Proc. Nat. Acad. Sci. USA
Vol. 71, No. 12, pp. 4752–4756, December 1974

The Regulation of Protein Synthesis in Mammalian Cells VI. Soluble and Polyribosome Associated Components Controlling *In Vitro* Polypeptide Initiation in HeLa Cells*

(endogenous activity/actinomycin inhibition/*in vivo* regulation/polyribosome initiation inhibition/soluble initiation stimulation)

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Communicated by James E. Darnell, Jr., September 16, 1974

ABSTRACT **The *in vitro* initiation of polypeptides on endogenous polyribosomes has been studied in extracts from HeLa cells. Regulation of the rate of initiation of polypeptides can be examined. In these experiments an assay using [^{35}S]fMet-tRNA$_f^{Met}$ has been developed, and the system further characterized.**

The system has been separated into a fraction containing polyribosomes with subunits and a fraction containing soluble components. The regulation of initiation has at least two distinct components.

There is one factor in the soluble fraction which develops a stimulated response after protein synthesis has been inhibited in intact cells. This stimulation does not require new RNA synthesis during the period of cell "stress."

A second component is associated with ribosomes. This factor is necessary for the initiation of polypeptides on endogenous polyribosomes. It disappears gradually when cells are exposed to actinomycin. The disappearance is first manifested by an inability of polyribosomes to respond to stimulated supernatants. This unstable component, which decays in the presence of actinomycin, has no apparent counterpart in systems that measure initiation on exogenous mRNA.

The control of the translation of a relatively stable population of messenger RNA molecules appears to be a significant aspect of regulation of protein synthesis in mammalian cells. Several lines of experimentation suggest that an important site of control is the process of initiation (1–12). In exponentially growing HeLa cells, it has been possible to induce enhanced rates of initiation *in vivo* (2, 10) by exposing cells to a period of decreased protein synthesis caused by elevated temperature, treatment with cycloheximide, or starvation for an essential amino acid. We shall refer to cells that have been exposed to conditions inhibiting protein synthesis as "stressed." We shall show that stressing cells results directly in activating a soluble factor in the cytoplasm which leads to enhanced rates of initiation.

A second distinct phenomenon is the gradual loss of the capacity of polyribosomes to initiate polypeptides both *in vivo* and *in vitro* when new RNA synthesis is blocked by actinomycin. The lesion occurs prior to the loss of significant amounts of mRNA and appears due to a block by the drug of the appearance of a component necessary for proper initiation on polyribosomes. Furthermore, the loss of this factor in actinomycin during "stress" prevents polyribosomes from responding to the activated soluble fraction. We believe this ribosome-associated factor can be demonstrated only on endogenous polyribosomes from cells with active RNA metabolism (Reichman, Goldstein, and Penman, manuscript in preparation).

In order to study the biochemical events involved in the cellular control of the initiation of translation, an *in vitro* system reflecting the *in vivo* state was needed. Recently, such a system has been developed using HeLa cells (11). The initiation of new polypeptides on endogenous polyribosomes was measured with Edman's degradation technique. The extracts show enhanced rates of initiation when prepared from cells that had been subjected to prior inhibition of protein synthesis. Most important, the cellular response is abolished by the presence of actinomycin during the pretreatment which suggests that the *in vitro* system reflects the phenomena previously measured *in vivo*. The initiation assay has since been simplified, using [^{35}S]fMet-tRNA$_f^{Met}$ derived from mammalian sources.

Despite the serious instability of the system, we have been able to effect the first steps in fractionation. Using high-speed techniques, we divided the extracts into a polyribosome plus subunit fraction and a supernatant fraction. Thus, it has been possible to separate the activation that occurs in the supernatant following "stress," and the decay of the ribosome-associated factor following the block of RNA synthesis with actinomycin.

MATERIALS AND METHODS

Cells and Cell Culture. HeLa S3 cells were grown in suspension conditions in Eagle's medium supplemented with 7% horse serum at a concentration of 4×10^5 cells per ml (13).

