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Metallothionein gene activation in the earthworm (*Lumbricus rubellus*)

M. Höckner [a, b, *], R. Dallinger [a], S.R. Stürzenbaum [b]

[a] University of Innsbruck, Institute of Zoology, Innsbruck, Austria
[b] King's College London, Faculty of Life Sciences & Medicine, London, United Kingdom

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ABSTRACT

In order to cope with changing environmental conditions, organisms require highly responsive stress mechanisms. Heavy metal stress is handled by metallothioneins (MTs), the regulation of which is evolutionary conserved in insects and vertebrates and involves the binding of metal transcription factor 1 (MTF-1) to metal responsive elements (MREs) positioned in the promoter of MT genes. However, in most invertebrate phyla, the transcriptional activation of MTs is different and the exact mechanism is still unknown. Interestingly, although MREs are typically present also in invertebrate MT gene promoters, MTF-1 is notably absent. Here we use *Lumbricus rubellus*, the red earthworm, to study the elusive mechanism of *wMT-2* activation in control and Cd-exposed conditions. EMSA and DNase I footprinting approaches were used to pinpoint functional binding sites within the *wMT-2* promoter region, which revealed that the cAMP responsive element (CRE) is a promising candidate which may act as a transcriptional activator of invertebrate MTs.

1. Introduction

The ability to respond to sudden changes within the (micro) environment requires effective and sensitive gene transcription mechanisms [1]. For example, heavy metal stress triggers the induction of metallothioneins (MTs) [2], a gene family coding for small, cysteine-rich metal binding proteins which constitute an integral part of the metal detoxification process [3,4]. In most organisms the transcriptional activation of MTs is driven via the metal transcription factor 1 (MTF-1) which binds to metal responsive elements (MREs) positioned within the MT promoter [5]. This mechanism is highly conserved from insects to mammals [6], however, most invertebrates (other than insects) lack a MTF-1 orthologue [7,8], despite the fact that most contain MREs within their respective promoters [9].

Only few have studied the transcriptional activation of MTs in invertebrates. For example, in yeast MT expression is induced via the copper-mediated binding of Ace1 to MREs [10,11]. In contrast, *Caenorhabditis elegans* ELT-2 binds to GATA elements in the mtl-1 and mtl-2 promoter region leading to cell specific MT expression. However this mechanism does not confer metal induced transcription and therefore other factors are thought to regulate metal dependent MT transcription in *C. elegans*, likely in a repressor-mediated manner [12]. Other metal-dependent MT regulators include TATA motifs [13], Nrf1 [14], AMT1 in copper specific MT regulation [15,16], and C/EBPalpha [17].

Although the knowledgebase surrounding the regulation of non-model invertebrate MTs is scarce, there is ample evidence that MT expression is closely correlated with the exposure to toxicological insults [18]. Earthworm MTs have been studied in some detail, however, the mechanistic detail surrounding the gene activation is still unknown [19–21]. Although the Cd-inducible wMT-2 gene of the earthworm *Lumbricus rubellus* was shown to contain three MREs in the promoter region [21], MTF-1 seems to be absent, a notion which is based on comprehensive experimental and in silico searches [9].

Therefore, the present study sets out to define functional binding sites within the earthworm MT promoter to identify putative transcriptional activators of the wMT-2 gene.

2. Materials and methods

2.1. Preparation of cytosolic and nuclear protein

The posterior tip of the worms was cut and homogenized in buffer A (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9) and

* Corresponding author. University of Innsbruck, Institute of Zoology, Innsbruck, Austria.
E-mail addresses: martina.hoeckner@uibk.ac.at (M. Höckner), Reinhard.Dallinger@uibk.ac.at (R. Dallinger), stephen.sturzenbaum@kcl.ac.uk (S.R. Stürzenbaum).

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centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet resuspended in 300 µl buffer A and left on ice for 10 min before the pellet was again homogenized and centrifuged at 3000 rpm for 30 min at 4 °C. The supernatant contained the cytosolic fraction which was kept on ice. The pellet was then resuspended in 300 µl buffer C (20 mM Hepes, 1.5 mM $MgCl_2$, 420 mM KCl, 25% Glycerol, pH 7.9) and rotated for 30 min at 4 °C. The supernatant contained the nuclear proteins. After measuring the protein concentration on a NanoDrop (Thermo Scientific, USA), the cytosolic and nuclear protein was shock frozen in liquid nitrogen and stored at −80 °C.

