

Stabilization of Interferon Messenger RNA Activity by Treatment of Cells with Metabolic Inhibitors and Lowering of the Incubation Temperature

(human cells/poly(I)·poly(C)/induction and superinduction/translation control)

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ABSTRACT Interferon production was induced in a strain of human diploid foreskin cells with poly(I)·poly(C). Cycloheximide was included in the culture medium at the time of addition of the inducer. Actinomycin D was added to the cultures 4 or 5 hr later, before the inhibition of protein synthesis was reversed at 6 hr. Thus, subsequent interferon synthesis had to be directed by messenger RNA synthesized before addition of actinomycin D. The amount of interferon produced after such treatment was about 50-fold greater than in cells induced with poly(I)·poly(C) but not treated with inhibitors. Experiments using cordycepin suggested that in spite of the continued presence of the inducer the synthesis of the bulk of interferon messenger RNA was completed within the first 2 hr of exposure of cells to poly(I)·poly(C) and cycloheximide. Transcription of interferon messenger RNA was apparently not affected when interferon synthesis was suppressed to various degrees by different inhibitors of protein synthesis, indicating the independence of transcription and translation. The high rate of interferon synthesis after the reversal of cycloheximide action was more sustained at 32° than at 37°. The rate of decrease of overall protein synthesis in cells treated with actinomycin D and then incubated either at 32° or 37° showed a similar dependence on incubation temperature, suggesting that the stability of messenger RNA (or of another actinomycin D-sensitive component required for protein synthesis) was greater at the lower temperature.

Experiments with metabolic inhibitors in cultures of rabbit or human cells suggested that the process of interferon induction by poly(I)·poly(C) requires *de novo* synthesis of interferon mRNA and its translation into the interferon protein (reviewed in ref. 1). In cultures induced with poly(I)·poly(C) and incubated in the presence of cycloheximide for several hours, the removal of cycloheximide was followed by a burst of interferon production, coinciding with the rapid resumption of protein synthesis after the reversal of cycloheximide action. The amount of interferon produced after such a short exposure of induced cultures to cycloheximide was greater than in control cultures induced with poly(I)·poly(C) but not treated with cycloheximide. The yield of interferon was enhanced even further in cultures to which actinomycin D was added 30-60 min before the reversal of cycloheximide action (2, 3).

The paradoxical enhancement of interferon production by judicious treatment with inhibitors of protein and/or RNA synthesis has been referred to as "superinduction"—a term originally coined for the paradoxical enhancement of tyrosine aminotransferase levels in hydrocortisone-induced cells by treatment with actinomycin D (4). Many other instances of increased enzyme activity after addition of actinomycin D to cells actively synthesizing a specific enzyme have been re-

ported (reviewed in ref. 5). This phenomenon, most thoroughly studied in the tyrosine aminotransferase system, has been ascribed to the preferential inhibition by actinomycin D of a regulatory protein exerting negative control over enzyme synthesis at a posttranscriptional level (6), although alternative interpretations have also been suggested (7, 8).

The experiments described in this communication sought to analyze the events occurring during the superinduction of interferon production. The results suggested that the treatments with metabolic inhibitors resulting in enhanced interferon synthesis lead to a stabilization of interferon mRNA. A somewhat similar effect was produced by the lowering of incubation temperature during translation of interferon mRNA. The rate of decline of overall cellular protein synthesis in cultures treated with actinomycin D was also slower at the lower incubation temperature.

MATERIALS AND METHODS

Cell Cultures. A strain of diploid human fibroblasts (FS-4), derived in this laboratory from a single neonate foreskin, was used throughout. This cell strain has been identified as a high interferon producer (Havell and Vilček, in preparation). The cells were grown in Eagle's minimal essential medium (MEM) with 5 or 10% heated fetal-calf serum as described (9). Confluent, 12-day-old cultures grown in 60-mm plastic petri plates were used in all experiments. The maintenance medium consisted of MEM with 2% heated fetal-calf serum or 0.5% human plasma-protein fraction (Armour Pharmaceutical Co., Chicago, Ill.), as specified. The volume of medium was 5 ml per plate during growth and 2 ml per plate during the experimental procedures. The incubation was in a humidified CO₂ incubator at 37°, unless specified.

Interferon Assays. The semi-micromethod for titration of human interferon has been described (9). Interferon yields are expressed in terms of reference units based on the 69/19 British reference standard for human interferon.

