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Induction of phase II enzymes and *hsp70* genes by copper sulfate through the electrophile-responsive element (*EpRE*): insights obtained from a transgenic zebrafish model carrying an orthologous *EpRE* sequence of mammalian origin

Daniela Volcan Almeida · Bruna Félix da Silva Nornberg · Laura A. Geracitano · Daniela Martí Barros · José Maria Monserrat · Luis Fernando Marins

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Abstract We have evaluated the homology of the electrophile-responsive element (*EpRE*) core sequence, a binding site for the Nrf2 transcription factor, in the proximal promoters of the mouse and zebrafish glutathione-*S*-transferase (*gst*), glutamate cysteine ligase catalytic subunit (*gclc*) and heat shock protein 70 (*hsp70*) genes. The *EpRE* sites identified for both species in the three analyzed genes showed a high similarity with the putative *EpRE* core sequence. We also produced a transgenic zebrafish model carrying a transgene comprised of the luciferase (*luc*) reporter gene under transcriptional control of a mouse *EpRE* sequence. This transgenic model was exposed to copper sulfate, and the reporter gene was significantly activated. The endogenous *gst*, *gclc* and *hsp70* zebrafish genes were analyzed in the *EpRE-Luc* transgenic zebrafish and showed an expression pattern similar to that of the reporter transgene used. Our results demonstrate that *EpRE* is conserved between mouse and zebrafish for detoxification-related genes and that the development of genetically modified models using this responsive element to drive the expression of reporter genes can be an important tool in understanding the action mechanism of aquatic pollutants.

Keywords Electrophile-responsive element · Glutamate cysteine ligase catalytic subunit · Glutathione-*S*-transferase · HSP70 · Luciferase and transgenic fish

D. V. Almeida · B. F. da Silva Nornberg · L. A. Geracitano · D. M. Barros · J. M. Monserrat · L. F. Marins (✉)
Programa de Pós-graduação em Ciências Fisiológicas, Fisiologia Animal Comparada, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande – FURG, Av. Itália km 8, CEP 96201-900 Rio Grande, RS, Brazil
e-mail: dqmluf@furg.br

Introduction

Food metabolism and the respiratory process generate reactive electrophiles that can, directly or indirectly, affect the physiological function of macromolecules, such as DNA, proteins and lipids (Halliwell 2001). Cells have acquired, during evolution, a complex defense mechanism against the electrophile-induced toxicity through a very coordinated induction of many genes and proteins (Talalay et al. 2003). This response is regulated, mainly, by the antioxidant-responsive element (*ARE*) or the electrophile-responsive element (*EpRE*),both of which are present in the regulatory region of genes encoding for enzymes of phase II (for review, see Itoh et al. 2004). The *EpRE* core sequence (5′-RTGAYnnnGC-3′) was characterized as a minimal sequence required for basal and xenobiotic inducible expression by Rushmore et al. (1991). The *EpRE* is recognized by the nuclear transcription factor erythroid 2 (NF-E2)-related factor 2 (Nrf2). Under non-induced conditions, Nrf2 is retained in the cytoplasm by the protein Keap1,

which is associated to the actin filaments. An increase in electrophile/oxidative stress results in the dissociation of Nrf2 from Keap1, allowing translocation of Nrf2 to the nucleus and binding to *EpRE* and a consequent activation of target genes (Lee and Surh 2005; Nguyen et al. 2003). The main genes regulated by *EpRE/Nrf2* include genes encoding phase II enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1) (Rushmore et al. 1991), the glutathione *S*-transferase Ya subunit (Gsta1) (Frilling et al. 1990), heme oxygenase 1 (HO-1) (Alam et al. 1995) and γ-glutamate cysteine ligase (Gcl) (Mulcahy et al. 1997). Additionally, chaperone proteins, such as heat shock protein (Hsp70), have also been identified as being regulated by *EpRE*-mediated mechanisms (Kwak et al. 2003), which together with the ubiquitin/proteasome system play an essential role in the response to stress (Beckmann et al. 1990; Parsell and Lindquist 1993).

