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HIF-1-mediated activation of transferrin receptor gene transcription by iron chelation

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ABSTRACT

Treatment with iron chelators mimics hypoxic induction of the hypoxia inducible factor (HIF-1) which activates transcription by binding to hypoxia responsive elements (HRE). We investigated whether HIF-1 is involved in transcriptional activation of the transferrin receptor (TfR), a membrane protein which mediates cellular iron uptake, in response to iron deprivation. The transcription rate of the TfR gene in isolated nuclei was up-regulated by treatment of Hep3B human hepatoma cells with the iron chelator desferrioxamine (DFO). The role of HIF-1 in the activation of TfR was indicated by the following observations: (i) DFO-dependent activation of a luciferase reporter gene in transfected Hep3B cells was mediated by a fragment of the human TfR promoter containing a putative HRE sequence; (ii) mutation of this sequence prevented stimulation of luciferase activity; (iii) binding to this sequence of HIF-1α, identified by competition experiments and supershift assays, was induced by DFO. Furthermore, in mouse hepatoma cells unable to assemble functional HIF-1, inducibility of TfR transcription by DFO was lost and TfR mRNA up-regulation was reduced. These results, which show the role of HIF-1 in the control of TfR gene expression in conditions of iron depletion, give insights into the mechanisms of transcriptional regulation which concur with the well-characterized post-transcriptional control of TfR expression to expand the extent of response to iron deficiency.

INTRODUCTION

Iron is an essential element required for cell multiplication and several other important cellular functions. Iron acquisition is mediated by the transferrin receptor (TfR), a membrane protein which internalizes diferric transferrin through receptor-mediated endocytosis. TfR expression is strongly influenced by intracellular iron levels: iron deprivation activates TfR synthesis whereas the opposite occurs when cells are iron replete (1). Analysis of the molecular mechanisms underlying the regulation of this gene by iron initially indicated a role for transcriptional control, as shown by experiments in which actinomycin D prevented the rise in TfR mRNA levels triggered by iron chelation (2) and by more direct nuclear run-on assays in iron-deficient and iron-loaded erythroid cells (3). Reporter gene assays of TfR promoter activity confirmed the role of the transcriptional response (4). However, the discrepancy between the extent of transcriptional induction (3- to 5-fold) and mRNA accumulation (10- to 20-fold) indicated the existence of a strong post-transcriptional control and led to the discovery of the IRE–IRP interaction, which is the major regulatory mechanism not only of TfR expression but also of intracellular iron homeostasis (5,6). In fact, the control of TfR mRNA levels by iron involves the interaction of iron regulatory proteins (IRP) with iron responsive elements (IRE) in the 3′-end of mRNA. In iron-depleted cells, IRP binding protects TfR mRNA from degradation and, at the same time, prevents ferritin mRNA translation, thus enhancing iron availability. The inverse occurs under conditions of iron overload, so that IRP link the expression of iron proteins to the cellular iron status (5,6). Since the discovery and characterization of the IRE/IRP system, studies of the regulation of TfR gene expression have focused on the post-transcriptional response whereas transcriptional control has been somewhat neglected, with the notable exception of proliferation-dependent regulation of TfR expression (reviewed in 7). In fact, higher rates of TfR gene transcription have been demonstrated in proliferating cells (8). In addition, the growth-dependent interaction of nuclear factors with mitogen-responsive regions in the TfR promoter has been outlined in detail (9–11).

Hypoxia inducible factor (HIF-1), which activates the transcription of a number of genes that may help the cell to adapt to reduced oxygen supply, is the major component of the molecular response to hypoxia (reviewed in 12–15). HIF-1 is a heterodimeric DNA binding complex composed of an α and a β subunit; the latter is constitutively expressed and is identical to the aryl hydrocarbon receptor nuclear translocator. Hypoxia results in the stabilization of HIF-1α enabling it, upon dimerization, to bind hypoxia responsive elements (HRE) in target genes.

