

# Identification of an iron–hepcidin complex

Sébastien FARNAUD<sup>\*1</sup>, Chiara RAPISARDA<sup>†</sup>, Tam BUI<sup>‡</sup>, Alex DRAKE<sup>‡</sup>, Richard CAMMACK<sup>‡</sup> and Robert W. EVANS<sup>§</sup>

<sup>\*</sup>School of Biosciences, University of Westminster, New Cavendish Street, London W1W 6UW, U.K., <sup>†</sup>Nutritional Sciences Division, King's College London, London SE1 1UL, U.K., <sup>‡</sup>Molecular Biophysics Group, Pharmaceutical Science Research Division, Franklin–Wilkins Building, King's College London, London SE1 9NH, U.K., and <sup>§</sup>Biosciences, School of Health Sciences and Social Care, Heinz Wolff Building, Brunel University, Uxbridge, Middlesex UB8 3PH, U.K.

Following its identification as a liver-expressed antimicrobial peptide, the hepcidin peptide was later shown to be a key player in iron homeostasis. It is now proposed to be the 'iron hormone' which, by interacting with the iron transporter ferroportin, prevents further iron import into the circulatory system. This conclusion was reached using the corresponding synthetic peptide, emphasizing the functional importance of the mature 25-mer peptide, but omitting the possible functionality of its maturation. From urine-purified native hepcidin, we recently demonstrated that a proportion of the purified hepcidin had formed iron–hepcidin complexes. This interaction was investigated further by computer modelling and, based on the sequence similarity of hepcidin with metallothionein, a three-dimensional model of hepcidin, containing one atom of iron, was constructed. To characterize these complexes further, the interaction with iron was analysed using different spectroscopic methods. Monoferric hepcidin was

identified by MS, as were possibly other complexes containing two and three atoms of iron respectively, although these were present only in minor amounts. UV/visible absorbance and CD studies identified the iron-binding events which were facilitated at a physiological pH. EPR spectroscopy identified the ferric state of the bound metal, and indicated that the iron–hepcidin complex shares some similarities with the rubredoxin iron–sulfur complex, suggesting the presence of Fe<sup>3+</sup> in a tetrahedral sulfur co-ordination. The potential roles of iron binding for hepcidin are discussed, and we propose either a regulatory function in the maturation of pro-hepcidin into active hepcidin or as the necessary link in the interaction between hepcidin and ferroportin.

**Key words:** ferroportin, haemochromatosis, hepcidin, iron homeostasis, propeptide maturation.

## INTRODUCTION

In searching for antimicrobial peptides in the human liver, the 25-amino-acid protein LEAP-1 (liver-expressed antimicrobial peptide-1), also called hepcidin, was identified in human blood ultrafiltrate [1] and in urine [2]. Although several bacterial and yeast species exhibited sensitivity on treatment with the corresponding synthetic peptide, the results of previous studies proposed a predominant role for hepcidin in regulating iron homeostasis [3]. In mice, expression levels of hepcidin were found to be inversely proportional to the iron content in their food, and over-expression of hepcidin resulted in iron deficiency anaemia [4]. In humans, hepcidin expression was found to be decreased in patients with haemochromatosis and is affected by hypoxia and inflammation, suggesting a key role in iron homeostasis under various pathophysiological conditions [5–7]. Hepcidin has now been shown to bind to the iron exporter ferroportin, leading to a decreased export of cellular iron into the blood circulation [8]. In identifying such a receptor for hepcidin, its role was defined as the hormone controlling iron uptake from the intestine and macrophages.

In humans, the active peptide is derived from the cleavage of the C-terminus of an 84-amino-acid precursor [2]. A predicted signal-sequence cleavage site would produce a 60-amino-acid residue propeptide with an unknown function. Pro-hepcidin was identified in plasma, and several studies have tried to relate the level of propeptide to the level of hepcidin, showing, for example, enhanced levels of pro-hepcidin in patients with chronic renal insufficiency and a decreased level in patients with hereditary haemochromatosis and patients with renal anaemia [9]. On the other hand, Kemna et al. [10] recently demonstrated that the level

of hepcidin is increased during inflammation and decreased in patients with iron deficiency and thalassaemia major, whereas serum pro-hepcidin levels displayed no significant difference between all of the groups tested. A consensus site for the convertase family of mammalian propeptide-processing enzymes has been identified at Arg<sup>59</sup>, and furin, a member of this family of proprotein convertases which is largely expressed in the liver, has recently been shown to mediate the post-translational processing of hepcidin [11]. In cell culture, no pro-hepcidin could be detected in the medium under control conditions, whereas inhibition of furin activity resulted in secretion of the propeptide into the medium, demonstrating the role of furin in cellular maturation of the active peptide. Although the pro-region of hepcidin is therefore proposed to facilitate peptide transit through subcellular compartments and prevent the potentially toxic effect of the mature cationic peptide, a role for pro-hepcidin in the regulation of the production of active hepcidin cannot be excluded.

