Biometals
https://doi.org/10.1007/s10534-020-00246-0

In silico analysis of putative metal response elements (MREs) in the zinc-responsive genes from *Trichomonas vaginalis* and the identification of novel palindromic MRE-like motif

Julio César Torres-Romero · José Luis Villalpando · Julio Lara-Riegos · Jesús Valdés · Elisa Azuara-Liceaga · Antonio Euan-Canto · César López-Camarillo · Maria Elizbeth Alvarez-Sánchez

Received: 13 May 2020 / Revised: 23 June 2020 / Accepted: 7 September 2020

Abstract Zinc is an essential micronutrient that plays an important role as a co-factor to several proteins, including zinc-responsive transcription factors. *Trichomonas vaginalis* is able to survive in the presence of high zinc concentrations in the male urogenital tract. Several genes in *T. vaginalis* have been shown to respond to changes in zinc concentrations, however, the zinc-dependent mechanism remains undetermined. Recently, we identified in *T. vaginalis* the zinc finger protein, TvZNF1, which is an ortholog of the mammal metal transcription factor (MTF1). We searched for several of the zinc-responsive genes in *T. vaginalis* to determine whether if they contain metal response elements (MRE), *cis*-acting DNA elements that specifically bind MTF1. Six highly conserved over-represented sequence motifs (TvMREs), which share similarity with other eukaryotic MREs, were identified in the zinc-responsive genes in *T. vaginalis*. We also demonstrated that some of the TvMREs assemble as divalent complexes either as two closely spaced TvMREs or as two overlapping TvMREs forming a palindromic-like sequence: TGCC(N3)GGCA. Electrophoretic mobility shift assays were used to detect the zinc-dependent binding of TvZNF1 and nuclear proteins from *T. vaginalis* to this specific palindromic motif. Our results support a novel mechanism used by *T. vaginalis* for the transcriptional regulation of associated zinc-responsive genes through a MTF1/MRE-like system.

Keywords MRE · *Trichomonas vaginalis* · Zinc-responsive genes · Zinc · MTF1

J. C. Torres-Romero · J. Lara-Riegos · A. Euan-Canto
Laboratorio de Bioquímica y Genética Molecular, Facultad de Química, Universidad Autónoma de Yucatán (UADY), Calle 43 S/N entre calle 96 y calle 40, Colonia Inalámbrica, CP 97069 Mérida, Yucatán, Mexico

J. L. Villalpando · E. Azuara-Liceaga · C. López-Camarillo · M. E. Alvarez-Sánchez (✉)
Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México (UACM), San Lorenzo # 290, Col. Del Valle, CP 03100 México City, Mexico
e-mail: elizbeth2@gmail.com; maria.alvarez@uacm.edu.mx

J. Valdés
Departmento de Bioquímica, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México City, Mexico

Introduction

Zinc is an essential micronutrient since playing an important role in catalytic and structural functions of proteins involved in diverse key metabolic processes, including cell signaling and transcription regulation (Maret 2017). Proteins that participate in the zinc uptake and export through the plasma membrane and other cellular membrane compartments (e.g. ZIP and ZnT transporters), or its utilization and storage (e.g.

Published online: 13 September 2020

metallothionein), regulate the cellular zinc homeostasis. The expression of these proteins is mainly co-regulated at the transcriptional level in response to fluctuations in cytosolic zinc concentrations, thus the encoding genes are known as zinc-responsive genes (Choi and Bird 2014).

When the intracellular zinc concentration is increased, MTF1 is translocated to the nucleus and binds to MREs located on target genes. It has been suggested that the regulatory outcome depends on the MRE location on the target gene. MREs have been reported in the promoter regions of the mouse *ZnT1* gene or *metallothionein* genes in diverse species (Langmade et al. 2000; Günther et al. 2012; Choi and Bird 2014). By the other hand, a transcription inhibition by RNA polymerase blockage could occur due to the interaction between MTF1 and MREs located in the coding region of certain genes, as the previously identified in the fish and mouse *ZIP10* genes or the human and murine *selenoprotein H* genes (Zheng et al. 2008; Stoytcheva et al. 2010; Lichten et al. 2011).

MREs are short DNA sequence motifs with a conserved 5′-TGCRCNC-3′ core with five free bases on either side (5′-nnnnnTGCRCNCnnnnn-3′) resulting in a 17-bp sequence (Francis and Grider 2018). Although, only the first 5 bases of the MRE core are required to interact with the four N-terminal zinc fingers in MTF1, this binding activity can be affected by the 5′- and 3′-flanking MRE region regions (Chen et al. 1999; Francis and Grider 2018). For example, GC-rich sequences in the 3′-flanking MRE region can take part in the MTF1 binding, even in cells without zinc supplementation (low-zinc MRE motif)(Koizumi et al. 1999; Wang et al. 2004), although it has been reported that in some cases, these GC-rich sequences, can be located in 5′-flanking MRE regions, rather than in 3′ (Stoytcheva et al. 2010). Particularly, it has been reported that the presence of a second MRE in the complementary strand located upstream from the 5′-flanking MRE region is required for a better interaction of MTF1 in the presence of zinc (Wang et al. 2004).

Trichomonas vaginalis, a protozoan parasite, is capable of surviving in two different microenvironments: the female urogenital tract with high iron content during the menstrual cycle and the male urogenital tract with high zinc concentrations in the prostatic fluid. There are cases reported of men that exhibit low concentrations of zinc (< 1.6 mM) with a higher incidence of trichomoniasis symptoms (Krieger and Rein 1982).

