Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 688–692, February 1991
Cell Biology

Regulation of ferritin and heme oxygenase synthesis in rat fibroblasts by different forms of iron

(translational control/iron-regulated proteins/metabolic regulation/iron regulatory element-binding protein/chelatable iron)

RICHARD S. EISENSTEIN*†, DORIS GARCIA-MAYOL†, WARREN PETTINGELL†, AND HAMISH N. MUNRO*†

*U.S. Department of Agriculture, Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111; and †Division of Toxicology, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by Hamish N. Munro, October 31, 1990

ABSTRACT **Synthesis of the iron-storage protein ferritin is thought to be regulated at the translational level by the cytosolic content of chelatable iron. This response to iron is regulated by the iron-modulated binding to ferritin mRNAs of a repressor protein, the iron regulatory element-binding protein. From measurements made in a cell-free system, regulation of the iron regulatory element-binding protein has been recently suggested to involve direct interaction with hemin. The following observations on the synthesis of ferritin and of heme oxygenase (HO), the heme-degrading enzyme, in rat fibroblasts or hepatoma cells lead us to conclude that chelatable iron is a direct physiological regulator of ferritin synthesis in intact cells: (*i*) the inhibitor of heme degradation, tin mesoporphyrin IX, reduces the ability of exogenous hemin to induce ferritin synthesis but enhances HO synthesis; (*ii*) the iron chelator desferal suppresses the ability of hemin to induce synthesis of ferritin but not of HO; (*iii*) the heme synthesis inhibitor succinylacetone does not block iron induction of ferritin synthesis; (*iv*) there is no apparent relationship between the ability of various metalloporphyrins to inactivate the iron regulatory element-binding protein in cell-free extracts and their capacity to induce ferritin synthesis in intact cells; (*v*) administered inorganic iron significantly induces the synthesis of ferritin but not of HO; (*vi*) addition of δ-aminolevulinic acid to stimulate heme synthesis represses the ability of inorganic iron to induce ferritin synthesis while activating HO synthesis. Taken together, our results demonstrate that (*i*) release of iron by HO plays an essential role in the induction of ferritin synthesis by heme and (*ii*) chelatable iron can regulate ferritin synthesis independently of heme formation.**

The iron-storage protein ferritin occurs as a hollow shell composed in vertebrates of 24 subunits of two types, H (M_r ≈ 21,000; H-F) and L (M_r 19,000–20,000; L-F) (1). By storing iron in a safe but available form, ferritin provides a mechanism for coping with the essential but potentially toxic nature of iron. Iron regulates ferritin gene expression at multiple levels (1–3). Translational control of ferritin synthesis involves the binding of a repressor protein, the iron regulatory element-binding protein (IRE-BP), to a highly conserved sequence, the iron regulatory element, within the 5′ untranslated region of ferritin mRNAs (4, 5). On administering iron, the IRE-BP is released, and translation of the stored mRNAs provides more ferritin to sequester incoming iron.

The biological form(s) of intracellular iron directly activating ferritin mRNA translation is unclear, being either heme (6) or *non*heme "chelatable" iron (7, 8). [Hemin refers to ferric protoporphyrin (PP). In this paper heme refers to iron PP of unspecified oxidation state. Chelatable iron refers to intracellular nonheme iron that is accessible to chelating agents such as desferal. All references to chelatable iron are meant, in the context of this paper, to be synonomous with nonheme iron.] Heme and chelatable iron appear to have specific roles in regulating the synthesis of several proteins involved in cellular iron use (9–13). Hemin blocks the capacity of the highly purified rabbit liver IRE-BP to repress translation of ferritin mRNA in a cell-free system (6). However, unlike its effect in cell culture, the iron-chelator desferal failed to block hemin action in the cell-free translation system, suggesting differences in regulation of ferritin mRNA translation *in vitro* as compared with cells in culture. However, cell-culture studies have not fully explored the extent to which administered heme or chelatable iron must first be metabolically interconverted to one or the other form to activate ferritin synthesis. The fact that desferal suppresses the iron-dependent induction of ferritin synthesis (8) favors chelatable iron as the relevant form but does not exclude the possibility that this iron has first to increase cellular heme content before ferritin synthesis is induced.

By comparing the effects of chelatable iron and heme upon the synthesis of heme oxygenase (HO), the rate of synthesis of which is regulated by heme (11, 13), with that of H-F and L-F, we have examined the roles of these forms of iron in regulating ferritin synthesis in fibroblasts and hepatoma cells. (*i*) We examined the extent to which heme and chelatable iron must be metabolically interconverted to activate synthesis of ferritin and of HO. (*ii*) We compared the ability of hemin and other metalloporphyrins (MPs), some of which are not metabolized (14, 15), to inactivate the IRE-BP *in vitro* with their capacity to induce ferritin and HO synthesis in fibroblasts. We present evidence suggesting that chelatable iron is an obligatory factor in inducing ferritin synthesis in intact cells and that this effect of hemin on ferritin synthesis is related to its capacity to donate iron to the chelatable iron pool through the action of HO.