The *EpRE/Nrf2* signaling pathway seems to be conserved among vertebrates. A comparison between zebrafish (*Danio rerio*) and mouse (*Mus musculus*) Nrf2 proteins has demonstrated high similarity among critical domains, which are very important for Nrf2 to exert its transcriptional regulatory function (Kobayashi et al. 2002). Six Neh (Nrf2-ECH homology) domains are shared by both mouse and zebrafish Nrf2, which implies a common mechanism of gene regulation (Itoh et al. 1995). Indeed, it has been demonstrated by gene knockdown analysis that Nrf2 is essential for some phase II enzymes in zebrafish (Kobayashi et al. 2002; Suzuki et al. 2005). In addition, Carvan et al. (2000, 2001) have produced zebrafish reporter cell lines transfected with transgenes containing *EpRE* from the mouse *gsta1* promoter. The exposure of these reporter cell lines to several classes of environmental pollutants resulted in a dose-dependent transgene induction, which suggests a conserved mechanism since the zebrafish Nrf2 seems to be able to recognize an *EpRE* sequence of mammalian origin. These authors have proposed the utilization of transgenic zebrafish carrying a reporter gene under *EpRE* control as sentinel for aquatic pollution.

In fact, fish have been considered to be a very useful model for ecotoxicology studies because these vertebrates are very sensitive to chemical toxicity and are valuable comparative animal models (Hill et al. 2005). The utility of fish as sentinels for environmental pollution has been expanded since transgenic fish have been designed to harbor reporter genes, such as luciferase (*luc*) or green fluorescent protein (*gfp*), driven by promoters that are responsive to chemical exposure. An additional advantage is that this technology can result in fish models carrying identical transgenes to those found in other organisms, thereby further increasing the possibility of extrapolation across species (Winn 2001).

We have compared, for the first time, elements of the *EpRE* core sequence in conserved regions of the zebrafish—*gst*, the *gclc* catalytic subunit and *hsp70* promoters—with their mouse homologs. In addition, we produced transgenic zebrafish carrying a genetic construct comprised by the *luc* reporter gene under the transcriptional control of a mouse *EpRE*-containing promoter. This in vivo model was exposed to copper sulfate and the expression of the endogenous *gclc*, *gst* and *hsp70* genes was compared to the induction of the reporter transgene.

Materials and methods

In the search for the *EpRE* core sequence in the analyzed genes, we identified sequences containing *gst*, *gclc* and *hsp70* proximal promoters at GenBank (http://www.ncbi.nlm.nih.gov). For the mouse *gst*, *gclc* and *hsp70*, we used sequences AC159313, AC160124 and AF109906, respectively; for zebrafish *gst*, *gclc* and *hsp70*, we used sequences AL929536, CR376766 and BX511232, respectively. Within these sequences, the transcription starting point for each gene was identified and the upstream 1500 bp were selected. The potential transcription factor binding sites for Nrf2 (RTGAYnnnGC or CGnnnYAGTR) were localized using the MatInspector program (Quandt et al. 1995). The *mEpREmt1* plasmid (hereafter named *EpRE-Luc*), kindly provided by Dr. M.J. Carvan III (Great Lakes WATER Institute, Milwaukee, WI), was used to produce the EpRE-mediated Luc. This plasmid contains a single *EpRE* from the mouse *gsta1* enhancer region (−754 to −714) fused to the minimal mouse *mt1* promoter and cloned into the pGL3-Basic (Promega, Madison, WI) firefly luciferase (*luc*) reporter gene construct (Carvan et al. 2000). Adult wild-type zebrafish obtained from a commercial supplier were kept in a closed water circulation system at 28°C under a 14/10-h (light/