It has been reported that *T. vaginalis* grown in high zinc conditions (1.6 mM) expresses differential proteins in comparison to parasites grown in normal conditions, which include two proteinases: TvCP39 and TvMP50, fimbrin (TvFim1), and adenosylhomocysteinase (TvAHCY) (Vazquez-Carrillo et al. 2011; Puente-Rivera et al. 2017). Also, we have demonstrated that the addition of sub-toxic extracellular zinc to the *T. vaginalis* culture resulted in a downregulation in the transcription of the ZIP (Slc39) transporters: *TvZIP1, TvZIP3, TvZIP5, TvZIP7*, and the ZnT (CDF/Slc30) transporters: *TvZnT1* and *TvZnT7*. In contrast, *TvZnT3* transcription was upregulated in the presence of zinc (Fernández-Martín et al. 2017; Torres-Romero et al. 2018).

Recently, we characterized a zinc finger protein in *T. vaginalis* (TvZNF1) and demonstrated that it was able to bind to a mammalian MRE sequence *in vitro*, which suggests that *T. vaginalis* possesses a transcriptional regulatory mechanism mediated by a MTF1/MRE-like system, similar to the one controlling cellular zinc metabolism in different eukaryotic organisms (Villalpando et al. 2017). Additionally, we identified a metallothionein in *T. vaginalis*, which is transcriptionally induced by elevated concentrations of divalent metals, including zinc, and contains atypical MRE-like motifs (TGTNCNC) in their 5′-UTR (Netzahualcoyotzi et al. 2019). However, a trichomonal regulatory mechanism that mediates zinc-induced transcriptional up-regulation has not been identified. Herein, we show the evidence that the zinc-responsive genes in *T. vaginalis* possess DNA motifs that may function as putative MREs. This led us to discover the presence of a palindromic-like motif that is capable of being specifically bound by both trichomonal nuclear proteins and by the recombinant trichomonal zinc finger protein TvZNF1.

Materials and methods

Bioinformatics analyses

An in silico approach was applied to screen potential MRE motifs in a set of zinc-responsive genes in *T. vaginalis*, based on the hypothesis that conserved

regulatory motifs are shared among the co-regulated or co-expressed genes under certain environmental conditions (Van Loo and Marynen 2009). For this study, the sequence of 13 genes in *T. vaginalis*, which code for proteins or transcripts that have been reported exhibiting differential expression in the presence of zinc, were retrieved from TrichDB (http://trichdb.org/trichdb/): cysteine-proteinase of 39 kDa *TvCP39* (TVAG_298080), adenosylhomocysteinase *TvAHCY* (TVAG_210320), fimbrin *TvFim1* (TVAG_351310), metallo-proteinase *mp50* (TVAG_403460), metallothionein *tvmt-1* (TVAG_220940), Zinc finger protein *tvznf1* (TVAG_458980), ZnT (CDF/Slc30) transporters: *TvZnT1*, *TvZnT3* and *TvZnT7* (TVAG_132220, TVAG_285720 and TVAG_283320; respectively), ZIP (Slc39) transporters: *TvZIP1*, *TvZIP3, TvZIP5* and *TvZIP7* (TVAG_372990, TVAG_053760, TVAG_273550 and TVAG_495850; respectively). To address the identification of the MRE-like motifs within the mentioned above genes, the Find Individual Motif Occurrences (FIMO) tool from MEME (Multiple Em for Motif Elicitation) was used (Grant, Bayley and Noble 2011). Significant motifs were found using the previously published 7-mer MRE motifs: TGCNCNC, TGTNCNC, TGCNCNG, and TGCNGNC within two sets: within − 1000 nucleotides relative to the predicted translation start sites (for genes with zinc-induced upregulation) or complete coding regions (for genes with zinc-induced downregulation). MEME was also used to generate sequence logos for each discovered motif (Bailey et al. 2009).

Trichomonas vaginalis culture

T. vaginalis HGMN01 isolate was grown in culture flasks containing Diamond´s trypticase-yeast extract-maltose (TYM) medium pH 6.2 supplemented with 10% (v/v) heat-inactivated horse serum (Gibco). The medium was replaced daily and the parasites with 1 week of growth, in the logarithmic phase, were used in all assays.

Nuclear protein extract

Nuclear extracts (NE) were prepared from HGMN01 trophozoites grown overnight in the presence of 1.6 mM $ZnCl_2$ (Sigma). Briefly, 20×10^6 trophozoites were harvested, washed twice with cold phosphate buffered saline, pH 6.8, and suspended in 400 µL cold Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) containing protease inhibitors (Complete, Roche Molecular Biochemicals) and incubated for 15 min at 4 °C.

Afterwards, trophozoites were centrifuged at 8000×*g* for 5 min at 4 °C. Pellet (Nucleus) were lysed by incubation and agitation in a vortex for 40 min at 4 °C in 100 µL Buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) in the presence of protease inhibitors. After centrifugation at 17,000×*g* for 10 min at 4 °C, the supernatant corresponding to NE was aliquoted and stored at − 70 °C until use (Carvjal-Gamez et al. 2011). Protein concentration was determined by the Bradford method (Kruger 2009).

Electrophoretic mobility shift assays (EMSA)

The EMSA assays were performed as previously described (Villalpando et al. 2017). Briefly, primers used in EMSA assays were as follows: 5′-CGAGACACACATGCACACGCACACAGG-3′ (for the consensus MRE sequence, MRE2) (Lichtlen et al. 2001) and 5′-AGCTACTTTCGATGCCGCTGGCATT-3′ (for the putative palindromic-like MRE sequence on *mp50* promoter: 2XTvMRE*pal-mp50*). Double-stranded DNA probes were end-labeled with [γ-^{32}P] ATP by T4 Polynucleotide Kinase (New England Biolabs), followed by purification with spin columns (Qiagen 28306) according to the manufacturer's instructions. The EMSA binding reactions were performed by incubating 1 µL (20,000 c.p.m.) of the end-labeled probe with 15 µg of protein extracts (NE) or 44 µM of recombinant TvZNF1 (rTvZNF1) in ice for 20 min. The samples were separated by 6% polyacrylamide gel at 100 V for 6 h. The gels were then fixed and dried, followed by autoradiography.

