The haemochromatosis protein HFE induces an apparent iron-deficient phenotype in H1299 cells that is not corrected by co-expression of β_2 -microglobulin

Jian WANG*, Guohua CHEN* and Kostas PANTOPOULOS*†¹

*Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, 3755 Cote-Ste-Catherine Road, Montreal, Quebec, Canada H3T 1E2, and

†Division of Experimental Medicine, Faculty of Medicine, McGill University, Montreal, Quebec, Canada

HFE, an atypical MHC class I type molecule, has a critical, yet still elusive function in the regulation of systemic iron metabolism. HFE mutations are linked to hereditary haemochromatosis type 1, a common autosomal recessive disorder of iron overload. Most patients are homozygous for a C282Y point mutation that abrogates the interaction of HFE with β_2 -microglobulin (β_2 M) and, thus, impairs its proper processing and expression on the cell surface. An H63D substitution is also associated with disease. To investigate the function of HFE we have generated clones of human H1299 lung cancer cells that express wild-type, C282Y or H63D HFE under the control of a tetracycline-inducible promoter. Consistent with earlier observations in other cell lines, the expression of wild-type or H63D, but not C282Y, HFE induces an apparent iron-deficient pheno-

type, manifested in the activation of iron-regulatory protein and concomitant increase in transferrin receptor levels and decrease in ferritin content. This phenotype persists in cells expressing wild-type HFE after transfection with a β_2 M cDNA. Whereas endogenous β_2 M is sufficient for the presentation of at least a fraction of chimeric HFE on the cell surface, this effect is stimulated by approx. 2.8-fold in β_2 M transfectants. The co-expression of exogenous β_2 M does not significantly affect the half-life of HFE. These results suggest that the apparent iron-deficient phenotype elicited by HFE is not linked to β_2 M insufficiency.

Key words: ferritin, iron-responsive element, iron-regulatory protein, transferrin receptor.

INTRODUCTION

Hereditary haemochromatosis type 1 (HH) is a very common autosomal recessive disorder of iron metabolism [1,2]. Its hallmarks are the loss of feedback regulation of dietary iron absorption in the duodenum and the inability of reticuloendothelial cells to retain appropriate quantities of iron. As a result, after saturation of serum transferrin (Tf), iron progressively accumulates in tissue parenchymal cells and eventually leads to organ dysfunction and damage.

The 'haemochromatosis gene' encodes an atypical MHC class I type protein [3], now known as HFE. A vast majority (> 80%) of HH patients carry an HFE C282Y point mutation, with an estimated approx. 10–15% carrier frequency among Caucasians of Northern European ancestry. This mutation abrogates a disulphide bridge between C282 and C225 within the $\alpha 3$ domain of HFE. The disruption of the bridge, in turn, prevents the association of the mutation with β_2 -microglobulin (β_2 M), which is indispensable for processing and maturation. Consequently, C282Y HFE fails to be expressed properly on the cell surface [4,5].

C282Y HFE homozygotes have a high risk of developing clinical symptoms of HH [6]. Another HFE point mutation, H63D, has been associated with a moderate risk for HH, especially in C282Y/H63D compound heterozygotes [7]. Further uncommon HFE mutations and polymorphisms have been linked to HH, including missense, frameshift and nonsense mutations [2]. A homozygous mutation at an intron–exon boundary of the HFE gene resulting in aberrant splicing has also been linked to HH [8].

The crucial role of HFE in the regulation of body iron metabolism was established with the targeted disruption of the *hfe* gene and the recapitulation of the HH phenotype in *hfe*^{−/−} mice [9–11]. It was shown earlier that β_2 M knockout (β_2 m^{−/−}) mice develop iron overload [12,13] phenotypically similar to HH [14]. The deletion of a large fragment of *hfe* results in more severe iron loading compared with targeted introduction of the C282Y mutation [10]. In addition, the degree of iron loading appears to vary between *hfe*^{−/−} [15] or β_2 m^{−/−} [16] mice of different genetic backgrounds, suggesting the existence of gene modifiers. This view is supported by crossing *hfe*^{−/−} mice with mice carrying mutations in other genes of iron metabolism, which yielded various degrees of iron loading in the progeny [17].

Despite the progress in the characterization of HFE, its molecular and physiological functions remain elusive. Crystallographic studies showed that unlike typical MHC class I molecules, HFE is probably not involved in antigen presentation because its putative ligand-recognizing domains $\alpha 1$ and $\alpha 2$ form a relatively narrow groove [18]. Along these lines, disruption of these domains in *hfe*^{−/−} mice is not associated with any obvious immunological abnormality [11]. An important functional hint was provided by the finding that HFE forms complexes with the Tf receptor (TfR) [19,20] and thereby modulates the TfR cycle [21–23], a major route for cellular iron uptake [24]. *In vitro*, the HFE–TfR interaction occurs readily at pH 7.5 (reflecting conditions on the cell surface), but is abrogated at pH 6.0 (reflecting conditions in the endosome) [18]. The crystal structure of HFE complexed with the extracellular portion of TfR shows that the interaction involves the $\alpha 1$ and $\alpha 2$ domains of HFE and induces conformational changes in TfR [25]. The HFE–TfR interaction accounts

Abbreviations used: EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; HH, hereditary haemochromatosis; IRE, iron-responsive element; IRP1, iron-regulatory protein 1; β_2 M, β_2 -microglobulin; 2-ME, 2-mercaptoethanol; Tf, transferrin; TfR, Tf receptor.

¹ To whom correspondence should be addressed (e-mail kostas.pantopoulos@mcgill.ca).

for the co-localization of HFE with TfR in the plasma membrane and in perinuclear regions of HeLa cells [26]. In addition, the HFE-TfR interaction is necessary for the co-localization of HFE with TfR in the apical membrane and the endosomal compartment of polarized HuTu-80 cells [27].

In vitro binding assays showed that HFE competes with Tf for binding to TfR [28], implying that HFE modulates negatively the TfR cycle. Overexpression of HFE in HeLa [22,29,30], HEK-293 [31] and HuTu-80 [27] cells correlates with decreased ferritin and increased TfR levels [22,27,29,30]. The expression of ferritin and TfR is co-ordinately regulated post-transcriptionally at the levels of mRNA translation and stability respectively by binding of iron-regulatory proteins (IRP1 and IRP2) to 'iron-responsive elements' (IREs) within their mRNAs [32,33]. IRE-binding assays showed that HFE expression activates IRPs to bind to cognate IREs [29,30,34] as a result of a decrease in the intracellular 'labile iron pool' [34]. All these results are consistent with a view that expression of HFE yields an iron-deficient phenotype. This interpretation was, however, challenged in a recent report, where the apparent iron-deficient phenotype of HFE-expressing TRVb-1 cells was corrected by co-expression of β_2 M [35].

