Iron-dependent regulation of the divalent metal ion transporter

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Abstract The first step in intestinal iron absorption is mediated by the H^+-coupled Fe^{2+} transporter called divalent cation transporter 1/divalent metal ion transporter 1 (DCT1/DMT1) (also known as natural resistance-associated macrophage protein 2). DCT1/DMT1 mRNA levels in the duodenum strongly increase in response to iron depletion. To study the mechanism of iron-dependent DCT1/DMT1 mRNA regulation, we investigated the endogenous expression of DCT1/DMT1 mRNA in various cell types. We found that only the iron responsive element (IRE)-containing form, which corresponds to one of two splice forms of DCT1/DMT1, is responsive to iron treatment and this responsiveness was cell type specific. We also examined the interaction of the putative 3′-UTR IRE with iron responsive binding proteins (IRP1 and IRP2), and found that IRP1 binds to the DCT1/DMT1-IRE with higher affinity compared to IRP2. This differential binding of IRP1 and IRP2 was also reported for the IREs of transferrin receptors, erythroid 5-aminolevulinate synthase and mitochondrial aconitase. We propose that regulation of DCT1/DMT1 mRNA by iron involves post-transcriptional regulation through the binding of IRP1 to the transporter's IRE, as well as other as yet unknown factors. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Divalent cation transporter 1; Divalent metal ion transporter 1; Natural resistance associated macrophage protein 2; Iron responsive element; Iron regulatory protein; Iron

1. Introduction

Iron is an essential nutrient in all living cells, yet it is toxic when present in excess due to the generation of reactive oxygen species [1,2]. Human iron metabolism is dependent on efficient cycling of iron from senescent to newly formed red cells [3], and less than 0.05% of the total body iron is acquired or lost each day, demonstrating the effectiveness with which iron is conserved. Therefore, iron levels in the body are tightly controlled via regulation of intestinal absorption, circulating erythrocytes, and storage in bone marrow, liver and spleen.

Recently, we identified divalent cation transporter 1/divalent metal ion transporter 1 (DCT1/DMT1) as the major intestinal iron absorptive mechanism [4,5]. It is a widely expressed H^+-coupled divalent metal ion transporter that accepts a variety of divalent metal ions [4]. It is strongly expressed in the duodenum where it mediates apical intestinal iron uptake [4,6]. In rat duodenum, DCT1/DMT1 mRNA is expressed primarily in the lower segments of the villi but not at the villus tips or in the deep crypt. Its mRNA levels increased 50–100-fold in rats kept on an iron-deficient diet for 3 weeks [4], indicating that this metal ion transporter plays a crucial role in body iron homeostasis. Crypt cells, which take up iron from the blood via the transferrin receptor (TfR), are known to be the body iron sensors/responders. The current concept is that, in response to increased body iron demand, crypt cells may be 'programmed' to upregulate this transporter's expression as they mature to become villus cells [24–26]. As part of this regulatory mechanism, cellular accumulation of iron may lead to dissociation of iron regulatory protein (IRP) from the DCT1/DMT1-IRE (iron responsive element), resulting in degradation of its mRNA to cease intestinal iron absorption.

Two spliced products were identified for DCT1/DMT1 in several species [4,5,7]. One transcript contains a sequence that resembles an IRE within its 3′-UTR [4], whereas the alternatively spliced counterpart lacks this sequence and encodes a protein that is seven amino acids longer at the C-terminus [8].

The IRE-like sequence of the rat duodenal DCT1/DMT1 mRNA is predicted to fold into a stem–loop structure which resembles the IRE present in tandem in the 3′-UTR of the TfR mRNA, and as a single copy in the 5′-UTR of ferritin mRNAs [4]. Studies of TfR revealed that interaction of the TfR-IREs with IRP1 and IRP2 protects the transcript against endonucleolytic cleavage [9,10]. The binding of IRPs to the IRE in the 5′-UTR of ferritin mRNA leads to the suppression

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Abbreviations: DCT1, divalent cation transporter 1; DMT1, divalent metal ion transporter 1; IRE, iron responsive element; IRP, iron regulatory protein

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of translational initiation. In addition to both ferritin H- and L-chain mRNAs, functional IREs have been found in the 5′-UTR of other transcripts, including the mRNA of erythroid 5-aminolevulinate synthase (eALAS), mitochondrial aconitase (m-aconitase), subunit b of *Drosophila melanogaster* succinate dehydrogenase, and, as revealed recently, the basolateral iron transporter IREG1/Ferroportin1/MTP1 [11–19].

The previously characterized IREs share important sequence and structural similarities, or consensus elements, characterized by a hexanucleotide loop (CAGUGN), and a single unpaired or bulged C that is separated from the loop by five complementary base pairs. Mutational studies [20–23] revealed that the addition or subtraction of base pairs from this region or the disruption of base-pairing through mismatches greatly diminished the ability of IRP1 and IRP2 to bind to the RNA element. The initial interpretation was that the IRE hairpin loop contributes more to IRP1 binding, and the internal loop/bulge region contributes more to IRP2 binding [21,35], although further studies are needed to address this hypothesis.

