Hemin-independent control of globin synthesis in Friend erythroleukemia cells induced to differentiate

(rabbit reticulocytes/in vitro protein synthesis/mRNA-depleted lysates/control of translation)

EVAN A. STRINGER AND CHARLOTTE FRIEND

The Mollie B. Roth Laboratory, Center for Experimental Cell Biology, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029

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ABSTRACT A hemin-independent translational inhibitor that prevents synthesis of rabbit globin when uninduced Friend leukemia (FL) cell and rabbit reticulocyte lysates are mixed [Cimadevilla, J. M. & Hardesty, B. (1975) Biochem. Biophys. Res. Commun. 63, 931–937] cannot be detected in FL cells induced to differentiate. Mixing of lysates of FL cells induced with hexamethylene bisacetamide or aminonucleoside of puromycin and rabbit reticulocytes does not cause inhibition of rabbit globin synthesis. Induction also results in the cells acquiring sensitivity to the inhibitor from uninduced FL cells. A reduction in total protein synthesis is observed when uninduced and induced FL cell lysates are mixed. Inhibition does not result from competition by an excess of uninduced FL cell mRNA species for the translational machinery because uninduced FL cell lysates retain their inhibitory activity after treatment with micrococcal nuclease. Rabbit globin mRNA recovered from rabbit reticulocyte lysates that have been incubated with lysates of uninduced FL cells can still be translated effectively, indicating that inhibition does not result from modification of other species of mRNA by uninduced FL cell lysates. A switch to hemin-dependent translational control does not follow induction of differentiation. The rate of amino acid incorporation in induced FL cell lysates—like that in uninduced FL cell lysates—is unaffected by omission of exogenous hemin from the system. Its presence is not required to prevent activation of heme-regulated inhibitor. From these data, it is clear that the control of protein synthesis in FL cells—whether or not they are induced—is different from that regulated by hemin in normal erythroid cells.

Friend erythroleukemia (FL) cells are virus-transformed murine erythroblasts that grow in continuous culture and can be induced to differentiate by treatment with various different compounds. The morphological changes induced mimic those seen in normal erythropoietic transition from proerythroblasts to orthochromophilic erythroblasts. They are accompanied by a decrease in proliferative capacity and the expression of late erythroid events such as an increase in iron uptake and heme synthesis, accumulation of hemoglobin, and the appearance of erythrocyte-specific membrane antigens (for review, see refs. 1–3). Thus, this system allows the molecular control of erythrodifferentiation to be examined.

In reticulocytes—the normal counterpart of erythroleukemia cells—the synthesis of globin, together with that of other less abundant proteins, depends on the presence of hemin. Unless exogenous heme is added to lysates of rabbit reticulocytes, there is an abrupt decline in the rate of protein synthesis after a few minutes (4–7). Hemin blocks the formation of a translational inhibitor by preventing the conversion of an inactive proinhibitor to an active inhibitor (8–10). The hemin-regulated inhibitor (HRI) possesses a protein kinase activity that can specifically phosphorylate the initiation factor eIF-2 and inhibit the initiation of new protein chains (11–14).

The fact that hemin, at concentrations required to prevent activation of HRI in rabbit reticulocytes, stimulates the accumulation of globin mRNA and globin protein (15) suggests that induction in FL cells also may be regulated via the hemin/HRI mechanism. On the other hand, evidence that the mechanism controlling protein synthesis may be different from that in reticulocytes comes from reports that the uninduced FL cell lysates (i) are not affected by the addition of hemin or HRI, and (ii) possess hemin-independent translational inhibitors that prevent the synthesis of rabbit globin (16–18).

These differences could be reconciled if the switch to a program of differentiation involved a modulation of hemin-independent inhibitors and the activation of hemin-dependent translational controls. Therefore, the behavior of induced FL cell lysates was examined to determine whether—like uninduced lysates—they still have the ability to inhibit globin synthesis in hemin-containing rabbit reticulocyte lysates but—unlike uninduced cells—develop a hemin requirement for protein synthesis.

