Expression of the two mRNA isoforms of the iron transporter Nrmap2/DMTI in mice and function of the iron responsive element

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Nramp2/DMT1 is a transmembrane proton-coupled Fe^{2+} transporter. Two different mRNAs are generated by alternative splicing; isoform I contains an iron responsive element (IRE), whereas isoform II does not. They encode two proteins differing at their C-terminal end and by their subcellular localization. IRE-mediated stabilization of isoform I mRNA is thought to stimulate DMT1 expression in response to iron deficiency. We have measured the two mRNAs by real-time quantitative PCR in several mouse tissues, in normal conditions or following injection of phenylhydrazine, a potent haemolytic agent. Isoform I mRNA is expressed in the duodenum and is induced by stimulation of erythropoiesis, whereas the non-IRE isoform is mostly induced in erythropoietic spleen. Surprisingly, both isoforms are highly expressed in the kidney and are not regulated by erythropoiesis. To evaluate the role of the IRE in regulating isoform I mRNA stability, in response to variations in cell iron status, several constructs were made in pCDNA3 with either a normal or a mutated IRE placed at the 3′ end of a stable mRNA. These constructs were transfected into HT29 cells and mRNAs were analysed after growing cells in the presence or absence of exogenous iron. There was no difference in the level of expression of the different messages, suggesting that the IRE does not regulate stability of isoform I mRNA. The half-life of the endogenous IRE-mRNA was also measured following actinomycin D addition in iron- or desferrioxamine-treated cells. Decay of the mRNA was very similar in both conditions. These results suggest that additional transcriptional regulations at the promoter level, or iron-dependent regulation of alternative splicing are likely to participate in the induction of isoform I mRNA by iron deficiency.

Key words: bivalent metal transporter 1, erythropoiesis, iron responsive element, mRNA stability, Nramp.

INTRODUCTION

Iron transport through biological membranes requires internalization of Fe(III)–transferrin (Tf) complexes bound to specific receptors, or active transport through membrane-bound divalent cation transporters belonging to different families of proteins, including the Nramp (natural resistance associated macrophage protein) family. The first *Nramp* gene identified in mammals was found to be associated with the *Bcg* locus in mice [1] and to confer resistance to experimental infections by intracellular parasites [2]. Nramp1 is only expressed in macrophages and neutrophils and its exact function is not yet fully elucidated [3]. Nramp2, now referred to as DMT1, was subsequently cloned by two different strategies and found to play a role in transport of Fe^{2+} through biological membranes [4, 5]. Proteins of the Nramp family are highly conserved throughout evolution. In *Arabidopsis thaliana*, there are five genes coding for Nramp proteins, which fall into two different classes inversely regulated by iron status of the cell [6]. *Saccharomyces cerevisiae* also expresses two Nramp homologues SMF1 and SMF2 which have been shown to transport Mn^{2+} [7] and Fe^{2+} [8]. By expression into *Xenopus* oocytes, mammalian DMT1 was shown to be a pH-dependent proton-coupled transporter of Fe^{2+}, as well as a transporter of Zn^{2+}, Mn^{2+} and several other bivalent metals [5]. A point mutation (G185R) in predicted transmembrane domain 4 in DMT1 in *mk* mice [4] and in Belgrade rats [9] is responsible for iron deficiency and hypochromic microcytic anaemia, resulting from a defect in intestinal iron absorption and impaired iron utilization in erythropoietic cells.

Mammalian DMT1 exists as two different isoforms resulting from alternative splicing of the 3′ terminal exons. Utilization of the proximal polyadenylation site present in exon 16 gives rise to isoform I mRNAs, which contains a putative iron responsive element (IRE) in its 3′ non-coding region. Alternative splicing using a 5′ splice acceptor site present in exon 16 and the 3′ splice site of exon 17 gives rise to isoform II mRNA devoid of IRE (Figure 1). The DMT1 isoforms also differ in their last C-terminal 18 (isoform I) or 25 (isoform II) amino acids and are found in different subcellular compartments. Although it is not yet clear whether the two isoforms can co-exist within the same subcellular compartment, it has been clearly demonstrated that in duodenal enterocytes, isoform I is present at the apical side of the cells [10] whereas isoform II was found to co-localize with recycling endosomes in several non-intestinal cell lines [11,12]. Isoform II has also been found in nuclei of sympathetic neurons [13]. Expression of the IRE-containing isoform is highly inducible by iron deficiency, at least in duodenal cells [10,14]. In genetic haemochromatosis, where the patients have increased intestinal absorption as a results of a mutation in the HFE gene [15], isoform I mRNA is induced as compared to normal individuals, both at the mRNA and at the protein level [16]. In HFE mutant mice, contradictory results have been reported on the induction of DMT1 in duodenal enterocytes [17,18]. Based on its homology to the transferrin receptor (TfR) IREs and on its position in the 3′ non-coding region of the mRNA, it has been proposed, although never demonstrated, that the DMT1 IRE stabilizes the mRNA in response to iron deprivation. TfR mRNA half-life changes from 30–45 min in iron-replete cells, to more than 3 h in

Abbreviations used: CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRE, iron responsive element; IRP, iron regulatory protein; Nramp, natural resistance associated macrophage protein; TfR, Fe(III)-transferrin receptor.