Incorporation of Labeled Amino Acid. Cultures were incubated in leucine-free MEM with either 0.5 μ Ci of uniformly labeled L-[¹⁴C]leucine (0.50 mCi/0.025 mg; New England Nuclear Corp., Boston, Mass.) or with 10 μ Ci of L-[³H]leucine (30 Ci/mol; New England Nuclear Corp., Boston, Mass.). Incorporation was stopped after 60 min by washing the cells three times with ice-cold phosphate-buffered saline (PBS). The cells were then lysed in 2 ml of 0.01 M ethylene-diaminetetraacetic acid-acetate buffer (pH 5.0) containing 0.5% sodium dodecyl sulfate. Lysates were then 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 5

Abbreviations: MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline, pH 7.4.

TABLE 1. Interferon yields from cells treated with cordycepin during the induction phase*

Time of treatment with cordycepin (hr)	Interferon yield
0-6	240
1-6	2,560
2-6	15,360
3-6	30,720
4-6	30,720
5-6	15,360
None	10,240

* FS-4 cells were stimulated at 0 hr by the addition of poly(I)·poly(C) (100 µg/ml) in the presence of cycloheximide (50 µg/ml) in serum-free MEM. Actinomycin D (1 µg/ml) was added to all cultures at 5 hr. The concentration of cordycepin was 100 µg/ml. At 6 hr, all cultures were washed and replenished with inhibitor-free MEM containing 2% fetal-calf serum. Interferon yields were determined in culture fluids collected at 24 hr.

ml each of 5% trichloroacetic acid. Trichloroacetic acid-insoluble radioactivity was measured in a liquid scintillation counter.

Chemicals. Poly(I)·poly(C) was obtained from the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Cycloheximide was purchased from the Upjohn Co., Kalamazoo, Mich.; cordycepin and puromycin dihydrochloride were from the Sigma Chemical Co., St. Louis, Mo.; actinomycin D was from Calbiochem, Los Angeles, Calif.

RESULTS

Transcription of Interferon mRNA in Cells Induced in the Presence of Cycloheximide. Interferon production in cultures of rabbit or human cells begins within 1-2 hr after a brief exposure to poly(I)·poly(C); it reaches a peak in 3-4 hr and rapidly declines to undetectable levels thereafter (9, 10). The addition of actinomycin D to rabbit kidney-cell cultures

up to 1 hr after stimulation with poly(I)·poly(C) greatly reduced interferon production while its addition at 2 hr or later had no inhibitory effect (10, 11). The inhibitory effect of actinomycin was likely to be due to the suppression of mRNA synthesis since the adenosine analogue, toyocamycin, had no inhibitory effect on interferon production at dose levels that greatly reduced ribosomal RNA synthesis while leaving cytoplasmic mRNA synthesis relatively unaffected (12).

Table 1 shows the results of an experiment that sought to establish the time needed for mRNA synthesis in cells stimulated with poly(I)·poly(C) in the presence of cycloheximide. The inhibitor of protein synthesis and poly(I)·poly(C) were kept in all cultures for 6 hr. In addition, groups of cultures were treated with cordycepin, (3'-deoxyadenosine) at the indicated intervals. To stop further RNA synthesis, all cultures were treated with actinomycin D at 5 hr. The culture fluids with the inhibitors were removed at 6 hr; the cultures were thoroughly washed and replenished with inhibitor-free medium to allow for interferon synthesis directed by mRNA accumulated in the first 6 hr.

Cordycepin preferentially inhibits the synthesis of cytoplasmic mRNA, apparently by interfering with the synthesis of polyadenylate and the transport of mRNA from the nucleus to the cytoplasm (13, 14). Although we cannot rule out that the synthesis of other species of RNA was also affected, the results shown in Table 1 can most likely be interpreted to mean that the bulk of interferon mRNA synthesis in the cultures took place within the first 2 hr of the addition of poly(I)·poly(C), despite the fact that the inducer was present in the cultures during the entire 6-hr period.

