# Transcriptional regulation of the tartrate-resistant acid phosphatase (TRAP) gene by iron

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Tartrate-resistant acid phosphatase (TRAP) was first identified in cells from patients with hairy cell leukaemia. Subsequently, it has been found in other leukaemias, B-lymphoblastoid cell lines, osteoclasts and subsets of normal lymphocytes, macrophages, and granulocytes. Recent data indicate that TRAP and porcine uteroferrin, a placental iron-transport protein, represent a single gene product. However, the intracellular role of TRAP is unknown. We used a full-length human placental TRAP cDNA probe to examine TRAP expression in human peripheral mononuclear cells (PMCs). TRAP mRNA increased 50–75-fold after 24 h in unstimulated PMC cultures. Cell-fractionation experiments indicated that monocytes were the main cell population accounting for increased TRAP mRNA transcripts, and this was confirmed by histochemical staining for TRAP enzyme activity. Because expression of other iron-binding and -transport proteins is controlled by iron availability, we examined the role of iron in regulating TRAP expression. Increase of TRAP mRNA transcripts in PMCs was inhibited by 50 $\mu$M desferrioxamine, a potent iron chelator. The 5′ flanking region of the TRAP gene was cloned from a mouse genomic library. In preliminary transient transfection experiments, it was determined that the 5′-flanking region of the TRAP gene contained iron-responsive elements. Therefore, a series of stably transfected HRE H9 cell lines was developed bearing genetic constructs containing various segments of the murine TRAP 5′ promoter region driving a luciferase reporter gene. Treatment of transfectants with 100 $\mu$g/ml iron-saturated human transferrin (FeTF) was performed to assess iron responsiveness of the constructs. Constructs containing a full-length TRAP promoter (comprising base pairs −1846 to +2) responded to FeTF with a 4–5-fold increase of luciferase activity whereas constructs containing only base pairs −363 to +2 of the TRAP promoter did not respond. Constructs containing 1240 or 881 bp of the TRAP promoter gave only a 1.5- to 2-fold increase of luciferase activity with FeTF. In all cases, increase of luciferase activity was blocked by desferrioxamine. Cells transfected with another luciferase construct driven by a simian virus 40 promoter did not show any increase of luciferase activity with FeTF. These data indicate that expression of TRAP is regulated by iron and that this regulation is exerted at the level of gene transcription. The transfection experiments also suggest that the region of the TRAP 5′-flanking sequence between base pairs −1846 and −1240 contains an iron regulatory element.

## INTRODUCTION

Tartrate-resistant acid phosphatase (TRAP), the type 5 isoenzyme of acid phosphatase, is a glycoprotein with an apparent $M_r$ of 34000 [1]. TRAP is distinguished from other acid phosphatase isoenzymes by the fact that its phosphoprotein phosphatase activity is not inhibited by L(+)-tartrate [2].

TRAP, normally detected as a minor isoenzyme in spleen, lung, liver and bone, can become the dominant isotype in cells affected by pathological conditions such as Gaucher's disease or hairy cell leukaemia [2–5]. Elevated TRAP levels also may be observed in spleens of patients with Hodgkin's disease [6], in cells of patients with various B- or T-cell leukaemias [6], and in sera of patients undergoing active bone turnover [7]. Among normal cells, TRAP has been identified in osteoclasts and in subsets of lymphocytes, macrophages and granulocytes [2,3].

TRAP contains two atoms of iron bound per molecule and human TRAP shares approx. 85% sequence identity with porcine uteroferrin [8]. Like TRAP, uteroferrin contains two iron atoms per molecule and has tartrate-resistant phosphoprotein phosphatase activity [1,8,9]. Uteroferrin is secreted by uterine endometrial cells under the influence of progesterone and is hypothesized to play a role in placental iron transport [9]. Lord et al. [10], Grimes et al. [11], and Ling and Roberts [12] have all observed a single band when hybridizing mouse and rat genomic DNA with a human TRAP cDNA clone, indicating that TRAP and uteroferrin represent a single gene product, a finding confirmed by direct nucleotide sequencing [12]. The intracellular function of TRAP is unknown, although it may have a critical role in the bone resorptive process of osteoclasts [13].

