MECHANISM OF IRON INDUCTION OF FERRITIN SYNTHESIS

Josef Zähringer, A.M. Konijn, B.S. Baliga, and H.N. Munro

Laboratory of Physiological Chemistry
Department of Nutrition and Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

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SUMMARY

Polyribosomes and mRNAs from normal and iron-treated rats were isolated, and their relative capacities for apoferritin synthesis in a cell-free system were measured. *In vitro* synthesis of apoferritin was 2-fold greater when polyribosomes or mRNA from iron-treated rats was used, whereas total protein synthesis was unchanged. This finding shows that one mechanism by which iron administration stimulates apoferritin synthesis is by increasing the amount of apoferritin mRNA in polyribosomes.

INTRODUCTION

There are several advantages to using ferritin, an iron-storage protein, in studies of the regulatory mechanism of protein synthesis in mammalian systems (1). The level of ferritin in the liver can be substantially increased by raising the intracellular concentration of iron in the tissue (2). Although the exact site of iron action is presently unknown, available evidence indicates that this stimulation by iron is actinomycin-D independent, suggesting a post-transcriptional mechanism (2-6). Recently, Drysdale and Shafritz (7) observed that addition of iron to a cell-free system stimulates the conversion of apoferritin subunits to holoferritin, which would indicate a post-translational mechanism. In a previous study, in which polyribosome species engaged in liver apoferritin synthesis were identified by specific binding of ^{125}I-antiferritin, we showed that the binding of the antibody to polyribosomes was greatly enhanced by pretreatment of the animals with iron (8).

This paper explores the mechanism by which iron induces ferritin synthesis. First, polysomes from normal and iron-treated rats were prepared and incubated in a cell-free system. The amount of apoferritin synthesized by these polysomes was compared using immunoprecipitation with ferritin antibody. Second, the mRNAs from these polysomes were isolated and incubated in heterologous cell-free systems prepared from Krebs II ascites tumor cells and from wheat germ. The capacity of the two mRNA preparations to synthesize apoferritin was compared. Our results demonstrate that iron increases the proportion of ferritin-mRNA in the total polysomal mRNA population of the liver.

MATERIALS AND METHODS

All chemicals used were of analytical grade: L-leucine (4,5-^{3}H[N]), specific activity 30-50 Ci/mmole, was obtained from New England Nuclear, Boston, Mass. Oligo-(dt)-cellulose was purchased from Collaborative Research, Inc., Waltham, Mass. Wheat germ was kindly provided by Dr. Bryan Roberts, and purified rat liver ferritin and ferritin antibody were generously supplied by Dr. M. Linder (Massachusetts Institute of Technology).

Rat liver polysomes were prepared (9) from male albino rats (100-200 g), which had received either ferric ammonium citrate (400 mg/100 g body wt.) or saline (control) intraperitoneally 5 hr before sacrifice (2). The incubation conditions for polysomes in cell-free systems containing [^{3}H]leucine and pH 5 enzyme have been previously described (9).

In order to obtain mRNA, total RNA was extracted from liver polyribosomes with phenol-chloroform-isoamyl alcohol (50:50:1) in presence of 1% SDS (10). Poly(A)-containing mRNA was then obtained by affinity chromatography of the total RNA on oligo-(dt)-cellulose columns (10), except that 20 mM Hepes buffer (pH 7.4) was used instead of Tris buffer. Translation of liver mRNA was performed with Krebs II ascites extract as described by Aviv and Leder (10) or with a preincubated S_{30} extract of wheat germ (11). Total incorporation of ^{3}H-leucine was measured according to the method of Mans and Novelli (12).