Hereditary hemochromatosis (HH) is a common autosomal recessive disorder. Its major causes are missense mutations (mostly C282Y) in the gene encoding HFE, a major histocompatibility complex class I-like protein. In patients with HH, the crypt iron sensor/responder is thought to be disturbed, as suggested by findings in *HFE* knockout mice, in which DCT1/DMT1 is upregulated, despite high serum and liver iron levels [27]. Consistent with this, DCT1/DMT1 mRNA expression in duodenal mucosa is increased in HH patients homozygous for the C282Y mutation [28,40].

IRP1 activity in the mucosal cells at the villus tips and duodenal iron absorption from the lumen show parallel changes in iron deficiency [29]. Duodenal crypt cells may sense the iron supply from the blood side and adjust the IRP activity. IRPs have been found to be more active in duodenal cells in HH patients [30]. We hypothesize that IRP activity is closely related to iron absorption via DCT1/DMT1, and that it accounts for excessive iron absorption in patients with HH.

Differential splicing in the DCT1/DMT1 transporter gene may also be important in regulating iron homeostasis. Lee et al. demonstrated that both splice variants of DCT1/DMT1 are ubiquitously expressed at various levels and ratios in human tissues. The brain appears to express the highest ratio of IRE to non-IRE form, and spleen, thymus and pancreas the highest ratio of non-IRE to IRE form [7].

The present study was designed to test the role of the IRE/IRP system in controlling DCT1/DMT1 expression. We have investigated the ability of the DCT1/DMT1-IRE to bind to IRP1 and IRP2, and studied the iron-dependent mechanism of regulation of DCT1/DMT1 mRNA at the transcriptional and post-transcriptional levels in Caco-2 (intestinal cells) and Hep3B (liver cells) and in HeLa cells.

2. Materials and methods

2.1. Enzymatic preparation of RNA oligonucleotides for gel-shift analysis

The RNAs used in this study were prepared enzymatically by transcribing from DNA templates with T7 RNA polymerase using a published method [20]. The oligodeoxynucleotide templates, which were purchased commercially (Operon), each included both the bottom strand sequence of the 17-nucleotide T7 RNA polymerase promoter and IRE sequence to generate either of the following RNA sequences: ferritin H-chain (Fer) IRE (5′-GGG AGA GGA UCC UGC UUC AAC AGU GCU UGG ACG GAU CC-3′), a 38-nucleotide IRE having a sequence derived from the human Fer IRE in its 5′-UTR, and DCT1/DMT1 (5′-GGG AGA AGC CAU CAG AGC CAG UGU GUU UCU AUG GUU-3′), a 36-nucleotide sequence derived from the non-consensus IRE found in the 3′-UTR of both human and rat DCT1/DMT1. The condition of the experiment was shown in ref. [20].

2.2. RNA gel-shift assays

All competition reactions were prepared as follows: ^{33}P-labelled wild-type Fer IRE at a final concentration of 36 nM was mixed with either a 1-, 2-, 5-, 10-, or 100-fold excess (36, 73, 182, 364, or 3640 nM) of unlabelled competitor RNA (either Fer IRE or DCT1/DMT1-IRE) in the presence of 4.5% (v/v) glycerol, 0.1 mg/ml yeast tRNA (non-specific competitor), 3 mM DTT, 1 U/ml RNase Inhibitor (5 Prime 3 Prime, Inc.), 40 mM KCl, 20 mM Tris–HCl (pH 7.5), and 18–30 nM IRP1 (Fig. 2A) or IRP2 (Fig. 2B). The experiment and the calculation of the dissociation constant (K_d) for the IRE–IRP interaction are described in ref. [20].

2.3. Northern analysis

Endogenous DCT1/DMT1 and transfected DCT1/DMT1 in Caco2 and/or Hep3B cells were analyzed by Northern blot analysis. Poly $(A)^+$ RNA (2–3 μg each well) was separated on a 1% formaldehyde/1% agarose gel and blotted onto a Nylon transfer membrane. Digoxigenin (DIG)-labelled anti-sense transcripts were synthesized using DIG RNA labelling kit (Roche) from a PCR fragment for the probes. A 777-bp fragment (bases 936–1713, AF008439) was used to detect both isoforms of human DCT1/DMT1 on resulting Northern blots. Hybridization and washing conditions using ^{32}P-labelled probes were as described previously [4]. For DIG-labelled Northern analysis, hybridization was at 68°C for overnight, and filters were processed according to the manufacturer's instructions (CDP Star, Roche).

