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Reexamination of the electrophile response element sequences and context reveals a lack of consensus in gene function

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Abstract

The electrophile response element (EpRE) is essential for regulation of many genes involved in protection against toxic agents. Putative EpRE core sequences (TGAnnnnGC) are localized in 5'-flanking regions (5'-UTR) of these genes but specificity of the internal bases and whether location affects function has not been refined. The catalytic subunit of human glutamate cysteine ligase (GCLC) gene is well documented to be under EpRE regulation and four sequences having an EpRE "consensus" sequence were reported with only one (EpRE 4) responsive to electrophiles. Using GCLC as a model, we asked whether the internal variable or flanking nucleotides and the location of the sequence were required for functional activity in response to 4-hydroxynonenal (HNE). We found that thirteen putative EpRE core sequences (TGAnnnnGC) were localized in 5'-UTR of GCLC and confirmed that EpRE 4 showed both constitutive and HNE-inducible activity. Four other sequences exhibited only constitutive activity while other putative EpREs demonstrated no activity. Nucleotide mutagenesis demonstrated specific requirements for internal and flanking nucleotides that were specific for the electrophilic response and that a TRE-like sequence within EpRE was essential for basal (non-electrophile -dependent) activity. Furthermore, EpRE 4 relocated to positions of other putative EpREs maintained activity but moving other EpREs to the EpRE 4 location did not. Thus in GCLC, specific flanking and internal nucleotides within EpRE were far more important for function than previously described while location did not influence activity. These two findings bring into question the meaning of the phrase, "consensus sequence" for this important *cis* element.

Keywords

EpRE; Nrf2; consensus sequence; oxidative stress; antioxidant response element; glutamate cysteine ligase

Introduction

The electrophile response element (EpRE), also called the antioxidant response element (ARE), is involved in the up-regulation of many antioxidant/detoxifying genes, including glutathione S-transferase (GST) [1], heme oxygenase-1 (HO-1) [2], NAD(P)H quinone oxidoreductase 1 (NQO-1) [3] and both subunits of glutamate cysteine ligase (GCL) [4,5]. Upon exposure to oxidants and other electrophiles, a transcription factor called nuclear factor erythroid-2 related factor 2 (Nrf2) is activated and recruited to the EpRE after heterodimerizing with other

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transcription factors such as Maf G/F/K and c-Jun [6,7]. The formation of EpRE-Nrf2 complex leads to increased transcription and expression of target genes. Based on the sequence of the functional EpREs identified in these genes, a common core sequence of EpRE, i.e., 5'-TGAnnnnGC-3' has been proposed and used for the identification and investigation of potential EpRE-regulating genes.

GCL is the rate-limiting enzyme for *de novo* synthesis of glutathione (GSH), the most abundant non-protein thiol in cells. GSH plays critical roles in antioxidant protection, detoxification of xenobiotics, metabolism of leukotrienes, and the cell cycle regulation[8]. Transcription and translation of both the catalytic and modifier subunits of GCL, namely GCLC and GCLM, are up-regulated in response to oxidative/electrophilic stimuli [9–11]. The induction of human GCLC has been demonstrated to be mediated through Nrf2/EpRE pathways. Nrf2 knockdown or mutation of a distal EpRE in the 5'-flanking region of GCLC gene abrogated both the constitutive and inducible expression of GCLC [4,12]. The Ep RE sequences in GCLC have been extensively investigated [4] and served as models for studying EpRE regulation.

Based on the initially proposed core sequence of EpRE, four EpRE sequences were reported in the promoter of GCLC but only one was reported to be functional in GCLC induction[4]. The finding that several EpREs are present in a gene but only one is inducible also occurs in other EpRE-regulating genes. Both human NAD(P)H quinone oxidoreductase (NQO2) and GCLM genes have 6 putative EpRE sequences in the 5'-flanking region, though only one was functional in regulating gene induction [5,13]. Little is known however, about what allows some EpRE sequences to be functional while others are not. Using the EpREs in GCLC gene as the model, we investigated the effects of the flanking nucleotides of EpRE core sequence, the nucleotides embedded between the –TGA- and –GC- boxes, and the location of EpRE in the gene, on EpRE activity. Upon reexamination of the 5'-flanking region of GCLC gene promoter, we found thirteen putative EpRE sequences. Nonetheless, the results of reporter assays suggest that the most important sequence in regulating GCLC expression is the one identified earlier. More importantly, the new results suggest that both the flanking nucleotides and the embedded nucleotides of EpRE are critical for EpRE activity but that changing the location of that functional sequence within the promoter region does not decrease its responsiveness. It should be noted, that even if the sequences in the database are incorrect, the analysis of the requirements for the bases in the studies here are valid as they involve synthesized and resequenced sequences.