To study the function of HFE in cellular iron metabolism and investigate the role of β_2 M, we have generated human H1299 lung cancer cells expressing either wild-type HFE, or the disease-associated C282Y or H63D mutants, in a tetracycline-inducible manner. The H1299 cells were recently employed by us to express a constitutive IRP1 mutant [36] and turned out to provide a reliable model for studies on cellular iron metabolism.

MATERIALS AND METHODS

Plasmid constructions

The cDNAs encoding wild-type, C282Y and H63D human HFE [4] were PCR-amplified with 5'-GATCGGATCCACCATGGGCCCGCGAGCCAGGCCGCGCTT-3' and 5'-CGTAGGATCCTTATCAAGCGTAATCTGGAACATCGTATGGGT-TACTCACGTTTCAGCTAAGACGTA-3' and cloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.). This manipulation retains the *Bam*H1 site at the 5' end of HFE [4], introduces a second *Bam*H1 site downstream of the stop codon and replaces the C-terminal FLAG described in [4] with a haemagglutinin (HA) epitope. The 1 kb *Bam*H1/*Bam*H1 inserts were excised and subcloned into the *Bam*H1 site of pUHD10-3 that contains a tetracycline-inducible hCMV minimal promoter [36] to yield pUHD-HFE_{wt}, pUHD-HFE_{C282Y} and pUHD-HFE_{H63D} respectively. Constructs with the correct orientation were identified by restriction analysis and sequenced to confirm the presence of the mutations.

Generation of HFE-expressing cell lines

The pUHD-HFE_{wt}, pUHD-HFE_{C282Y} or pUHD-HFE_{H63D} plasmids were co-transfected with the puromycin resistance pBabe in tTA-H1299 cells [36] by the calcium phosphate method. Stable transfectants were selected in media containing 2 μ g/ml of puromycin, 250 μ g/ml of G418 and 2 μ g/ml of tetracycline. Representative clones, referred to as HFE_{wt}, HFE_{C282Y} or HFE_{H63D}, were isolated for further characterization. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) tetracycline-free foetal bovine serum (Clontech), 2 mM glutamine, 100 units/ml of penicillin and 0.1 ng/ml of streptomycin. Expression of HFE is induced on removal of tetracycline (tet-off system).

Western blotting

Cells were lysed directly in Laemmli sample buffer (unless otherwise indicated) and lysates immediately boiled for 5 min [36]. Equal aliquots were resolved by SDS/PAGE [11% (w/v) gels] and proteins transferred on to nitrocellulose filters. The blots were saturated with 10% non-fat milk in PBS and probed with the culture supernatant of 12CA5 hybridoma cells (containing HA antibodies) or with TfR (Zymed, San Francisco, CA, U.S.A.), ferritin (Roche, Indianapolis, IN, U.S.A.), β -actin (Sigma) or β_2 M (Santa Cruz) antibodies. Dilutions for TfR and β -actin antibodies were 1:1000 and 1:500 for ferritin and β_2 M antibodies. After a wash with PBS containing 0.1% (v/v) Tween 20, the blots with HA and TfR monoclonal antibodies were further incubated with rabbit anti-mouse IgG (1:4000 dilution). The blots with ferritin and β -actin antibodies were incubated with goat anti-rabbit IgG (1:5000 dilution) and the blots with the β_2 M antibody with rabbit anti-goat IgG (1:3000 dilution). Detection of the peroxidase-coupled secondary antibodies was performed with the ECL[®] method (Amersham, Piscataway, NJ, U.S.A.). The blots were quantified by densitometric scanning.

Northern blotting

Cells were lysed with the Trizol reagent (Gibco BRL, Burlington, ON, Canada) and RNA was prepared according to the manufacturer's recommendation. Total cellular RNA (10 μ g) was electrophoretically resolved on denaturing agarose gels, transferred on to nylon membranes and hybridized to radiolabelled human TfR or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes.

Tf-binding assay

The cells were scraped, suspended in medium and incubated at 37 °C with different concentrations of FITC-conjugated diferric human Tf (Molecular Probes). The Tf-binding capacity of TfR was determined by analysing fluorescent cells with FACS as in [37]. Curve-fitting, non-linear regression analysis and calculations of the binding constants were performed with the GraphPad Prism software (version 2.0).

⁵⁵Fe uptake experiments

Generation of ⁵⁵Fe-Tf and cellular uptake experiments of ⁵⁵Fe-Tf were done according to methods described in [37] for ⁵⁹Fe-Tf.

Analysis of HFE expression on the cell surface

The cells were washed twice, suspended in PBS and incubated for 2 h at 4 °C with 1:100 diluted 10G4 monoclonal antibody against HFE (kindly provided by Dr Yang, R. W. Johnson Pharmaceutical Research Institute, San Diego, CA, U.S.A.). The cells were washed extensively to remove unbound primary antibody, incubated for another 2 h with 1:100 diluted FITC-conjugated anti-mouse IgG (Sigma) and analysed by FACS.

Electrophoretic mobility-shift assay (EMSA)

The cells were lysed in 'cytoplasmic lysis buffer' (1% Triton X-100, 40 mM KCl, 25 mM Tris/HCl; pH 7.4). Analysis of IRE binding by EMSA was performed as in [38].

Transient transfections

Transient transfections with the pBA- β 2M plasmid (a gift of Dr Caroline Enns, Oregon Health and Science University, Portland,

OR, U.S.A.) for expression of human $\beta_2\text{M}$ were performed with the Lipofectamine Plus™ reagent (Gibco BRL). Transfection efficiency was assessed by parallel transfection of the pEGFP-C1 vector (Clontech) and analysis of cells expressing the green fluorescent protein by FACS.

Metabolic labelling and immunoprecipitation

Cells were metabolically labelled for 2 h with (50 $\mu\text{Ci/ml}$) *trans*-[^{35}S]label, a mixture of 70:30 [^{35}S]methionine–cysteine (ICN, Montreal, QC, Canada), washed twice with PBS and solubilized in lysis buffer (1% Triton X-100, 300 mM NaCl and 50 mM Tris/HCl; pH 7.4). Equal amounts of trichloroacetic acid-insoluble radioactivity were subjected to quantitative immunoprecipitation with antibodies against ferritin (Roche) or TfR (Zymed) as in [37]. In pulse–chase experiments, the radioactive medium was removed after labelling and the cells were chased in Dulbecco's modified Eagle's medium supplemented with additives. Subsequently, the cells were lysed as above and 500 μg of cytoplasmic lysates was subjected to quantitative immunoprecipitation with an HA monoclonal antibody (from 12CA5 hybridoma cells). Immunoprecipitated proteins were analysed by SDS/PAGE and visualized by autoradiography. Radioactive bands were quantified by phosphor imaging.