Cell Fractionation. Cells were harvested by centrifugation, washed thoroughly with cold Earle's saline, and recentrifuged. The pellet was resuspended in 1.5 volume of lysing medium [75 mM KCl, 1.5 mM $Mg(OAc)_2$, 6 mM dithiothreitol, 10 mM K-Hepes (*N*-2-hydroxy ethylpipirazine-*N'*-2-ethane sulfonic acid), pH 7.1] and allowed to stand on ice for 10 min. The cells were then disrupted in a Potter homogenizer and centrifuged at $8000 \times g$ for 8 min (or $2000 \times g$ for 2 min for decay experiments) to remove nuclei and mitochondria. This postmitochondrial supernatant was freshly prepared for all experiments. Where indicated, the supernatant fractions were further fractionated by ultracentrifugation in a Spinco angle

Abbreviation: ATA, aurintricarboxylic acid.

* Paper V in this series is ref. 10.

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TABLE 1. *Inhibition of in vitro initiation of protein synthesis by ATA*

tRNA species	Incorporation of [^{35}S]methionine	
	−ATA	+ATA
$tRNA_f^{Met}$	6,446	1,299
$tRNA_m^{Met}$	12,925	13,050

A lysate from prestressed (with cycloheximide) cells was incubated in the presence and absence of 100 μM ATA as described in *Materials and Methods*. Incorporation of [^{35}S]methionine into trichloroacetic-acid-precipitable protein from beef liver $tRNA_m^{Met}$ and $tRNA_f^{Met}$ was measured in cpm/sample.

50 rotor for 15 min at 50,000 rpm. Two milliliter microtubes, half-filled, were used. These conditions pelleted 95% of the ribosomal subunits.

Conditions of In Vitro Incubation. The postmitochondrial supernatant fraction makes up 60% of the final reaction volume of 0.10 ml. The reactions are incubated in the presence of 1 mM ATP, 0.2 mM GTP, 4.0 mg/ml of creatine phosphate, 0.4 mg/ml of creatine phosphokinase, and 0.1 mM methionine. In addition 0.1 μmol of $Mg(OAc)_2$, 20 μmol of K-Hepes at pH 7.1, and 72 μmol of KCl are added to balance the final salt concentrations. Five microliters of [^{35}S]fMet-$tRNA_f^{Met}$ containing 115,000 cpm are added to start the incubation, which is carried out for 10 min at 25°. When initiation was to be inhibited specifically, ATA (aurintricarboxylic acid) at a concentration of 100 μM was used and the ATA insensitive incorporation was subtracted from the total. The reactions were terminated by the addition of 0.1 N NaOH and incubated at 37° for 5 min. Ten percent trichloroacetic acid containing sodium pyrophosphate and casamino acids was then added. The precipitate was collected by centrifugation, dissolved in 0.1 N NaOH, reprecipitated with trichloroacetic acid, and collected on Millipore filters. The filters were assayed for radioactivity by liquid scintillation counting. Protein determinations were by the procedure of Lowry *et al.* (14). Each reaction contained extract from 2×10^7 cells.

Preparation of [^{35}S]fMet-$tRNA_f^{Met}$. RNA was prepared from beef liver by extraction with phenol (15). High-molecular-weight RNA was removed by precipitation with 2 M NaCl. The soluble RNA was partially purified on a benzoylated DEAE-cellulose column in order to separate the two methionine tRNA peaks (16). The partially purified $tRNA_f^{Met}$ was acylated with [^{35}S]methionine and formylated with a crude *Escherichia coli* synthetase preparation according to RajBhandary and Ghosh (17). The final product was used at 10–15,000 cpm/μg of tRNA.

HeLa tRNA was prepared by extracting with phenol the cell supernatant from a 150,000 × *g* centrifugation. The total tRNA was acylated and formylated with the *E. coli* enzyme preparation as described above. Any unformylated Met-tRNA was deacylated by $CuSO_4$ treatment according to Schofield and Zamecnik (18), leaving [^{35}S]fMet-$tRNA_f^{Met}$ that was more than 90% formylated. The tRNA was used at 40,000 cpm/μg of tRNA.

