

Translation of ferritin light and heavy subunit mRNAs is regulated by intracellular chelatable iron levels in rat hepatoma cells

(hemin/deferroxamine/diethylenetriaminepentaacetic acid)

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ABSTRACT Acute administration of iron to rats has been previously shown to induce liver ferritin synthesis by increasing the translation of inactive cytoplasmic ferritin mRNAs for both heavy (H) and light (L) subunits by mobilizing them onto polyribosomes. In this report rat hepatoma cells in culture are used to explore the relationship of this response to intracellular iron levels. After adding iron as ferric ammonium citrate to the medium, latent ferritin H- and L-mRNAs were extensively transferred to polyribosomes, accompanied by increased uptake of [³⁵S]methionine into ferritin protein. Because total cellular levels of L- and H-mRNA were not significantly changed by exposure to iron, the increased ferritin mRNAs on polyribosomes most probably come from an inactive cytoplasmic pool, consistent with the inability of actinomycin-D and of cordycepin to inhibit iron-induced ferritin synthesis. When deferroxamine mesylate, an intracellular iron chelator, was added after the addition of iron to the medium, ferritin mRNA on the polyribosomes was reduced, while the free messenger pool increased, and ferritin synthesis diminished. In contrast, the extracellular iron chelator diethylenetriaminepentaacetic acid failed to inhibit the induction of ferritin protein synthesis. Addition of iron in the form of hemin also caused translocation of mRNA to polyribosomes, a response that could be similarly quenched by deferroxamine. Because hemin does not release chelatable iron extracellularly, we conclude that the level of chelatable iron within the cell has a regulatory role in ferritin synthesis through redistribution of the messenger RNAs between the free mRNA pool and the polyribosomes.

Ferritin is a ubiquitous iron-storage protein consisting in mammals of two types of subunit, a heavier H (M_r 21,000) and a lighter L (M_r 19,000) type. In rat liver, synthesis of the protein shell can be induced by iron administration (1). Because this is not inhibited by actinomycin D or cordycepin (2, 3), control must be exerted at a post-transcriptional level. Following administration of iron to the rat, mRNAs for each subunit are mobilized from cytoplasmic pools of free messenger molecules onto polyribosomes where they commence translation (3-5). It has been assumed that an increase in intracellular free iron triggers the activation of blocked ferritin mRNAs. A significant intracellular pool of readily chelatable iron has been postulated by several investigators such as Jacobs (6). However, using K-562 cells in culture, Bottomley *et al.* (7) found that, after 2-hr incubation with [⁵⁹Fe]transferrin, 85% of the ⁵⁹Fe label was recovered in ferritin, 7% of the label was found in heme, and 8% of the label remained unidentified. Furthermore, Bridges and Hoffman (8) showed that deferroxamine can remove iron from ferritin in cell cultures, indicating that chelation is not confined to free iron pools. Here, we explore the role of

chelatable intracellular iron in determining the translatability of the latent ferritin mRNAs.

MATERIALS AND METHODS

Cell Culture. Rat hepatoma cells (FT0-2B; see *Acknowledgements*) were grown with 95% viability in equal parts of Dulbecco's modified Eagle's minimal essential medium (DMEM) and Ham's F-12 medium supplemented (9) with L-glutamine, penicillin, streptomycin, and 10% fetal calf serum (GIBCO Laboratories). Cells (5×10^7 per flask) were (i) iron-loaded with 114 μ M iron as ferric ammonium citrate (pH 7.0) or 100 μ M hemin or (ii) chelated with 470 μ M deferroxamine (Desferal; CIBA-Geigy) or 810 μ M diethylenetriaminepentaacetic acid (DTPA).

