THE INDUCTION OF INTERFERON AND ITS MESSENGER RNA IN HUMAN FIBROBLASTS

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SUMMARY. RNA from human fibroblasts containing mRNA for human interferon was isolated and translated with fidelity in chick fibroblast cells. RNA stimulating the synthesis of human interferon was found to sediment predominantly at 7-10S and bind to oligo(dT)-cellulose. The rate of interferon synthesis in human fibroblasts was compared to the amount of interferon specific mRNA synthesized both in cells induced by poly(I · C) and poly(I · C) in the presence of metabolic inhibitors (superinduced cells). While the production of interferon was markedly increased in superinduced cells, the measurable amount of human interferon mRNA was only slightly higher in superinduced cells when compared to those induced with poly(I · C) alone.

The mechanism of interferon induction by poly(I · C) in human fibroblasts still remains unclear. As in many other inducible systems (1), the yield of interferon can be enhanced by the judicious employment of metabolic inhibitors during the induction period (2). This superinduction effect suggests that post-transcriptional levels of control may be involved in the regulation of interferon synthesis (3).

It has been shown that interferon specific mRNA isolated from mouse and monkey cells can be translated with fidelity in heterologous cell systems (4). In the experiments described, this assay was applied to the human system under various conditions of induction with analysis of the levels of interferon induced by poly(I · C) in human fibroblasts in comparison to the amounts of interferon mRNA synthesized in these cells.

MATERIALS AND METHODS. Poly(I · C) complex, obtained from P. L. Biochemicals, Inc., had a molecular weight higher than $2x10^5$, and a molar extinction coefficient of $4.5x10^3$ at 260nm (in 0.01M sodium phosphate, pH 7.0). Oligo(dT)-cellulose was purchased from Collaborative Research, Inc.

Human fibroblast cells (passage 8-15), previously identified as high

interferon producers, were used in all studies (3). They were grown in MEM*, supplemented with 5% FBS, and used for interferon induction 12 days after seeding. Interferon was stored at -70°C (pH 5.0) and assayed collorimetrically (5) employing Vesicular stomatitis virus (New Jersey serotype) as a challenge virus. The activity is given in research reference units.

RESULTS. Performing a sensitive assay of human interferon mRNA enabled us to directly measure and compare the rate of interferon production with the synthesis of interferon mRNA, both in poly(I · C) induced and superinduced cells. Human fibroblasts begin to produce interferon within 2-3 hrs. of induction; the rate reaches its maximum at 6 hrs. and declines to insignificant levels by 24 hrs. (Fig. 1A). While the kinetics of induction were principally the same under conditions of our experiments in both examined systems, the extent of interferon production was markedly enhanced in superinduced cells. The question, therefore, arises - What are the differences in the translatable interferon mRNA present in these two systems. When RNA was prepared from human fibroblasts induced by poly (I · C) (under conditions identical to those used for study of the rate of interferon synthesis) at different times after induction and assayed on chick cells, the kinetics of assayable mRNA production paralled the production of interferon (Fig. 1B). In poly(I · C) induced cells, both interferon production and synthesis of interferon mRNA plateau at 6 hrs. after induction. In superinduced cells, no stabilization of the interferon mRNA was observed, as it was recently suggested (3).

However, there was a quantitative difference between the amount of interferon and its assayable mRNA when these two systems were compared. The levels of human interferon produced by chick systems should correspond to the total amount of interferon mRNA present in the human cells at any corresponding time after induction. These interferon levels may best be compared to the inter-

*Abbreviations Used: 1) MEM - Eagle's minimal medium (with Earle's salts)
2) FBS - Fetal bovine serum
3) PBS - 0.02M phosphate buffer, pH 7.4 containing 0.14M NaCl
4) DEAE-dextran - diethylaminoethyl-dextran