2.2. Electric mobility shift assay (EMSA)

Earthworms were exposed to soil substrate spiked with 100 mg/kg $CdCl_2$ for 7 days at 15 °C and nuclear and cytosolic proteins were isolated as stated above. The EMSA reactions were conducted using the 2nd generation DIG Gel Shift Kit (Roche, Switzerland) according to the user manual with some modifications. The binding reaction contained 10 mM Hepes, 50 mM $NaCl_2$, 0.1 mM EDTA, 1 mM $MgCl_2$, 1 mM DDT, 4.6% glycerol, 1 µg polyD-IC, poly L-Lysine, 10 µg protein, 0.155 pmol probe, and 2 mM $ZnCl_2$ in a total volume of 20 µl. The binding reaction was incubated for 20 min at room temperature. When using a cold probe (100 pmol/µl) for the competition experiment, the protein was pre-incubated with the competitor for 10 min at room temperature. The binding reaction was then separated on a 5% precast TBE gel (Biorad, USA), blotted onto a positively charged nylon membrane (Roche, Switzerland) and luminescence detected by film exposure.

2.3. DNase I footprinting

A 299 bp fragment of the *wMT-2a* promoter region (−135 to −433 from the transcription start site (TSS)) including all three MREs was PCR amplified and cloned into the pGEM-T vector (Promega, USA). A FAM-labeled T7 primer (Microsynth, Switzerland) was used in a PCR approach together with a promoter specific primer (5′ ACCGATTCACAAGATCACACA 3′) to generate a FAM-labeled probe (368 bp). The PCR conditions were as follows: 1x Titanium Taq PCR buffer, 10 mM ea. dNTPs, 0.4 µM primer, 1x Titanium Taq polymerase (Clontech, US), plasmid containing the *wMT-2* promoter in a 50 µl approach; 95 °C 5 min initial denaturation, 95 °C 30 sec, 55 °C 30 sec, 68 °C 30 sec (35 cyles) 68 °C 5 min. The probe was then gel-purified (gel purification kit from Qiagen, Netherlands) and the DNA concentration was measured using a NanoDrop (Thermo Scientific, USA). Cytosolic and nuclear protein was extracted as described above from control and Cd-exposed worms (50 mg/kg $CdCl_2$ for 14 days at 15 °C). Protein (20 µg), binding buffer (as described for the EMSA approach), 2 mM $ZnCl_2$, 2% polyvinyl alcohol, and 1 µg poly dA-T was incubated for 10 min at room temperature. The probe (500 ng) was then added, brought to a total volume of 30 µl and left on ice for 30 min. DNase I (0.1 U) (Life Technologies, USA), 10x buffer and 20 µl of distilled water were added and incubated for 5 min at room temperature. The DNase I digest was stopped by heating to 75 °C for 10 min.

The sample was then purified using PCR purification columns (Qiagen, Netherlands) and eluted in 20 µl elution buffer. The sample (5 µl), 0.1 µl GeneScan™ 500 LIZ™ dye Size Standard (Life Technologies, USA), and 4.9 µl Hi-Di™ Formamide was mixed, incubated for 5 min at 95 °C, separated on an ABI 3130 genetic analyzer (Life Technologies, USA) and analyzed by means of the Peakscanner software v1.0 (Life Technologies, USA). The plasmid which was used to PCR amplify the probe was sequenced with the Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit (Affymetrix, USA). 0.1 µl GeneScan™ 500 LIZ™ dye Size Standard (Life Technologies, USA) and 4.9 µl Hi-Di™ Formamide (Life Technologies, USA) was added and run on an ABI 3130 genetic analyzer (Life Technologies, USA). The footprints were then assigned to the DNA sequence of the promoter region. The negative control contained 20 µg bovine serum albumin (BSA) (Sigma-Aldrich) instead of the earthworm protein. The DNase I footprinting approach was carried out with minor modifications as specified by Zianni et al. [22].