2.4. Cell culture and transient transfections

Caco2 cells (AATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 μmol/l non-essential amino acids, 100 U/l penicillin, 100 μg/l streptomycin and 1 mM sodium pyruvate. Hep3B cells were cultured in α-MEM instead of DMEM, under the same conditions. Cells were plated and grown for an additional 3 days before any further treatment [100 μM hemin, 100 μg/ml ferric ammonium citrate (FAC; Sigma), or 100 μM desferrioxamine (Df; Sigma) for 16–18 h]. Transfections were performed using FuGENE 6 (Roche) or Lipofectamine (Gibco BRL) according to the manufacturers' protocol. After transfection, cells were treated as described above.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using the 'RNAClean' reagent (Hybaid) and was further purified through an 'RNeasy' mini column (Qiagen). To remove residual DNA from the samples, a DNase I digestion was performed using the Qiagen RNase-free DNase set. First strand of cDNA was obtained using the Superscript Reverse Transcription Kit (Gibco BRL). As a negative control, a sample without reverse transcriptase was included. 1/20th of the cDNA mix was used as a template for the PCR amplification with Taq Polymerase (Perkin Elmer) at 95°C for 30 s, 55°C for 90 s, 72°C for 45 s. Samples were amplified for 22 cycles for endogenous DCT1/DMT1 and TfR cDNAs, and 20 cycles for GAPDH. Primer sets used were: for endogenous DCT1/DMT1, 5′-CAG ATG ACA GTG TTT CTG GA-3′ and 5′-GCA ATG GCT GAG CCA ATG AC-3′; for TfR, 5′-CGA TAA CAG TCA TGT GGA GA-3′ and 5′-AGT AAC TGT TGC AGC CTT AC-3′; for GAPDH, 5′-CCA TGG AGA AGG CTG GGG-3′ and 5′-CAA AGT TGT CAT GGA TGA CC-3′. The expected size for endogenous DCT1/DMT1 is 486 bp, TfR 525 bp, and GAPDH 195 bp. For the HeLa cell experiment, another primer pair for detecting endogenous DCT1/DMT1 was used. These primers were 5′-AAC CCA GCC AGA GCC AGG TA-3′ and 5′-CCC CCT TTG TAG ATG TCC AC-3′. The expected size for this fragment is 349 bp. Ethidium bromide-stained gels were scanned and quantified using the Fuji FLA2000 FluorImager. Data for DCT1/DMT1 mRNA levels were normalized with respect to GAPDH levels.

2.6. Promoter-deficient luciferase reporter gene

In order to investigate whether the DCT1/DMT1 promoter region is responsive to iron-dependent regulation, we have transfected the constructs containing the human DCT1/DMT1 promoter region subcloned into a promoter-deficient pGL3-Basic vector containing the luciferase reporter gene (Promega). As described in ref. [31], plasmid p(−3120/+145) Luc contains a 3.2-kb *Eco*RI–*Hin*dIII fragment comprising nucleotides −3120 to +15 of the DCT1/DMT1 promoter region. Similarly, p(−1849/+145) Luc, p(−714/+145) Luc, and p(−246/+145) Luc were generated by progressive deletion from p(−3120/+145) Luc (Fig. 4A). Hep3B and/or Caco2 cells were transiently transfected with luciferase constructs and β-galactosidase or Renilla luciferase constructs. After transfection, cells were treated with 100 μ Df or 200 μg/ml FAC for 20–48 h (Fig. 4B). Luciferase assays were performed according to the manufacturer's protocol and the data were normalized against β-galactosidase or Renilla luciferase activity (Promega).

2.7. Quantitative RT-PCR

We used the LightCycler PCR instrument (Roche). Selective amplification of the two DCT1/DMT1 splice forms was achieved by subtype-specific primers, and the oligonucleotide sequences and the details for RT-PCR were as described elsewhere (Rolfs et al., manuscript in review). The values for each sample were normalized to β-actin to account for differences in mRNA amount in the reactions.

2.8. Radiotracer uptake studies

Hep3B cells were cultured on 100-mm plates to about 70% confluency. Cells were transfected with mouse DCT1/DMT1-IRE or DCT1/DMT1 non-IRE forms which was constructed in the pMT2 vector. Two days after transfection, radiotracer uptake experiments were performed by incubating cells for 20 min in 10 ml standard uptake medium (100 mM NaCl, 10 mM HEPES and 1 mM ascorbic acid, pH 6.2) along with 10 μM [^{55}Fe]$FeCl_2$ (specific activity for $^{55}FeCl_3$ from NEN Life Science Products, Inc. was 40.4 mCi/mg) at 37°C. Cells were gently washed twice with ice-cold PBS, resuspended in 0.5 ml water, and then the 0.5 ml of the suspension was added to 10 ml of scintillation fluid for scintillation counting [36].

3. Results

As shown previously, DCT1/DMT1 mRNA is upregulated 50–100-fold in the duodenum of iron-deprived rats, compared to normal rats [4]. In all other tissues examined, we only observed about a ∼1.5–3-fold upregulation in iron deficiency, suggesting that the intestine is much more responsive to iron.

DCT1/DMT1 has a putative 3′-IRE which has consensus features [11–19] of an IRE, including a six-nucleotide motif in the stem–loop head (CAGUGN) and a single unpaired bulge C (Fig. 1). Interestingly, it contains an unusual unpaired U in the upper stem that could potentially distort the structure of the IRE and interfere with IRP binding. To determine whether the DCT1/DMT1-IRE is conserved among different species, we PCR-amplified and sequenced the corresponding IRE region from different species. The IRE is conserved in rat, mouse, rabbit, and human (Fig. 1), supporting the concept that the DCT1/DMT1-IRE may be involved in the regulation of this metal ion transporter via the IRE/IRP system.