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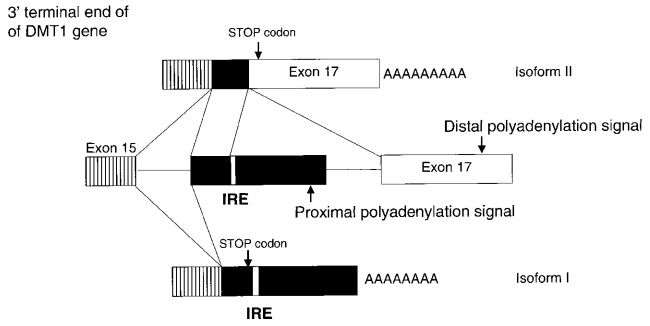


Figure 1 Schematic representation of the three terminal exons of human DMT1 gene and formation of the alternative splicing leading to the two isoform mRNAs

Isoform I mRNA results from pre-mRNA termination and polyadenylation at the end of exon 16. Alternative splicing using the 5′ splice acceptor site present in exon 16 and the 3′ donor site of exon 17 gives rise to isoform II mRNA.

cells treated with an iron chelator [19]. The 3′ untranslated region of TfR mRNA contains a rapid turnover determinant and five IREs that mediate these changes in mRNA stability [20]. Iron-mediated destabilization of the TfR mRNA requires a minimum of three of these IREs. Binding of iron regulatory proteins (IRPs) to these IREs when the cell iron status is low results in stabilization of the mRNA, whereas iron-mediated inactivation of the IREs leads to its rapid degradation. A similar mechanism might operate to regulate DMT1 mRNA in conditions of iron deficiency.

The aim of our work was to study the tissue-specific distribution of the two DMT1 isoform mRNAs in normal mice or following stimulation of erythropoiesis, and to study the function of the putative IRE present in the 3′ non-coding region of isoform I mRNA in iron-mediated regulation of DMT1 expression.

MATERIALS AND METHODS

Animals

Normal DBA2 × C57Bl6 mice were used for tissue mRNAs quantification. C57Bl6 × 129SV mice were used for quantification of DMT1 mRNAs following stimulation of erythropoiesis. Three intraperitoneal injections of phenylhydrazine (80 μg each), a potent haemolytic agent, were performed on three consecutive days. Animals were killed two days after the final injection. In these conditions, the spleen becomes highly erythropoietic.

Cell cultures

Human HT29 colon carcinoma cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Iron supplementation was obtained by growing the cells in the presence of ferric ammonium sulphate (100 μM) for 24 h, and iron depletion by growing the cells in the presence of desferrioxamine (100 μM) for the same period of time. To assess DMT1 mRNA stability, iron-or desferrioxamine-treated cells were washed once, resuspended in fresh medium in the presence of 0.4 μM actinomycin D (Sigma) and harvested at different times following addition.

RNA extraction and reverse transcription

Total RNA was extracted from mouse organs or from cultured cells using RNA Plus Extraction Solution Kit (Quantum Biotechnologies, Illkirch, France). The reverse transcriptase reaction was performed using 2 μg of total RNA, random hexamers and the Reverse Transcription Kit (Invitrogen Life Technologies, Cergy-Pontoise, France).

Real-time quantitative PCR

The two DMT1 mRNAs were measured by real-time quantitative PCR method using an ABI PRISM 7700 instrument (PE Applied Biosystems, Courtaboeuf, France), in several mouse tissues from both normal and phenylhydrazine-treated mice. PCR primers were designed using the Primer Express Software (PE Applied Biosystems). After reverse transcription, a quantity of cDNA corresponding to 20 pg of total RNA was amplified by PCR. For each sample, three distinct amplifications were carried out in parallel, in order to amplify DMT1 isoform I cDNA, isoform II cDNA, and the ribosomal protein S14 cDNA as a reference gene. The primers used for amplification are indicated in Table 1. The SYBR® Green PCR Core Reagents Kit (PE Applied Biosystems) was utilized for fluorescent detection of cDNA. For each tissue, DMT1 mRNA values were normalized to the mean value of S14 mRNA calculated from six independent determinations in the corresponding tissue.

For quantification, we used Standard Curve Method (User Bulletin 2, ABI PRISM 7700 Sequence Detection System). Briefly, for each DMT1 isoform and for ribosomal protein S14, an absolute standard curve was obtained by blotting the cycle of threshold (Ct) obtained following PCR amplification of serial dilutions of a known quantity of a plasmid containing the corresponding cDNA. Plasmid concentration was deduced from the absorbance at 260 nm, and converted into the number of copies using the molecular mass of the DNA. Ribosomal protein S14 mRNA was used as reference gene and results are expressed as number of DMT1 mRNA copies per 20 pg of total RNA. Each value is the mean of the results obtained for six different mice.

