J. Kelly, Jr. for their help with the manuscript. The work was supported by grants from the National Institutes of Health (AI 10944-03) and the American Cancer Society, Maryland Division. P.M.P. is a scholar of the Leukemia Society of America, Inc.

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