Gel electrophoretic retardation assays were performed to study whether the IRE binding proteins IRP1 and IRP2 can interact in vitro with the DCT1/DMT1-IRE. Purified IRP1 or IRP2 was incubated with the ^{33}P-UTP-labelled RNA oligonucleotides containing IREs derived from the Fer 5′-UTR (Fig. 2). Binding was determined in the presence or absence of increasing amount of unlabelled IRE competitor sequence and the mixture was analyzed by gel retardation assays (Fig. 2). The experiments show that both IRP1 and IRP2 interact with the DCT1/DMT1-IRE, but the affinities of the interactions were lower than those of Fer IRE. IRP1 interacted with the DCT1/DMT1-IRE with higher affinity than IRP2. Based on competitor analysis, the K_d values for the DCT1/DMT1-IRE interactions were 91 pM for IRP1 and 750 pM for IRP2. In comparison, the K_d values for Fer IRE were 30 and 29 pM, respectively.

To further test the role of the IRE/IRP system in regulating DCT1/DMT1, we investigated whether the DCT1/DMT1 mRNA expressed endogenously in intestinal Caco2 or liver Hep3B cells is upregulated in response to iron depletion (Df addition) or diminished in response to iron overload (FAC addition). In experiments involving Caco2 cells, Df or FAC treatment was performed 10 days after the cells were fully confluent and differentiated. In experiments involving Hep3B cells, Df or FAC treatment was performed after the cells reached 80% confluence. The expected size of the human DCT1/DMT1-IRE and non-IRE forms are ∼4.1 and 3.9 kb [7], respectively. A distinct band at ∼4.0 kb was observed in Hep3B, Caco2 and HeLa cells (Fig. 3), particularly in the iron-deficient state. As is shown in Fig. 5A,B, the non-IRE form is expressed at much lower levels compared to the IRE form, indicating that the observed band must correspond to the IRE form. Fig. 3A,B shows substantial increases of the 4-kb band in the iron-depleted condition for both Caco2 and

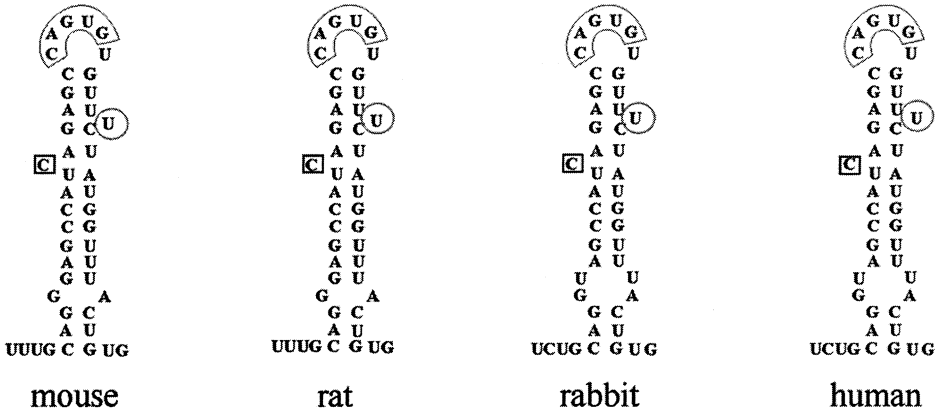

Fig. 1. Conservation of the IRE stem–loop structure in the 3′-UTR of DCT1/DMT1 mRNA among different species. In all of the five species examined, the stem–loop structure consists of a loop comprising five nucleotides, CAGUG, and an unpaired bulge C in the stem. The unpaired U, which is indicated by a circle, is unique in the DCT1/DMT1 IREs.