Real-time quantitative PCR was also used to measure isoform I mRNA in HT29 cultured cells at different times following actinomycin D addition. A different set of primers was used for PCR amplification (see Table 1) and the results were normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA in HT29 cells. Results are the mean of two independent DMTI mRNA quantifications performed in duplicate for each of four culture experiments.

Table 1 Oligonucleotides used for real-time quantitative PCR

mRNA	Forward primer	Reverse primer
Murine S14 ribosomal protein	5′ CAGGACCAAGACCCCTGGA	5′ ATCTTCATCCCAGAGCGAGC
Murine DMT1 isoform I	5′ TGTTTGATTGCATTGGGTCTG	5′ CGCTCAGCAGGACTTTCGAG
Murine DMT1 isoform II	5′ TCCTGGACTGTGGACGCTC	5′ GGTGTTCAGAAGATAGAGTTCAGG
Human DMT1 isoform I	5′ GGGCATGTCCTTCCTGGACT	5′ GGTGTTCAGAAGATAGAGTTCAGGC
Human GAPDH	5′ GAAGGTGAAGGTCGGAGTC	5′ GAAGATGGTGATGGGATTTC

Gel retardation assay

A 61 bp fragment containing the mouse DMT1 IRE was generated by PCR using the following oligonucleotides: forward primer 5′GTCTTTGCAGGGAGCCATC; reverse primer 5′TGGCTATGTTCACACAGTAAACC. The PCR product was subcloned into the pGEM-T vector. The construct was linearised by *Not*1 and used as a template to generate a [^{32}P]sense RNA probe. A human H ferritin IRE probe was synthesized from pIL2-CAT as previously described [21] and used as a positive control. Gel retardation assays were performed using 3×10^5 cpm of either the H ferritin or the DMT1 probe and 5 μg mouse macrophage cytoplasmic extracts. Molar excess of unlabelled DMT1 or H ferritin IRE were used as competitors.

DNA constructs

An initial construct was made in pcDNA3, containing the coding sequence of the human L ferritin subunit and the SV40 polyadenylation signal (pLFt). This construct was shown to express a stable message, in an iron-independent fashion. A small 60-nucleotide fragment, containing a normal (pLFt-IRE) or a mutated (pLFt-IRE mut) IRE sequence, was inserted downstream of the L ferritin stop codon and before the polyadenylation signal. The IRE fragment was generated by annealing two 39-base oligonucleotides, IRE-*Xho*I (5′ GGCCGCTCGAGCCATCAGAGCCAGTGTGTTTCTATGGTT) and IRE-*Xba*I (5′ GCTCTAGAGCTATGTTCACACAGTAAACCATAGAAACAC), which overlapped over 14 nucleotides. The non-overlapping regions were extended using Klenow fragment of DNA polymerase I and dNTPs. The resulting double-stranded fragment was digested by *Xba*I and *Xho*I and inserted into pLFt, downstream of the L ferritin stop codon.

To generate the mutated IREs, the IRE-*Xho*I oligonucleotide was annealed to IRE-mut1 (5′ GCTCTAGAGCTATGTAGACACAGTAAACCATAGAAACAC) which introduced a GAA $\rightarrow$ GCT mutation into a GAA consensus signal, identified by homology with an endonuclease cleavage site from the TfR mRNA (see Figure 4), and the IRE-*Xba*I oligonucleotide was annealed to IRE-mut2 (5′ GGCCGCTCGAGCCATCAGAGCCGGTGTGTTTCTATGGTT) which introduced a A $\rightarrow$ G mutation in the IRE loop.

Transient transfections and ribonuclease protection assays

These pLFt-IRE constructs were transiently transfected into 60–70 %-confluent HT29 colon carcinoma cells by lipofection, using Fugen Transfection Reagent (Roche, Meylan, France) and 4 μg DNA. 24 h after transfection, the cells were treated with either ferric ammonium sulphate (100 μM) or desferrioxamine (100 μM) for a further 24 h.

For quantification of endogenous or transfected L ferritin mRNA, a cDNA fragment overlapping the human L ferritin ATG start codon was used to generate a 150 bp antisense RNA probe, using SP6 polymerase in the presence of [^{32}P]UTP. Total RNA (5 μg) from transfected or control non-transfected cells was hybridized with 3×10^5 cpm of each probe in 80 % formamide hybridization buffer overnight at 55 °C. Following RNase A, RNase T1 and Proteinase K digestion, the protected fragments were separated on a denaturating 8 % polyacrylamide gel. The endogenous L ferritin mRNA was protected over 129 bases, whereas the mRNA transcribed from the transfected plasmid was protected over 75 bases. Radioactivity associated with the bands was quantified using an Instant Imager (Packard Instruments, Rungis, France). The radioactivity in the transfected mRNA was normalized by that of the endogenous L ferritin mRNA, which was assumed to be constant.

Statistical analysis

Statistical significance was evaluated using the non-parametric Mann–Whitney test for comparisons between two groups. A value of $P < 0.05$ was considered as significant. GraphPad Prism software (GraphPad Software, San Diego, CA) was used for statistical evaluation.

