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Bioinformatic analysis of the metal response element and zinc-dependent gene regulation via the metal response element-binding transcription factor 1 in Caco-2 cells

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Abstract The purpose of this study was to determine the correlation between the position or number of metal regulatory elements (MREs) near gene transcriptional or translational start sites, and the strength of metal response element-binding transcription factor 1 (MTF-1) regulation. A secondary analysis was performed in silico on published results measuring the effects of Zn and MTF-1 on transcriptional regulation of genes (n = 120) in the Caco-2 cell line. MRE sequence variations throughout the human genome were sorted using a position weight matrix. Three null hypotheses (H_0) were tested: (1) there is no correlation between the number of MREs and MTF-1 transcriptional strength, (2) there is no correlation between the distance of the MRE upstream from the transcriptional start site (TSS) and MTF-1 transcriptional strength, and (3) there is no correlation between the distance of the MRE downstream from the translational start site (TrSS) and MTF-1 transcriptional strength. Spearman correlation was used to test for significance ($p < 0.05$). From our results we rejected the first H_0; we observed a significant correlation between the total number of MRE sequences − 7Kbp upstream from the TSS, within the 5′ untranslated region, and + 1Kbp downstream from the TrSS, versus the strength of MTF-1 regulation ($r = 0.202$; $p = 0.027$). The second and third H_0 were accepted. These results expand our understanding of the role of the MRE in Zn-dependent gene regulation. The data indicate that Zn influences the transcriptional control of gene expression beyond maintaining intracellular Zn homeostasis.

Keywords MTF-1 · MRE · Zinc · Nutrigenomics · Transcription factor

Introduction

It is estimated that 2800 human proteins or about 10% of our proteome is composed of metalloproteins that require zinc (Zn) for biological function, regulation of activities, or structural purposes (Andreini et al. 2006; Klug 2010). The large majority of Zn-binding proteins are involved in the regulation of gene expression, with the most abundant class being Zn-finger proteins (Klug 2010). Intracellular Zn homeostasis is controlled through the coordination of Zn with the metal response element-binding transcription factor 1 (MTF-1), a 72.5 kDa Zn-finger protein in the Cys_2-His_2 family of transcription factors. MTF-1 has traditionally been understood in the context of positive

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transcriptional regulation of metallothionein (MT) and *SLC30A1* gene product in response to fluctuating intracellular Zn concentrations, and the resultant maintenance of Zn homeostasis driven by the expression of these genes (Stuart et al. 1984; O'Halloran 1993; Langmade et al. 2000; Saydam et al. 2002). However, the physiological role of MTF-1 is more wide-ranging. MTF-1 regulates gene expression in response to metal ion exposure, hypoxia, oxidative stress, and/or elevated temperature (Andrews 2001; Lichtlen and Schaffner 2001). Its regulatory effects have been implicated in the cellular antioxidant response (Stoytcheva et al. 2010), insulin synthesis (Huang et al. 2010; Li et al. 2014), growth and development (Grider et al. 2017), immunity and inflammation/hypoxia (Cramer et al. 2005; Murphy et al. 2005, 2008), iron and copper homeostasis (Selvaraj et al. 2005; Balesaria et al. 2010; Troadec et al. 2010), and epigenetic histone modification (Okumura et al. 2011). It has been suggested that MTF-1 is a master regulator of microRNA (miRNA) expression, along with c-Myb, NF-Y, Sp1, and AP-2α (Lee et al. 2007). MTF-1 is also currently a target for drug discovery and development to treat epileptogenesis (Van Loo et al. 2015), osteoarthritis (Kim et al. 2014), and prion disease (Bellingham et al. 2009).

As the name suggests, MTF-1 binds to the metal response element (MRE), a cis-regulatory element which consists of a highly conserved 5′-TGCRCNC-3′ core (R = A or G, N = A, C, G, or T) (Günther et al. 2012b). This seven-nucleotide consensus sequence is fairly ubiquitous in the genome and occurs over 2 million times (Kearse et al. 2012). The 5′ and 3′ flanking regions of the MRE may affect transcriptional activity (Koizumi et al. 1999), and there is the potential that a longer sequence beyond the 7-mer core sequence can alter the affinity and function of MTF-1 binding to the MRE.