RESULTS

In this report, we first show that the assay actually measured polypeptide initiation on endogenous polyribosomes. We then demonstrate that the initiation rate in the total extract can be manipulated by pretreating the cells. The alterations in *in vitro* activity follow those previously seen *in vivo*. Finally, we show the results of fractionating the total extract into polyribosomes and supernatant.

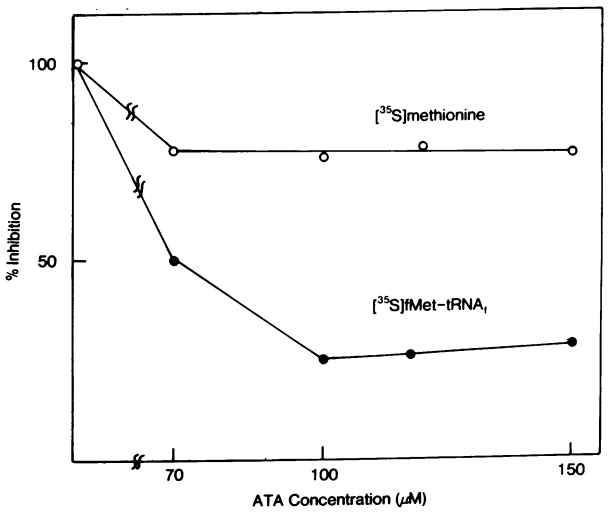

FIG 1. Inhibition of *in vitro* initiation by ATA. A lysate from prestressed (with cycloheximide) HeLa cells was prepared and incubated as described in *Materials and Methods* in the presence of various amounts of ATA. Incorporation of [^{35}S]methionine into trichloroacetic-acid-precipitable protein from beef liver [^{35}S]fMet-$tRNA_f$ (●) or from free methionine (○) was measured.

It is worth emphasizing that the system used here differs from many others. Polypeptide initiation on endogenous polyribosomes is measured in an extract derived from growing cells. We believe this approach shows aspects of protein synthesis regulation not seen in studies on endogenous polyribosomes from nongrowing cells (reticulocytes) or in extracts from growing cells where initiation is measured on exogenous mRNA.

In a previous report, it was shown that N-terminal amino acids are labeled in these extracts only if methionine is the radioactive donor (11). This labeling was completely suppressed by concentrations of pactamycin that only had a small effect on labeling internal amino acids.

The advantages of using labeled, formylated Met-$tRNA_f^{Met}$ to measure initiation are several. The formylated form of the tRNA should be more resistant to deacylation during the *in vitro* incubation. Also, this procedure is considerably simpler than the lengthy Edman degradation techniques for determining the amount of initiation. In the initial experiments, $tRNA_f^{Met}$ was first purified on benzoylated DEAE-cellulose and then charged and formylated with an *E. coli* enzyme preparation. Later, a simplified technique was adopted in which total tRNA was charged and formylated, and then all nonformylated Met-tRNAs were selectively deacylated in copper sulfate (18). Both methods of preparing radioactive tRNA give essentially the same results.

The results in Fig. 1 and Table 1 show that the formylated Met-$tRNA_f^{Met}$ derived from beef liver enters only into a reaction which is inhibited by ATA, a selective inhibitor of initiation. In Fig. 1, the inhibition by ATA of incorporation from fMet-$tRNA_f^{Met}$ is compared to that of the total incorporation of free methionine. As shown previously, the free methionine enters principally into internal positions. The incorporation