dark) photoperiod. Freshly fertilized eggs were collected for microinjection, and the *EpRE-Luc* plasmid was injected into fertilized eggs before the first cleavage. One-cell embryos were injected following the general protocol recommended by Vielkind (1992) using an IM-30 motorized picoinjector (Narishige, Japan) to inject approximately 300 pl of DNA solution, representing a total of 10^6 copies of the transgene per injected embryo. Microinjected embryos were incubated at 28°C until hatching. *EpRE-Luc* transgenic larvae were exposed to copper sulfate (Sigma, Brazil) 72 h after fertilization in 20 ml of acclimated tap water in an all-glass container. Three replicate treatments (3 × 20 larvae) were exposed to 0.02 mg l^{-1} copper sulfate for 24 h. After exposure, larvae were killed and immediately frozen at −80°C for RNA extraction.

The expression of the *luc*, *gst*, *gclc* and *hsp70* genes was evaluated by semiquantitative reverse transcriptase (RT)-PCR from *EpRE-Luc* transgenic larvae. Following copper sulfate exposure, larvae were pooled in 1.5-ml microtubes (20 larvae per tube), and total RNA was extracted using TRIzol reagent (Invitrogen, Brazil) according to the protocol suggested by the manufacturer. RNA concentration was quantified on a Qubit Fluorometer (Invitrogen, Brazil). The Quant-iT RNA Assay kit (Invitrogen, Brazil) was used, and calibration was performed using a two-point standard curve. Approximately 2 μg of total RNA from each pool was utilized as the template for the RT-PCR with the AP primer [5′-GG CCACGCGTCGACTAGTAC(T)17-3′; Invitrogen, Brazil]. The complementary DNA (cDNA) synthesis was carried out using the enzyme RT SuperScript III (Invitrogen) according to the protocol suggested by the manufacturer. All PCR reactions were carried out in a 12.5-μl reaction volume containing 1.25 μl 10× PCR buffer, 0.2 μ*M* of each primer, 0.2 m*M* of each dNTP, 0.75 m*M* $MgCl_2$, 0.5 U Platinum Taq DNA polymerase (Invitrogen) and 1 μl cDNA solution. In order to be able to normalize the data, we used *β-actin* gene expression as an internal control. Specific primers, fragment length and annealing temperature for each gene are listed in Table 1. Amplification products were separated on a 1% (w/v) agarose gel and stained with Sybr Safe (Invitrogen). Band densitometry was performed with 1Dscan EX software ver. 3.1 (Scanalytics, Rockville, MD).

Differences between controls and treated samples in terms of gene expression were determined using Student's *t* test for statistical comparisons. The statistical test used a fixed type I error of 5% ($\alpha = 0.05$).

Results and discussion

The *EpRE*-mediated gene expression represents an important factor in the cellular detoxifying process. Considering that this responsive element is present in promoter regions of several genes coding for phase II enzymes and chaperones, such as Hsp70, it seems obvious that this system includes very intricate and coordinated mechanisms. The *EpRE* sequence has

Table 1 Nucleotide sequences of the PCR primers used to assay gene expression by semiquantitative reverse transcriptase-PCR

Gene	Primer	Sequence	Fragment length (bp)	Annealing temperature (°C)
luc	LUC-F	5′-GGTCCTATGATTATGTCCG-3′	450	56
	LUC-R	5′-GGCCTTTTATGAGGATCTCT-3′		
gst	ZFGST-FOR	5′-CAGTTGCCTAAATTTGAAGATGG-3′	329	60
	ZFGST-REV	5′-AGATTCAGCAGGAGATCGAACAG-3′		
gclc	ZFGCL1-F	5′-AGGCCTGAGCTATGCAGC-3′	914	60
	ZFGCL3-R	5′-GTGGTCCGATTCGTTCTCA-3′		
hsp70	ZFHSP70-F	5′-ATTGACCTGGGCACCACCT-3′	612	60
	ZFHSP70-R	5′-ATGGTCAGGATGGACACGT-3′		
β-actin	ZFBAC-F	5′-CCCTTGTTCACAATAACCT-3′	380	53
	ZFBAC-R	5′-TCTGTTGGCTTTGGGATTCA-3′		