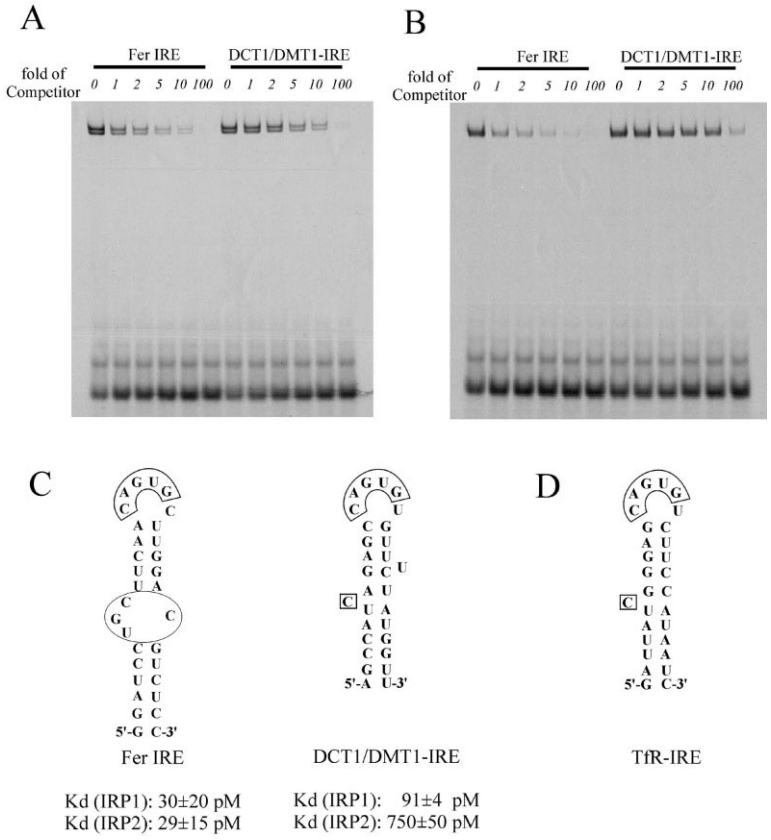

Fig. 2. Gel-shift assay to demonstrate that both IRP1 and IRP2 interact with the DCT1/DMT1-IRE. A: Competition-based gel-shift assay showing IRP1 binding to Fer IRE and DCT1/DMT1-IRE (see sequences in C). B: Competition-based gel-shift assay showing IRP2 binding to Fer IRE and DCT1/DMT1-IRE. ^{33}P-labelled wild-type Fer IRE (36 nM) was mixed with a 1-, 2-, 5-, 10-, or 100-fold (36, 73, 182, 364, or 3640 nM) excess of unlabelled competitor RNA (either Fer IRE or DCT1/DMT1-IRE). Experimental details are described in Section 2. C: The DCT1/DMT1-IRE and IRP1 interaction shows a higher affinity than the DCT1/DMT1-IRE and IRP2 interaction, and K_d values were 91 pM for IRP1 and 750 pM for IRP2, based on the band-shift competition assays using consensus Fer IRE. D: The TfR-IRE used in ref. [21] is shown. Parts C and D highlight the differences in IRE stem structure: the Fer IRE stem has an internal loop shown by the circle (C, left), whereas IREs of DCT1/DMT1 and TfR have simple bulge Cs (C, right, and D).

Hep3B cells, with the most dramatic responses for Caco2 cells (~50-fold increase when comparing Df and FAC treatments). Equal RNA loading was validated by either β-actin probing (Fig. 3A) or ethidium bromide staining of the 18S and 28S rRNA (Fig. 3B).

To evaluate the regulation of endogenously expressed DCT1/DMT1 in different cell types, we performed semi-quantitative PCR using probes which amplify both the IRE and non-IRE forms of DCT1/DMT1. Caco2 cells were treated with either Df (100 μM) or hemin (100 μM) for 16 h. A representative gel showing the alteration of the PCR products is shown in Fig. 3C. The levels of the endogenously expressed DCT1/DMT1 and TfR mRNAs in Caco 2 cells were ~10-fold upregulated after Df treatment, when comparing with hemin treatment (Fig. 3C). In contrast, HeLa cells showed a three- to five-fold change in TfR mRNA when comparing Df with hemin treatment, but no changes were observed for DCT1/DMT1 (Fig. 3D). This is in striking contrast to Caco2 cells. The data indicate that the DCT1/DMT1 response is cell type specific.

We also studied whether DCT1/DMT1 may be regulated at the transcriptional level. To address this question, we prepared a series of DCT1/DMT1 promoter–luciferase reporter constructs covering the promoter region up to nucleotide −1849 (Fig. 4A) and −3120 (data not shown). Analysis of the constructs in transfected Hep3B, HepG2 and HeLa cells treated with Df and FAC revealed slightly decreased promoter activity compared to no iron treatment (Fig. 4B). In two independent experiments we observed no response to iron treatment (Df versus FAC) (Fig. 4B and data not shown).

To verify which splice form of endogenous DCT1/DMT1 is iron responsive, we performed quantitative RT-PCR using subtype-specific primers (Fig. 5A). Only the IRE form of DCT1/DMT1 was responsive to iron both in Hep3B and Caco2 cells, and Caco2 cells exhibited a more dramatic iron responsiveness to iron depletion (Fig. 5A). Following Df treatment of Caco2 cells, the expression level of the IRE was ~400-fold higher compared to the non-IRE form. The difference between Df and FAC treatment of the IRE form is consistent with the data of the Northern analysis in Fig. 3A,B. In contrast, the non-IRE form of DCT1/DMT1 was expressed at much lower levels (Fig. 5B). In Hep3B control (no iron treatment), the expression level of the IRE form was almost 100-fold higher compared to the non-IRE form (Fig. 5A).

Given the large difference between the expression levels of the IRE and non-IRE forms, the question arises as to whether there is any functional difference between the two isoforms. Iron transport studies were performed using ^{55}Fe (Fe^{2+}) and Hep3B cells transfected transiently with cDNAs encoding the DCT1/DMT1-IRE or non-IRE forms. No difference in iron

