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A Cell Free System from HeLa Cells Active in Initiation of Protein Synthesis†

L. A. Weber, E. R. Feman, and C. Baglioni*

ABSTRACT: A cell free system programmed by endogenous mRNA and active in initiation of protein synthesis has been obtained from HeLa cells by adding 25-100 μM hemin to the medium used to homogenize the cells. Hemin stabilizes the initiation activity of the extract, which otherwise decays rapidly even at 0°C. The role of hemin in promoting initiation has been examined by fractionating the extracts into ribosomes and postribosomal supernatant (S150). An extract prepared without hemin or the S150 obtained from this extract inhibits protein synthesis of the extract containing hemin by about 30%. The ribosomes prepared from extracts containing hemin are active in initiation of protein synthesis, whereas the ribosomes obtained from the extracts prepared without hemin show little or no initiation. These results have suggested that addition of hemin prevents the formation of an inhibitor of initiation in the S150 and at the same time protects from inactivation an initiation factor associated with ribosomes or ribosomal subunits. Addition of 2 mM GTP to HeLa extracts stabilizes the initiation activity, though to a smaller degree than hemin. The effects of hemin and GTP are not additive, suggesting that they may act on the same target molecule, though possibly by different mechanisms. The mechanism of action of GTP is discussed in view of similar observations made in the rabbit reticulocyte cell free system.

In order to study translational control mechanisms in a cell free system it is necessary to have available a cell extract active in initiation of new polypeptide chains. A very active cell free system can be obtained from rabbit reticulocytes and several features of the regulation of α and β globin synthesis have been studied in this system (Lodish, 1974). However, it has been difficult to obtain initiation in vitro with extracts of HeLa cells, due to a rapid loss of activity during cell fractionation (Reichman and Penman, 1973). This problem has partially been overcome by preparing extracts by high-speed techniques (Goldstein et al., 1974). These extracts cannot, however, be fractionated by conventional biochemical techniques and cannot be frozen without loss of the ability to initiate new polypeptides. This is somewhat unfortunate, since there is a wealth of observations on the regulation of protein synthesis in HeLa cells which have been made by Penman and his associates

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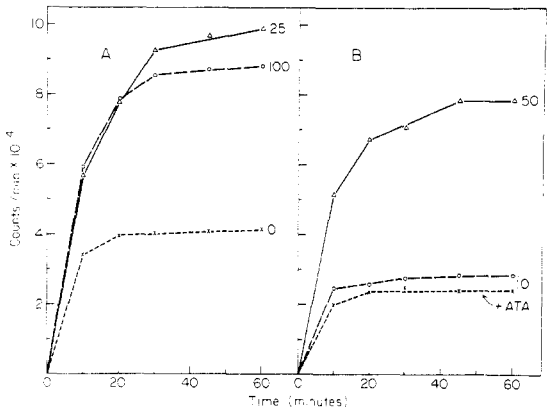

FIGURE 1: Protein synthesis with fresh (A) and frozen and thawed (B) extracts of HeLa cells. The extracts are prepared and tested as described under Experimental Procedures with no hemin added or with hemin at concentrations between 25 and 100 μM. Protein synthesis with fresh extracts containing 0, 25, and 100 μM hemin is shown in A. The frozen and thawed extracts are tested also in the presence of 100 μM ATA (B); protein synthesis of extracts containing hemin is identical with that of extracts not containing hemin in the presence of this concentration of ATA. The cpm/10-μl aliquots of incubation mixture are shown.

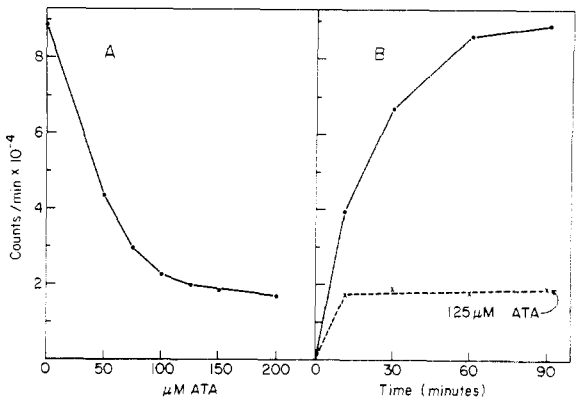

FIGURE 2: Inhibition of protein synthesis by ATA with extracts containing 50 μM hemin. A dose response curve to ATA inhibition is shown in A; aliquots are taken for counting after a 60-min incubation. The time course of inhibition of protein synthesis by 125 μM ATA is shown in B.