Ferritin Protein Synthesis by Intact Cells. Cells were incubated with either iron and/or chelators and then in a methionine-free medium (RPMI 1640, GIBCO) with 25 μ Ci (1 Ci = 37 GBq) of [³⁵S]methionine per ml at 37°C for 1 hr. The cells were harvested, washed, and solubilized in buffer A (1% Triton X-100/0.5% Nonidet P-40/0.15 M NaCl/10 mM Tris-HCl, pH 7.2/5 mM EDTA with 0.2 mM phenylmethylsulfonyl fluoride to prevent proteolysis). [³⁵S]Methionine incorporation into trichloroacetic acid-precipitated protein, used as a measure of total protein synthesis, was unaffected due to different treatments. [³⁵S]Methionine-labeled ferritin was immune-precipitated (10, 11). Lysate samples (4×10^6 counts in total protein) were treated with 250 μ l of 10% (wt/vol) protein A staphylococcus (IgG-sorb; Enzyme Center, Malden, MA) and 25 μ l of fetal calf serum for 3 hr at 4°C and centrifuged at 10,000 rpm (Sorvall SA 600) for 10 min. The supernatant was treated with antiferritin antibody or antialbumin antibody (ICN) overnight at 4°C in buffer A, followed by IgG-sorb to isolate the antibody-antigen complex, and then washed three times in buffer A containing 0.1% NaDodSO₄. Ferritin or albumin was released by boiling for 5 min in the appropriate electrophoresis buffer and applied to 15% polyacrylamide gels containing 6 M urea/0.1% NaDodSO₄/0.1 M sodium phosphate, pH 7.2, or electrophoresed on 15% polyacrylamide gels according to the method of Laemmli (12). Gels prepared by either method were fluorographed (EN³HANCE; New England Nuclear) and autoradiographed.

Extraction of Total RNA from Hepatoma Cells. Total RNA was extracted according to the method of Chirgwin *et al.* (13). After cell lysis in 4 M guanidium thiocyanate, the lysate was precipitated with 0.75 volume of ethanol and centrifuged at 10,000 rpm (Sorvall SA 600) for 10 min. The pellet was resuspended in 1-2 ml of 7.5 M guanidium hydrochloride and precipitated with 0.5 volume of ethanol. The RNA pellet was phenol-extracted and ethanol-precipitated, yielding about 1 mg of total RNA from 10^8 cells.

Polysome Fractionation of Hepatoma Cytoplasmic RNA.

Using a slight modification of the method of Aziz and Munro (4), the cells (5×10^7 to 1×10^8) were harvested by lysis for 10 min at 4°C in 1 ml of buffer B [0.15 M KCl/10 mM Tris·HCl, pH 7.2/0.5% Nonidet P-40/150 µg of cycloheximide per ml/20 mM dithiothreitol/10 mM MgCl₂/100 units per ml of ribonuclease inhibitor (RNasin)]. Mitochondria and nuclei were pelleted for 10 min in an Eppendorf centrifuge and the postmitochondrial supernatant, constituted with KCl to 0.25 M, was layered onto 32 ml of a 10%–50% sucrose gradient buffered with 20 mM Hepes buffer, pH 7.2/0.25 M KCl/10 mM MgCl₂/20 mM dithiothreitol containing 150 µg of cycloheximide per ml, 100 units of RNasin per ml, and 0.5 µg of heparin per ml (the latter two being RNase inhibitors) in a Beckman model SW-27 ultracentrifuge tube (25 × 89 mm) and spun for 4 hr at 26,000 rpm. The gradients were separated into 14 fractions using an ISCO (Lincoln, NB) fractionator with an absorbance monitor to trace the polyribosome profile of the gradient. The RNA from each fraction was phenol-extracted and run on an agarose gel for ethidium bromide staining (10 µg/ml) and RNA blotting (4, 14).

RNA Blotting. RNA was prepared from either sucrose-gradient fractions or from total RNA extracts and was RNA blotted by standard procedures (4, 14) using Amersham nylon bond membranes instead of nitrocellulose filters. These were hybridized in 20 ml of a buffer containing 50% formamide/5× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7)/10 mM sodium phosphate, pH 7/0.1% NaDodSO₄/50 µg of salmon sperm DNA per ml/1× Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin) for 1 hr at 42°C. Probes of rat ferritin L-cDNA (11) or rat ferritin H-cDNA or mouse actin-cDNA were randomly prime-labeled to a specific activity of 10^8 cpm per µg of DNA and 0.05 µg DNA was used for hybridization in the same buffer at 42°C for 12 hr and then washed in 0.5× SSC/0.1% NaDodSO₄ buffer at 55°C three times for ferritin probes, and in 1× SSC in 0.1% NaDodSO₄ at 50°C for the actin probe. The filters were autoradiographed for 1–2 days at –70°C. At the stringencies described above, there was no cross-hybridization between rat H- and L-mRNAs with the other cDNA probe.

