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Relationship between interferon production and interferon messenger RNA synthesis in human fibroblasts

(cell-free protein synthesis/*Xenopus* oöcytes/messenger RNA translation/superinduction)

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ABSTRACT **Poly(A) containing mRNA prepared from poly(rI)·poly(rC)-induced human fibroblasts stimulated [^{14}C]leucine incorporation into protein in wheat germ cell-free extracts. For the translation of interferon mRNA into a biologically active product, the presence of spermine was essential. The protein synthesized *in vitro* fulfilled the criteria for human interferon—namely, its antiviral activity was species specific, and its activity was completely neutralized by antiserum to human fibroblast interferon.**

The amount of interferon synthesized in human fibroblasts induced by poly(rI)·poly(rC) (normal induction) and poly(rI)·poly(rC) in the presence of cycloheximide (superinduction) was compared to the amount of translatable interferon mRNA both in the wheat germ cell-free system and the *Xenopus* oöcyte system. Although the production of interferon after the termination of transcription by actinomycin D was markedly increased in superinduced cells, the measurable amount of interferon mRNA as assayed in the oöcyte system was only slightly higher in superinduced cells than in cells induced with poly(rI)·poly(rC) alone. When compared in the wheat germ cell-free system, however, the translational product of mRNA preparation from cells induced with poly(rI)·poly(rC) alone was inactive while that from superinduced cells was active.

The study of regulation of genetic expression in eukaryotic cells requires isolation, characterization, and quantitation of separate gene products. The interferon system involves induction of an antiviral protein, interferon, by a well-defined inducer and its subsequent interaction with the cell which leads to development of the antiviral state (1). The high biological activity of interferon (at least 10^9 biological units/mg of protein) provides a special advantage for studying the regulated expression of mammalian genes because even small amounts of interferon mRNA as well as its translational product can be detected by a bioassay in which decrease in virus yield is measured (2).

The synthesis of interferon, which is not detectable in uninfected cells, can be induced in cultured cells by viral infection or by some polyribonucleotide complexes [e.g., poly(rI)·poly(rC)] (3). The regulatory steps involved in the transcription of interferon mRNA and its translation have been analyzed predominantly in terms of susceptibility to metabolic inhibitors. It has been shown that production of interferon could be considerably enhanced by judicious use of inhibitors of protein or RNA synthesis during the induction process (superinduction) (4–6), and it was suggested that, as with many other inducible responses, the production of interferon was regulated post-transcriptionally (7). However, there are a number of problems involved in the unambiguous interpretation of these experiments, since inhibitors may have multiple effects on cellular metabolism (8). Hence, to investigate cellular control involved in interferon induction, it is necessary to quantitate mRNA levels directly.

In the current study, the amounts of interferon mRNA induced by poly(rI)·poly(rC) in human fibroblasts were compared to the amount of mRNA induced by poly(rI)·poly(rC) in the presence of cycloheximide (superinduced cells). Interferon mRNA was assayed by its translation in *Xenopus* oöcytes and in a wheat germ cell-free protein synthesizing system. The *in vitro* system was found to be highly efficient and quantitative. The results presented indicate that interferon mRNAs isolated from human fibroblasts induced by poly(rI)·poly(rC) in the presence and absence of cycloheximide can stimulate the synthesis of interferon in *Xenopus* oöcytes equally well, but their translational capacities differ significantly in the cell-free wheat germ system.

MATERIALS AND METHODS

Induction and mRNA Preparation. Human foreskin fibroblast cells (obtained from J. Vilcek) were grown in roller bottles in Eagle's minimal medium supplemented with 5% (vol/vol) fetal bovine serum and gentamicin at 50 μg/ml (9). Ten- to 12-day-old cultures were induced with poly(rI)·poly(rC) (100 μg/ml) in 0.01 M phosphate buffer, pH 7.4/0.15 M NaCl in the presence of cycloheximide (50 μg/ml). After 2 hr of incubation at 37°, the medium was replaced with Eagle's minimal medium containing cycloheximide (50 μg/ml). Four hours later, the cells were harvested, washed with the phosphate-buffered saline, and stored at −70° until the time of RNA extraction. Control cells were treated with cycloheximide but not with poly(rI)·poly(rC).