The addition of cordycepin at the later intervals, particularly at 3 or 4 hr, increased interferon production, i.e., it had an additional superinducing effect. In this respect the action of cordycepin also resembles that of actinomycin D; the latter inhibitor produced a maximal increase of interferon production when it was added 3 or 4 hr after the exposure of FS-4 cells to poly(I)·poly(C) and cycloheximide (Havell and Vilček, in preparation). We also determined that cordycepin

TABLE 2. Effects of cycloheximide and puromycin on interferon production and on the transcription of interferon messenger RNA

Inhibitor of protein synthesis used	[³ H]Leucine incorporation*		Interferon yield		
	cpm × 10 ⁻³	% Control	In the presence of inhibitors†	After removal of inhibitors‡	In uninduced cells
None	435	(100)	512	ND	<4§
Cycloheximide (50 µg/ml)	7.5	1.7	256	24,576	ND
Puromycin (10 µg/ml)	16.7	3.7	128	16,384	ND
Cycloheximide (50 µg/ml) + puromycin (10 µg/ml)	1.0	0.2	8	16,384	<4¶

* Cells were labeled for 1 hr, starting 30 min after the onset of treatment with inhibitor(s). The results, expressed as cpm per culture, have been multiplied by 10⁻³.

† Cultures were treated with the appropriate inhibitor(s) in serum-free MEM. Poly(I)·poly(C) (100 µg/ml) was added to all cultures 30 min after the onset of this treatment. At 1 hr the cultures were washed free of poly(I)·poly(C) and replenished with medium containing the same concentration of inhibitor(s). The fluids were collected at 6 hr, dialyzed to remove the inhibitors, and assayed for interferon.

‡ Cultures were treated with the appropriate inhibitor(s) in serum-free MEM. Poly(I)·poly(C) (100 µg/ml) was added 30 min after the onset of inhibitor treatment. Actinomycin D (1 µg/ml) was added to all cultures at 4 hr. At 6 hr the cultures were washed free of the inhibitors and poly(I)·poly(C), and replenished with MEM containing 0.5% human plasma-protein fraction. Fluids were collected for interferon assays 24 hr after the removal of the inhibitors. ND, not done.

§ Same as †, except for the omission of poly(I)·poly(C).

¶ Same as ‡, except for the omission of poly(I)·poly(C).

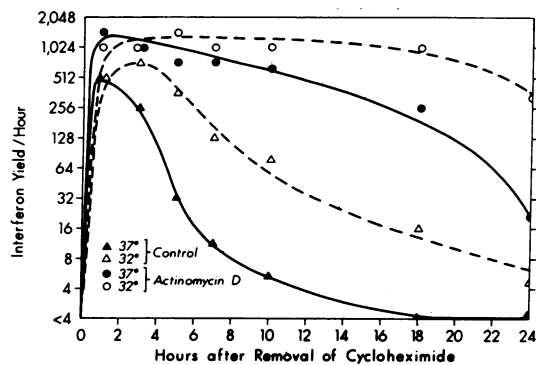


FIG. 1. Effect of incubation temperature and actinomycin D on the kinetics of interferon production. A group of cultures was induced at 37° with poly(I)·poly(C) (100 μ g/ml) in the presence of cycloheximide (50 μ g/ml). Half the cultures were treated with actinomycin D (1 μ g/ml) at 4 hr. All cultures were washed at 6 hr and replenished with inhibitor-free MEM containing 0.5% human plasma-protein fraction. Half of the actinomycin D-treated cultures and half of the cultures not treated with actinomycin D were then further incubated at 32°; the incubation of the remaining cultures continued at 37°. At the indicated intervals fluids were collected and the cultures were washed and replenished with fresh medium warmed to 37° or 32°. The determined interferon yields in the samples were divided by the number of hr that had elapsed from the collection of the previous sample.

caused superinduction when added at 4 hr to cultures that never received actinomycin D, although the increase in the interferon yield was somewhat less than in cultures treated at 4 hr with either actinomycin D or a combination of actinomycin D and cordycepin (not shown).

Apparent Independence of Transcription and Translation of Interferon mRNA. The finding that interferon mRNA transcription occurred in the presence of cycloheximide suggested that this process can proceed while translation is suppressed. However, cycloheximide is relatively inefficient in suppressing poly(I)·poly(C)-stimulated interferon production in cultures of rabbit or human cells (10, 15–17). Therefore, it seemed worthwhile to compare the interferon yields from cultures induced in the presence of cycloheximide and/or puromycin. One set of cultures was used to determine the interferon yields produced in the presence of the inhibitors of protein synthesis. In other groups, the production of interferon was measured only after reversal of the action of the inhibitors of protein synthesis and addition of actinomycin D (Table 2).