RESULTS

Distribution and Total Amounts of Ferritin mRNAs in Untreated and Iron-Treated Hepatoma Cells. It has been established with rat hepatoma cells (15), rat liver cells (16, 17), and human leukemic cells (18) in culture that nontransferrin-bound iron effectively enters the intracellular iron pool where it induces ferritin synthesis and affects transferrin receptor dynamics (19). Our hepatoma cells were divided into untreated control samples and those exposed to ferric ammonium citrate for 2–4 hr in order to show that nonpolysomal ferritin H- and L-mRNA is abundantly present and is mobilized by adding iron to the medium. After incubation, each cell population was separated on sucrose gradients to yield polyribosomes, monosomes, ribosomal subunits and a postpolyribosome fraction in which any free mRNA would be present. The identity of each fraction was confirmed from the UV absorption spectrum of the gradient and ethidium bromide staining for ribosomal RNAs (data not shown).

Total RNA extracted from each of the 14 gradient fractions was RNA blotted and hybridized with ³²P-labeled H- or L-cDNA probes (Fig. 1 A and B). In the untreated controls, most of the L- and H-mRNA occurred at the top of their respective gradients in the cytosol fraction, whereas the iron-treated cells showed an extensive shift of both mRNAs into the polysome fraction of the gradients similar to the response of rat liver ferritin (4).

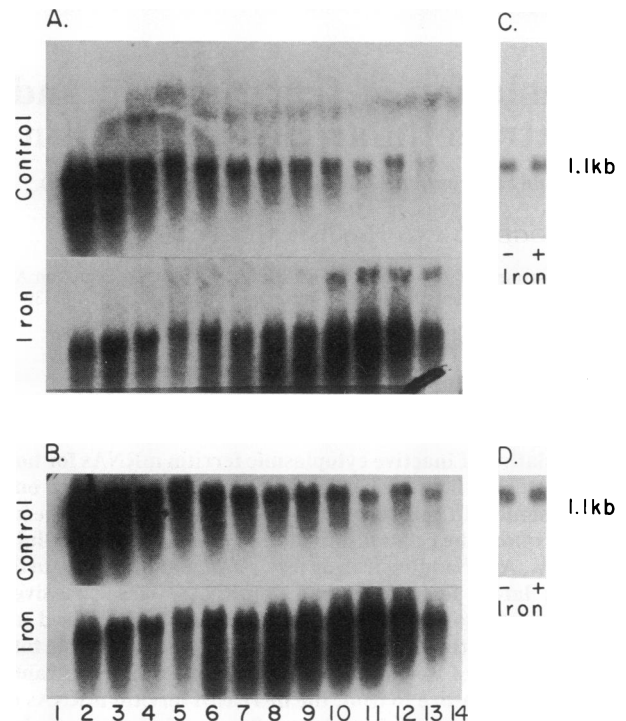


FIG. 1. Distribution of ferritin L-subunit mRNA (A) and H-subunit mRNA (B) on polyribosome gradients of rat hepatoma cells incubated without or with 2-hr treatment with 114 µM ferric ammonium citrate. Cytosolic homogenates were resolved on sucrose gradients and collected in 14 fractions. As judged by the UV absorbance profile and by ethidium bromide staining after RNA separation on a 1.2% agarose gel, fractions 1 and 2 were free of ribosomal subunits; fractions 3, 4, and 5 represented ribosomal subunits and monosomes, and polyribosomes were present in fractions 6–14. Each fraction was RNA blotted and sequentially probed with ³²P-labeled L-ferritin cDNA and H-ferritin cDNA. Total amounts of ferritin L (C) and H (D) subunit mRNAs isolated from rat hepatoma cells grown without (–) or with (+) iron salts for 2.5 hr. Total RNA was isolated after cell incubation, and 20-µg samples were RNA blotted prior to probing with ferritin L-subunit (C) and H-subunit (D) cDNAs.

To determine whether the cell content of each subunit mRNA is affected by iron administration, total RNA was extracted from control and from cells treated with iron for 2.5 hr, RNA blotted, and probed with H- and L-cDNAs labeled with ³²P to the same activity. Total cell ferritin mRNAs, representing steady-state amounts, were apparently unaffected (Fig. 1 C and D), a finding confirmed by the failure of actinomycin D and cordycepin to suppress the stimulation of [³⁵S]methionine-incorporation into ferritin after adding iron to the medium (Fig. 4).

