Inhibition of transferrin receptor 1 transcription by a cell density response element

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TfR1 (transferrin receptor 1) mediates the uptake of transferrin-bound iron and thereby plays a critical role in cellular iron metabolism. Its expression is coupled to cell proliferation/differentiation and controlled in response to iron levels and other signals by transcriptional and post-transcriptional mechanisms. It is well established that TfR1 levels decline when cultured cells reach a high density and in the present study we have investigated the underlying mechanisms. Consistent with previous findings, we demonstrate that TfR1 expression is attenuated in a cell-density-dependent manner in human lung cancer H1299 cells and in murine B6 fibroblasts as the result of a marked decrease in mRNA content. This response is not associated with alterations in the RNA-binding activity of iron regulatory proteins that are indicative of a transcriptional mechanism. Reporter assays reveal that

the human TfR1 promoters contains sequences mediating cell-density-dependent transcriptional inhibition. Mapping of the human and mouse TfR1 promoters identified a conserved hexanucleotide 5'-GAGGC-3' motif with notable sequence similarity to a previously described element within the IGF-2 (insulin-like growth factor-2) promoter. We show that this motif is necessary for the formation of specific complexes with nuclear extracts and for cell-density-dependent regulation in reporter gene assays. Thus the TfR1 promoter contains a functional 'cell density response element' (CDRE).

Key words: cell density, iron responsive element (IRE), iron regulatory protein (IRP), iron metabolism, transferrin receptor 1 (TfR1).

INTRODUCTION

TfR1 (transferrin receptor 1) is a homodimeric type II transmembrane glycoprotein that mediates the uptake of transferrin-bound plasma iron into tissues [1,2]. It is expressed in many cell types and carries out an indispensable function in iron acquisition by erythroid cells, the major iron consumers in mammals. TfR1^{-/-} mouse embryos exhibit severe defects in erythropoiesis and neuronal development and die before day E12.5 of gestation [3]. TfR1 binds with high affinity to iron-loaded transferrin on the cell surface and internalizes the cargo by an endocytic pathway involving clathrin-coated pits [1,2]. Acidification of the late endosome promotes the release of iron from transferrin, which is transported across the endosomal membrane by the bivalent metal transporter and utilized in metalloproteins. Excess iron is stored and detoxified in ferritin, a cytosolic protein with a shell-like structure composed of H- and L-chains [4]. Iron's potential toxicity is based on its capacity to catalyse the generation of free radicals which damage cell constituents and promote disease [5,6]. The expression of TfR1 and ferritin is regulated in response to iron levels by the IRE (iron responsive element)/IRP (iron regulatory protein) system [7,8]. In iron-replete cells, TfR1 mRNA undergoes nucleolytic degradation and the mRNAs encoding H- and L-ferritin are readily translated. When iron is scarce, IRP1 and IRP2 bind to IREs within the 3' and 5' untranslated regions of TfR1 and ferritin mRNAs respectively. These interactions stabilize TfR1 mRNA and inhibit translation of H- and L-ferritin mRNAs, resulting in increased iron uptake and reduced storage. The IRP-mediated stabilization of TfR1 mRNA is coupled with transcriptional activation via a hypoxia response element within the TfR1 promoter,

which provides a binding site for hypoxia-inducible factor 1 [9–11].

The expression of TfR1 also depends on cell proliferation/differentiation and is tightly linked to metabolic iron requirements [1]. Stimulation of quiescent cells by mitogens, cytokines or growth factors activates TfR1 expression mainly at the transcriptional level [12–14]. A region approx. 100 bp upstream from the transcription start site is crucial for mitogen-responsive and basal TfR1 transcription [12,15]. This region contains an AP1 (activator protein 1)-like sequence and a putative GC-rich SP1 (transcription factor SP1)-binding site that co-operatively mediate mitogen-dependent transcriptional activation [13]. It also contains an Ets-binding site and a cAMP-response element-like motif, which account for tissue-specific TfR1 transcription in differentiating erythroid cells [16,17]. This response is crucial to meet the exceptionally high demand for iron by these cells [1].