Material and methods

Chemicals and reagents

Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO). Luciferase activity assay kit, pGL-3 basic vector, and pGL-3 promoter vector, were from Promega (Madison, WI). FuGENE 6 transfection reagent was from Roche (Indianapolis, IN). Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA). 4-hydroxynonenal was ordered from Cayman Chemical (Ann Arbor, MI). All chemicals used were at least analytical grade.

Cell culture and treatment

A human bronchial epithelial cell line (HBE1 cell) was cultured in collagen-coated dishes in 5% CO₂ at 37°C as described by Harper et al. [14]. Cells were transfected when 70–80% confluent. Twenty-four hours after transfection, cells were treated with HNE or ethanol for an additional 24h before being harvested.

Construction of Plasmids

The oligonucleotides containing the putative EpRE sequences were designed with an Mlu I and an Xho I cutting site on its 5'- and 3'-end, respectively (Table 1). Oligos were cloned into pGL-3 promoter vector after the vector was digested with corresponding restriction enzymes.

To construct plasmid m4 GCLC-Luc, two DNA fragments were first amplified with-3802/GCLC-Luc as the PCR template. The two fragments were annealed before being cloned into pGL-3 basic vector. The primers used were: for DNA fragment 1, sense 5'-GTACGCGTCACAGGCAGATCCGCGGGTTT-3'(Mlu I), antisense 5'-GCAGATCTCTCCCCGCGCGCAGCGCCTCCC -3' (Bgl II); for DNA fragment 2, sense 5'-CGAGATCTGCGGGCCCGCGCAGtcCGTGGC-3'(Bgl II), antisense 5'-ACTCGAGTCTTTGCGTCCGCTAGCT-3' (Xho I). The plasmid -3802/GCLC 5'-luc, which was first constructed by cloning 3802 bp nucleotides upstream of transcription start site of the human GCLC gene into pGL3-basic luciferase vector [4], was kindly provided by Professor Dale A. Dickinson.

Plasmids, EpRE4/n1-Luc, EpRE4/n6-Luc, EpRE n1/E4-Luc, EpRE n6/E4-Luc EpRE E3/E4-Luc, were made by using m4 GCLC-Luc as the template. First, two DNA fragments were amplified. For the first fragment, the primers were: sense 5'-GTACGCGTCACAGGCAGATCCGCGGGTTT-3'(Mlu I), antisense 5'-GCAGATCTCTGTGAGACTTGGGACGA (EpRE4/n1-Luc), 5'-GCAGATCTACGATAGCTGAAGGGGAA (EpRE4/n6-Luc), 5'-GCAGATCTCTCCCCGCGCGCAGCGCCTCCC -3' (for EpREn1/E4-Luc, EpREn6/E4-Luc, and EpRE 3/E4-Luc) (underlined nucleotides are Bgl II sites); for the second fragment, the primers were: sense 5'-GCAGATCTACAAAGCGCTGAGTCACGGGGAGTGAAGTGTTC -3' (EpRE4/n1-Luc), 5'-GCAGATCTACAAAGCGCTGAGTCACGGGGAGTGAAAACACTCCTC-3' (EpRE4/n6-Luc), 5'-GCAGATCTCAGTAAGTGATGGAGCCTAGAGTCGCTGCGCGCGC-3' (EpRE n1/E4-Luc), 5'-CAGATCTACAGCCCTGATGGAGCTGTCTGCGCTGCGCGCGC-3' (EpRE n6/E4-Luc), 5'-GCAGATCTGCGGGCCCGCGCAGTCACGTGGCGCGCTGCGCGCGC (EpRE3/E4-Luc) (underlined nucleotides are Bgl II sites), antisense 5'-A-CTCGAGTCTTTGCGTCCGCTAGCT-3' (Xho I). Next the two fragments were digested with Bgl II and annealed. Then the annealed fragment was cloned into pGL-3 basic vector.

Transfection of plasmids and assay of luciferase and β -galactosidase activity

Cells were transfected with plasmids (0.5 μ g/well) using FuGENE 6 transfection reagent in 12-well plates, and β -galactosidase (β -Gal) plasmid (1/10 of total amount of plasmid) was co-transfected to normalize the transfection efficiency. Twenty-four hours after transfection, the medium was replaced and cells were treated with/without HNE. After treatment, cells were rinsed with 1X PBS and then lysed with M-PER mammalian protein extraction reagent (Pierce). After centrifugation, the supernatant was used for determination of the activity of luciferase and β -galactosidase with procedures described previously [15].