RESULTS

Tetracycline-inducible expression of wild-type, C282Y and H63D HFE in H1299 cells

Human H1299 lung cancer cells expressing the transactivator tTA [36] were transfected with cDNAs encoding HA-tagged wild-type, C282Y or H63D HFE under the control of a tetracycline-inducible promoter (tet-off system). Representative clones HFE_{wt}, HFE_{C282Y} and HFE_{H63D} were utilized for further characterization. First, the expression of HFE was analysed by Western blotting with an HA antibody (Figure 1). Removal of tetracycline from the medium for 3 days results in a profound induction of wild-type, C282Y and H63D HFE expression (Figure 1, upper panel), whereas levels of control β -actin remain unchanged (lower panel). In agreement with other reports [5,29,30], wild-type HFE migrates in two bands with apparent molecular masses of approx. 45 and approx. 48 kDa (lane 2) respectively, which quite probably represent partially and fully glycosylated polypeptides [5,23,26]. By contrast, the 48 kDa band is absent from C282Y HFE and a faster migrating band of approx. 42 kDa appears below the predominant approx. 45 kDa polypeptide

Figure 1 Tetracycline-inducible expression of HFE in H1299 cells

Human tTA-H1299 lung cancer cells were stably transfected with HA-epitope tagged wild-type human HFE or with disease-associated H63D or C282Y HFE point mutants. Cells (1×10^6) of each representative clone HFE_{wt} (lanes 1–2), HFE_{H63D} (lanes 3–4) or HFE_{C282Y} (lanes 5–6) were plated in 100 mm dishes and grown for 3 days without (–) or with (+) 2 $\mu\text{g/ml}$ tetracycline. The expression of HFE was analysed by Western blotting with antibodies against the HA tag (upper panel). The filter was also probed with an antibody against β -actin (lower panel).

Figure 2 Wild-type and H63D HFE stimulate TfR expression

Cells (1×10^6) of each representative clone HFE_{wt} (A), HFE_{H63D} (B) or HFE_{C282Y} (C) were plated in 100 mm dishes and grown for 1–4 days without (–) or with (+) 2 $\mu\text{g/ml}$ of tetracycline. The expressions of HFE, TfR and control β -actin were analysed by Western blotting. The filters were probed with antibodies against HA (upper panel), TfR (middle panel) and β -actin (lower panel).

(lane 6). The H63D mutant gives rise to all three variants, with the 45 kDa form being predominant (lane 4). The fast migrating band of approx. 42 kDa may correspond to a degradation product or to a completely non-glycosylated form.

Expression of wild-type and H63D HFE results in an apparent iron-deficient phenotype

The effects of wild-type and mutant forms of HFE in the expression of TfR were evaluated in a time-course experiment. HFE_{wt}, HFE_{C282Y} and HFE_{H63D} cells were grown in the presence or absence of tetracycline for 1–4 days and TfR steady-state levels were analysed by Western blotting. The prolonged (≥ 2 days) expression of wild-type and H63D HFE correlate with a time-dependent increase in TfR (Figures 2A and 2B). However, the expression of C282Y HFE does not appear to stimulate TfR

Figure 3 Effects of HFE expression in the association of Tf with TfR

Cells (1×10^6) of each representative clone HFE_{wt} (A), HFE_{H63D} (B) or HFE_{C282Y} (C) were plated in 100 mm dishes and grown for 3 days without (—) or with (+) 2 μ g/ml of tetracycline. Aliquots of 0.25×10^6 cells (in duplicates) were suspended in medium and tumbled for 30 min at 37 °C with 2.5, 5, 7.5, 10, 12.5, 25, 50 or 100 μ g/ml FITC-conjugated diferric Tf (Molecular Probes). Subsequently, the cells were washed extensively with PBS containing 0.1% BSA to remove excess FITC label, fixed in 3.7% formaldehyde and analysed by FACS. The relative fluorescence corresponding to cell-associated FITC-Tf after induction of HFE expression (▲) or before (■) is plotted against the indicated concentrations of free FITC-Tf. The binding constants $K_{cell association}$ (in nM) and B_{max} are shown in the insets. Non-linear regression analysis and curve-fitting were done with the GraphPad Prism software (version 2.0). * $P < 0.05$ versus control.

Figure 4 Effects of HFE expression in the IRE-IRP system

Cells (1×10^6) of each representative clone HFE_{wt}, HFE_{H63D} or HFE_{C282Y} were plated in 100 mm dishes and grown for 3 days without (—) or with (+) 2 μ g/ml of tetracycline. (A) Analysis of TfR (upper panel) and GAPDH (lower panel) mRNA by Northern blotting. (B) EMSA with 40 μ g cytoplasmic extracts and 25000 c.p.m. 32 P-labelled IRE probe in the absence (upper panel) or presence (lower panel) of 2% 2-ME. Only the positions of the IRE-IRP complexes are shown (arrows). (C) The cells were treated overnight with or without 100 μ M desferrioxamine, and metabolically labelled with 35 S-methionine-cysteine for 2 h. Cytoplasmic extracts, normalized for trichloroacetic acid-insoluble radioactivity, were subjected to quantitative immunoprecipitation with 5 μ l ferritin antibody (Roche). Immunoprecipitated material was resolved by SDS/PAGE (11% gel), and ferritin (arrow) was visualized by autoradiography. (D) Analysis of ferritin (upper panel) and β -actin (lower panel) by Western blotting.

expression (Figure 2C). Under all experimental conditions, the levels of β -actin remain unaffected (lower panels). The increase in the steady-state levels of TfR after expression of wild-type and H63D HFE is consistent with a cellular response to iron deficiency. To characterize better the molecular basis of this response, we first examined the role of HFE in the Tf-binding properties of TfR. To this end, cells grown for 3 days in the presence or absence of tetracycline were incubated with different concentrations of FITC-conjugated Tf and analysed by FACS (Figure 3). Under these conditions, the total cell-associated

FITC-Tf corresponds to steady-state levels of cell-surface-bound and internalized ligand [37]. The binding constants $K_{cell association}$ and B_{max} were computed by curve-fitting and non-linear regression analysis. Expression of wild-type HFE results in a small but significant ($P < 0.05$) increase in $K_{cell association}$ from 10 to 29.5 nM and in the B_{max} from 1.2 to 1.8 (Figure 3A). By contrast, expression of C282Y HFE leads to insignificant changes of the $K_{cell association}$ from 18.2 to 15.3 nM and the B_{max} from 1.6 to 1.7 (Figure 3C). Expression of H63D HFE results in insignificant changes of the $K_{cell association}$ from 15.3 to 12.1 nM and in a

Table 1 HFE inhibits the uptake of $^{55}\text{Fe-Tf}$

HFE_{wt} cells (4.5×10^5), grown for 3 days without (–) or with (+) 2 $\mu\text{g/ml}$ of tetracycline, were incubated for 6 h with 10 μM human $^{55}\text{Fe-Tf}$ (948 c.p.m./pmol). After a $3 \times$ wash with ice-cold PBS, half of the cells were lysed in 0.1% SDS. The other half was lysed in a buffer containing 1% Triton X-100, 300 mM NaCl and 50 mM Tris/HCl (pH 7.4) and subjected to quantitative immunoprecipitation with 5 μl of ferritin antibody (Roche). Total cellular and ferritin-associated ^{55}Fe was determined by liquid-scintillation counting. Values correspond to triplicate samples.