Since treatment with the iron chelator desferrioxamine (DFO) mimics the effect of oxygen deprivation on a number of hypoxia responsive genes (12–15), we decided to evaluate the role of HIF-1 in transcriptional activation of the TfR gene under conditions of iron deprivation.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

MATERIALS AND METHODS

Cell lines and culture conditions

Hep3B human hepatoma cells were grown in minimal essential medium (MEM) and Hepa-1 c1c7 and c4 mouse hepatoma cells in αMEM. Media were supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin. Cell lines were maintained in a humidified incubator at 37°C in 5% CO_2, 95% air. Cells were exposed to 100 μM DFO or 100 μM $CoCl_2$ (Sigma, Milano, Italy) for 20 h.

Northern blot analysis

Total cellular RNA was isolated as described (16) and equal amounts of RNA were electrophoresed under denaturing conditions. To confirm that each lane contained equal amounts of total RNA, the rRNA content in each lane was estimated in the ethidium bromide stained gels by laser densitometry. RNA was transferred to Hybond-N filters (Amersham Co., Milano, Italy) which were hybridized with ^{32}P-labeled human TfR cDNA pTR10 (17) or mouse TfR cDNA pTfR-2 (18). Quantitative determination was achieved by direct nuclear counting using an InstantImager (Packard Instruments Co., Milano, Italy) and the values were calculated after normalization to the amount of rRNA.

Nuclear transcription assay

Nuclei were purified and incubated for *in vitro* transcription as described (19). ^{32}P-labeled RNA elongated *in vitro* was purified and equal amounts of trichloroacetic acid-precipitable radioactivity for each sample was hybridized to TfR (17) and ferritin L subunit (20) cDNAs fixed on nitrocellulose filters. Hybridization signals were evaluated by direct nuclear counting using an InstantImager and normalized to the values of ferritin L subunit after subtraction of the background values represented by the hybridization signals of the empty plasmid pGEM (Promega, Milano, Italy).

Plasmid constructs

To construct the pTfRB-luc clone, a 455 bp fragment was amplified from the no. 9 derivative of plasmid pcD-TR1 (21) using oligonucleotides corresponding to positions –439 to –424 and +2 to +16 as 5′ and 3′ primers, respectively. The amplified product was blunted and inserted into the *Sma*I site of the pGL2 vector (Promega, Milano, Italy). Mutation of the putative HRE sequence 5′-TACGTGC-3′ in the pTfRB-luc plasmid with replacement of the bases TACGT by AATTC to construct pTfRBm-luc was by PCR-based site-directed mutagenesis using the ExSite Mutagenesis kit (Stratagene, Milano, Italy). All constructs were verified by DNA sequencing.

Transient transfection assay

Subconfluent Hep3B and Hepa-1 cells were transiently co-transfected using the calcium phosphate method with 10 μg of a 50:1 mixture of pGL2 constructs and pRL-SV40 reporter vector containing Renilla luciferase which was used to normalize for transfection efficiency. After recovery for 48 h the cells were subjected to the various treatments. Cells were collected, washed and lysed using the reporter lysis buffer (Promega, Milano, Italy). Luciferase activities were then measured in a Lumat LB 9501 luminometer (Berthold) using the Dual-Luciferase Reporter Assay System (Promega, Milano, Italy) according to the manufacturer's instructions.

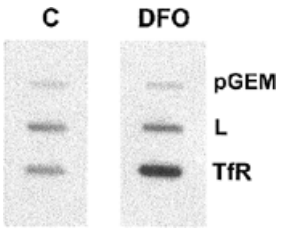

Figure 1. Nuclear run-on assay of TfR transcription. Equal amounts of ^{32}P-labeled RNA synthesized in vitro by isolated nuclei purified from Hep3B cells left untreated (C) or treated with 100 μM DFO for 20 h were hybridized to panels of the indicated DNA probes (pGEM, pGemini; L, ferritin L subunit; TfR, transferrin receptor) immobilized on nitrocellulose filters. The autoradiogram shown is typical of three different experiments.