Our data show that FL cells, after induction, lose their ability to inhibit rabbit globin synthesis and, in the process, become sensitive to the action of the hemin-independent inhibitors of uninduced FL cells. However, induction does not result in a switch to hemin-dependent translational control. These results suggest that modulation of hemin-independent translational inhibitors may provide an alternative pathway to differentiation in the neoplastic cells.

MATERIALS AND METHODS

Establishment, growth conditions, and characterization of the subclone 5-86 of the prototype FL cell line 745A have been described (19). Cells were seeded at a concentration of 1×10^5 per ml and were maintained in a basal medium/Eagle (GIBCO no. 420-1100) supplemented with 10% fetal calf serum (Reheis, Kankankee, IL), penicillin, and streptomycin. The cultures were kept at a constant temperature of 37°C in an atmosphere of 5% CO_2 in air.

Aminonucleoside of puromycin (AMS) (Sigma) and hexamethylene bisacetamide (HMBA) (a kind gift of Roberta Reuben and Yair Gazitt) were added to cultures at concentrations of 10 μg/ml and 5 mM, respectively, at the time of seeding. The cultures were scored for benzidine-positive (B+) cells 4–5 days after the initiation of the cultures by the wet benzidine method of Orkin et al. (20).

Preparation of Lysates. Rabbit reticulocyte lysates were prepared by the method of Ranu and London (21). Lysates of FL

Abbreviations: AMS, aminonucleoside of puromycin; FL, Friend leukemia; HMBA, hexamethylene bisacetamide; HRI, hemin-regulated inhibitor.

cells, harvested after 4–5 days of growth in medium with and without inducers, were prepared by the same method, except that total lysis of the cells was accomplished by additional mechanical disruption in a Dounce homogenizer. Lysates were stored in small aliquots in liquid nitrogen.

Protein Synthesis in Cell-Free Lysates. Immediately after thawing, the lysates were made 50 μg/ml in creatine phosphokinase and 5 mM creatine phosphate.

Amino acid incorporation was measured by using a modification of the procedure of Ranu and London (21). Reaction volumes of 80 μl contained 20 mM Tris·HCl (pH 7.5), 80 mM potassium acetate, 0.5 mM magnesium acetate, unlabeled amino acids at concentrations related to their frequency in rabbit globin, [^{35}S]methionine (4,000 cpm/pmol), 10 μg of creatine phosphokinase, and 5 mM creatine phosphate. Hemin was added to give a final concentration of 15 μM to prevent activation of HRI in lysates of rabbit reticulocytes. The reaction mixture was incubated at 30°C for a total of 45 min. Aliquots of 8 μl were removed at 15-min intervals and treated according to Kramer *et al.* (13). The aliquots were diluted in 1.0 ml of 0.5 M NaOH/1.5% H$_2$O$_2$, incubated at 37°C for 10 min, brought up to 5% in trichloroacetic acid, and incubated for a further 10 min at 90°C. After cooling in ice for 10 min, the precipitate that formed was collected on nitrocellulose filters (0.45-μm pore size, type HAWP, Millipore) and was washed with three 5-ml volumes of 5% trichloroacetic acid. Radioactivity was measured by liquid scintillation in 10 ml of counting fluid containing 4 g of 2,5-diphenyloxazole (POP) and 40 mg of 1,4-bis[2(5-phenyl-oxazolyl)]benzene (POPOP) per liter of toluene.

Measurement of Rabbit Globin Synthesis. The products synthesized in the cell-free systems were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis in gels containing 15% polyacrylamide according to Schreier *et al.* (22). After staining with Coomassie brilliant blue, the gels were destained in methanol/acetic acid/H$_2$O, and then they were cut into 2-mm slices, treated with 0.5 ml of NCS solubilizer (Amersham) at 50°C for 2 hr, and cooled; the radioactivity was counted in the scintillation fluid indicated above.

Preparation of mRNA-Deficient Lysates. The mRNA contained in 100-μl aliquots of lysates was digested by treatment with micrococcal nuclease (Sigma) according to Pelham and Jackson (23).

Purification of mRNA. Globin mRNA was purified from rabbit reticulocytes by phenol/chloroform/isoamyl alcohol extraction followed by chromatography on a oligo(dT)-cellulose column, as outlined by Aviv and Leder (24) and Krystosek *et al.* (25).