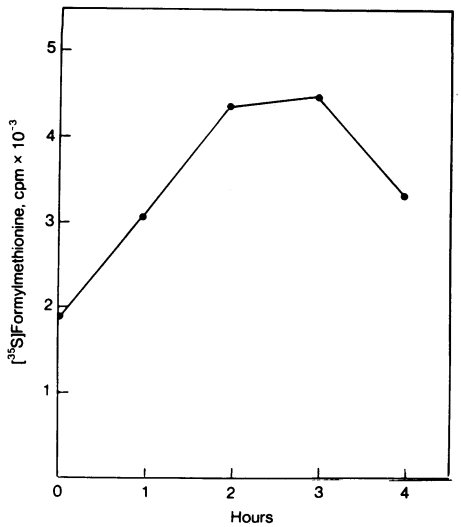

FIG. 2. Effect of increasing times of cycloheximide pretreatment *in vivo* on *in vitro* initiation. HeLa cells were incubated *in vivo* in 1 μg/ml of cycloheximide for various lengths of time. Cycloheximide was then washed out and postmitochondrial supernatant fractions were prepared and assayed for the incorporation of [^{35}S]methionine into trichloroacetic-acid-precipitable material from HeLa cell [^{35}S]fMet-tRNA$_f$ according to *Materials and Methods*.

from fMet-tRNA$_f^{Met}$ is severely inhibited by the ATA, while total incorporation is relatively insensitive. The small amount of methionine radioactivity incorporated in high concentrations of ATA is believed due to a contamination of this preparation with charged Met-tRNA$_m^{Met}$. Later experiments in which the unformylated tRNAs had been selectively deacylated gave background incorporation in the presence of ATA which was essentially negligible (approximately 100 cpm). Thus, the incorporation from fMet-tRNA$_f^{Met}$ appears to be exclusively due to the initiation of new polypeptides.

TABLE 2. *Stimulation of in vitro initiation after prestressing HeLa cells*

Exp.	Treatment	Specific activity [^{35}S]fMet-tRNA$_f^{Met}$
1	Cycloheximide	7610
	Cycloheximide + actinomycin	1155
	Control	1070
2	−Leucine	5875
	−Leucine + actinomycin	2540
	Control	2770
3	42° treatment	5563
	42° + actinomycin	2540
	Control	3330

Cells were incubated in the presence of 1–2 μg/ml of cycloheximide with and without 4 μg/ml of actinomycin for 2 hr. Cells were starved for leucine with and without 4 μg/ml of actinomycin for 2.5 hr. A third batch of cells was exposed to 42° temperature for 2.33 hr with and without 4 μg/ml of actinomycin. Lysates were then prepared from the treated cells and control cells for each experiment according to *Materials and Methods*. Incorporation of [^{35}S]methionine into trichloroacetic-acid-precipitable material from beef [^{35}S]fMet-tRNA was measured.

TABLE 3. *Subcellular fractionation of stimulatory activity produced by cycloheximide pretreatment*

Source of ribosomal pellet	Source of supernatant fraction	
	Cycloheximide	Control
Cycloheximide	6656	2702
Control	6256	2628

Postmitochondrial supernatant fractions were prepared from cells that had been incubated for 2 hr in 1 μg/ml of cycloheximide and from untreated control cells. Ribosomal pellets and postribosomal supernatant fractions were then prepared according to *Materials and Methods*. These fractions were recombined as indicated in the table and assayed for their ability to incorporate [^{35}S]methionine into trichloroacetic-acid-precipitable material from HeLa cell [^{35}S]fMet-tRNA$_f$ according to *Materials and Methods*. The numbers are ^{35}S cpm/sample.

A direct demonstration that only fMet-tRNA$_f^{Met}$ contributes to the initiation reaction is shown by the data in Table 1. ATA severely inhibits incorporation originating from purified fMet-tRNA$_f^{Met}$. The incorporation from Met-tRNA$_m^{Met}$, which should be only into internal polypeptide positions, is completely insensitive to the drug. Similar results have been obtained with pactamycin (which has a greater effect on elongation than ATA). It is concluded that measuring the incorporation of radioactivity from fMet-tRNA$_f^{Met}$ into alkali-stable, trichloroacetic-acid-precipitable material is an accurate measure of the amount of initiation of polypeptide synthesis occurring in these extracts.

