JOURNAL OF VIROLOGY, May 1971, p. 588-594
Vol. 7, No. 5

Printed in U.S.A.

Post-Transcriptional Control of Interferon Synthesis

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Received for publication 11 February 1970

Low to moderate doses of cycloheximide had a stimulatory effect on interferon production in rabbit kidney cell cultures treated with double-stranded polyinosinate-polycytidylate (poly I:poly C). A very marked stimulation occurred in the presence of a dose of cycloheximide inhibiting amino acid incorporation into total cellular protein by about 75%. Higher doses of cycloheximide caused a shift in interferon release towards later intervals and a gradual decrease in the overall degree of stimulation. An even greater increase in the amount of interferon produced was observed if cells were treated with cycloheximide for only 3 to 4 hr immediately after their exposure to poly I:poly C. Under the latter conditions, a rapid burst of interferon production occurred after the reversal of cycloheximide action. Treatment with a high dose of actinomycin D before the reversal of cycloheximide action caused a further increase and a marked prolongation of interferon production. It is postulated that inhibitors of protein synthesis suppress the accumulation of a cellular regulatory protein (repressor) which interacts with the interferon messenger ribonucleic acid mRNA and thereby prevents its translation. Therefore, active interferon mRNA can apparently accumulate in rabbit kidney cells which, after exposure to poly I:poly C, are kept in the presence of an inhibitor of protein synthesis. Some of this accumulated interferon mRNA can be translated during a partial block of cellular protein synthesis, but its most efficient translation occurs after the reversal of the action of the protein synthesis inhibitor.

Youngner and co-workers (14–16) were the first to show a paradoxical effect of cycloheximide and other inhibitors of protein synthesis on endotoxin- or polyinosinate-polycytidylate (poly I:poly C)-stimulated interferon production in mice. Instead of decreasing the amount of interferon produced, the serum interferon titers were actually higher in animals which had also been injected with inhibitors of protein synthesis. Youngner and co-workers postulated that interferon produced in the presence of inhibitors of protein synthesis was not newly synthesized but preformed, i.e., either simply released by the inducer or derived from an inactive interferon precursor.

In earlier reports (11 to 13), we described similar paradoxical effects of inhibitors of ribonucleic acid (RNA) and protein synthesis on the release of interferon in cultures of rabbit kidney cells stimulated with poly I:poly C. For example, cells treated with poly I:poly C and then maintained in the continuous presence of cycloheximide produced about 3 to 10 times more interferon than control cultures. On the basis of kinetic studies we concluded that these effects can most likely be explained by the inhibition of a cellular regulatory protein (repressor) which controls interferon production (12).

After our initial reports, Tan et al. (9) found that if cycloheximide was present in rabbit kidney cell cultures for only 3 to 4 hr after poly I:poly C treatment and then removed, the amount of interferon produced after the removal of cycloheximide was even greater than the amount of interferon made in cultures maintained in the continuous presence of cycloheximide. On the basis of these data, along with experiments employing actinomycin D, Tan et al. concluded that interferon messenger RNA (mRNA) apparently had accumulated in rabbit kidney cells during cycloheximide treatment and that this mRNA was translated once the block of protein synthesis had been lifted.

The results to be reported here supplement our earlier observations and those of Tan et al. It will be shown that the still hypothetical repressor of interferon synthesis is most likely to act by preventing the translation of the interferon mRNA.

MATERIALS AND METHODS

Cell cultures. Rabbit kidney cell cultures, were prepared by trypsinization of fresh kidneys from 2 to 4-week-old rabbits. All experiments were done in secondary cultures grown to confluency in 60-mm plastic petri dishes at 36 C in a humidified atmosphere

with 5% CO_2. Eagle's minimum essential medium (MEM) supplemented with 10 or 2% heated fetal calf serum was used as growth medium or maintenance medium, respectively.

Interferon titrations. Twofold dilutions of the tested fluids were prepared in maintenance medium. Duplicate cultures were incubated with 2 ml of each of the dilutions for 18 to 20 hr and then inoculated with about 100 plaque-forming units of vesicular stomatitis virus. The virus was adsorbed onto cells for 1 hr. Thereafter, the cultures were overlaid with warm maintenance medium (at 43 C) containing 1% agar. For counting viral plaques, the cells were stained with neutral red about 48 hr after inoculation. Dose response curves were constructed by plotting the per cent of control plaque count against the log dilution of the tested fluid. An internal laboratory rabbit interferon standard was included with each assay. This internal standard had been calibrated against a reference rabbit interferon standard prepared by Monto Ho and received through the courtesy of the Reference Reagents Branch, National Institutes of Health, Bethesda, Md. All interferon yields represent interferon titers per 2 ml, corrected to this standard.