Iron availability is known to influence expression, largely through post-transcriptional mechanisms, of proteins such as transferrin receptor and ferritin, which are involved in iron transport, uptake and storage [14]. Because TRAP/uteroferrin represents an iron-containing protein that may also be involved in iron transport, we considered the possibility that iron also might regulate expression of TRAP. We used a full-length human placental TRAP cDNA probe [8] to examine expression of TRAP mRNA by human peripheral mononuclear cells (PMCs) and the role of iron in regulating this expression.

## MATERIALS AND METHODS

### Preparation of human PMC populations and cell cultures

PMCs were isolated from venous blood of healthy volunteers by density sedimentation on ficoll-Hypaque [15]. PMCs were separ-

Abbreviations used: TRAP, tartrate-resistant acid phosphatase; PMC, peripheral mononuclear cell; FeTF, iron-saturated human transferrin; DF, desferrioxamine; TFR, transferrin receptor; PKC, protein kinase C.

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ated into adherent and nonadherent cell populations by two successive incubations in T-150 polystyrene flasks. For each incubation, PMCs, $2.5 \times 10^6$ per ml, were incubated in RPMI medium containing 10% heat-inactivated fetal bovine serum (FBS) for 1 h at 37 °C in a 5% (v/v) $CO_2$ atmosphere. To assess TRAP expression, adherent or nonadherent PMC subpopulations were incubated for various times at 37 °C in 5% $CO_2$ in RPMI containing 200 mM L-glutamine and 10% heat-inactivated FBS with no additions or 50 $\mu$M desferrioxamine (DF). TRAP enzyme activity was evaluated using cytospin preparations of PMC cultures stained by standard cytochemistry methods [2].

### RNA hybridization analyses

Total cellular RNA was isolated by a modification of the guanidinium isothiocyanate technique [16]. Integrity of RNA was assessed by visualization of ethidium bromide-stained gels. Total cellular RNA was fractionated by electrophoresis through a 1% (w/v) agarose gel containing formaldehyde, then transferred to nitrocellulose filters presoaked in 20× SSC (SSC = 0.15 M NaCl, 15 mM sodium citrate dihydrate). The filters were hybridized with $^{32}$P-oligolabelled cDNA probes. After washing, the filters were exposed to Kodak XAR film at −70 °C with Dupont Cronex intensifying screens. Relative intensities of blots on autoradiographs were quantified by scanning densitometry.

Two cDNA probes were used: a full-length human placental TRAP cDNA probe of 1412 bp [8], and a 4.1 kb fragment from the bovine phPKC-$\beta$ 1-15-Eco clone which detects human PKC-$\beta$ mRNA [17].

### Construction of TRAP–luciferase chimeric gene constructs

The murine TRAP gene was isolated from a mouse spleen genomic library [18]. A 2 kb *Sma*I fragment containing the 5′-flanking region was subcloned into the pBluescript II KS-vector to produce the plasmid pSma5 and the nucleotide sequence of this fragment was determined (GenBank accession number M85212). Construction of a TRAP–luciferase fusion gene (pKB5) was accomplished by insertion of TRAP sequences from the region −1846 to +2 bp into the *Kpn*I/*Bgl*II sites of a promoter-deficient pGL2 basic vector containing the luciferase reporter gene [18]. Other TRAP–luciferase fusion constructs (pk5SP8, −1240 to +2 bp; pk5STt2, −881 to +2 bp; pk5SSt1, −330 to +2 bp) were developed by progressive 5′ deletion of the TRAP promoter region.