Genes: *hsp*, Heat shock protein; *luc*, luciferase; *gst*, glutathione *S*-transferase; *gclc*, glutamate cysteine ligase catalytic subunit

been recognized as a primary binding site for Nrf2, which is a very conserved transcription factor among vertebrates (Kobayashi et al. 2002). Within this context, we have demonstrated that the *EpRE* sequence is a common element within different promoters of *gst*, *gclc* and *hsp70* genes. In order to determine the position of the *EpRE* core sequences, the proximal promoters (1500 bp) from mouse and zebrafish *gst*, *gclc* and *hsp70* genes were analyzed. The results obtained are shown in Table 2. Two *EpRE* core sequences were identified for the mouse *gsta1* promoter, as previously described by Prestera et al. (1993) at position −743 to −734 and −728 to −719, respectively. However, the promoter analysis carried out in our study recognized two additional *EpRE* sequences at position −913 to −904 and −148 to −139, respectively. For the zebrafish ortholog gene, we found only two *EpRE* sequences—at positions −1331 to −1322 and −749 to −740, respectively.

Analysis of the mouse *gclc* proximal promoter revealed an *EpRE* site at position −731 to −722, while in the zebrafish ortholog two *EpRE* sites at −453 to −444 and −355 to −346, respectively, were found. Our analysis of the mouse *hsp70* promoter revealed an *EpRE* at −502 to −493, while in the zebrafish ortholog, this element appears at −690 to −681. The *EpRE* sites identified for both species in all three genes compared showed a high similarity with the putative *EpRE* core sequence that could be a potential binding site for the Nrf2 transcription factor (Table 2). This observation suggests that the *EpRE/Nrf2* signaling pathway is a shared detoxifying mechanism for all genes analyzed here. Nevertheless, it is interesting to notice that the comparison between the mouse and zebrafish *gclc* proximal promoter identified two *EpRE* sites for zebrafish while the mouse only has one. Conversely, the *gst* promoter analysis indicated four mouse *EpRE* sequences against only two for the zebrafish orthologous promoter. This loss and gain of transcription factor binding sites probably has to do with the evolution of these genes in terms of their role within the cellular complex detoxification mechanism in mouse and zebrafish.

In addition to comparing proximal promoters in order to establish the relationship of the *EpRE/Nrf2* signaling pathway between mouse and zebrafish for the different proteins involved with detoxification mechanisms, we also produced a transgenic zebrafish model carrying a transgene comprised of reporter genes of luciferase under transcriptional control of a mouse *EpRE* sequence. As expected, all genes (*luc*, endogenous *gst*, *gclc* and *hsp70*) were induced in terms of mRNA transcript quantity after metal exposure. This observation suggests that the *gst*, *gclc* and *hsp70* genes are under the same regulatory system mediated by the *EpRE* sequence located at the proximal promoters. No mortality was observed in any of our experiments. In comparison to the control group, which was not exposed to copper, exposure to copper increased the level of *Luc* mRNA (Fig. 1a) by

Table 2 Analysis of the *EpRE* core sequences in the mouse (*Mus musculus*) and zebrafish (*Danio rerio*) *gst*, *gclc* and *hsp70* proximal promoters (1500 bp)