MATERIALS AND METHODS

Special Reagents. MPs (Porphyrin Products, Logan, UT) were dissolved as described (16), except 1 M Hepes, pH 7.3, was used. Freshly prepared 10 mM stock solutions of MPs were made and used in subdued light. Ferric pyridoxal isonicotinoylhydrazone (FePIH) and ferric citrate were used as described (17). δ-Aminolevulinic acid (ALA) (Porphyrin Products) was freshly made with 20 mM Hepes, and the pH was adjusted to 7.5.

Cell Culture. Rat-2 fibroblasts (18) were grown in 60-mm dishes in Dulbecco's minimal essential medium (DMEM) (low glucose, plus sodium pyruvate; GIBCO/BRL) with 5% heat-inactivated fetal bovine serum, gentamicin (50 μg/ml),

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ALA, δ-aminolevulinic acid; FePIH, ferric pyridoxal isonicotinoylhydrazone; HO, heme oxygenase; H-F, H ferritin subunit; IP, immunoprecipitation; IRE-BP, iron regulatory element-binding protein; MeP, mesoporphyrin; L-F, L ferritin subunit; MP, metalloporphyrin; PP, protoporphyrin.

and L-glutamine. FTO-2B rat hepatoma cells were grown (8) in 60-mm dishes with gentamicin. Only cells in logarithmic phase were used.

Rate of Protein Synthesis. Cells were preincubated with an iron source or various MPs for a specified period. The medium was removed, the cells were rinsed with DMEM minus methionine, and 1.5 ml of labeling media [DMEM-Met plus L-[^{35}S]methionine at 50–200 μCi/ml (1 Ci = 37 GBq)] plus the original iron source or MP was added for 30 min. Cells were rinsed twice with phosphate-buffered saline plus 1 mM methionine and then were directly solubilized in buffer A (8) plus 10 μg/ml each of leupeptin and pepstatin A (Boehringer Mannheim). Centrifugation at 15,000 × *g* for 15 min removed insoluble material. Total protein synthesis was measured by trichloroacetic acid precipitation (19). H-F, L-F, and HO were immunoprecipitated from cell extracts (10^6–10^7 cpm) by using anti-human ferritin antiserum (Boehringer Mannheim) or anti-rat liver-HO IgG (14). Extracts were precleared with fetal bovine serum (20), and antibody–antigen complexes were collected with protein-A Sepharose (Pharmacia) (21). Proteins were analyzed on 15–20% SDS/PAGE (22) gradient gels with fluorography (23), and the amount of [^{35}S]methionine in immunoprecipitated proteins was determined (20).

RESULTS

Can Hemin Induce Synthesis of Both Ferritin and HO? Hemin induced the synthesis of several proteins in fibroblasts (Fig. 1*A*, compare lane 2 with 1), including the H-F and L-F subunits and HO (Fig. 1*B*, lanes 4 and 6). Addition of 50 μM hemin induced ferritin synthesis as early as 1 hr; this rate of synthesis increased for up to, at least, 6 hr when it was 30-fold greater (Fig. 1*C*). In contrast, after 3 hr HO synthesis rose to a maximum of 13-fold, declining sharply to 4-fold by 6 hr (Fig. 1*C*). With 100 μM hemin HO synthesis declined less sharply after 3 hr (see Fig. 6). At 5 μM, hemin induced ferritin and HO synthesis but at levels much lower than seen with 50 or 100 μM (R.S.E., D.G.-M., and H.N.M., unpublished work). The kinetics of induction of HO followed by ferritin are compatible with the premise that iron first released from heme by the action of HO then stimulates ferritin synthesis.