### Transfection assays

The rabbit endometrial cell line, HRE H9, was used for these experiments because it efficiently expresses TRAP and other cell lines that do so are not available [19]. HRE H9 cells were plated at a density of $2 \times 10^6$ cells/100 mm dish in $\alpha$ minimal essential medium with 10% FBS 24 h before transfection using the $CaHPO_4$ method (Stratagene). For transfections, 15 $\mu$g caesium chloride gradient-purified plasmids were used. The neomycin-resistant plasmid PWL neo (Stratagene) was co-transfected with TRAP–luciferase plasmids at a 1:3 molar ratio. Cells were exposed for 12 h to the $CaHPO_4$/DNA precipitate, washed twice with serum-free medium, fed with fresh culture medium supplemented with 10% FBS, then incubated for 48 h at 37 °C in 5% $CO_2$. G418 treatment (400 $\mu$G/ml) was applied 48 h after removal of DNA from the cultures and was maintained for 30 days before beginning the experiments. Stable clones bearing constructs of interest were derived by limiting dilution. To test response to iron, stably transfected cell lines were incubated for 48 h with or without FeTF (100 $\mu$g/ml) or DF (50 $\mu$M). After incubation, the cell monolayer was washed twice with phosphate-buffered saline and incubated at room temperature for 15 min with 0.3 ml cell-lysis reagent [25 mM Tris, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*NNN′N′*-tetra-acetic acid, 10% (v/v) glycerol, and 1% (v/v) Triton X-100]. The monolayer was scraped and spun briefly in a microcentrifuge to pellet the debris. Luciferase activity was assayed in the supernatant by mixing a 20 $\mu$l aliquot with 100 $\mu$l of luciferase assay reagent (20 mM tricine, 1.07 mM $MgCO_3$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 $\mu$M coenzyme A, 470 $\mu$M luciferin and 530 $\mu$M ATP). Light emission was measured for 20 s of integrated time using a Turner TD-20e Luminometer and following the manufacturer's instructions (Promega, Madison, WI, U.S.A.).

### Studies of mRNA stability

To determine the half-life of luciferase mRNA in transfected cells, HRE H9 cells bearing the pKB5 construct were incubated for 48 h under standard conditions with or without FeTF (100 $\mu$g/ml). Actinomycin D (5 $\mu$g/ml) was then added and aliquots of cells were removed at various times. RNA was extracted and subjected to hybridization analysis using a $^{32}$P-oligolabelled luciferase probe. Autoradiography was performed and relative intensities of blots were quantified by scanning densitometry.

## RESULTS

### Expression of TRAP mRNA in PMC cultures

Figure 1 illustrates a representative experiment to examine the appearance of TRAP mRNA transcripts in human PMC cultures as a function of incubation time. TRAP mRNA, barely detectable in freshly isolated PMCs, increased dramatically between 0 and 16 h of culturing, reaching levels 50–75 times that of baseline after 24 h. By contrast, PKC-$\beta$ transcripts did not change under the same conditions. This pattern was seen consistently and repeatedly (12 experiments) and occurred in unstimulated PMC cultures. When phytohaemagglutinin was added to the cultures,

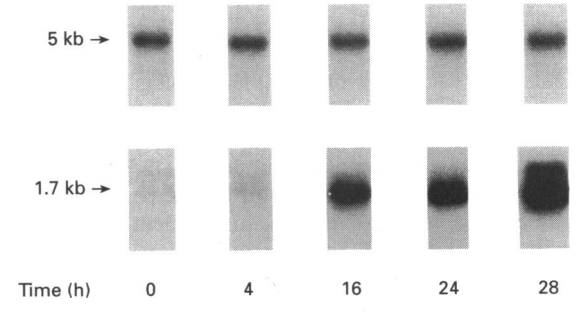


**Figure 1 Expression of TRAP mRNA by human PMCs**

PMCs were cultured under conditions described in the Materials and methods section for the times indicated. Cells then were harvested; total RNA was extracted and separated by formamide agarose gel electrophoresis, and transferred to nitrocellulose filters. The filters were hybridized with $^{32}$P-oligolabelled cDNA probes for TRAP (1.7 kb mRNA transcript) or PKC-$\beta$ (5.0 kb transcript).