Effect of Deferoxamine on Ferritin mRNA Distribution. To determine the role of intracellular chelatable iron on the activation of H- and L-mRNAs for ferritin, deferoxamine was added in molar excess to the cell cultures, both with and without iron treatment (Fig. 2A). The sucrose gradient distribution of L-mRNA in the untreated control samples occurred mostly at the top of the gradient (row I). After 4-hr iron treatment, row II shows that a large proportion of the mRNA had become polysome-associated. In row III, deferoxamine addition 2 hr after adding iron caused complete reversal of the action of iron despite its continuing presence for the remaining 2 hr of incubation. In row IV, deferoxamine was added alone for the first 2 hr of incubation, and the cells were then exposed to iron as well for the last 2 hr of incubation; this prevented the iron-induced mRNA translocation onto polysomes. Finally, as a control procedure, the

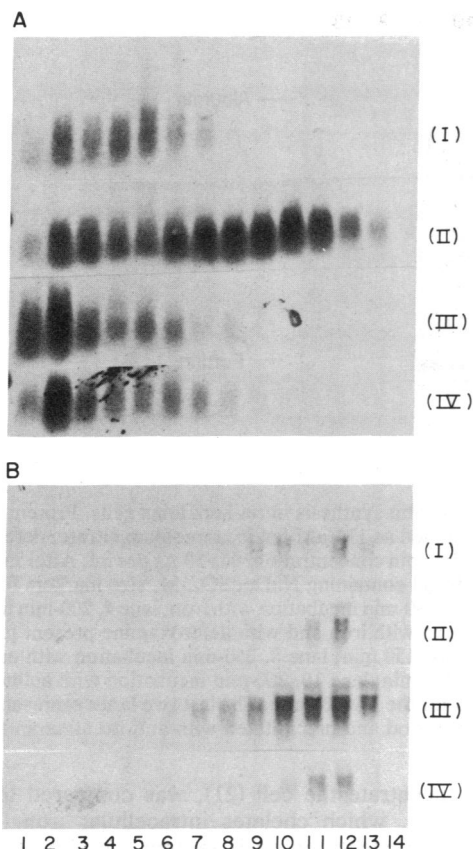


FIG. 2. Distribution of ferritin L-subunit mRNA (A) and actin mRNA (B) in polyribosome gradients from rat hepatoma cells treated with iron alone or with iron and deferoxamine. The cells were incubated with no additions to the medium (row I); with 114 μM ferric ammonium citrate for 4 hr (row II); with iron for 4 hr, and 470 μM deferoxamine present over the final 2 hr (row III); and, finally, with deferoxamine for 4 hr during the last 2 hr of which the cells were also exposed to ferric ammonium citrate (fourth row). Sucrose gradients were run and RNA phenol-extracted for RNA blotting as described in Fig. 1. (A) Each filter was probed with L-ferritin cDNA. (B) The filters were stripped and reprobed with mouse actin cDNA.

same samples were probed with actin cDNA (Fig. 2B). In contrast to ferritin mRNA, this 1.5-kilobase (kb) mRNA was fully polysome-associated in the untreated cells (row I), and the profile was unaffected by iron or deferoxamine treatment.

Effect of Hemin Addition on Ferritin mRNA Activation. It

has been shown with cells in culture that hemin iron becomes rapidly available intracellularly where it induces ferritin synthesis (10) and also down-regulates transferrin receptor populations (19), an effect that can be prevented by deferoxamine due to chelation of the intracellular iron. Accordingly, rat hepatoma cells were incubated in the presence of hemin with or without a molar excess of deferoxamine over a 4-hr period (Fig. 3). Ferritin H-mRNA became associated with polyribosomes when only hemin was added, but not in the presence of deferoxamine. Similar changes were observed with L-cDNA as the probe (data not shown). Because deferoxamine does not chelate iron from hemin *in vitro* (19), these results imply that hemin acts by liberating its iron intracellularly to augment these pools.