2.4. qPCR

Worms were exposed to 50 mg $CdCl_2$/kg dry soil for 14 days at 15 °C. The posterior tip was cut and stored in RNA later and kept at −20 °C. Total RNA was isolated using TRIZol (Life Technologies, USA) and RNA concentration determined in triplicates using the Ribogreen® assay (Life Technologies, USA). RNA (450 ng) was reverse transcribed to cDNA using M-MLV, RNase H Minus (Promega, USA). Quantitative Real Time PCR was performed with SYBR®Green (Life Technologies, USA) and the absolute copy number of wMT-2 cDNA per 10 ng of total RNA was calculated according to a standard curve. The qPCR reaction contained 2 µl cDNA, 2 µl 10x BSA, 2 µl of each primer (9 µM forward, 3 µM of the reverse primer; a primer matrix was previously run to determine the optimal primer concentrations) and 10 µl SYBR®Green and assessed on an ABI 7500 RealTime PCR analyzer (Life Technologies, USA).

2.5. Western blot

Earthworms were exposed to 50 mg $CdCl_2$/kg dry soil for 7 days at 15 °C, then shock frozen in liquid nitrogen and the posterior tip was cut for total protein extraction. The tissue was homogenized using an UltraTurrax (IKA, Germany) in 300 µl lysis buffer (80 mM potassium citrate, 5 mM MgAcetate, 20 mM HEPES pH 7.5). The homogenate was centrifuged for 5 min at maximum speed and protein concentration was determined on a NanoDrop (Thermo Scientific, USA).

Total protein (50 µg) was separated on a 10% TGX gel (Biorad, USA) for 45 min at 150 V. The gel was blotted (semidry) at 25 V, 200 mA for 30 min to an Immun-Blot® PVDF membrane (Biorad, USA) and blocked for an hour with 5% dry milk in TBS 0.1%Tween (TBS-T). The membrane was incubated with the primary antibody (wMT-2 against *L. rubellus* [20]) overnight at 4 °C and after three TBS-T wash steps, the secondary antibody was incubated for one hour at room temperature (goat anti rabbit, HRP tagged; Abcam, UK). After three TBS-T wash steps, ECL (GE Healthcare, Switzerland) was added and the chemiluminescent signal was detected using a chemidoc (Biorad, USA).

2.6. Transcription factor binding site prediction

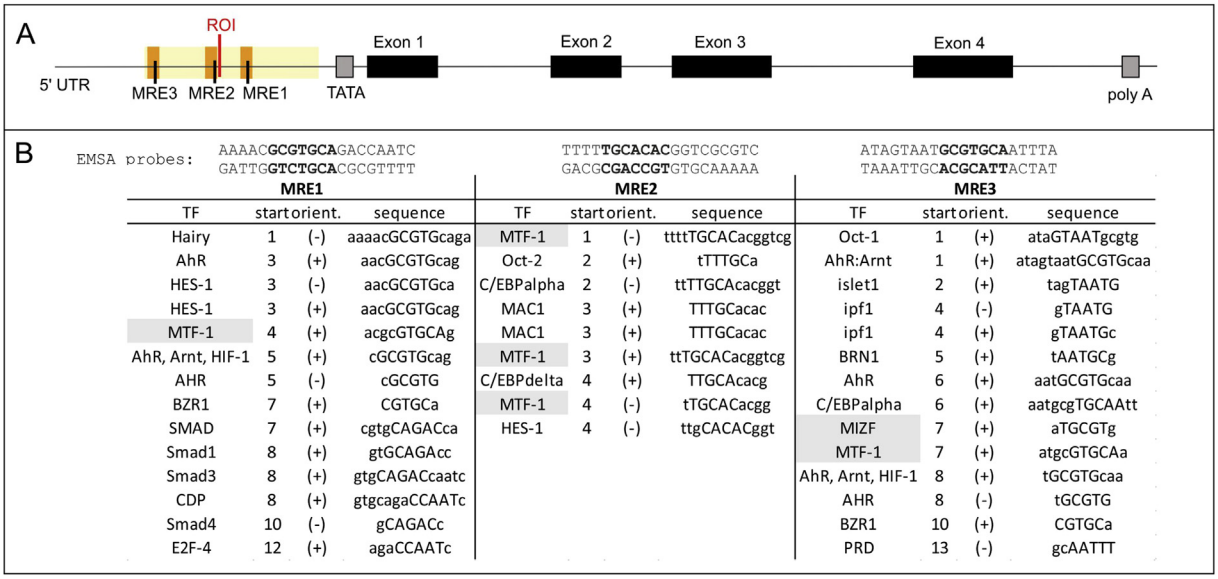
TRANSFAC® (Biobase) was used to identify putative TF binding sites using the match algorithm (v2014.4). The cut-off was set to a core score of 1.0 and a matrix score of 0.9 [23].

