Overexpression of the Ferritin H Subunit in Cultured Erythroid Cells Changes the Intracellular Iron Distribution

By Virginie Picard, Françoise Renaudie, Catherine Porcher, Matthias W. Hentze, Bernard Grandchamp, and Carole Beaumont

To test the hypothesis that variations in H- and L-subunit composition in the ferritin shell affect intracellular iron metabolism, we established stable transfectants of mouse erythroleukemia cells overexpressing the H-ferritin subunit. Analyses were performed on individual clones of transfected cells induced to differentiate with hexamethylenebisacetamide (HMBA). The results showed that there was a reduction in the amount of hemoglobin produced, in inverse relationship with the level of H-subunit overexpression. Incorporation of [2-14C]glycine into heme was reduced by 20% to 30% in the clones overexpressing H-ferritin subunit compared with control clone. However, the reduction in hemoglobin production was not reversed by addition of heme precursors (δ-aminolevulinic acid or iron) or by hemin itself. A reduced accumulation of β-globin mRNA was also observed, which could account for the impaired hemoglobin synthesis. Furthermore, synthesis of the endogenous L-ferritin subunit was greatly repressed. Gel retardation assays performed on cytoplasmic extracts of transfected cells using an iron-responsive element (IRE) as a probe revealed that in overexpressing cells, the iron-regulatory protein (IRP) had a conformation with a high RNA-binding affinity, thus leading to translational repression of the endogenous L-ferritin synthesis. These data suggest that an increased formation of H-rich isoferritins leads to a rapid chelation of the regulatory iron pool. While the mechanism underlying the reduction in β-globin mRNA remains to be elucidated, this study provides direct evidence for the role of IRP-mediated regulation of ferritin expression in erythroid cell metabolism.

Ferritin is a multimer of 24 subunits of two types, named H and L, that can assemble in various proportions, thus generating a wide range of isoforms.1 The H-subunit has a ferroxidase activity that catalyzes the oxidation of Fe2+ into Fe3+, thus facilitating iron incorporation into the ferritin molecule;12 whereas the L-subunit increases the rate of iron core formation.13 Changes in the subunit composition of the ferritin molecule result mostly from transcriptional regulation of the H-subunit gene, as there is little evidence of a transcriptional regulation of the L-gene besides a moderate increase in response to iron in the liver.14 Multiple stimuli have been shown to increase the transcription of the H-gene, such as tumor necrosis factor,15 c-myc,16 or onset of erythropoietic differentiation.17 The accumulation of H-rich isoferritins during the maturation of red blood cells is likely to affect the intracellular distribution and the availability of iron for heme synthesis, although the exact role of ferritin in the control of these processes is still a matter of debate. Conflicting data have been obtained showing that iron incorporated into ferritin either can be used for heme synthesis19 or remains poorly available.1,20 However, these studies have been performed on either normal or transformed cells or on cells at various stages of differentiation that are likely to have different isoferritin profiles.

To clarify the role of ferritin in the control of intracellular iron metabolism, we decided to artificially alter the subunit...
composition of the ferritin molecule in mouse erythroleukemia (MEL) cells by establishing stable transfectants overexpressing the H-subunit. This study provides some evidence that accumulation of H-rich isoforms alters the intracellular iron distribution.

MATERIALS AND METHODS

Plasmid construction. A 6.6-kb EcoRI fragment containing the entire murine H-ferritin gene, 1 kb of promoter region, and 2.6 kb of 3′ flanking sequence was subcloned into pGEM T7 f (+) (Promega, Madison, WI; Fig 1). The IRE was subsequently mutated by replacing the Eagl-Stf II fragment of the 5′ untranslated region with a synthetic oligonucleotide, where two bases (C and A in positions 16 and 17) essential for IRP binding were deleted (Fig 2A).

