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Iron Loading Increases Ferroportin Heterogeneous Nuclear RNA and mRNA Levels in Murine J774 Macrophages[1,2]

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Abstract

The transmembrane protein ferroportin is highly expressed in tissue macrophages, where it mediates iron export into the bloodstream. Although ferroportin expression can be controlled post-transcriptionally through a 5′ iron-responsive element in its mRNA, various studies have documented increased ferroportin mRNA levels in response to iron, suggesting transcriptional regulation. We studied the effect of iron loading on levels of macrophage ferroportin mRNA, as well as heterogeneous nuclear RNA (hnRNA), the immediate product of *ferroportin* gene transcription. J774 cells, a mouse macrophage cell line, were incubated for 0, 3, 6, 9, 12, and 24 h in medium supplemented or not with 200 μmol/L iron. Quantitative RT-PCR was used to measure steady-state levels of ferroportin mRNA and hnRNA. Ferroportin mRNA levels increased by 12 h after iron treatment, reaching 6 times the control levels after 24 h. Changes in ferroportin mRNA levels were paralleled by similar changes in the levels of ferroportin hnRNA. Time course studies of ferroportin mRNA and hnRNA abundance after incubating cells with the transcriptional inhibitor actinomycin D revealed that ferroportin mRNA has a half-life of ~4 h and that iron loading does not stabilize ferroportin mRNA or hnRNA. Collectively, these data are consistent with the hypothesis that iron increases macrophage ferroportin mRNA levels by inducing transcription of the *ferroportin* gene. J. Nutr. 139: 1–5, 2009.

Introduction

Resident macrophages of the spleen, liver, and bone marrow play a central role in whole-body iron balance by recycling systemic iron (1). These macrophages, collectively known as the reticuloendothelial (RE)[3] system, recycle iron by ingesting old or damaged erythrocytes and catabolizing the hemoglobin to liberate iron. The liberated metal is either stored in the cell or exported into the bloodstream. Each day in the average adult human, RE macrophages recycle 20–25 mg of iron, an amount that is 10–20 times more than the intestine absorbs from the diet.

Export of iron from the macrophage is mediated by ferroportin (2), a transmembrane iron transport protein located on the cell surface (3). In mice engineered to lack ferroportin, iron accumulates in hepatic and splenic macrophages, indicating that ferroportin is the primary, if not only, iron export protein in RE cells (4). The critical role of ferroportin in macrophage iron efflux is further highlighted by an ever-growing number of clinical reports of ferroportin mutations associated with pathologic macrophage iron accumulation (5). Some of these mutations lead to a loss of ferroportin-mediated iron export, as indicated by cell culture studies (6,7).

A key advance in our understanding of ferroportin in iron metabolism came from the discovery of hepcidin, a peptide hormone that regulates systemic iron homeostasis (8). Produced and secreted into the circulation by the liver, hepcidin binds to ferroportin at the cell surface and causes its internalization and degradation (2,9). This post-translational degradation of ferroportin by systemic hepcidin therefore determines the amount of iron released into the plasma. In the absence of hepcidin (10), cellular mechanisms primarily control ferroportin expression. One of these mechanisms includes post-transcriptional regulation through iron-regulatory proteins (IRP), which bind to mRNA stem loop structures called iron-responsive elements (IRE). Ferroportin mRNA contains an IRE in its 5′ untranslated region similar to the mRNAs encoding ferritin L and ferritin H, 2 subunits of the iron storage protein ferritin (11). The post-transcriptional regulation of ferritin expression by IRE-IRP interactions is well characterized (12). Under low iron conditions, IRP bind to the 5′ IRE in ferritin mRNA and block its translation. When iron levels are high, IRP are either degraded or fail to bind IRE with high affinity, allowing for translation. The 5′ IRE of ferroportin shows a similar iron-dependent regulation of translation in the mouse macrophage cell line,

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[3] Abbreviations used: Fe-NTA, ferric nitrilotriacetic acid; hnRNA, heterogeneous nuclear RNA; IRE, iron-responsive element; IRP, iron-regulatory protein; qRT-PCR, quantitative RT-PCR; RE, reticuloendothelial.
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RAW 264.7 (13). In vivo, iron levels in RE macrophages are normally high, because these cells regularly ingest senescent erythrocytes. Under these high iron conditions, IRP degrade and no longer exert post-transcriptional control through IRE interactions. A more important determinant of macrophage ferroportin expression in this state, therefore, would be the pool of ferroportin mRNA available for translation.