Ferritin synthesized in these cell-free systems was identified by SDS-polyacrylamide electrophoresis following either chemical purification or immunoprecipitation. For chemical purification, the incubation products were supplemented with an excess of carrier ferritin and purified by the procedure of Linder and Munro (13). For immunoprecipitation, the ferritin incubation products (both released protein and nascent peptide chains) were precipitated by excess ferritin antiserum after addition of 10 µg of carrier ferritin. The immunoprecipitate was washed twice with a solution containing 1% Triton X-100, 1% DOC, 10 mM nonradioactive leucine, 10 mM Na_2HPO_4, and 150 mM NaCl, and then centri-

fuged through a discontinuous gradient (0.5 M: 1.2 M sucrose containing the above detergent solution) (14, 15). The tubes were frozen, the bottom 3 mm were cut off, and the immunoprecipitate was dissolved in 25 µl of a solution containing 0.062 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol (v/v), and 5% 2-mercaptoethanol for electrophoresis. The immunoprecipitate was then analyzed on a SDS-polyacrylamide gradient (10-15%) gel, which was prepared and run essentially as described by Maizel (16). The gel was stained to show protein bands, then sliced and analyzed for radioactivity as described in the legend to Fig. 1.

The concentration of RNA solutions was determined, assuming $E^{1\%}_{260}$ nm to be 250. Protein was determined according to the method of Lowry *et al.* (17).

RESULTS AND DISCUSSION

First, the incubation products of a system in which liver mRNA was added to the wheat germ extract were tested for specific ferritin synthesis by immunoprecipitation followed by SDS-polyacrylamide gel elecrophoresis (Fig. 1). Protein staining of the gel showed only ferritin subunits, and the light and heavy chains of the antibody. The major radioactivity peak co-migrated with the major apoferritin subunit, which had a mol. wt. of 19,000; minor radioactivity peaks present between 10,000 and 16,000 mol. wt represent known subunit components of ferritin (18). The absence of other radioactive peaks demonstrates that our method of immunoprecipitation and SDS gel separation provides a pure product and confirms the authenticity of the ferritin synthesized in our cell-free systems.

When polysomes from untreated rats were incubated in the presence of [^{3}H]leucine, uptake of radioactivity into apoferritin averaged 0.23% of the total protein (Table 1). Incorporation of radioactivity was substantially increased to 0.53% when polysomes from iron-treated rats were used. Since iron treatment did not affect uptake of radioactivity into total protein, this result implies that iron specifically stimulates only apoferritin synthesis. This observation clearly demonstrates that iron treat-

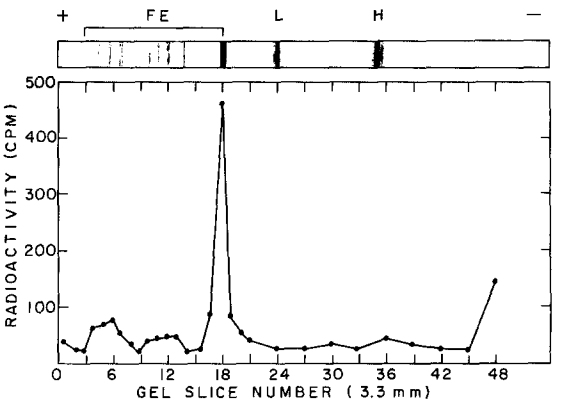

Figure 1. SDS-polyacrylamide gradient gel analysis of released apoferritin chains recovered from immunoprecipitated cell-free products of wheat germ S_{30} fraction incubated with liver mRNA.

The wheat germ S_{30} fraction was incubated with the liver mRNA in the presence of [^{3}H]leucine. Ten µg of carrier ferritin were added to 100 µl of 105,000 × *g* supernatant of the incubated wheat germ reaction mixture, containing 225,000 CPM of total radioactivity. Following immunoprecipitation and purification on a discontinuous sucrose gradient, the purified immunoprecipitate was dissolved in SDS buffer, and run on a SDS-polyacrylamide gradient (10-15%) gel. After destaining, the gel was sliced into 3.3-mm pieces, which were placed in vials containing 100 µl H_2O_2, and incubated at 50°C overnight. Then 700 µl of protosol were added, and incubation was continued for 1 hr. Each vial was counted with 10 ml of toluene/PPO/POPOP.

Abbreviations: FE = ferritin subunits; L = L-chain, H = H-chain of immunoglobulin.

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ment increases the number of polysomes synthesizing ferritin, presumably by increasing the polysomal ferritin mRNA content.