^{55}Fe [pmol (10^6 cells) $^{-1}$ (6 h) $^{-1}$]	tet (+)	tet (–)
Total cellular	220.0 $\pm$ 8.1	161.4 $\pm$ 6.6*
Ferritin-associated	101.6 $\pm$ 14.5	90.1 $\pm$ 4.0

* $P < 0.01$ versus control (Student's t test).

significant ($P < 0.05$) increase of the B_{max} from 2.3 to 2.8 (Figure 3B). We conclude that in H1299 cells, expression of the wild-type, but not C282Y or H63D, HFE slightly lowers the affinity of TfR for its ligand Tf.

The expression of TfR is regulated, at least in part, at the level of mRNA stability [32]. Northern-blot analysis reveals that cells expressing wild-type and H63D HFE for 3 days have dramatically increased levels of TfR mRNA (Figure 4A, upper panel, lanes 1–4). By contrast, expression of C282Y HFE is not associated with any appreciable alterations in TfR mRNA (lanes 5–6). HFE does not have any effect on control GAPDH mRNA levels (lower panel). Since the stabilization of TfR mRNA is mediated by IRE–IRP interactions [32], we evaluated the effects of HFE expression on IRE-binding activity, which reflects intracellular iron status. Cells were grown in the presence or absence of tetracycline for 3 days, and cytoplasmic lysates were analysed by EMSA with an IRE probe. Removal of tetracycline leads to a notable increase in IRE-binding activity in cells expressing wild-type and H63D HFE (Figure 4B, upper panel, lanes 1–4), but has no effect in cells expressing C282Y HFE (lanes 5–6). As human IRE–IRP1 and IRE–IRP2 complexes co-migrate in EMSAs [32,33], we refer to the protein(s) responsible for this activity as ‘IRP’. A treatment of the cell extracts with 2% (v/v) 2-mercaptoethanol (2-ME), known to activate latent IRP1 preferentially [32], serves as a loading control (lower panel). The profound activation of IRE binding by this treatment also implies that the largest fraction of ‘IRP’ probably corresponds to IRP1.

We next addressed the role of HFE in the expression of ferritin, which is regulated at the translational level by IRE–IRP interactions [32]. After metabolic labelling of cells with ^{35}S -methionine–cysteine, ferritin synthesis was analysed by immunoprecipitation (Figure 4C). Expression of wild-type or H63D HFE after the removal of tetracycline clearly inhibits ferritin synthesis (lanes 1–2 and 5–6), whereas C282Y HFE fails to do so (lanes 9–10). A treatment of the cells with the iron chelator desferrioxamine leads to an almost complete block of ferritin synthesis (lanes 3–4, 7–8 and 11–12). Finally, we analysed the effects of HFE in ferritin content. Western-blot analysis shows that expression of wild-type or H63D, but not C282Y, HFE for 3 days is associated with a profound decrease in ferritin levels (Figure 4D, upper panel), without affecting levels of β -actin (lower panel). In conclusion, wild-type or H63D, but not C282Y, HFE promotes an apparent iron-deficient phenotype in H1299 cells, manifested by increased IRP activity and concomitant stabilization of TfR mRNA and inhibition of ferritin synthesis. This leads to an increase in TfR expression and decrease in ferritin content.

Figure 5 Transfection of a human $\beta_2\text{M}$ cDNA into HFE_{wt} cells promotes maturation and cell-surface expression of wild-type HFE

HFE_{wt} cells (1×10^6) were transiently transfected with or without the pBA- $\beta_2\text{M}$ plasmid, plated in 100 mm dishes and grown for 3 days without (–) or with (+) 2 $\mu\text{g/ml}$ of tetracycline. (A) The cells were lysed in ‘cytoplasmic lysis buffer’. Extracts (5 μg) from control non-transfected and from $\beta_2\text{M}$ -transfected cells were analysed by Western blotting with antibodies against HA (upper panel), $\beta_2\text{M}$ (second panel), TfR (third panel) or β -actin (lower panel). (B) Control non-transfected and $\beta_2\text{M}$ -transfected cells (0.5×10^6) suspended in PBS were incubated for 2 h at room temperature with 1:100 diluted 10G4 monoclonal antibody [23]. After extensive washing, the cells were incubated for another 2 h with 1:100 diluted FITC-conjugated anti-mouse IgG (Sigma) and analysed by FACS.

Expression of wild-type HFE impairs uptake of $^{55}\text{Fe-Tf}$

Having established that wild-type HFE stimulates cellular responses to iron deficiency, we directly addressed the role of HFE expression in the uptake of $^{55}\text{Fe-Tf}$ and the storage of ^{55}Fe in ferritin. Cells grown for 3 days with or without tetracycline were incubated with 10 μM $^{55}\text{Fe-Tf}$ for 6 h and cell-associated radioactivity was measured by liquid-scintillation counting. Expression of HFE on removal of tetracycline results in a significant ($P < 0.05$) inhibition of $^{55}\text{Fe-Tf}$ uptake by approx. 20% (Table 1). Analysis of ferritin-associated radioactivity by quantitative immunoprecipitation reveals that HFE-expressing cells have a slightly reduced capacity (by approx. 10%) to store ^{55}Fe in ferritin. This effect is, however, non-significant ($P > 0.05$). We conclude that wild-type HFE impairs the cellular uptake of $^{55}\text{Fe-Tf}$, despite the increase in TfR expression.