Results

It is well known that transcriptional regulation of zinc-responsive genes is mediated by *cis*-acting metal response elements (MREs). Therefore, we retrieved the promoter sequences or the full-length coding

regions of the trichomonal genes that are upregulated or downregulated by zinc, respectively. First, we considered, that there may exist known as conserved MRE motifs (**TGC**N**C**N**C**) present in the regions of each of the 13 zinc-responsive genes in *T. vaginalis*. A conserved motif **TGC**N**C**(T/A)**C** (TvMRE1) was found nine times in the DNA sequences of 7 of the 13 genes analyzed (Fig. 1a). Almost all putative TvMRE1 motifs were located in the coding regions of downregulated genes by zinc, as expected for this type of regulation. Only *tvznf1*, that is upregulated by zinc, showed a conserved TvMRE1 motif in its promoter region. Also, the *TvAHCY* gene carried three conserved MRE motifs in its coding region and two of them were followed by a semi-conserved GC-rich region at either end (Fig. 1b), which, as it has been reported, is required for a better MTF1 binding (Francis and Grider 2018).

By in silico analysis, we previously reported a putative MRE in the metallothionein gene in *T. vaginalis* with the atypical signature: **TG**T(T/A)**C**N(**C**/A) (Netzahualcoyotzi et al. 2019). Thus, we extended our approach with a manual construction of the novel trichomonal MRE, conserving the "TGT" as the beginning of our motif and the last four bases of the consensus core, which includes the cytosines at positions 5 and 7: TGTNCNC, designated as TvMRE2 (Fig. 2a). The FIMO program was used to search for the TvMRE2 motif in the two training sets of the zinc-responsive genes in *T. vaginalis*. Interestingly, the occurrences of the TvMRE2 motif sequence was higher in the coding sequences (Fig. 2b). By contrast, the analysis of the promoter region of *tvmt-1*, the only downregulated gene by zinc, exhibited three TvMRE2 motifs in its UTR. These TvMRE2 motifs in *tvmt-1* are concordant with three of the five that were previously

A

B

Gene	Motif Position	Strand	Alignment
TvZnT1	1177/1183	+	TCGGC**TGCCCAC**ACGTC
TvZnT1	384/390	+	CTTAT**TGCACAC**GAAAA
TvZIP7	20/14	-	GCGTC**ATGCTCAC**CAAAA
TvZnT7	720/714	-	ATTGT**TGCTCCC**ATGAT
tvznf1	-147/-141	+	TTCTA**TGCTCTC**ATACA
TvZIP3	792/786	-	TTCCA**TGCTCTC**TCCTT
TvAHCY	901/907	+	CAATC**TGCGCTC**TCCAGG
TvAHCY	841/847	+	AGGGC**TGCGCTC**AATCC
TvAHCY	673/667	-	GAGTT**TGCCCTC**CTTCT

Fig. 1 Identification of conserved MRE motifs in *T. vaginalis*. **a** Logo representation, generated by MEME software, of the enriched motif for consensus 7 bp core sequence: TGCNCNC (TvMRE1), generated from similar sequences found, either coding or promoter region, from each of the 13 zinc-responsive genes from *T. vaginalis*. **b** Partial alignment of TvMRE1 motif-containing regions in the two training sets mentioned above. Boldface letters indicate the core sequences and the GC-rich sequences flanking motif core are underlined

A

B

Gene	Motif Position	Strand	Alignment
tvmt-1	-169/-175	-	TTCAT**TGTTCAC**TCAAA
tvmt-1	-278/-272	+	TTCGG**TGTACTC**TCAAT
tvmt-1	-402/-408	-	CGCTA**TGTTCAC**TGCCA
TvCP39	62/68	+	GGCAAC**ATGTTCAC**AGGCG
TvCP39	133/127	-	GCACGG**TTGTGCTC**CTGGA
TvCP39	579/573	-	TTTGA**TGTGCCC**TTAGC
TvCP39	825/819	-	CAAGA**TGTGCCC**CAGGA
TvAHCY	549/555	+	CTCGC**TGTCCTC**AAGCA
TvAHCY	1371/1377	+	CTCGA**TGTCCAC**CTTA
TvZnT7	625/631	+	CTGCA**TGTGCCC**TTTAT
TvZnT7	924/918	-	TTCAT**TGTCCAC**GAGAA
TvZnT1	822/828	+	GGTCG**TGTTCTC**TTAGA
TvZIP5	76/82	+	TATCG**TGTCCTC**TATTC
TVZIP7	2/8	+	AGAAA**TGTTCAC**TTTTG
TvFim1	-29/-35	+	AAAAG**TGTCCGC**TTTAT

Fig. 2 Identification of trichomonal specific MREs motifs. **a** Logo representation, generated by MEME software, of the enriched motif for consensus 7 bp core sequence: TGTNCNC (TvMRE2), generated from similar sequences found, either coding or promoter region, from each of the 13 zinc-responsive genes from *T. vaginalis*. **b** Partial alignment of TvMRE1 motif-containing regions in the two training sets mentioned above. Boldface letters indicate the core sequences and the GC-rich sequences flanking motif core are underlined

reported by us, confirming the validity of our first approach.