RESULTS

Tissue-specific distribution of DMT1 mRNAs

To assess the tissue-specific distribution of the two DMT1 mRNAs, we performed real-time quantitative PCR measurements on total RNA from several mouse tissues. For each tissue, three distinct amplifications were carried out, in parallel, to amplify both DMT1 isoforms and S14 ribosomal protein mRNA as a reference gene. Although this reference mRNA was initially considered as having the same level of expression in all tissues, it became rapidly evident that this was not the case. The amount of mRNA encoding the S14 ribosomal protein clearly represented a broad range of tissue-specific levels (Table 2). Therefore, for each tissue, we chose to normalise the DMT1 mRNA levels by the average of the values obtained for S14 mRNA from 6–12 independent PCR amplifications for the corresponding tissue.

The results obtained for DMT1 mRNAs show that both isoforms are ubiquitously expressed, with 2- to 5-fold variations in the level of expression between the different organs. Surprisingly, both isoforms are 10–15 times more abundant in the kidney than in other organs (Figure 2). Although differences were not always significant, due to experimental variability, the non-IRE isoform II was significantly higher in the kidney than in heart, duodenum and bone marrow. The IRE-containing isoform I mRNA was significantly higher in the kidney than in lung, spleen, bone marrow and testis.

Both iron deficiency and active erythropoiesis can stimulate intestinal iron absorption. Since DMT1 mRNA, and especially isoform I, is known to be induced by iron deficiency in duodenal enterocytes, we tested the effect of increased erythropoiesis in conditions of normal iron stores on DMT1 expression. DMT1 mRNAs were measured using an ABI PRISM 7700 instrument in duodenum, kidney and spleen from six phenylhydrazine-treated mice and six control mice (Figure 2). Phenylhydrazine is a potent haemolytic agent that induces spleen erythropoietic activity. The genetic background of these mice was mixed C57Bl6 $\times$ 129SV and differed from the one used in the previous experiment. The

Table 2 S14 mRNA levels in different mouse tissues

Results are the means $\pm$ S.E.M. of separate measurements performed on 6–12 mice.

Tissue	mRNA level (copies per 20 pg of total RNA)
Duodenum	142 $\pm$ 39.5
Liver	2543 $\pm$ 154
Heart	2817 $\pm$ 192
Lung	1738 $\pm$ 827
Kidney	2930 $\pm$ 559
Brain	2797 $\pm$ 931
Spleen	3410 $\pm$ 380
Bone marrow	86 $\pm$ 20
Thymus	709 $\pm$ 414
Testis	2743 $\pm$ 143

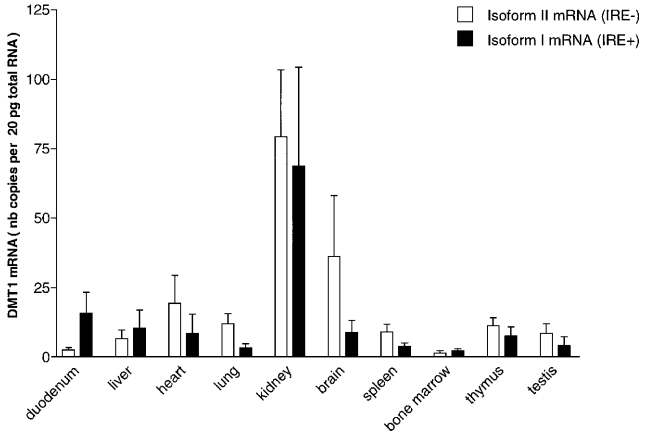


Figure 2 Real-time PCR quantification of DMT1 mRNAs in several mouse tissues

Several organs were excised from six normal mice, of congenic inbred strain DBA2 × C57Bl6, and total RNA was extracted using RNA Plus extraction kit. Real-time PCR was performed using oligonucleotides specific for each mRNA isoform and S14 mRNA was amplified as a reference gene. SYBR® Green was used for fluorescent detection of cDNA and PCR amplification was followed in real time in a ABI PRISM 7700 instrument. DMT1 quantities were normalized to the amount of S14 mRNA for the corresponding tissues (values are shown in Table 2) and are expressed as number (nb) of copies per 20 pg total mRNA. Results are the mean of duplicate measurements performed for six mice. For isoform II mRNA, significant differences were found in kidney versus duodenum ($P = 0.028$), heart ($P = 0.0022$) or bone marrow ($P = 0.028$). For isoform I, significant differences were found in kidney versus lung ($P = 0.028$), spleen ($P = 0.028$), bone marrow ($P = 0.028$) or testis ($P = 0.028$).