The density of cultured cells strongly affects their proliferation rate, differentiation status and promotes alterations in gene expression. Usually sparse cells grow exponentially and cease division at confluence, this is accompanied by a decrease in the expression of growth related genes including those encoding TfR1 [18,19], bFGF (basic fibroblast growth factor) [20,21] and IGF-2 (insulin-like growth factor-2) [22,23]. Although increasing cell density is generally associated with transcriptional silencing of bFGF and IGF-2 genes [20,23], the mechanism for cell-density-dependent inhibition in TfR1 expression has not been studied thus far. In the present study we report the identification of a CDRE (cell density response element) within the TfR1 promoter, consisting of a conserved hexanucleotide 5'-GAGGC-3' core motif which is necessary for negative regulation of TfR1 transcription in a cell-density-dependent manner.

Abbreviations used: bFGF, basic fibroblast growth factor; CDRE, cell density response element; DFO, desferrioxamine; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; IGF-2, insulin-like growth factor-2; IRE, iron responsive element; IRP, iron regulatory protein; TfR1, transferrin receptor 1.

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MATERIALS AND METHODS

Cell culture

Human H1299 lung cancer cells and murine B6 fibroblasts were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS (foetal bovine serum), 2 mM glutamine, 100 µg/ml penicillin and 0.1 ng/ml streptomycin. H1299 clones expressing a tetracycline-inducible FLAG-tagged IRP1_{C437S} mutant (HIRP1_{mut} cells) [24] were maintained in DMEM containing 2 µg/ml tetracycline, 2 µg/ml puromycin and 250 µg/ml G418. To evaluate density effects, an increasing initial number of cells was plated onto 100 mm or 60 mm culture dishes. After 2–3 days in culture the cells were trypsinized and counted on a hemacytometer. The cell density was expressed as the number of trypsinized cells per mm² of culture dish. Phase-contrast images prior to trypsinization were obtained using a Carl Zeiss AxioVision microscope.

Northern blotting

Cells were washed twice in cold PBS and lysed with the TRIzol[®] reagent (Invitrogen). RNA was purified according to the manufacturer's instructions. Total cellular RNA (10 µg) was electrophoretically resolved on a denaturing agarose gel, transferred onto nylon membrane and hybridized to radiolabelled human TfR1 or β -actin cDNA probes. The radioactive bands were visualized by exposure of the blot onto X-ray film and quantified by phosphorimaging.

Western blotting

Cells were washed twice in cold PBS and lysed in cytoplasmic lysis buffer [1% (v/v) Triton X-100, 40 mM KCl, 25 mM Tris/HCl, pH 7.4]. Cell debris was cleared by centrifugation and protein concentration was measured with the Bradford reagent (Bio-Rad). Cell lysates (30 µg) were resolved by SDS/PAGE on 7.5% gels and proteins were transferred onto nitrocellulose filters. The blots were saturated with 10% milk in PBS containing 0.1% (v/v) Tween 20 (PBS-T) and probed with antibodies against TfR1 (Zymed, 1:1000 dilution) or β -actin (Sigma, 1:1000 dilution) diluted in PBS-T. Following a wash with PBS-T, the blot containing anti-TfR1 antibody was incubated with peroxidase-coupled rabbit anti-(mouse IgG) (Sigma, 1:4000 dilution) and the blot with anti- β -actin antibody was incubated with peroxidase-coupled goat anti-(rabbit IgG) (Sigma, 1:5000 dilution). Peroxidase-coupled antibodies were detected by ECL[®] (Amersham Biosciences, Piscataway, NJ, U.S.A.). Immunoreactive bands were quantified by densitometric scanning.

