Priming Increases the Amount of Interferon mRNA in Poly(rI).poly(rC)-treated L Cells

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SUMMARY

Priming by mouse interferon pre-treatment resulted in an accumulation of interferon mRNA in poly(rI).poly(rC)-treated L cells, starting early in the period of interferon synthesis. On electrophoresis, the priming activity of an interferon preparation co-migrated with the antiviral activity, which suggests identity of the functional principle(s) for these activities.

INTRODUCTION

Pre-treatment of animal cells with interferon often brings about an enhanced interferon synthesis (priming effect) when the cells are stimulated for interferon production afterwards. Previous reports (Stewart II et al. 1971, 1973; Barmak & Vilcek, 1973) suggested that the priming and the antiviral activities were distinguishable functions residing on the same molecule of interferon. This conclusion was drawn from the observations that (1) the primed state was not always accompanied by the antiviral state in interferon-treated cells and (2) the development of the primed state was not blocked by the addition of cycloheximide at a concentration inhibiting the antiviral activity of interferon.

From experiments with metabolic inhibitors, Barmak & Vilček (1973) suggested that priming increased the rate of interferon mRNA synthesis. In a previous study (Saito et al. 1976), we detected larger amounts of interferon mRNA in primed cells than in unprimed control cells. These results imply that interferon stimulates the transcriptory process(es) of interferon synthesis. In our studies we used the mouse encephalomyelitis virus, GD-7, to stimulate formation of interferon. It was therefore desirable to rule out the possibility that the interferon pre-treatment might increase interferon yields by preventing the virus from causing a general inhibition of cellular macromolecular syntheses. We now report the results of studies in which a synthetic RNA, poly(rI).poly(rC), instead of live virus, was used as an interferon inducer. However, since the yield of interferon in response to this inducer was far less than that in response to the virus, there was insufficient extractable interferon mRNA activity for assay by the method originally described by De Maeyer et al. (1972). We therefore developed a more sensitive method involving Xenopus laevis oocytes, which produced mouse interferon with high efficiency under the direction of micro-injected interferon mRNA. Our results again show that enhanced interferon synthesis in response to priming is accompanied by augmented production of its mRNA.

METHODS

Cells and viruses. Mouse L cells (CCL strain) were grown in Eagle’s minimum essential medium (MEM) supplemented with 5% calf serum and antibiotics. The preparation and cultivation of primary chick embryo (PCE) cells was described previously (Kohno et al.
Cells of the 7M-1 clone of BHK-21 were grown as described by Kohno et al. (1968). Rabbit RK-13 cells were cultivated in MEM containing 5% calf serum. The Miyadera strain of Newcastle disease virus (NDV) was propagated in the chorio-allantoic cavity of embryonated hen's eggs. Seed pools of Sindbis and vesicular stomatitis viruses were prepared from infected PCE cells. Mouse encephalomyelitis virus, GD-7, was grown in BHK-21 cells. These viruses were used without purification.

Preparation and partial purification of mouse interferon. NDV-induced L cell interferon was prepared as described previously (Saito et al. 1976). Priming of interferon synthesis in response to poly(rI).poly(rC) was carried out as follows. One-day-old monolayers of L cells grown in glass Roux bottles (700 ml) were exposed for 7 h to 300 units (30 ml/bottle) of interferon. The cells in each bottle were then washed once with 20 ml Hanks balanced salt solution (BSS) and incubated for 1 h with 5 ml of MEM containing 10 μg/ml of poly(rI), poly(rC) and 100 μg/ml of diethylaminoethyl (DEAE)-dextran. The cells were washed once with BSS to remove the inducer and further incubated at 37 °C in 30 ml MEM containing 2% calf serum. This medium was harvested at 12 h and constituted the crude interferon. In some experiments, crude interferon (3 × 10^8 units/mg protein) was concentrated by ammonium sulphate precipitation and purified to about 3 × 10^5 units/mg protein by carboxymethyl (CM)-Sephadex chromatography as reported by Kawakita et al. (1978). An additional purification with poly(rU)-Sepharose affinity chromatography (De Maeyer-Guignard et al. 1977) to about 3 × 10^6 units/mg protein was carried out in the indicated experiments.