3. Results

The promoter region of the wMT-2 gene (Fig. 1A) was scanned for putative binding sites within the EMSA probes using the TF prediction tool from TRANSFAC®, Biobase (Fig. 1B).

The Cd responsiveness of wMT-2 was verified on the transcriptional and protein level by showing a strong up-regulation of the wMT-2 transcript (Fig. 2A) and the resultant protein (Fig. 2B).

The EMSA studies demonstrated that proteins from the nuclear extract were not able to bind to the three probes, each containing the MRE binding site flanked by 13 base pairs of promoter region (Fig. 1). In contrast, proteins from the cytosolic fraction returned a $ZnCl_2$ dependent band shift for each of the



MRE1

TF	start	orient.	sequence
Hairy	1	(-)	aaaacGCGTGcaga
AhR	3	(+)	aacGCGTGcag
HES-1	3	(-)	aacGCGTGca
HES-1	3	(+)	aacGCGTGcag
MTF-1	4	(+)	acgcGTGCAg
AhR, Arnt, HIF-1	5	(+)	cGCGTGcag
AHR	5	(-)	cGCGTG
BZR1	7	(+)	CGTGCa
SMAD	7	(+)	cgtgCAGACca
Smad1	8	(+)	gtGCAGAcc
Smad3	8	(+)	gtgCAGACcaatc
CDP	8	(+)	gtgcagaCCAATc
Smad4	10	(-)	gCAGACc
E2F-4	12	(+)	agaCCAATc

MRE2

TF	start	orient.	sequence
MTF-1	1	(-)	ttttTGCACAcggtcg
Oct-2	2	(+)	tTTTGCa
C/EBPalpha	2	(-)	ttTTGCAcacggt
MAC1	3	(+)	TTTGCacac
MAC1	3	(+)	TTTGCacac
MTF-1	3	(+)	ttTGCACacggtcg
C/EBPdelta	4	(+)	TTGCAcacg
MTF-1	4	(-)	tTGCACacgg
HES-1	4	(-)	ttgCACACggt

MRE3

TF	start	orient.	sequence
Oct-1	1	(+)	ataGTAATgcgtg
AhR:Arnt	1	(+)	atagtaatGCGTGcaa
islet1	2	(+)	tagTAATG
ipf1	4	(-)	gTAATG
ipf1	4	(+)	gTAATGc
BRN1	5	(+)	tAATGCg
AhR	6	(+)	aatGCGTGcaa
C/EBPalpha	6	(+)	aatgcgTGCAAtt
MIZF	7	(+)	aTGCGTg
MTF-1	7	(+)	atgcGTGCAa
AhR, Arnt, HIF-1	8	(+)	tGCGTGcaa
AHR	8	(-)	tGCGTG
BZR1	10	(+)	CGTGCa
PRD	13	(-)	gcAATTT

Fig. 1. A. Schematic overview of the wMT-2 gene. The yellow box indicates the corresponding promoter region of the DNase I footprinting probe. The orange boxes highlight the respective location of the EMSA probes. B. Putative transcription factor (TF) binding sites predicted by the TF prediction tool (TRANSFAC®, Biobase) which are located within the EMSA probe regions. ROI: Region of Interest. "MTF-1" indicates the presence of MRE motifs; note: an MTF-1 orthologue is not present in the earthworm genome. Zinc finger TFs are highlighted in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