Divergent MRE sequences that contain only one mismatch in the consensus core motif either at 5 or at 7 positions (principally a substitution by G instead of the conserved C) have been previously reported (Varela-Nallar et al. 2006; Qi et al. 2007; Talavera-Montañez et al. 2019). To determine whether these divergent MRE motifs could be present in the zinc-responsive genes in *T. vaginalis*, we used the dataset and a search was performed using FIMO software with a p-value of $1e^{-05}$. The analysis revealed two novel trichomonal motifs: **TGC**(T/C)**C**(A/K)G (TvMRE3) and **TGC**(C/A)G(C/G)**C** (TvMRE4), which are closely related to the consensus MRE, but they contain a G > C substitution (underlined) at the positions mentioned earlier (Fig. 3, parts A and C). A total of 30 putative DNA motifs (14 for TvMRE3 and 16 for TvMRE4) appear in the analyzed regions of 10 of the 13 zinc-responsive genes in *T. vaginalis* (Fig. 3, parts B and D). Interestingly, all TvMRE3 and TvMRE4 motifs exhibited a peculiar pattern characterized by having a GC-rich sequence in at least three of the last four bases of the consensus core. Also, among the 30 TvMRE3-4 motifs, a conserved motif **TGCC**(G/**C**)H(**C**/G), designated here as **TGCC** motif, was found 11 times in five genes and was likewise found to be present in their coding regions (90.9%).

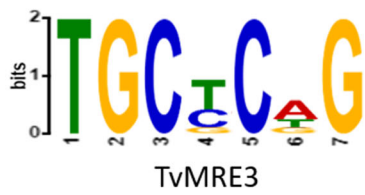
We also manually searched for novel trichomonal MRE motifs containing the conserved "**TGC**" sequence at the beginning of the motif, and the GC-rich sequence in the last four bases of the 7-mer core sequence. The analysis of the zinc-responsive genes from *T. vaginalis* resulted in the identification of two GC-rich conserved motifs: **TGC**(C/G)G(C/G)(T/A) and **TGC**(C/G)(T/A)(G/C)(**C**/G), termed TvMRE5 and TvMRE6, respectively (Fig. 4, parts A and C). The TvMRE5 motif was subsequently found within 5 of the 13 zinc-responsive genes in *T. vaginalis*, almost all present within the coding sequences (Fig. 4b). The TvMRE6 motif was found seven times, where the *TvAHCY* gene carried four of them (Fig. 4d). Particularly, among all TvMRE motifs, TvMRE5-6 motifs displayed the most conserved flanking GC-rich region around the consensus core.

Since it has been reported that the number and orientation of the motifs could be associated with pronounced effects on gene expression, we combined our data to establish a curated dataset with all potential TvMRE motifs to find specific patterns of response to zinc. In particular, we took into account the previously reported pattern of two MRE motifs within 18–20 base pairs (GTGTGCAN_{4-6}TGCGCAC), in a divergent orientation (2XMREs-c) and an MRE with an extended flanking sequence of consensus (TTT**TGC**G**C**A**C***GGC*ACTAAAT), which are related to the MTF1 binding under high or low zinc concentrations, respectively (Wang et al. 2004). We particularly noticed that an arrangement given by two close TvMREs motifs (2XTvMREs-c), separated by 4–5 base pairs, was found nine times in the DNA sequences of 5 of the 13 genes analyzed (Fig. 5A). The nine 2XTvMREs-c patterns are composed of two different TvMRE motifs that do not overlap and exhibit a random orientation (Fig. 5a). Most 2XTvMREs-c patterns were identified in the coding sequences of the involved genes, except for a pattern exhibited in the *tvmt-1* gene (Fig. 5b).

Interestingly, we noted a second rearrangement involving two TvMREs in opposite strands. Analysis of these regions shows that, in general, there are two TvMRE motifs partially overlapping at their 3′ end, displaying a palindromic-like sequence of 11 base pairs (2XTvMREs-*pal*), which was present four times in opposite DNA strands of zinc-responsive genes: *mp50*, *TvZnT1*, *TvZnT7* and *TvAHCY* in *T. vaginalis* (Fig. 6a). To find a consensus pattern of this palindromic-like sequence, the significant overlapping TvMREs motifs were analyzed using the MEME software. The sequence logo shows a well-conserved motif of 11-mer with the sequence: **TGCC**(**C**/G)N(**C**/K)**GGCA** (Fig. 6b), which is consistent with the overlapping of mainly **TGCC** motifs previously mentioned as part of the TvMRE3 and TvMRE6 motifs. Interestingly, we found two other overlapping sequences in *TvZnT1* and *TvZnT7* genes that consist of a TvMRE with an extended flanking sequence that consists of a second MRE at the same strand either an antisense (5′-**TGCC**C**A**C**A**C**GT**-3′) or in tandem (5′-**TGC**A**C**T**GCC**GCT-3′) orientation, respectively. Both overlapped sequences contain, at least, one TGCC motif (dashed underline).

Lastly, since the 2XTvMREs-*pal* motif found in the *mp50* gene (2XTvMRE-*palmp50*) was the only one in the promoter region of a zinc-responsive gene from *T. vaginalis*, we decided to use this sequence to partially characterize the specific DNA binding capability by an EMSA assay using both the recombinant trichomonal

A



C

B

Gene	Motif Position	Strand	Alignment
TvAHCY	498/492	-	GGAAC**TGCTCC**GGCGTT
TvAHCY	600/606	+	GTCGC**TGCCCAG**ATGAA
TvAHCY	777/771	-	TCAGA**TGCGCGG**TTGAT
TvAHCY	1099/1105	+	AGAAC**TGCCCA**GGCATC
TvZnT7	514/521	+	TCTCA**TGCACTG**CCGCT
TvZnT7	652/646	-	AAGGA**TGCACTG**TAAAA
TvZnT7	930/936	+	ATGAA**TGCACTG**GAGAA
TvZnT7	953/959	+	GATGA**TGCTCGG**TAAAT
TvZnT1	838/844	+	AAACA**TGCCCTG**AGCAG
TvZnT1	848/842	-	CTATC**TGCTCAG**GGCAT
TvZIP5	150/144	-	ATACC**TGCTCCG**AGAAA
TvZIP5	356/362	+	AAGTA**TGCTCAG**TGATG
TvZIP7	453/447	-	GAAAA**TGCGCTG**ACAAT
TvCP39	236/230	-	GCCTTG**TGCTCGG**CCTTG