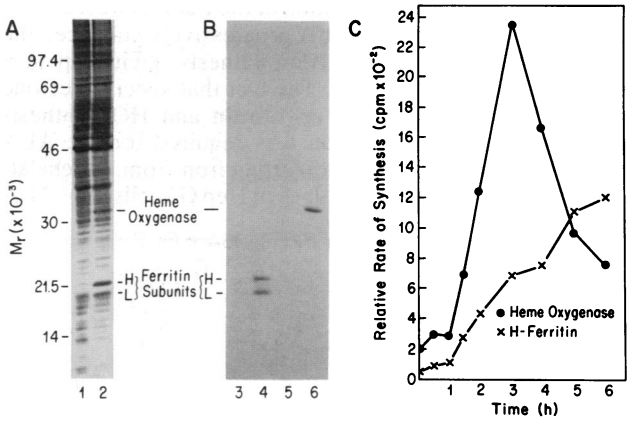

FIG. 1. Hemin induction of ferritin and HO synthesis in rat fibroblasts. (*A*) Fibroblasts were grown without (lane 1) or with (lane 2) 100 μM hemin for 7 hr and 45 min. Cells were then pulse-labeled with [^{35}S]methionine (NEN), as described. Positions of H-F and L-F, as well as of HO, are indicated. (*B*) Immunoprecipitation (IP) of H-F and L-F (lanes 3 and 4) and HO (lanes 5 and 6) from extracts of fibroblasts treated without (lanes 3 and 5) or with (lanes 4 and 6) 50 μM hemin for 1 hr and 45 min and then pulse-labeled with [^{35}S]methionine, as indicated. (*C*) Fibroblasts were exposed to 50 μM hemin for the indicated times, the amount of H-F and L-F or HO synthesis was determined by IP, and the cpm in each band was measured by scintillation counting. Relative rate of synthesis is defined here as cpm of [^{35}S]methionine incorporated into ferritin or HO per 2 × 10^6 trichloroacetic acid-precipitable cpm.

Is Liberation of Iron from Heme Necessary for Induction of Ferritin Synthesis by Exogenous Hemin? Some MPs other than heme, such as Sn PP and Sn mesoporphyrin (MeP), can also induce HO synthesis—yet are potent inhibitors of HO activity both *in vivo* and *in vitro* (14, 24, 25). Because their inhibitory effects exceed their inductive effects, administration of Sn PP to rats resulted in a 17-fold induction in liver of HO protein, but HO activity *decreased* by >90% (14). We used Sn MeP because it is more potent *in vivo* than Sn PP (25). With induction of HO synthesis as a measure of Sn MeP uptake, 250 μM of this MP alone increased HO synthesis 5-fold after 4 hr without affecting [^{35}S]methionine incorporation into total protein (R.S.E., D.G.-M., and H.N.M., unpublished work). To exploit the inhibitory action of Sn MeP on HO activity, fibroblasts were pretreated with 250 μM Sn MeP for 2 or 4 hr before adding 50 μM hemin. At 1, 2, 4, or 6 hr after hemin addition, rates of HO and ferritin synthesis were determined. Sn MeP plus hemin was more effective than hemin alone in inducing HO synthesis (Fig. 2*A*). Furthermore, even after 10 hr, 250 μM Sn MeP alone was less effective in inducing HO synthesis than was 50 μM hemin for 2 hr (R.S.E., D.G.-M., and H.N.M., unpublished work). Hemin alone slightly induced ferritin synthesis within 2 hr—the rate increasing for 4–6 hr (Fig. 2). In cells pretreated with Sn MeP, however, the induction of ferritin synthesis by hemin lagged significantly, and the synthesis rate did not reach the level seen with hemin alone (Fig. 2). This effect of Sn MeP was dose-dependent; a 4-hr pretreatment with 10, 50, or 250 μM Sn MeP inhibited the ability of 50 μM hemin given for 2 hr to induce ferritin synthesis by 32, 49, and 79%, respectively (R.S.E., D.G.-M., and H.N.M., unpublished work). Thus, the inhibitor of heme breakdown, Sn MeP, delayed induction of ferritin synthesis by hemin while simultaneously increasing HO synthesis.

Do Iron Chelators Differentially Affect Induction of Ferritin and of HO Synthesis by Hemin? Chelation of iron with desferal suppresses induction of ferritin synthesis by hemin in hepatoma cells (8). Similarly, we found desferal effective in fibroblasts whether given before or after hemin addition. Added with hemin, a 10-fold molar excess of desferal reduced the induction of ferritin synthesis by 85% (Fig. 3 *A* and *C*) without blocking the hemin-dependent induction of HO (Fig. 3 *A* and *B*) and without affecting [^{35}S]methionine incorporation into protein (R.S.E., D.G.-M., and H.N.M., unpub-

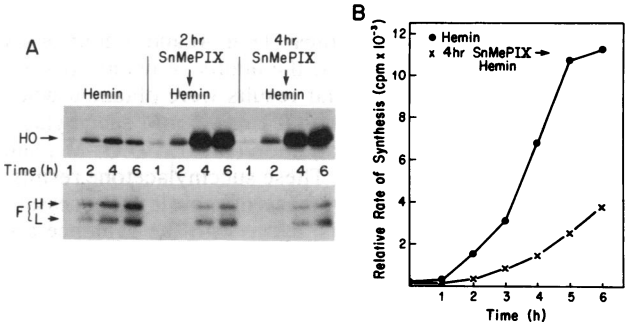

FIG. 2. Release of iron from heme is necessary for induction of ferritin synthesis by exogenous hemin. (*A*) Fibroblasts were treated without or with Sn MeP IX (SnMeP IX, 250 μM) for 2 or 4 hr. The medium was removed and replaced with one containing 50 μM hemin or 50 μM hemin plus 250 μM Sn MeP for 1, 2, 4, or 6 hr. Amount of ferritin or HO synthesized was determined by IP, as described in Fig. 1. (*B*) Fibroblasts were pretreated without (●) or with (×) 250 μM Sn MeP for 4 hr and then with 50 μM hemin (●) or 50 μM hemin plus 250 μM Sn MeP (×) for the times shown. H-F synthesis was measured after IP; L-F showed a similar response.