^{14}C-leucine incorporation. Cultures were incubated at 36 C with 1 ml of leucine-free MEM containing 2% dialyzed, heated fetal calf serum and 0.5 μCi of uniformly labeled ^{14}C-L-leucine (311 μCi/μmole; New England Nuclear Corp., Boston, Mass.). Incorporation was stopped after 30 min by rapidly washing the cells with cold saline. The cells were then dissolved in 2 ml of 1 M NaOH. The protein content of the resulting lysate was determined by its absorbance at optical density $(OD)_{280}$. A portion of the lysate was neutralized and made to 10% with trichloroacetic acid. The resulting precipitates were collected on Whatman GF/C glass fiber filters (Reeve Angel, Clifton, N.J.) and washed four times with 10 ml each of 5% trichloroacetic acid. Trichloroacetic acid-insoluble radioactivity was measured in a liquid scintillation counter. Specific activities are expressed as counts per minute per OD_{280}.

Chemicals. Poly I:poly C (double-stranded sodium salt, lyophilized, lot 2027) was purchased from P-L Biochemicals, Milwaukee, Wis. It was dissolved in phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM phosphate, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2 \cdot 6\,H_2O$; *p*H 7.4) at 45 C and kept at 4 C until used. The concentration of the stock solution was 1 mg/ml. For use on cell cultures, the stock solution was diluted to the desired concentraton in warm PBS at 37 C.

Cycloheximide (Acti-Dione) was purchased from The Upjohn Co., Kalamazoo, Mich. Pactamycin (lot 5292-THP-93-12) was obtained through the courtesy of the Cancer Chemotherapy National Center, National Cancer Institute, Bethesda, Md. Actinomycin D (lot 940051) was purchased from Calbiochem, Los Angeles, Calif.

RESULTS

Effects of inhibitors of protein synthesis on interferon production. Earlier studies have demonstrated that, in rabbit kidney cell cultures exposed to poly I:poly C, most interferon was released between 2 and 4 hr after exposure to the polynucleotide complex. Thereafter, further interferon production rapidly ceased. In the continuous presence of moderate doses of cycloheximide, interferon production did not stop at about 4 hr after poly I:poly C treatment, but, instead, its release continued at a high rate for at least several more hours. As a result, the total yield of interferon was greatly increased in the presence of cycloheximide, although amino acid incorporation into total cellular protein was inhibited by over 90% (11, 12).

Table 1 shows the effect of different concentrations of cycloheximide on interferon production in the first 4 hr and between 4 and 22 hr after poly I:poly C treatment. Also shown is the effect of the same cycloheximide concentrations on the incorporation of ^{14}C-leucine into total cellular protein. All concentrations of cycloheximide, with the exception of the highest one employed, enhanced total interferon production. However, as the concentration of cycloheximide

TABLE 1. *Effect of different concentrations of cycloheximide on poly I: poly C-stimulated interferon production*

Cycloheximide concn (μg/ml)	Interferon yield[a]			^{14}C-leucine incorporation[b]	
	Between 1 and 4 hr	Between 4 and 22 hr	Total yield	Counts per min per OD_{280}	Control (%)
None	1,320	83	1,403	6,257	
0.08	1,920	450	2,370	2,893	46.2
0.4	5,100	3,320	8,420	1,435	23.0
2.0	2,550	5,000	7,550	580	9.3
10.0	1,450	5,700	7,150	287	4.6
50.0	760	4,300	5,060	148	2.4
250.0	155	1,100	1,255	ND[c]	

[a] All cultures were treated with 50 μg of poly I:poly C per ml for 1 hr and washed free of extracellular poly I: poly C. They were then incubated with the indicated concentrations of cycloheximide in minimum essential medium (MEM) with 2% fetal calf serum. Fluids were collected 4 hr after exposure to poly I: poly C, and the cultures were washed and replenished with media containing the same concentration of cycloheximide. Fluids were again collected 22 hr after poly I: poly C treatment. Before titrating the interferon content, all fluids were dialyzed to remove the cycloheximide.