RNA was extracted as described previously (2). Cells from 30 roller bottles were homogenized in 0.2 M Tris·HCl, pH 9.0/0.05 M NaCl/0.01 M Na_2EDTA/0.5% (wt/vol) sodium dodecyl sulfate. The homogenate was extracted with an equal volume of phenol saturated with the same buffer. The aqueous phase was extracted three times with a phenol/chloroform mixture, 1:1 (vol/vol). DNA was removed by precipitation with 1 volume of ethanol containing 2% (wt/vol) potassium acetate. RNA was precipitated with 2.5 volumes of ethanol. Single-stranded RNA was precipitated with 2 M LiCl in the cold overnight. The precipitate was dissolved in 10 mM Tris·HCl, pH 7.5/0.5 M NaCl and passed through a column of oligo(dT)-cellulose (Collaborative Research Inc.) equilibrated with the same buffer. The RNA fraction that eluted with 10 mM Tris·HCl, pH 7.5, was precipitated with ethanol and used as mRNA.

Translation in Wheat Germ Extracts. Extracts of raw wheat germ (General Mills, Minneapolis, MN) were prepared according to Roberts and Paterson (10). A standard *in vitro* reaction mixture contained per 50 μl: 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.6), 2 mM dithiothreitol, 80 mM KCl, 2 mM magnesium acetate, 1 mM ATP, 25 μM GTP, 7.5 mM creatine phosphate, 1 μg of creatine phosphokinase, 15 μl of S-30, 100 μM spermine, 0.5 μCi of [^{14}C]leucine (299 mCi/mmol), 0.04 mM each of 19 nonradioactive amino acids and, when indicated, 2 μg of mRNA.

After incubation for 45 min at room temperature, the reaction mixture was diluted with Eagle's minimal medium supplemented with 5% fetal bovine serum and used for the interferon assay. For measuring the time course of [^{14}C]leucine incorporation, 10-μl samples were withdrawn at indicated time intervals and radioactivity was measured as described (10).

Translation in *Xenopus* Oöcytes. Oöcytes were obtained and microinjected with RNA solutions as described recently (2); mRNA was dissolved in 50 μl of L-15 medium and 20 oöcytes were injected for each experiment. Injected oöcytes were incubated at room temperature for 24 hr and then were homogenized and centrifuged at 15,000 × *g* for 20 min. The supernatant was assayed for antiviral activity in Eagle's minimal medium containing 15% fetal bovine serum.

Interferon Assay. Interferon titers were measured both by reduction in virus yield and by the colorimetric method of Finter (11). Vesicular stomatitis virus, New Jersey serotype, was used as a challenge virus. One unit of interferon was defined as the minimal amount required to decrease the virus yield by 50%. The titers are given in international reference standard units; 1 unit of international reference standard unit was titrated as 0.33 unit in our assay.

Anti-interferon sera were obtained from G. Gallasso (National Institutes of Health) and had been prepared in rabbits by using either human fibroblast interferon [poly(rI)·poly(rC) induced] or human leukocyte interferon (induced with Sendai virus) as antigens. A 1024-fold dilution of antiserum to fibroblast interferon completely neutralized 10 units of fibroblast interferon; however, an 8-fold dilution of this serum did not affect the activity of 10 units of leukocyte interferon. There was a partial crossreactivity in the antiserum to leukocyte interferon: a 10,000-fold dilution of it neutralized 10 units of leukocyte interferon and a 240-fold dilution neutralized 10 units of fibroblast interferon.

RESULTS

Standardization of Wheat Germ Cell-free System for Interferon Synthesis. For quantitative studies of molecular events regulating the synthesis of interferon, the cell-free system is most appropriate. It was shown previously that human interferon mRNA can be translated with fidelity in cell-free ribosomal systems (2, 12). Because a number of eukaryotic and viral mRNAs were found to be efficiently translated in a wheat germ cell-free extract (10, 13), we examined the possibility of using this system for *in vitro* mRNA-directed synthesis of biologically active interferon.