Like many transcription factors, MTF-1 resides in the cytoplasm and, upon activation, translocates into the nucleus (Smirnova et al. 2000; Saydam et al. 2001). A domain near the C-terminus of MTF-1 regulates its homodimerization, which is important for metal-induced transcriptional activity. Interestingly, although Zn increases the transcriptional activity of MTF-1, it does not enhance dimerization of MTF-1. Copper (Cu) activates MTF-1 binding to a lesser degree than Zn but enhances dimerization more than Zn to synergistically enhance gene regulation (Günther et al. 2012a).

MTF-1 is conserved across metazoan species and has been characterized in human (Brugnera et al. 1994), mouse (Andrews et al. 2001), capybara (Lindert et al. 2008), *Drosophila melanogaster* (Zhang et al. 2001), pufferfish (Maur et al. 1999), zebrafish (Hogstrand et al. 2008), trout (Dalton et al. 2000), and chicken (Laity and Andrews 2007). MTF-1 knockout mice die in utero around day 14 from liver decay and edema. It is not known whether MTF-1 has a primary role in embryonic liver development, or if this is a cascading effect resulting from impaired cellular pathways and/or increased sensitivity to heavy metal content at this stage (Günes et al. 1998; Lichtlen et al. 2001; Wang et al. 2004). It is important to note that mouse and capybara MTF-1 (mMTF-1) is less sensitive to Zn-induced mMRE binding and subsequent regulation than human MTF-1 (Brugnera et al. 1994; Lindert et al. 2008). Interestingly, changing only three amino acids in the protein sequence of mMTF-1 to resemble the human counterpart in the nuclear export signal of the acidic activation domain will augment its metal inducibility to nearly match that of human MTF-1 (Lindert et al. 2009).

MTF-1 may also exhibit preferential binding to the "N" base in 5′-TGCRCNC-3′ depending on the metal stimuli. Cadmium treatment in *Drosophila* genomic tiling arrays (ChIP-chip) conferred to MTF-1 a binding preference to a C or T in the "N" position of the MRE, and Cu treatment gave a preference to G or A in that position, with little overlap between the two. Therefore, permutations of the core MRE motif could function to regulate genes which are expressed in response to various mineral stimuli other than Zn (Sims et al. 2012).

Of the six Zn fingers in MTF-1, the N-terminal fingers 1–4 have a high affinity for both Zn and the MRE. These fingers bind to the TGCRC side of the MRE sequence. Fingers 5 and 6 have a weaker affinity for the MRE and bind to the 3′ end of the sequence. However, all six fingers contribute to the ability of MTF-1 to form a complex with the MRE. It has also been suggested that fingers 5 and 6 can modulate the specificity and affinity of MTF-1 for the MRE (Labbé et al. 1991; Chen et al. 1999). MTF-1 exhibits the highest affinity binding to the sequence 5′-TGCACTC-3′, labeled MREd (Labbé et al. 1991; Stoytcheva et al. 2010).

MTF-1 also exhibits transcriptional regulation when binding to an MRE downstream from the transcription start site (TSS) of a gene. Human selenoprotein H (*SELENOH*) is downregulated by the action of MTF-1 binding to an MRE in the 5′ untranslated region (UTR) of the gene, 92 base pairs (bp) upstream from its translational start site (TrSS) (Stoytcheva et al. 2010). Similarly, an MRE within the first exon was shown to be responsible for downregulation of ZIP10 (*SLC39A10*) in zebrafish (Zheng et al. 2008). This upstream regulation by MTF-1 may be related to the RNA polymerase II stalling mechanism in genes which require rapid transcriptional response in cells (Muse et al. 2007). However, the relevancy of transcription factors binding to exonic regulatory elements is not clear, and it is currently a subject of debate whether this binding is passive and nonfunctional, or if it can contribute to recruitment of the transcription machinery (Stergachis et al. 2013; Xing and He 2015; Agoglia and Fraser 2016).