(McCormick and Penman, 1969; Goldstein and Penman, 1973). By exposing these cells to a period of decreased protein synthesis, these authors have been able to demonstrate an enhanced rate of initiation when the treatment which decreases protein synthesis ceases. It has been suggested that a soluble factor in the cytoplasm is responsible for this stimulation of initiation and it has been shown that incubation in the presence of actinomycin prevents this stimulation (Goldstein et al., 1974). The identification of the factor(s) responsible for the enhancement of initiation and for the actinomycin effect has not been possible thus far, because of the unstable initiation activity of the cell free system.

Jacobs-Lorena and Baglioni (1973) have reported that HeLa cell ribosomes are active in translating globin mRNA in a cell free system containing rabbit reticulocyte postribosomal supernatant. The HeLa ribosomes are thus capable of initiation under the conditions described by these authors (Jacobs-Lorena and Baglioni, 1973). This observation led us to suspect that some of the components present in the reticulocyte cell free system may be responsible for the initiating activity of HeLa ribosomes. The reticulocyte incubation medium has one component—hemin—which is not present in the incubation medium used by other authors to prepare HeLa cell free systems, and has another component—GTP—present at a much higher concentration (2 mM) than that used by other investigators (Celma and Ehrenfeld, 1974). We have thus tested the effect of hemin and of this high GTP concentration on initiation of protein synthesis by an HeLa cell free system programmed by endogenous mRNA.

Results

The Effect of Hemin on the Initiation of New Polypeptide Chains in the HeLa Cell Free System. A HeLa cell free system prepared as described in Experimental Procedures and tested within 5 min after breaking the cells, synthesizes protein linearly for 10 min or less and shows very little activity afterwards. Preliminary experiments showed that the addition of 50 μM hemin to the cell free incubation gives a low and variable level of stimulation of protein synthesis. However, when 25 or 100 μM hemin is included in the homogenization medium, considerable stimulation of protein synthesis is observed (Figure 1A). The rate of protein synthesis is linear for almost 30 min and the final amount of protein synthesized is at least threefold over that synthesized in the absence of hemin. Intermediate concentrations of hemin have been tested and the stimulation of protein synthesis is between that observed with 25 and 100 μM.

We have determined the conditions optimal for protein synthesis in the HeLa cell free system by assaying extracts prepared with 50 μM hemin at concentrations of K^+ between 50 and 90 mM and of Mg^{2+} between 2 and 5 mM, and by incubating the extracts at 26, 30, and 37°C. The ionic composition of the medium described in Experimental Procedures gives the best results. Protein synthesis proceeds at a slower rate at 26°C, but at an almost identical rate at 30 and 37°C; however, at 37°C protein synthesis stops after 30 min and we have thus used 30°C for our incubations.

We routinely freeze our extracts in liquid nitrogen. When an extract prepared without hemin is tested after freezing and thawing, it shows a loss of about 25% of its original activity. An extract containing 50 μM hemin loses less than 10% of its original activity upon freezing and thawing once (Figure 1B); however, extracts containing 25 μM hemin lose more than 50% of their activity when frozen. We therefore include 50 μM hemin in our extracts and avoid freezing and thawing them repeatedly.

The loss of activity observed upon freezing and thawing HeLa cell extracts in the absence of hemin is caused by an almost complete lack of reinitiation of protein synthesis. This is suggested by experiments in which aurintricarboxylic acid (ATA)[1] has been added. This inhibitor is reported to block initiation in a HeLa cell free system prepared without hemin at a concentration of 100 μM (Goldstein et al. 1974). This concentration of ATA has little effect on protein synthesis in extracts which have been frozen and thawed in the absence of hemin (Figure 1B).

In order to show that the stimulation of in vitro protein synthesis by hemin is a result of increased initiation, we have tested the effect of different concentrations of ATA on a HeLa cell extract containing hemin (Figure 2A). At ATA

[1] Abbreviations used are: ATA, aurintricarboxylic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; RBS buffer, 10 mM NaCl, 10 mM Tris (pH 7.4), and 1.5 mM $MgCl_2$.