In contrast to ferritin, the amount of macrophage ferroportin mRNA varies substantially with cellular iron status, increasing in iron loading and decreasing in iron deficiency (14–17). The variations in ferroportin mRNA levels may reflect differences in transcription rate or mRNA stability. Studies using the transcriptional inhibitor actinomycin D provide support that macrophage ferroportin is regulated transcriptionally by iron (15,16). In the present study, we examined the relationship between levels of ferroportin mRNA and pre-mRNA [heterogeneous nuclear RNA (hnRNA)]. The levels of specific hnRNA, which represent precursors of mature mRNAs, are increasingly being used as an indicator of transcriptional activity (18).

Materials and Methods

Cell culture and treatments. J774 cells, a murine macrophage cell line, were cultured in α-minimum essential medium (Mediatech) supplemented with 10% fetal bovine serum (Cambrex), penicillin, and streptomycin and incubated at 37°C in 5% CO_2. Cells were incubated in medium supplemented or not with 200 μmol/L ferric nitrilotriacetic acid (Fe-NTA) for 0, 3, 6, 12, and 24 h. Fe-NTA (molar ratio of 1:4) was prepared as a 20-mmol/L stock from NTA (Sigma-Aldrich) and ferric chloride hexahydrate. Transcription was inhibited by incubating cells with actinomycin D (1 mg/L; Sigma-Aldrich) for up to 8 h in the presence or absence of 200 μmol/L Fe-NTA.

Measurement of ferroportin mRNA and hnRNA abundance. Relative abundances of specific RNA were determined by using quantitative RT-PCR (qRT-PCR). Briefly, total cellular RNA was isolated from J774 cells by using RNABee (Tel-Test). Isolated RNA was treated with DNase I (Turbo DNA-*free* kit, Ambion) to remove any contaminating genomic DNA. First-strand cDNA was synthesized from the isolated RNA (1 μg) using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using iQ SYBRGreen Supermix (Bio-Rad) and an Applied Biosystems 7300 real-time PCR system. Quantitation of mRNA was determined by comparison to standard curves generated by 4 10-fold dilutions of standard cDNA. To investigate changes in transcriptional intermediates, we used qRT-PCR to measure steady-state levels of ferroportin hnRNA. Also called pre-mRNA, hnRNA is rapidly processed to mature mRNA and has been measured as an index of transcriptional activity (19,20). Abundance of ferroportin hnRNA was determined by using oligonucleotide primers that span the junction between *ferroportin* exon 3 and intron 3 (**Fig. 1**). Relative quantitation of hnRNA was determined by comparison to standard curves generated by 4 4-fold dilutions of standard cDNA. Levels of mRNA and hnRNA were normalized to that of 18S rRNA. Dissociation curve analysis of all PCR products revealed single peaks, indicating specific amplification products. All PCR amplification efficiencies were 100 ± 10%. For PCR amplification, the following oligonucleotide primers were used: ferroportin mRNA (f, 5′-CTACCATTAGAAGGATTGACCAGCTA-3′; r, 5′-ACTGGAGAACCAAATGTCATAATCTG-3′); ferroportin hnRNA (f, 5′-TGACTGGGTGGATAAGAATGC-3′; r, 5′-ATATTCCCTCCATTCCAGAGG-3′); ferritin L mRNA (f, 5′-CTACTCCGGATCAGCCATGAC-3′; r, 5′-AAGTTGACCAGGCGGTTCAC-3′); and 18S rRNA (f, 5′-CGAGGAATTCCCAGTAAGTGC-3′; r, 5′-CCATCCAATCGGTAGTAGCG-3′).

Statistical analyses. Data are expressed as means ± SEM where indicated. Data were analyzed by 2-way ANOVA with a Bonferroni post test, unless indicated otherwise. To account for unequal variances, data were log-transformed prior to analysis. Nontransformed data are shown in figures. Statistical analyses were performed by using Prism 4.03 (GraphPad) software.