Translation of human interferon mRNA in the wheat germ cell-free system into an active product requires the presence of spermine. Under the conditions used, the increase in Mg^{2+} concentration did not substitute for the presence of spermine (data not shown). The optimal concentration for spermine was critical and was found to be 80–100 μM (Fig. 1*A*). The presence of spermine, however, did not substantially affect the efficiency of translation of poly(A)-rich RNA when measured by [^{14}C]leucine incorporation into acid-insoluble precipitate (Fig. 1*B*). The poly(A)-rich RNA used was not homogeneous but contained a large number of mRNA species (N. B. K. Raj and P. M. Pitha, unpublished data); the template activity of the majority of the mRNA species may not be affected by spermine. Spermine also promotes the completion of translation; a requirement of spermine for efficient translation of avian myeloblastosis virus RNA and mouse interferon mRNA in the wheat germ cell-free system was noted earlier (14, 15).

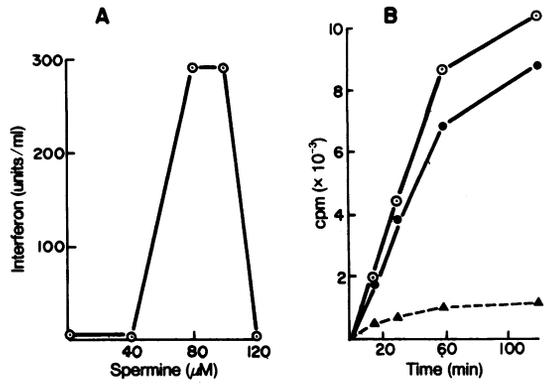

FIG. 1. Effect of spermine on the efficiency of translation of the RNA fraction containing interferon mRNA in wheat germ cell-free system. Translation and preparation of poly(A)-rich RNA from human fibroblast cells induced with poly(rI)·poly(rC) in the presence of cycloheximide were as described in *Materials and Methods*. (*A*) Incubation was for 45 min in a total volume of 100 μl containing 5 μg of RNA. At the end of incubation, the reaction mixture was assayed for interferon activity. (*B*) Incubation was in a total volume of 50 μl containing 2 μg of RNA; at each time point, 10-μl aliquots were withdrawn and radioactivity in the trichloroacetic acid-insoluble fraction was measured. ▲, no exogenous RNA; ⊙, incubation in the presence of 100 μM spermine; ●, incubation without spermine.

The antiviral activity was synthesized only in response to exogeneously added poly(A)-rich RNA isolated from the superinduced cells. The RNA from the uninduced cells or RNA fraction from the induced cells that did not bind to the oligo(dT)-cellulose column did not direct synthesis of the interferon-like product (Table 1). The fact that no interferon-like activity was synthesized in the absence of spermine and without appropriate incubation indicates that the observed activity was

Table 1. Human interferon synthesis in wheat germ cell-free extract stimulated by RNA fractions from poly(rI)·poly(rC)-induced human fibroblasts

RNA from	Oligo(dT)-cellulose RNA fraction*	Incubation time, min	Spermine	Interferon activity units/ml
—	None	45	+	<30
Human fibroblasts induced in presence of cycloheximide	Flow through	45	+	<30
	mRNA bound	45	+	480
	mRNA bound	0	+	<30
	mRNA bound	45	−	<30
Human fibroblasts treated with cycloheximide only	Flow through	45	+	<30
	mRNA bound	45	+	<30

* The RNA fractions (10 μg) were incubated with the wheat germ extract either in the presence (+) or absence (−) of 100 μM spermine, and the antiviral activity of the translational product was assayed as described in *Materials and Methods*.

Table 2. Neutralization of antiviral activity of translational product by antiserum against human interferon

Antiserum from	Interferon, units/ml
None	120
Fibroblasts*	<12
Leukocytes†	96

* Interferon produced in fibroblast cells induced by poly(rI)·poly(rC).

† Interferon produced in leukocytes induced with Sendai virus. Translation was carried out as described in *Materials and Methods*. Aliquots containing 120 units of interferon were incubated at 37° for 60 min in the presence of 1/50 dilution of antiserum against fibroblast or leukocyte interferon. The control sample was incubated in Eagle's minimal medium supplemented with 5% fetal bovine serum. All samples were then tested for antiviral activity.

not due to the contamination of interferon mRNA with interferon protein or poly(rI)·poly(rC).