Apart from its proposed interaction with ferroportin, the mechanism of hepcidin as an iron sensor still needs to be investigated. As a result of the low physiological concentration of hepcidin, together with the difficulties encountered in its purification, most of the work on hepcidin has been carried out using the corresponding synthetic peptide. In order to investigate the direct interaction of the peptide with iron, as observed for most of the proteins involved in iron metabolism, we analysed a small amount of the peptide isolated from urine [12]. Iron was found to be bound to a proportion of the purified peptide, which led us to build a three-dimensional model of iron-bound hepcidin [12]. This model, which is based on sequence similarities with the metallothionein protein family, is different from the solved structure of the synthetic peptide, but corresponds to the only

Abbreviations used: MMP: matrix metalloproteinase; TCEP, tris-(2-carboxyethyl)phosphine.

<sup>1</sup> To whom correspondence should be addressed (email s.farnaud@westminster.ac.uk).

published CD spectrum of the native peptide [2]. In the present study, the direct interaction of iron with hepcidin is investigated further by biophysical characterization of the iron-containing hepcidin complex.

## MATERIALS AND METHODS

### Peptide

The oxidized synthetic 25-mer hepcidin, purified to > 95% by HPLC, was purchased from Alpha Diagnostic (San Antonio, TX, U.S.A.) with its cysteine residues air-oxidized. The oxidized peptide was resuspended (final concentration of 0.5 mg/ml) in 10 mM Hepes (pH 7.5) and 100 mM NaCl. To reduce the peptide, TCEP [tris-(2-carboxyethyl)phosphine]·HCl was added to the solution at a final concentration of 2.5 mM. Iron was added from a stock solution of Fe<sup>3+</sup> HCl (ferric iron solution; 0.54 mM).

### MS analysis

Data were obtained using a Waters Micromass MS Technologies MALDI (matrix-assisted laser-desorption/ionization) micro MX<sup>TM</sup> mass spectrometer operating with a positive polarity in a linear mode.  $\alpha$ -Cyano-4-hydroxycinnamic acid was used as the sample matrix.

### EPR analysis

Samples were frozen in quartz tubes (3 mm internal diameter) at 12 K; microwave power, 20 mW; frequency, 9.379 GHz; magnetic field modulation amplitude, 1 mT; and frequency, 100 kHz.

### Absorption and CD analysis

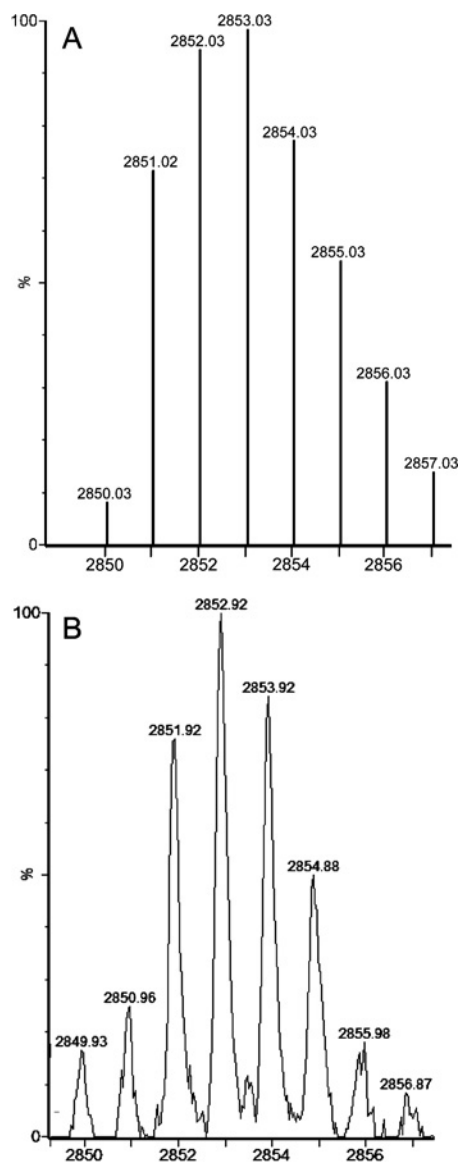
All spectra were acquired using the Applied Photophysics Chirascan spectropolarimeter, and 10 mm and 0.5 mm Suprasil rectangular cells were employed. Measurements were performed with a stepsize of 0.5 nm and time-per-point of 1.5 s in the visible and near-UV (750–230 nm) regions and 3.0 s in the far-UV (260–190 nm) region. The instrument was flushed continuously with pure evaporated nitrogen throughout the experiment. All CD and UV spectra were buffer-baseline corrected.