Response of Ferritin Protein Synthesis to Iron. To confirm that the above changes in mRNA distribution were associated with differences in ferritin synthesis, the cells were labeled with [^{35}S]methionine for the last 60 min of incubation under various conditions of iron and/or deferoxamine treatment. The immunoprecipitated labeled ferritin was isolated on NaDodSO₄ gels in which both ferritin subunits comigrated as a single discrete band (Fig. 4). Lanes 1 (90-min incubation) and 2 (200-min incubation) are untreated controls for lanes 3 (90-min iron exposure) and lane 4 (200-min iron exposure). The gel shows increased ferritin synthesis only after the longer period of exposure to iron (lane 4) and a further increase in intensity when incubation with iron was extended to 260 min (lane 5). Lane 6 represents incubation with iron for 200 min, the chelator deferoxamine being added during the last 90 min. This treatment demonstrated less incorporation than in its iron-treated control (lane 4). Lane 7 was incubated in medium similar to that of lane 5 (with iron for 260 min) but with addition of deferoxamine for the last 150 min; incorporation of the labeled amino acid was even more effectively reduced by the presence of the chelator for the longer period. Finally, an experiment (lanes 8–11) was done using inhibitors of transcription (actinomycin D) and of RNA processing (cordycepin). The inhibitors were added 60 min before giving iron, and the [^{35}S]methionine was added during the last 60 min of a 200-min incubation. Controls for iron-treatment for 200 min without these inhibitors are provided by lanes 2 and 4. Lanes 8 and 9 represent cells treated with cordycepin (20) and incubated for 200 min in the absence or presence of iron, respectively, while lanes 10 and 11 provide a similar comparison of synthesis in the absence or presence of iron when actinomycin D had been added to each sample prior to incubation for 200 min. The data confirm that neither inhibitor in any way diminishes synthesis of ferritin subunits in

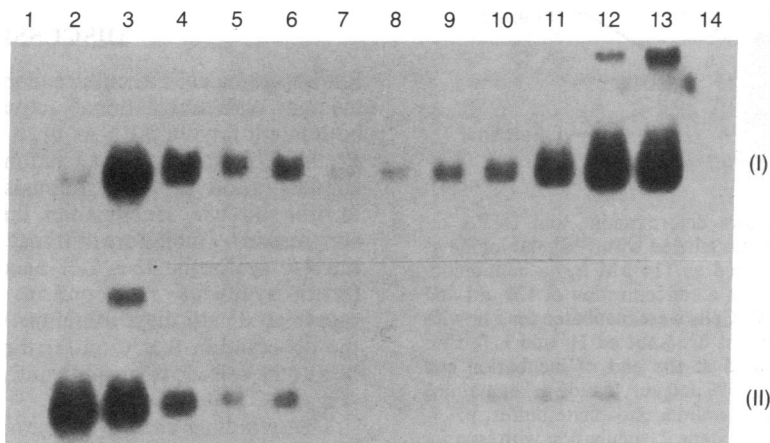


FIG. 3. Comparison of the distribution of ferritin H-mRNA in sucrose gradients isolated from rat hepatoma cells grown in the presence of 100 μM hemin (I) or 100 μM hemin and 470 μM deferoxamine (II). Gradients were run and the mRNA from each of the 14 fractions were phenol-extracted and RNA blotted with the ferritin H subunit probe.