The final construct was generated by inserting two additional fragments: the 1.9-kb HindIII fragment from the β-globin locus control region (LCR),2 containing the erythroid-specific enhancer HS II, and the 1.1-kb XhoI-SalI fragment isolated from the pMCI-Neo vector (Stratagene, La Jolla, CA) containing the Neo selection gene under control of the thymidine kinase (TK)-promoter.

Cell cultures and stable transfections. Clone 745 of MEL cells was grown in minimal essential medium (MEM; GIBCO-BRL, Paris, France) supplemented with 10% fetal calf serum (FCS). For induction, cells were diluted to 2 × 10^5/mL and grown for 24 hours. Hexamethylenbisacetamide (HMBA) was then added to the culture medium at 5 mmol/L final concentration, and the cells were grown for an additional 3 or 4 days. In some cases, the induction was performed in the presence of ferric ammonium citrate (100 µmol/L, 17% iron), desferroxamine (100 µmol/L), δ-aminolevulinic acid (δ-ALA; 500 µmol/L), or hemin (10 µmol/L).

For stable transfection experiments, 2 × 10^7 mid-log phase MEL cells were harvested, washed twice in nonsupplemented MEM, and resuspended in 0.8 mL of medium containing 20 µg of plasmid linearized with XhoI. The cells were electroporated at 240 V and 960 µF using a BioRad Gene-Pulser (Bio-Rad, Ivvy, France). Cells were then resuspended in culture medium and grown for 48 hours before addition of neomycin (G418) at 800 µg/mL final concentration. To isolate individual clones of transfected cells, neomycin-resistant cells were grown on methylcellulose in the presence of the Neo vector (Stratagene, La Jolla, CA) containing the Neo selection gene under control of the thymidine kinase (TK)-promoter.

RNA analysis. For RNA analysis, MEL cells were induced for 4 days with HMBA. Cells were harvested and washed twice in phosphate-buffered saline (PBS). The total RNA was extracted using RNazole B (Bioprobe Systems, Paris, France), as previously described.

Five micrograms of total RNA was used for a reverse-transcription reaction, using the Moloney murine leukemia virus (MMLV) reverse transcriptase at 42°C for 30 minutes. Of this reaction, 5 µL was then taken for polymerase chain reaction (PCR) amplification, in the presence of 12 pmol of two oligonucleotides (named HNC and FHM, which carries a fluorescent label) located on either side of the ferritin IRE (Fig 2A). The PCR proceeded for 5 minutes at 95°C, then for 30 cycles consisting of 15 seconds at 95°C, 30 seconds at 68°C, and 30 seconds at 72°C, followed by 5 minutes at 72°C. The PCR products were loaded on an automatic sequencing machine (ALF; Pharmacia, Uppsala, Sweden). The respective proportions of the two PCR fragments originating from the transfected mRNA and from the endogenous mRNA and differing by only two bases were evaluated using Fragment Manager software (Pharmacia), which calculates the surface under the fluorescence peaks corresponding to each PCR fragment.23 Nucleotide sequences of the oligonucleotides used in the PCR reaction were as follows: HNC, 5′ GGCCCGCCGG-GGGTCGGGAGGCTTGACGACC 3′; HNC, 5′ AGAAGTTCT-CGTCTAGATCG 3′.

Fig 2. H-ferritin mRNA quantifications in transfected cells. (A) Nucleotide sequence of the 5′ untranslated region of the H-ferritin mRNA. The IRE is boxed, and the two nucleotides that are deleted in the expression vector are indicated (arrows). Sequences of the two primers used in PCR amplification are underscored. (B) Typical plot of the end sequence obtained by analysis of the PCR products on ALF sequencing machine. The example shown corresponds to clone 205 and to the control clone transfected with the porphobilinogen deaminase gene. In the H-ferritin–transfected clone, two peaks are observed, corresponding, respectively, to the exogenous (98 bp) and to the endogenous (88 bp) mRNA amplification, whereas only the 88-bp fragment is obtained in the control clone.