Co-expression of wild-type HFE and $\beta_2\text{M}$ does not reverse the apparent iron-deficient phenotype

Recent experiments in TRVb-1 cells led to the proposal that some of the effects that have been observed in cell lines overexpressing HFE may be alleviated by co-expression of $\beta_2\text{M}$ [35]. To address the role of $\beta_2\text{M}$ in HFE-mediated regulation of cellular iron metabolism, H1299 cells expressing wild-type HFE were transiently transfected with a human $\beta_2\text{M}$ construct. A parallel transfection with a construct encoding the green fluores-

Figure 6 Pulse-chase of wild-type HFE in the absence or presence of transfected β_2 M

HFE_{wt} cells (1×10^6) were transiently transfected with or without the pBA- β_2 M plasmid and plated in 100 mm dishes. After 3 days of growth without tetracycline, the cells were metabolically labelled for 2 h with 35 S-methionine–cysteine and chased in unlabelled medium for the indicated time points. Cytoplasmic lysates (500 μ g) were subjected to quantitative immunoprecipitation with saturating amounts of the HA antibody (supernatant from 12CA5 hybridoma cell culture). Immunoprecipitated HFE_{wt} was analysed by SDS/PAGE (11% gel) and visualized by autoradiography. The radioactive bands (inset) of non-transfected control cells (**A**) and β_2 M transfectants (**B**) were quantified by phosphor imaging. The percentage of residual radioactivity is plotted against time. Open and closed bars correspond to the radioactivity of the precursor and mature polypeptides of approx. 45 and approx. 48 kDa respectively.

cent protein and analysis by FACS revealed a transfection efficiency of 85 %. Non-transfected control and β_2 M-transfected cells were grown in the presence or absence of tetracycline for 2 days and extracts were prepared for Western-blot analysis (Figure 5A). Probing with a β_2 M antibody shows that non-transfected control cells express endogenous β_2 M (second panel, lanes 1–2) and, as expected, its levels are dramatically increased in β_2 M transfectants (lanes 3–4). Probing the filter with an HA antibody to detect HFE reveals that expression of β_2 M results in enrichment of the mature 48 kDa polypeptide (upper panel, cf. lanes 2 and 4), consistent with the role of β_2 M in HFE processing. However, it should be noted that endogenous β_2 M is sufficient to promote maturation of a fraction of transfected HFE (Figures 2A and 5A). Importantly, the presence of excessive levels of β_2 M has no effect on the increase in TfR steady-state levels associated with HFE expression (Figure 5A, third panel). The expression of control β -actin remains unchanged (lower panel).

Having established that transient transfection of β_2 M promotes maturation of tetracycline-induced HFE, we assessed whether this correlates with increased expression of HFE on the cell surface. To this end, we utilized the monoclonal antibody 10G4, which recognizes an epitope on the heavy chain of HFE [23]. The cells were first incubated with the 10G4 antibody, subsequently with FITC-conjugated anti-mouse IgG and analysed by FACS. In agreement with the results in Figure 5(A), removal of tetracycline results in detection of HFE on the cell surface, which is stimulated approx. 2.8-fold in β_2 M transfectants (Figure 5B).

Co-expression of β_2 M in TRVb-1 cells resulted in a dramatic increase in HFE half-life, prevented its degradation within 2 h and promoted its maturation [35]. To address whether this is a more general phenomenon, control or β_2 M-transfected HFE-expressing H1299 cells were metabolically labelled for 2 h with

35 S-methionine–cysteine and subsequently chased with unlabelled medium for different time intervals. The fate of HFE was analysed by quantitative immunoprecipitation from cell extracts normalized for protein concentration. This experiment shows that in the absence of transfected β_2 M, the bulk of the 45 kDa polypeptide, corresponding to precursor HFE, is degraded within 8 h with a half-life of 2–4 h (Figure 6A). At zero time, HFE is exclusively detected in the precursor form of 45 kDa and only a small fraction (approx. 10 %) is converted into the mature 48 kDa form within 4–8 h. The presence of transfected β_2 M does not result in dramatic alterations in the half-life of HFE (Figure 6B). However, it clearly stimulates its maturation. This is evident at zero time, where the mature 48 kDa form represents approx. 14 % of total radioactivity. Importantly, the mature fraction is significantly enriched during the chase and after 4 h it represents greater than 50 % of the residual radioactivity.

We finally examined whether overexpression of β_2 M affects the function of HFE in modulating the IRE–IRP regulatory system. Control and β_2 M-transfected cells were grown in the presence or absence of tetracycline for 2 days and cytoplasmic extracts were prepared for analysis of IRE-binding activity by EMSA (Figure 7A). The expression of HFE correlates with an increase in IRP activity (upper panel, lanes 1–2), and this pattern is not affected in β_2 M transfectants (lanes 3–4). Analysis of the same extracts in the presence of 2-ME confirms equal loading (lower panel).

To address the role of HFE and β_2 M in the expression of proteins encoded by IRE-containing mRNAs, control and β_2 M-transfected cells were grown in the presence or absence of tetracycline for 2 days and metabolically labelled with 35 S-methionine–cysteine. The synthesis of TfR and ferritin were analysed by quantitative immunoprecipitation (Figure 7B). As

Figure 7 Transfection of HFE_{wt} cells with β_2 M does not correct the apparent HFE-dependent iron-deficient phenotype

HFE_{wt} cells (1×10^6) were transiently transfected with or without the pBA- β_2 M plasmid, plated in 100 mm dishes and grown for 3 days without (—) or with (+) 2 μ g/ml of tetracycline. **(A)** Cytoplasmic extracts (40 μ g) were analysed by EMSA with 25000 c.p.m. 32 P-labelled IRE probe in the absence (upper panel) or presence (lower panel) of 2% 2-ME. Only the positions of the IRE-IRP complexes are shown (arrows). **(B)** The cells were metabolically labelled with 35 S-methionine-cysteine for 2 h. Cytoplasmic extracts, normalized for trichloroacetic acid-insoluble radioactivity, were subjected to simultaneous quantitative immunoprecipitation with 2 μ l of Tfr (Zymed) and 5 μ l of ferritin antibodies (Roche). Immunoprecipitated material was resolved by SDS/PAGE (11% gel). Tfr and ferritin (arrows) were visualized by autoradiography.

expected, on the basis of the previous results, expression of HFE in the presence or absence of β_2 M results in stimulation of Tfr (upper panel) and inhibition of ferritin synthesis (lower panel). Thus, expression of exogenous β_2 M in H1299 cells fails to antagonize the responses elicited upon expression of HFE.

DISCUSSION

A tetracycline-inducible system for expression of wild-type and disease-linked forms of HFE in H1299 cells was established to study the function of the HH gene in cellular iron metabolism. Expression of wild-type HFE is associated with an increase in Tfr mRNA (Figure 4A) and protein (Figure 2A) steady-state levels, stimulation of IRP activity (Figure 4B) and reduction in ferritin synthesis (Figure 4C) and content (Figure 4D), which are hallmarks of an iron-deficient phenotype. These findings are in agreement with results obtained in HeLa [22,29,30], HEK-293 [31] and HuTu-80 [27] cells. We also show here that expression of the C282Y HFE mutant fails to elicit these responses (Figures 2C and 4), whereas in this context, H63D HFE essentially behaves as wild-type (Figures 2B and 4). The results with the mutants are not only in agreement, but also extend observations of previous studies [27,31], since they directly address the effects of C282Y or H63D HFE on the IRE-IRP system.