The purpose of this study was to determine whether the number or location of MRE are correlated with the degree of Zn-dependent transcriptional regulation as mediated through MTF-1. Three null hypotheses (H_0) were tested: (1) there is no correlation between the number of MREs and MTF-1 transcriptional strength, (2) there is no correlation between the distance of the MRE upstream from the transcriptional start site and MTF-1 transcriptional strength, and (3) there is no correlation between the distance of the MRE downstream from the translational start site and MTF-1 transcriptional strength. The results from this study will further our understanding of the role of Zn in transcriptional regulation of gene expression, as well as in other Zn-dependent cellular, biochemical and physiological processes.

Materials and methods

All analyses were performed using the human hg38 genome assembly (Dec. 2013, Genome Reference Consortium GRCh38). A search was performed to determine the genomic positions of the MRE consensus sequence with five bases added to the 5′ and 3′ ends (5′-nnnnnTGCRCNCnnnnn-3′) (Geneious; v10.2.3) (Kearse et al. 2012). The resulting sequences (n = 2,214,485) were applied to a position weight matrix for analysis (R v3.4.3; seqLogo v1440) (Fig. 1)

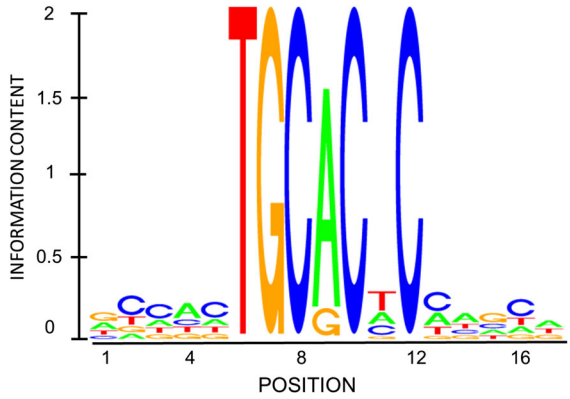

Fig. 1 Sequence logo of the genome-wide instances of TGCRCNC with five free bases on either side (5′-nnnnnTGCRCNCnnnnn-3′). There is a strong preference for A in the "R" position and a preference for T in the "N" position. The sequence made by the predominate base at each position coincides with the most commonly occurring 7-mer MRE sequence in the genome, TGCACTC, and the most common 17-mer sequence, GCCACTGCACTCCAGCA

(Schneider and Stephens 1990; Bembom 2017). Chi square analysis using Excel CHISQ.DIST was used to determine significance ($p \leq 0.05$) at each position (Table 1).

The genes that were analyzed in this investigation were obtained from Hardyman et al. (2016) which identified 120 protein coding genes within the Caco-2 human colonic adenocarcinoma cell line whose expression were affected by cellular Zn status and the presence/absence of MTF-1. Expression of these genes was defined as a ratio for the effect of MTF-1 on their expression: [downregulation (< 1); upregulation (> 1); no effect (1)] (Hardyman et al. 2016). The "absolute strength" of MTF-1 effect, controlling for direction of regulation, is defined as the absolute value of one minus the expression ratio, and was determined using the following formula:

$$\text{Strength of MTF-1 effect on gene expression (directional)} = \frac{\text{fold change with control siRNA(MTF-1 available)}}{\text{fold change with MTF-1 siRNA(without MTF-1)}}$$

$$\text{Absolute strength of MTF-1 effect on gene expression} = \left|1 - \frac{\text{fold change with control siRNA(MTF}-1\text{ available)}}{\text{fold change with MTF-1 siRNA(without MTF-1)}}\right|$$

A recent study reported that MTF-1 transcriptional regulation also occurs when an MRE is located downstream of the gene TrSS (Stoytcheva et al. 2010). Our search range in base pairs was chosen

Table 1 Position weight matrix of all instances of nnnnnTGCRCNCnnnnn in the human genome (n = 2,214,485)