Fig 1. Construction of H-ferritin expression vector. The entire H-ferritin gene mutated in the IRE (black box; exons) was placed under control of the erythroid-specific enhancer HS II (stippled box). The Neo selection gene with a TK-promoter (dark stippled box) confers neomycin resistance to the transfected clones. B, BamHI; H, HindIII; E, EcoRI; S, SalI; X, XhoI.

In vitro synthesis of mouse ferritin subunits. Mouse ferritin H-
and L-subunits were synthesized in vitro as previously described. Briefly, sense mRNA from pMLF27 or pMHFA1 (cDNA clones for mouse H- and L-subunits) were transcribed in large amounts using T7 polymerase, and the mRNAs were translated in vitro in a reticulocyte lysate (Promega) in the presence of 35S-methionine (400 Ci/mmol/L). The encoded subunits were analyzed directly by polyacrylamide gel electrophoresis under denaturing conditions.

**Metabolic labeling and immunoprecipitation of ferritin.** Transfected MEL cells were induced to differentiate with HMBA for 4 days. The cells (10^6) were harvested, washed twice, resuspended in 5 mL of methionine-free MEM supplemented with 10% FCS, and incubated for 2 hours at 37°C. 35S-methionine (75 μCi) was then added to the culture, and the cells were incubated for an additional 2 hours. At the end of the labeling period, the cells were washed twice in ice-cold PBS buffer and lysed in immunoprecipitation buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X-100, 1% sodium dodecyl sulfate [SDS]) by three freeze-thaw cycles. After centrifugation, the supernatant was cleared by addition of nonimmune rabbit serum and 1% protein A-Sepharose (Pharmacia), and the 35S-labeled ferritin was immunoprecipitated from the cleared cell lysate using antiferritin antibodies and loaded on a 12% SDS-polyacrylamide gel in parallel with the subunits synthesized in vitro.

**Preparation of cytoplasmic extracts and bandshift experiment.** Cells were induced for 4 days with HMBA. During the last 4 hours, they were treated with either 100 μmol/L ferric ammonium citrate or 100 μmol/L desferoxamine. Cells were washed twice with ice-cold PBS buffer. The pellet was lysed with ice-cold buffer (40 mmol/L KCl, 25 mmol/L Tris HCl pH 7.4, 1% Triton X-100, 0.3 mmol/L phenylmethylsulfonyl fluoride [PMSF], 0.7 g/L aprotinin) for 30 minutes at 4°C and centrifuged. Supernatant was collected and immediately frozen in liquid nitrogen. Protein concentration was determined by a BioRad protein assay.

Gel retardation assays were performed as previously described using a human ferritin H-subunit IRE probe. Briefly, equal aliquots (15 μg) of cellular extracts were incubated with 3,000 cpm of probe at 22°C; where indicated, 2% (vol/vol) β-mercaptoethanol was added to the extract 2 minutes before the addition of the probe. After 30 minutes, 3 mg/mL heparin was added for an additional 10 minutes. Analysis of RNA/protein complexes by nondenaturing gel electrophoresis and autoradiography was performed as previously described.

**Hemoglobin quantifications.** Cells grown in the presence of HMBA, 6-ALA, and ferric ammonium citrate, either alone or in various combinations, were collected, washed twice in ice-cold PBS buffer, and lysed in 50 mmol/L Tris, pH 7.8, by three freeze-thaw cycles. Protein content of the supernatant was measured using a BioRad protein assay. For hemoglobin determination, a volume corresponding to 20 μg of proteins was added to 250 μL of 1% benzidine (prepared in 90% acetic acid) and 250 μL of 1% H2O2. The reaction was allowed to proceed for 20 minutes and then was stopped by addition of 500 μL of 50% acetic acid. After 10 minutes, the optical density was read at 515 nm in a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan), and hemoglobin concentrations in the samples were calculated from the standard curve plotted from solutions of known hemoglobin content.