[b] Cells were incubated for 30 min with the indicated concentration of cycloheximide and diluted in MEM with 2% fetal calf serum. They were then incubated with ^{14}C-leucine for 30 min in the presence of cycloheximide.

[c] Not done.

increased, there was a shift in maximum interferon release towards later intervals.

These results can be best explained on the basis of a combined effect of cycloheximide on both the synthesis of a cellular regulatory protein and on the production of interferon. On one hand, cycloheximide appeared to inhibit the synthesis of the regulatory protein and, therefore, caused a stimulation of interferon production. On the other hand, the observed delay in interferon release and the decrease in the overall amount of interferon produced in the presence of increasing doses of cycloheximide are consistent with the idea that protein synthesis is required for interferon production or, at least, that maximum interferon yields are only obtained if cellular protein synthesis is not inhibited below a certain critical level.

Cycloheximide is known to suppress protein synthesis by interfering with peptide elongation and, usually to a lesser extent, by affecting initiation (1, 3, 7). It acts as a selective inhibitor of protein synthesis on cytoplasmic 80*S* ribosomes of eukaryotic cells, although not affecting protein synthesis on the smaller mitochondrial ribosomes (4). However, cytoplasmic protein synthesis is not completely inhibited even by extremely high cycloheximide concentrations (6). It therefore seemed worthwhile to compare the action of another inhibitor of protein synthesis which both has a different mechanism of action and is more efficient in arresting protein synthesis. We employed pactamycin, an inhibitor affecting primarily the initiation of protein synthesis in both prokaryotic and eukaryotic cells (2).

The effects of cycloheximide and pactamycin on interferon production are shown in Table 2. A stimulation of interferon production was again observed in cultures kept in the continuous presence of cycloheximide (group 2) but not in those maintained in the continuous presence of pactamycin (group 5). The addition of pactamycin suppressed the stimulation of interferon production by cycloheximide (groups 3 and 7). The greatest stimulation of interferon production was found if cycloheximide had been present in the cultures only during the first hours after poly I:poly C treatment (group 4). In the latter group, the removal of cycloheximide was followed by the release of large quantities of interferon. The exposure of cells to pactamycin, followed by its removal at 4 hr, resulted in a marked but less dramatic stimulation of interferon production (groups 6 and 8).

Together, these results support the view that the suppression of cellular protein synthesis after poly I:poly C treatment promotes the accumulation of functional interferon mRNA. The latter could be efficiently translated after the reversal of the inhibitory effect of cycloheximide. Less interferon was produced after the removal of pactamycin, apparently because its inhibitory

TABLE 2. *Effect of combined treatment with inhibitors of protein synthesis on poly I:poly C-stimulated interferon production*[a]

Group	Treatment (between 1 and 4 hr after exposure to poly I:poly C)	Interferon yield (between 1 and 4 hr after exposure to poly I:poly C)	Treatment (between 4 and 22 hr after exposure to poly I:poly C)	Interferon yield (between 4 and 22 hr after exposure to poly I:poly C)	Total interferon yield
1	None	500	None	130	630
2	Cycloheximide	700	Cycloheximide	5,900	6,600
3	Cycloheximide	700	Cycloheximide + pactamycin	1,850	2,550
4	Cycloheximide	700	None	22,500	23,200
5	Pactamycin	60	Pactamycin	305	365
6	Pactamycin	60	None	5,900	5,960
7	Cycloheximide + pactamycin	≤40	Cycloheximide + pactamycin	240	240–280
8	Cycloheximide + pactamycin	≤40	None	6,500	6,500–6,540

[a] Cultures were treated with 50 μg of poly I: poly C per ml for 1 hr and thoroughly washed. They were then incubated in minimum essential medium with 2% fetal calf serum with or without the inhibitor(s) of protein synthesis, as indicated. Culture fluids were collected 4 hr after the addition of poly I:C, and the cultures were washed and replenished with fresh media with inhibitor(s) added as indicated. The final concentrations of cycloheximide and pactamycin were 2 μg/ml and 0.5 μg/ml, respectively. The effect of these inhibitor concentrations on ^{14}C-leucine incorporation is shown in Table 3.

effect on cellular protein synthesis was not completely reversible (Table 3).