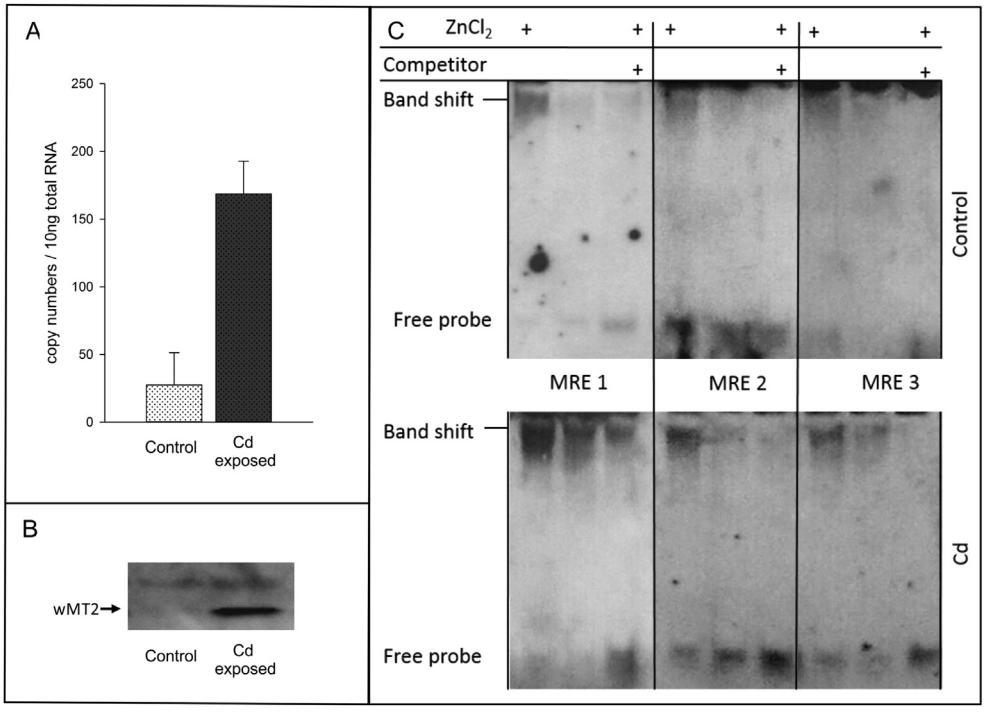


Fig. 2. A. Quantitative RealTime PCR confirmed the induction of wMT-2 gene expression in Cd-exposed samples. B. wMT-2 protein expression was also shown to be induced in Cd-exposed individuals. A polyclonal antibody raised against *L. rubellus* wMT-2 was used [20]. C EMSA reactions applying cytosolic protein fraction revealed zinc dependent shifts for all three probes (MRE1-3) applied. Competition experiments were also conducted for MRE1-3 (3rd lane, respectively).

three probes (Fig. 2C). Unlabeled probe was used to define the specificity of the protein binding as band shifts were drastically reduced or absent in the negative control reactions (Fig. 2C). All EMSA results were confirmed in three independent biological replicates.

DNase I footprinting was applied across 299 bp of the wMT-2 gene promoter (including all three MRE binding sites) to reveal putative protein binding sites. BSA was used as a negative control (in lieu of earthworm protein extract), which uncovered the cleavage pattern due to unspecific binding and/or preferred cleavage of DNase I. Footprints which were present in the BSA negative control were therefore deemed invalid. Although methodical restrictions were encountered, a footprint spanning a 10 bp nucleotide region was observed (Fig. 3) and defined as the region of interest (ROI). DNase I footprinting experiments were repeated and validated in five independent replicates.