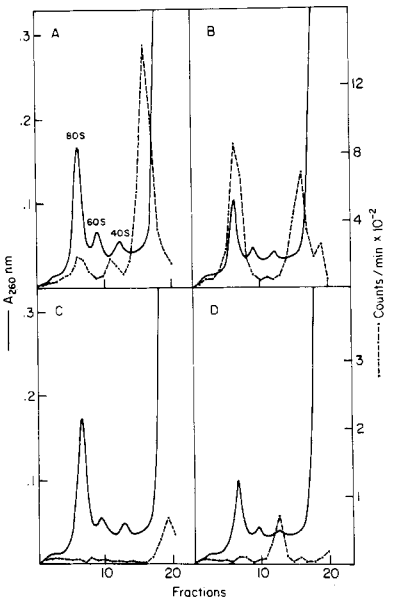

FIGURE 3: Binding to initiation complexes of [^{3}H]histone mRNA and [^{3}H]met-tRNA$_f$. HeLa cell free systems prepared from cells homogenized without hemin (A and C) or with 50 μM hemin (B and D) have been incubated as described under Experimental Procedures. The top panels (A and B) show the binding of histone mRNA to initiation complexes; the bottom panels (C and D) show the met-tRNA$_f$ binding.

concentrations higher than 100 μM only elongation of growing polypeptide chains takes place (Steward et al., 1971). The addition of ATA at these concentrations leads to more than 80% inhibition of protein synthesis in extracts prepared in the presence of hemin. The kinetics of protein synthesis is also drastically affected; in the presence of ATA protein synthesis proceeds for 10 min or less, whereas it proceeds for up to 60 min in the control (Figure 2B).

The specific effect of hemin on the maintenance of initiating activity is directly demonstrated by experiments described in Figure 3. The ability of cell free systems to bind trace amounts of ^{3}H-labeled messenger RNA to ribosomes after 5 min of incubation at 30°C is greatly diminished in the absence of hemin (Figure 3A) and maintained in its presence (Figure 3B). The small peak observed at fractions 11-12 is likely to be a complex of mRNA with the 40S subunit. Heywood and Thompson (1971) have indeed shown that ^{32}P-labeled myosin mRNA attaches to 40S subunits in a binding reaction with unfractionated salt-washed ribosomes.

In the reticulocyte cell free system hemin has been reported to be required for the interaction of met-tRNA$_f$ with the native 40S ribosomal subunit (Legon et al., 1973). Hemin appears to act in a similar way in the HeLa cell free system. Extracts without hemin rapidly lose the ability to bind detectable amounts of radioactive met-tRNA$_f$ to the 40S subunit (Figure 3C), while hemin appears to preserve this activity (Figure 3D).

The Mechanism of Action of Hemin. We have established that it is necessary to add hemin when HeLa cells are broken and that unlike in the reticulocyte cell free system (Zucker and Schulman, 1968), the later addition of hemin will not restore the activity of cell extracts. In the experiment described in Figure 4 we have homogenized HeLa cells in the absence of hemin and then have added hemin before preparation of the postmitochondrial fraction or at different times after obtaining this fraction. The extracts

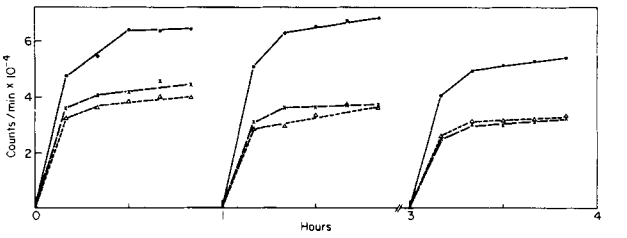

FIGURE 4: Protection of initiation activity of HeLa extracts by hemin. An extract is prepared from cells in the absence of hemin. To one aliquot 50 μM hemin is added after homogenizing the cells (●—●), whereas another aliquot of extract is kept in ice for the times indicated; time 0 is 20 min after cell homogenization. The extract prepared without hemin is then tested in the presence of 50 μM hemin (× - - ×) added at the beginning of the incubation or in the absence of hemin (Δ - - - Δ). Protein synthesis is assayed as described under the legend to Figure 1.

have been kept in ice and tested immediately and at hourly intervals. The activity of each extract has been compared to the extract without hemin.

Addition of hemin approximately 20 min after homogenizing the cells gives a small stimulation of protein synthesis, but addition after 1 or 2 hr to extracts kept at 0°C has practically no effect (Figure 4). Once hemin is added to an extract the initiating activity of the extract seems stabilized, so that there is no loss of activity after 1 hr and 20 min and a 10% loss of activity after 3 hr and 20 min at 0°C.

These results can be explained by the stabilization of a factor necessary for initiation of protein synthesis by the presence of hemin. Alternatively, hemin may prevent the formation of an inhibitor which inactivates a factor necessary for initiation. A precedent for both mechanisms of action exists in the rabbit reticulocyte cell free system. Addition of hemin prevents the formation of an inhibitor of initiation which prevents the association of met-tRNA$_f$ with the native 40S ribosomal subunit (Legon et al., 1973). Hemin also has been shown to directly activate initiation factors isolated from reticulocytes, brain or liver (Raffel et al., 1974).