Construction of plasmids

Luciferase reporter plasmids were generated by inserting a 32-bp BglII/HindIII annealed oligonucleotide fragment, containing wild-type (wtTR-D) or mutated (mutTR-D) CDRE of murine TfR1, into the corresponding sites of the O-Fluc vector [25]. These are located upstream of a minimal *c-fos* promoter and a luciferase coding-sequence. The oligonucleotide sequences were: (a) wtTR-D, 5'-AGCTTAAGGTTAGGCGGAGGGCGACAGGCCAA-3' (sense) and 5'-GATCTTGGCCTGTGCGCCCTCCGCCTAACCTTA-3' (antisense); (b) mutTR-D, AGCTTAAGGTTAGGCG-TTTTTTGACAGGCCAA-3' (sense) and 5'-GATCTTGGCCTGTCAAAAAACGCCTAACCTTA-3' (antisense). The nucleotides corresponding to wild-type or the mutant CDRE core motif are underlined. The TR-282 and TR-118 plasmids containing human TfR1 promoter fragments [16] were kindly given by Drs Chun Nam Lok and Prem Ponka (Montreal, Canada).

Transient transfections and dual luciferase reporter assays

Firefly luciferase reporter plasmids (2 µg each) were cotransfected with 0.04 µg of *Renilla reniformis* luciferase control vector pRL-CMV (Promega) into H1299 cells by using Lipofectamine[™] Plus reagent (Invitrogen). Dual luciferase assays were performed in cell lysates according to the manufacturer's instructions (Promega).

RNA gel-shift assays

Cytoplasmic lysates were analysed for IRE-binding activity with a radiolabelled RNA probe containing ferritin IRE, as previously described [26].

DNA gel-shift assays

Nuclear cell extracts were prepared as in [27]. The annealed wtTR-D oligonucleotide fragment (as described above) was labelled with [α -³²P]dCTP by using the Klenow fragment of DNA polymerase I. The specific radioactivity was 10⁷ c.p.m./µg. The labelled probe (50000 c.p.m.) was incubated for 20 min at room temperature with 5 µg of nuclear extracts, in the presence or absence of 300 ng of unlabelled competitor oligonucleotides (wtTR-D and mutTR-D). The reaction buffer contained 20 mM Hepes pH 7.9, 25 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 2 mM spermidine, 0.5 mM dithiothreitol, 0.025% NP-40 (Nonidet P40), 10% glycerol, 100 ng of poly(dI:dC) and 2 µg of BSA. The products of the binding reactions were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel. Radiolabelled bands were visualized by autoradiography.

RESULTS AND DISCUSSION

Inhibition of TfR1 expression in dense cultures of H1299 and B6 cells

The impact of cell density on the expression of TfR1 was assessed in cell lines of human and mouse origin. Firstly we utilized HIRP1_{mut} clones, which are derived from human H1299 lung cancer cells and express the constitutive IRP1_{C437S} mutant under the control of a tetracycline inducible promoter (tet-off system) [24]. The cells were seeded at initial numbers of 1, 2 or 3 × 10⁶ per 100 mm culture dish and after 3 days of growth in the presence or absence of 2 µg/ml tetracycline reached densities of 500, 800 or 1200 cells/mm² (Figure 1A). Tetracycline had no appreciable effect on cell growth. The steady-state levels of TfR1 were evaluated by Western blotting (Figure 1B). As expected, in the absence of tetracycline, TfR1 expression was stimulated 2-fold (Figure 1B, compare lanes 1 and 4) as a result of IRP1_{C437S} induction [24]. The increase in cell density inversely correlated with TfR1 content (Figure 1B, upper panel), whereas levels of control β -actin remained unchanged (Figure 1B, lower panel). In quantitative terms, the increase in cell density from 500 to 1200 cells/mm² (corresponding to almost 100% confluence) was associated with an approx. 50% decrease in TfR1 expression.