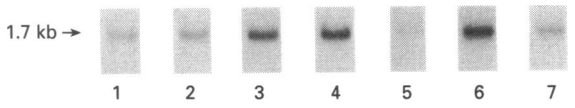


**Figure 2 Cell-fractionation experiments and effect of DF on TRAP mRNA expression**

Unfractionated PMCs, and adherent or nonadherent subsets were cultured under conditions described in the Materials and methods section for 24 h. Cells then were harvested; total RNA was extracted and separated by formamide agarose gel electrophoresis, and transferred to nitrocellulose filters. The filters were hybridized with a $^{32}$P-oligolabelled TRAP cDNA probe to detect the 1.7 kb TRAP mRNA transcript. Lane 1, unfractionated PMCs, time 0; lane 2, nonadherent PMCs, 24 h; lane 3, unfractionated PMCs, 24 h; lane 4, adherent PMCs, 24 h. In a parallel experiment, unfractionated PMCs were incubated without (lane 6) or with (lane 7) 50 $\mu$M DF for 24 h, after which RNA was extracted, fractionated and hybridized to the $^{32}$P-labelled TRAP cDNA probe.

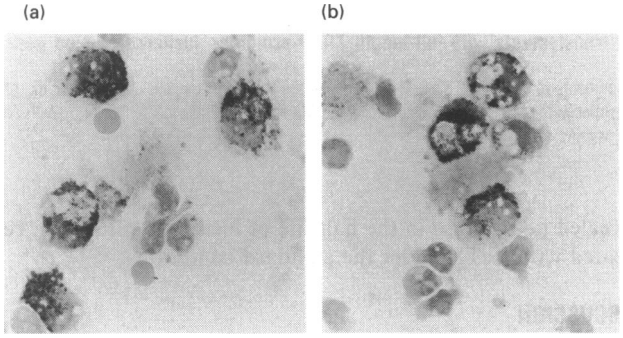


**Figure 3 TRAP enzyme activity in cultured PMCs**

PMCs were cultured under conditions described in Materials and methods for 24 h. Cells were harvested; cytospin slides were made and then were stained for TRAP activity (**b**) with or (**a**) without addition of 1 mM L-(+)-tartrate.

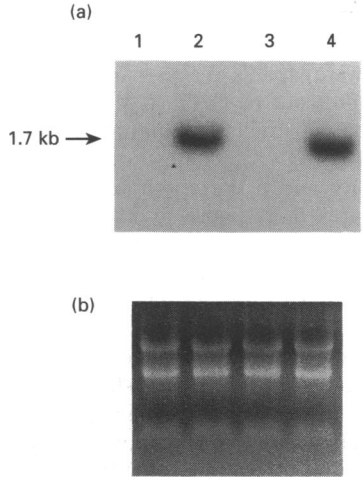


**Figure 4 Effect of DF and ferrioxamine on TRAP mRNA expression by PMCs**

Unfractionated PMCs were cultured for 48 h under conditions described in the Materials and methods section. Cells then were harvested; total RNA was extracted and separated by formamide agarose gel electrophoresis, and transferred to nitrocellulose filters. The filters were hybridized with a $^{32}$P-oligolabelled TRAP cDNA probe to detect the 1.7 kb TRAP mRNA transcript. Both the autoradiograph (**a**) and the corresponding ethidium bromide-stained gel (**b**) are shown. Lane 1, PMCs, time 0; lane 2, 48 h untreated; lane 3, 48 h + DF, 50 $\mu$M; lane 4, 48 h + ferrioxamine, 50 $\mu$M.

this increase of TRAP mRNA transcripts was not observed (results not shown).

Cell-fractionation experiments were performed to determine which PMC subpopulation was responsible for the increase of TRAP mRNA expression. Representative results are shown in Figure 2 (lanes 1–5). These data indicate that the adherent PMC subpopulation was solely responsible for the full increase of TRAP mRNA transcripts observed in these cultures. To confirm and to ascertain whether the TRAP protein was expressed in these cells, cytospin slides were prepared from 24 h cultures and stained for TRAP enzyme activity by a standard cytochemical method [2]. Results, shown in Figure 3, confirm the results of the RNA-hybridization data from the cell-fractionation experiments by demonstrating the presence of substantial TRAP activity in only those cells with monocyte–macrophage morphology, and not in lymphoid cells. In addition, these data indicate that the TRAP mRNA was actively translated into functional protein under these conditions.