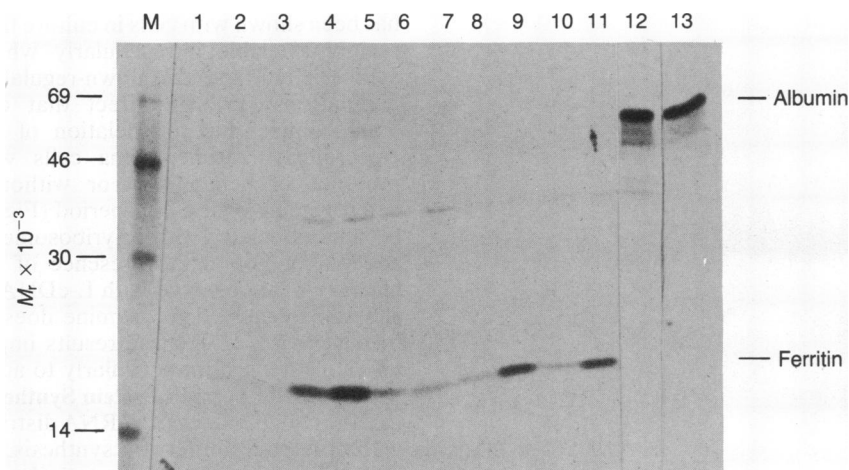


FIG. 4. Effects of deferoxamine, actinomycin D, and cordycepin on iron-induced ferritin synthesis in rat hepatoma cells. Protein synthesis was measured by adding [35 S]methionine for the last hr of each incubation. Iron was added as 114 μ M ferric ammonium citrate, deferoxamine concentration was 470 μ M, actinomycin D concentration was 0.05 μ g per ml, and cordycepin concentration was 50 μ g per ml. After incubation, the ferritin in each sample was immunoprecipitated and run on a 15% polyacrylamide gel containing NaDodSO₄. M, size markers for subunit proteins. Lane 1, 90-min incubation (control); lane 2, 200-min incubation (control); lane 3, 90-min incubation with iron; lane 4, 200-min incubation with iron added; lane 5, 260-min incubation with iron added; lane 6, 200-min incubation with iron and with deferoxamine present for the last 90 min; lane 7, 260-min incubation with iron and with deferoxamine present for the last 150 min; lane 8, 260-min incubation with cordycepin present; lane 9, 260-min incubation with cordycepin and with iron added for the last 200 min; lane 10, 260-min incubation with actinomycin D present; lane 11, 260-min incubation with actinomycin D present and with iron present for the last 200 min. The last two lanes represent albumin synthesized without iron (lane 12) and with iron (lane 13) over a 200-min incubation period and precipitated with anti-rat albumin antibody.

response to iron addition to the medium (cf. refs. 2 and 3). As a control procedure for the specificity of iron action, samples were incubated for 200 min with or without iron, and then albumin was precipitated with anti-rat albumin antibody. Lanes 12 (control) and 13 (iron treatment) show that albumin synthesis is unaffected by this prolonged iron treatment.

In a second experiment (Fig. 5), the action of the iron chelator diethylenetriaminepentaacetate (DTPA), which

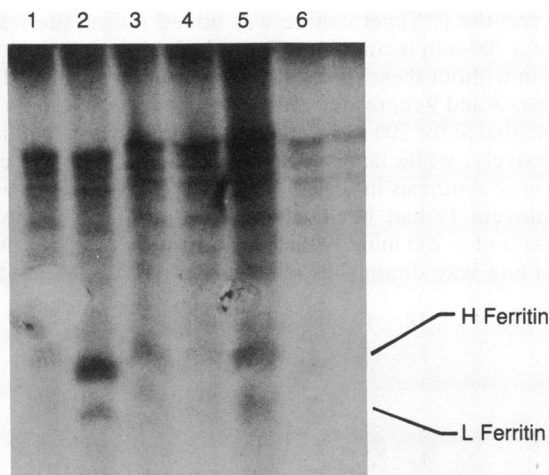


FIG. 5. Effect of the chelators deferoxamine and DTPA on iron-induced biosynthesis of ferritin labeled with [35 S]methionine in rat hepatoma cells. Iron was added as 114 μ M ferric ammonium citrate, deferoxamine was added in a concentration of 470 μ M and DTPA concentration was 820 μ M. Cells were incubated for 5 hr with [35 S]methionine present for the last hr. Labeled H- and L-ferritin subunits were immunoprecipitated at the end of incubation and separated by electrophoresis in a 15% polyacrylamide gel containing 0.1% NaDodSO₄/6 M urea/0.1 M sodium phosphate buffer, pH 7. Lane 1, control incubation for 5 hr; lane 2, incubation with iron for 5 hr; lane 3, incubation with iron for 5 hr and with deferoxamine for the last 2.25 hr; lane 4, incubation with only deferoxamine for 2.25 hr; lane 5, incubation with iron for 5 hr and with DTPA for the last 2.25 hr; lane 6, incubation with DTPA alone for 2.25 hr.