The mechanism by which HFE promotes iron deficiency when expressed in mammalian cell lines is not fully understood, but it is very likely related to its ability to interact with the Tfr physically. This interaction occurs shortly after biosynthesis while both proteins are still in the ER/cis-Golgi [23,26] and persists on the cell surface and in the endosomal compartments during endocytosis [27]. On the basis of *in vitro* binding studies [28], it is reasonable to hypothesize that HFE may compete with

Tf for binding to Tfr *in vivo*. We show here that expression of wild-type HFE decreased the apparent affinity of diferric FITC-Tf for Tfr by approx. 3-fold (Figure 3A) and resulted in a 50% increase in the number of functional receptors on the cell surface ($B_{\max}$). By contrast, expression of C282Y HFE had no appreciable effect on the Tf-Tfr interaction (Figure 3C), whereas H63D did not affect the $K_{\text{cell association}}$ but increased the $B_{\max}$ by approx. 20% (Figure 3B).

These results are in line with those of Feder et al. [21] obtained with HEK-293 cells. However, it is questionable whether the HFE-mediated increase in $K_{\text{cell association}}$ contributes to the apparent iron deficiency of H1299 cells, considering that the cells are grown in saturating Tf concentrations (normal plasma concentration is approx. 50 μ M with approx. 10% in the diferric form [39]; therefore, a medium supplemented with 10% foetal bovine serum is conjectured to contain approx. 500 nM diferric Tf). Alternative hypotheses on how HFE modulates the Tfr cycle include a negative function in endocytosis [23] or the release/transport of iron across the endosomal membrane [22]. Both hypotheses could provide an explanation on the HFE-mediated inhibition in the cellular uptake of isotopically labelled Fe-Tf (Table 1) [22,29,30], despite the increased expression of Tfr (Figure 2A) [22,27,29,30]. Interestingly, Table 1 shows that HFE-expressing cells largely retain their capacity to store freshly internalized 55 Fe in ferritin, despite the marked reduction in ferritin content (Figure 4D). This suggests that the remaining part of the ferritin pool is far from being saturated and provides a flexible reservoir for storage of excess iron.

The negative role of HFE in iron acquisition has been documented in different cell models, all involving non-physiological overexpression of HFE (wild-type or mutants). This view was recently challenged by data showing reversal of the HFE-dependent iron-deficient phenotype by co-expression of HFE together with β_2 M in TRVb-1 cells [35]. We examined the validity of this scenario in our model system and found that H1299 cells express endogenous β_2 M, which is sufficient to promote maturation and cell-surface expression of a fraction of chimeric HFE. Nevertheless, this fraction is significantly enriched after transfection of a β_2 M cDNA (Figures 5A and 5B). The contribution of exogenous β_2 M to HFE maturation is also evident in the pulse-chase experiment shown in Figure 6. It should be noted that the half-life of the precursor HFE remains largely unaffected by the presence of the transfected β_2 M (Figure 6).

Despite its clear effects in HFE biogenesis, exogenous β_2 M fails to antagonize HFE-induced iron deficiency in H1299 cells. Under our experimental conditions, HFE stimulates IRP activity, Tfr synthesis and accumulation, and inhibits ferritin synthesis, regardless of whether it is co-expressed with β_2 M (Figures 5A and 7). These results are in sharp contrast with those of Waheed et al. [35], where co-expression of β_2 M dramatically stabilized HFE in TRVb-1 cells. As a result, the HFE-induced increase in Tfr and decrease in ferritin expression were blunted, and this was associated with an enhancement in the rate of Tfr recycling. It should be noted that TRVb-1 cells are derived from Chinese-hamster ovary cells, lack endogenous Tfr and are stably transfected with a human Tfr cDNA. Experiments were performed with human HFE and β_2 M [35]. Conceivably, the discrepancies observed between TRVb-1 and H1299 cells could be attributed to variations in the experimental settings and/or species- and cell-type-related differences.

Our results provide some insights on the role of HFE and β_2 M in cellular iron metabolism. However, they do not give a clue as to how HFE controls iron homeostasis in the body, nor on the pathogenesis of HH. Probably, a key to understanding

mechanistic aspects of iron overload at the systemic level is the observation that the duodenal crypt cells of HH patients are iron-deficient [40,41]. These cells are precursors of mature absorptive enterocytes. Their iron status determines the capacity of mature enterocytes to transport dietary iron and deliver it to the circulation, by a process involving the apical and basolateral transporters DMT1 and ferroportin 1 respectively [42]. The crypt cells express TfR and HFE, and take up iron from plasma Tf. Recent experiments showed that this pathway is impaired in HFE knockout mice [43], in agreement with the results from HH patients.

The reticuloendothelial system is another important player in systemic iron metabolism because macrophages are instrumental in the recycling of iron from senescent erythrocytes [42]. Like duodenal crypt cells, the macrophages from HH patients are not only spared from iron overload, at least at the early stages of the disease, but in fact appear to be iron-deficient [44]. Interestingly, expression of wild-type HFE in monocytes from HH patients carrying the C282Y mutation resulted in the normalization of iron loading from Tf [45].

Taken together, the findings in duodenal crypt cells and macrophages support a view where HFE plays a positive role in the loading of these cells with iron. We speculate that whereas HFE inhibits iron uptake when overexpressed in cell culture models ([22,27,29–31] and the present study), it may perform the opposite function in highly specialized cells of the gastrointestinal tract and in macrophages. Along these lines, the unique intracellular and perinuclear staining of HFE in duodenal crypt cells as opposed to the membrane staining in epithelial cells of the oesophagus, stomach and colon is worth considering [46]. Conceivably, a cell-type-specific function of HFE may depend on the activity of other factors. A very good candidate is the recently described antimicrobial peptide hepcidin, which appears to function as a hormonal regulator for iron absorption [47,48].

Note added in proof (received 17 January 2003)

While this paper was under review, Drakesmith et al. [49] showed that HFE inhibits iron efflux from THP-1 monocyte/macrophage cells.

We thank Dr An-Sheng Zhang and Dr Caroline Enns for the β_2 M cDNA, Dr Young Yang for the 10G4 antibody, Franca Sicilia for technical assistance with FACS, and Dr Joan Buss, Dr Antonis Koromilas and Dr Prem Ponka for critical reading of the manuscript. J.W. holds a fellowship from the Canadian Institutes of Health Research (CIHR). K.P. is a scholar of CIHR and a researcher of the Canada Foundation for Innovation (CFI). This work was supported by a grant from the CIHR.