## Effect of DF on TRAP mRNA expression

Having established the pattern of TRAP mRNA expression by cultured PMCs, we next addressed the role of iron availability in regulation of this expression. To screen for a potential role of iron, we incubated PMCs under identical conditions to those used earlier except for the addition of the intracellular iron chelator DF. As shown in Figure 2 (lanes 6 and 7) and Figure 4, addition of 50 $\mu$M DF to the PMC cultures effectively abolished the expected increase of TRAP mRNA transcripts. By contrast, ferrioxamine had no effect (Figure 4). The expression of $\alpha$-actin mRNA transcripts was not reduced by DF (results not shown). These data strongly suggest that increase of TRAP mRNA transcripts in PMC cultures is dependent upon the intracellular availability of iron rather than any toxicity of DF.

## Transfection experiments and response of TRAP–luciferase fusion genes to iron availability

To test directly the role of iron in expression of TRAP mRNA transcripts, we constructed a series of mouse TRAP–luciferase fusion genes in the plasmid pGL2 basic and performed transfections into the cell line HRE H9. To assess response to iron, we treated cells with human iron-saturated (diferric) transferrin (FeTF). Radioligand-binding studies with $^{125}$I-FeTF by methods we have described previously [16] were first carried out to confirm that human FeTF interacts with HRE H9 cells. Results of these studies indicated $\sim 6 \times 10^4$ high-affinity ($K_a = 3.2 \times 10^8$ M$^{-1}$) FeTF-binding sites per cell (results not shown).

The 1846 bp 5′-flanking region of the mouse TRAP gene has previously been shown to have promoter activity [18]. We initially performed transient transfections with constructs containing various segments of the TRAP 5′-flanking region. These experiments indicated that this region does contain DNA sequences that respond to FeTF with increased transcriptional activity.

Because of inherent variation in transfection efficiency with transient transfection experiments, we developed stable HRE H9 transfectants by co-transfection with PWL neo and selection in G418. We established stable lines bearing the luciferase fusion gene constructs listed in Table 1. FeTF stimulated 3.7-fold increase of luciferase activity in cells stably transfected with constructs containing the 1846 bp fragment of the TRAP 5′-flanking DNA. In addition, these FeTF-treated stable transfectants had increased levels of luciferase mRNA compared to untreated control transfectants (Figure 5). These increases were

**Table 1 Results of transfection assays with TRAP–luciferase gene constructs**

Stably transfected HRE H9 cells carrying the indicated gene constructs were established as described in the Materials and methods section, then incubated for 48 h with no additions, with FeTF (100 $\mu$g/ml), or with FeTF and 50 $\mu$M DF. Luciferase activity in cell lysates was then determined. Relative luciferase activity was determined by setting the activity of untreated transfectants at 1.0. Results for each gene construct are means ± S.E.M. of three separate determinations.

| Gene construct | TRAP sequence (bp) | Relative luciferase activity | |
|---|---|---|---|
| | | FeTF | FeTF + DF |
| pkB5 | −1846 to +2 | 3.7 ± 0.8 | 0.9 ± 0.1 |
| pk5SP8 | −1240 to +2 | 2.0 ± 0.1 | 1.0 ± 0.1 |
| pk5STt2 | −881 to +2 | 1.3 ± 0.1 | 0.8 ± 0.1 |
| pk5SSt1 | −330 to +2 | 0.9 ± 0.1 | 1.0 ± 0.1 |

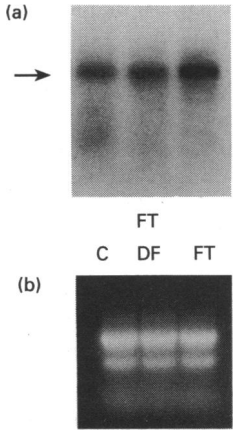


**Figure 5 Effect of FeTF on luciferase mRNA transcripts in stable transfectants with full-length TRAP-promoter–luciferase fusion genes**