Despite the high degree of inhibition of cellular protein synthesis afforded by cycloheximide or puromycin, as measured by [3 H]leucine incorporation, interferon yields produced in the presence of these inhibitors were reduced only by 50 or 75%, respectively, as compared to the amount of interferon produced in the absence of any inhibitor treatment. (Nevertheless, as it will be explained in the *Discussion*, these findings can be reconciled with the assumption that even the interferon produced in the presence of inhibitors of protein synthesis is newly synthesized interferon.) The combination of cycloheximide and puromycin was much more effective in inhibiting interferon production. In spite of these differences in the direct action on interferon production, the treatment of cells with both inhibitors of protein synthesis separately, or with the combination of the two inhibitors, resulted in very similar

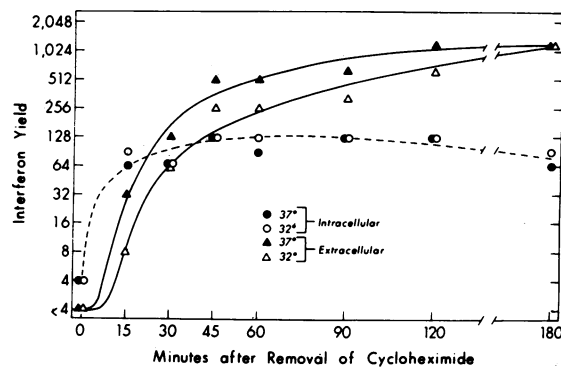


FIG. 2. Intracellular synthesis and release of interferon at 32° and 37°. A group of cultures was induced at 37° as described in Fig. 1, except that actinomycin D was added to all cultures at 4 hr. At the indicated intervals, fluids were collected from cultures incubated for the assay of extracellular interferon at 32° and 37°. The cells were washed with ice-cold PBS and subjected to 5 cycles of rapid freezing and thawing in 1 ml of PBS. The resulting cell extract was used for the assay of intracellular interferon.

interferon yields from cultures that were allowed to produce interferon after the reversal of inhibitor action. Thus, similar levels of interferon mRNA were apparently synthesized and available for translation in all groups of cultures treated with inhibitors of protein synthesis despite the varying degrees of suppression of the translation of the interferon mRNA afforded by these inhibitors.

Promoting Effect of Lowered Incubation Temperature. In the experiment shown in Fig. 1, all cultures were induced with poly(I)·poly(C) in the presence of cycloheximide at 37°. Two

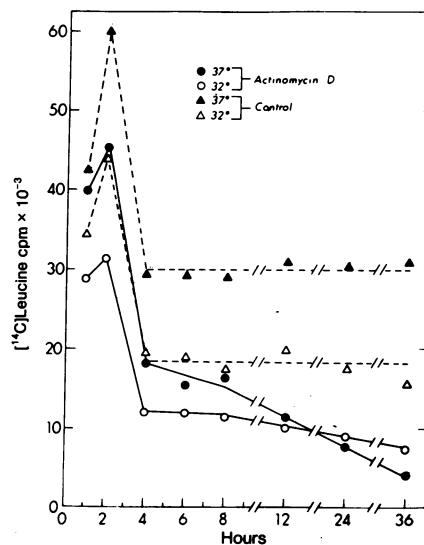


FIG. 3. Effect of incubation temperature and actinomycin D on cellular protein synthesis. Groups of cultures were treated at 37° for 1 hr with actinomycin D (1 μ g/ml) in serum-free MEM or with control MEM. At the end of this treatment (time 0) the cultures were washed, replenished with MEM containing 0.5% human plasma-protein fraction, and further incubated at either 37° or 32°. At the indicated intervals, groups of cultures were labeled for 1 hr with [14 C]leucine at the respective temperature and processed as described in *Methods*. The results are expressed as cpm per culture.

TABLE 3. Effect of inhibitors of protein synthesis on the production of interferon at 32° in superinduced cells*

Treatment at 22 hr after removal of cycloheximide†	Interferon yield at hr after treatment‡	
	1	7
None	512	4,096
Cycloheximide	384	128
Puromycin	256	32

* FS-4 cells were induced at 37° with poly(I)·poly(C)(100 µg/ml) in the presence of cycloheximide (50 µg/ml). Actinomycin D (1 µg/ml) was added to all cultures at 4 hr. At 6 hr the cultures were washed, replenished with inhibitor-free MEM containing 0.5% human plasma-protein fraction, and shifted to 32°.

† Medium was removed from all cultures 22 hr after the removal of inducer and inhibitors. (The interferon yield in the pooled media at this time was 16,384). The cultures were washed, and fresh 32° medium containing cycloheximide (50 µg/ml), puromycin (10 µg/ml), or no inhibitor was added.