D

Gene	Motif Position	Strand	Alignment
TvAHCY	76/70	-	TTCCT**TGCGGCC**AAGGAC
TvAHCY	198/204	+	CCAGAC**TGCCGTC**CTCA
TvAHCY	282/288	+	ACAGC**TGCAGCC**GCTAT
TvAHCY	330/324	-	ATACC**TGCTGGC**TTTTC
TvAHCY	742/748	+	ACGGC**TGCCGCC**ATTCT
TvAHCY	766/760	-	GTTGA**TGCCGTC**GATAA
TvAHCY	835/829	-	GCCCT**TGCCGAC**ATCGCC
TvZnT7	624/618	-	GGGCACA**TGCAGCC**ATAAG
TvZnT7	879/885	+	GATTC**TGCCGGC**GGCA
TvZnT7	889/883	-	AACAA**TGCCGCC**GGCAG
TvCP39	451/445	-	ACCAT**TGCAGCC**ATAGC
TvCP39	505/499	-	GAACT**TGCCGCC**CTGGT
TvZIP1	126/132	+	CTTT**TGCTGGC**GGAGT
TvZIP3	63/69	+	GCAAT**TGCAGGC**TTGT
TvZIP7	207/213	+	ATTAC**TGCTGGC**TTGGC
TvZnT3	-160/-166	-	TTCCT**TGCCGAG**GGAAT

Fig. 3 Identification of the TvMRE3 and TvMRE4 motifs. **a**, **c** Show the highest-scoring enriched motifs for consensus 7 bp core sequence: TGC(T/C)C(A/K)G (denominated TvMRE3) and TGC(C/A)G(C/G)C (denominated TvMRE4), both generated from similar sequences found, either coding or promoter region, from each of the 13 zinc-responsive genes from *T. vaginalis*. **b**, **d** Partial alignment of TvMRE3 and TvMRE4 motifs-containing regions in the two training sets mentioned above, respectively. Boldface letters indicate the core sequences and the GC-rich sequences flanking motif core are underlined

zinc finger protein TvZNF1 and the nuclear proteins from this parasite. The 2XTvMRE-*palmp50* probe forms a complex with the recombinant TvZNF1 of similar mobility to that which binds a conserved MRE (Fig. 7a). Similar results were obtained using nuclear extracts from *T. vaginalis* grown in high zinc conditions, where both 2XTvMRE-*palmp50* and mammal MRE probes form, at least, three complex bands with similar mobility (Fig. 7a, lanes 3 and 4). Next, we tested whether the novel TvMRE-*pal* sequence binds to DNA in a zinc-dependent manner. The EMSA assay was repeated using the standard reaction with additional increasing concentrations of EDTA, a very potent zinc-chelating agent. Figure 7b shows that the capability of the 2XTvMRE*pal-mp50* probe to bind to nuclear proteins in *T. vaginalis* grown in high zinc conditions was inhibited by EDTA in a concentration-dependent manner, with a decrease in the intensity of the complexes from the lower concentration tested until the complete abolishing at 100 µM of EDTA. Together, these results support the idea that *T. vaginalis* exhibits conserved and variant MRE sequences (in a novel palindrome-like rearrangement) that, through the interaction with TvZNF1, could mediate be mediating the zinc-responsive transcription of several genes.

A

C

B

Gene	Motif Position	Strand	Alignment
TvAHCY	943/937	-	CCTCGA**TGCGGCG**GACC
TvAHCY	1207/1213	+	TTGGC**TGCGCTA**CAGGT
TvZnT7	519/525	+	TGCAC**TGCCGCT**CACTC
TvZnT7	1242/1248	+	GAGAGC**TGCCGGA**AAGC
TvCP39	44/50	+	GCTGGA**TGCGCGA**GACAGG
TvCP39	325/331	+	GCCAG**TGCGGCT**CCTGC
TvZIP7	459/465	+	TTTTC**TGCCGGA**ACATT
mp50	-164/-158	+	TTCGA**TGCCGCT**GGCAT

D

Gene	Motif Position	Strand	Alignment
TvAHCY	104/110	+	GGAAA**TGCCAGG**TCTCA
TvAHCY	415/409	-	GGCCCT**TGCCATC**TGGCC
TvAHCY	538/544	+	CACA**TGCGTCC**TCGCT
TvAHCY	1108/1102	-	CTTGA**TGCCTGG**GCAG
TvZnT3	-120/-126	+	TACTC**TGCGTCC**CCCGC
TvCP39	898/904	+	TGGC**TTGCATCC**CAACA
mp50	-154/-160	-	TTGAA**TGCCAGC**GGCAT

Fig. 4 Identification of GC-rich trichomonal MRE motifs. **a**, **c** Show the highest-scoring enriched motifs for consensus 7 bp core sequences: TGC(C/G)G(C/G)(T/A) (denominated TvMRE5) and TGC(C/G)(T/A)(G/C)(C/G) (denominated TvMRE6), both generated from similar sequences found, either coding or promoter region, from each of the 13 zinc-responsive genes from *T. vaginalis*. **b**, **d** Partial alignment of TvMRE5 and TvMRE6 motifs-containing regions in the two training sets mentioned above, respectively. Boldface letters indicate the core sequences and the GC-rich sequences flanking motif core are underlined

Discussion

Among human parasitic infections, protozoan infections are the leading causes of morbidity and mortality. Protozoan parasites have evolved several specific pathogenicity mechanisms to survive within their hosts. Comprehension of these mechanisms, as the molecular mechanisms controlling gene regulation of pathogenesis-related proteins, could help us to improve protozoan infection prevention, control and therapeutics (Gomez et al. 2010).