Species	Gene	Core sequence[a]	Position	Strand	Sequence
Danio rerio	*gclc*	RTGAYnnnGC	−453 to −444	(−)	**ATGAC**aac**GC**
		RTGAYnnnGC	−355 to −346	(−)	**GTGAC**tca**GC**
	hsp70	RTGAYnnnGC	−690 to −681	(+)	**GTGAC**gcg**GC**
	gst	RTGAYnnnGC	−749 to −740	(+)	**ATGAT**aga**GC**
		CGnnnYAGTR	−1331 to −1,322	(−)	**CG**cca**CAGTG**
Mus musculus	*gclc*	CGnnnYAGTR	−731 to −722	(+)	**CG**tgg**TAGTG**
	hsp70	CGnnnYAGTR	−502 to −493	(+)	**CG**caa**CAGTG**
	gst	RTGAYnnnGC	−913 to −904	(−)	**ATGAT**ctg**GC**
		RTGAYnnnGC	−743 to −734	(+)	**ATGAC**att**GC**
		RTGAYnnnGC	−728 to −719	(+)	**GTGAC**aaa**GC**
		RTGAYnnnGC	−148 to −139	(+)	**ATGAT**tg**GC**

[a] R represents A or G; Y represents C or T

about 1.39-fold ($P < 0.05$), that of the endogenous zebrafish *gclc* gene (Fig. 1b) by 1.23 fold ($P < 0.05$); there was an even higher induction for the zebrafish *gst* gene—3.38 fold ($P < 0.05$) (Fig. 1c). The *hsp70* gene expression analysis showed an increase in the mRNA level (Fig. 1d) of about 1.59 fold that of the control group ($P < 0.05$). These results indicate a specific binding of the zebrafish Nrf2 transcription factor to the *EpRE* of mouse origin and corroborate the hypothesis that this signaling pathway is fully conserved among vertebrates (Kobayashi et al. 2002). Carvan et al. (2001) have already demonstrated that zebrafish transcription factors are able to recognize the mouse *EpRE* sequence in a dose-dependent manner in zebrafish cells exposed to several pollutants.

Even though there are no published studies on transgenic fish harboring a genetic construct comprised by a reporter gene under transcriptional control of the orthologous *gclc* promoter, there is a recent study also demonstrating that a mammalian *hsp70* promoter can be recognized by fish transcription factors. Seok et al. (2006) produced transgenic zebrafish carrying a transgene comprised by a human *hsp70* promoter driving the gene coding for enhanced green fluorescent protein (eGfp). These authors demonstrated that this model is sensitive enough to detect copper sulfate at low concentration (0.066 mg l^{-1}), which is below the median lethal concentration established for zebrafish larvae (0.08 mg l^{-1}). We detected induction of the *luc* reporter gene at an even lower level of copper sulfate (0.02 mg l^{-1}). However,