## RESULTS

In order to mimic an intracellular interaction between iron and the peptide under the conditions which would be encountered initially following peptide translation in the ER (endoplasmic reticulum), the reduced form of the peptide was chosen to be assessed for iron binding. However, on resuspension of the synthetic freeze-dried reduced peptide, the peptide was found to be partially insoluble. It was therefore decided to use the oxidized form of the synthetic peptide resuspended in the same dilution buffer, but without a reducing agent, and to proceed with its reduction after resuspension.

### MS analysis

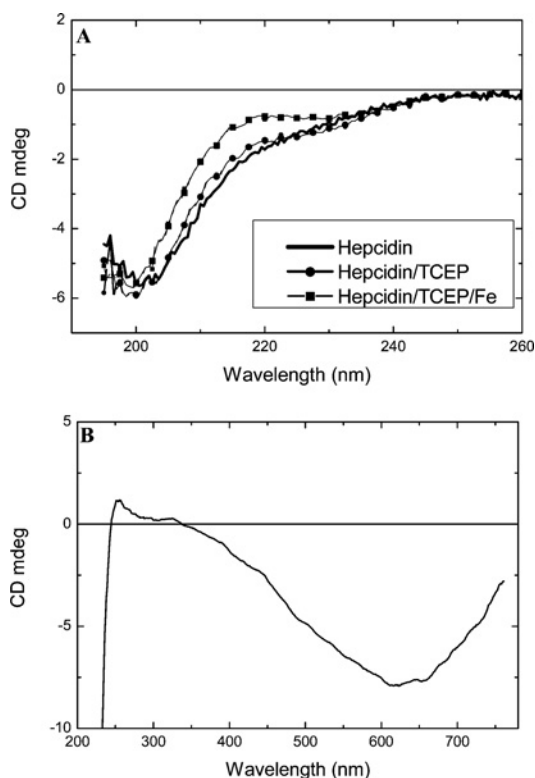
After confirming the oxidized state of the synthetic 25-mer peptide (0.18 mM) by MS analysis (results not shown), the peptide was then reduced using TCEP at a final concentration of 2.5 mM. After 30 min of incubation, complete reduction of the peptide was confirmed by MS analysis by the presence of a peak at 2797 Da (results not shown). It is noteworthy that, although direct solubilization of the freeze-dried reduced peptide was not successful, the peptide resulting from the reduction of the



**Figure 1** MS spectra of hepcidin peptide incubated with iron

After addition of iron to the reduced peptide, a peak at 2853 Da, corresponding to the mass of iron-bound hepcidin, was detected. The y-axes show the percentage intensity in relation to the highest peak shown. The predicted distribution of the relative isotopic abundance (A) corresponds to the distribution obtained in the experimental spectrum (B). The value at the top of each peak is the mass of the peak in Da.

solubilized oxidized peptide was perfectly soluble. Iron was added to the newly reduced peptide successively in three batches of Fe<sup>3+</sup> HCl to a final concentration of 0.54 mM. MS analysis of the mixture indicated that a proportion of the peptide was found to be iron-bound, with a peak at 2853 Da (Figure 1). The validity of the results was confirmed by the corresponding distribution of the relative isotopic abundance between the obtained and predicted profiles (Figure 1). The molecular mass also indicates that iron binding to the peptide does not result in deprotonation and suggests a chelate complex, as observed with other metal complexes [13] such as rubredoxin [14] and ferredoxin I [15]. Although the largest proportion of the iron-containing complex identified corresponds to the molecular mass of a monoferric iron–hepcidin complex, other species of molecular mass, corresponding to iron–hepcidin with two and three atoms of iron bound per peptide, were



**Figure 2** CD spectra of hepcidin in the presence and absence of iron

(A) In the far-UV region, no changes can be observed in CD after the addition of TCEP and reduction of the peptide, and only a minor increase can be measured after addition of iron. (B) The CD spectrum of reduced hepcidin alone has been subtracted from the CD spectrum of reduced hepcidin with added iron; the prominent negative peak in the visible region at 650 nm confirms that iron is bound to the peptide.

also obtained in minor amounts (results not shown), suggesting the existence of several forms of iron–hepcidin.