A concentration of 2 μg of cycloheximide per ml, which inhibited cellular protein synthesis by about 90%, was employed in the experiment shown in Table 4. The drug was added 1 hr after poly I:poly C treatment and kept in the cultures for varying periods of time. The amount of interferon produced in the presence of cycloheximide and after its removal from the cultures was compared. The results show that a relatively slow release of interferon occurred in the presence of cycloheximide. (Note that in the absence of cycloheximide, most interferon is released within the first 4 hr after exposure to poly I:poly C; cf. Tables 1 and 2.) In each case, the release of greater quantities of interferon occurred after the removal of cycloheximide.

Superinduction of interferon production by actinomycin D. It was noted earlier that whereas the addition of a high dose of actinomycin D prior to or up to 1 hr after the exposure of cells to poly I:poly C suppressed interferon production, the same treatment at 3 to 5 hr after poly I:poly C enhanced subsequent interferon production (9, 13). Table 5 shows the interferon yield from cultures which were treated with poly I:poly C, then incubated in the presence of cycloheximide, and, finally, exposed to different doses of actinomycin D. The optimal concentration of actinomycin D (3 μg/ml) caused an additional almost fivefold stimulation of interferon production.

Certain predictions could be made on the basis of the preceding experiments. As mentioned

TABLE 3. *Effect of cycloheximide and pactamycin on ^{14}C-leucine incorporation and the reversal of their inhibitory effects*

Treatment[a]	^{14}C-leucine incorporation			
	In the presence of drug[b]		After removal of drug[c]	
	Counts per min per OD_{260}	Control (%)	Counts per min per OD_{260}	Control (%)
None	34,900		29,500	
Cycloheximide	2,980	8.6	29,600	100.0
Pactamycin	1,520	4.4	10,920	36.9
Cycloheximide + pactamycin	1,680	4.8	8,580	29.0

[a] Concentrations of cycloheximide and pactamycin were the same as those used in the experiment shown in Table 2.

[b] Cells were treated for 30 min with the indicated inhibitor(s) diluted in minimum essential medium with 2% fetal calf serum. They were then incubated with ^{14}C-leucine for 30 min in the presence of the respective inhibitor concentrations.

[c] Cells were treated for 90 min with the respective inhibitor(s) diluted in minimum essential medium with 2% fetal calf serum, washed, and incubated for 1 hr in inhibitor-free media before incubating with ^{14}C-leucine for 30 min.

TABLE 4. *Effect of varying the time of cycloheximide treatment on interferon yield*[a]

Time of cycloheximide treatment (hr after exposure to poly I:C)	Interferon yield during cycloheximide treatment	Interferon yield after removal of cycloheximide	Total interferon yield in 24 hr
None			1,520
1–2	715	5,580	6,295
1–4	2,520	11,400	13,920
1–6	4,430	11,400	15,830
1–8	6,300	7,200	13,500
1–24	6,100		6,100

[a] All cultures were treated with 50 μg of poly I:poly C per ml for 1 hr and washed free of extracellular poly I:poly C thereafter. They were then incubated with 2 μg of cycloheximide per ml in minimum essential medium with 2% fetal calf serum for the time indicated. Fluids were collected at the end of incubation with cycloheximide. The cultures were then washed, replenished with cycloheximide-free media, and incubated until 24 hr after exposure to poly I: poly C when fluids were again collected. To remove cycloheximide, fluids were dialyzed before titrating their interferon content.

TABLE 5. *Superinduction of interferon production after the removal of cycloheximide and treatment with different doses of actinomycin D*[a]

Dose of actinomycin D (μg/ml)	Interferon yield
None	9,600
0.1	13,600
0.3	19,600
1.0	22,800
3.0	47,600
10.0	15,200

[a] All cultures were treated with 50 μg of poly I:poly C per ml for 1 hr and thoroughly washed thereafter. They were then incubated with 2 μg of cycloheximide per ml in minimum essential medium with 2% fetal calf serum. Four hours after the exposure of cells to poly I: poly C, the cultures received the indicated concentrations of actinomycin D and were incubated for 30 min. The cells were then washed free of cycloheximide and actinomycin D and replenished with inhibitor-free media. Interferon yields were measured in culture fluids collected 22 hr after poly I:poly C treatment.