The activity of the antiviral product (288 units/ml) was completely destroyed by trypsin treatment (<24 units/ml); it was not affected by ribonuclease treatment (288 units/ml). The antiviral substance produced in the wheat germ system was species specific: human fibroblasts (288 units/ml), but not mouse L cells (<24 units/ml) or rat cells (<24 units/ml), were protected against virus replication. Antiserum against human fibroblast interferon completely neutralized the antiviral activity of the synthesized protein, but the antiserum against human leukocyte interferon had only a slight neutralization effect (Table 2). This is in agreement with the results of others (16, 17) who showed that, although the antiserum against human fibroblast interferon is monospecific, the antiserum against human leukocyte interferon crossreacts slightly with human fibroblast interferon. Thus, these results plus the species specificity of the antiviral activity produced suggest that interferon was synthesized in the wheat germ system by translation of its mRNA.

We also examined whether in the wheat germ system the *de novo* synthesis of human interferon was directly proportional to the amount of interferon mRNA added. Fig. 2 shows a linear increase in the amount of interferon synthesized in the presence of exogenous RNA (isolated from superinduced cells) between 2.5 and 10 μg per reaction mixture. This indicates that, within these limits, the synthesis of interferon is limited only by the amount of exogenously added mRNA, which makes this system suitable for the quantitation of human interferon mRNA. The linearity in interferon production is probably a function of the RNA preparation used; it was recently shown that synthesis of

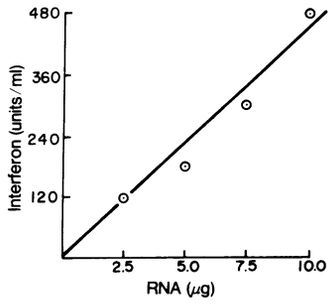

FIG. 2. Translational activity of interferon mRNA in the wheat germ cell-free system. Translation was performed as described in *Materials and Methods* in the presence of 100 μM spermine. Incubations with poly(A)-rich fraction of RNA isolated from superinduced cells were for 45 min at room temperature.

Table 3. Interferon yields from cells induced by poly(rI)·poly(rC) in presence and absence of metabolic inhibitors

	Interferon yield, units/ml/per hr		
Hours	Poly(rI)·poly(rC)*	Poly(rI)·poly(rC)† + actinomycin D	Poly(rI)·poly(rC)‡ + cycloheximide + actinomycin D
0–3	40	40	<20
3–6	20	20	80
6–9	8	16	1280
9–12	<4	16	1280
12–24	<4	8	320

Data from a representative experiment. In all cases, the medium was dialyzed before assay for interferon activity.

* Cells were induced with poly(rI)·poly(rC) (100 μg/ml) in phosphate-buffered saline for 2 hr at 37°. Poly(rI)·poly(rC) solution was then removed, and the cells were washed and overlayed with Eagle's minimal medium. Interferon medium (3 ml per dish) from two duplicate cultures was pooled at times indicated and the cells were overlayed with Eagle's minimal medium supplemented with 2% fetal bovine serum.

† Cells were induced as above. Actinomycin D (2 μg/ml) was added to the culture medium at 5 hr after induction. At 6 hr, the medium was removed, the cells were washed, and Eagle's minimal medium with 2% fetal bovine serum was added. Interferon was collected as described above.

‡ Cells were induced by poly(rI)·poly(rC) in the presence of cycloheximide (50 μg/ml). This medium was removed at 2 hr after induction and replaced with Eagle's minimal medium containing cycloheximide (50 μg/ml). Actinomycin D (2 μg/ml) was added at 5 hr. At 6 hr after induction, the medium containing metabolic inhibitors was removed and replaced with Eagle's minimal medium containing 2% fetal bovine serum.

mouse interferon in the wheat germ system was not proportional to the amount of added RNA (15). Under the same conditions, poly(A)-rich RNA fraction isolated from the normally induced human fibroblast in the same concentration range (2.5–10 μg per reaction mixture) did not stimulate the synthesis of detectable amounts of human interferon.