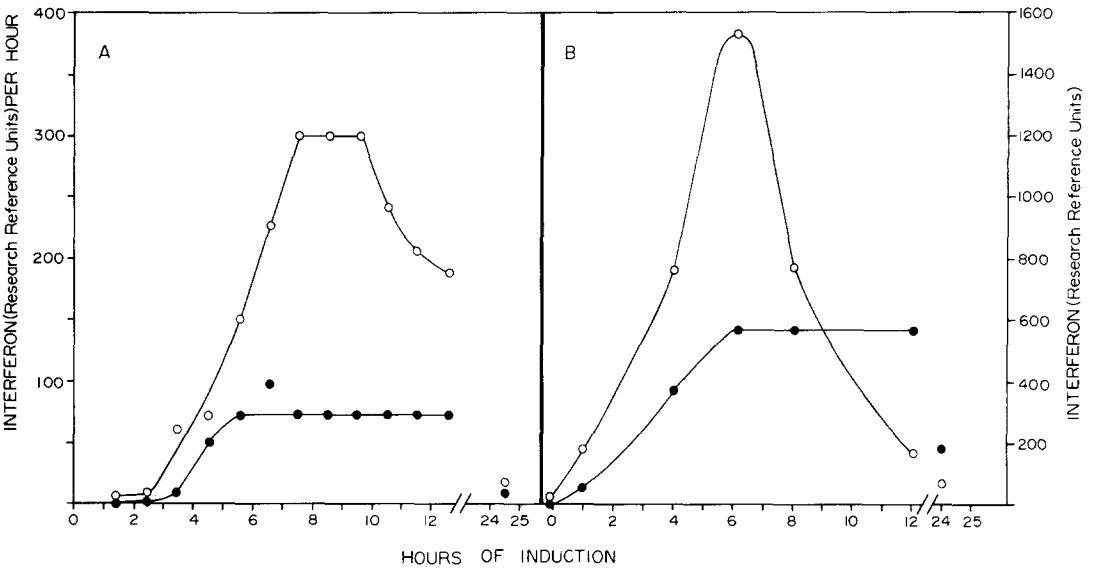

Fig. 1 - Time course of the induction of interferon and its mRNA. (A) Human fibroblasts (passage 9) were induced with poly(I · C) (100µg/ml) in PBS (•). Identical monolayer cultures were superinduced by including cycloheximide (50µg/ml) in the poly(I · C) solution followed by cycloheximide (50µg/ml) in MEM for 5 hrs. with actinomycin D (1µg/ml) added at hrs. 5½ to 6 (o) (3). In superinduced cultures at times up to 6 hrs., the cycloheximide in MEM was replaced by MEM supplemented with 2% FBS for the hour of interferon production. Interferon containing media (2ml) was collected and pooled from two (60mm diameter) petri dishes per point per hour. (B) Total cellular RNA was prepared by direct extraction of cell monolayers in ½ gal. roller bottles at the indicated times with an equal mixture of 15ml cold buffer 0.2M Tris-HCl, pH 9.0, 0.05M NaCl, 0.01M disodium ethylenediamine-tetraacetate acid and 0.5% sodium dodecyl sulfate and 15ml buffer saturated phenol and chloroform mixture (1:1, v/v). The aqueous phase was reextracted 5 times followed by the addition of sodium acetate to 0.2M and precipitated with 2.5 volumes of cold ethanol (7). Following centrifugation, the nucleic acid precipitates were dissolved in 0.2M sodium acetate and LiCl was added to 2M. After standing at 4°C overnight, the single stranded RNA was pelleted by centrifugation, dissolved in water and stored at -70°C. RNA fractions containing mRNA specific for human interferon were translated in primary chick fibroblast cells, which were seeded at a density of $1x10^5$ cells per 60mM petri dish 3 days before use (4). For the assay, cells were treated with actinomycin D (0.075µg/ml) for 6 hrs., washed, and incubated with the mixture of tested RNA (50µg/ml) and DEAE-dextran (50µg/ml) in PBS for 1 hr. at 37°C. After washing, cells were incubated with MEM (2% FBS) for an additional 4 hrs., then the medium was collected, stored overnight at 4°C, and interferon assayed in human fibroblast cells (5). Chick cell assays of RNA from poly(I · C) induction alone (•) and superinduced cellular RNA (o) are shown for various induction times.