In order to test whether the increased proportion of apoferritin synthesizing polyribosomes reflects more mRNA for apoferritin, these experiments were extended to cell-free systems that depended on exogenous mRNA for protein synthesis. Poly(A)-containing RNA was extracted from the liver polysomes of control and iron-treated rats and the ability of this mRNA to incorporate [^{3}H]leucine into apoferritin was tested in wheat germ and Krebs II as-

Table 1. Ferritin Synthesis in a Cell-free System Containing Liver Polysomes from Normal and Iron-injected Rats.

	Incorporation/Incubation		
	Total Protein Radioactivity (cpm)	Radioactivity in Ferritin (cpm)	Ferritin Ratio: ——————— X 100 Total Protein
Normal	162,900	381	0.23 ± 0.01
Iron-injected	142,875	752	0.53 ± 0.03

The ferritin was purified by immunoprecipitation and incorporation into ferritin was determined by measuring the radioactivity under the major ferritin peak (see Figure 1). The results are the mean of 6 experiments ± S.E.

cites systems. The Mg^{++} and K^{+} optima for both systems were essentially as described previously (10, 11). Amino acid incorporation into protein in the wheat germ system depended extensively on the addition of exogenous mRNA, there being no radioactive peak of ferritin on the SDS gel when liver mRNA was omitted from the incubation. When mRNA was added to the wheat germ system, [^{3}H]leucine incorporation was stimulated 25- to 50-fold over endogenous mRNA activity, whereas the ascites system showed only 4- to 6-fold stimulation in response to liver mRNA. Nevertheless, acrylamide electrophoretic analysis revealed that the amount of apoferritin synthesized in the presence of mRNA was 0.2 to 0.5% of the total liver protein made in both the ascites and wheat germ systems.

Table 2 shows the effect of previous iron administration on the proportion of apoferritin synthesized by liver mRNA in such cell-free systems. The apoferritin synthesized by liver mRNA in

Table 2. Synthesis of Ferritin in Cell-free Extracts of Wheat Germ and Krebs Ascites Tumor Cells by Liver mRNA Isolated from Normal and Iron-injected Rats.

	Incorporation/Incubation		Ferritin Ratio: $\frac{\text{Ferritin}}{\text{Total Protein}} \times 100$
	Total Protein (cpm)	Ferritin (cpm)	
Wheat Germ			
Normal	226,000	461	0.25 ± 0.01
Iron-injected	239,000	856	0.40 ± 0.03
Krebs Ascites			
Normal	47,000	110	0.23 ± 0.02
Iron-injected	60,000	240	0.40 ± 0.04

Incubation conditions are described in the text. Radioactivity in ferritin in the wheat germ system was measured as described in Figure 1. The radioactivity of ferritin in the Krebs ascites system was measured after chemical isolation (13). In both systems, isolation was followed by SDS gel electrophoresis. The results are the mean of 5 experiments ± S.E.

in the cell-free system was calculated from the amount of [^{3}H]leucine incorporated into the major apoferritin subunit obtained on SDS electrophoresis, and was expressed as a percentage of total incorporation of [^{3}H]leucine. In the wheat germ system, the mRNA caused twice the proportion of apoferritin synthesis as did liver mRNA from control rats. Additional evidence for the synthesis of authentic apoferritin in response to mRNA was obtained from the Krebs ascites system in which the ferritin was recovered from the reaction products by chemical purification in the presence of excess unlabeled ferritin. The apoferritin recovered from direct immunoprecipitation followed by SDS-acrylamide gel electrophoresis

was indistinguishable from the material purified chemically on Sephadex G-200. Identical results were obtained regardless of the systems or methods used in translation of mRNA (Table 2).

Several lines of evidence strongly indicate the presence of a regulatory mechanism that utilizes iron to stimulate translation of ferritin mRNA. Among compounds considered to act within such a regulatory mechanism, mRNA was excluded from consideration on the basis of the resistance of induction to actinomycin-D treatment. However, our results show that iron in some manner increases the amount and/or availability of apoferritin mRNA in polysomes even if it is not being newly synthesized. The 2-fold magnitude of the increase in polysomal abundance of ferritin mRNA is less than the much larger increase in apoferritin labeling observed *in vivo* after the same dose of iron (2). This finding suggests that control is also exerted at the translational level, as suggested by Drysdale and Shafritz (7).

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