Quantitation of Interferon mRNA in Poly(rI)·Poly(rC) Induced and Superinduced Human Fibroblasts. It has been shown previously (18) that the kinetics of interferon and its mRNA induction were practically the same in human fibroblasts induced and superinduced by poly(rI)·poly(rC); in poly(rI)·poly(rC)-induced cells, synthesis of both interferon and its mRNA reached their maxima at 6 hr after the start of induction. To compare the amount of interferon mRNA present in human fibroblasts with the amount of interferon produced by these cells under conditions of normal and superinduction, human fibroblasts were either induced with poly(rI)·poly(rC) alone or in the presence of cycloheximide and both cultures were treated 5 hr later with actinomycin D for 1 hr to terminate the transcription. The yield of interferon before and after termination of RNA synthesis was compared with the relative amount of translatable interferon mRNA present in the cells at 6 and 8 hr after induction (Tables 3 and 4). It can be seen that while the rate of interferon synthesis during the first 6 hr of induction in normally induced and superinduced cells did not differ greatly, the difference in the amount of interferon produced in these two systems after termination of transcription by actinomycin D was large. In normally induced cells there was a decline in interferon synthesis between 6 and 9 hr after induction; in the same time interval, in superinduced cells the rate of interferon synthesis increased by 100-fold.

If interferon synthesis were regulated at the transcriptional

Table 4. Interferon produced by translation of mRNA isolated from cells induced and superinduced by poly(rI)·poly(rC)

Translational system	RNA,* μg	Induction time, hr	Interferon titer				
				Normally induced		Superinduced	
			Uninduced	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Wheat germ	10	6	<30	<30	<30	488	480
Xenopus oöcytes	5	6	<15	4500		6000	
(n = 20)	5	8			3600		4800
	2.5	8			1800		1800

Cells were induced by poly(rI)·poly(rC) (100 μg/ml) in phosphate-buffered saline in the presence (50 μg/ml) or absence of cycloheximide for 2 hr at 37°. Cells were washed and overlayed with Eagle's minimal medium with or without cycloheximide (50 μg/ml) (superinduced cells). Actinomycin D (2 μg/ml) was added to superinduced cells, 5 hr after induction, for 1 hr. Cells were harvested at 6 or 8 hr after induction and RNA was prepared as described in *Materials and Methods*. Exps. 1 and 2 were done with two different preparations of mRNA.
* Per 100 μl of reaction mixture of cell-free wheat germ system or per 50 μl of L-15 medium.

level, then the rate of interferon synthesis during this time interval should reflect the amount of interferon mRNA present in the cells at that time. The poly(A)-rich fraction of the total cellular RNA isolated from normally and superinduced cells was therefore assayed for its ability to direct the synthesis of biologically active interferon both in the wheat germ system and *Xenopus* oöcytes (Table 4). In oöcytes, the injected mRNA was being translated for 24 hr and thus approximately 20 times more biologically active product was synthesized compared to the wheat germ system in which the translation is completed in 45 min. The interferon mRNA from superinduced cells was translated with fidelity in both systems. The mRNA isolated from poly(rI)·poly(rC)-induced cells, however, was not translated into biologically active product in the wheat germ system whereas in oöcytes its translation was not greatly impaired. Thus, under conditions such that the amount of interferon synthesized in superinduced cells was 35-fold higher than in normally induced cells, the amount of interferon mRNA as assayed by its translation in oöcytes was only slightly (25%) higher in superinduced cells than in those induced by poly(rI)·poly(rC) alone. It is important to point out that the translation in oöcytes was not done under saturation conditions; the amount of interferon synthesized was proportional to the amount of injected mRNA (Table 4). These results indicate that not all interferon mRNA induced by poly(rI)·poly(rC) in human fibroblast cells is translated in these cells.

Although in normally induced cells the interferon synthesis declines by 6 hr after induction, the detectable amount of interferon mRNA at 6 and 8 hr after induction was approximately the same (Table 4). This suggests that the shut-off in interferon synthesis (Table 3) in normally induced cells is not due to the fast degradation of interferon mRNA.

The synthesis of interferon in the wheat germ system was found to be directly proportional to the amount of exogenous interferon mRNA used (Fig. 2) and therefore should detect a 25% decrease in the amount of interferon mRNA. Hence, no biologically active product was synthesized, within the limits of detection, by translation of interferon mRNA from poly(rI)·poly(rC)-induced cells.