Trichomonas vaginalis, the etiological agent of human trichomoniasis, requires several nutrients for growth, metabolism, and virulence. This parasite survives in the male tract in an adverse microenvironmental situation due to the high zinc concentration (> 1.6 mM) which is toxic and lethal (Krieger and Rein 1982). In contrast, at sub-toxic levels, zinc co-regulates the expression of several pathogenesis-related proteins and zinc homeostasis-related genes (Vazquez-Carrillo et al. 2011; Puente-Rivera et al. 2017; Fernández-Martín et al. 2017; Torres-Romero et al. 2018). However, the molecular mechanisms that control this zinc-induced differential expression in *T. vaginalis* is still poorly understood.

In most organisms, the gene expression of proteins associated with zinc metabolism is dependent on the MTF1/MRE system. We recently identified a trichomonad zinc finger protein (TvZNF1) which was able to bind to specific mammalian MRE sequences. Because of this, it was of particular interest to examine whether if MRE or MRE-like sequences are present in zinc-responsive genes previously identified in *T. vaginalis*, to establish a plausible zinc-dependent gene regulation mechanism in this parasite, at the transcriptional level.

A characteristic of the MRE and MRE-like sequences is their location, either in the promoter region or downstream of the transcription start site of metal-responsive genes that mediate induction or repression of gene expression by MTF1 binding, respectively (Stoytcheva et al. 2010; Zheng et al. 2008; Lichten et al. 2011). To test the latter hypothesis, we performed an in silico search for potential

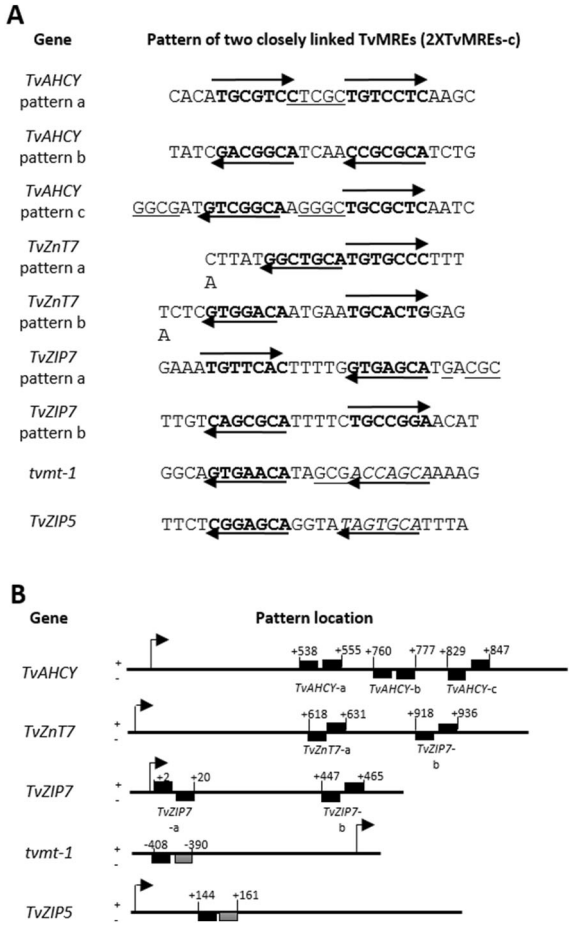


Fig. 5 Identification of a pattern of two closely linked TvMREs. **a** Shows the sequences containing two closely spaced TvMREs (2XTvMREs-c). Arrows indicate the random orientation of TvMRE motifs (boldface letters). The TvMRE-like sequences are indicated in italic letters. **b** Location of the 2XTvMREs-c pattern and strand orientation (+ or −) relative to the transcription start site (indicated by small arrows pointing to the right) with TvMREs represented by black boxes are shown. The gray boxes represent TvMRE-like sequences. The nucleotide positions are specified at the upper of the boxes

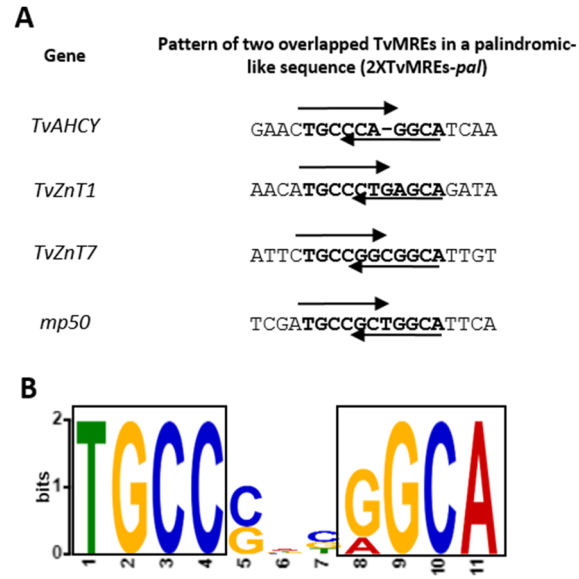


Fig. 6 Identification of a palindromic sequence generated by overlapping two TvMREs. **a** Shows the sequences containing a pattern of two overlapped TvMREs in a palindromic-like sequence (2XTvMREs-*pal*). The arrows indicate the individual TvMREs motifs (boldface letters) in a partially overlapping palindromic orientation. **b** Sequence logo, generated by MEME, of the enriched palindromic motif identified in the regions of trichomonal *AHCY*, *ZnT1*, *ZnT7* and *mp50* genes

MREs. An average of 5 trichomonal MRE (TvMRE) motifs per gene (65/13) was found the zinc-responsive genes in *T. vaginalis*. It was clear that the genes down-regulated by zinc exhibited more TvMRE motifs in their coding sequences in comparison to the promoter regions of genes with zinc-induced up-regulation in this parasite, suggesting a possible relationship between the number of MREs and the transcriptional control.