REFERENCES

- Andrews, N. C. and Levy, J. E. (1998) Iron is hot: an update on the pathophysiology of hemochromatosis. *Blood* **92**, 1845–1851
- Fleming, R. E. and Sly, W. S. (2002) Mechanisms of iron accumulation in hereditary hemochromatosis. *Annu. Rev. Physiol.* **64**, 663–680
- Feder, J. N., Gnirke, A., Thomas, W., Tsuchihashi, Z., Ruddy, D. A., Basava, A., Dormishian, F., Domingo, R. R., Ellis, M. C., Fullan, A. et al. (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat. Genet.* **13**, 399–408
- Feder, J. N., Tsuchihashi, Z., Irrinki, A., Lee, V. K., Mapa, F. A., Morikang, E., Prass, C. E., Starnes, S. M., Wolff, R. K., Parkkila, S. et al. (1997) The hemochromatosis founder mutation in HLA-H disrupts β_2 -microglobulin interaction and cell surface expression. *J. Biol. Chem.* **272**, 14025–14028
- Waheed, A., Parkkila, S., Zhou, X. Y., Tomatsu, S., Tsuchihashi, Z., Feder, J. N., Schatzman, R. C., Britton, R. S., Bacon, B. R. and Sly, W. S. (1997) Hereditary hemochromatosis: effects of C282Y and H63D mutations on association with β_2 -microglobulin, intracellular processing, and cell surface expression of the HFE protein in COS-7 cells. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12384–12389
- Olynyk, J. K., Cullen, D. J., Aquilia, S., Rossi, E., Summerville, L. and Powell, L. W. (1999) A population-based study of the clinical expression of the hemochromatosis gene. *N. Engl. J. Med.* **341**, 718–724
- Gochee, P. A., Powell, L. W., Cullen, D. J., Du Sart, D., Rossi, E. and Olynyk, J. K. (2002) A population-based study of the biochemical and clinical expression of the H63D hemochromatosis mutation. *Gastroenterology* **122**, 646–651
- Steiner, M., Ocran, K., Genschel, J., Meier, P., Gerl, H., Ventz, M., Schneider, M. L., Buttner, C., Wadowska, K., Kerner, W. et al. (2002) A homozygous HFE gene splice site mutation (IVS5+1 G/A) in a hereditary hemochromatosis patient of Vietnamese origin. *Gastroenterology* **122**, 789–795
- Zhou, X. Y., Tomatsu, S., Fleming, R. E., Parkkila, S., Waheed, A., Jiang, J., Fei, Y., Brunt, E., Ruddy, D. A., Prass, C. E. et al. (1998) HFE gene knockout produces mouse model of hereditary hemochromatosis. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2492–2497
- Levy, J. E., Montross, L. K., Cohen, D. E., Fleming, M. D. and Andrews, N. C. (1999) The C282Y mutation causing hereditary hemochromatosis does not produce a null allele. *Blood* **94**, 9–11
- Bahram, S., Gilfillan, S., Kühn, L. C., Moret, R., Schulze, J. B., Lebeau, A. and Schümann, K. (1999) Experimental hemochromatosis due to MHC class I HFE deficiency: immune status and iron metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13312–13317
- de Sousa, M., Reimao, R., Lacerda, R., Hugo, P., Kaufmann, S. H. E. and Porto, G. (1994) Iron overload in β_2 -microglobulin-deficient mice. *Immunol. Lett.* **39**, 105–111
- Rothenberg, B. E. and Volland, J. R. (1996) β_2 knockout mice develop parenchymal iron overload: a putative role for class I genes of the major histocompatibility complex in iron metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1529–1534
- Santos, M., Schilham, M. W., Rademakers, L. H. P. M., Marx, J. J. M., de Sousa, M. and Clevers, H. (1996) Defective iron homeostasis in β_2 -microglobulin knockout mice recapitulates hereditary hemochromatosis in man. *J. Exp. Med.* **184**, 1975–1985
- Fleming, R. E., Holden, C. C., Tomatsu, S., Waheed, A., Brunt, E. M., Britton, R. S., Bacon, B. R., Roopenian, D. C. and Sly, W. S. (2001) Mouse strain differences determine severity of iron accumulation in Hfe knockout model of hereditary hemochromatosis. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2707–2711
- Sproule, T. J., Jazwinska, E. C., Britton, R. S., Bacon, B. R., Fleming, R. E., Sly, W. S. and Roopenian, D. C. (2001) Naturally variant autosomal and sex-linked loci determine the severity of iron overload in β_2 -microglobulin-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 5170–5174
- Levy, J. E., Montross, L. K. and Andrews, N. C. (2000) Genes that modify the hemochromatosis phenotype in mice. *J. Clin. Invest.* **105**, 1209–1216
- Lebrón, J. A., Bennet, M. J., Vaughn, D. E., Chirino, A. J., Snow, P. M., Mintier, G. A., Feder, J. N. and Bjorkman, P. J. (1998) Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. *Cell* (Cambridge, Mass.) **93**, 111–123
- Parkkila, S., Waheed, A., Britton, R. S., Bacon, B. R., Zhou, X. Y., Tomatsu, S., Fleming, R. E. and Sly, W. S. (1997) Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13198–13202
- Waheed, A., Parkkila, S., Saarnio, J., Fleming, R. E., Zhou, X. Y., Tomatsu, S., Britton, R. S., Bacon, B. R. and Sly, W. S. (1999) Association of HFE protein with transferrin receptor in crypt enterocytes of human duodenum. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 1579–1584
- Feder, J. N., Penny, D. M., Irrinki, A., Lee, V. K., Lebron, J. A., Watson, N., Tsuchihashi, Z., Sigal, E., Bjorkman, P. J. and Schatzman, R. C. (1998) The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1472–1477
- Roy, C. N., Penny, D. M., Feder, J. N. and Enns, C. A. (1999) The hereditary hemochromatosis protein, HFE, specifically regulates transferrin-mediated iron uptake in HeLa cells. *J. Biol. Chem.* **274**, 9022–9028
- Salter-Cid, L., Brunmark, A., Li, Y., Leturcq, D., Peterson, P. A., Jackson, M. R. and Yang, Y. (1999) Transferrin receptor is negatively modulated by the hemochromatosis protein HFE: implications for cellular iron homeostasis. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 5434–5439
- Ponka, P. and Lok, C. N. (1999) The transferrin receptor: role in health and disease. *Int. J. Biochem. Cell Biol.* **31**, 1111–1137
- Bennett, M. J., Lebron, J. A. and Bjorkman, P. J. (2000) Crystal structure of the hereditary haemochromatosis protein HFE complexed with transferrin receptor. *Nature* (London) **403**, 46–53
- Gross, C. N., Irrinki, A., Feder, J. N. and Enns, C. A. (1998) Co-trafficking of HFE, a nonclassical major histocompatibility complex class I protein, with the transferrin receptor implies a role in intracellular iron regulation. *J. Biol. Chem.* **273**, 22068–22074
- Ramalingam, T. S., West, A. P., Lebron, J. A., Nangiana, J. S., Hogan, T. H., Enns, C. A. and Bjorkman, P. J. (2000) Binding to the transferrin receptor is required for endocytosis of HFE and regulation of iron homeostasis. *Nat. Cell Biol.* **2**, 953–957