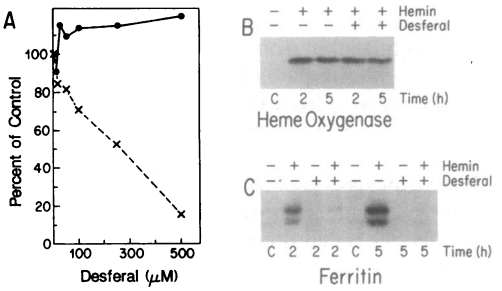

FIG. 3. Different effect of desferal on hemin induction of ferritin and HO synthesis. (*A*) Fibroblasts were treated for 4 hr with 50 μM hemin without or with various concentrations of desferal. Rate of H-F (×) or HO (●) synthesis was expressed as % of values for hemin alone. The cpm incorporated into H-F and HO without hemin was 330 and 310 cpm, respectively. With hemin and without desferal the cpm incorporated into H-F and HO was 6090 and 2620 cpm, respectively. Desferal (500 μM, 4 hr) given alone had a minor effect. (*B*) HO synthesis was determined in fibroblasts treated with no additions (lanes labeled C refer to control cells); 50 μM hemin for 2 hr, 50 μM for 5 hr, 50 μM hemin plus 250 μM desferal for 2 hr, and 50 μM hemin plus 250 μM desferal for 5 hr. Desferal did not affect the cpm incorporated into HO with hemin. (*C*) Fibroblasts were treated with no additions (lanes labeled C), 50 μM hemin for 2 hr, 250 μM desferal for 2 hr, 50 μM hemin plus 250 μM desferal for 2 hr, 50 μM hemin for 5 hr, 250 μM desferal for 5 hr, and 50 μM hemin for 2 hr and then 50 μM hemin plus 250 μM desferal for another 3 hr. In *A*, *B*, and *C*, the synthesis of ferritin and HO was determined by IP.

lished work). A 5-fold molar excess of desferal, added 2 hr after hemin, greatly reduced the induction of ferritin synthesis (Fig. 3*C*). Similarly, picolinic acid (5 mM), which affects ferritin and transferrin-receptor synthesis in other cells (26, 27), inhibited ferritin synthesis in fibroblasts by 99% without reducing synthesis of HO or total protein (R.S.E., D.G.-M., and H.N.M., unpublished work). Thus, the hemin-dependent inductions of ferritin and HO are differentially sensitive to iron chelators.

Is *de Novo* Synthesis of Heme Required for Induction of Ferritin Synthesis by Exogenous Hemin? Heme entering the cell may be rapidly degraded by HO, releasing its iron. Recycling of this iron into newly synthesized heme may be necessary for inducing ferritin synthesis. Thus, the desferal effect could be from inhibition of heme synthesis. Accordingly, we tested the effect of inhibiting heme synthesis with succinylacetone, the potent inhibitor of ALA dehydratase (28). Pretreatment with 1 mM succinylacetone for 1 hr failed to block the ability of exogenous hemin (or inorganic iron, see Fig. 5) to induce ferritin synthesis (Fig. 4) and in four of six experiments slightly enhanced the ability of hemin to stimulate ferritin synthesis. Similar results were obtained when fibroblasts were pretreated with 0.1 or 0.5 mM succinylacetone for 24 hr before hemin addition (R.S.E., D.G.-M., and H.N.M., unpublished work). These succinylacetone results, which suggest that *de novo* synthesis of heme is not necessary for inducing ferritin synthesis, agree with the evidence obtained with Sn MeP and desferal. Taken together, such results suggest that iron released from heme is the agent responsible for stimulating ferritin synthesis.

Can Chelatable Iron Induce Synthesis of Ferritin and of HO? To compare the relative ability of chelatable iron to induce ferritin and HO synthesis, we used FePIH, a compound capable of donating its iron directly to the chelatable iron pool more extensively than diferric transferrin (29). In contrast to hemin (Fig. 5 *A* and *D*), FePIH at 65 μM induced ferritin synthesis after 1, 2, or 4 hr (Fig. 5*E*) without appreciably stimulating HO synthesis (Fig. 5*B*). After 6 hr FePIH slightly stimulated HO synthesis but only to 5 or 10% of the level seen with hemin (R.S.E., D.G.-M., and H.N.M., unpublished