We have assayed HeLa extracts for the formation of an inhibitor by mixing experiments. In the first experiment we have incubated separately extracts obtained from cells homogenized with hemin (+H extract) or without hemin (−H extract), and a mixture of equal volumes of the +H and −H extracts. Both extracts are kept for 1 hr at 0°C, while aliquots are centrifuged to obtain a postribosomal supernatant (S150). In this experiment the expected level of protein synthesis in the incubation containing a mixture of +H and −H extract should be intermediate between those obtained with the individual extracts, if hemin is only involved in the stabilization of an initiation factor. However, we have observed a 30% inhibition of protein synthesis with respect to the expected values in the incubation containing a mixture of +H and −H extract (Figure 5A). It seems thus likely that an inhibitor of protein synthesis is formed in the −H extract while it is kept at 0°C and that this hypothetical inhibitor is capable of decreasing the activity of the +H extract in the mixing experiment.

In order to localize the inhibitor to either the S150 or to the ribosomal fraction, we have tested a +H extract in the presence of +H S150 and −H S150. The dilution of the +H extract with an equal volume of +H S150 causes a reduction of about 50% in the protein synthesized per unit volume of the incubation, because the polysomes are diluted

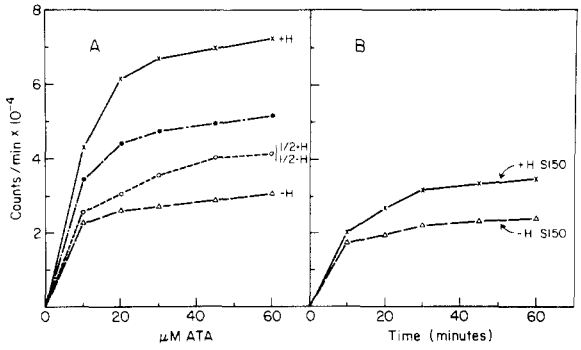

FIGURE 5: Inhibition of protein synthesis by extracts or postribosomal supernatant (S150) not containing hemin. A fresh extract has been prepared with 50 μM hemin or without hemin, and kept in ice for 1 hr, while an aliquot has been centrifuged for preparation of S150 (see Experimental Procedures). The extracts are assayed for protein synthesis as described under the legend of Figure 1. (A) Incubations with extract containing 50 μM hemin (+H); extract without hemin (−H); a mixture of equal volume of the two extracts (½+H − ½−H); and the expected incorporation (●—●) if protein synthesis with the mixture of the two extracts were the average of the incorporation obtained with each separate extract. (B) Incubations with an equal volume of extract containing 50 μM hemin and of S150 obtained from the extract with hemin (+H S150) or without hemin (−H S150).

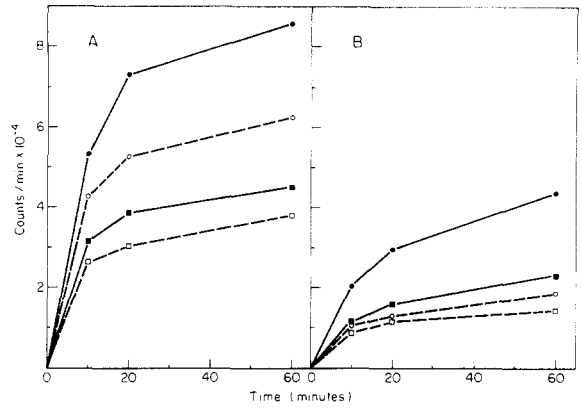

FIGURE 6: Protein synthesis with ribosomes and S150 obtained from extracts prepared with or without hemin. Ribosomes and S150 are prepared as described under Experimental Procedures; the ribosomes have been resuspended at 4 mg/ml. (A) The incubations contain 0.4 vol of polysomes and 0.4 vol of S150; (B) the incubations contain 0.2 vol of ribosomes and 0.6 vol of S150. (●—●) Incubations of ribosomes and S150 from extracts with 50 μM hemin; (○—○) incubations with ribosomes with hemin and S150 without hemin; (■—■) incubations with ribosomes without hemin and S150 with hemin; and (□ - - - □) incubations with ribosomes and S150 without hemin.