Titration of interferon. Mouse interferon was titrated by yield reduction of GD-7 virus haemagglutinin (HA) in L cells (Oie et al. 1972). One unit was the interferon concentration at which the HA yield was reduced by 10^-6 and corresponded to 0.3 research reference units (NIH No. G002-904-511). Results presented here are standardized in terms of this reference preparation. The antiviral state in PCE cells was determined by a HA yield reduction method with Sindbis virus (Oie et al. 1972) and that in RK-13 cells by an infectivity yield reduction method with vesicular stomatitis virus.

SDS-polyacrylamide gel electrophoresis (PAGE). This was done by a modification of the method described by Stewart II (1974) and 10% polyacrylamide gel in 4 mm × 100 mm columns (7 mA/column). Interferon partially purified by a combination of CM-Sephadex and poly(rU)-Sepharose chromatography was dialysed against 0.01 M-phosphate buffer containing 1% SDS for 12 h prior to electrophoresis. In those experiments indicated, 1% 2-mercaptoethanol was included in the dialysis buffer. Addition of urea was avoided in all pre-treatments. The treated interferon was then heated at 100 °C for 1 min. In order to assay the fractionated material, extracts were made. Gels were cut into discs 2 mm thick, each of which was pulverized in 1 ml of MEM containing 5% calf serum and antibiotics and incubated overnight at room temperature. Molecular weight standards were treated and electrophoresed exactly as interferon samples, except that they were stained with Coomassie brilliant blue.

Assay of priming activity. Extracts from gels were diluted 20-fold with MEM containing 2% calf serum and added to monolayers of L cells in plastic plates (Linbro, 24 wells, diam. 18 mm, 0.5 ml/well). After 7 h incubation the cells were treated with poly(rI).poly(rC) and interferon yields were determined after overnight incubation.

Antiserum against mouse interferon. Two albino rabbits were immunized with partially purified NDV-L cell interferon (approx. 3 × 10^6 units/mg protein). After a first intramuscular dose of 3 × 10^6 units mixed with complete Freund's adjuvant, each rabbit received intravenous injections of 3 × 10^6 units from the third to fourteenth week. Serum was obtained at the fifteenth week. Serum from a normal rabbit was obtained at the same time. In both cases the sera were heated at 56 °C for 30 min before storage at -20 °C.
Mouse interferon priming

Fig. 1. Kinetics of primed and unprimed interferon production. ○—○, Primed culture; ●—●, unprimed culture.

anti-interferon serum, diluted 1:1000, was mixed with an equal vol. containing 15 units of partially purified interferon and completely neutralized its antiviral activity during incubation for 1 h at 37 °C. The serum did not neutralize a human leukocyte interferon preparation.

Extraction of RNA and its translation in oocytes. Cellular RNA was extracted by hot phenol as described elsewhere (Saito et al. 1976). In some experiments, the RNA was further fractionated into adenine-rich molecules by oligo(dT)-cellulose chromatography (Aviv & Leder, 1972). The *Xenopus laevis* oocytes were obtained and treated according to the method reported by Gurdon et al. (1971). RNA preparations were dissolved in the injection buffer (88 mM-NaCl, 15 mM-tris-HCl, pH 7.6) and injected into oocytes (50 nl/cell, 300 to 360 μg RNA/ml). The concentration of RNA was adjusted by monitoring its absorbance at 260 nm. Usually 10 to 20 oocytes were used per RNA sample. The oocytes were incubated in 0.1 ml of modified Barth solution (Gurdon, 1976) at room temperature. As only a part of the synthesized interferon was released into the medium, a homogenate of the cells and the medium was assayed for interferon after clarification by centrifugation at 1500 g for 20 min).

Chemicals. Poly(rI).poly(rC) was purchased from Miles Laboratories Inc, Elkhart, Ind., U.S.A.; DEAE-dextran, CM-Sephadex and poly(rU)-Sepharose from Pharmacia Fine Chemicals Inc; oligo(dT)-cellulose from Collaborative Research Inc; the mol. wt. standards (phosphorylase A, bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome c) from Schwartz–Mann Co.