Stably transfected HRE H9 cells containing full-length TRAP promoter (base pairs −1846 to +2)–luciferase fusion genes were established as described in the Materials and methods section, then incubated for 48 h with or without FeTF (100 $\mu$g per ml) or DF (50 $\mu$M). Cells were harvested; RNA was extracted, then analysed by hybridization to a $^{32}$P-oligolabelled luciferase probe. Both the autoradiograph (**a**) and the corresponding ethidium bromide-stained gel (**b**) are shown. The arrow indicates the position of migration of the luciferase transcripts.

blocked by DF, indicating a necessary role for iron in stimulating luciferase activity from the fusion gene construct. Constructs containing −1240 to +2 and −881 to +2 of the TRAP promoter region gave only 1.3–2.0-fold increases in luciferase activity when cells were treated with FeTF. And there was no significant increase of luciferase activity in stable transfectants containing the −363 to +2 bp TRAP promoter fragments. Cells transfected with plasmids in which the luciferase gene was driven by an SV40 promoter (pGL2 promoter) expressed high baseline luciferase activity but this activity did not increase in response to FeTF.

To examine the possibility that an untranslated mRNA region may have contributed to the increase of luciferase transcripts induced by FeTF in stable transfectants containing the −1846 to +2 bp segment of TRAP 5′-flanking DNA, we carried out studies of mRNA stability (Figure 6). The results of these studies

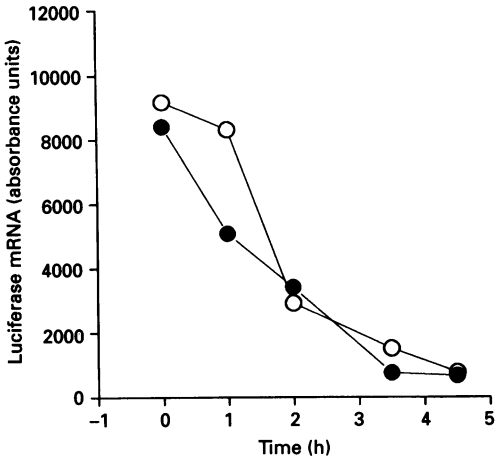


**Figure 6 Effect of FeTF on stability of luciferase mRNA transcripts in HRE H9 transfectants with full-length TRAP-promoter–luciferase fusion genes**

Experiments to determine the half-life of luciferase mRNA in transfectants bearing the pKB5 construct were performed as described in the Materials and methods section. ○, With FeTF; ●, without FeTF.

revealed no increase in the half-life of luciferase mRNA in cells treated with FeTF under the standard conditions.

## DISCUSSION

We have examined regulation of TRAP expression in human PMCs. A marked increase of expression of TRAP mRNA and functional protein occurred in the adherent cell component of 24 h unstimulated human PMC cultures. Increase of TRAP mRNA transcripts was inhibited by DF, a potent intracellular iron chelator. By contrast, ferrioxamine had no effect, indicating that the inhibition by DF was attributable to iron deprivation rather than a nonspecific effect on cell differentiation. HRE H9 cells transiently transfected with chimeric gene constructs containing mouse TRAP 5′-flanking DNA linked to the firefly luciferase gene were capable of expressing high levels of luciferase activity in the presence of FeTF. Cells stably transfected with TRAP-luciferase constructs containing 1846 bp of the mouse TRAP 5′-flanking DNA demonstrated a 4-fold increase of luciferase activity and a corresponding increase of luciferase mRNA transcripts in response to FeTF whereas cells stably transfected with constructs containing only 363 bp of the 5′-flanking TRAP DNA demonstrated no increase. Transfectants bearing constructs with intermediate lengths of the TRAP 5′-flanking DNA (1240 or 881 bp) gave more modest (1.3–2.0-fold) increases of luciferase activity. The increase in luciferase activity in FeTF-treated cultures was abolished by DF. These data provide strong evidence that intracellular iron levels are critically involved in regulation of TRAP expression and that this regulation is mediated at the level of gene transcription.