Software and statistical analysis

EpRE sequences in the 5'-flanking region of GCLC were analysed with Gene runner (version 3.05, Hastings Software, Inc). All data were expressed as the mean $\pm$ standard error. Sigma Stat software was used for statistical analysis and statistical significance was accepted when $p < 0.05$. Comparison of variants between experimental groups was performed with ANOVA and the Tukey test.

Results

Putative EpRE sequences in 5'-flanking region of human GCLC

To reexamine the roles of the different EpREs in the 5'-flanking region of human GCLC, we used Gene Runner (version 3.05). Table 2 shows the 13 putative EpRE sequences that were identified in the 3802bp sequence based on the initially proposed core sequence for EpRE. Some EpRE sequences were in a reverse orientation in the gene but all EpREs were shown in TGA ? GC direction in Table 2. These putative EpREs shared the similar core sequence (TGAnnnnGC) that was composed of a –TGA - and a –GC- box and four nucleotides between them. The nucleotides embedded between–TGA - and –GC- boxes, and the flanking nucleotides on both the 5' - and 3'-end of the core sequence, varied among EpREs.

Not all putative EpREs were functional in regulating GCLC gene expression

The activity of these putative EpRE sequences were investigated by cloning about 40 bp oligonucleotides containing individual EpRE into a luciferase reporter (pGL3 promoter vector) driven by Simian virus 40 promoter and then transiently transfecting the constructed plasmid into HBE1 cells. Figure 1 showed that the reporters containing EpRE 1, n7, EpRE 3, EpRE 4, and n8 had significantly higher activity compared with that of the vehicle vector (pGL3 promoter vector). Among them, the EpRE 4-containing reporter exhibited the highest activity. These data suggest that EpRE 1, n7, EpRE3, EpRE4, and n8 exhibit constitutive activity to increase gene expression while other putative EpRE sequences do not.

To determine how these putative EpRE sequences responded to oxidative stress, reporters constructed from these EpREs were transiently transfected into HBE1 cells and then the cells were treated with HNE (15 μ M) for 24h. Figure 1 showed that only the activity of the EpRE 4-containing reporter was increased by HNE treatment. The activity of reporters constructed from other putative EpREs did not change upon the exposure to HNE. These EpRE-containing reporters showed a similar response to DMNQ (10 μ M) treatment (data not shown), suggesting that these putative EpREs exhibit different response to oxidative stress even though they share a common core sequence (TGAnnnnGC).

Effects of flanking nucleotides on EpRE activity

To test whether the different activity of these putative EpREs is due to the variety of the nucleotides flanking the EpRE core sequence, reporters with nucleotide mutation on the flanking regions were made. Table 3 showed the relative reporter activity when these reporters were transiently transfected in HBE1 cells for 24h with or without HNE exposure.

Mutation of the 5'-flanking nucleotides of EpRE 4 (5'-C⁸C⁷T⁶C⁵C⁴C³C²G¹-3') from G¹ to T¹, C⁵ to T⁵, T⁶ to C⁶, or the 3'-flanking nucleotides (5'-G¹C²T³T⁴T⁵-3') from G¹ to T¹, T³, T⁴, and T⁵ to C³, C⁴, C⁵, respectively, significantly decreased the constitutive activity and abolished the HNE-inducible activity of EpRE4, indicating that these flanking nucleotides are required for EpRE activity. Mutations of other flanking nucleotides of EpRE 4 did not affect its activity.

To confirm the critical role of flanking nucleotides G¹, C⁵, T⁶ on the 5'-end, G¹, T³, T⁴, and T⁵ on the 3'-end, in EpRE activity, the corresponding nucleotide in the flanking region of EpRE 3, n1, and n8 were mutated to that of EpRE 4. Upon these mutations EpRE 3 and n8 but not n1 became inducible by HNE, revealing that other nucleotides than those in the flanking regions are also required for EpRE function. Interestingly, after mutation of those necessary flanking nucleotides, EpRE 4 retained about 40% of the original constitutive activity, suggesting that the phorbol 12-O-tetradecanoate 13-acetate responsive element (TRE)-like element in EpRE 4 is responsible for part of the constitutive activity of EpRE 4.