‡ The culture fluids were collected 1 hr after the preceding treatment. The cultures were then again washed and replenished with fresh media containing inhibitors as above. These fluids were collected after an additional 6 hr of incubation at 32°. All fluids were dialyzed before the interferon assays.

groups of cultures received actinomycin D 4 hr after the addition of inducer while the other two groups were not treated with actinomycin. All cultures were washed and replenished with inhibitor-free medium 6 hr after induction. One group of actinomycin-treated cells and one group not treated with actinomycin were shifted to 32° while the remaining two groups of cultures were incubated at 37°. The kinetics of interferon production were determined separately in the four groups of cultures.

All cultures showed a rapid initial burst of interferon production after the reversal of cycloheximide action but the rates of decline of interferon production were strikingly different in the four groups. Both the addition of actinomycin D or the shift to 32° markedly prolonged the time during which interferon was produced at a high rate in the cultures—as measured by its accumulation in the culture fluid. The overall interferon yields were also increased. The effects of low incubation temperature and actinomycin D were additive, suggesting that they acted by different mechanisms.

Is the Sustained High Rate of Interferon Production at 32° Due to the Continued Translation of Interferon mRNA? The intracellular synthesis of interferon in rabbit kidney-cell cultures precedes its appearance in the culture fluid by about 20–30 min and the release of interferon from cells is inhibited at 4° (18, 19). Therefore, it seemed necessary to rule out the possibility that the sustained high rate of interferon production at 32° was due to the slower release of interferon from the cells at the lower temperature.

A set of FS-4 cultures was induced with poly(I)·poly(C) in the presence of cycloheximide at 37°. After the reversal of cycloheximide action, cultures were incubated at either 32° or 37°. Interferon levels in the culture fluids and in cell extracts were determined at different intervals up to 3 hr after the removal of cycloheximide (Fig. 2). In the first 15 min interferon levels were higher in the cell extracts than in the culture fluids. No significant differences were seen in the levels of intracellular interferon at the two temperatures. There appeared to be a slight delay in the appearance of extracellular

interferon at 32° as compared to 37°. However, this small delay in the release would not be sufficient to account for the marked differences in the rates of interferon production at the two temperatures shown in Fig. 1.

The experiment shown in Table 3 served to confirm the conclusion that the sustained high rate of interferon production at 32° was the result of continued intracellular interferon synthesis at this temperature. Cultures induced in the presence of cycloheximide were allowed to synthesize interferon in the absence of cycloheximide at 32°. Fresh medium containing cycloheximide, puromycin, or no inhibitor was added to groups of cultures 22 hr after the original reversal of cycloheximide action. The amount of interferon produced was determined within 1 and 7 hr after this treatment. It was expected that the release of the intracellular interferon synthesized before the addition of the inhibitors would be completed within the first hour of the treatment with the inhibitors. However, further synthesis, as measured by the amount of interferon produced into the medium 6 hr later, should be greatly reduced in the presence of cycloheximide or puromycin. These predictions were borne out by the experimental results.

Effect of Incubation Temperature on the Rate of Decline of Cellular Protein Synthesis in Actinomycin-Treated Cells. The following experiment was devised to determine whether the rate of decline of overall cellular protein synthesis after the arrest of RNA synthesis would also be affected by incubation temperature. A group of cultures was treated with actinomycin D for 60 min while another group received control medium. After this treatment, half of the actinomycin-treated and control cultures were shifted to 32°, the rest of the cultures were kept at 37°. At various intervals thereafter groups of cultures were labeled with [¹⁴C]leucine for 60 min at the respective temperatures (Fig. 3).

Amino-acid incorporation increased initially in all groups and peaked at 2 hr. This increase was undoubtedly the result of the preceding medium change. In actinomycin-free cells, protein synthesis then proceeded at a steady rate between 4 and 36 hr. [¹⁴C]Leucine incorporation during this period was about 40% less at 32° than at 37°, reflecting the less efficient functioning of the protein-synthesizing machinery at the lower temperature, which was also confirmed in other experiments (Havell and Vilček, in preparation). In actinomycin D-treated cells, on the other hand, the rate of synthesis declined at an approximately linear rate between 4 and 36 hr. The rate of this decline was greater at 37° than at 32°. The half-life of this function (i.e., the time required for the rate of incorporation determined at 4 hr to decrease by 50%) was calculated to be 20 and 43 hr, at 37° and 32°, respectively.