above, cycloheximide and other inhibitors of protein synthesis are believed to suppress the production of a cellular repressor. The latter, in turn, appears to act by inactivating the interferon mRNA which accumulates in cells after poly I:poly C treatment. It follows that active interferon mRNA can accumulate in the presence of inhibitors of protein synthesis and that this mRNA is rapidly translated after reversal of the blockade of protein synthesis. If this interpretation is correct, then soon after the removal of the protein synthesis inhibitor, the cellular repressor will also accumulate and further interferon synthesis should be rapidly "turned off." Thus, the removal of cycloheximide would be expected to lead to a rapid burst of interferon production. The addition of actinomycin D just before the removal of cycloheximide would inhibit further synthesis of both the interferon and repressor mRNA. If the repressor indeed acts by preventing the existing interferon mRNA from being translated, then one would expect that actinomycin D will prevent the rapid cessation of interferon production after the removal of cycloheximide.

These predictions were fully born out in the experiment shown in Fig. 1. It could be calculated on the basis of this experiment that the half-life of the interferon mRNA in the group of cultures treated with actinomycin D is about 3.5 hr. This is the time which elapsed from the addition of actinomycin D until the rate of interferon release attained in the first hour after the removal of cycloheximide had dropped by 50%. Conceivably, this is a very rough calculation which, among other things, does not take into account the possible effects of actinomycin D on mRNA stability.

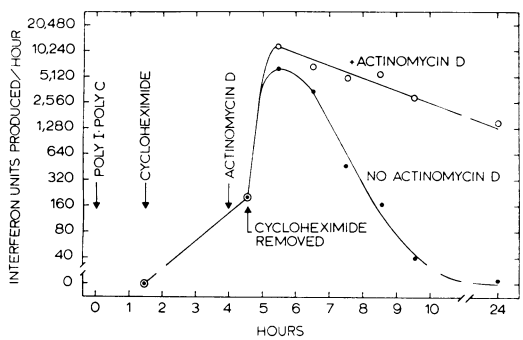

FIG. 1. *Post-transcriptional control of interferon production. Rabbit kidney cell cultures were treated with 50 μg of poly I:poly C per ml for 1 hr and then washed and fed with minimum essential medium (MEM) supplemented with 2% fetal calf serum. At the indicated time all cultures received 10 μg of cycloheximide per ml. At 4 hr after exposure to poly I:poly C, one group of cultures was treated with 5 μg of actinomycin D per ml. All cultures were washed at 4.5 hr and replenished with inhibitor-free MEM with 2% fetal calf serum. At the intervals indicated, the culture fluids were collected, the cultures were washed and fed with fresh medium. To remove the inhibitors, the 4.5-hr samples were dialyzed before titrating their interferon content.*

DISCUSSION

Since interferon has not been completely purified, it is impossible to study the mechanism of interferon production by specific labeling of the interferon protein in the course of its synthesis and processing by the cells. Ultimately, such studies will have to be performed in order to substantiate the conclusions based on less direct investigations employing metabolic inhibitors, such as the present one.

The pivotal question is whether poly I:poly C-stimulated interferon in rabbit kidney cell cultures is newly synthesized upon induction or preformed. The results of the present study suggest that maximum interferon yields are produced only when cellular protein synthesis is not inhibited below a certain critical level. This conclusion is supported by the results shown in Table 2. It finds additional support in the kinetics of interferon release observed in cultures which had been exposed to poly I:poly C, then kept in the presence of cycloheximide, and, finally, incubated in cycloheximide-free medium. Under the latter conditions, the rate of interferon release observed in the first hour after cycloheximide removal was about 20 times higher than that observed before the removal of cycloheximide (Fig. 1). These results do not support the idea that poly I:poly C-induced interferon in rabbit kidney cell cultures is preformed. The subsequent conclusions are, therefore, based on the postulate that either all of this interferon, or the major portion of it, is newly synthesized on induction.

We have further postulated that interferon synthesis in rabbit kidney cells is controlled by a cellular repressor acting at a post-transcriptional level. This conclusion is based mainly on the results shown in Fig. 1. It is virtually impossible that synthesis of interferon mRNA could have taken place after treatment with 5 μg of actinomycin D per ml. Therefore, all interferon synthesis after this treatment must have been directed by interferon mRNA which had accumulated before the addition of actinomycin D. The fact that treatment with actinomycin D resulted in an enhanced and prolonged interferon production implies that actinomycin D somehow promoted

the translation of interferon mRNA already present in the cell at the time of actinomycin D treatment. In other words, actinomycin D inhibited a cellular function which otherwise would have prematurely terminated the translation of the interferon mRNA. Experiments with inhibitors of protein synthesis have suggested that this turning off of the interferon mRNA is the function of a cellular protein which, for convenience, has been referred to as a "repressor."