### UV absorption and CD spectroscopy

UV absorption and CD spectroscopy were used to investigate further the ability of the peptide to bind iron by comparing the spectra obtained with the different peptides before and after the addition of iron. When absorbance in the near-UV region was compared between the oxidized and reduced peptides, no major changes were observed; only a slight decrease around 275 nm, which probably relates to the reduction of the four disulfide bridges (results not shown). The CD spectrum of the oxidized peptide before the addition of iron did not possess any positive ellipticity in the far-UV region, suggesting the peptide possessed a disorganized structure, similar to the original spectrum published previously for the native peptide [2], but with a different secondary structure content from that described for the synthetic peptide [16]. In order to detect any changes in conformation following reduction of the peptide, CD spectra were measured after the addition of TCEP. No changes were observed, indicating that the reducing agent had no effect on the peptide secondary structure (Figure 2). Following each successive addition of  $\text{Fe}^{3+}$  HCl to the reduced peptide, a slight increase in absorbance was observed at approx. 280 nm, which is typical of a charge-transfer transition between the metal and the peptide following the binding of iron (results not shown). The same event was followed by measuring its CD. In the far-UV region, only a small increase in CD was observed after the addition of iron (Figure 2A). In the near-UV

region, the presence of two distinct positive peaks at approx. 250 and 300 nm, and a more prominent negative peak in the visible region at 650 nm, all confirm that iron was bound to the peptide (Figure 2B). Following the addition of different components to the mixture, variations in the pH of the solution had to be considered as a result of the lowering effect of TCEP on the pH, since the addition of  $\text{Fe}^{3+}$  HCl to the solution was calculated to lower the pH of the solution to approx. pH 3. In order to re-establish conditions closer to physiological conditions, the pH was increased to 7.2 by adding crystals of Trizma base to the solution. The pH increase had an immediate effect, with a drastic amplification of all of the variations described above following the addition of iron (results not shown).