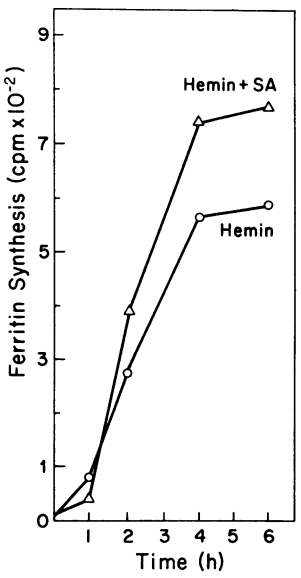

FIG. 4. *De novo* synthesis of heme is not necessary for induction of ferritin synthesis. Fibroblasts were pretreated with (△) or without (○) succinylacetone (SA, 1 mM) for 1 hr, and then the medium was replaced with one containing 50 μM hemin with (△) or without (○) 1 mM succinylacetone. Ferritin synthesis was determined by IP, as described in Fig. 1. The potent inhibitor of ferrochelatase, *N*-methyl PP, gave similar results.

work). Furthermore, pretreatment of cells with succinylacetone at 1 mM for 1 hr failed to block induction of ferritin synthesis by FePIH (Fig. 5*F*). These experiments provide strong evidence that ferritin synthesis can be induced without significant induction of HO, presumably because intracellular heme levels were not sufficiently elevated by FePIH to affect HO synthesis.

Does the Rate of Heme Synthesis Affect the Ability of Chelatable Iron to Induce Synthesis of Ferritin or HO? ALA was added to fibroblasts to stimulate heme synthesis, as it appears to do in a variety of cell types (30, 31), through bypassing ALA synthase, an enzyme limiting the rate of heme production (32). Fibroblasts responded to ALA by reducing ferritin synthesis (Table 1) presumably due to diversion of chelatable iron into heme. Addition of 13 μM FePIH stimulated H-F synthesis by 4.9-fold (Table 1) while increasing HO synthesis by only 1.7-fold. Addition of ALA with FePIH resulted in a dose-dependent decrease in ferritin synthesis with complete inhibition of the FePIH effect at 1000 μM (Table 1). In contrast, ALA progressively enhanced the ability of FePIH to induce HO synthesis, giving up to a 3.5-fold stimulus at 1000 μM. The fact that succinylacetone blocked the action of ALA on ferritin and HO synthesis indicates that heme formation was required for the ALA effect. Thus, by presumably diverting iron from the chelatable iron pool into heme, as it does in Hep G2 cells (31), ALA

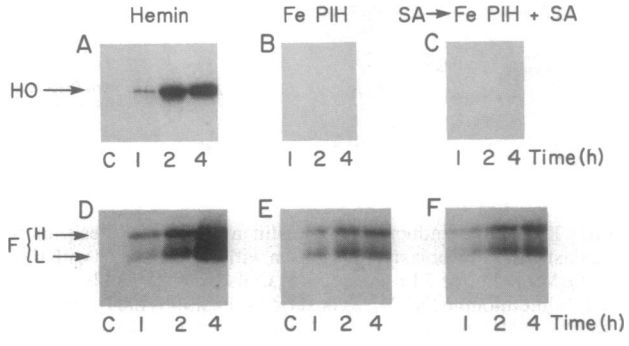

FIG. 5. Differential effect of heme and chelatable iron in inducing ferritin and HO synthesis. (*A* and *D*) Fibroblasts were treated with 50 μM hemin for 1, 2, or 4 hr. (*B* and *E*) Fibroblasts were treated with 65 μM FePIH for 1, 2, or 4 hr. (*C* and *F*) Fibroblasts were treated with succinylacetone (SA) for 1 hr and then with 65 μM FePIH with SA for 1, 2, or 4 hr. The rate of synthesis of ferritin (*D*, *E*, and *F*) and HO (*A*, *B*, and *C*) was determined by IP, as described in Fig. 1. C, untreated cells.

Table 1. Contrasting effects of ALA on the iron-dependent induction of ferritin and of HO synthesis in rat fibroblasts

FePIH (13 μM)	ALA, μM	SA (1 mM)	H-F synthesis, cpm	HO synthesis, cpm
Exp. 1				
−		−	650	ND
−	1000	−	105	ND
Exp. 2				
−		−	140	170
−	1000	−	ND	320
+		−	690	290
+	100	−	270	560
+	500	−	110	590
+	1000	−	130	1010
+		+	520	460
+	100	+	530	480
+	500	+	460	480
+	1000	+	480	450

In experiment 1, 10×10^6 cpm was used to determine ferritin synthesis by IP. In experiment 2, 4×10^6 cpm was used to determine rates of ferritin and HO synthesis by IP. ND, not determined. Higher levels of FePIH further stimulated ferritin synthesis but were without significant effect on HO synthesis. Incubations were 4 hr in length. SA, succinylacetone.

altered the relative ability of FePIH to induce ferritin synthesis as compared with HO synthesis.