Nuclear extract and electrophoretic mobility shift assay (EMSA)

The TfR-18 probe corresponding to the sequences from nucleotide position –93 to –75 relative to the transcription start site in the human TfR gene (AGCG**TACGTGC**CTCAGGA) was labeled with [γ-^{32}P]ATP by means of T4 polynucleotide kinase. The W18 oligonucleotide was synthesized according to Wang and Semenza (22). EMSA using 10 μg protein of Hep3B nuclear extracts prepared as described (22) was performed in 10 mM Tris, pH 7.8, 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 5 mM dithiothreitol and 20 μg/ml poly(dI-dC). After preincubation for 5 min at room temperature and addition of the labeled probe, samples were incubated for 20 min prior to electrophoresis. For supershift assay, 1 μg of OZ15 monoclonal antibody to HIF-1α (NeoMarkers, Union City, CA) was added and the binding reaction mixture was incubated at 4°C for 90 min.

RESULTS

Run-on transcription analysis

The role of HIF-1 in the response of TfR to DFO was studied in Hep3B hepatoma cells, which are extensively used to investigate the regulation of genes associated with hypoxic stress. We initially tested whether transcriptional regulation of the TfR gene in response to iron deprivation was present in hepatoma cells. In fact, previous evidence of transcriptional activation (3) has been obtained in erythroid cells, which express TfR at very high levels and show distinct features of TfR regulation (23). To directly assess this point, we measured TfR gene transcription in isolated nuclei. Figure 1 shows that the transcription rate of the TfR gene is stimulated by DFO. On the other hand, as previously demonstrated in K562 cells (24), transcription of the gene for ferritin L subunit was not affected by DFO. Quantification of three separate experiments showed a 3-fold induction of TfR gene transcription.

Transcriptional activity of the TfR promoter

We then analyzed the structure of the human TfR gene to determine whether any HRE were present. Search with the

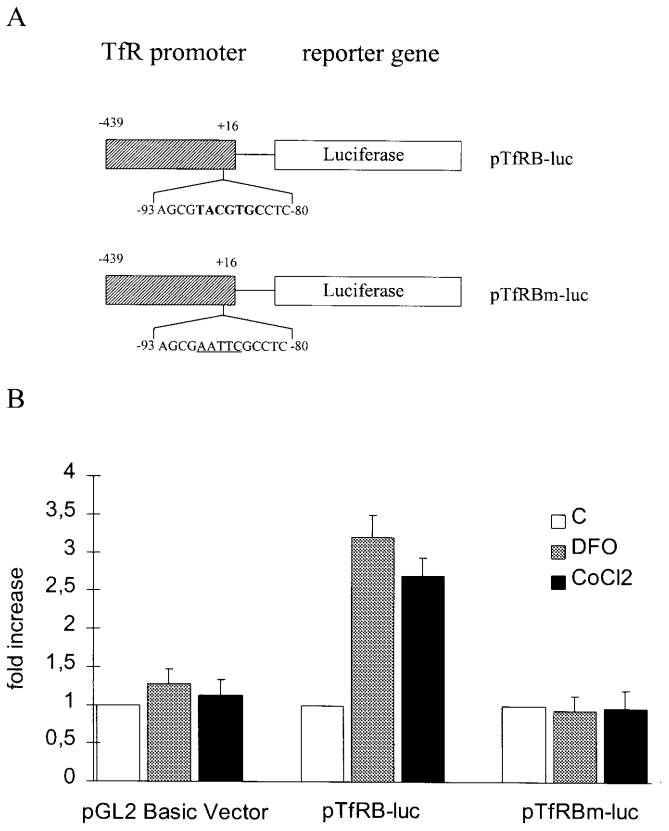

Figure 2. Reporter gene activation assay of TfR transcription. (**A**) Structures of reporter gene constructs. A 455 bp fragment of the TfR promoter (pTfRB-luc) was cloned in front of the firefly luciferase gene. A sequence containing the putative HRE is shown expanded with the core motif in bold (pTfRB-luc). This sequence was mutated in pTfRBm-luc (underlined). (**B**) Transient expression assay. Hep3B cells were co-transfected with reporter plasmids and control vector pRL-SV40 which contains the Renilla luciferase gene. After treatment with DFO or $CoCl_2$ for 20 h, luciferase activity was determined, corrected for transfection efficiency according to the Renilla luciferase activity and normalized to the normoxic relative luciferase activity arbitrarily defined as 1. All values represent means ± SD of three independent experiments.