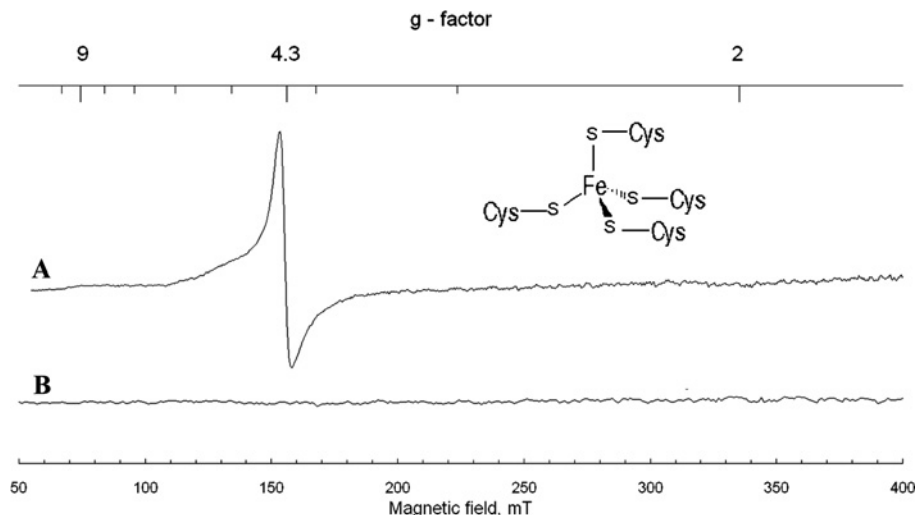
### EPR spectroscopy

In order to identify the nature of the iron complex, the reduced peptide solution containing iron was subjected to EPR analysis. The observation of the spectrum indicated that iron was in its ferric state, with a  $g$ -factor value of approx. 4.3 (Figure 3). Such a prominent absorption derivative near  $g = 4.3$  is typical of high-spin  $\text{Fe}^{3+}$  in a centre of rhombic symmetry, and arises from the  $S = \pm 3/2$  Kramers doublet of the  $S = 5/2$  ion. This is found in non-haem iron proteins such as transferrin, ferrichrome or rubredoxin, which have single iron centres [17]. In the small bacterial iron–sulfur protein rubredoxin, the tetrahedral iron centre is chelated by four cysteine residues (Figure 3) [18]. Additional signals may be observed at  $g = 9$ , corresponding to the  $S = \pm 1/2$  and  $S = \pm 5/2$  Kramers doublets [19], but these are expected to be of lower amplitude; a broad feature was detected in this region, which may represent broadening due to strain effects.

### DISCUSSION

Hepcidin has been shown to play an important role in the regulation of iron homeostasis [20]. After binding to the iron transporter ferroportin, hepcidin facilitates its endocytosis, hence preventing iron distribution from the intestine and macrophages within the body [8]. The majority of this work has been performed using the corresponding synthetic peptide, therefore demonstrating the importance of the primary structure in its function, but omitting other structural properties that could have functional importance in its regulation. In order to study the structural and functional relevance of its native state, a pilot study was carried out using urine-purified native hepcidin where we reported the isolation of iron-bound hepcidin [12]. Following such findings, iron binding to hepcidin was investigated further using the synthetic corresponding peptide, and the results are reported in the present study.

In order to mimic an iron-binding event that would occur during maturation of the peptide, the reduced form of hepcidin was chosen for use in binding assays. However, since the freeze-dried synthetic reduced peptide was found to be partially insoluble, the oxidized form was therefore used as starting material and was reduced after solubilization. The CD profile obtained for the solubilized oxidized peptide was similar to the only published spectra of native hepcidin [2], with both spectra being characteristic of a peptide with no organized secondary structure [21]; in contrast with the published structure of the synthetic peptide, which showed a distorted  $\beta$ -sheet structure [16]. The reduction of the four disulfide bridges did not have a major effect on the secondary structure of the peptide, since the spectra were comparable, but it allowed the binding of iron following the addition of  $\text{Fe}^{3+}$  HCl, as indicated by MS detection of the components, with masses corresponding to ferric–hepcidin complexes. Although



**Figure 3** EPR spectra of iron-bound hepcidin

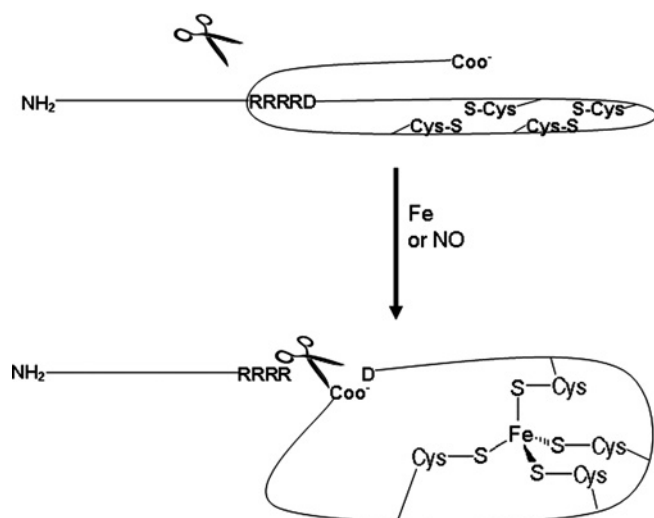
The signal at  $g = 4.3$ , which is present in the iron/hepcidin solution (**A**) and is absent in the iron-only solution (**B**), is characteristic of a rubredoxin-type signal, with  $\text{Fe}^{3+}$  in tetrahedral sulfur co-ordination as illustrated above the spectra.