**[2,14C]Glycine incorporation into heme.** The cells were collected by centrifugation and resuspended in fresh MEM culture medium. Identical aliquots of 250 μL of cell suspension (10^6 cells) were distributed into a six-well microtiter plate, and one aliquot was retained for protein assay. To each well, 2 μCi of [2,14C]glycine (57 mCi/mmol/L) was added. At indicated time intervals, the cell aliquots were collected and washed twice with PBS, and heme was extracted using the procedure of Ponka et al. Briefly, the cell pellets were resuspended into 50 μL distilled water and stored overnight at −80°C. The proteins were precipitated by adding 1 mL cold 3% acid acetone (3 mL HCl for 97 mL acetonone). After centrifugation, the supernatant was kept apart, and the pellets were washed once with acid acetone. The supernatants were pooled, and 15 mL distilled water was added. After 90 minutes, the solutions were filtered through a glass microfiber filter (GF/C; Whatman, Maidstone, UK). Filters were dissolved in Econofluor-2 (Dupont, Paris, France) and counted.

**RESULTS**

**Establishment of stable transfectants of MEL cells.** To investigate the role of the H-ferritin subunit in the control of intracellular distribution of iron during erythropoietic differentiation, we stably transfected erythroleukemia cells with a plasmid carrying the mouse H-ferritin gene with some modifications (Fig 1). (1) The gene was placed under the control of hypersensitive site II (HS II), an erythroid-specific enhancer from the human β-globin LCR, to stimulate the production of the H-ferritin mRNA. (2) The gene was mutated in the IRE by a two-base deletion, so that the synthesis of the H-subunit would be uncoupled from feedback regulation by iron. This mutation also allowed us to observe the expression of the transfected gene by PCR amplification after reverse transcription of the mRNA (RT-PCR). Using two oligonucleotides located on either side of the deletion (Fig 2A), we amplified two PCR fragments of 88 bp and 86 bp originating, respectively, from the endogenous or from the transfected H-ferritin mRNA. The PCR was loaded on an automatic sequencing machine where the two fragments, differing by only two bases, could be separated from each other and quantitated individually (Fig 2B). We analyzed eight individual clones of transfected cells that produced various amounts of exogenous mRNA. One clone (205), which produced the highest amount of mutated H-ferritin mRNA, was retained for further analysis.

**Overexpression of the H-ferritin subunit downregulates the synthesis of the endogenous ferritin.** To verify that the mutation introduced into the IRE of the transfected gene actually leads to H-ferritin overexpression, we performed metabolic labeling experiments on induced cells. A clone transfected with the mouse porphobilinogen deaminase gene was used as a control (UR). Both UR and clone 205 were induced over 4 days with HMBA and then incubated for 2 hours in a methionine-depleted medium supplemented with 35S-methionine. At the end of the incubation period, the same amount of trichloro-triacetic acid (TCA)-precipitable radioactivity was immunoprecipitated using antiferritin antibodies and protein A-Sepharose. Immunoprecipitates were loaded on a denaturing polyacrylamide gel (Fig 3A) in parallel with either purified mouse liver ferritin or with H- and L-subunits synthesized in vitro. In these conditions, it can be clearly seen that the rate of H-subunit biosynthesis is threefold to fourfold greater in clone 205 than in the control clone. Remarkably, the amount of L-subunit produced in the transfected clone was reduced.

The concomitant increase in the H-subunit and decrease in the L-subunit biosynthetic rates that occurs in the transfected cells is likely to result in a drastic change in the H-subunit:L-subunit ratio in the ferritin shell. The reduction in L-subunit biosynthesis was not due to diminished L-ferritin mRNA levels, as there was no difference in the amount
Ferritin biosynthesis.

A

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<th>H</th>
<th>205</th>
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<td>H</td>
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Northern blot.