RESULTS

Expression of Inhibitory Activity in FL Cell Lysates. Because induced FL cells, like reticulocytes, synthesize large amounts of globin, it was of interest to determine whether the hemin-independent inhibitor of globin synthesis found in uninduced FL cells is retained after induction. FL cells were induced to differentiate by treatment with either HMBA—an inducer which stimulates ornithine decarboxylase activity—or AMS—an inducer which does not stimulate this activity (26).

As shown in Fig. 1A (and Table 1), when lysates of uninduced FL cells were mixed with lysates of rabbit reticulocytes, the synthesis of rabbit globin was inhibited, whereas the synthesis of proteins from uninduced FL cells proceeded. This selective discrimination of uninduced FL cells in favor of the synthesis of their own proteins suggests that there are hemin-independent elements involved in the control of translation.

In contrast, such elements were not detected in the induced cells (Fig. 1B), which lose the ability to inhibit protein synthesis in rabbit reticulocytes. When equal amounts of amino acid in-
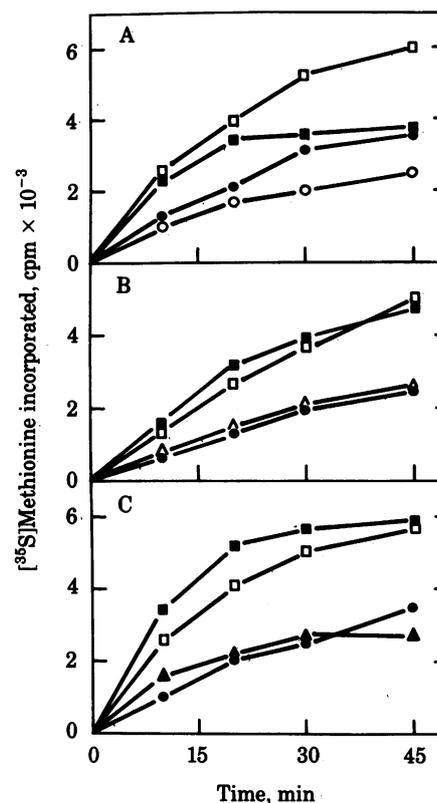

FIG. 1. Effect of FL cell lysates on protein synthesis in rabbit reticulocytes. Amino acid incorporation by 150-μg lysates of each of (*A*) uninduced FL cells (○——○), (*B*) HMBA-induced FL cells (△——△), and (*C*) induced cells (▲——▲) was measured alone and in combination with rabbit reticulocyte lysate (900 μg) (●——●) as described. □——□, Theoretical additive value of amino acid incorporation by FL cell plus rabbit reticulocyte lysates; ■——■, observed additive value.

corporating activity from lysates of rabbit reticulocytes and lysates of FL cells induced to differentiate by HMBA are mixed together, protein synthesis was not affected. The total incorporation of [^{35}S]methionine into protein is equal to the sum of the values obtained for the individual lysates. Similarly, when lysates of FL cells induced by AMS were mixed with rabbit reticulocytes, the total incorporation obtained was at least additive (Fig. 1C).

When the differentiated FL cells lose their ability to express the inhibitor, they acquire sensitivity to its action. The mixing of uninduced FL cell lysates with lysates of the cells induced to differentiate by HMBA (Fig. 2A) or AMS (Fig. 2B) caused a decrease in the total amount of amino acid incorporated. These results were obtained with hemin added to the assay mixtures; however, identical results were observed when hemin was omitted (data not shown).