the highest proportion of iron-containing hepcidin complexes identified were monoferric, complexes of masses corresponding to diferric and triferric complexes were also identified, but in minor amounts. The increase in absorbance observed following the increase in pH indicates a stronger affinity for iron at neutral pH, which could relate to a weaker protonation of the cysteine residues. Although the partial precipitation of ferric iron at higher pH values cannot be ignored and could also be responsible to some degree for the change in absorbance, the differences observed in the CD profiles in the visible region after the addition of iron confirmed iron binding to the peptide. The binding of iron to the peptide was further substantiated by EPR analysis, where the presence of a spectrum implies the detection of iron in the ferric state. In addition, the shape of the spectrum characteristic of rubredoxin, a small protein found in various sulfur-metabolizing bacteria, strongly suggests an iron–sulfur complex involving one atom of iron chelated by four cysteine residues. Rubredoxin is a small protein, containing one atom of iron bound to the sulfur atoms of four cysteine residues, forming an iron–sulfur complex with tetrahedral symmetry, which has been proposed to be involved in electron transfer [22]. A redox function for hepcidin has never been suggested, but a role for the peptide in the oxidation of ferrous to ferric iron could relate to its interaction with the ferrous iron transporter ferroportin, although such a function would have to be clarified. The question of the role of iron binding to hepcidin therefore remains unresolved.

The demonstrated activity of injected iron-free synthetic hepcidin [23] implies that iron could only participate directly in the peptide interaction with ferroportin if its binding to hepcidin occurred prior to or through its interaction with ferroportin. Although iron binding to the oxidized synthetic peptide could not be achieved *in vitro* (results not shown), the nature of the chelate complex suggests that the reduction of the four disulfide bridges might not be necessary under physiological conditions to allow iron to bind to the peptide. In addition, since the presence of free iron is quite unlikely, its interaction with hepcidin could involve a carrier, for which ferroportin would be a prime candidate. Therefore an interaction of iron with the oxidized mature peptide at a later stage and following its secretion cannot be excluded but remains to be demonstrated.

A role for iron binding, distinct from the function of hepcidin as a ligand for ferroportin, might be found in the regulation of hepcidin peptide maturation. Although the present study has been carried out using the synthetic peptide equivalent to the 25-mer hepcidin, iron binding might be considered not only with regards to the active peptide, but also its precursor pro-hepcidin. Apart from the eight cysteine residues present in the 25-mer corresponding to the active peptide, the pro-hepcidin sequence does not contain any other cysteine residues, so it is therefore reasonable to assume that the described iron–sulfur cluster would not be compromised. In the model of iron binding inside the cell, binding of iron could play a role in regulating maturation of the propeptide, a model based on existing mechanisms such as the ‘cysteine switch’ activation mechanism of the MMP (matrix metalloproteinase) family, where the mechanism relies on unmasking of the cleavage site following metal binding to a cysteine residue on the protease [24]. A similar mechanism could be involved in the maturation of pro-hepcidin into hepcidin; although, in contrast with MMPs, it is the binding of iron, and not its dissociation, that would allow the unmasking of the RRRRD cleavage site. Such a mechanism would depend on the local iron content, as at low iron concentrations the cleavage site of the folded iron-free propeptide would be inaccessible to the convertase, whereas at high iron concentrations, iron binding to the propeptide would engender sufficient conformational changes to permit access to the cleavage site and lead to maturation of the propeptide into the active peptide, followed by its secretion (Figure 4).

To conclude, we propose two hypotheses for the role of iron binding to hepcidin that both imply another direct role of iron in the regulation of its absorption. In the first instance, iron could be directly involved in the interaction between hepcidin and ferroportin, with a selective interaction of hepcidin and only ferroportin actively transporting iron, which would explain the different effects of hepcidin observed between enterocytes and macrophages [25]. In the second mechanism, following binding of iron, the conformational changes sustained by pro-hepcidin would lead to the unmasking of the cleavage site and therefore to its accessibility to the convertase furin, leading to maturation of the active 25-mer peptide. Such a mechanism of regulation at the



**Figure 4** Hypothetical mechanism of maturation of hepcidin

In the absence of ligand, the cleavage site is masked by the pro-domain; but in the presence of ligand, displacement of the pro-domain following a conformational change as a result of the binding of a ligand, i.e Fe or NO, to the cysteine residues allows the convertase to access the cleavage site, leading to the maturation of pro-hepcidin into the active 25-mer hepcidin.

post-translational level is not a substitute for the more complex mechanism of regulation described at the transcriptional level, but is simply a complementary mechanism of a first response to subtle variations in iron levels in the cell, as observed with other hormone mechanisms.

We thank the Medical Research Council for the research studentship awarded to C. R.

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Received 21 February 2008/17 April 2008; accepted 1 May 2008

Published as BJ Immediate Publication 1 May 2008, doi:10.1042/BJ20080406