In fibroblasts, the effect of ALA on ferritin synthesis depended on the amount of added iron. At higher levels of FePIH (65 μM), ALA still enhanced HO synthesis but was without effect on ferritin synthesis, suggesting that the increased rate of heme formation induced by ALA could no longer reduce the size of the chelatable iron pool enough to impair induction of ferritin synthesis (R.S.E., D.G.-M., and H.N.M., unpublished work). The effects of ALA on ferritin synthesis have also been seen with hepatoma cells (Table 2), except that ALA was effective at both FePIH doses, perhaps indicating that hepatoma cells synthesize heme at higher rates than do fibroblasts. Finally, the ALA effect on ferritin and HO synthesis was seen when hemin was the iron donor; 1000 μM ALA reduced by 85% induction of ferritin synthesis by 5 μM hemin while increasing HO synthesis a further 20% (R.S.E., D.G.-M., and H.N.M., unpublished work).

Do MPs Other Than Hemin Affect Ferritin and HO Synthesis in Cell Culture? We (R.S.E., W.P., and H.N.M., unpublished work), and others (6, 33), have observed that many MPs can inactivate the IRE-BP *in vitro*. To relate the ability of MPs to inactivate the IRE-BP *in vitro* with their ability to induce ferritin synthesis in intact cells, we tested whether Co PP could induce ferritin synthesis in fibroblasts. Co PP was used because it is as effective as hemin in inactivating the IRE-BP *in vitro* but, unlike hemin, Co PP cannot be degraded by HO

Table 2. Suppression by ALA of the iron-dependent induction of ferritin synthesis in rat hepatoma cells

FePIH, μM	ALA, μM	L-F synthesis, cpm	H-F synthesis, cpm
		420	280
	1000	280	200
13		1290	930
13	100	750	440
13	500	300	155
13	1000	160	100
65		3670	2130
65	100	2200	1240
65	500	800	490
65	1000	900	550

Incubations were 4 hr in length, and IP used 4×10^6 cpm.

(24, 34). Hemin and Co PP each effectively induced HO synthesis within 2 hr (Fig. 6), and hemin induced ferritin synthesis within 2 hr. Co PP also induced ferritin synthesis, but the response was much less than that for hemin (Fig. 6): at 6 hr hemin had induced ferritin synthesis by 17-fold, but Co PP had only a 2.5-fold effect.

In contrast to Sn PP, Co PP is a much less effective inhibitor of HO activity *in vivo*. Administration of Co PP to rats increases hepatic HO activity considerably (14) and decreases cellular cytochrome P450 levels (35). To determine whether induction of HO enzyme activity by Co PP leads to an accumulation of chelatable iron, perhaps released from the breakdown of endogenous heme, we examined the effect of desferal on the ability of Co PP to induce ferritin synthesis. Simultaneous addition of desferal blocked the ability of Co PP to induce ferritin synthesis, suggesting that this MP does not directly activate ferritin synthesis (Fig. 6). Finally, Ni PP and Zn PP (at 100 μM for 6 hr), both potent inactivators of the IRE-BP *in vitro*, failed to induce ferritin synthesis in fibroblasts ((R.S.E., D.G.-M., and H.N.M., unpublished work). In conclusion, of the MPs that inactivate the IRE-BP *in vitro*, only hemin significantly induces ferritin synthesis by cells in culture.

DISCUSSION

Our results provide supporting evidence that chelatable iron can regulate ferritin synthesis by intact cells without heme participation. By using both hemin and inorganic iron and by manipulating the flow of iron between the intracellular chelatable iron and heme pools in the experimental design, our results suggest that the extent of induction of ferritin synthesis is directly related to the size of the chelatable iron pool. This conclusion is supported by several findings. (*i*) With hemin as the iron source, HO was induced concurrently with ferritin, indicating that iron was being actively released from heme during this time. (*ii*) As suggested by experiments with Sn MeP or by ALA addition, release of iron from heme is necessary for maximal induction of ferritin synthesis. (*iii*) In the presence of intracellular iron chelators, exogenous hemin induces synthesis of HO but not of ferritin. (*iv*) Synthesis of heme appears unnecessary for hemin or FePIH to stimulate ferritin synthesis. (*v*) Direct donation of iron to the intracellular chelatable iron pool, by FePIH, significantly induced ferritin synthesis but was not a good inducer of HO. (*vi*) ALA greatly decreased the ability of FePIH to induce ferritin synthesis while synergistically activating HO synthesis, suggesting that diversion of iron into heme is not necessary for and, in fact, impedes the FePIH-dependent stimulation of ferritin synthesis. Taken together, these results indicate that