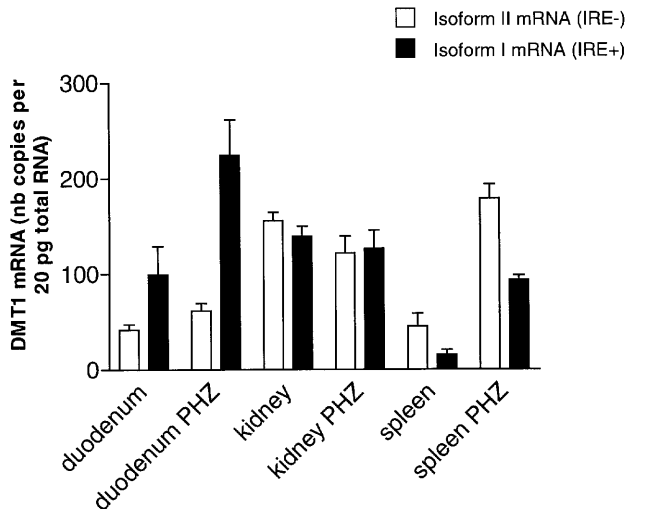


Figure 3 Real-time PCR quantification of DMT1 mRNAs in phenylhydrazine-treated mice

Three intraperitoneal injections of 80 μg of phenylhydrazine were performed on three consecutive days. Six normal and six treated mice of mixed C57Bl6 × 129SV genetic background were killed two days after the final injection. Kidney, spleen and duodenum were excised and total RNA was extracted using a RNA Plus kit. DMT1 mRNAs were quantified by real-time PCR as described in Figure 2. Phenylhydrazine treatment (PHZ) induced a significant increase in isoform II mRNA in spleen ($P = 0.0022$) and in isoform I mRNA in duodenum ($P = 0.0026$).

DMT1 mRNA values in these non-treated mice were much higher than those obtained in the DBA2 × C57Bl6 congenic inbred strain (Figure 2), although the relative proportion between the two isoforms remained the same. However, stimulation of

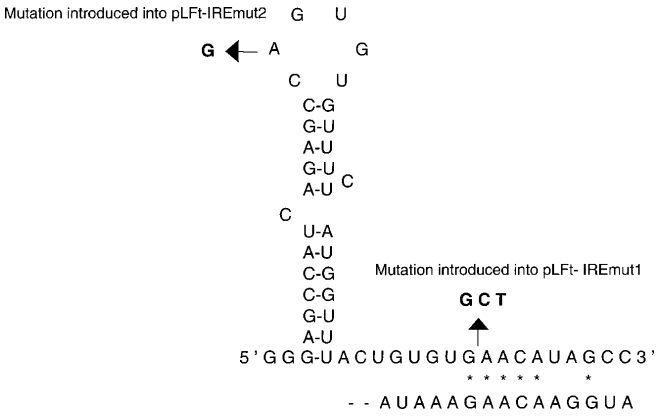


Figure 4 Predicted structure of the IRE present in the 3′ non-coding region of DMT1 isoform I mRNA

The nucleotide sequence with the predicted stem-loop structure of the IRE is shown. The endonucleolytic cleavage site present in the transferrin receptor mRNA is shown and conserved nucleotides with the sequence immediately downstream of the DMT1 IRE are identified with asterisks. Mutations that have been introduced in the constructs shown on Figure 6 are also indicated.

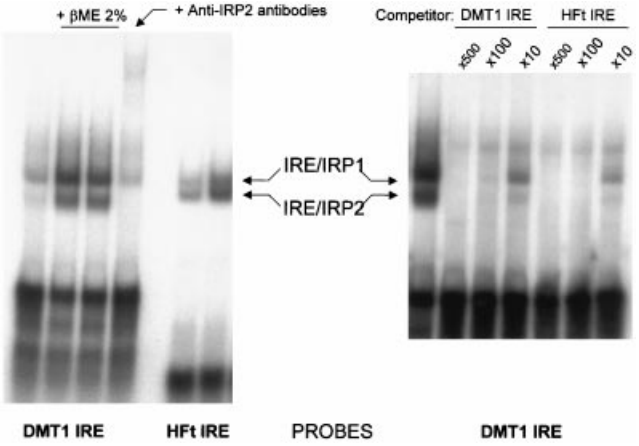


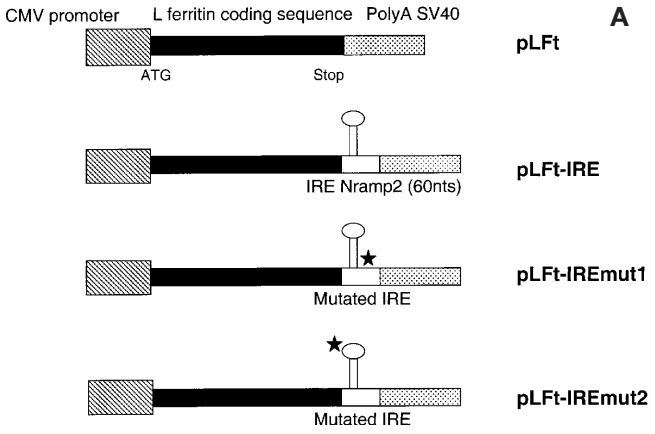
Figure 5 Gel retardation assays of ferritin and DMT1 IRE