To better understand the underlying mechanism, the levels of TfR1 mRNA were analysed by Northern blotting and quantified by phosphorimaging relative to control β -actin mRNA. Increasing cell density negatively affected TfR1 mRNA expression in cells grown with tetracycline (Figure 1C). TfR1 mRNA content was decreased by more than 90% when cell density increased from 500 to 800 cells/mm² (Figure 1C, lanes 1 and 2) and dropped further to almost undetectable levels at 1200 cells/mm² (Figure 1C, lane 3). Similar results were obtained using parent H1229 cells (results not shown). The removal of tetracycline

Figure 1 Decrease in TfR1 expression in dense HIRP1_{mut} cells

HIRP1_{mut} cells ($1, 2$ or 3×10^6) were plated in 100 mm culture dishes in the presence or absence of 2 µg/ml tetracycline (tet) and after 3 days reached densities of 500, 800 or 1200 cells/mm² respectively. **(A)** Phase contrast images at the indicated cell densities (magnification 75×). **(B)** Analysis of TfR1 (top) and β-actin (bottom) expression by Western blotting. **(C)** Analysis of TfR1 mRNA (top) and β-actin mRNA (bottom) expression by Northern blotting.

Figure 2 Decrease in TfR1 expression in dense B6 cells

B6 cells ($2, 4, 8$ or 12×10^6) were plated in 100 mm culture dishes and after 2 days reached densities of 1250, 2500, 3000 or 3150 cells/mm² respectively. **(A)** Phase contrast images at the indicated cell densities (magnification 67×). **(B)** Analysis of TfR1 (top) and β-actin (bottom) expression by Western blotting. **(C)** Analysis of TfR1 mRNA (top) and β-actin mRNA (bottom) expression by Northern blotting. Where indicated cells were treated overnight with 100 µM DFO.

relieved the suppression of IRP1_{C437S}, which in turn stabilized TfR1 mRNA (Figure 1C, lanes 4–6), as shown previously [24]. We then utilized murine B6 fibroblasts, which are smaller and grow faster than HIRP1_{mut} and parent H1299 cells. B6 cells were seeded at initial numbers of 2, 4, 8 or 12×10^6 per 100 mm culture dish and within 2 days reached densities of 1250, 2500, 3000 or

3150 cells/mm² respectively (Figure 2A). The increase in cell density correlated with a dramatic inhibition in TfR1 expression (Figure 2B). Thus the signal corresponding to TfR1 almost disappeared at high confluence (Figure 2B, lanes 3 and 4), but as expected the expression of β-actin was not affected by alterations in cell density (Figure 2B, lower panel). The expression of

Figure 3 Effects of cell density on IRE-binding activity

An increasing number of H1299 (A) or B6 (B) cells were plated in 100 mm culture dishes (as described in Figures 1 and 2) and allowed to grow for 3 or 2 days respectively, to reach the indicated densities. Cytoplasmic extracts from H1299 cells (30 µg) or B6 cells (10 µg) were analysed using a gel-shift assay with a ³²P-labelled RNA probe containing a ferritin IRE sequence, in the absence (top) or presence (bottom) of 2% 2-mercaptoethanol (2-ME), which activates latent IRP1 [30]. The positions of IRE-IRP1 complexes and of excess free probe are indicated by arrows. The ratios of the intensity of IRE-IRP1 complexes formed in the absence (upper panel) or presence of 2% 2-ME (bottom panel) were determined by phosphorimaging.

TfR1 mRNA was likewise negatively regulated by cell density (Figure 2C) and dropped to negligible levels at high confluence (lanes 3–4). An overnight treatment with the iron chelator DFO (desferrioxamine) boosted TfR1 mRNA levels and antagonized their cell-density-dependent decrease (lanes 5–8).

Collectively these results confirm previous observations in HL-60 myeloid leukaemia cells [18,19] and furthermore, demonstrate that the cell-density-dependent negative regulation of TfR1 expression is a more general phenomenon and reflects a decline in TfR1 mRNA content.

The cell-density-dependent inhibition of TfR1 mRNA expression is not caused by a decrease in IRE-binding activity

IRP1 and IRP2 are known to protect TfR1 mRNA from degradation by binding at the IREs within its 3' untranslated region [7,8]. Thus it is conceivable that the cell-density-dependent inhibition of TfR1 mRNA expression might be associated with a decrease in IRE-binding activity and under such conditions unprotected TfR1 mRNA would be destabilized. To examine this possibility H1299 and B6 cells were seeded at increasing densities and IRE-binding activity was analysed in cytoplasmic extracts by an RNA gel-shift assay (Figure 3). The increase in density did not appreciably affect IRE-binding activity in H1299 cells (Figure 3A). Similar results were obtained using B6 cells (Figure 3B), nevertheless, post-confluent B6 cells displayed an approx. 70% reduction in IRE-binding activity (Figure 3B, lane 4) compared with sub-confluent counterparts (Figure 3B, lanes 1–3).