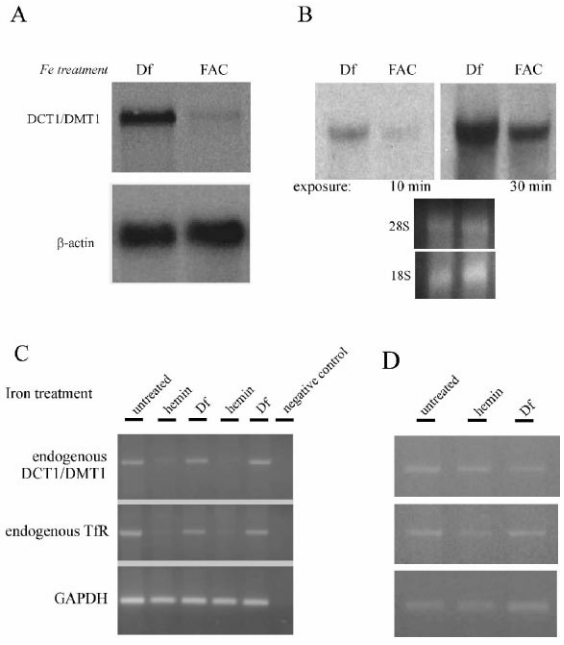

Fig. 3. Examination of DCT1/DMT1 regulation in culture by Northern analysis and RT-PCR. Response of endogenous DCT1/DMT1 mRNA levels to 100 μM Df and 100 μg/ml FAC in the Caco2 intestinal cell line (A) and Hep3B hepatoma cell line for 10 min and 30 min (B). β-Actin and ethidium bromide staining of rRNA were included as loading controls. Semi-quantitative PCR for the DCT1/DMT1-IRE form and TfR RNAs in Caco2 (C) and HeLa cells (D) treated with hemin or Df. GAPDH was measured as a control. In panel C, lanes 2 and 3 and lanes 4 and 5 represent duplicate experiments.

uptake was observed for the two isoforms (Fig. 5C). We also found no difference in subcellular localization in these cells (data not shown).

4. Discussion

DCT1/DMT1 mRNA and its protein are strongly (50–100-fold) upregulated in the duodenum of iron-deficient animals [4,6,27], and the level of regulation was higher in intestine compared to other tissues such as liver, kidney, heart and brain (see ref. [4]). Thus, iron entry in the duodenum is tightly regulated by dietary iron availability and body iron requirement.

To study the mechanism of DCT1/DMT1 regulation, we used various cultured cells expressing the transporter. We observed a large (up to 50-fold) iron responsiveness to iron chelation with Df in endogenous DCT1/DMT1 mRNA expressed in either intestinal Caco2 or liver Hep3B cells with more dramatic changes in Caco2 cells (Figs. 3A,B and 5A). There was no significant regulation of endogenous DCT1/DMT1-IRE in HeLa cells (Fig. 3D) or LMTK fibroblasts, as shown earlier [32]. The Northern analyses of tissues from normal and iron-deficient rats, which show the highest responses in the duodenum [4], indicate that the regulation of DCT1/DMT1 mRNA expression is cell type- and organ-specific. Iron responsiveness in Caco2 cells also increased with cell confluence (data not shown). Taken together, these data show that DCT1/DMT1 mRNA is highly responsive to cellular iron in cell types of intestinal or hepatic origin, whereas, in HeLa cells, it is unresponsive, suggesting that DCT1/DMT1 mRNA regulation depends on as yet unknown cell- or tissue-specific factors and possibly also on cell differentiation, in addition to the IRE/IRP system.

Interestingly, the predominant spliced product of DCT1/DMT1 mRNA in Hep3B and Caco2 cells is the IRE form (Fig. 5A), indicating that there is efficient preferential splicing of the IRE form, which has total 16 exons, compared to 17 exons for the non-IRE form [7]. Both spliced products transported iron efficiently in transfected Hep3B cells (Fig. 5C). Also, both isoforms were expressed in the plasma membrane and later endosomal compartments (data not shown and ref. [32]). Thus, given the current information available, the two isoforms have similar or identical roles in vivo, but the majority of DCT1/DMT1 in Hep3B and Caco2 cells exists in the IRE form.

Furthermore, as shown in Fig. 5B, the expression of the non-IRE form was not altered significantly by iron treatment, whereas the IRE form was highly responsive (Fig. 5A). The specific regulation of the IRE-splice product suggests that iron regulation may not be due to transcriptional regulation of the DCT1/DMT1 gene. Studies of promoter/reporter constructs (up to ~3 kb upstream of DCT1/DMT1 5′-UTR) also did not demonstrate any iron-dependent transcription of the human DCT1/DMT1 gene (Fig. 4B). Lee et al. showed that the human DCT1/DMT1 5′-regulatory region contains two CCAAT boxes, but no TATA box. This regulatory region also contains five potential metal response elements, three potential SP1 binding sites and a single γ-interferon regulatory element [7]. Our constructs contained all of these elements. However, we could find neither a significant increase in promoter activity in the presence of an iron chelator nor suppression by divalent metals such as iron (Fig. 4B), zinc and cadmium (data not shown). Given these results, it is less likely that regulation of DCT1/DMT1 occurs at the transcriptional level, although it cannot be completely excluded that distant upstream enhancer elements may confer iron responsiveness.