- 28 Lebrón, J. A., West, Jr, A. P. and Bjorkman, P. J. (1999) The hemochromatosis protein HFE competes with transferrin for binding to the transferrin receptor. *J. Mol. Biol.* **294**, 239–245
- 29 Riedel, H. D., Muckenthaler, M. U., Gehrke, S. G., Mohr, I., Brennan, K., Herrmann, T., Fitscher, B. A., Hentze, M. W. and Stremmel, W. (1999) HFE downregulates iron uptake from transferrin and induces iron-regulatory protein activity in stably transfected cells. *Blood* **94**, 3915–3921
- 30 Corsi, B., Levi, S., Cozzi, A., Corti, A., Altamare, D., Albertini, A. and Arosio, P. (1999) Overexpression of the hereditary hemochromatosis protein, HFE, in HeLa cells induces an iron-deficient phenotype. *FEBS Lett.* **460**, 149–152
- 31 Roy, C. N., Carlson, E. J., Anderson, E. L., Basava, A., Starnes, S. M., Feder, J. N. and Enns, C. A. (2000) Interactions of the ectodomain of HFE with the transferrin receptor are critical for iron homeostasis in cells. *FEBS Lett.* **484**, 271–274
- 32 Hentze, M. W. and Kühn, L. C. (1996) Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8175–8182
- 33 Theil, E. C. and Eisenstein, R. S. (2000) Combinatorial mRNA regulation: iron regulatory proteins and iso-iron responsive elements (iso-IREs). *J. Biol. Chem.* **275**, 40659–40662
- 34 Roy, C. N., Blemings, K. P., Deck, K. M., Davies, P. S., Anderson, E. L., Eisenstein, R. S. and Enns, C. A. (2002) Increased IRP1 and IRP2 RNA binding activity accompanies a reduction of the labile iron pool in HFE-expressing cells. *J. Cell. Physiol.* **190**, 218–226
- 35 Waheed, A., Grubb, J. H., Zhou, X. Y., Tomatsu, S., Fleming, R. E., Costaldi, M. E., Britton, R. S., Bacon, B. R. and Sly, W. S. (2002) Regulation of transferrin-mediated iron uptake by HFE, the protein defective in hereditary hemochromatosis. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 3117–3122
- 36 Wang, J. and Pantopoulos, K. (2002) Conditional de-repression of ferritin synthesis in cells expressing a constitutive IRP1 mutant. *Mol. Cell. Biol.* **22**, 4638–4651
- 37 Caltagirone, A., Weiss, G. and Pantopoulos, K. (2001) Modulation of cellular iron metabolism by hydrogen peroxide. Effects of H₂O₂ on the expression and function of iron-responsive element-containing mRNAs in B6 fibroblasts. *J. Biol. Chem.* **276**, 19738–19745
- 38 Mueller, S. and Pantopoulos, K. (2002) Activation of iron regulatory protein-1 (IRP1) by oxidative stress. *Methods Enzymol.* **348**, 324–337
- 39 Ponka, P., Beaumont, C. and Richardson, D. R. (1998) Function and regulation of transferrin and ferritin. *Semin. Hematol.* **35**, 35–54
- 40 Pietrangelo, A., Rocchi, E., Casalgrandi, G., Rigo, G., Ferrari, A., Perini, M., Ventura, E. and Cairo, G. (1992) Regulation of transferrin, transferrin receptor, and ferritin genes in human duodenum. *Gastroenterology* **102**, 802–809
- 41 Pietrangelo, A., Casalgrandi, G., Quaglino, D., Gualdi, R., Conte, D., Milani, S., Montosi, G., Cesarini, L., Ventura, E. and Cairo, G. (1995) Duodenal ferritin synthesis in genetic hemochromatosis. *Gastroenterology* **108**, 208–217
- 42 Andrews, N. C. (2000) Iron homeostasis: insights from genetics and animal models. *Nat. Rev. Genet.* **1**, 208–217
- 43 Trinder, D., Olynyk, J. K., Sly, W. S. and Morgan, E. H. (2002) Iron uptake from plasma transferrin by the duodenum is impaired in the Hfe knockout mouse. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 5622–5626
- 44 Cairo, G., Recalcati, S., Montosi, G., Castrusini, E., Conte, D. and Pietrangelo, A. (1997) Inappropriately high iron regulatory protein activity in monocytes of patients with genetic hemochromatosis. *Blood* **89**, 2546–2553
- 45 Montosi, G., Paglia, P., Garuti, C., Guzman, C. A., Bastin, J. M., Colombo, M. P. and Pietrangelo, A. (2000) Wild-type HFE protein normalizes transferrin iron accumulation in macrophages from subjects with hereditary hemochromatosis. *Blood* **96**, 1125–1129
- 46 Parkkila, S., Waheed, A., Britton, R. S., Feder, J. N., Tsuchihashi, Z., Schatzman, R. C., Bacon, B. R. and Sly, W. S. (1997) Immunohistochemistry of HLA-H, the protein defective in patients with hereditary hemochromatosis, reveals unique pattern of expression in gastrointestinal tract. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2534–2539
- 47 Nicolas, G., Bennoun, M., Devaux, I., Beaumont, C., Grandchamp, B., Kahn, A. and Vaulont, S. (2001) Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 8780–8785
- 48 Nicolas, G., Bennoun, M., Porteu, A., Mativet, S., Beaumont, C., Grandchamp, B., Sirtio, M., Sawadogo, M., Kahn, A. and Vaulont, S. (2002) Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 4596–4601
- 49 Drakesmith, H., Sweetland, E., Schimanski, L., Edwards, J., Cowley, D., Ashraf, M., Bastin, J. and Townsend, A. R. (2002) The hemochromatosis protein HFE inhibits iron export from macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 15602–15607

Received 14 October 2002/13 November 2002; accepted 4 December 2002

Published as BJ Immediate Publication 4 December 2002, DOI 10.1042/BJ20021607