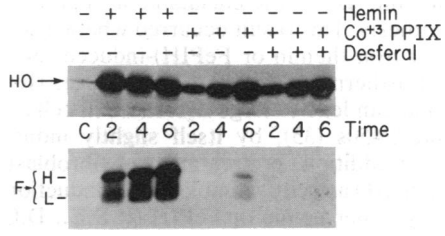

FIG. 6. Effects of cobalt PP IX and hemin on synthesis of ferritin and HO. Fibroblasts were incubated with 100 μM hemin with 100 μM Co PP, or with 100 μM Co PP plus 100 μM desferal for 2, 4, or 6 hr. Ferritin and HO synthesis was determined by IP, as described in Fig. 1. Proceeding from left to right the cpm incorporated into HO was: 330 (control cells); 7480, 8240, and 6730; 1840, 4170, and 9130; 2630, 5380, and 9570. X-ray fluorescence analysis (Oneida Research Services, Whitesboro, NY) indicated that the Co PP contained levels of iron (<50 ppm) not sufficient to have caused the observed effects on ferritin synthesis.

chelatable iron can activate ferritin synthesis without heme participation.

Our results with Sn MeP, desferal, succinylacetone, and ALA suggest that the iron derived from heme activates ferritin synthesis but that the heme molecule itself is not the primary agent responsible for inducing ferritin synthesis. In support of this we found that other MPs, although they could effectively inactivate the IRE-BP *in vitro*, were not effective inducers of ferritin synthesis in cell culture. This observation, coupled with the fact that Co and Zn PP apparently are not broken down *in vivo* (14, 15), argues against intact MPs, such as heme, being inducers of ferritin synthesis *in vivo*.

Another important question concerns whether or not heme regulates ferritin synthesis *in vivo* independently of chelatable iron. Our observation that a variety of MPs may inactivate the IRE-BP in liver extracts agrees with the results of Haile *et al.* (33) who, in addition, found that hemin inactivates a variety of nucleic acid-binding proteins other than the IRE-BP and that the mechanism of hemin action *in vitro* appeared to differ from its effects in cell culture. Coupled with our cell-culture experiments it appears that some caution should be exercised in concluding that heme is a primary regulator of ferritin synthesis. Furthermore, because (*i*) our results with ALA indicate that low levels of chelatable iron, but *not* intracellularly synthesized heme, stimulate ferritin synthesis, and (*ii*) given the fact that all cell types examined contain HO activity (36), it seems reasonable to conclude that chelatable iron can be a primary regulator of ferritin synthesis. However, whether heme has a more direct role in inducing ferritin synthesis in other cell types, such as those of the reticuloendothelial system, which generate large amounts of heme from senescent red cells, remains to be determined.

Further, why on an equimolar basis is hemin more effective than FePIH in stimulating ferritin synthesis in fibroblasts? Heme, when at high levels, may facilitate the action of iron in inducing ferritin synthesis by providing a second level at which the IRE-BP is inactivated. This effect could include a direct interaction of heme with the IRE-BP (6) or could involve an effect of heme on ferritin synthesis at other posttranscriptional levels (37). Finally, in K-562 cells, a cell type with many transferrin receptors, on a molar basis diferric transferrin is several times *more* effective than hemin in activating ferritin synthesis (38). Thus, differential transport rates of heme and nonheme iron into cells may influence the relative ability of these two iron forms to induce ferritin synthesis.

Our results also indicate that changes in heme turnover may influence the size of the chelatable iron pool and, as such, affect the rate of ferritin synthesis. Thus, we observed that inhibitors of heme degradation, which should presumably reduce the size of the chelatable iron pool, suppressed ferritin synthesis. This result occurred whether we measured the basal or iron (hemin or FePIH)-induced rate of ferritin synthesis. Furthermore, Co PP, which induces HO activity *in vivo* (14) and can lead to large decreases in cellular levels of cytochrome P450s (35), by itself slightly induced ferritin synthesis. In addition, pretreatment of fibroblasts with Co PP, to induce HO activity, stimulated the induction of ferritin synthesis by either hemin or FePIH (R.S.E., D.G.-M., and H.N.M., unpublished work), a result again consistent with the proposal that increased rates of heme degradation increase the size of the chelatable iron pool. ALA reduced the ability of FePIH and hemin to induce ferritin synthesis, presumably by diverting chelatable iron into heme. Thus, the effect of ALA is similar to that of iron chelators in that both of these agents appear capable of reducing the amount of available iron in the chelatable iron pool. Similarly, PP IX acts like picolinic acid in its effect on transferrin-receptor synthesis (27). Thus, as in Hep G2 cells (31), a dynamic relationship apparently exists between the intracellular-heme and chelatable-iron pools.

We thank Drs. A. Kappas and G. Drummond for graciously providing antibodies against rat liver heme oxygenase and for Sn MeP IX and Drs. H. Schulman and P. Ponka for kindly providing pyridoxal isonicotinoylhydrazone. We also thank Drs. A. Bomford, P. Sinclair, S. Sassa, A. Kappas, I. London, and J.-J. Chen for helpful discussions and comments on the manuscript.