feron produced in human fibroblasts after the transcription has been terminated by actinomycin D. From this reason, human fibroblasts either induced by poly (I · C) or superinduced were treated with actinomycin D (1µg/ml) 5½ hrs. after induction for 30 min., and interferon was collected 24 hrs. post induc-

tion. Under these conditions, the superinduced cells produced approximately 30 times more interferon (5,000 units) than those cells induced with poly(I · C) alone (180 units); however, the amount of assayable interferon mRNA was only 2½ times higher in superinduced cells (1,500 units) than in the poly(I · C) induced cells (600 units), when the RNAs were isolated 6 hrs. post induction. Within the limits of the chick cell assay, these results indicate that not all interferon mRNA which is induced by poly(I · C) in human fibroblasts is translated in these cells.

Fractionation of the RNA containing interferon mRNA by velocity sedimentation revealed that the majority of interferon specific mRNA sedimented between 7-10S (Table I). However, biological activity was also found sedimenting with the 18S ribosomal RNA fraction, thus indicating that both small and large mRNAs may be present in this system. In control experiments not shown, treatment of the RNA preparations with RNAase or sodium hydroxide, completely

Table I. Gradient Fractionation of Cellular RNAs

Cellular RNA prepared at hour 6 of superinduction as described in the legend to Fig. 1 was fractionated by velocity sedimentation essentially as described for the preparation of hemoglobin mRNA (8). One hundred A_{260} units of RNA in 1ml were layered over 37ml of 10-30% ribonuclease-free sucrose (Schwarz-Mann) gradient and centrifuged at 27,000 rpm in a SW-27 rotor (Beckman) at 1.5°C for 20 hrs. Fractions were collected and pooled in comparison to markers of human fibroblast tRNA, and 18 and 28S ribosomal RNAs. The 18-28S fraction included any larger RNAs from the bottom of the tube. Peak fractions were concentrated to 1ml by dilution in gradient buffer (0.01M Tris-HCl, pH 7.5, 0.05M NaCl and 0.01M disodium ethylenediamine-tetraacetate acid) and centrifugation for 30 hrs. at 55,000 rpm in a 60 Ti rotor. The peak fractions were then recentrifuged in 10-30% sucrose gradients as described above, reconcentrated by centrifugation in the 60 Ti rotor and ethanol precipitated. Fifty µg of each RNA was assayed by application to chick cells as described in the legend to Fig. 1.

	Interferon Produced In Chick Cells (units)	
Fraction	Experiment 1	Experiment 2
4 - 7S	6	< 6
7 - 10S	1024	1536
10 - 18S	512	768
18 - 28S	32	< 6

destroyed the ability of the RNA to code for human interferon in chick cells, but treatment with RNAase-free-DNAase or pronase was without any effect. These controls confirm the earlier finding that the observed effect is an RNA mediated response (4).

When the biologically active RNA fraction from induced cells was chromatographed on oligo(dT)-cellulose, and further assayed in chick cells, all the interferon coding activity was absorbed on the column (Table II). In control

Table II. Interferon Produced in Response to Fractionated RNAs Prepared From Poly(I · C) Induced Human Fibroblasts

Unfractionated cellular RNA was prepared as described in the legend to Fig. 1 from human fibroblasts at hour 6 of the superinduction procedure. This RNA (100 A_{260} units) was applied to a column containing 2g of oligo(dT)-cellulose in 0.5M NaCl, 0.01M Tris-HCl buffer, pH 7.5 (9). The fraction not retained was ethanol precipitated and assayed on chick cells as non-poly(A)-containing RNA, while the fraction retained in high salt buffer was eluted in 0.01M Tris-HCl (pH 7.5) and assayed as poly(A) containing RNA. In a control experiment, chick cells were treated with poly(I · C).