This response could partially contribute to the decrease in TfR1 mRNA levels, however, it may represent a cell-specific effect as it is not observed in H1299 cells. Considering that in dense cultures of both H1299 and B6 cells a profound decrease in TfR1

Figure 4 Cell-density-dependent inhibition of TfR1 gene promoter activity

(A) Schematic representation of TR-282 and TR-118 luciferase reporter constructs. The indicated promoter fragments from the human TfR1 gene (grey boxes) were cloned upstream from a promoter-less firefly luciferase coding-sequence (white boxes). (B) TR-282 and TR-118 were cotransfected with a *Renilla* luciferase control vector, pRL-CMV, into H1299 cells. After 24 h, 3.5, 7 or 10.5 × 10⁶ cells were reseeded in 60 mm culture dishes and allowed to grow for 3 days reaching densities of 500, 800 or 1200 cells/mm² respectively. Cell lysates were analysed by dual luciferase assays. Values correspond to mean ± S.E.M. for triplicate samples from a representative of two independent experiments. ***P* < 0.01 versus control (Student's *t* test).

expression occurs under conditions where IRE-binding activity remains unchanged, we conclude that the downregulation of TfR1 mRNA is not based primarily on compromised IRP-dependent stabilization. Our results are in agreement with earlier experiments, which showed that the 3' untranslated region of TfR1 mRNA is not involved in the negative regulation of TfR1 in growth-arrested cells [28].

Cell-density-dependent inhibition of promoter activity in the TfR1 gene

Since the expression of TfR1 mRNA is regulated not only by post-transcriptional, but also by transcriptional mechanisms [28,29], we investigated whether the activity of the TfR1 promoter is affected by alterations in cell density. Therefore we utilized reporter constructs containing fragments of the human TfR1 promoter upstream of a promoter-less firefly luciferase coding-sequence, which were previously employed to assess responses to hypoxia [9] and erythroid differentiation [16]. The TR-282 and TR-118 constructs encompass a human TfR1 promoter region between nt -282 and +14, and nt -118 and +14 relative to the transcription start site respectively (Figure 4A). The proximal (-118 to +14) promoter region is GC-rich and includes basal/mitogen-responsive promoter elements [12], a hypoxia response element [9,10] and sequences required for TfR1 transcriptional activation during erythroid differentiation [16]. Preliminary transfection experiments in H1299 cells showed a robust, greater than 500-fold, induction of luciferase activity compared with control promoter-less pGL2-basic vector.

To analyse whether TfR1 promoter sequences mediate cell-density-dependent transcriptional regulation TR-282 and TR-118 were transiently cotransfected using a *Renilla* luciferase control vector into H1299 cells. After 24 h the cells were trypsinized and reseeded, reaching increased densities within 3 days. Subsequently, cell extracts were prepared for dual luciferase

Figure 5 CDREs within human and mouse Tfr1 promoters

The numbers indicate nt positions relative to transcription start site. The sequence of the previously identified [23] regulatory element within IGF-2 promoter is shown below. CDREs within the human and mouse Tfr1 promoters are underlined.