Base	Position																
	1	2	3	4	5	6 (T)	7 (G)	8 (C)	9 (R)	10 (C)	11 (N)	12 (C)	13	14	15	16	17
A	0.309	0.134	0.199	0.487	0.186	0	0	0	0.889	0	0.273	0	0.300	0.439	0.195	0.182	0.229
C	0.129	0.508	0.479	0.175	0.490	0	0	1	0	1	0.218	1	0.455	0.177	0.214	0.493	0.464
G	0.391	0.151	0.151	0.165	0.154	0	1	0	0.111	0	0.056	0	0.069	0.171	0.437	0.124	0.146
T	0.171	0.207	0.171	0.173	0.170	1	0	0	0	0	0.453	0	0.176	0.214	0.155	0.201	0.161

based on data from Hardyman et al. (2016) and Stoytcheva et al. (2010). The Integrative Genomics Viewer (IGV; v2.3.90) was used to plot all TGCRCNC sequences that occur within − 7Kbp upstream of the TSS, as well as within the 5′ UTR and + 1Kbp downstream from the TrSS (Robinson et al. 2011; Thorvaldsdóttir et al. 2013). For genes with multiple MRE sequences, only the closest MRE to the TSS or TrSS was considered in the distance analyses. Only MRE sequences on the same DNA strand as the associated gene were considered. Spearman correlation used to test for significance was performed using GraphPad Prism (v7.04).

Results

Within the human genome, the 'R' and 'N' bases within the MRE consensus core sequence are most frequently 'A' and 'T', respectively (Fig. 1). The resultant sequence TGCACTC (MREd) is also the most commonly occurring MRE core sequence in the genome (Fig. 2). The MRE consensus sequence was extended by 5 bases in the 5′ and 3′ directions; there are 732,932 permutations of the 17-mer MRE present in the genome. The most common 17-mer MRE sequence (10.0% of total) is GCCACTGCACTC-CAGCC (Fig. 1). The majority of the 17-mer MRE sequence permutations (72.8%) occur singly in the genome.

The maximum range upstream for counting the MRE sequences was determined by searching for the MRE positions upstream from the TSS of genes; certain MTF-1-dependent genes within the working gene list exhibited their first MRE − 7Kbp upstream from their TSS. The lower position in the range was determined by searching for the MRE positions + 1Kbp downstream from the TrSS of genes in which there were no MREs in their 5′UTR. This search strategy took into consideration genes such as *ATP6V1B1* and *HIST1H2BD* which exhibited an ∼ 20% change in expression following treatment with the MTF-1 siRNA, yet lacked an MRE either upstream or in their 5′ UTR. Therefore, the MREs downstream of their TrSS, + 477 and + 755 bp respectively, appear to be the most likely candidates as functional binding sites for MTF-1 (Stoytcheva et al. 2010). The position of the MRE within *KRT17* was > + 1Kbp upstream from the TrSS, and it exhibited a 0.79-fold change in expression, however this gene is treated as a special case (Fig. 3).

There is a significant correlation between the number of MRE motifs in the region of − 7Kbp from the TSS to + 1Kbp from the TrSS, versus the absolute strength of MTF-1 regulation (n = 118, r = 0.205, p = 0.0260; Table 2). No significant correlations were observed in null hypotheses 2 or 3 as defined above.

The possibility that a MRE sequence longer than the 7-mer consensus sequence is more predictive of MTF-1 transcriptional gene regulation was explored. A 17-mer MRE, RCCAYTGCACTCYAGCC (R = A or G, Y = C or T) was used to search − 7Kbp upstream from the TSS of the 120-gene cohort. This 17-mer sequence is a combination of six of the most common permutations of the 17-base MRE sequence, and accounts for 19.5% of the total instances in the genome. Of the genes identified as actively regulated by MTF-1, a significant number (n = 63, 52.5%) had one or more instances of this motif in the range from − 7Kbp upstream to the TSS [χ^2 (1, N = 120) = 71.492, $p < 0.0001$].