1. Eisenstein, R. S., Bettany, A. J. E. & Munro, H. N. (1990) in *Metal-Ion Induced Regulation of Gene Expression*, eds. Eichorn, G. L. & Marzilli, L. G. (Elsevier, New York), pp. 91–138.
2. Zahringer, J., Baliga, B. S. & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 857–861.
3. White, K. & Munro, H. N. (1988) *J. Biol. Chem.* **263**, 8938–8942.
4. Leibold, E. A. & Munro, H. N. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2171–2175.
5. Walden, W. E., Daniels-McQueen, S., Brown, P. H., Gaffield, L., Russell, D. A., Bielser, D., Bailey, L. C. & Thach, R. E. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9503–9507.
6. Lin, J.-J., Daniels-McQueen, S., Patino, M. M., Gaffield, L., Walden, W. E. & Thach, R. E. (1990) *Science* **247**, 74–77.
7. White, G. P., Bailey-Wood, R. & Jacobs, A. (1976) *Clin. Sci. Mol. Med.* **50**, 145–152.
8. Rogers, J. & Munro, H. N. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2277–2281.
9. Casey, J. L., Hentze, M. W., Koeller, D. M., Caughman, S. W., Roualt, T. A., Klausner, R. D. & Harford, J. B. (1988) *Science* **240**, 924–928.
10. Elferink, C. J., Sassa, S. & May, B. K. (1988) *J. Biol. Chem.* **263**, 13012–13016.
11. Alam, J., Shibahara, S. & Smith, A. (1989) *J. Biol. Chem.* **264**, 6371–6375.
12. Ward, J. H., Jordan, I., Kushner, J. P. & Kaplan, J. (1984) *J. Biol. Chem.* **259**, 13235–13240.
13. Cable, E., Greene, Y., Healey, J., Evans, C.-O. & Bonkovsky, H. (1990) *Biochem. Biophys. Res. Commun.* **168**, 176–181.
14. Sardana, M. K. & Kappas, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2464–2468.
15. Kutty, R. K. & Maines, M. D. (1987) *Biochem. J.* **246**, 467–474.
16. Chen, J. J. & London, I. M. (1981) *Cell* **26**, 117–122.
17. Ponka, P., Schulman, H. M. & Wilczynska, A. (1982) *Biochim. Biophys. Acta* **718**, 151–156.
18. Topp, W. C. (1981) *Virology* **113**, 408–411.
19. Eisenstein, R. S. & Harper, A. E. (1984) *J. Biol. Chem.* **259**, 9922–9928.
20. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), p. 426.
21. Jagus, R. (1987) *Methods Enzymol.* **152**, 296–304.
22. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
23. Chamberlain, J. P. (1979) *Anal. Biochem.* **98**, 132–135.
24. Yoshida, T. & Kikuchi, G. (1978) *J. Biol. Chem.* **253**, 8479–8482.
25. Drummond, G. S., Galbraith, R. A., Sardana, M. K. & Kappas, A. (1987) *Arch. Biochem. Biophys.* **255**, 64–74.
26. Testa, U., Louache, F., Titeux, M., Thomopoulos, P. & Rochant, H. (1985) *Br. J. Haematol.* **60**, 491–502.
27. Louache, F., Testa, U., Pelicci, P., Thomopoulos, P., Titeux, M. & Rochant, H. (1984) *J. Biol. Chem.* **259**, 11576–11582.
28. Tschudy, D. P., Hess, R. A. & Frykholm, B. C. (1981) *J. Biol. Chem.* **256**, 9915–9923.
29. Ponka, P. & Schulman, H. M. (1985) *J. Biol. Chem.* **260**, 14717–14721.
30. Drew, P. D. & Ades, I. Z. (1989) *Biochem. J.* **262**, 815–821.
31. Iwasa, F., Sassa, S. & Kappas, A. (1989) *Biochem. J.* **262**, 807–813.
32. Andrew, T., Riley, P. G. & Dailey, H. A. (1990) in *Biosynthesis of Heme and Chlorophylls*, ed. Dailey, H. A. (McGraw-Hill, New York), pp. 163–200.
33. Haile, D. J., Roualt, T. J., Harford, J. B. & Klausner, R. D. (1990) *J. Biol. Chem.* **265**, 12786–12789.
34. Yoshinaga, T., Sassa, S. & Kappas, A. (1982) *J. Biol. Chem.* **257**, 7778–7785.
35. Drummond, G. S. & Kappas, A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2384–2388.
36. Maines, M. H. (1988) *FASEB J.* **2**, 2557–2568.
37. Mattia, E., Blaauwen, J. D., Ashwell, G. D. & van Renswoude, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1801–1805.
38. Mattia, E., Josie, D., Ashwell, G., Klausner, R. D. & van Renswoude, J. (1986) *J. Biol. Chem.* **261**, 4587–4593.