Figure 6 The 5'-GAGGGC-3' motif within the Tfr1 promoter is crucial for binding of nuclear factors

B6 cells ($2, 4$ or 8×10^6) were plated in 100 mm culture dishes and after 2 days reached densities of 1250, 2500 or 3000 cells/mm² respectively. Cells were lysed and nuclear extracts (10 µg) were analysed by a gel-shift assay with a ³²P-labelled DNA probe (wtTR-D), in the presence or absence of 300 ng of unlabelled competitors (wtTR-D or mutTR-D). The positions of the specific DNA-protein complexes A and B, and of excess free probe, are indicated by arrows.

assays (Figure 4B). Luciferase activity derived from the TR-282 construct decreased significantly ($P < 0.01$) by approx. 30–50 % in a cell-density-dependent manner. By contrast, luciferase activity derived from the TR-118 construct did not change considerably under similar conditions. These results show that increasing cell density attenuates Tfr1 promoter activity. Furthermore, they suggest that the sequences within the human Tfr1 promoter accounting for cell-density-dependent regulation are located between nucleotides –282 and –118 relative to the transcription start site and outside of the earlier defined basal/mitogen-responsive promoter elements (–118 to +14) [12].

Tfr1 promoter contains a functional CDRE

Dai et al. [23] identified and characterized a *cis*-element within the P3 promoter of IGF-2 that plays a critical role in the cell-density-dependent downregulation of its transcription [23]. The core motif of this element consists of the hexanucleotide 5'-GA-

Figure 7 The 5'-GAGGGC-3' motif within the Tfr1 promoter is necessary for cell-density-dependent reporter gene regulation

(A) Schematic representation of wtTR-D-Fluc and mutTR-D-Fluc reporter constructs. A 32 bp wild-type or mutant oligonucleotide fragment, corresponding to sequences from the mouse Tfr1 promoter, was cloned upstream from a minimal c-fos promoter and a firefly luciferase gene (grey and white boxes respectively). The wild-type 5'-GAGGGC-3' core motif (in wtTR-D-Fluc) and the mutant sequence 5'-TTTTT-3' (in mutTR-D-Fluc) are underlined. (B) wtTR-D-Fluc and mutTR-D-Fluc were cotransfected using a *Renilla* luciferase control vector pRL-CMV into H1299 cells. After 24 h, 3.5, 7 or 10.5 × 10⁶ cells were reseeded in 60 mm culture dishes and allowed to grow for 3 days reaching densities of 500, 800 or 1200 cells/mm² respectively. Cell lysates were analysed by dual luciferase assays. Values correspond to mean ± S.E.M. for triplicate samples from a representative of two independent experiments. ** $P < 0.01$ versus control (Student's *t* test).

GGGC-3'. By examining the –282 to –118 region of the human Tfr1 promoter we noticed the presence of the same sequence at positions –155 to –150 (Figure 5). Further analysis revealed that the hexanucleotide 5'-GAGGGC-3' motif is conserved within the murine Tfr1 promoter. Thus both human and murine Tfr1 promoters contain an element implicated in cell-density-dependent attenuation of IGF-2 transcription [23].

We reasoned that the 5'-GAGGGC-3' motif might likewise be necessary for the cell-density-dependent regulation of Tfr1 transcription. This was examined by a series of experiments. First, a ³²P-labelled 32-bp oligonucleotide corresponding to sequences of the murine Tfr1 promoter including the 5'-GAGGGC-3' motif was analysed by a DNA gel-shift assay using nuclear extracts of B6 cells grown at various densities. Two DNA-protein complexes were formed and are referred to as A and B in Figure 6. The interactions visualized by this assay were specific, as complex-formation was antagonized in the presence of a 60-fold molar excess of unlabelled wild-type (Figure 6, wtTR-D, lanes 3, 6 and 9), but not mutant, competitor (Figure 6, mutTR-D, lanes 4, 7 and 10), in which the 5'-GAGGGC-3' motif was replaced with 5'-TTTTT-3'.

The intensity of the signal appeared stronger at higher cell densities (Figure 6, compare lanes 2, 5 and 8), suggesting that the 5'-GAGGGC-3' motif may constitute a binding site for negative regulators. It should, however, be noted that gel-shift assays with a probe corresponding to the IGF-2 regulatory element and nuclear

extracts from Caco2 cells showed decreased binding activity with increasing cell density [23]. It is conceivable that the cell-density-dependent binding of *trans*-acting factors to the 5'-GAGGGC-3' motif may be cell-type specific. In addition, it could be affected by the flanking sequences. Although future work is expected to clarify these issues, the results in Figure 6 clearly show that the 5'-GAGGGC-3' motif, within the context of TfR1 promoter sequences, is involved in binding to nuclear factor(s).