First, we considered that the MRE sequences in trichomonal genes may exist as known conserved MRE motifs: TGCRCNC. Analysis of the sequences of each of the 13 zinc-responsive genes in *T. vaginalis* revealed nine putative MREs in six different trichomonal genes and the MEME motif-based sequence analysis revealed one novel motif denominated as TvMRE1: TGCNCNC (Fig. 1). Interestingly, a consensus motif was already reported in the murine *ZIP11* gene (Yu et al. 2013), which differs from the conserved MRE core at the fourth base and exhibits the same signature as the one identified as TvMRE1 in this paper.

Only 2 TvMRE1 motifs: TGCACAC and TGCGCTC, which were identified once and twice times, respectively, showed a perfect match with the conserved MRE core, which have been previously denominated as MREe and MREc (Francis and Grider 2018). The two TGCGCTC motifs were identified in the *TvAHCY* gene, which also exhibited a third motif: TGCCCTC, whose sequence is identical to an MRE found in the murine *ZIP11* gene (Yu et al. 2013). Although it has been reported that there is no correlation between the number of MREs and MTF1 transcriptional strength, particularly the *TvAHCY* gene

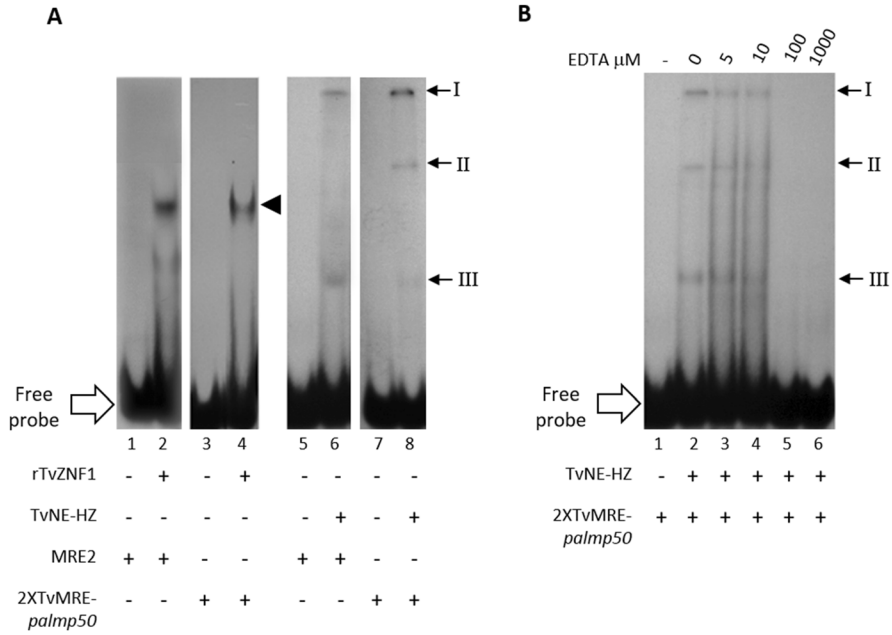


Fig. 7 Analysis of binding activity of a palindromic TvMRE probe to recombinant TvZNF1 and trichomonal nuclear proteins by EMSA. **a** Gel-shifting assays to detect DNA-protein complexes formation between MRE2 probe, as a positive control (lanes 2 and 6) and the trichomonal 2XTvMRE-*pal* probe (2XTvMRE-*palmp50*; lanes 4 and 8) with rTvZNF1 protein (lanes 2 and 4) or with *T. vaginalis* nuclear extracts from parasites grown in high zinc conditions (TvNE-HZ; lanes 6 and 8). Free probes, as negative control, are shown (lanes 1, 3, 5 and 7). **b** Shows the zinc-dependent interaction between the TvNE-HZ proteins and the 2XTvMRE-*palmp50* probe by chelation with EDTA for 15 min in the binding reaction

exhibited the three conserved TvMRE1 motifs at the coding region, which is consistent with the hypothesis of transcriptional repression by the trichomonal ortholog of MTF1, TvZNF1.

To search for novel trichomonal MRE sequences, the regions from the set of zinc-responsive genes in *T. vaginalis* were extracted and analyzed using atypical MRE signatures. First, we searched using the TGTNCNC signature, which differs from the conserved MRE core at the third base, coming from the previously MRE-like sequences identified in the metallothionein gene in *T. vaginalis* (*tvmt-1*). In the present paper, we identified 15 TGTNCNC motifs (TvMRE2) in 8 different genes (Fig. 2), confirming three previously identified in the *tvmt-1* gene. It has been previously reported that the sequences known not to bind MTF1 in mammals exhibited preferentially purines (A or G) in the third base of the core sequence (Wang et al. 2010), supporting a functional MRE-like sequence given by a substitution of a T instead of a C in this specific position for the trichomonal TvMRE2 motifs.