B

Fig 3. Expression of ferritin subunits and mRNA in transfected cells. (A) Metabolic labeling experiments on HMBA-induced cells using \(^{35}S\)-methionine. The same amounts of TCA-precipitable counts were used to immunoprecipitate \(^{35}S\)-labeled ferritin from clone 205 and from a control clone transfected with an unrelated plasmid (UR). Immunoprecipitates were loaded on an SDS-polyacrylamide gel in parallel with molecular weight markers (M), purified rat liver ferritin (L), and with H- and L-ferritin subunits synthesized in vitro from their respective cDNAs. (B) Northern blot of total RNA (10 \(\mu\)g) from two individual transfected clones (106 and 205) and from the total MEL population (C) hybridized with a ferritin L-subunit cDNA probe.

Hemoglobin biosynthesis is inhibited in transfected cells. To explore these possibilities, we examined the effects of H-ferritin overexpression on terminal differentiation of MEL cells, which is heralded by increased hemoglobin biosynthesis. We measured the hemoglobin content using a benzidine/H\(_2\)O\(_2\) assay on cell extracts from individual clones of cells transfected with either the mutated H-ferritin gene or with an unrelated gene and from the total MEL cell population after 4 days of induction in the presence of HMBA. For individual clones, the level of hemoglobin accumulation was plotted against the level of mRNA produced from the transfected gene.

The results shown in Fig 5 patently reveal an overall reduction in the amount of hemoglobin that accumulates in the various clones tested, in inverse relationship with the amount of H-mRNA from the transfected gene. The control value (C) represents the mean of the hemoglobin content from the clone transfected with an unrelated plasmid and nontransfected MEL cells.

Overexpression of H-ferritin increases the RNA binding activity of IRP. The repression in the L-subunit synthesis that occurs in the transfected cells, in the absence of any noticeable reduction in the amount of the corresponding mRNA, suggests that translation of the endogenous L-subunit mRNA is repressed in these cells. To explore this possibility, we performed gel retardation assays using a radiolabeled IRE probe and cytoplasmic extracts from various MEL transfecants. Three different controls were used: (1) a clone transfected with an unrelated gene (UR), (2) the original population of MEL cells, and (3) a clone that had spontaneously lost the ability to differentiate in response to inducers (REV). In each case, the cells were grown for 3 days with HMBA to allow maximum overexpression of the subunit and were then grown for an additional 18 hours in the presence of ferric ammonium citrate as iron source or deferoxamine, a potent iron chelator. The results of a typical experiment are shown in Fig 4, where it can be seen that, in all three controls (REV, MEL, UR), the IRP RNA-binding activity is reduced in iron-treated cells (Fig 4A, lanes Fe) to almost background levels. In contrast, in clone 205, a strong band is observed in both deferoxamine and iron-treated cells, suggesting that addition of iron does not significantly suppress the RNA-binding activity of the IRP in these cells. The additional, faster-migrating complex is likely to represent binding of the IRE probe to IRP\(_2\), a second IRE-binding protein from rodent cells. A second gel retardation assay was performed in the presence of \(\beta\)-mercaptoethanol to estimate the total amount of IRP. Intensities of the band appeared very similar in the various extracts (Fig 4B), suggesting that overexpression of the H-subunit does not affect the synthesis of IRP but, rather, increases its function as an IRE-binding protein.

It is, therefore, tempting to speculate that the drastic change in the subunit composition of the ferritin molecules in these cells leads to a reduced intracellular free iron pool and that some other aspects of cellular metabolism, like heme synthesis, might also be affected.

Heme synthesis is reduced in cells overexpressing the H-ferritin subunit. We performed kinetic measurements of [2-\(^{14}\)C]glycine incorporation into heme in transfected cells (UR and 205) induced to differentiate for 3 days in the presence of HMBA. The results presented in Fig 6 show that in induced cells, the rate of [2-\(^{14}\)C]glycine incorporation into heme is reduced by 20% to 30% in the clone that overexpresses the H-ferritin subunit as compared with the control clone. However, it is not clear whether this reduced rate of heme synthesis is sufficient to account for the defect in hemoglobin production.