MatInspector v.2.2 program revealed a sequence at –90 to –83 relative to the transcription start site (5′-TACGTGC-3′) matching consensus HRE and close to a CACA repeat (around –70) which seems to be necessary for hypoxic inducibility (12). To assess the role of HIF-1 in DFO-mediated transcriptional activation of TfR gene expression, a 455 bp fragment (pTfRB-luc) of the human TfR promoter region was cloned in front of luciferase and the resulting construct was transiently transfected into Hep3B cells. The activity of pTfRB-luc was compared with that of a similar construct (pTfRBm-luc) in which the HRE sequence had been mutated (Fig. 2A). In agreement with previous results which showed that a short region of the promoter is sufficient for expression of the TfR gene (25), the cloned fragment imparted efficient transcription of the promoterless reporter gene in transfected Hep3B cells, as demonstrated by a several hundred-fold induction of basal luciferase activity over that of the pGL2 Basic vector. Exposure to DFO stimulated luciferase expression 3-fold, as revealed by quantification of three experiments (Fig. 2B). Treatment with $CoCl_2$, as a positive control for HIF-1-mediated activation (12–15), induced a slightly lower response. No elevation was detected in cells transfected with the control pGL2 DNA. Importantly, the mutation almost completely prevented the DFO- and $CoCl_2$-stimulated increase in luciferase activity (Fig. 2B), thus suggesting that this sequence is a functional HRE in the response of TfR to DFO and $CoCl_2$.

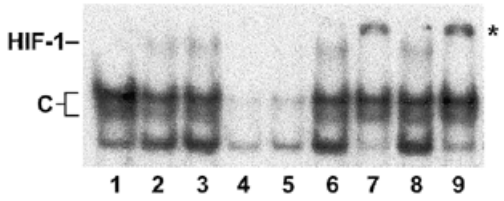

Figure 3. Analysis of HIF-1 DNA binding activity. Mobility shift assay. Nuclear extracts prepared from Hep3B cells left untreated (lane 1) or treated with 100 μM DFO (lanes 2, 4 and 5) or $CoCl_2$ (lane 3) for 20 h were incubated with radioactive TfR-18 in the absence (lanes 1–3) or presence of a 400-fold molar excess of unlabeled TfR-18 (lane 4) and W-18 (lane 5) oligonucleotides. The position of complexes containing constitutive (C) or HIF-1 binding activity is indicated. Supershift assay. DNA binding activities of nuclear extracts prepared from Hep3B cells treated with 100 μM DFO (lanes 6 and 7) or $CoCl_2$ (lanes 8 and 9) for 20 h were assayed as described above in the absence (lanes 6 and 8) or presence (lanes 7 and 9) of OZ15 monoclonal antibody against HIF-1α. The position of supershift complexes is indicated by the asterisk. The autoradiogram shown is representative of three different experiments.

DNA binding activity to the TfR HRE

To investigate whether the HRE sequence found in the TfR gene promoter was bound by HIF-1, EMSAs were performed. Figure 3 shows that the TfR-18 probe detected a constitutively expressed DNA binding activity (C) as well as a DNA binding activity (HIF-1) present in DFO- and $CoCl_2$-treated cells (lanes 2 and 3) but absent in untreated cells (lane 1). Competition with both unlabeled TfR-18 (lane 4) and an oligonucleotide corresponding to the HRE present in the erythropoietin enhancer (W-18, lane 5) inhibited the binding of both the constitutive and inducible complexes, thus demonstrating the specificity of the interaction and also suggesting the involvement of HIF-1. Supershift assays, in which nuclear extracts from DFO- and $CoCl_2$-treated cells (lanes 7 and 9, respectively) were incubated with a monoclonal antibody to HIF-1α prior to the mobility shift assay, confirmed that HIF-1α interacts with the HRE sequence of the TfR gene.