We considered the possibility that the effect of iron might be exerted in part at a post-transcriptional level. Primer extension analysis of mouse TRAP poly(A)$^+$ RNA demonstrated a transcription start site at position −552 bp [18]. Therefore, we cannot exclude the possibility that an untranslated mRNA region may be present in the luciferase transcripts encoded by some of the fusion gene constructs used in these experiments. However, two observations argue against a significant translational control mechanism mediated by such an untranslated mRNA region. First, two constructs, PK5SP8 and PK5STt2, contain the coding

sequences for the potential untranslated region, yet neither showed as substantial an increase of luciferase activity in response to FeTF as did construct pKB5, which contained the entire 1846 bp 5′-TRAP DNA (Table 1). The 4-fold increase of luciferase activity induced by FeTF treatment of pKB5 indicates that the effect of iron is exerted predominantly at base pairs −1846 to −1240 of the 5′-flanking region, a segment that does not encode transcribed sequences. Second, the mRNA stability studies demonstrated no prolongation of the half-life of luciferase mRNA when FeTF was present in the incubations (Figure 6). These observations are additional evidence that the effect of iron is at the level of gene transcription.

TRAP, therefore, joins other eukaryotic genes whose expression is regulated by iron. The best understood members of this group are transferrin receptor (TFR) and ferritin, in whose cases iron regulation occurs predominantly at a post-transcriptional level [14]. However, there also are reports of iron effects on TFR and ferritin gene transcription, albeit of a considerably more modest degree. For example, Rao et al. [20] used nuclear run-on assays to demonstrate increase or decrease of TFR transcription in K562 cells in the presence of DF or haemin respectively. In addition, iron may stimulate ferritin transcription in some cell types [21,22]. For example, White and Munro [21] treated rats with ferric ammonium citrate and demonstrated 3-fold increase of L-ferritin transcription by liver nuclei [21]. Besides the examples of iron regulation of TRF and ferritin expression, there are a number of reports of regulation of gene transcription by iron-containing compounds such as haem, haemopexin, or ferritin [23–27]. In this regard, however, the roles of iron versus protoporphyrin or protein moieties of these compounds remain unresolved.

We have shown previously that the protein kinase C (PKC)-$\beta$ gene is transcriptionally regulated by iron in the T-lymphoblastoid cell line, CCRF-CEM [28]. In those studies, nuclear run-on assays demonstrated an approx. 7-fold increase in the rate of PKC-$\beta$ gene transcription within 3 h of treatment with FeTF. Increase in gene transcription was associated with sustained (up to 72 h) steady-state levels of PKC-$\beta$ mRNA transcripts and increase in both PKC enzyme activity and phorbol dibutyrate binding [28]. The requirement for iron in this regulation was established by showing that DF but not ferrioxamine abolished the FeTF-stimulated increase of PKC-$\beta$ transcripts and that neither gallium transferrin nor apo-transferrin stimulated a similar increase. Subsequently, we have shown that iron stimulates PKC-$\beta$ transcription in many haematopoietic cell lines [29], and most recently we have constructed PKC-$\beta$–luciferase fusion genes, whose expression is regulated by iron [30]. These data indicate that both PKC-$\beta$ and TRAP are regulated by iron at the transcriptional level. As indicated in Figure 1, however, it is also clear that different sets of circumstances are required for PKC-$\beta$ or TRAP gene transcription to be initiated or terminated in PMCs. Therefore, iron appears to represent only one factor in apparently complex gene regulatory pathways.

The mechanism by which the transcriptional regulation of TRAP expression by iron is mediated is unknown. The TRAP 5′-flanking DNA has been sequenced as far as −1846 bp [18]. The sequence contains numerous candidate transcription factor binding sequences, including those for AP1 and H-APF-1. Although a putative iron response element has been identified in the 5′-flanking region of the porcine uteroferrin gene [31], no candidate iron response elements have been identified in the TRAP 5′-flanking region. It therefore seems likely that a novel iron-responsive transcription regulatory mechanism is involved.

In summary, we have demonstrated that expression of the TRAP gene is regulated by iron. This iron regulation is mediated at the level of gene transcription. Iron responsiveness is contained within a 1846 bp fragment of the 5′-flanking region of the mouse TRAP gene. Based on the results of transfection experiments with TRAP–luciferase fusion genes, the DNA region between base pairs −1846 and −1240 appears to be most critical for conferring iron responsiveness.

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