Results

Iron loading increases ferroportin mRNA and hnRNA levels in the macrophage. To assess the effect of iron on ferroportin pre-mRNA and mRNA abundance, we used qRT-PCR to measure steady-state levels of ferroportin hnRNA and mRNA in J774 mouse macrophages treated with 200 μmol/L Fe-NTA for 0, 3, 6, 9, 12, and 24 h. This amount of Fe-NTA maximally induces ferroportin mRNA expression in J774 cells, as determined by Northern blot analysis (16). In response to iron treatment, ferroportin mRNA abundance increased progressively over time. By 12 h, ferroportin mRNA levels increased 3-fold over controls ($P < 0.01$), reaching 5-fold higher levels ($P < 0.001$) after 24 h (**Fig. 2***A*). A similar temporal response and magnitude of increase was observed in ferroportin hnRNA levels (Fig. 2*B*).

Effect of iron loading on ferroportin mRNA and hnRNA stability. The parallel increases in ferroportin hnRNA and mRNA levels suggest that iron increases *ferroportin* gene transcription. Nonetheless, it is also possible that iron increases steady-state transcript levels by message stabilization. To examine this possibility, J774 cells were incubated with or without 200 μmol/L Fe-NTA for 12 h, followed by treatment with the transcriptional inhibitor actinomycin D for 0, 2, 4, 6, and 8 h. Treatment of iron-loaded cells with actinomycin D decreased ferroportin mRNA levels over time compared with cells not treated with actinomycin D (**Fig. 3***A*). In these same cells, actinomycin D treatment decreased ferroportin hnRNA levels more potently (Fig. 3*B*). After 2 h of treatment, ferroportin hnRNA levels decreased by >80% ($P < 0.001$). Decreases in macrophage ferroportin mRNA and hnRNA levels after acti-

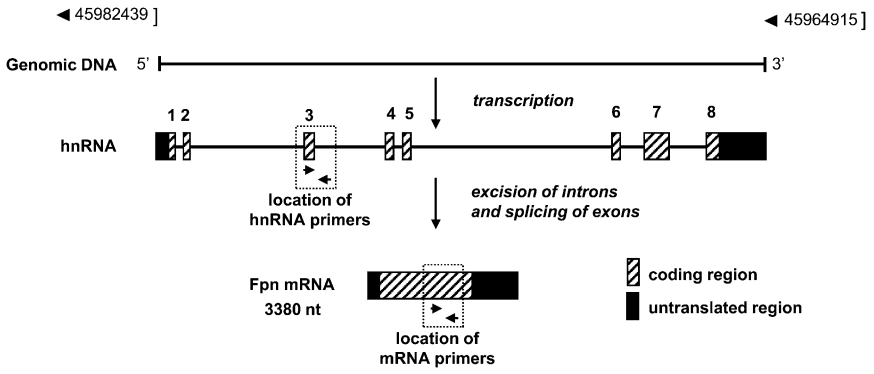

FIGURE 1 Diagram of the mouse *ferroportin* gene, hnRNA, and mRNA. The mouse *ferroportin* gene is located in a 17,524-bp region (NC_000067) on the minus strand of chromosome 1, positions 45964915–45982439. Levels of ferroportin hnRNA were determined by using qRT-PCR and primers targeting a region that spans the 3rd intron-exon junction. Ferroportin mRNA (NM_016917) abundance was determined by using qRT-PCR and primers targeting a region in exon 6.