does not penetrate the cell (21), was compared to that of deferoxamine, which chelates intracellular iron. A phosphate-urea gel electrophoresis system (11) separated the H and L subunits of ferritin and demonstrated that both subunits responded similarly to the various treatments. The cells were incubated for 5 hr, during the last 60 min of which their proteins were labeled by addition of [35 S]methionine. Lane 1 is a control incubation without inhibitors, whereas lane 2 shows augmented ferritin synthesis due to the presence of iron. Lane 3 demonstrates that addition of deferoxamine during the last 2.25 hr of iron treatment reduced synthesis, whereas deferoxamine addition for a similar period without iron (lane 4) left ferritin synthesis as in the control cells (lane 1). Lane 5 shows the effect of adding the external chelator DTPA to iron-exposed cells during the last 2.25 hr of incubation, while lane 6 represents treatment with this chelator in the absence of iron. DTPA failed to reduce the increased ferritin synthesis due to iron addition (lane 2), implying that chelation of intracellular iron is essential for suppression of iron induction of ferritin synthesis.

DISCUSSION

Rat hepatoma cells in culture respond to iron addition to the medium with translational activation through transferring both latent ferritin mRNAs onto polyribosomes (Fig. 1 A and B), thus confirming the translational activation following iron administration to intact animals (3-5). The synthesis of ferritin subunits accompanies this shift (Fig. 4) and is not suppressed by inhibitors of transcription, indicating that new mRNA synthesis does not account for the iron-induced ferritin synthesis. This confirms similar evidence on intact rats treated with these inhibitors (2, 3). Also in agreement is the observation that total ferritin H- and L-mRNAs in the hepatoma cells do not significantly increase on iron treatment (Figs. 1 C and D).

These findings and the earlier observations (3-5) imply that latent cytoplasmic messenger RNA is present in abundance and is available for specific translational activation by iron followed by increased synthesis of new ferritin subunits of both types. The mechanism of mRNA recruitment demands

the storage of message in a repressed state that can be activated either by an iron-dependent signal causing derepression of an mRNA-protein complex or alternatively by iron-induced mRNA activation through interaction of the latent mRNA with an iron-sensitive protein followed by transport onto the polyribosomes. There is precedence for mRNA storage under specific circumstances, such as oocytes before fertilization (22), where the model involves stabilization of the mRNA by proteins that are derepressed by removal when the active message is required on fertilization.

The response to addition of the chelator deferoxamine throws some light on the role of iron. Iron-induced synthesis of ferritin protein (Fig. 4) and the iron-induced mRNA shift to polysomes (Fig. 2) were inhibited within 1.5–2 hr after adding the chelator to the medium, indicating sensitivity of the mechanism to iron removal. This time course seems incompatible with the presence of an iron-protein complex stable for long periods as the translation activator; such a complex would be expected to persist for a longer time. The mechanism of mRNA activation must, therefore, be attributed directly to changes in the levels of chelatable intracellular iron, possibly including ferritin iron. Deferoxamine is the preferred iron chelator, because it enters the cell (7), whereas DTPA is restricted to extracellular chelation (21). Deferoxamine depletes the intracellular iron pool as evidenced by (i) a shift in transferrin receptor synthesis and display (21, 23), (ii) depletion of intracellular ferritin iron (7, 8), and (iii) the decrease in the iron-regulated rate of ferritin synthesis (24).

To ensure that the iron causing the changes in regulation of ferritin mRNA translation is exclusively intracellular, we demonstrated (Fig. 3) that the addition of hemin to the medium activates the latent ferritin mRNA through intracellular iron release. In agreement, Mattia *et al.* (10) showed that hemin is a very effective inducer of K-562 cell ferritin synthesis in culture (see also Figs. 4 and 5). Rouault *et al.* (19) found that hemin added to cultured cells also decreases transferrin receptor biosynthesis. Because this action is suppressed by deferoxamine, they conclude that heme iron is released into the intracellular iron pool. Deferoxamine does not directly chelate heme iron when mixed together *in vitro*, implying that the effect of deferoxamine occurs only after heme has released its iron intracellularly (19). The lack of an action of the extracellular iron chelator DTPA on iron-stimulated ferritin synthesis (Fig. 5) confirms that intracellular iron levels are controlling factors in maintaining translation of H- and L-mRNAs for ferritin. The form of this intracellular iron is unknown, but it should be noted that 87% of ⁵⁹Fe added to K-562 cells is deposited within 2 hr into the cell ferritin, but this labeled iron remains chelatable (7).

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