The observed regulation of DCT1/DMT1 may involve RNA stabilization through binding of IRP1 and/or IRP2 to

the putative IRE in DCT1/DMT1. We show that DCT1/DMT1-IRE can bind both IRP1 and IRP2, but the affinity for IRP1 was considerably higher (Fig. 2), and the low affinity of the DCT1/DMT1 IRE for IRP2 may fall to within a range that is insufficient to mediate regulation [20]. The IRP1 affinity (K_d of 91 ± 4 pM) is somewhat lower than that of Fer IRE (whose K_d is ~30 ± 20 pM). The latter is similar to the IRP1 K_d values for TfR or eALAS [21,35]. Ke et al. also showed that the Fer IRE is recognized equally by both IRP1 and IRP2. However, if the internal loop was mutated to form a simple bulge C (analogous to that in Fig. 2D), decreased IRP2 binding affinity was observed [21]. The simple bulge C structure is a common feature of the IREs of DCT1/DMT1 (Fig. 2C, right), TfR (Fig. 2D), eALAS [21] and m-aconitase IREs [21], all of which exhibit low IRP2 affinity, whereas the proposed Fer IRE internal loop/bulge structure (Fig. 2C) appears to be different from the single bulge C described above [39,21]. This suggests that the regulation of DCT1/DMT1, TfR, eALAS and m-aconitase are more dependent on IRP1 than IRP2. IRP1/IRP2 ratios vary significantly in different cell types. IRP1 levels are higher in liver, kidney and intestine [34], suggesting that the cell type- and organ-specific expression of DCT1/DMT1 may correlate with the organ-specific distribution of IRP1 and IRP2 [37,38]. Future studies, including the analysis of the phenotype caused by targeted disruption of the IRP1 gene, will be required to determine the exact

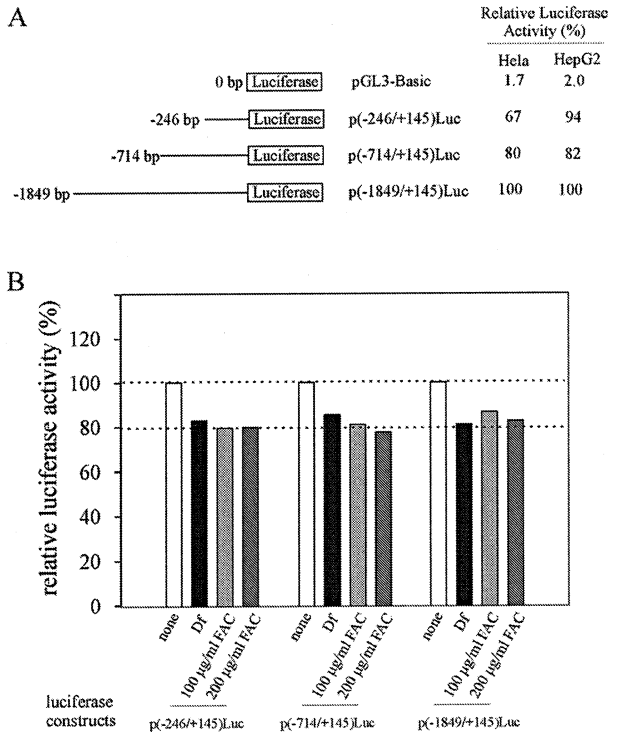

Fig. 4. Examination of the transcriptional regulation of expression of DCT1. A: Luciferase constructs of the human DCT1/DMT1 gene promoter placed upstream of the luciferase reporter gene. The pGL3-basic vector (Promega) was used in these experiments. DCT1/DMT1 5′-promoter deletions were made as described in Section 2, and ref. [31]. B: Hep3B cells were transiently transfected with luciferase constructs and β-galactosidase or Renilla luciferase constructs. After transfection, cells were treated with 100 μM Df or 100–200 μg/ml FAC for 20–48 h. Each bar indicates relative luciferase activity in Hep3B cells transfected with luciferase reporter constructs shown in A.