Base composition may play a significant role in the binding affinity of MTF-1 to the MRE. To test this, a 17-base position weight matrix made from MREs

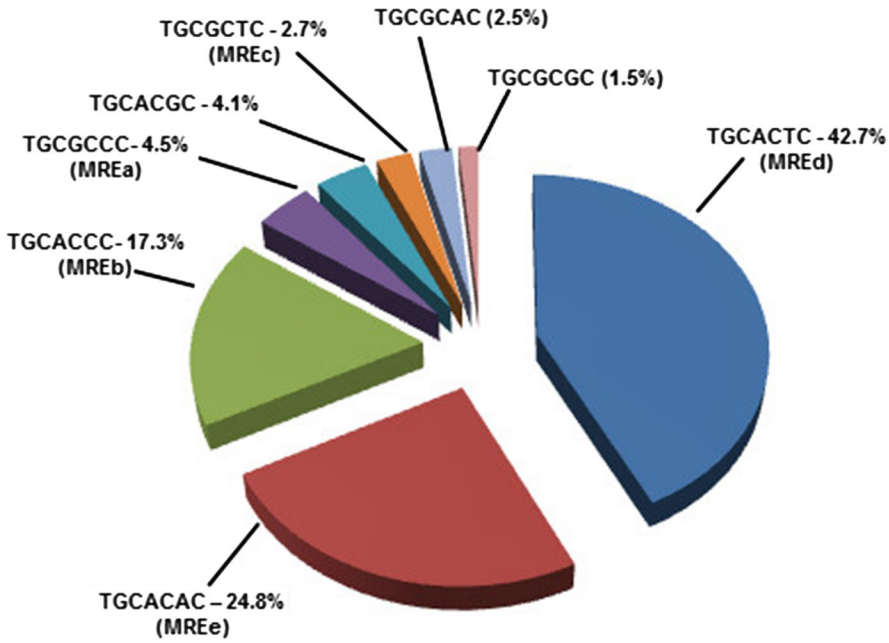

Fig. 2 Permutations of the MRE consensus sequence 5′-TGCRCNC-3′ in the human genome. Equivalent MRE names sometimes used are given in parenthesis. TGCACTC or MREd, which has been demonstrated to exhibit the highest affinity binding to MTF-1 (Stoytcheva et al. 2010), is also the most prevalent in the human genome

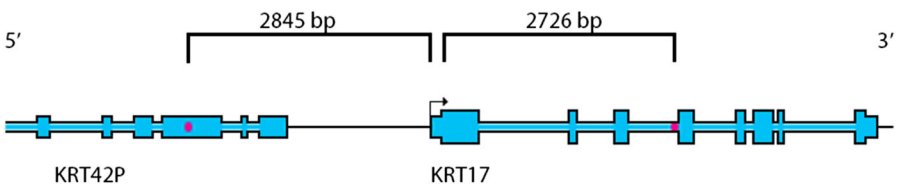

Fig. 3 *KRT17* (chr17:41,617,440-41,626,630) exhibits a 0.79-fold change ratio in MTF-1 control versus knockdown conditions. Large bars indicate coding sequence regions, dots indicate MRE positions. There is an MRE 2726 bp downstream from the *KRT17* TrSS, and the MRE 2845 bp upstream from the TSS is in an exon of the neighboring gene, *KRT42P*. Neither of these sites conform to currently understood mechanisms for MTF-1 binding and regulation

Table 2 Results of Spearman correlation tests comparing distance and quantity of MRE sequences versus the strength of MTF-1 effects on the regulation of associated genes

Spearman correlation test	Directional strength MTF-1			Absolute strength MTF-1		
	n	R	p (two-tailed)	n	R	p (two-tailed)
Upstream MRE distance	111	− 0.04555	0.6350	111	− 0.07074	0.4606
Downstream MRE distance	50	− 0.07241	0.6173	50	0.1325	0.3591
Total number of MRE instances	118	− 0.06677	0.4725	118	0.205	0.0260[a]