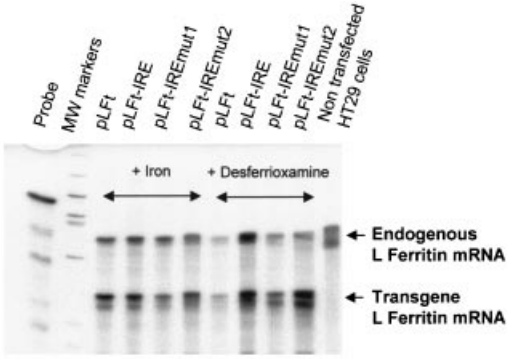
Gel retardation assays were performed using either the H ferritin (HFt) IRE or the DMT1 IRE as probe with cytoplasmic extracts from mouse cell lines. Both probes can form complexes with IRP1 and IRP2. Identity of the IRE/IRP2 complex was ascertained using anti-IRP2 antibodies which specifically supershifted this complex. Relative affinity of the ferritin IRE and the DMT1 IRE to bind IRPs was tested by competition experiments using the corresponding unlabelled probe in molar excess. βME, β-mercaptoethanol.

erythropoiesis following acute haemolysis significantly increased the expression of the IRE-containing mRNA in the duodenum and of the non-IRE isoform in erythropoietic spleen (Figure 3). This is in agreement with the respective putative function of DMT1 in these two tissues. Treatment with phenylhydrazine has no effect on either isoform mRNA in the kidney.

In vitro functional studies of the DMT1 IRE

The structure of the putative IRE found in the 3′ non-coding region of isoform I mRNA is depicted in Figure 4, together with the mutations introduced into the pLFt constructs used in Figure 6. It has been proposed that this IRE could stabilize DMT1





Quantification of transgene mRNA using an Instant Imager

	pLFt	pLFt-IRE	pLFt-IREmut1	pLFt-IREmut2
Iron	16800	13800	10400	14400
Desferrioxamine	14300	12400	18500	22400

Figure 6 Transfection of IRE-containing constructs and quantification of mRNA by ribonuclease protection assay

(**A**) Schematic representation of the DNA constructs used in transient transfections. The L ferritin coding sequence was inserted downstream of the CMV promoter in pCDNA3 vector and this construct was named pLFt. A 60 nucleotide (nts) double-stranded oligonucleotide overlapping the DMT1 IRE was inserted between the ferritin coding sequence and the SV40 polyadenylation sequence, to generate pLFt-IRE construct. Oligonucleotides containing a mutation in the endonucleolytic cleavage site (pLFt-IREmut1) or in the IRE loop (pLFt-IREmut2) were also inserted in place of the normal IRE. The mutations are shown in Figure 4. (**B**) Ribonuclease protection assay of HT29 transfected cells, following treatment with iron or desferrioxamine. Transfected HT29 cells were grown for 24 h in the presence of either iron (ferric ammonium citrate 100 μM) or desferrioxamine (100 μM) prior to RNA extraction and quantification. Total RNA (5 μg) was hybridized to 3×10^5 cpm antisense RNA probe. Following hybridization and RNAse digestion, the protected fragments were separated on a denaturing 8% polyacrylamide gel. A single protected fragment is observed in non-transfected cells corresponding to the endogenous L ferritin mRNA. A smaller protected fragment is also visible in transfected cells, corresponding to the mRNA transcribed from the transfected construct. Radioactivity of the protected fragment was measured in an Instant Imager. Relative values obtained for the transfected mRNA were normalised to the values for the endogenous mRNA and results are shown in the table below the gel.

mRNA in conditions of iron deficiency, by analogy with the regulation of TfR mRNA. In this study, we demonstrate that this IRE is functional *in vitro*. An RNA probe with the DMT1 IRE sequence can form complexes with both IRP1 and IRP2 in a gel retardation assay using mouse cytoplasmic extracts (Figure 5).

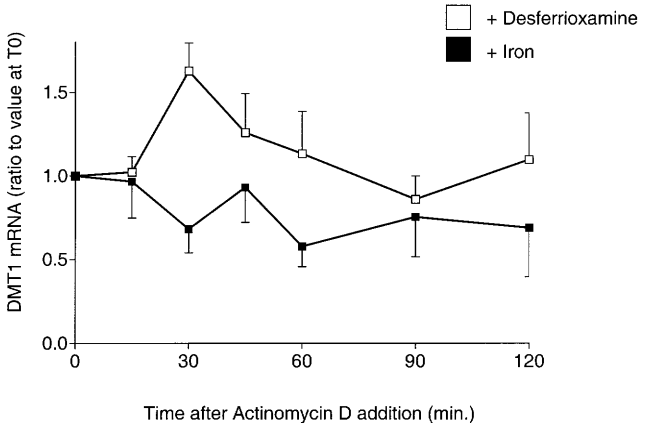


Figure 7 Half-life of DMT1 isoform I mRNA in HT29 cells

HT29 cells were cultured for 24 h in the presence of iron (ferric ammonium citrate, 100 μM) or desferrioxamine (100 μM). Cells were then washed, resuspended in fresh medium containing actinomycin D (0.4 μM) and harvested at different times following addition, for up to 2 h. Total RNA was extracted and endogenous DMT1 isoform I mRNA was measured by real-time quantitative PCR. Results were normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase measured in parallel. This mRNA was considered as stable within the time frame of the experiment. Values are expressed as a ratio to DMT1 mRNA at the time of actinomycin D addition and are the means of four independent culture experiments. For each culture, mRNA was quantified twice in duplicate. The means plus (desferrioxamine) or minus (iron) S.E.M. are shown.