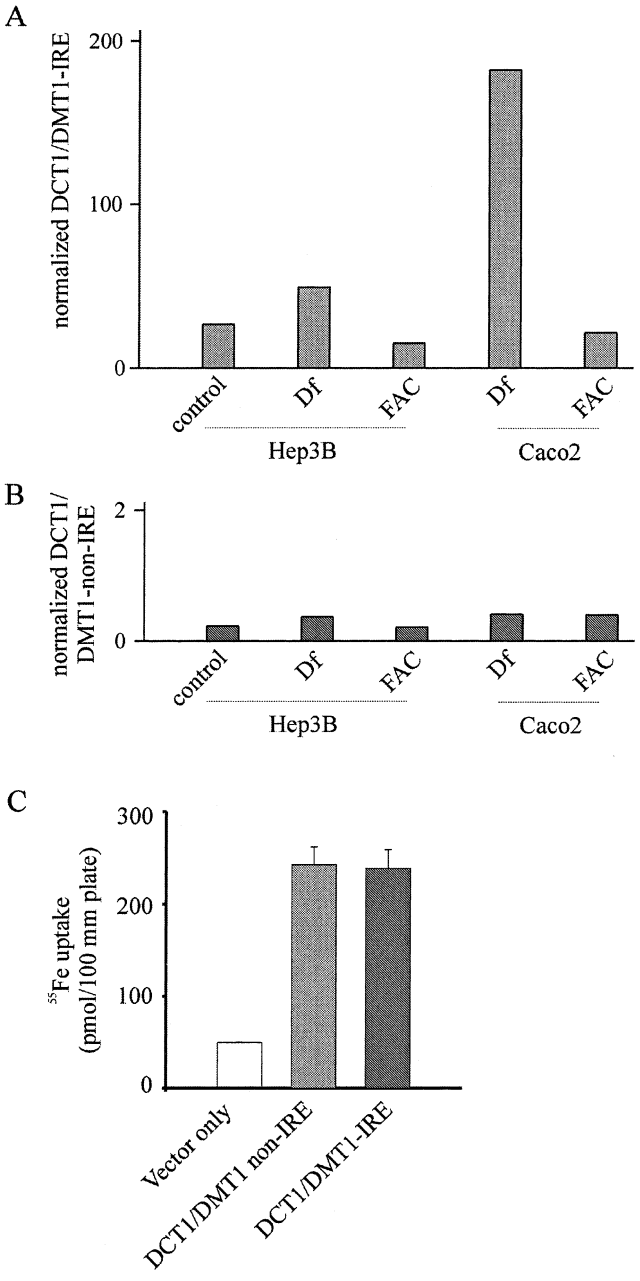

Fig. 5. A,B: Evaluation of the iron responsiveness of the IRE and non-IRE forms of DCT1 by quantitative PCR and (C) ^{55}Fe uptake by the IRE- and non-IRE forms of DCT1/DMT1 in Hep3G cells. Quantitative RT-PCR for DCT1/DMT1-IRE (A) and DCT1/DMT1 non-IRE (B) with mRNA from Hep3B and Caco2 cells treated with Df, FAC or no treatment. The Y axis indicates DCT1/DMT1 expression levels normalized by β-actin. The values for each sample were normalized to β-actin to account for differences in mRNA amount in the reactions. C: ^{55}Fe uptake in Hep3G cells transfected with either of the two isoforms of DCT1/DMT1.

role of IRP1 in regulating DCT1/DMT1-mediated iron transport.

As shown in Figs. 1 and 2C, the hairpin loop structure is conserved in all IREs reported to date. The only remarkable differences in these IREs are the stem structure, in particular the internal loop/bulge in Fer, the C bulge in the other IREs and the additional U bulge in DCT1/DMT1. These variations in structure may determine IRP2 binding [21,35] and thus the

differential binding ratio of IRP1 and IRP2. Because IRP1 binding affinity is lower in DCT1/DMT1-IRE compared to Fer IRE shown in Fig. 2C, the additional U bulge in DCT1/DMT1 may potentially further decrease IRP2 binding affinity.

Experiments with transiently transfected DCT1/DMT1 *c*-myc constructs containing the 3′-UTR IRE did not exhibit significant iron-dependent mRNA regulation in either Caco2 or Hep3B cells (data not shown). This apparent lack of responsiveness may have been due to overexpression of DCT1/DMT1-IRE. This may have compromised iron responsiveness because excessive IRE-mRNA can saturate the IRP system, including IRPs and putative nucleases or cell differentiation-specific factors. Because Casey et al. used in their TfR-IRE studies constructs with the TfR promoter itself, their experiments avoided overexpression and, therefore, mimicked the in vivo situation more closely [10]. Alternatively, lack of responsiveness may have been due to missing 5′- and/or 3′-untranslated sequences.

In summary, our results demonstrate that endogenous DCT1/DMT1 expression in Caco2 and Hep3B is highly responsive to iron treatment, consistent with the observed response in rat and human duodenum [4,28]. Responsiveness depends on cell type and cell confluence, suggesting that cell- and differentiation-specific factors may be involved in regulation. We show that IRP1 binds with high affinity to the DCT1/DMT1-IRE, whereas IRP2 binds with much lower affinity. Our studies show that only the DCT1/DMT1 splice form containing the IRE is responsive to iron, both in cultured cells (Fig. 5A) and in human intestine (Rolfs et al., manuscript in review). We propose that DCT1/DMT1 mRNA is regulated through stabilization by the IRE/IRP system, in analogy to TfR mRNA. However, the exact mechanisms which confer cell type- and/or differentiation-specific regulation remain yet to be elucidated.

In vivo, DCT1/DMT1 mRNA stabilization is likely the predominant mechanism for iron-dependent regulation, allowing massive accumulation of the IRE splice product during iron starvation. The transfer of iron from the intestinal lumen into the blood requires not only DCT1/DMT1 in the brush border membrane but also an iron exit mechanism at the basolateral side of enterocytes. The molecular identity of this mechanism has recently been unraveled. It consists of an iron transporter, called IREG1/Ferroportin1/MTP1 [17–19], and a multi-copper oxidase, called hephaestin [33]. Interestingly, IREG1/Ferroportin1/MTP1 also has an IRE located in the 5′-UTR [17–19], in analogy to ferritin where the 5′-IRE regulates translation in an iron-dependent manner. Abboud and Haile demonstrated that MTP1 IRE was functional [19]. We propose that the IREs in DCT1/DMT1 and IREG1/Ferroportin1/MTP1 play a crucial role in maintaining continuous transepithelial iron transport in the intestine. If serum iron levels are low, DCT1/DMT1 will be expressed maximally in the brush border membrane of enterocytes. We hypothesize that IREG1/Ferroportin1/MTP1 mRNA is on translational or transcriptional arrest until DCT1/DMT1 delivers iron into enterocytes to trigger its expression and localization in the basolateral membrane and to facilitate transfer of iron into the blood.

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