The search using the other two MRE-like sequences: TGCNCNG and TGCNGNC, which are examples of MREs that mismatch the consensus MRE core motif in one nucleotide at the 5 or 7 position, as well as a manual search with a GC-rich content at positions 4 to 7, gave us a total of 45 putative MRE sequences that were aligned and results in four conserved motifs: TvMRE3, TvMRE4, TvMRE5 and TvMRE6 (Figs. 3 and 4). The consensus sequence of the TvMRE3 motif, **TGC**(T/C)**C**(A/K)G, is consistent with the MRE-like sequences previously reported. For example, Talavera-Montañez and col. reported a potential MRE: **TGCACA**G, located in the promoter region of the *Myogenin* gene, which is capable of interacting with MTF1 (Talavera-Montañez et al. 2019). On the other hand, the TvMRE4 motif consists of a consensus sequence: **TGC**(C/A)G(C/G)**C**, which is in agreement with the MRE-like sequences reported in the promoter regions of the rat *Prnp* (**TGC**GG**TC**) and bean *PvSR2* (**TGC**AG**GC**) genes (Varela-Nallar et al. 2006; Qi et al. 2007).

TvMRE5 and TvMRE6 motifs exhibit G or C nucleotides in the last four positions of the MRE core,

with an exception either at position 7 or at position 5, respectively. This type of sequences was already reported as MRE-like motifs. For example, the **TGC**GCG*T* sequence reported as an MRE motif in *Drosophila mojavensis* (Yepiskoposyan et al. 2006) is closely related to the TvMRE5 motifs: **TGC**GCGA and **TGC**GGC*T*, located in the coding region of the *TvCP39* gene; or **TGC**CGC*T*, located in the promoter region of the *mp50* gene. For the TvMRE6 motifs, the consensus sequence **TGC**(C/G)(T/A)(G/C)(**C**/G) is in agreement with a previous MRE-like sequence: **TGC**GTGG, identified in the *Selh* genes from 4 eukaryotic species, one in the promoter region and three downstream of the transcription start site (TSS) (Stoytcheva et al. 2010).

It has been reported that the 5′ and 3′ flanking regions of the MRE core can affect the MTF1 binding activity in response to heavy metals. For example, a semi-conserved GC-rich region at 3´ end, which includes the Cytosine in position 7 of the core followed by a consensus (G/C)(G/T)C(C/A) sequence in positions 8–10, it has been shown for genes that respond to zinc, cadmium and copper (Wang et al. 2004; Sims et al. 2012; Francis and Grider 2018). Additionally, in some cases this GC-rich region can be found in the 5′ end flanking the MRE core (Stoytcheva et al. 2010). In the present study, we have shown that around 70% (45/65) of the putative MREs identified in *T. vaginalis* possess a GC-rich sequence either in the 5′ or 3′ flanking regions or even in both ends (Figs. 1, 2, 3 and 4). This suggests that the conservation of this flanking region could be related to the response to zinc of these trichomonal genes.

Particularly, in response to zinc and the MTF1 binding activity, it has been reported that conserved MRE sequences can be flanked in the 5′ end by a second MRE in the opposite strand in an arrangement of two closely spaced MREs (2XMREs: GTGTGCAN$_{4-6}$TGCGCAC) (Wang et al. 2004). Here, we showed that at least 5 zinc-responsive genes in *T. vaginalis* exhibited a similar rearrangement of two closely spaced TvMREs (2XTvMREs-c) with a random orientation and spaced by 4–5 bases (Fig. 5). Notably, four of these genes are homologous to the zinc transporters that belong to the ZIP (*TvZIP5* and *TvZIP7*) and ZnT (*TvZnT7*) families and to the metallothionein, which have been shown to be the proteins primarily involved in zinc homeostasis and metabolism in almost all organisms (Kimura and Kambe 2016).

Considering the large number of motifs identified (65 total) in this study, including numerous divergent/hypothetical MRE-like sequences, only four sequences involving two overlapped TvMRE motifs were found. This partially overlapping rearrangement resembles to a palindrome-like sequence: **TGCC**(**C**/G)N(**C**/K)**GGCA** (2XTvMRE-*pal*; Fig. 6). It has been reported a palindromic sequence: CACTCCC(CC)GGGAGTG (ZTRE), involved in zinc-induced transcriptional repression of several genes, including members of the ZnT family (Coneyworth et al. 2012). To our knowledge, this is the first report of a palindromic-like sequence involving MRE sequences.

Although two genes from the ZnT family identified in *T. vaginalis*, whose transcriptional expression is repressed by zinc, exhibited the 2XTvMRE-*pal* motif. Notably, only one gene with zinc-induced upregulation, *mp50*, contains a 2XTvMRE-*pal* motif. To test the possible MTF1 binding capability, we performed an EMSA assay. Our results demonstrate that the 2XTvMRE-*pal* motif present in the *mp50* gene is a target for both the recombinant TvZNF1 and the nuclear proteins in *T. vaginalis* grown in high zinc conditions and this binding is dependent on the zinc content demonstrated by EDTA chelating (Fig. 7). Although the biological functionality of this particular TvMRE motif needs to be investigated in depth, this is the first approach that posits that trichomonal MREs may exist in an arrangement that combines the *T. vaginalis*-specific sequences and that these selected *tvznf1*-specific sequences may occur naturally in trichomonal genes encoding proteins which are not directly involved in zinc homeostasis.

In conclusion, subject to the search for other zinc-responsive trichomonad MRE-carrying genes with specific functions, our results suggest that *T. vaginalis* possesses a MTF1/MRE-like system for the control of gene expression of associated zinc-responsive genes through TvZNF1, that at high zinc conditions, interacts with specific MRE sequences inducing or blocking the transcription depending on their location either at promoter or at coding region, respectively.

Acknowledgements This work was supported by grant from Autonomous University of Mexico City awarded to MEAS. We acknowledge Brenda Herrera Villalobos and Laura Isabel

Vazquez Carrillo and Alfredo Padilla Barberi from the Autonomous University of Mexico City UACM for their technical assistance.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest regarding the publication of this article.

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