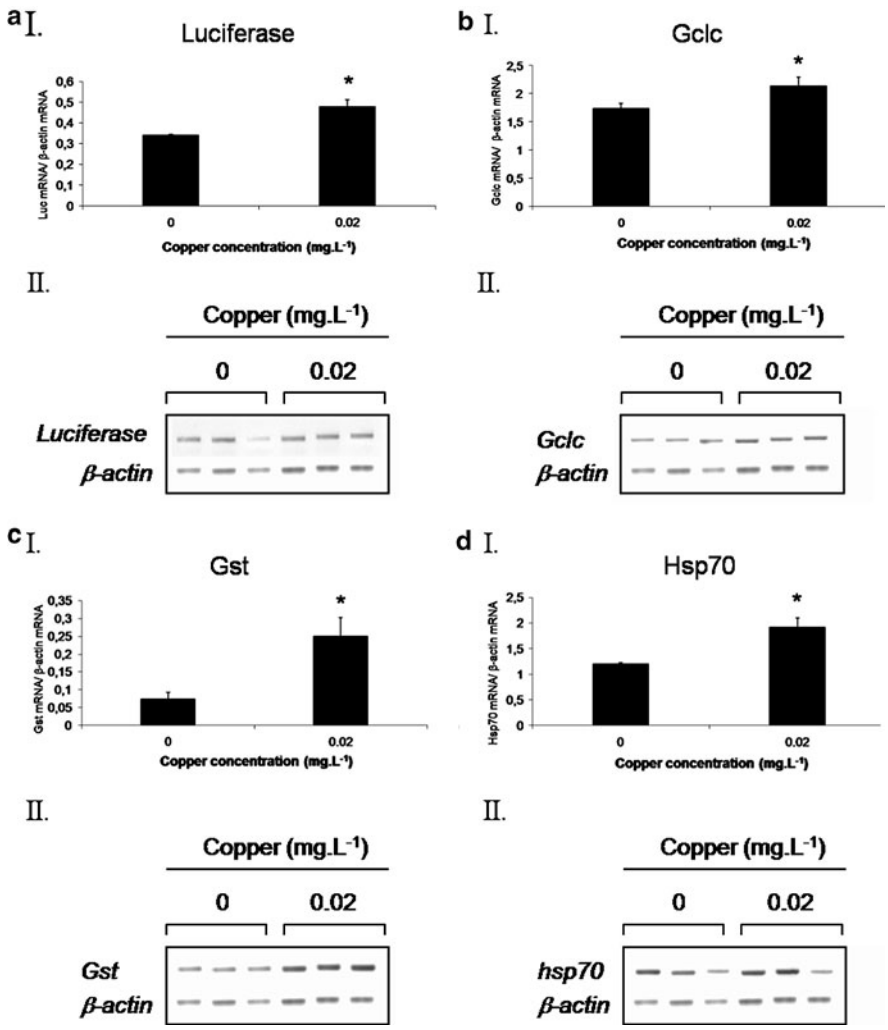

Fig. 1 Effects of exposure to copper sulfate in terms of luciferase (*luc*, **a**),glutamate cysteine ligase catalytic subunit (*gclc*, **b**), glutathione *S*-transferase (*gst*, **c**) and heat shock protein (*hsp70*, **d**) gene expression on the electrophile-responsive element (*EpRE*)-*Luc* transgenic zebrafish larvae. *I* Graphic representation of the quantification of the bands shown below, normalized according to *β-actin* expression and analyzed by band densitometry. *II* Levels of mRNA for all genes were measured by reverse transcriptase PCR analysis following treatment with copper sulfate at a concentration of 0.02 mg l^{-1} for a period of 24 h. Data are expressed as mean ± standard error ($n = 3$). *Asterisks* (*) indicate significant differences ($P < 0.05$)

this discrepancy cannot be associated with the *EpRE* origin of the two transgenes used in both studies, but with the different methods for gene expression analysis applied. In our study, we used RT-PCR to quantify expression of reporter gene (*luc*), while Seok et al. (2006) only detected eGfp expression through the epifluorescence microscope. Blechinger et al. (2002) used a similar approach to monitor the effect of cadmium in a stable germline transgenic zebrafish containing a zebrafish *hsp70*-regulated eGfp construct. The authors demonstrated that the transgene responds in a dose-dependent manner and is sensitive enough to detect cadmium at doses below the median lethal concentration.

In conclusion, the results of our study demonstrate that *EpRE* is conserved between mouse and zebrafish in terms of the *gst*, *gclc* and *hsp70* genes. We also produced transgenic zebrafish containing an *EpRE-Luc* construct and analyzed the endogenous *gst*, *gclc* and *hsp70* zebrafish genes. The gene expression analysis quantified by RT-PCR showed an induction of the endogenous genes as well as the transgene used. Therefore, since *EpRE* is conserved between fish and mammals and is present in the regulatory region of genes encoding for detoxification-related proteins, the development of genetically modified models using this responsive element driving the expression of reporter genes can be an important tool towards gaining an understanding of the action mechanism of aquatic pollutants. The use of a reporter gene, such as GFP, could make this technology more cost-effective, since GFP can be detected without any external substrate and chemical or radioactive labels. This could permit a wider ecotoxicological approach in terms of pollutant concentration and exposure time.

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