Effects of embedded nucleotides on EpRE activity

The importance of the nucleotides embedded between –TGA - and –GC- box to EpRE activity was investigated by mutating these embedded nucleotides. Mutation of the embedded nucleotides in EpRE 4 (5'-C¹T²C³A⁴-3') from C¹ to A¹, C³ to T³ abolished both its constitutive and inducible activity, while mutating the nucleotides from T² to C², or A⁴ to G⁴ did not affect EpRE 4 activity. As above, EpRE n1 remained inactive upon mutation of some necessary flanking nucleotides to those of EpRE 4. When the embedded nucleotides T¹ and G³ of EpRE n1 were mutated to C¹ and C³, respectively, EpRE n1 became active and shows both constitutive and inducible activity. These data indicate that in the embedded nucleotides, C¹ and C³ are critical for EpRE function.

Effect of location in the gene on EpRE activity

We studied EpRE location on its function in gene regulation by relocating EpRE 4 (about 26 bp oligonucleotide containing EpRE 4) into positions of EpRE n1 or n6 in EpRE4-mutated GCLC reporter (i.e. the original EpRE 4 was mutated). Mutation of the –TGA - box in EpRE 4 significantly decreased the constitutive activity of the reporter and abrogated the HNE-inducible activity (Figure 2). Replacing EpRE n1 or n6 with EpRE 4 maintained GCLC reporter activity. In contrast, replacing EpRE 4 with EpRE n1, n6, or EpRE 3 in the GCLC reporter significantly reduced the constitutive activity and abrogated the HNE-inducible activity. Overall these data suggest that relocation of the functional EpRE sequence in the 5'-flanking region of GCLC gene does not affect its gene regulating activity.

Discussion

The principal question addressed in this study was whether the functionality of EpRE sequence was determined by the core sequence, the flanking bases and/or the location of the sequence in the promoter. We used human GCLC as it has been well studied and EpRE was shown to be clearly involved in its induction by oxidative stimuli [12,16,17]. In the present study, 13 putative EpRE sequences were present in the human GCLC promoter based on the EpRE core sequence initially proposed (5'-TGAnnnnGC-3'), but only one (EpRE 4) was inducible. Similarly, more than one EpRE core sequences were also frequently found in the promoters of many other EpRE-regulating genes, although normally only one was functional. Herein we demonstrated that besides the EpRE core sequence (TGAnnnnGC), some flanking nucleotides were required for the EpRE activity in the human GCLC gene. It was also found that the embedded TRE-like sequence in EpRE 4 was responsible for part of its constitutive activity. On the other hand, EpRE location in the 5'-flanking region did not appear to be important.

Early studies on EpRE function proposed that functional EpREs shared the consensus sequence as 5'-TGAnnnnnGC-3' [18–20]. Among the 13 putative EpREs identified in the GCLC promoter, only EpRE 4 was essential for induction by HNE (Figure 1), suggesting that although the initially proposed EpRE consensus sequence is helpful to identify putative EpREs, it cannot precisely tell what EpRE is functional. Wasserman and Fahl systematically investigated the essential nucleotides for EpRE induction in rat GST-Ya and suggested that the EpRE consensus sequence should be 5'-TMAnnRTGAYnnnGCRWWW-3' (where M=A or C, R=A or G, Y=C or T, W=A or T) [21]. This consensus sequence was consistent with the functional EpRE sequences in many other genes, including genes of mice GST-Ya, rat NQO-1, human NQO-1, and rat GST-P [21]. The sequence of EpRE 4 in GCLC gene had a similar core sequence (5'-RTGAYnnnGCR-3') as proposed by Wasserman *et al* [21]. The flanking nucleotides on both the 5'- and 3'-end of EpRE 4 were however, different from that in Wasserman's consequence. In the fourth position of the 5'-lanking region of EpRE 4 there was a cytosine instead of an adenosine (5'-T⁶C⁵C⁴C³C²G¹-3' vs. 5'-T⁶M⁵A⁴n³n²R¹-3'). Mutagenesis of C⁴ to T did not affect the EpRE 4 function, suggesting this cytosine was not essential for EpRE function (Table

3). Nioi *et al.* reported that C³ in the 5'-end of the flanking region of EpRE consequence of mouse NQO-1 was required for the induction [22]. In GCLC however, it was not essential since mutation of C³ to T did not change EpRE 4 activity (Table 3).