These results were further validated using a luciferase reporter assay (Figure 7). The wtTR-D or mutTR-D oligonucleotides were cloned upstream of a minimal *c-fos* promoter and a firefly luciferase coding-sequence. The resulting wtTR-D-Fluc or mutTR-D-Fluc reporter constructs (Figure 7A) were cotransfected using a *Renilla* luciferase control vector into H1299 cells. The cells were plated to reach increasing density and after 3 days, lysates were analysed by dual luciferase assays. The activity corresponding to the wtTR-D-Fluc construct decreased significantly ($P < 0.01$) by 50% in dense cultures, whereas the activity corresponding to the mutTR-D-Fluc remained largely unresponsive to alterations in cell density (Figure 7B). Thus the 5'-GAGGGC-3' motif is necessary for cell-density-dependent regulation of luciferase reporter constructs and displays the hallmarks of a functional CDRE.

Concluding remarks

Collectively the data presented in Figures 6 and 7 show that the 5'-GAGGGC-3' motif is a CDRE, necessary for cell-density-dependent inhibition of TfR1 transcription, by analogy to a similar element in IGF-2 promoter [23]. The identification and characterization of *trans*-acting factors is expected to shed light on the underlying mechanism. Finally, we observed the presence of a 5'-AGGGC-3' sequence at positions -652 to -647 of the human bFGF promoter. We do not know whether this truncated motif also functions as a CDRE, nevertheless, the promoter region from -650 to the transcription start site of bFGF was reported to mediate negative cell-density-dependent regulation in astrocytes [20]. Further phylogenetic and mutational analysis is expected to define the exact CDRE boundaries and sequence requirements. This would also contribute to the understanding of whether a CDRE-based mechanism for cell-density-dependent transcriptional attenuation is shared by additional growth-related genes.