Effect of mRNA Depletion on Inhibitory Activity of FL Cell Lysates. To eliminate the possibility that the inhibition of protein synthesis was a result of uninduced FL cell mRNA competing for the mRNA recognition and binding sites more effectively than other species of mRNA, the assays for inhibitory activity were repeated after the lysates had been treated with micrococcal nuclease. The results obtained with the mRNA-depleted FL cell lysates parallel those obtained with the lysates containing intact mRNA (Figs. 1 and 2). As seen in Fig. 3, nuclease-treated uninduced FL cell lysates retained the ability to inhibit protein synthesis when added to lysates of rabbit reticulocytes or to lysates of HMBA- or AMS-induced FL cells, and nuclease-treated lysates of HMBA- (Fig. 4) or AMS-induced (Fig. 5) FL cells remained without an effect on protein synthesis in lysates of rabbit reticulocytes or uninduced FL cells. In all
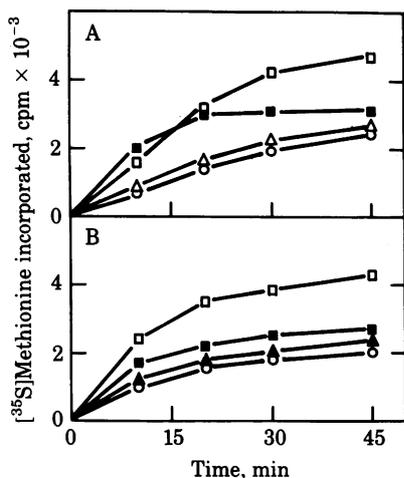
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FIG. 2. Effect of uninduced FL cell lysates on protein synthesis in induced FL cells. Amino acid incorporation by 150 μg of uninduced FL cell lysates was measured alone (○——○) and in combination with 150 μg of either (*A*) HMBA-induced FL cell lysate (△——△) or (*B*) AMS-induced FL cell lysate (▲——▲). □——□, Theoretical additive value of amino acid incorporation by uninduced FL cell lysate plus induced FL cell lysate; ■——■, observed additive values. (Reprinted from ref. 16 with permission of Raven Press.)

cases, the nuclease-treated lysates by themselves no longer supported amino acid incorporation due to the digestion of their mRNA. The fact that nuclease treatment did not modify the activity of the inhibitor suggests that it may be protein in nature.

Effect of FL Cell Lysates on Rabbit Globin Synthesis. To quantitate the inhibitory effect of FL cell lysates, rabbit globin synthesis was monitored by subjecting aliquots of individual lysates and mixtures of FL cell and rabbit reticulocyte lysates to polyacrylamide gel electrophoresis in the presence of
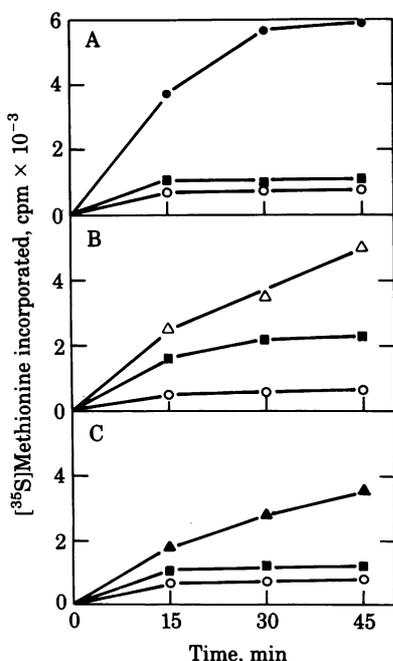

FIG. 3. Effect of nuclease treatment on activity of lysates of uninduced FL cells. Amino acid incorporation by lysates of each of (*A*) rabbit reticulocytes (●——●), (*B*) HMBA-induced FL cells (△——△), and (*C*) AMS-induced FL cells (▲——▲) was measured alone and in combination with mRNA-depleted uninduced FL cell lysates obtained by treatment with micrococcal nuclease (○——○), as outlined in Figs. 1 and 2. ■——■, Observed amino acid incorporation resulting from mixing mRNA-depleted and untreated lysates.
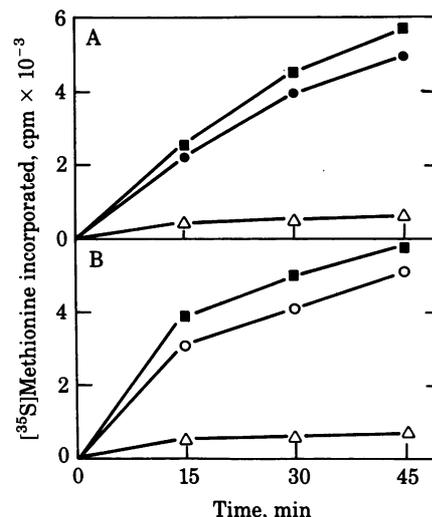