This repressor could function in one of two possible ways. (i) It could act as a ribonuclease causing premature degradation of the interferon mRNA, or (ii) it could combine with the interferon mRNA to form a complex which would prevent the interferon mRNA from being translated. Although our experiments cannot distinguish between these two possible mechanisms of repressor action, it seems unlikely that a ribonuclease would show specificity for only one type of mRNA. Therefore, we favor the latter mechanism. A similar translation control mechanism was proposed by Tomkins et al. (10) to explain paradoxical effects of actinomycin D on steroid hormone-induced enzyme synthesis. In at least one other instance the binding of a protein to mRNA was directly shown to suppress mRNA translation (8).

It is not fully understood why inhibitors of protein synthesis, such as cycloheximide, appear to suppress preferentially repressor synthesis over interferon synthesis. Our earlier suggestion that cycloheximide-resistant rabbit kidney cell interferon might be synthesized on mitochondrial rather than cytoplasmic ribosomes (12) could not be confirmed. Several selective inhibitors of mitochondrial protein synthesis, including chloramphenicol and ethidium bromide, failed to affect interferon production in rabbit kidney cells (J. Vilček and M. H. Ng, *unpublished results*). Following are two suggested explanations that seem worth exploring. (i) The repressor protein could be labile and rapidly turned over. Therefore, cycloheximide treatment could delay the accumulation of a critical repressor concentration needed to prevent the translation of interferon mRNA. (ii) It is possible that cycloheximide exerts a differential effect on the synthesis of various mRNA species and that the synthesis of repressor mRNA is inhibited by cycloheximide, whereas that of interferon mRNA is not. Such differential effect on mRNA synthesis is not inconceivable, as Muramatsu et al. (5) have recently reported that the synthesis of nucleolar RNA is suppressed by cycloheximide to a much greater extent than extranucleolar RNA synthesis. In any case it is the accumulation of functional interferon mRNA which might explain the curious paradox of how enhanced interferon production can take place when cellular protein synthesis is greatly reduced.

It cannot be ruled out that an additional as yet unrecognized post-translational control mechanism operates in interferon production. For instance, the cellular degradation of interferon could also be suppressed by metabolic inhibitors. However, in view of the results presented in this paper, it no longer seems necessary to postulate the existence of a separate post-translational regulatory mechanism and of two types of interferon—one newly synthesized and one derived from a preexisting precursor (11)—to explain the paradoxical effects of metabolic inhibitors on interferon production in rabbit kidney cells.

Although our present experiments were all performed in rabbit kidney cell cultures with poly I:poly C used as interferon inducer, other evidence suggests that the results of this work may be applicable to other cells and other polyanionic interferon inducers, including viral RNA. Tan et al. (9) have reported a stimulation of Newcastle disease virus-induced interferon production in rabbit kidney cells by cycloheximide. More recently, Myers and Friedman (*personal communication*) observed paradoxical effects of inhibitors of RNA and protein synthesis on interferon production in a strain of human cells, which in many ways resemble our earlier and present results. Our own observations on the stimulatory effects of actinomycin D and cycloheximide on interferon production in human cells will be reported elsewhere (Havell and Vilček, Bacteriol. Proc., p. 195, 1971). Apart from their theoretical interest, these studies can be useful in devising methods for the production of large quantities of interferon in cell cultures.

While this paper was in preparation, we learned of a related independent study by Tan, Armstrong, and Ho (*personal communication*). Their main conclusion about the need for protein synthesis in poly I:poly C-stimulated interferon production in rabbit kidney cell cultures is in agreement with the results of this study.

On the basis of experiments in human cell cultures, Myers and Friedman (*personal communication*) have suggested the existence of a translation control mechanism for interferon synthesis that is in accord with the interpretation advanced in this paper.

ACKNOWLEDGMENTS

We thank Fermina Varacalli and Juan Casanova for skilled technical assistance.

This investigation was supported by U. S. Public Health Service grant AI-07057 from the National Institute of Allergy

and Infectious Diseases and by a grant from the Irwin Strassburger Memorial Medical Foundation. J. V. is the recipient of U. S. Public Health Service Career Development award 5-K4-AI-38784.

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