RESULTS

Basic characteristics of priming on the interferon synthesis induced by poly(rI).poly(rC)

Preliminary experiments revealed that pre-treatment with mouse interferon (3 to 3000 units, for 6 to 7 h) increased the interferon yield of L cells in response to poly(rI).poly(rC) by 10- to 20-fold. The level of priming, in terms of the amount of interferon synthesized, was not related to the concentration of interferon applied within the above range. Based on these results, we treated L cells with 300 units of interferon for 7 h to prime interferon production.

Fig. 1 shows the time course of the appearance of interferon in the medium of primed and unprimed L cells in Roux bottles. The rate of synthesis was enhanced by priming, but not its duration. During the observation period in Fig. 1, the primed and induced cells maintained their original levels of total protein and RNA synthesis, as estimated by incorporation
of tritiated leucine and uridine into acid-insoluble material (data not shown), indicating that the priming did not lead to an overall stimulation of transcription or translation.

**SDS-PAGE of antiviral and priming activities**

To identify the molecule(s) responsible for the antiviral and priming activities, a partially purified interferon preparation (3 × 10⁶ units/mg protein) was applied on SDS-PAGE and fractions were assayed for both activities. Results are shown in Fig. 2. As frequently reported by others (Stewart II, 1974; Stewart II et al. 1977; De Maeyer-Guignard et al. 1978), SDS-PAGE separated two major components corresponding to mol. wt. 19,000 and 35,000 in our study. As seen in the figure, these peaks were associated with priming activity, although the migration profile was less clear-cut than that of antiviral activity. This was presumably due to the fact that the amount of interferon produced by primed cells did not increase when the level of pre-treating interferon was raised beyond 3 units.

**Comparison of NDV- and poly(rI), poly(rC)-induced interferons by SDS-PAGE**

To see whether or not the molecular constituents of the interferon produced by L cells which had been primed and exposed to poly(rI), poly(rC) were identical to those of NDV-induced L cell interferon, the experiments summarized in Fig 3(a) and (b) were carried out. By comparing Fig. 3(a) with Fig. 2, one can assume that both stimuli induce L cells to produce the same molecular species of interferon.

Stewart II (1974) and Stewart II et al. (1977) reported that the fast moving component lost its antiviral potency upon reduction with 2-mercaptoethanol. Likewise, as seen in Fig. 3(b), this reducing agent destroyed the antiviral potency of our 19,000 mol. wt. component. A corresponding peak in the SDS-PAGE profile of NDV-induced interferon also disappeared after reduction (data not shown).
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Fig. 3. SDS-PAGE of poly(rI).poly(rC)-induced interferon (a) before and (b) after reduction with 2-mercaptoethanol. Poly(rI).poly(rC)-induced interferon was concentrated and partially purified by CM-Sephadex and poly(rU)-Sepharose chromatography to 1.5 x 10⁴ units/mg protein. Before SDS-PAGE, one half of the sample was treated with 2-mercaptoethanol (see Methods). One hundred and fifty units of each interferon sample were applied to each gel. ●—●, Interferon activity; O—O, mol. wt. standards.

Based on these results, we suggest that there is no qualitative difference between the interferon mRNA induced in L cells by exposure to the synthetic inducer or in response to the live virus.

Quantification of interferon mRNA in Xenopus oocytes

Even with priming, poly(rI).poly(rC) induced only a few hundred units of interferon in the culture medium, which was roughly one-hundredth of the yield of interferon when L cells were stimulated by GD-7 virus (Saito et al. 1976). It was not unexpected, therefore, that the method which had been developed by De Maeyer et al. (1972) and successfully employed in our previous study (Saito et al. 1976) failed to demonstrate interferon mRNA in L cells treated with poly(rI).poly(rC). We therefore needed to develop a more sensitive method for determining the activity of the mRNA. This was achieved by a combination of the translational system of Xenopus oocytes and fractionation of mRNA by oligo(dT)-cellulose chromatography; this detected about 5% of the total extracted RNA and 100% of interferon mRNA (data not shown). The antiviral activity of the material produced by the oocytes was expressed in L cells, but not in RK-13 or in PCE cells. Moreover, no antigenic difference was discernible by neutralization tests between interferons produced by oocytes and by L cells after stimulation with NDV or poly(rI).poly(rC) (data not shown).

Results presented in Fig. 4(a), (b), (c) served to standardize experimental conditions for the assay. In these experiments an RNA sample extracted from NDV-infected L cells was used.