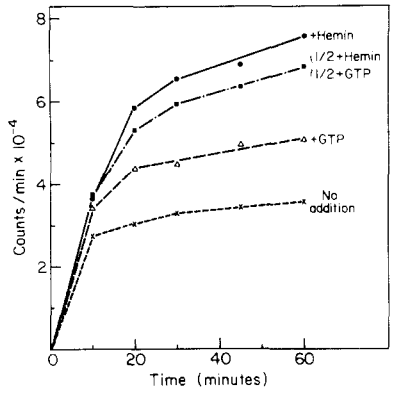

FIGURE 7: Protein synthesis with extracts prepared with 2 mM GTP. Extracts are prepared by adding 2 mM GTP and 2 mM $Mg(OAc)_2$, 50 μM hemin, or with no addition. The incubations are carried out with these extracts or by mixing an equal volume of the extract containing GTP and of the extract containing hemin. See Figure 1 for conditions of incubation.

by ½. The addition of the −H S150 causes a 30% inhibition of protein synthesis with respect to the incubation of the +H extract mixed with the +H S150 (Figure 5B). Quantitatively, the same inhibition of protein synthesis can be obtained with the complete −H extract or with the −H S150. This suggests that at 0°C an inhibitor of protein synthesis is formed in extracts prepared without hemin. This inhibitor is effective in inhibiting protein synthesis in extracts prepared in the presence of hemin.

We have tested different extracts for the formation of the inhibitor under different conditions. By simply freezing and thawing an extract prepared without hemin a maximum amount of inhibitor is formed. Incubation of the extract or of the S150 at 0 or 30°C does not result in additional formation of inhibitor as tested by the assay described above.

We have established that ribosomes prepared from extracts containing hemin are markedly more active in initiation of protein synthesis than ribosomes prepared from extracts without hemin. Initiation in these assays is defined by protein synthetic activity beyond the initial 10 min of incubation, when elongation of already initiated polypeptides takes place, and by control experiments in the presence of ATA (not shown) in which this activity is abolished. Ribosomes and S150 have been prepared from +H and −H extracts and tested in all combinations (Figure 6). The assays have been carried out at two different ratios of ribosomes to S150, one close to that of the unfractionated extracts (Figure 5A) and the other at threefold excess of S150 (Figure 6B). Protein synthesis is most active when ribosomes and S150 from the +H extract are used. The incubations containing +H ribosomes and −H S150 show 30 and 54% inhibition of protein synthesis at the low and high ratio of S150 to ribosomes, respectively. The incubations of −H ribosomes with −H S150 show a 20 and 34% inhibition, relative to the incubations of −H ribosomes with +H S150.

The Effect of 2 mM GTP on Initiation of Protein Synthesis. The addition of 2 mM GTP to the homogenizing medium has also been found to increase the activity of the HeLa cell free system (Figure 7); 2 mM GTP is not quite as effective as hemin, since it gives a 50% stimulation of protein synthesis over that of an extract without additions. We have investigated whether hemin and GTP act in a similar manner in two experiments. First, by mixing an extract prepared with hemin with one prepared with GTP we have observed an intermediate activity, though closer to that of the extract with hemin alone (Figure 7). In the other experiment we have prepared two extracts by homogenizing HeLa cells with hemin alone or with hemin and 2 mM GTP; the activity of these extracts is identical. If hemin and GTP were stabilizing initiation by completely different mechanisms, we might expect that the two compounds added together would stimulate protein synthesis above the level observed with hemin alone. This argument is valid if initiation is not limited by other factors, which would prevent us from detecting a synergistic effect.

We have not investigated the effect of other concentrations of GTP, or that of other purines. It seems possible that these will prove equally effective in view of the observation of Legon et al. (1974) that a variety of purines are able to overcome the inhibition of protein synthesis observed in reticulocyte lysates incubated in the absence of hemin. This

block in initiation can also be overcome by the addition of an initiation factor which forms a complex with GTP and methionyl-tRNA$_f$ (Kaempfer and Kaufman, 1972).

Discussion

The purpose of this communication is to describe the preparation of a cell free system from HeLa cells active in initiation of protein synthesis. We have thus examined in detail the conditions necessary to obtain initiation in this system and have defined the optimal ionic composition of the incubation medium. A preliminary attempt to clarify the role of hemin has also been carried out; this study has shown that hemin prevents the formation of an inhibitor in the postribosomal supernatant and at the same time stabilizes the initiation of ribosomes.

We feel that it is quite important to have a cell free system from HeLa cells active in initiation of protein synthesis, since these cells have been used in numerous experiments directed at studying the regulation of protein synthesis in intact cells (McCormick and Penman, 1969; Goldstein and Penman, 1973). With the availability of an active and stable cell free system it is now possible to fractionate cells which have been subjected to periods of decreased protein synthesis and to identify the cellular components responsible for the increased rate of initiation observed after this treatment (Goldstein et al., 1974). In addition, other regulatory mechanisms which have been described in intact HeLa cells can now be studied in a fractionated cell free system. We are presently investigating the regulation of histone synthesis in this system. A block in DNA replication inhibits histone synthesis and causes a parallel disappearance of histone mRNA from polyribosomes; the histone mRNA is not found in the postribosomal supernatant and is presumably degraded (Borun et al., 1975). The HeLa cell free system can be used to study this regulatory mechanism in vitro.