FIG. 4. Effect of nuclease treatment on activity of lysates of HMBA-induced FL cells. Amino acid incorporation by lysates of (*A*) rabbit reticulocytes (●——●) and (*B*) uninduced FL cells (○——○) was measured alone or in combination with mRNA-depleted HMBA-induced FL cell lysates (△——△) obtained by treatment with micrococcal nuclease, as outlined in Figs. 1 and 2. ■——■, Observed amino acid incorporation resulting from mixing mRNA-depleted and untreated lysates.

NaDodSO$_4$. The results are summarized in Table 1. The addition of uninduced FL cell lysates to rabbit reticulocyte lysates decreased rabbit globin synthesis by 80%, whereas induced FL cell lysates did not have the same effect. When HMBA-induced FL cell lysates were added to rabbit reticulocytes lysates, globin synthesis was enhanced. The fact that nuclease-treated lysates of AMS-induced FL cells did not inhibit synthesis in rabbit reticulocytes (Fig. 5A) suggests that the 20% reduction in globin synthesis observed in the experiment in Table 1 resulted from a large endogenous pool of mRNA molecules in the induced FL cell lysates competing with globin mRNA.

Effect of Uninduced Cell Lysates on Rabbit Globin mRNA. To ensure that inhibition of rabbit globin synthesis by uninduced FL cells did not result from modification of globin mRNA, mRNA from a mixture of uninduced FL cells and rabbit reticulocyte lysates was isolated and translated in a mRNA-de-
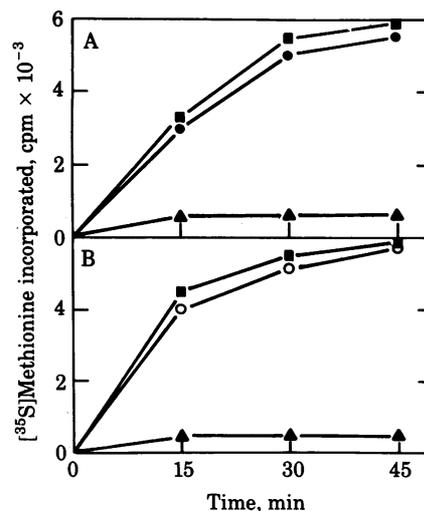

FIG. 5. Effect of nuclease treatment on activity of lysates of AMS-induced FL cells. Experiments in Fig. 4 were repeated by using mRNA-depleted lysates of AMS-induced FL cells (▲——▲) in place of mRNA-depleted HMBA-induced FL cell lysates. Remaining symbols are the same as in Fig. 4.

Table 1. Effect of FL cell lysates on rabbit globin synthesis

Lysate	Rabbit globin synthesized,* pmol	Inhibition, %
Rabbit reticulocyte	10.0	—
Uninduced FL cell	—	—
HMBA-induced FL cell	—	—
AMS-induced FL cell	—	—
Rabbit reticulocyte + uninduced FL cell	2	80
Rabbit reticulocyte + HMBA-induced FL cell	12.7	—
Rabbit reticulocyte + AMS-induced FL cell	8.0	20

Reaction mixtures identical to those used in Fig. 2 were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis as described. The amount of [^{35}S]methionine-labeled protein migrating in the region of rabbit globin was quantitated in each case.
* Mean of three estimations.

pleted rabbit reticulocyte amino acid incorporating system. Analysis of the products of translation by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 6) shows that a large proportion of the proteins synthesized migrated in the region of rabbit globin, indicating that rabbit globin mRNA could still be translated. The efficiency of translation of the mixture of mRNA species was similar to that of globin mRNA isolated from rabbit reticulocytes alone (data not shown).

Effect of Hemin on Protein Synthesis in Induced FL Cell Lysates. Because FL cells, after being induced to actively synthesize hemoglobin, do not appear to retain the hemin-independent inhibitor that prevents protein synthesis in rabbit reticulocytes, it was of interest to determine whether protein synthesis had become dependent on hemin.