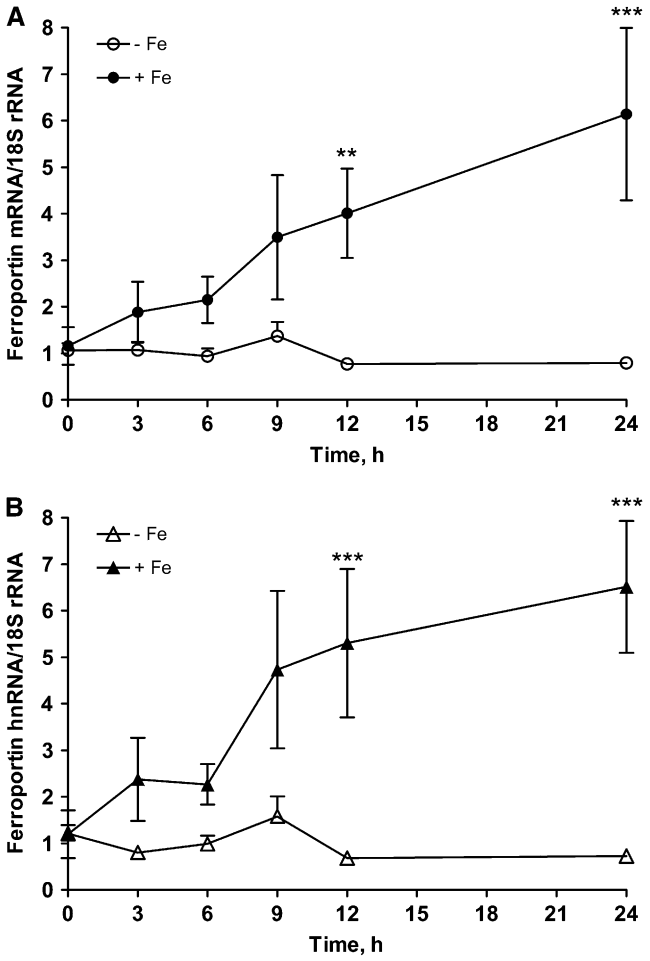

FIGURE 2 Steady-state levels of ferroportin mRNA and hnRNA increase in J774 macrophages after treatment with iron. J774 cells were incubated for the times indicated in medium supplemented (+Fe) or not (−Fe) with 200 μmol/L Fe-NTA. Relative levels of ferroportin mRNA (*A*) and hnRNA (*B*) were determined using qRT-PCR. Values are means ± SEM of 3 independent experiments. Asterisks indicate a difference between the +Fe and –Fe-treated cells at the times indicated, $***P < 0.001$, $**P < 0.01$.

nomycin D treatment were similar in the presence or absence of Fe-NTA (Fig. 3*A* vs. 3*C* and Fig. 3*B* vs. 3*D*). These results indicate that iron loading does not stabilize ferroportin mRNA or hnRNA and that the half-life of ferroportin mRNA is ~4 h.

Effect of iron loading on ferritin L mRNA levels. Treatment of J774 cells with 200 μmol/L Fe-NTA has been shown to markedly increase levels of the iron-storage protein ferritin L (16), but in that study, ferritin L mRNA levels were not measured. In the present study, we found that steady-state levels of ferritin L mRNA did not change during the first 12 h of iron treatment but increased 2-fold after 24 h (**Fig. 4**). In contrast to ferroportin, we were unable to measure ferritin L hnRNA levels, even by using 2 different primer sets that were able to amplify genomic DNA efficiently.

Discussion

We used qRT-PCR to measure levels of ferroportin hnRNA and mRNA in response to iron loading. Given that hnRNA intermediates represent transient mRNA precursors, their steady-state levels reflect their rates of synthesis (transcription) and processing (excision of introns and splicing of exons). Because processing of hnRNA is a rapid process, steady-state hnRNA levels are widely thought to reflect transcription rate (18) and are sometimes used as a surrogate for the nuclear run-on assay (21). We found that treating J774 macrophages with iron progressively increased steady-state levels of both ferroportin mRNA and ferroportin hnRNA over time. By 12 h after iron treatment, ferroportin mRNA and hnRNA levels increased 4- and 5-fold, respectively, over control levels and remained elevated after 24 h. The parallel increases in ferroportin hnRNA and mRNA levels suggest that iron loading increases *ferroportin* gene transcription. However, it is possible that increases in the levels of ferroportin hnRNA (and ultimately mRNA) in response to iron result not from transcriptional activation but from increased stability of its pre-mRNA, as has been observed for the expression of the dihydrofolate reductase gene during growth (22). To determine whether iron increases the stability of ferroportin pre-mRNA, we blocked transcription in the presence or absence of added iron and then measured changes in steady-state ferroportin hnRNA levels over time. Our results show that after blocking transcription, ferroportin hnRNA and mRNA levels decreased rapidly and dramatically, especially in the presence of iron, indicating that iron does not stabilize ferroportin pre-mRNA. Moreover, these results provide evidence that transcription is required to increase ferroportin hnRNA and mRNA levels in response to iron. It therefore appears that iron increases ferroportin expression primarily by increasing transcription.