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REFERENCES

- Ponka, P. and Lok, C. N. (1999) The transferrin receptor: role in health and disease. *Int. J. Biochem. Cell Biol.* **31**, 1111–1137
- Aisen, P. (2004) Transferrin receptor 1. *Int. J. Biochem. Cell Biol.* **36**, 2137–2143
- Levy, J. E., Jin, O., Fujiwara, Y., Kuo, F. and Andrews, N. C. (1999) Transferrin receptor is necessary for development of erythrocytes and the nervous system. *Nat. Genet.* **21**, 396–399
- Torti, F. M. and Torti, S. V. (2002) Regulation of ferritin genes and protein. *Blood* **99**, 3505–3516
- Pietrangolo, A. (2003) Iron-induced oxidant stress in alcoholic liver fibrogenesis. *Alcohol* **30**, 121–129
- Papanikolaou, G. and Pantopoulos, K. (2005) Iron metabolism and toxicity. *Toxicol. Appl. Pharmacol.* **202**, 199–211
- Pantopoulos, K. (2004) Iron metabolism and the IRE/IRP regulatory system: an update. *Ann. N.Y. Acad. Sci.* **1012**, 1–13
- Hentze, M. W., Muckenthaler, M. U. and Andrews, N. C. (2004) Balancing acts; molecular control of mammalian iron metabolism. *Cell* **117**, 285–297
- Lok, C. N. and Ponka, P. (1999) Identification of a hypoxia response element in the transferrin receptor gene. *J. Biol. Chem.* **274**, 24147–24152
- Tacchini, L., Bianchi, L., Bernelli-Zazzera, A. and Cairo, G. (1999) Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. *J. Biol. Chem.* **274**, 24142–24146
- Bianchi, L., Tacchini, L. and Cairo, G. (1999) HIF-1-mediated activation of transferrin receptor gene transcription by iron chelation. *Nucleic Acids Res.* **27**, 4223–4227
- Miskimins, W. K., McClelland, A., Roberts, M. P. and Ruddle, F. H. (1986) Cell proliferation and expression of the transferrin receptor gene: promoter sequence homologies and protein interactions. *J. Cell Biol.* **103**, 1781–1788
- Ouyang, Q., Bommakanti, M. and Miskimins, W. K. (1993) A mitogen-responsive promoter region that is synergistically activated through multiple signalling pathways. *Mol. Cell. Biol.* **13**, 1796–1804
- Seiser, C., Teixeira, S. and Kühn, L. C. (1993) Interleukin-2-dependent transcriptional and post-transcriptional regulation of transferrin receptor mRNA. *J. Biol. Chem.* **268**, 13074–13080
- Casey, J. L., Di Jeso, B., Rao, K., Rouault, T. A., Klausner, R. D. and Harford, J. B. (1988) The promoter region of the human transferrin receptor gene. *Ann. N.Y. Acad. Sci.* **526**, 54–64
- Lok, C. N. and Ponka, P. (2000) Identification of an erythroid active element in the transferrin receptor gene. *J. Biol. Chem.* **275**, 24185–24190
- Marziali, G., Perrotti, E., Ilari, R., Lulli, V., Coccia, E. M., Moret, R., Kuhn, L. C., Testa, U. and Battistini, A. (2002) Role of Ets-1 in transcriptional regulation of transferrin receptor and erythroid differentiation. *Oncogene* **21**, 7933–7944
- Chitambar, C. R., Massey, E. J. and Seligman, P. A. (1983) Regulation of transferrin receptor expression on human leukemic cells during proliferation and induction of differentiation. Effects of gallium and dimethylsulfoxide. *J. Clin. Invest.* **72**, 1314–1325
- Taetle, R., Ralph, S., Smedsrud, S. and Trowbridge, I. (1987) Regulation of transferrin receptor expression in myeloid leukemia cells. *Blood* **70**, 852–859
- Moffett, J., Kratz, E., Florkiewicz, R. and Stachowiak, M. K. (1996) Promoter regions involved in density-dependent regulation of basic fibroblast growth factor gene expression in human astrocytic cells. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2470–2475
- Galy, B., Maret, A., Prats, A. C. and Prats, H. (1999) Cell transformation results in the loss of the density-dependent translational regulation of the expression of fibroblast growth factor 2 isoforms. *Cancer Res.* **59**, 165–171
- Singh, P., Dai, B., Yallampalli, U., Lu, X. and Schroy, P. C. (1996) Proliferation and differentiation of a human colon cancer cell line (CaCo2) is associated with significant changes in the expression and secretion of insulin-like growth factor (IGF) IGF-II and IGF binding protein-4: role of IGF-II. *Endocrinology* **137**, 1764–1774
- Dai, B., Wu, H., Holthuisen, E. and Singh, P. (2001) Identification of a novel cis element required for cell density-dependent down-regulation of insulin-like growth factor-2 p3 promoter activity in Caco2 cells. *J. Biol. Chem.* **276**, 6937–6944
- 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
- Wang, J., Shou, J. and Chen, X. (2000) Dickkopf-1, an inhibitor of the Wnt signaling pathway, is induced by p53. *Oncogene* **19**, 1843–1848
- Mueller, S. and Pantopoulos, K. (2002) Activation of iron regulatory protein-1 (IRP1) by oxidative stress. *Methods Enzymol.* **348**, 324–337
- Andrews, N. C. and Faller, D. V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19**, 2499
- Owen, D. and Kühn, L. C. (1987) Noncoding 3' sequences of the transferrin receptor gene are required for mRNA regulation by iron. *EMBO J.* **6**, 1287–1293
- Casey, J. L., Di Jeso, B., Rao, K., Klausner, R. D. and Harford, J. B. (1988) Two genetic loci participate in the regulation by iron of the gene for the human transferrin receptor. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1787–1791
- Cairo, G., Recalcati, S., Pietrangolo, A. and Minotti, G. (2002) The iron regulatory proteins: targets and modulators of free radical reactions and oxidative damage. *Free Radical Biol. Med.* **32**, 1237–1243

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