TfR gene expression in HIF-1β-deficient hepatoma cells

To further investigate whether HIF-1 is involved in transcriptional stimulation of the TfR gene, we made use of a mouse hepatoma cell line harboring a non-functional HIF-1β subunit (26). The mutant Hepa-1 c4 cells and the parental Hepa-1 c1c7 were transfected with pTfRB-luc and treated with DFO and $CoCl_2$. Figure 4A shows that luciferase activity was markedly stimulated by iron chelation and $CoCl_2$ in the wild-type cells (2.6- and 1.9-fold, respectively), whereas in the Hepa-1 c4 mutant cell line the extent of induction was much lower.

Since IRP activity is not affected in the mutant cell line (27), northern blot analysis (Fig. 4B) demonstrated a marked up-regulation of TfR mRNA content in both mouse hepatoma cell

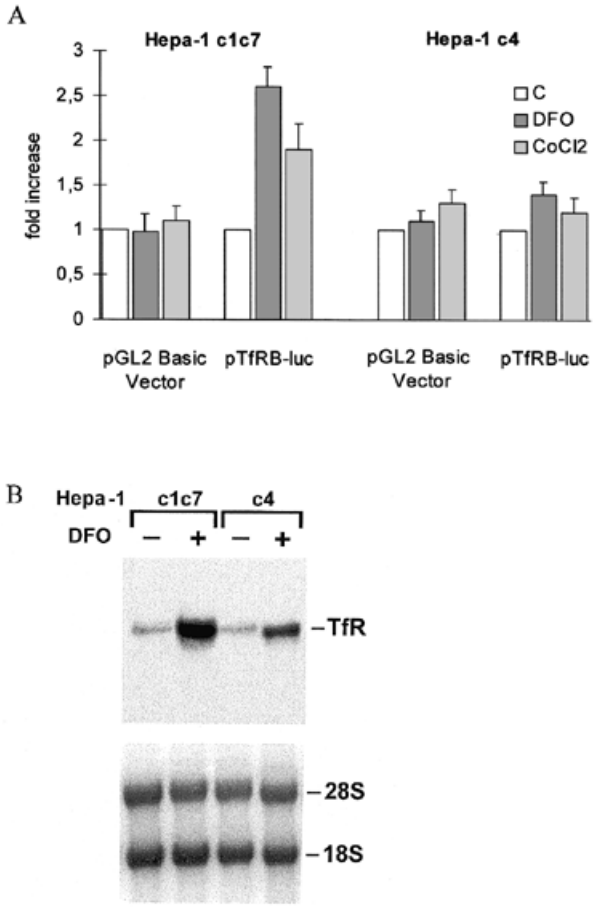

Figure 4. Regulation of TfR expression in wild-type and mutant Hepa-1 cells. (**A**) Reporter gene activation assay of TfR transcription. Wild-type Hepa-1 c1c7 and mutant c4 cells were co-transfected with reporter plasmids and control vector pRL-SV40 which contains the Renilla luciferase gene. Treatments and evaluation of luciferase activity were as described in the legend to Figure 2. All values represent means ± SD of at least three independent experiments. (**B**) Northern blot analysis of TfR mRNA levels. A filter with equal amounts of total cellular RNA, as revealed by ethidium bromide fluorescence of rRNAs, was hybridized with the mouse TfR cDNA as indicated in Materials and Methods. RNA was isolated from Hepa-1 c1c7 and c4 cells left untreated or treated with 100 μM DFO for 20 h. The autoradiogram shown is representative of three independent experiments.

lines, but quantification of three different experiments showed that TfR gene expression was stimulated 3-fold as much in Hepa-1 c1c7 cells as in their HIF-1β-deficient counterpart. These findings suggest therefore that HIF-1 is involved in transcriptional stimulation of the endogenous TfR gene and that in cells capable of assembling HIF-1 increased transcription of the TfR gene contributes with IRP-mediated post-transcriptional control to the accumulation of a higher amount of TfR mRNA.