To clarify this point, we supplemented the culture media with 6-ALA, iron, or hemin. Indeed, two enzymatic steps in the heme biosynthetic pathway could be affected by a reduction in the availability of iron: ALA-synthase, the first enzyme of the pathway whose expression in erythroid cells is iron-regulated via an IRE, and ferrochelatase, which catalyzes the insertion of Fe\(^{2+}\) into protoporphyrin IX. Thus, overexpression of the H-subunit could repress translation of cALA-S-mRNA, and the limited iron pool could impair the function of ferrochelatase.

To test these possibilities, we performed kinetics of hemo-
globin accumulation in transfected cells (205 for H-ferritin overexpression and UR for control) induced to differentiate in the presence of HMBA with or without the addition of δ-ALA, exogenous iron (added as ferric ammonium citrate), or a combination of both. Every 2 days, an aliquot of the cell suspension was harvested, and hemoglobin concentration was measured using the benzidine/H$_2$O$_2$ assay. Results are presented in Fig 7, where it can be seen that in clone 205, the hemoglobin content remains well below that of the control clone throughout the induction period and that supplementation of the culture medium with δ-ALA, iron, or both compounds does not restore hemoglobin synthesis in the transfected clone.

We also tested the ability of hemin to restore hemoglobin production in three different clones (106, 204, 205) with various levels of transgene expression (see Fig 5). Hemin addition to culture media during the last 24 hours of induction leads to an increase in hemoglobin accumulation in both H-overexpressing and control cells, although the hemoglobin level in the transfected clones always remained below that in control cells (Table 1).

The fact that supplementation of the culture medium with a combination of δ-ALA and iron or with the hemin molecule itself did not allow the hemoglobin content of the transfected cells to return to a normal level suggests that depletion of the regulatory iron pool and the subsequent reduction in heme synthesis also impairs the production of globin chains. To test this hypothesis, we measured β-globin mRNA accumulation during HMBA-induced differentiation of both transfected and nontransfected cells.

Accumulation of β-globin mRNAs is diminished in H-ferritin–overexpressing clones. Total RNA was extracted from clones 106, 204, and 205. One microgram of total RNA and serial dilutions were loaded on a nylon membrane and were hybridized with $^{32}$P-labeled β-globin cDNA as a probe. After
mainly arising from correlation between isoferritin profiles used the presence of an erythroid-specific enhancer upstream that the L-ferritin subunit is associated with long-term iron turnover. We present evidence that the H-subunit contributes and iron distribution in the cells. It has been proposed reduction in the rate of heme synthesis.

previous biochemical studies performed on recombinant fer-

tion in the IRE to promote synthesis of the H-subunit, inde-

pendently of the iron status of the host cells. The amount of exogenous mRNA produced in the transfected cells was

though between the amount of exogenous H mRNA and the reduc-

tion in the amount of mRNA (Fig 8). These results suggest that the deficit in hemoglobin synthesis that occurs in relation to H-subunit overexpression may also be the consequence of a deficiency in β-globin mRNA production.

DISCUSSION

This report presents the first evidence that changing isofer-

ritin synthesis in cells alters intracellular iron distribution. Previous biochemical studies performed on recombinant ferritins had shown that subunit composition of the ferritin shells affects the rate of iron uptake in vitro and that H- and L-subunits have cooperative roles in the uptake and storage of iron. However, indications that H- and L-subunits exhibit functional differences in vivo were indirect, mainly arising from correlation between isoferritin profiles and iron distribution in the cells. It has been proposed that the L-ferritin subunit is associated with long-term iron storage, while the H-ferritin subunit allows high internal iron turnover. We present evidence that the H-subunit contributes to rapid chelation of the regulatory iron pool, thereby preventing the formation of the Fe-S cluster of the IRP and inducing its constitutive activation.