Though we have carried out only preliminary experiments on the role of hemin in initiation of protein synthesis, we would like to discuss some obvious implications of our present observations. When the effect of hemin on initiation of globin synthesis in reticulocytes was first described, it was suggested that hemin could have a regulatory role by adjusting the rate of globin synthesis to that of heme (Zucker and Schulman, 1968). This role of hemin is unlikely since hemin affects the synthesis of all the proteins made by the reticulocyte lysate (Mizuno et al., 1972; Lodish and Desalu, 1973). Moreover, hemin stimulates protein synthesis to a small extent in intact ascites cells (Beuzard et al., 1973) at relatively high concentrations, and in the ascites cell free system (Beuzard et al., 1973; Matthews, 1972) at concentrations similar to those which we have used.

These observations and our present results suggest that hemin may have a more general role in initiation of protein synthesis than previously thought. Two major questions remain unanswered, however: (1) does hemin or a related compound have a specific biological role in initiation of protein synthesis; and (2) what is the mechanism of action of hemin or of a related compound? The first question can be answered only by direct chemical analysis of the initiation factors of eukaryotic cells. If a protoporphyrin-containing compound is found associated with a specific initiation factor, this will provide support to the notion that hemin or a related molecule has a biological role in protein synthesis, particularly if it can be shown that the activity of the factor is dependent upon its association with hemin.

A mechanism of action for the stimulation of protein synthesis by hemin has been proposed by Raffel et al. (1974). These authors have shown that a factor obtained from brain or liver can overcome the block in initiation seen in the reticulocyte cell free system incubated in the absence of hemin. The activity of the brain factor is strongly stimulated upon preincubation with hemin which mediates the formation of an active high mol wt complex from lower mol wt components. Raffel et al. (1974) suggest that a low concentration of hemin may be necessary for all tissues to satisfy the requirement for active protein synthesis. In reticulocyte extracts hemin may become limiting because it is consumed by being incorporated into hemoglobin; in HeLa or other cells hemin may be present in too low concentrations to provide an extract active in initiation when the cells are homogenized. There is no clear-cut evidence, however, that hemin is the biological compound responsible for the activation of a specific initiation factor.

Our results show that hemin acts by stabilizing the initiation activity of ribosomes and by preventing the formation of an inhibitor in the postribosomal supernatant. It seems possible that hemin acts on the same molecule(s) in both ribosomes and supernatant, since some initiation factors are found both in the supernatant and on ribosomes (Schreier and Staehelin, 1973). The inhibitory activity observed in the −H S150 may be explained by competition by an inactive initiation factor with the active initiation factor associated with +H ribosomes. The inactive factor may participate in the formation of an inactive initiation complex and thus block initiation.

The stimulation of initiation observed with 2 m*M* GTP has only been studied in a preliminary way. GTP can form an initiation complex with IF-MP, met-tRNA$_f$, and mRNA (Gupta et al., 1973). It may thus act by stabilizing this complex. However, purines like theophylline, caffeine, adenine, and 2-aminopurine are active in the reticulocyte cell free system in overcoming the inhibition of initiation by lack of hemin (Legon et al., 1974). The observation that cAMP can also overcome the inhibition caused by lack of hemin in the reticulocyte cell free system may provide a unifying hypothesis for the mechanism of action of GTP and purines. These are inhibitors of phosphodiesterase (Burk, 1968; Froelich and Rachlemer, 1972; Rozengurt and Pardee, 1972), whereas GTP stimulates adenylate cyclase by interacting with noncatalytic regulatory sites of the enzyme (Rodbell et al., 1971; see for other reference, Cuatrecasas et al., 1975). The fact that cAMP is active in the reticulocyte cell free system at concentrations higher than the likely cellular levels may not be incompatible with this hypothesis, since the phosphodiesterase activity of the reticulocyte extract has not been determined and most of the cAMP may be rapidly degraded. Experiments in the HeLa cell free system are now in progress to test this hypothesis.