Although ferroportin mRNA levels increased markedly 12 h after iron treatment, ferritin L mRNA levels did not change. Similar responses for ferroportin and ferritin L mRNA have been reported in studies of bone marrow-derived macrophages loaded with erythrocyte iron (14). It is important to note that, despite the lack of change in ferritin L mRNA abundance in response to iron, macrophage ferritin L protein levels increase markedly (14). This demonstrates that macrophage ferritin L expression is regulated mostly in a post-transcriptional manner by iron. We did, however, observe a 2-fold increase in ferritin L mRNA levels after loading cells with iron for 24 h. Because we were unable to measure ferritin L hnRNA, it seems that the increase in ferritin L mRNA levels in response to iron results from an increase in mRNA stability (23) rather than an increase in transcription (24,25).

The differential responses of ferritin L and ferroportin mRNA to iron loading may relate to their relative abundances. By our qRT-PCR analyses of J774 macrophages, we estimate that basal ferritin L mRNA levels are at least 3 orders of magnitude greater than ferroportin mRNA levels. This estimate is consistent with a serial analysis of gene expression in human monocyte-derived macrophages, which identified ferritin L as the single most abundantly expressed mRNA of 35,000 different transcripts (26). With such a large supply of pre-existing ferritin L mRNAs, the production of sufficient ferritin L protein in response to iron loading can occur without requiring additional transcription. Ferroportin message, in contrast, is not abundant in J774 cells. Thus, in response to an iron challenge, production of sufficient ferroportin would require increased steady-state levels of ferroportin mRNA, which, as we show here, likely results from increased transcription. It is probable that the transcriptional activation of *ferroportin* by iron is the reason why ferroportin mRNA is readily detectable only in tissues actively involved in iron transport or storage (i.e. spleen, liver, duodenum, and placenta) (11,27).