Enhanced rates of polypeptide initiation in extracts prepared from prestressed cells are demonstrated by the data in Table 2. Protein synthesis was inhibited during the pretreatment of the cells either by incubating cells with a low level of cycloheximide (1–2 μg/ml), by removing the essential amino-acid leucine, or by exposing the cells to supraoptimal temperature (42°). A cytoplasmic extract is rapidly prepared and nuclei and mitochondria removed in a single step of centrifugation. The preparation obtained is able to initiate protein synthesis on endogenous polyribosomes at a higher rate than a comparable preparation from untreated control cells. This increased ability to initiate protein synthesis is apparently completely suppressed by the simultaneous addition of actinomycin to the cells during the pretreatment. The time course for the development of the cycloheximide-induced enhanced reaction is the same with the tRNA assay as it was when measured previously (11) using Edman degradation (Fig. 2). There was no difference in the rate of deacylation of added [^{35}S]fMet-tRNA$_f^{Met}$ by the two lysates. In addition, increasing the concentration of [^{35}S]fMet-tRNA$_f^{Met}$ to saturation level does not change the degree of stimulation of *in vitro* initiation caused by prior *in vivo* prestressing. This insures that the [^{35}S]fMet-tRNA$_f^{Met}$ assay is a true measurement of extent of initiation and is not affected by the rate of deacylation or by changes in pool size of charged tRNA.

Previous evidence from both *in vivo* studies and *in vitro* ability to initiate protein synthesis has indicated that some metabolic activity involving RNA is necessary to maintain proper initiation rates in HeLa cells. This process is inhibited by actinomycin (2, 10, 11). When this phenomenon was examined in detail in *in vivo* experiments, it was found that the actinomycin concentration required for half-maximum effect was 0.1–0.2 μg/ml. This was true for (*1*) actinomycin-induced

TABLE 4. *Subcellular fractionation of lysates pretreated with cycloheximide and with cycloheximide plus actinomycin D*

Source of ribosomal pellet	Source of supernatant fraction: Cycloheximide	Control	Cycloheximide plus actinomycin D
Cycloheximide	4338	3690	8060
Control	3326	2998	7122
Cycloheximide + actinomycin	1772	2438	2494

Postmitochondrial supernatant fractions were prepared from cells that had been incubated for 2.5 hr in 1 μg/ml of cycloheximide with and without added actinomycin D at 4 μg/ml and from untreated cells. Ribosomal pellets and postribosomal supernatant fractions were then prepared according to *Materials and Methods*. These fractions were recombined as indicated in the table and assayed for their ability to incorporate [^{35}S]fMet-tRNA$_f$ according to *Materials and Methods*. Numbers are cpm/sample.

decay of protein synthesis, (*2*) response to the lesion introduced when cells are incubated at the supraoptimal temperature of 42°, and (*3*) inhibition of induction by cycloheximide of protection against the lesion at 42°. The data in Fig. 3 show that actinomycin added to cells during the period of prestress inhibits the development of an enhanced rate of initiation *in vitro*. The dose response to the drug is similar to that found in the *in vivo* experiments. This suggests that the *in vitro* measurements reflect the metabolic response of the intact cell.

The dependence of initiation on the Mg^{++} concentration has been examined for prestressed and control lysates (Fig. 4). A clear difference in behavior of the two systems is apparent. This different Mg^{++} dependence suggests an alteration in the initiation system and shows the importance of optimizing the magnesium concentration for *stimulation* rather than simply for initiation.

Unfortunately, the lysates described here have proven to be exceedingly unstable. Activity is lost even at 4° with half-life of about 1 hr. This precludes, for the present, elaborate fractionation procedures. However, a quantitative separation of ribosomes and subunits from soluble components is possible by high-speed centrifugation in small centrifuge tubes. Lysates from control and cycloheximide-prestressed cells were reconstituted from their component supernatant and ribosomal fractions. The data in Table 3 show that reconstituted lysates exhibit the same degree of stimulation of *in vitro* initiation as do nonfractionated lysates.