Experimental Procedures

Preparation of the HeLa Cell Extract. Cells are grown in Joklik's modifies Eagle Medium for spinner cultures (G.I.B.Co.) and harvested at 5×10^5 cells/ml. The cells are washed three times with spinner salts solution (G.I.B.Co.) and pelleted by centrifugation. To 1 vol of packed cells is added 2 vol of a buffer containing 10 m*M* KCl, 1.5 m*M* $Mg(OAc)_2$, 20 m*M* Hepes buffer (pH 7.4), and 0.5 m*M* dithiothreitol. After standing for 10 min in ice the cells are homogenized in a Dounce homogenizer. The homogenate is spun 4 min at 27000*g* and the supernatant removed and ei-

ther used immediately or frozen in liquid nitrogen. Hemin is added to the homogenate or the homogenization buffer as indicated in the text. A stock solution of 1 m*M* hemin is prepared by dissolving hemin in $^1/_{100}$ vol of 1 *N* KOH, followed by 0.6 vol of 20 m*M* Hepes buffer (pH 7.4) and 0.39 vol of water.

Protein Synthesis Assay. The incubation mixtures contain 7 vol of cell extract, 2 vol of energy mixture, and 1 vol of salt solution. The energy mixture contains 10 m*M* ATP, 1.5 m*M* GTP, 50 m*M* creatine phosphate, 300 units/ml of creatine phosphokinase (Sigma), 110 m*M* Hepes buffer (pH 7.4), and 4 m*M* dithiothreitol. The salt solution contains 700 m*M* KCl and 30 m*M* $Mg(OAc)_2$. The salt solution is dried down in experiments where other components are added to the incubation. Unlabeled amino acids minus lysine and [^{3}H]lysine (38.3 Ci/mmol; New England Nuclear) are routinely dried down in the tube used for the incubation and redissolved to a final concentration of 0.05 m*M* and 5 μCi/0.1 ml. The reaction is started by transferring the tube to a 30°C water bath; 10-μl aliquots are taken before starting the reaction and at the times indicated and spotted on 2.3-cm 3MM Whatman paper filters. The filters are air dried and dropped then in 5% trichloroacetic acid, boiled for 5 min, washed again twice, and dried. The filters are counted in 0.8% butyl-PBD (C.I.B.A.) in toluene. The radioactivity of the aliquot taken at time 0 is substracted from that of the other samples.

Preparation of Polysomes and Postribosomal Supernatant (S150). Fresh extract (0.5–1 ml) is centrifuged 60 min at 50000 rpm in a Ti-50 Spinco rotor. The supernatant is carefully removed and designated S150. The polysome pellet is resuspended in a volume of homogenization buffer corresponding to $^1/_3$ the volume of the extract using a small glass homogenizer. The polysome suspension is spun 10 min at 12000*g* to remove any unresuspended material and frozen in liquid nitrogen. The proportions of polysome suspension and S150 used in different incubations is indicated under the legends to the figures.

mRNA and met-tRNA$_f$ Binding Experiments. Labeled histone mRNA has been prepared by incubating HeLa cells synchronized in S phase by a double thymidine block with 50 μCi/ml of [5-^{3}H]uridine (New England Nuclear). Polysomes are isolated and the RNA extracted with phenol and fractionated by two successive sucrose density gradients (Weber, Williams, and Baglioni, in preparation); electrophoretic analysis on 4.5% acrylamide gels has shown that this RNA contains discrete RNA species migrating between 7 S and 9.5 S. These RNA species are translated into specific histones by an ascites cell free system (T. W. Borun, personal communication).

Met-tRNA$_f$ has been prepared from rabbit reticulocytes as previously reported by Shafritz and Anderson (1970) and charged with [^{3}H]methionine (3.0 Ci/mmol); the specific activity of the met-tRNA$_f$ is 1500 cpm/pmol. We thank Dr. Shafritz for the gift of a sample of met-tRNA$_f$.

The binding reactions have been carried out in a 0.1 ml volume. The cell free system described above (containing all 20 unlabeled amino acids at 0.05 m*M*) is incubated for 3 min at 30°C and 0.1 m*M* sparsomycin is added for 2 min, followed by the addition of 10 μl of either [^{3}H]+histone mRNA or [^{3}H]-met-tRNA$_f$ solution in water for 2 more min. The samples are diluted with 0.40 ml of ice-cold RSB buffer (10 m*M* NaCl, 10 m*M* Tris (pH 7.4), and 1.5 m*M* $MgCl_2$) and fractionated by centrifugation on 15–30% sucrose gradients in RSB for 16 hr at 22000 rpm in the SW-27 Beckman rotor; 0.8-ml fractions are collected and counted directly by addition of a mixture of 1 vol of Triton X-100, 2 vol of 0.8% butyl-PBD (C.I.B.A.), and 10% water for the mRNA binding experiments, or after precipitation of the samples with 1.5 ml of 2% cetyltrimethylammonium bromide and 0.25 mg of carrier yeast tRNA for the met-tRNA$_f$ binding experiments. The precipitates are collected on Millipore filters and counted as described above.