Protein synthesis in lysates of cells induced with HMBA and AMS was studied to determine whether the response to the addition of hemin (15 μM) was as sensitive as that of rabbit reticulocytes. In rabbit reticulocytes (Fig. 7A) hemin was required to maintain linear rates of incorporation of amino acids. In its absence, HRI was activated and protein synthesis was cut off after 10 min. In contrast, a lack of response to the addition of hemin—similar to that in uninduced FL cell lysates (B) (16–18)—was found in lysates of HMBA-induced (C) and AMS-induced (D) cells, although over 70% of the treated cells were benzidine-positive (B+), indicating synthesis and accumulation of hemoglobin. The addition of 5 mM HMBA and 10 μg of AMS
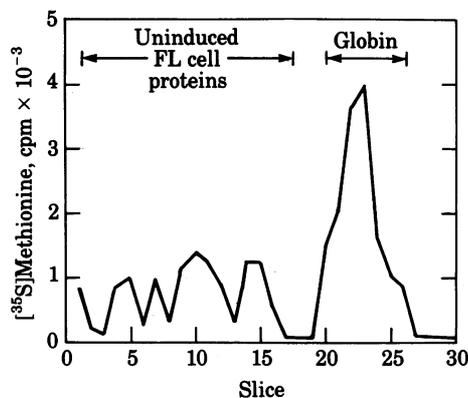

FIG. 6. NaDodSO$_4$/polyacrylamide gel electrophoresis analysis of products of translation of mRNA isolated from mixed lysates. mRNA isolated from a mixture of uninduced FL cell and rabbit reticulocyte lysates was translated in a mRNA-dependent rabbit reticulocyte amino acid incorporating system and the [^{35}S]methionine-labeled products were examined by electrophoresis.
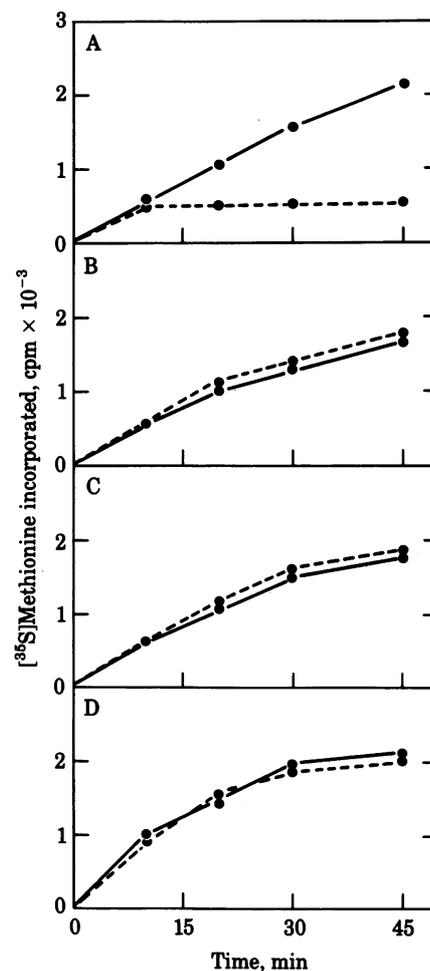

FIG. 7. Hemin requirement for reticulocytes but not for FL cell lysates. Amino acid incorporation was measured in the presence (●———●) and absence (●– – –●) of 15 μM hemin in lysates of (A) rabbit reticulocytes, (B) uninduced FL cells, (C) HMBA-induced FL cells, and (D) AMS-induced FL cells.

per ml—the concentrations used for induction—has no effect on amino acid incorporation in rabbit reticulocyte lysates (16), thus excluding the possibility that inducers alone could have directly affected the translational machinery.