Much greater insight is obtained when the supernatant and polyribosome fractions from the two sources are mixed. Prestressing cells with cycloheximide results in an enhanced capacity to initiate protein synthesis. This activity is largely found in the supernatant, but a variable portion is found in the ribosome fraction. Thus, the ribosomal fraction from prestressed cells will sometimes show an enhancement of initiation when mixed with supernatant from control cells. In such cases, the enhancing activity in the prestressed supernatant is correspondingly reduced. The behavior is as though a fixed amount of enhancing activity is induced, but its partition between ribosomes and supernatant can vary. The source of this variability is not known at present, and is not due to any of the more obvious variables, such as ionic conditions. In most experiments, the major portion of stimulatory activity

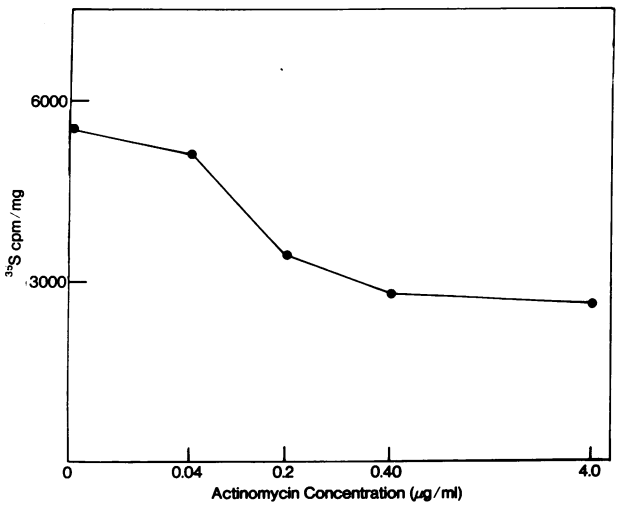

FIG. 3. Dose response of the suppression by actinomycin of cycloheximide-induced activity in whole cell lysates. HeLa cells were incubated *in vivo* in 1 μg/ml of cycloheximide and various amounts of actinomycin for 2 hr. The cells were washed thoroughly to remove the cycloheximide and postmitochondrial supernatant fractions were prepared and assayed for incorporation of [^{35}S]methionine into trichloroacetic-acid-precipitable material from beef liver [^{35}S]fMet-tRNA according to *Materials and Methods*.

is in the supernatant. Furthermore, it is shown below that altering the preincubation conditions of the cells produces stimulatory activity that is completely supernatant.

The most important results are obtained when the ribosomes and supernatant from cells exposed to actinomycin are examined (Table 4). It should be emphasized that although in these experiments the exposure to actinomycin was in the

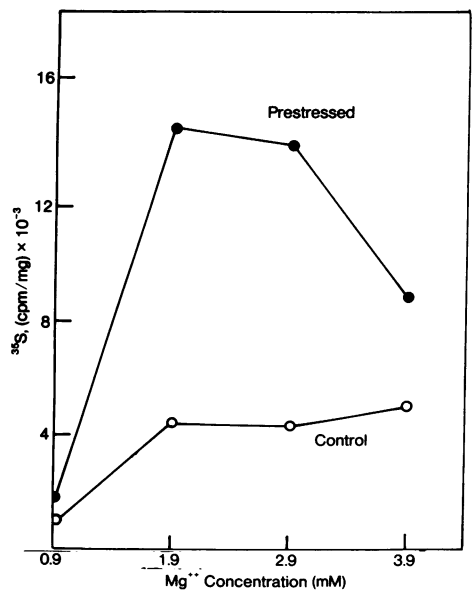

FIG. 4. Effect of Mg^{++} concentration on *in vitro* initiation. Lysates from prestressed (with cycloheximide) (●) and control (○) cells prepared according to *Materials and Methods* were incubated in various concentrations of added Mg^{++} for 10 min. Incorporation of [^{35}S]Met from HeLa cell fMet-tRNA$_f$ into trichloroacetic-acid-precipitable material was then determined. The contribution of endogenous Mg^{++} in the lysate is not estimated.

presence of cycloheximide, exactly the same results are obtained, for the ribosomes at least, when actinomycin is used alone.