Competition experiments using either DMT1 IRE or the H ferritin IRE show that these two IREs have similar affinity for IRP binding *in vitro*.

Role of IRE in the control of mRNA stability

To study the role of the DMT1 IRE in regulating mRNA stability following changes in the cell iron status, several DNA constructs were made and analysed by transient transfections.

The first construct (pLFt) contained the L ferritin coding sequence placed under the control of the cytomegalovirus (CMV) promoter (Figure 6A). The expression of this vector is not regulated by iron. We then inserted a 60-nucleotide fragment containing the normal IRE sequence (pLFt-IRE), or a mutated IRE (pLFt-IRE mut), between the L ferritin coding sequence and the polyadenylation signal.

One mutation (mut 1) abolishes a GAA motif located immediately 3′ of the stem-loop structure of the IRE and identical to an endonuclease-cleavage site identified in the TfR mRNA (Figure 4). The other mutation (mut 2) is an A → G change in the second position of the highly conserved 5′-CAGUG-3′ sequence of the IRE loop. The same mutation has been found in the L ferritin IRE in patients with the hereditary hyperferritinaemia-cataract syndrome and has been shown to abolish IRP binding to the IRE [22].

These constructs were transiently transfected into HT29 cells and the transfected cells were incubated in the presence of iron (ferric ammonium citrate, 100 μM) or desferrioxamine (100 μM) for 24 h prior to RNA analysis. The effect of changes in the cell iron status on endogenous or transfected ferritin mRNA synthesis was assessed by ribonuclease protection assay, using a L ferritin probe. This probe allowed simultaneous evaluation of the endogenous and the transgene L ferritin mRNAs (Figure 6B). Radioactivity associated with the protected fragments was quantified in an Instant Imager. Values for the bands corresponding to the transfected mRNA were normalised to those corresponding

to the endogenous L ferritin mRNA, used as an internal standard.

The results show that the presence of DMT1 IRE in the transgene mRNA did not change the level of expression, neither in conditions of iron depletion by desferrioxamine nor of iron supplementation (Figure 6B). Similar results were obtained by transfections into K562 cells (data not shown). In desferrioxamine-treated cells, there was no increase in the amount of transgene mRNA accumulating in pLFt-IRE transfected cells (12400 cpm), as compared to pLFt transfections (14300 cpm). Mutations that either abolished IRP binding or a putative endonuclease cleavage site had no effect on the level of transgene expression. Similarly, in iron-treated cells, both constructs, with or without IRE, had the same level of expression (13800 cpm versus 16800 cpm, respectively). This suggests that in our experimental conditions, the DMT1 IRE is not sufficient to confer iron-mediated regulation of mRNA stability to a reporter gene and apparently does not contain a rapid turnover determinant. Similar results were obtained when the entire 3′ non-coding region of the isoform I mRNA was inserted instead of just the IRE (results not shown).

Half-life of the isoform I mRNA

IRE motifs present in the 3′ non-coding region of the TfR mRNA are known to regulate the mRNA half-life in response to changes in iron status of the cells. The half-life of the IRE-containing mRNA was measured in HT29 colon carcinoma cells pre-treated for 48 h with either ferric ammonium citrate (100 μM) or desferrioxamine (100 μM). Measurements of DMT1 mRNA at different times following inhibition of transcription by actinomycin D showed that there was a slow decay of isoform I mRNA in iron-replete cells; 60% of the mRNA remained 120 min following inhibition of transcription. Surprisingly, in desferrioxamine-treated cells, there was an initial burst of isoform I mRNA following actinomycin D addition, followed by a progressive decay of the mRNA to the same rate as in iron-treated cells.

DISCUSSION

In this paper, we show that the level of expression of both DMT1 isoform mRNAs is tissue-specific and regulated by erythropoiesis. In addition, we show that the DMT1 IRE is functional *in vitro*, but, by using two different experimental approaches, we failed to demonstrate a role for the IRE in regulating mRNA stability in response to variations in cell iron status.

The most striking observation is the high level of expression of DMT1 in the kidney, where both isoforms are 10–15 times more abundant than in the other tissues tested. This is in agreement with what has been observed at the protein level. In duodenal enterocytes, the protein level is too low to be detected in normal conditions, and the protein is only seen in conditions of iron deficiency [10] or in anaemic *mk* mice [14]. This is consistent with the low level of expression we observe in the duodenum at the mRNA level. By immunohistochemistry using anti-DMT1 antibodies recognizing both isoforms, a strong immunostaining was observed in rat kidney, in both the cortex and outer medulla [23]. DMT1 protein was present intracellularly in the proximal tubules and at the apical side of cells in the distal convoluted tubules. Since we find that both isoforms are highly expressed in the kidney, it is possible that these two different intracellular distributions each correspond to different isoforms. In duodenal enterocytes, isoform I is only found at the apical side of the cells [10]. DMT1 function in kidney is not known but it could transport bivalent cations other than iron and participate in ion exchanges during urine formation.