The histone mRNA has a specific activity of 180000 cpm/μg and 0.03 μg is added in each binding assay; 5 pmoles of met-tRNA$_f$ is added in each assay. The binding assay follows with minor modifications the procedure described by Darnbrough et al. (1973). Sparsomycin is added to specifically bind mRNA to initiation complexes containing a single ribosome, since this drug prevents elongation, but does not inhibit initiation (Darnbrough et al., 1973). When sparsomycin is omitted from the incubations, histone mRNA becomes associated with polysomes (unpublished observations).

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Evidence of the Involvement of a 50S Ribosomal Protein in Several Active Sites†

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ABSTRACT: The functional role of the *Bacillus stearothermophilus* 50S ribosomal protein B-L3 (probably homologous to the *Escherichia coli* protein L2) was examined by chemical modification. The complex [B-L3·23S RNA] was photooxidized in the presence of rose bengal and the modified protein incorporated by reconstitution into 50S ribosomal subunits containing all other unmodified components. Particles containing photooxidized B-L3 are defective in several functional assays, including (1) poly(U)-directed poly(Phe) synthesis, (2) peptidyltransferase activity, (3) ability to associate with a [30S·poly(U)·Phe-tRNA] complex, and (4) binding of elongation factor G and GTP. The rates of loss of the partial functional activities during photooxidation of B-L3 indicate that at least two independent inactivating events are occurring, a faster one, involving oxidation of one or more histidine residues, affecting peptidyltransferase and subunit association activities and a slower one affecting EF-G binding. Therefore the protein B-L3 has one or more histidine residues which are essential for peptidyltransferase and subunit association, and another residue which is essential for EF-G·GTP binding. B-L3 may be the ribosomal peptidyltransferase protein, or a part of the active site, and may contribute functional groups to the other active sites as well.

The mechanism of protein biosynthesis can be represented as a series of binding interactions and partial reactions occurring on the ribosome, which can be separated operationally in vitro. Correspondingly, the ribosome is an assembly of about 50 macromolecular components, which can be separated and characterized. In order to understand the relationship between the functional complexity of protein synthesis and the structural complexity of the ribosome, it is necessary to identify the individual components which contribute to each of the functional activities.

The protein B-L3 of the large ribosomal subunit from *Bacillus stearothermophilus* has been identified as an essential ribosomal component by in vitro reconstitution studies (Fahnestock et al., 1973a). Particles reconstituted without B-L3 are defective in several functional assays, but contain all other ribosomal proteins as well as 5S and 23S RNA. It is difficult to draw any conclusions concerning direct functional roles of this protein based on such experiments, because of the high probability of relatively large scale structural perturbations caused by the omission of a component, and the demonstrable sensitivity of the functional activity of 50S subunits to structural changes (Fahnestock et al., 1973b).

Affinity labeling studies have identified a number of proteins of *Escherichia coli* ribosomes which are located close to the peptidyltransferase active center (for review, see Cantor et al., 1974). One of these, L2, is probably homologous to the *B. stearothermophilus* protein B-L3, since (1) both have direct binding sites on 23S RNA (Stöffler et al., 1971); (2) they have similar molecular weights and polyacrylamide gel electrophoretic mobilities; and (3) B-L3 cross-reacts immunologically with *E. coli* L2, but not with any of the other *E. coli* ribosomal proteins (Tischendorf et al., 1973). In view of the one-for-one functional homology between the 30S ribosomal proteins of *E. coli* and *B. stearothermophilus* which has been demonstrated by Higo et al. (1973) it is reasonable to assume that the structural homology between the 50S proteins B-L3 and L2 reflects a functional homology as well. These affinity labeling studies suggest the possibility that L2 (and therefore, presumably, B-L3) is the ribosomal peptidyltransferase, or at least may form part of the active site. In order to investigate this possibility I have subjected B-L3 to chemical modification in an effort to introduce limited structural and functional alterations into the 50S ribosomal subunit. The pH dependence of ribosomal peptidyltransferase activity suggests the involvement of a histidine residue in the active site (Fahnestock et al., 1970). Photooxidation in the presence of the dye rose bengal is the most selective method available for the modification of histidine residues in proteins (Means and Feeney, 1971), especially with regard to its total lack of reactivity toward amino groups, which are abundant in ribosomal proteins. The peptidyltransferase protein is likely to have a critical histidine residue and therefore to be sensitive to photooxidation. The experiments reported here demon-

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