Actinomycin does not prevent the appearance of the stimulated soluble activity. Rather, the supernatant fraction from cycloheximide–actinomycin-treated cells consistently appears superstimulated. We conclude that the appearance of the stimulatory activity is due to the inhibition of protein synthesis and does not require new RNA. The superstimulation is at present not understood and conceivably represents still another process.

The ribosomes from actinomycin-treated cells are somewhat less active than those from control cells when exposed to a control supernatant. This effect is magnified after longer treatments with actinomycin, as will be reported elsewhere. Here, the polyribosomes from actinomycin-treated cells appear to have just begun losing their normal initiating capacity.

The most striking observation is the inability of the ribosomes from actinomycin-treated cells to respond to the stimulated supernatants. Thus the block of RNA synthesis results in ribosomes gradually losing the ability to respond to stimulated supernatants. We propose that a factor, which is strictly associated with polyribosomes, is gradually lost during exposure to actinomycin. We further suggest that the unstable component of this factor is probably RNA, since its replenishment is blocked by actinomycin and not by cycloheximide. In all cases the mRNA in the polyribosomes appears normal. We term the putative factor an "activator," since its presence is necessary for polyribosome function. The polyribosome-associated component has no obvious counterpart in systems that initiate on exogenous mRNA.

DISCUSSION

In these experiments, we study the initiation of polypeptide synthesis on the endogenous polyribosomes of a cell synthesizing a wide spectrum of proteins, using a simplified assay based on [^{35}S]fMet-tRNA$_f^{Met}$. The system can show *in vitro* the alterations in rates of polypeptide initiation produced *in vivo*.

The factorology of mammalian protein synthesis is already complex and it is with great reluctance that we suggest additional components that regulate the initiation of translation. However, these results and others to be reported elsewhere lead to a seemingly inescapable conclusion: i.e., at least two controlling elements are present in this system which have no apparent counterpart in the known factors that have been described to date.

1. There is a soluble component that results in stimulation of the cytoplasmic supernatant whenever cellular protein synthesis is suppressed. Such an activity could be due to the decay of an unstable protein repressor. This stimulation appears to be general and we will show elsewhere that it stimulates initiation both on normal polyribosomes and on exogenous mRNA (Goldstein and Penman, in preparation). The superactivation, which occurs when actinomycin is also present during cell stress, is not understood. This could be due to a more complete elaboration of the activity developed by protein synthesis inhibition or, conceivably, is due to still another process.

2. The most significant finding reported here concerns the polyribosome-associated factor. This is a component of the endogenous protein synthetic system that has no counterpart in exogenous message systems. There appears to be a factor associated only with endogenous polyribosomes that is necessary for initiation. This polysome factor decays when actinomycin is administered to cells but is apparently unaffected by cycloheximide. The disappearance of this factor is first seen in the failure of polyribosomes from actinomycin-treated cells to respond to stimulated supernatants. Possibly the polysome factor becomes rate-limiting and the additional activity in the supernatant cannot be manifested. In a subsequent report, we will show that poliovirus infection in the presence of actinomycin leads to completely nonfunctional endogenous message. However, this same message, when extracted and fed back to the same extracts, functions as well as any other exogenous mRNA. Failure of endogenous message to function normally in cells in which the polyribosome factor has apparently decayed distinguishes protein synthesis on endogenous polyribosomes from that measured on exogenous mRNA.

There is a candidate for such a regulatory RNA. A 7S RNA has been found both on host polyribosomes and associated with the RNA of oncornoviruses (19).

We wish to thank Leslie Kenney and Laura Ransom for their valuable technical assistance, and Dr. Uttam L. RajBhandary, Massachusetts Institute of Technology, for his gift of *E. coli* synthetase enzyme. This work was supported by the National Institutes of Health (Grants 5-R01-CA08416 and CA12174) and by the National Science Foundation (Grant GB 37709X). One of us (E.S.G.) was supported by a Research Fellowship from the National Institutes of Health (1 F02 CA53661).

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