[a]There is a significant correlation between the absolute strength of MTF-1 effect on gene expression, defined as $\left|1 - \frac{\text{fold change with control siRNA(MTF-1available)}}{\text{fold change with MTF-1siRNA(withoutMTF-1)}}\right|$, and the total number of MREs associated with that gene

associated with MTF-1 responsive genes (Table S1a, 856 MREs, 120 genes, "expected") was compared against a position weight matrix made from MREs associated with MTF-1 unresponsive genes (Table S1b, 153 MREs, 7 genes, "observed") using a Chi squared test at each base position. There are significant differences in the relative weight of bases at every variable position in 5′-*nnnnn*TGCRC*N*C*nnnnn*-3′ excluding "R" [χ^2 (3, N = 153) > 7.815, $p < 0.05$]. However, the two most commonly

occurring 17-mer MRE sequences throughout the genome (GCCACTGCACTCCAGCC, ACCACTGCACTCCAGCC) remain the first and second most common MREs in both the responsive and unresponsive sets.

Discussion

MTF-1 functions as an intracellular Zn sensor by coordinating Zn and binding to the MRE within the promoters of MT and Zn transporters resulting in their transcription (Stuart et al. 1984; O'Halloran 1993; Langmade et al. 2000; Saydam et al. 2002). Several studies support a role for MTF-1 regulating genes other than those directly involved with cellular Zn homeostasis, including as a master regulator of miRNA gene expression (Andrews 2001; Lichtlen and Schaffner 2001; Lee et al. 2007). The results of this study are consistent with those indicating that MTF-1 is a Zn-dependent transcriptional regulator beyond MT and intracellular Zn homeostasis. Our study investigated the target for MTF-1, the MRE, and defined its prevalence within the human genome as well as correlating its location and abundance with the degree of expression in a Caco-2 gene cohort (Hardyman et al. 2016).

It has been previously suggested that base differences in the 5′ and 3′ flanking regions of the MRE core sequence contribute to the binding affinity of MTF-1 to the MRE, and thus can vary the strength of gene regulation (Koizumi et al. 1999). The number of MREs in this position analysis of bases was not large enough from which to draw conclusions, but the significant differences in base permutations within MREs of genes that have varied MTF-1 expression strength and responsivity is a subject of continued interest and relevance. It was interesting to note the proximity of MREs in genes shown to have no regulatory effects from MTF-1 (Table S1b). The presence of 109 instances of the MRE in the 5′ UTR of *ODZ4*, a gene unresponsive to MTF-1 regulation, raises the question of whether these MREs have any functional aspect. This discrepancy might be attributed to the base differences mentioned above. For example, in *ODZ4* the predominant base in the "N" position of its 109 MREs is A (42.1%, T = 28.6%), while in the MTF-1 responsive cohort of genes it is predominately T (40.5%, A = 25.0%).

For genes which have a nearby MRE but are unresponsive to MTF-1 regulation, there are other extraneous factors which could contribute to a lack of an effect. MTF-1 is hypothesized to form a transcriptional complex with p300 (or CBP) and Sp1 that is essential for gene regulation (Ogra et al. 2001; Li et al. 2008). Absence of, or interference with, either of these factors would likely disturb MTF-1 mediated regulation. MTF-1 also forms cascading regulatory circuits with miRNAs throughout the genome. For example, in a feed forward loop MTF-1 can target a gene for upregulation, while simultaneously upregulating one or more miRNAs that can post-transcriptionally suppress the products of that same target gene; this would appear as a net zero of regulatory effects (Arora et al. 2013). Although the idea was not explored here, the 3D structure of DNA can also potentially render some MREs more or less functional in terms of their MTF-1 binding and gene regulating capacity (Woringer et al. 2014).

The mechanism of action for MTF-1 binding to the MRE, and subsequently altering transcription, has yet to be fully elucidated. However, we have shown that for a large (n = 120) cohort of genes actively regulated by MTF-1, the number of MREs proximal to a gene affects its transcription. Additionally, not every gene with an MRE is regulated by MTF-1. There have not been identified extended MRE sequences that can definitively alter the function of MTF-1, but we have presented here a 17-mer sequence that appears in a significant number of MTF-1 active genes. These findings can serve to improve predictive analyses for genes, other than those directly involved with intracellular Zn homeostasis, that are regulated by MTF-1.

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