We also observed a marked reduction in the amount of hemoglobin that accumulates in the transfected cells in response to HMBA induction. Given the large number of individual clones that we analyzed and the good correlation between the amount of exogenous H mRNA and the reduction in hemoglobin content, it is likely that this effect is due to overexpression of the H-ferritin subunit rather than to a spontaneous loss of inducibility by HMBA of individual clones after long-term culture of MEL cells. Furthermore, the gel retardation assays that were conducted in parallel on normal cells, on a clone selected for resistance to HMBA-induced terminal cell differentiation, and on a clone transfected with a porphobilinogen deaminase gene clearly
gesting that the HS II is not sufficient to confer position-

Table 1. Effect of Hemin Supplementation on Hemoglobin Accumulation in 4-Day Induced Cells

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<td>+ Hemin</td>
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<tr>
<td>UR</td>
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<td>106</td>
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<td>205</td>
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Hemin was added to the culture media at day 3 (10 µmol/L final concentration).
Abbreviation: Hb, hemoglobin.
Fig 8. Quantification of β-globin mRNA accumulation in transfected cells. Total RNA (1 μg) and serial dilutions from the control clone (UR) and from the transfected clones 106, 204, and 205 were loaded in slots on a N'-Hybond membrane and hybridized with a 32P-β-globin probe. After autoradiography, intensity of the signals was scanned using OneScanner (Apple) and plotted against the amount of RNA.

showed that the reduction in the size of the regulatory iron pool was related to H-ferritin overexpression.

It has been shown that desferoxamine treatment of MEL or K562 cells leads to a marked reduction of iron incorporation into ferritin or into heme. As overexpression of H-ferritin subunits mimicks the effect of desferoxamine on the RNA-binding affinity of the IRP, a reduction in heme synthesis was also expected in the transfected clones. Indeed, overexpression of H-ferritin reduced [2-14C]glycine incorporation into heme by 20% to 30%. This moderate reduction in heme synthesis would suggest that eALA-S is not repressed to the same extent as ferritin synthesis, in agreement with data showing that the eALA-S IRE induces a lower degree of translational repression than the ferritin IRE. The rapid chelation of the regulatory iron pool by the H-rich isoferritins could also contribute to the reduction in heme synthesis, as previous studies performed either on reticulocytes or on MEL cells at various stages of differentiation have suggested that ferritin iron is poorly available for heme synthesis in erythroid cells. However, it is possible that the fraction of iron that is used for heme synthesis did not transit through ferritin.

Additional experiments revealed that this slower rate of heme synthesis is not sufficient to account for the defect in hemoglobin production. When the cells were induced to differentiate in the presence of exogenous δ-ALA and iron to bypass an IRP-mediated translational repression of eALA-S and a rapid chelation of the incoming iron, the hemoglobin-deficient phenotype was not reversible. Furthermore, addition of hemin during the final 24 hours of induction only partially overcame the deficiency in hemoglobin production in the transfected cells. There is some evidence that heme stabilizes globin mRNA. We and others have shown that when MEL cells are induced to differentiate in the presence of succinylacetone, an inhibitor of heme synthesis, there is a significant decrease in the level of α- and β-globin mRNAs. However, this effect can be reverted by the addition of exogenous heme, contrary to what is observed in our transfected cells. As the reduction in β-globin mRNA accumulation appears to be directly related to the level of overexpression of the H-subunit, it is conceivable that the H-ferritin itself, either as free subunit or in a polymeric form, represses the expression of globin chains or affects the erythroid differentiation program. A ferritin-like protein has been found in nuclear extracts of K562 cells, and there is some evidence that it can act as a repressor of adult β-globin gene expression. This would be consistent with the reduced β-globin mRNA accumulation in our transfected clones. Alternatively, a depleted regulatory iron pool could lead to the deficiency in the function of some nuclear factor. GATA-1 (Zn-finger erythroid factor) was shown to be able to use Fe3+ instead of Zn2+ in vitro to form a finger-like domain capable of binding to the recognition sequence with a somewhat higher affinity than the zinc complex. Although the evidence is still lacking that GATA-family proteins can form iron complexes in vivo, it is possible that a reduced regulatory iron pool could lead to an inactive GATA protein and a reduced expression of globin genes. Further experiments are needed to elucidate these points.

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