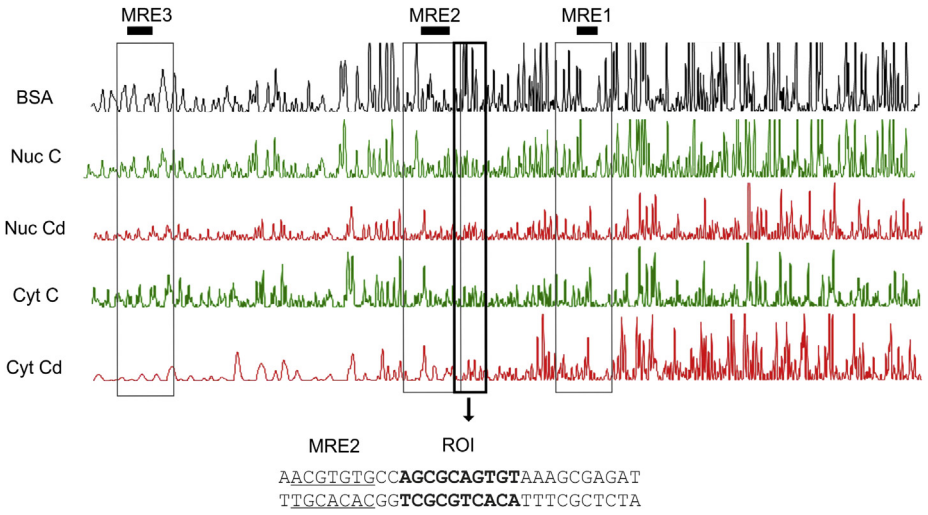


Fig. 3. DNase I footprinting chromatograms are shown for nuclear and cytosolic protein fractions in control and Cd-exposed earthworm samples as well as for the negative control (BSA). The grey boxes indicate the corresponding promoter regions which were used for EMSA experiments. The black box highlights the footprint observed in all samples (ROI). ROI: Region of Interest.

The results suggest the attachment of a transcription factor complex to the ROI, which by prediction from TRANSFAC® site search (Table 1) are possibly members of the cAMP responsive element-binding protein family (CREB).

4. Discussion

In mammals, fish and insects, the activation of MT gene transcription is driven by the metal transcription factor 1 (MTF-1) which binds to metal responsive elements (MREs) positioned in the

Table 1
Transcription factor (TF) prediction using the match algorithm (TRANSFAC®) for the Region of Interest (ROI) including 10 bp of the 5′ and 3′ flanking region. Light grey colors highlight the results for the ROI. The TF with the highest matrix score is shown in dark grey. "MTF-1" indicates the presence of MRE motifs; note: an MTF-1 orthologue is not present in the earthworm genome.

Factor	Position	Orientation	Consensus Sequence	Matrix Score
GEN_INI	8	(-)	AAATGtga	0,949
CREB1	9	(+)	aatgTGACGcgaccg	0,962
CREB	9	(-)	aatgTGACGcgacc	0,934
ATF-1	10	(+)	atgTGACGcgac	0,963
CREM	10	(-)	atgTGACGcga	0,942
TGA1	10	(+)	atgTGACGcga	0,938
CREB	10	(+)	atgTGACGcga	0,936
ATF	10	(+)	atgTGACGcgaccg	0,932
Whn	11	(+)	tgtGACGCgac	0,961
CREB1	11	(-)	tgTGACGcgacc	0,959
ATF-3	11	(-)	tgTGACGcgac	0,956
ATF-3	11	(+)	tgTGACGcgacc	0,946
AP-1	11	(+)	tGTGACgcgac	0,935
CREB	11	(+)	tgTGACGcgacc	0,935
ATF-4	11	(-)	tgTGACGcgacc	0,931
CREB	11	(+)	tgTGACGcgacc	0,904
CREB	12	(-)	gTGACG	0,999
ATF-1	12	(-)	gTGACGcga	0,968
CREB, ATF	12	(+)	gTGACGcga	0,959
HBP-1a	12	(-)	gTGACGcgac	0,915
ARG RI	13	(+)	tGACGC	0,988
StuAp	13	(-)	tgaCGCGAcc	0,962
HES-1	20	(+)	accGTGTGcaa	0,980
MTF-1	21	(+)	ccgtGTGCAa	0,997
C/EBPdelta	22	(-)	cgtgTGCAA	0,907

promoter region of MT genes. In contrast, although MREs are present in the promoter region of most invertebrates and yeast, they notably lack MTF-1 orthologues, thus suggesting that different mechanisms control the expression of these genes.

Previous reports have demonstrated that the expression of *wMT-2* from the earthworm *L. rubellus* is dose responsive to $CdCl_2$ [24,25]. The present study confirms that wMT-2 is transcriptionally and translationally induced upon Cd exposure in *L. rubellus* (Fig. 2A and B).