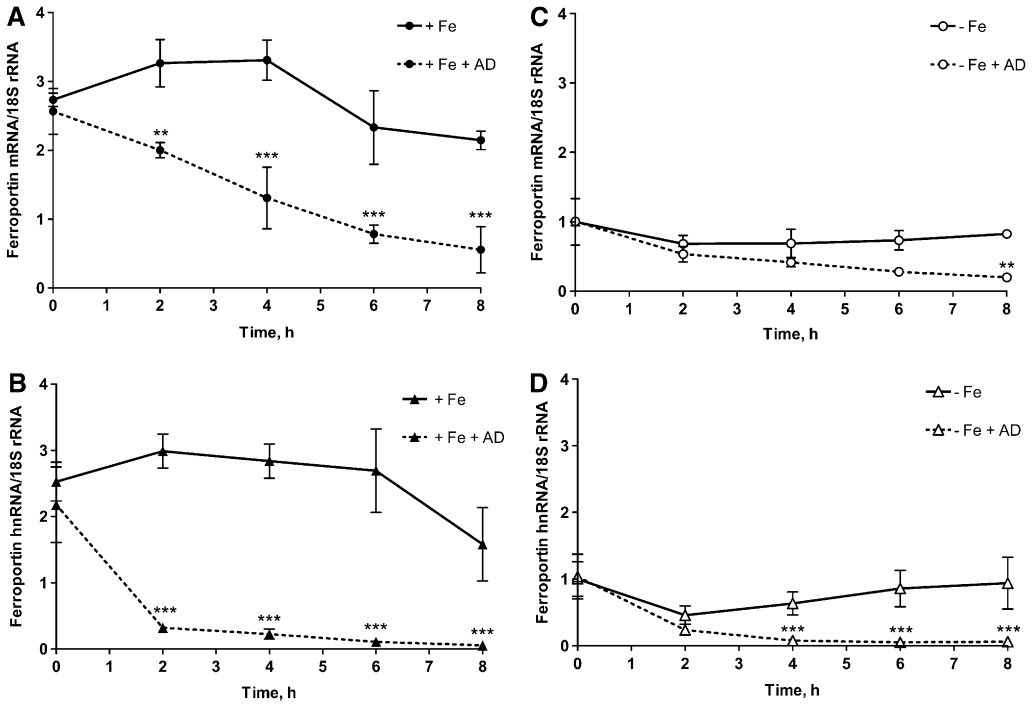

FIGURE 3 Effect of iron on ferroportin mRNA and hnRNA stability. J774 cells were incubated in medium supplemented (+Fe) or not (−Fe) with 200 μmol/L Fe-NTA. After 12 h, actinomycin D (0.001 g/L) was added (+AD) or not and cells were harvested every 2 h. Relative ferroportin mRNA (*A,C*) and hnRNA levels (*B,D*) were determined by qRT-PCR. Values are means ± SEM of 3 and 4 independent experiments, respectively. Asterisks indicate a difference between +AD-treated cells and controls at the times indicated, $***P < 0.001$, $**P < 0.01$.

The present study suggests that transcription is an important means of modulating ferroportin expression. If so, ferroportin transcription would be high in the usually iron-rich RE macrophage, whereas post-transcriptional regulation (i.e. suppression of translation) through IRE/IRP interactions would be low. Indeed, ferroportin transcription would be a primary determinant of macrophage ferroportin expression when hepcidin levels are low, such as in anemia (28), hypoxia (28), states of enhanced erythropoiesis (29), *HFE*-related hemochromatosis (30), and juvenile hemochromatosis (31).

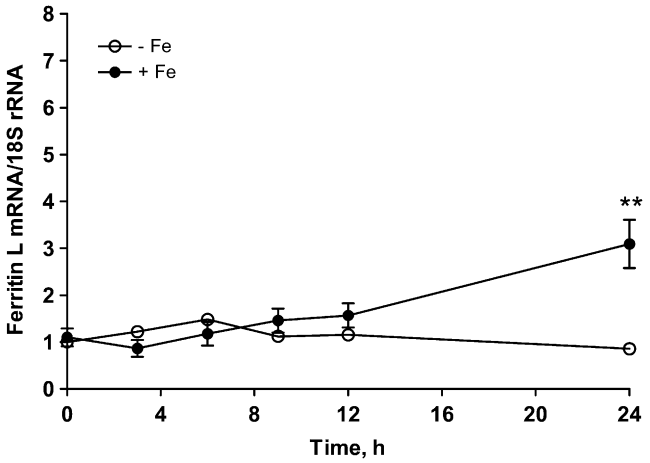

FIGURE 4 Effect of iron loading on steady-state ferritin L mRNA levels. J774 cells were incubated for the times indicated in medium supplemented (+Fe) or not (−Fe) with 200 μmol/L Fe-NTA. Relative transcript abundance of ferritin L was determined by qRT-PCR. All values are means of 3 independent experiments, except for the 24-h time point, which represents 6 independent experiments. At the 24-h time point, ferritin L mRNA levels were higher in the +Fe-treated cells than in controls, as determined by Student's *t* test, $**P < 0.01$.

Although iron loading consistently increases ferroportin mRNA levels in macrophages in vivo (17,32) and in cultured macrophages (2,14–16), it has the opposite or no effect in the duodenum (32), isolated enterocytes (33,34), or cultured intestinal cells (34–36). Moreover, iron deficiency has been shown to decrease ferroportin mRNA levels in macrophages (15,16) but increase ferroportin mRNA levels in intestinal cells (11,32–34). In one of these studies (34), the increase in ferroportin mRNA abundance was shown to involve transcription, as demonstrated by nuclear run-off analysis. Studies of isolated enterocytes from mice with nutritional and genetic iron deficiency indicate that intestinal ferroportin mRNA (and protein) levels respond to systemic signals rather than local iron concentration (33). We conclude from the present study that macrophage ferroportin expression responds to local iron concentrations (likely via transcription) in addition to systemic signals such as hepcidin. Future studies of cell type-specific regulation of ferroportin will be needed to identify the *cis* and *trans* regulatory elements involved in the iron-dependent regulation of *ferroportin* transcription.

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