The results further show that the interferon induction by poly(rI)·poly(rC) is the *de novo* induction process; no interferon mRNA was detected in the uninduced cells in either translational system.

DISCUSSION

The earlier findings (19–21) that the induction of interferon by poly(rI)·poly(rC) can be considerably enhanced by inclusion of metabolic inhibitors such as cycloheximide, actinomycin D, and 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole indicated that inhibition of macromolecular synthesis is linked to the superinduction of interferon production. In poly(rI)·poly(rC)-induced cells the interferon production was found to be shut off shortly after the induction; however, this "shut off" was prevented by the presence of inhibitors of RNA and protein synthesis, which caused the marked increase in interferon production. It previously has been shown that the amounts of interferon mRNA induced in normal and superinduced cells did not differ greatly (within the limits of the assay) in these two systems (18). However, the chick cell system which was used previously for the assay of interferon mRNA was rather insensitive and had a large biological variability. For quantitative studies of events regulating the synthesis of interferon, more sensitive methods of detecting and quantitating interferon mRNA were needed. *Xenopus* oöcytes translate interferon mRNA with both high efficiency and fidelity (2) and are therefore useful for detection of small amounts of interferon mRNA; also, the translation was shown to be dependent only on the amount of interferon mRNA injected and not to be affected by the presence of other RNA species. The present data suggest that the human interferon mRNA can also be translated with fidelity in the wheat germ cell-free extract.

The data presented show an obvious paradox. The production of interferon was much higher in superinduced cells than in normally induced cells. When the relative amounts of interferon mRNA present in the normally induced and superinduced cells were compared by translation in *Xenopus* oöcytes, the levels of interferon mRNA detected were practically the same. However, when compared by translation in the wheat germ system, a large difference in the detectable amount of interferon mRNA was observed. These results indicate that the interferon mRNA from normally induced cells is not translated effectively *in vivo* (in the producing cell) or *in vitro* (in the wheat germ cell-free system), but it can be translated in *Xenopus* oöcytes.

It is possible that, in normally induced and superinduced cells, approximately the same levels of interferon mRNA are present as poly(A)-rich molecules, but the majority of interferon mRNA induced by poly(rI)·poly(rC) is present in inactive form, and it is not translated into biologically active protein. The heterologous cell system such as *Xenopus* oöcytes or chick cells (9) can modify the inactive interferon mRNA, and interferon is synthesized, whereas the wheat germ system may not have the ability to activate this mRNA. The impairment of interferon mRNA can be prevented by the presence of cycloheximide during the induction period (superinduction) and therefore the interferon mRNA isolated from superinduced cells is translated

both in *Xenopus* oöcytes and in a wheat germ cell-free system. The difference in the efficiencies of translation of these mRNA molecules could also reflect a qualitative difference in interferon mRNAs transcribed in normally induced and superinduced cells [in human fibroblasts, asystemic genetic loci are required for interferon induction (22)]. Hence, other possibilities—e.g., that the mRNA fraction from normally induced cells may contain a translational inhibitor active in wheat germ systems—cannot be eliminated, and further studies are needed to elucidate the nature of the observed translational difference of the two kinds of interferon mRNA.

Finally, it should be pointed out that it is unlikely that the control element could be a simple mRNA competition such as suggested (23) for superinduction of ovalbumin synthesis. According to this hypothesis, under conditions of inhibition of RNA synthesis the relatively unstable mRNA would have broken down and the newly formed interferon mRNA molecules should be translated at an increasing rate because of more favorable competition for the rate-limiting factors. Our observations are not in accord with this hypothesis for the following reasons: (*i*) in the absence of cycloheximide, actinomycin D treatment of poly(rI)·poly(rC)-induced cells did not lead to a substantial increase in interferon synthesis; and (*ii*) the results from the oöcyte system indicate that the relative amounts of mRNA in normally induced and superinduced cells are comparable. This indicates that the difference observed in the wheat germ system cannot be due to the competition with the other cellular mRNA species. The recent results (19) on superinduction of interferon by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole are also inconsistent with the simple mRNA competition hypothesis.

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