We also observed that DMT1 mRNA levels differ with the genetic background of the mice, being 2–4 times higher in a mixed C57Bl6 × 129SV background (Figure 3) than in congenic inbred strain DBA2 × C57Bl6 (Figure 2). Variations in the level of expression of the DMT1 gene could contribute to the variability in tissue iron loads that has been observed between mice of different genetic background [24]. The ratio between the two isoforms, however, was the same in both experiments. Both isoforms were expressed at the same level in the kidney, whereas isoform I predominated in the duodenum and isoform II in the spleen.

Two signalling pathways regulate intestinal iron absorption. The 'store regulator' stimulates absorption in conditions of iron deficiency, and several proteins such as HFE [15] or hepcidin [25] are likely to participate in this regulation. The 'erythroid regulator' stimulates iron absorption following intra-medullar haemolysis or ineffective erythropoiesis, by some unknown mechanisms [26]. We wanted to assess the effect of erythropoiesis on DMT1 expression, in conditions of normal iron stores. We injected phenylhydrazine, a potent haemolytic agent, and followed DMT1 expression in duodenum, kidney and erythropoietic spleen. There was no change in either isoform expression in the kidney, whereas isoform I was induced in the duodenum and isoform II in the spleen. Induction of isoform I is likely to contribute to the increase in duodenal iron uptake which has been described following phenylhydrazine injection [27]. This pattern of expression correlates with the putative role of isoform I protein in iron uptake from the lumen by duodenal enterocytes, and putative role of isoform II in transfer of endosomal iron into the cytoplasm in erythroid cells. These results suggest that both tissue specificity and erythropoiesis regulate DMT1 transcription and alternative splicing.

The role of the IRE in isoform I mRNA stability is more difficult to elucidate. The folding of the DMT1 IRE is very similar to that of the TfR IRE, except for an additional unpaired cytosine on the 3′ descending part of the stem (Figure 4). Although this additional cytosine might destabilize the stem, the results obtained in the present study and by others [28] have shown, using gel retardation assay, that this IRE is functional *in vitro* and presents specific and high affinity binding for both IRP1 and IRP2.

Using two different experimental approaches, however, we failed to prove the IRE function in mediating mRNA decay in response to iron supplementation. Endonucleolytic cleavage of the 3′ untranslated region, rather than poly (A) tail shortening, has been found to be the first degradation step for a number of mRNAs. Examples are the mRNAs encoding TfR [20], the insulin-like growth factor II [29], or several short-lived mRNAs containing AUUA-rich motifs in their 3′ untranslated region [30]. The endonucleases that are involved in this degradation have not been identified. Conserved stem-loop structures are usually found upstream of the cleavage site and thought either to protect the mRNA from degradation [20] or to stabilize the cleavage site and favour endonucleolytic cleavage [29]. Site-specific cleavage of the TfR mRNA by endoribonuclease requires a minimum of three IREs and a GAA motif downstream of the most distal IRE. This GAA motif is also found downstream of the DMT1 IRE and we hypothesized that it could regulate DMT1 mRNA stability. In order to gain insight into the ability of the IRE, including the putative conserved cleavage site, to destabilize an exogenous mRNA in response to iron, we transfected several constructs into HT29 cells. A 60-nucleotide fragment corresponding to the IRE, was inserted downstream of the L ferritin coding sequence in a pCMV expression vector. Mutations were introduced in the IRE loop or in the endonucleo-

lytic cleavage site and their ability to prevent mRNA degradation was tested. The level of expression of the various constructs was compared in iron-treated or desferrioxamine-treated cells. Presence of the IRE, with either a normal or a mutated sequence, did not increase the amount of transgene mRNA present in conditions of iron deprivation as compared with non-IRE-containing transgene. Similarly, the IRE did not destabilize the reporter mRNA in conditions of iron supplementation. Since a specific RNA recognition site can be dependent on a large surrounding RNA region, we also tested the effect of the entire DMT1 3′ non-coding region in modulating mRNA stability, but still observed no effect. In addition, the half-life of the endogenous DMT1 IRE-containing mRNA appeared to be very similar in both iron supplementation and iron deficiency. It is noteworthy that IREG1 [31], also named ferroportin [32] or MTP1 [33], is a Fe^{2+} iron transporter expressed at the baso-lateral side of enterocytes that is also induced by iron deficiency. IREG1/ferroportin/MTP1 mRNA has an IRE structure in its 5′ non-coding region, which would be expected to down-regulate protein synthesis in response to iron deficiency by similarity with the ferritin IRE [19]. It is tempting to speculate that following depletion of iron stores the same mechanism would contribute to up-regulation of both DMT1, which imports iron at the apical side of enterocytes, and ferroportin, which exports iron at the baso-lateral side. This mechanism, which could be either transcriptional or post-transcriptional, remains to be identified.

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