A time-course study (Fig. 4a) revealed that the injected RNA stimulated one round of interferon synthesis in the oocytes, which terminated at about 6 h. Fig. 4(b) showed that the amount of interferon synthesized by the oocytes was linearly proportional to the amount of input RNA up to 14 ng/oocyte.

Protein synthesis in the oocytes in response to interferon mRNA may be inhibited by an overwhelming amount of other species of functional mRNAs contained in the inocula. In a further experiment the RNA sample exhibiting interferon mRNA activity (Fig. 4a, b) was diluted serially with an RNA solution of an equal concentration obtained from
Fig. 4. (a) Time-course of interferon production by oocytes. Each *Xenopus* oocyte received about 18 ng of the RNA sample. At each time indicated, 10 oocytes were homogenized with incubation medium. (b) Relationship between the amount of injected RNA and the yield of interferon. The RNA sample exhibiting interferon mRNA activity was appropriately diluted with injection buffer and injected into oocytes (20 oocytes/dilution, 50 nl/oocyte), which were homogenized at 12 h. (c) Dependence of the amount of synthesized interferon on the relative amount of input interferon mRNA. The RNA used in the experiments shown in (a) and (b) was diluted with an RNA solution of equal concentration prepared from non-induced L cells (see text). Eighteen ng of the mixed RNA were injected into an oocyte. Each point in the figure represents data from a pool of 10 such oocytes.

Fig. 5. Quantification of interferon mRNA in L cells during primed and unprimed interferon production. The RNA samples obtained as described in the text from primed (□) and unprimed (■) L cells were assayed for interferon mRNA activity by injection into oocytes (20 oocytes/sample, 15 ng/oocyte, 12 h incubation). Interferon production by primed (○—○) and unprimed (●—●) L cells was determined at hourly intervals by harvesting a portion of the culture medium.

uninfected L cells. An equal amount of each of these RNA mixtures was administered into oocytes, whose interferon production is plotted as a function of the dilution in Fig. 4(c). The linearity in dose-response relationship indicated that such an interference did not take place within the dilution range tested.

From all these results we concluded that the translational system was faithful and quantitative enough to enable relatively small amounts of interferon mRNA to be measured.

**Determination of interferon mRNA in L cells during primed and unprimed interferon synthesis**

L cells in Roux bottles were either primed or mock-treated with MEM and subsequently induced for interferon by poly(rI), poly(rC). At variable times thereafter, 10 bottles of cells of each group were disrupted and extracted from RNA. A part of the pooled culture media
was saved for assay of interferon potency. The RNA samples were further fractionated for mRNA and each RNA sample was injected into 20 oocytes to determine interferon mRNA content. A result of these experiments is shown in Fig. 5. The interferon mRNA was demonstrated only in primed cells, during the period of increasing interferon synthesis. In several similar experiments, RNA from unprimed cells failed to show interferon mRNA activity.

**DISCUSSION**

The result shown in Fig. 5 agrees with our previous finding (Saito et al. 1976) in that interferon mRNA could be extracted in relatively increased amounts from primed L cells. We propose, therefore, that the augmentation of interferon mRNA increases its availability to the protein synthesizing system of L cells, leading to an enhanced rate of synthesis and an increased final yield of interferon in primed cells. We further envisage the possibility that either acceleration of the transcription of the interferon gene, or inhibition of the process(es) inactivating interferon mRNA, may take place in the primed and induced cells.

However, currently available results, including those reported here, do not throw light on underlying mechanism(s). Moreover, the actual process through which primed cells gain a greater responsiveness to an interferon-forming stimulus by producing more interferon mRNA may depend on the nature of the stimulus. In consequence, when the priming effect is investigated with an interferon-synthesizing system initiated by a replicating agent with cell-killing potential (Stewart II et al. 1971; Saito et al. 1976), the antiviral function of interferon may contribute to the observed priming effect.

It is now possible to produce very highly purified mouse interferon (De Maeyer-Guignard et al. 1977, 1978) with a specific activity exceeding $10^9$ units/mg protein. The interferon preparation employed here was not ‘pure’ in this sense. However, since the priming activity co-migrated with the antiviral principle through SDS-PAGE (Fig. 2), it seems reasonable to attribute the priming activity to the interferon itself.

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**REFERENCES**


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