DISCUSSION

Uninduced FL cell lysates contain a nonhemin-regulated translational inhibitor that prevents rabbit globin mRNA from being translated while allowing the synthesis of uninduced FL cell proteins to continue (Fig. 1A and Table 1) (16–18). This suggests that the inhibitor may act at the level of mRNA recognition by causing a preferential selection of uninduced mRNA for translation. No such translational inhibitor can be detected in lysates of HMBA- or AMS-induced FL cells. It is either repressed or no longer present after induction of differentiation (Fig. 1B and C). It is interesting to note that some enhancement of globin synthesis occurs when HMBA-induced FL cell lysates are mixed with rabbit reticulocytes. With AMS-induced FL cell lysates, a small inhibitory effect is observed that is due to a large endogenous pool of mRNA molecules in these lysates competing with globin mRNA. When uninduced and induced FL cell lysates are mixed, the total protein synthesis observed is decreased (Fig. 2A and B), indicating that induced FL cells become sensitive to the hemin-independent inhibitor in lysates of uninduced FL cells.

Lodish has suggested that different species of mRNA com-

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peting to be translated may be a factor in translational control; mRNA molecules with a high affinity for the initiation factor or ribosome binding sites involved in the selection of mRNA will be translated before mRNA with lower affinities. Similarly, the more numerous species of mRNA will be translated preferentially in a population of mRNA molecules with similar affinities (27). To eliminate these factors in our studies, we assayed for inhibitory activity in mRNA-depleted lysates. The results obtained were the same whether or not the lysates were nuclease treated.

Micrococcal nuclease treatment did not diminish the ability of uninduced FL cell lysates to inhibit protein synthesis in lysates of rabbit reticulocytes (Fig. 3A) and induced FL cells (Fig. 3B and C), and nuclease-treated lysates of induced FL cells remained without inhibitory activity when added to lysates of either rabbit reticulocytes or uninduced FL cell lysates (Figs. 4 and 5).

The nature and site of action of the inhibitor is not known. Its resistance to micrococcal nuclease digestion suggests it may be a protein. It does not modify other species of mRNA because rabbit globin mRNA recovered from mixtures of lysates of rabbit reticulocytes and uninduced FL cells can still be translated effectively in a cell-free protein synthesizing system (Fig. 6). Evidence presented here suggests that initiation factors from uninduced FL cells have different properties in recognizing mRNA than those from differentiated normal erythroid cells. This possibility is supported by the findings of Kramer *et al.* (28). They have shown that crude initiation factor preparations from uninduced FL cells are able to support accurate translation of rabbit globin mRNA, using *Artemia salina* ribosomes but at a much lower efficiency than when the rabbit reticulocyte fractions are used. In line with our present findings is their observation that the initiation factor fraction from uninduced FL cells promoted peptide synthesis as efficiently as the corresponding fraction from rabbit reticulocytes.

Two translational inhibitors (A and B) have been detected in lysates of uninduced FL cells. Inhibitor A appears to block protein synthesis by preventing the binding of mRNA to the ribosome (18). Therefore, it could be similar to either the proteins associated with mRNA in embryonic chicken muscle (29) or mouse ascites cells (30) or low molecular weight translational control RNAs from chicken erythroblasts (31) that bind to and sequester mRNA molecules, rendering them inactive. Inhibitor B is a repressor containing protein kinase activity that phosphorylates the 38,000-dalton subunit of eIF-2 and prevents it from transferring the initiator Met-tRNA$_f$ to 40S ribosomal subunits (32). Inhibitor B is of particular interest because the site of action of its protein kinase is the same as that of HRI, but its activity is not regulated by hemin. It is not clear whether the specific inhibition of synthesis of rabbit globin and induced FL cell proteins by lysates of uninduced FL cells is a function of either of these inhibitors.

What is evident is that induced FL cells lose the ability to inhibit rabbit globin synthesis and, in the process, become sensitive to the action of hemin-independent inhibitors of uninduced FL cells. Induction does not, however, result in a switch to hemin-dependent translational control. The rate of amino acid incorporation in induced FL cell lysates—like that in uninduced lysates—is unaffected by the omission of exogenous hemin from the system (Fig. 7C and D), showing that its presence is not required to prevent activation of HRI-like inhibitors. Thus, the control system of protein synthesis in FL cells—whether or not they are induced—is different from that regulated by hemin in normal erythroid cells. The failure to regulate the ac-

tivity of these "normal" inhibitors may result in the inability of FL cells to differentiate. Our data suggest that modulation of hemin-independent translational inhibitors may provide an alternative pathway to differentiation.

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