	Human Interferon Produced In Chick Cells (units)	
	Experiment 1	Experiment 2
Unfractionated Cellular RNA (50μg)	512	36
Non-Poly(A) Containing RNA from Oligo(dT)-Cellulose Chromatography (50μg)	6	< 6
Poly(A) Containing Fraction from Oligo(dT)-Cellulose Chromatography (1.0μg)	512	384
Poly(I · C) Stimulation of Chick Cells 50μg DEAE-dextran plus 50μg of Poly(I · C)	< 6	< 6

experiments, no retention of poly(I · C) on oligo(dT)-cellulose was observed, nor did the deliberate application of poly(I · C) to chick cells and subsequent assay of the medium on human fibroblasts induce antiviral state.

These results indicate that human interferon specific mRNA contains poly(A) regions greater than 50 nucleotides long, since this is the minimum length required for stable binding of RNA to these columns (9); on the other

hand, it has been shown that mouse interferon specific mRNA induced by poly-(I · C) does not bind to oligo(dT)-cellulose, and the interferon production in mouse L cells was not inhibited by treatment with cordycepin (10). Since our preparations of mRNA were from human cells induced by poly(I · C) in the presence of metabolic inhibitors, we examined whether the poly(A) content of interferon mRNA was not specific for superinduced cells only. For this reason we measured the sensitivity of interferon production to the treatment with cordycepin in cells induced by poly(I · C) and poly(I · C) in the presence of metabolic inhibitors. The results in Table III show that in all examined systems, the interferon production was inhibited by the addition of cordycepin during the first 2 hrs. post induction. An identical observation for the superinduced system has recently been reported (3).

DISCUSSION. The present results indicate that poly (I · C) induces in human

Table III. The Effects of Cordycepin Addition During Interferon Induction

Interferon was induced by treatment of the cellular monolayers in 60mm diameter petri dishes with a solution of poly(I · C) [100μg/ml] in PBS for one hour at 37°C. MEM, supplemented with 2% FBS (2ml), was added with collection of the interferon containing media 18 hours post induction. For superinduction, cycloheximide (50μg/ml) was included in the poly(I · C) solution which was replaced with a solution of MEM (without serum) containing cycloheximide (50μg/ml) until the sixth hour of the induction followed by MEM supplemented with 2% FBS. Actinomycin D (1μg/ml) was added at hour 5½ for 30 minutes (2). Cordycepin (Sigma, 100μg/ml) was added at the indicated times and replaced with MEM, supplemented with 2% FBS, at hour 6 of the induction.

	Units of Interferon Produced		
Hours of Cordycepin Treatment	Poly(I · C) Alone	Poly(I · C) Plus Cycloheximide	Poly(I · C) Plus Cycloheximide Plus Actinomycin D
Control No Addition	128	6,400	36,200
0 - 6	32	8	25
1 - 6	16	4	30
2 - 6	256	400	400
3 - 6	300	3,200	12,800
4 - 6	128	12,800	51,200
5 - 6	128	6,400	25,600

fibroblasts formation of interferon specific mRNAs, which can be translated with fidelity in primary chick fibroblasts. Within the limits of this assay, it was shown that human interferon is coded for by a poly(A) containing mRNA sedimenting between 7 and 10S. Activity sedimenting with 18S ribosomal RNA may represent larger interferon mRNA or it may represent aggregation. Aggregation in sucrose gradients of purified mRNA coding for mouse myeloma light chains was recently reported (11). We have previously observed the molecular heterogeneity of interferon induced in human fibroblasts by poly(I · C) (12).

The close parallel between the rate of interferon production by human fibroblasts and the amount of interferon produced in response to interferon mRNA containing preparations in chick cells indicates that interferon, which is produced in response to the newly synthesized interferon mRNA, is immediately released from the cells to the medium. This observation makes the existence of preformed interferon highly improbable (13).

Finally, the results indicate that poly(I · C) induction of human fibroblasts results in levels of interferon mRNA that are capable of producing more interferon than is expressed in these cells, thus giving the first direct evidence that control of interferon production in human fibroblasts in response to poly(I · C) exists beyond the transcriptional level.

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