DISCUSSION

It is evident that iron deficiency up-regulates TfR expression post-transcriptionally via IRP-mediated control of mRNA stability (5,6); however, we provide here results from several lines of investigation to show that iron deprivation also stimulates TfR gene transcription through HIF-1α, the transcriptional activator of hypoxia-sensitive genes. This conclusion is based on the following observations. We showed that a well-conserved HRE sequence is present in the human TfR promoter region which confers inducibility by DFO to transfected constructs (4; present study). Indication that the TfR HRE is required to confer transcription activation in response to iron chelation was provided by the finding that, when the putative HRE was mutated in order to abolish HIF-1 binding, the response to DFO was lost almost completely. Results of both EMSA and supershift assays were also consistent with HIF-1 acting as a transactivating factor in the response of TfR to DFO. Analysis of TfR expression in a mutant mouse hepatoma cell line which fails to form the HIF-1 complex (26), and which therefore has been widely used to link hypoxia inducible expression with HIF-1, has provided additional evidence suggesting that assembly of a functional HIF-1 is required for activation of TfR gene transcription and full TfR expression in response to iron deprivation. Inducibility of cooperative factors often contributes to HIF-1-mediated responses; e.g. an AP-1 site in proximity to the HRE has been shown to be involved in full hypoxic response (12–15). Indeed, an AP-1 site, implicated in mitogenic stimulation of TfR expression (9,11), is located downstream of the HRE in the TfR promoter. However, two types of evidence suggest, but do not prove, that additional transcriptional factors do not have a role in DFO-induced transcription: (i) in agreement with Kvietikova *et al*. (28), EMSA assays did not show variations in AP-1 binding activity in extracts from DFO-treated Hep3B cells (data not shown); (ii) mutation of the HRE almost completely prevented the response to the iron chelator. Taken together these studies demonstrate therefore that the TfR HRE is sufficient to confer transcription activation in response to DFO and that TfR possesses the main properties shared by HIF-1α-regulated genes (15).

Multiple levels of regulation of gene expression are often combined to expand the range of response to stimuli. This seems to apply also to the control of iron storage and acquisition. In fact, we have shown that overlapping controls determine iron-dependent and independent variations in ferritin synthesis (16,19,29,30). Also, in the case of DFO-induced TfR activation, two molecular mechanisms seem to be involved: increased transcription may allow IRP-mediated stabilization of a higher amount of transcripts, thus achieving a greater response to conditions of iron deprivation and allowing the cell to react to a situation that may severely affect important functions. This is made evident by our finding that HIF-1β-deficient cells, which rely only on post-transcriptional regulation, accumulate lower levels of TfR mRNA than their wild-type counterpart in response to DFO treatment. The findings of the present study add complexity to the view we have about the regulation of iron homeostasis as they further show that multiple levels of regulation of gene expression are combined to broaden the range of response to variations in iron content.

Recent findings have provided strong evidence confirming the interaction of HIF-1 and iron. In fact, von Hippel–Lindau protein-mediated regulation of HIF-1 stability is iron dependent (31) and the effect of DFO on HIF-1-mediated gene induction is due to its capacity to chelate iron, although it is presently still obscure how low intracellular levels of the metal trigger HIF-1 activation. Moreover, HIF-1 has been shown previously to induce a broad set of target genes in response to

iron chelation, probably including two proteins involved in iron metabolism: i.e. heme oxygenase and transferrin, which have been shown to be activated by HIF-1 (32,33). The results of the present study, which show that TfR, a protein strategically involved in iron acquisition, is transcriptionally activated by DFO, provide an additional link between HIF-1 and iron homeostasis.

In addition to giving insights into previous observations about the role played by transcriptional control in the activation of TfR gene expression, our finding that iron chelation activates TfR transcription by means of HIF-1 suggests that iron deficiency and hypoxia, the two conditions known to increase iron uptake, operate at least in part by means of the same molecular pathway, i.e. HIF-1-mediated activation of TfR. Whether TfR gene transcription is actually also induced by hypoxia remains to be determined and is presently under investigation.

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NOTE ADDED IN PROOF

While this work was under review, HIF-1-mediated activation of transferrin receptor gene expression in DFO-treated erythroid cells was reported by Lok and Ponka [(1999) *J. Biol. Chem.*, **274**, 24147–24152].