DISCUSSION

Interferon synthesis represents a unique model of induced protein synthesis in eukaryotic cells. Its synthesis can be quite readily elicited in a wide variety of differentiated and undifferentiated cells. In the great majority of cell systems there appears to be no basal level of synthesis, as judged from the absence of any detectable interferon activity in uninduced cells. The major drawback of all studies involving interferon is the fact that its assay still has to rely solely on the demonstration of biological activity. Therefore, it remains impossible to measure *directly* the rates of synthesis and decay of either the interferon protein or its mRNA.

Many induced enzymes in various animal cells are subject to superinduction by actinomycin D (reviewed in ref. 5).

Superinduction has been most thoroughly studied with steroid-induced tyrosine aminotransferase in cultures of rat hepatoma cells (20). Induction in this system is characterized by about a 2-hr lag period, followed by gradual increase of enzyme activity to a plateau level which is about 10 times higher than the basal level of enzyme in uninduced cells. The induced level is maintained for as long as the inducer is present in the cultures. Addition of actinomycin D during the early stages of induction suppressed enzyme induction but its addition to fully induced cells resulted in about a 3-fold increase in enzyme activity.

Tomkins *et al.* (5, 6) have proposed that the increase in enzyme activity after the addition of actinomycin D is the result of enhanced enzyme synthesis. To explain the mechanism of this phenomenon they postulated the existence of a labile regulatory protein that prevents the translation and promotes degradation of enzyme mRNA. The synthesis of this regulatory protein is thought to be rapidly inhibited by actinomycin D, and treatment with this inhibitor thus causes the "rescue" of stable enzyme mRNA which would otherwise not be available for translation. This explanation has not been quite universally accepted. Reel and Keeney (7) proposed that superinduction of tyrosine aminotransferase can be explained by a decreased degradation of the enzyme in actinomycin D-treated cells. Schimke *et al.* (8) suggested that actinomycin treatment produces a relative increase in the concentration of stable mRNA which, in turn, can be more efficiently translated.

Poly(I)·poly(C)-stimulated interferon production could also be superinduced by the addition of actinomycin D late during the induction process (11). The resulting increase in interferon levels was small, not unlike that reported with other inducible systems. Superinduction of interferon by actinomycin appeared to involve new synthesis of interferon because it was blocked in the presence of puromycin.

The superinduction of interferon production through the action of inhibitors of protein synthesis (10), or through the combined action of inhibitors of protein synthesis and actinomycin D (2, 3), is much more striking; it can result in up to a 100-fold increase in interferon activity over that obtained in cultures exposed to the inducer alone (9, 16, 17). A similar superinducing action of inhibitors of protein synthesis has not been reported for other inducible systems.

The results reported in this communication suggest that cycloheximide and other inhibitors of protein synthesis act by increasing the amount of interferon mRNA available for translation in cells stimulated with poly(I)·poly(C). Although direct evidence is not available, it seems reasonable to assume that inhibitors of protein synthesis act by promoting the stability of interferon mRNA. Since various inhibitors of protein synthesis share this activity, the effect is likely to be indirect. Thus, our results essentially support the idea of a regulatory protein acting on the mRNA, as postulated by Tomkins *et al.* (5, 6).

Further evidence for post-transcriptional regulatory mechanisms comes from the experiment showing that the duration of the period during which interferon synthesis proceeds at a high rate after the reversal of cycloheximide action can be influenced by actinomycin D as well as by incubation temperature (Fig. 1). Actinomycin D is likely to act by preventing the synthesis of the regulatory protein after the reversal of cycloheximide action (3). The lowering of incubation temperature, on the other hand, may have a direct stabilizing effect on cellular mRNA (21), although stabilization of some

other actinomycin D-sensitive component that is rate-limiting in protein synthesis cannot be ruled out.

The conclusion that inhibitors of protein synthesis indirectly produce an increase in the amount of functional interferon mRNA available for translation offers an explanation of the relative resistance of poly(I)·poly(C)-induced interferon production to inhibitors of protein synthesis (10, 15-17, 22). It would appear from the results shown in Table 2 that cycloheximide or puromycin inhibited interferon production by only 50 or 75%, respectively, as compared to cells induced in the absence of inhibitors of protein synthesis. However, when a comparison is made between the amount of interferon produced in the presence of cycloheximide or puromycin and in similarly treated cultures *after the reversal of inhibitor action*, the interferon yields from the former cultures are only about 1% of the yields from the latter cultures. In other words, the potential to produce interferon is much greater in cells induced in the presence of inhibitors of protein synthesis (apparently as a result of increased levels of functional interferon mRNA) than in the control induced cells, and a simple comparison of the interferon yields with the rates of overall protein synthesis may be misleading.

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