Given that three MRE binding sites are positioned within 299 bp of the proximal *wMT-2* promoter [21], we tested their binding capacity by means of EMSA using cytosolic and nuclear protein extracts from control and Cd-exposed earthworms. Interestingly, only cytosolic proteins were able to produce a band shift in control and Cd-exposed samples, notably in a zinc dependent manner (Fig. 2C). This confirms that proteins present in the cytosolic fraction are able to bind to MREs. At this stage we are not able to exclude that (i) nuclear proteins may have leaked into the cytosolic fraction during the protein extraction procedure [26], or (ii) that the cytosolic fraction contains factors which are able to bind to the MRE regions but do not, or have not yet, translocated to the nucleus [27,28].

The data obtained from the DNase I footprinting study revealed that the cleavage pattern from the BSA negative control was, at large, too similar to the earthworm samples (Fig. 3). Although this precludes us to state with confidence whether the MREs are directly implicated in the regulation of the wMT-2 gene, the presence of specific binding factors in the cytosolic protein fraction suggests some involvement (Fig. 2C). Furthermore, the zinc dependent band shifts indicate that the bound factors are possibly zinc finger proteins. Putative binding sites of the EMSA probes, predicted by the TRANSFAC® TF tool (match algorithm, version 2014.4), revealed that the MRE sequences are the only zinc finger binding regions (Fig. 1B). It has been proposed that the underlying mechanism for Cd^{2+} induced gene induction by MTF-1 is due to the replacement of Zn^{2+} by Cd^{2+} from the MT protein followed by the activation by excess Zn^{2+} [6]. An analogous mechanism may drive the induction of the wMT-2 gene in *L. rubellus*, however via a different zinc finger transcription factor. The involvement of MTF-2, a further MRE binding protein postulated to be a putative transcriptional activator of some MT genes [7,29,30], is highly unlikely in the present case as it seems to be absent from the *L. rubellus* genome and transcriptome (unpublished data).

Setting aside the methodical restrictions of the DNase I footprinting, it was possible to identify a putative transcriptional regulator in control and Cd-exposed samples. The footprint spans the proximity of the MRE2 binding site and the match algorithm (TRANSFAC®) returned the highest matrix score for a member of the cAMP responsive element (CRE) binding protein (CREB) family (Table 1). Searching the *L. rubellus* genome database (http://badger.bio.ed.ac.uk/earthworm/) revealed several blast hits for CREB genes confirming their presence in the genome and transcriptome of the red earthworm. The presence of the footprint in both control and Cd-exposed samples suggests that the occupation of the binding site may not only be limited to the transcription of basal MT levels but may also be involved in metal-dependent MT gene activation.

CRE binding elements are also located in close proximity to the TATA box in the *C. elegans* mtl-2 gene and it has been hypothesized that they may be involved in replacing the role of MREs [31]. Indeed, CREB has previously been suggested to act as a transcriptional activator of MT genes [32]. CREB has also been linked to Cd exposure and calcium signaling. Elevated levels of Ca^{2+} increase the binding of CREB to the respective promoter elements of the GLutamate ASpartate Transporter (GLAST) upon Cd-exposure, thereby acting as a transcriptional repressor [33]. Cd also induces the binding of CREB motifs in rat astrocytes [33] and the increase of phosphorylated CREB is observed in human renal cells upon Cd exposure [34]. Furthermore, mitochondrial biogenesis is mediated by the activation of CREB upon stress and a transient decrease of ATP levels probably caused by an increased energy demand [35]. The involvement of CREB in several pathways of the transcriptional response to toxicological insults has been discussed by Jennings et al., 2013 [36].

In summary, we suggest that in *L. rubellus* the activation of the wMT-2 gene is, probably in combination with other factors, dependent on the binding of CREB. We hypothesize that CREB is involved in basal-level and Cd-induced *wMT-2* transcription and also acts as a transcriptional activator. This information furthers our understanding of the evolution of the molecular responses to heavy metal exposure in invertebrates and in doing so underlines how the study of non-model organisms can assist in filling in evolutionary gaps [37].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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