The present work showed that the 3'-flanking nucleotides of EpRE 4 were also critical for induction (Table 3, mutation data of EpRE 4, n1, and EpRE 3). This finding confirmed previous studies of the requirement of nucleotides in the 3'-flanking region for EpRE function [21,22]. Nonetheless, there were differences in EpRE 4 from Wasserman's EpRE consequence; EpRE 4 had a cytosine instead of an adenosine/thymidine in the second position of the 3'-flanking region (5'-G¹C²T³T⁴T⁵-3' vs. R¹W²W³W⁴W⁵, where R= A or G and W=A or T). Mutation of C² to T in the 3'-flanking region of EpRE 4 did not change its EpRE function, suggesting that nucleotide in this position can vary. The critical involvement of some nucleotides in the flanking regions of EpRE in EpRE function was further confirmed with mutation in EpRE 3, n1, and n8 (Table 3). In brief, some of the 5'- and 3'-flanking nucleotides were required for EpRE 4 induction by HNE. If the concept of an EpRE consensus sequence is still useful, it should, at least, be extended to the flanking region.

The initially proposed core EpRE sequence, 5'-TGAnnnnGC-3', was useful in finding putative EpRE sequence. This allowed us to demonstrate that a) not all EpREs with this core sequence were inducible by HNE (Figure 1) and b) mutation of the core sequence abrogated EpRE 4 induction (Table 3). Regarding the role of nucleotides embedded between the -TGA - and -GC- boxes on EpRE function, mutagenesis data showed that the cytosine (in EpRE 4) or guanosine (in EpRE 3) immediately following the -TGA - box, and the cytosine in the third position (5'-C¹T²C³A⁴-3') of the four embedded nucleotides, were required and other two nucleotides could be replaced without decreasing EpRE induction by HNE (Table 3). The critical role of cytosine at this position (the position immediately following the -TGA - box) for EpRE function has been well documented [18,21,22], but guanosine in this position was sufficient for EpRE function is the first reported. Consistent with our finding that the embedded C³ was necessary for EpRE 4 function, Nioi *et al.* reported that the cytosine in this position was critical for the induction of EpRE in mice NQO-1 gene (5'-TGAY¹n²C³n⁴GCR-3') [22]. However, this was in disagreement with Wasserman's EpRE consensus sequence model, in which the nucleotide in this position could be replaced without altering EpRE function[21]. It is unclear whether this inconsistency is gene or inducer specific. It also remains to be determined whether these embedded nucleotides change the binding and/or response to different transcription factors and thereby determine which signaling pathways are needed for GCLC induction. Nonetheless, we found here that the initially proposed core EpRE 4 sequence was necessary for induction by HNE and some flanking nucleotides on both the 5'- and 3'-ends were also required.

Another important finding in this study was that some EpRE core sequences displayed constitutive activity even though they were not responsive to HNE induction (Figure 1). Examination of the EpRE sequences demonstrated that these EpREs contained a TRE (EpREn8) or TRE-like element (EpRE 1, 3 and n7). Mutation of the -GC- box in EpRE 3 and n8 left a TRE-like and a TRE element respectively and these sequences still displayed modest activity to regulate gene expression (Table 3). It remains to be determined whether these TRE- or TRE-like elements are involved in the constitutive expression of the GCLC gene. Many EpRE core sequences contain such TRE or TRE-like element, such as the EpREs in human NQO-1 [19] and sulfiredoxin-1 [23]. These TRE or TRE-like elements were responsive to phorbol 12-O-tetradecanoate 13-acetate (TPA) but also critical for the EpRE induction by oxidant stimuli [19]. The EpRE 4 in human GCLC also contains a TRE-like element (5'-TGACTCA -3'). Mutation of the GC box in EpRE 4 made it a pure TRE-like element that showed constitutive activity, although it was not responsive to HNE induction. These data suggest that the TRE-like element in EpREs plays a role in the constitutive EpRE activity. It

has been demonstrated that at basic condition, EpRE 4 complexes was composed of Nrf2 and c-Jun [24], and this may explain the involvement of TRE/activating protein-1 (AP-1) binding site in the constitutive activity of EpRE 4. On the other hand, it is important to note that a recent study found that while c-Jun must be phosphorylated for transcriptional activation of a true AP-1, phosphorylation of c-Jun may be not essential and can sometime inhibit transcription that is dependent upon EpRE [25].

For a long time an EpRE consensus sequence has been sought so that it can be used to predict EpRE-regulated genes, identify EpRE binding proteins, and study gene polymorphism in EpRE. The finding here that some of the flanking and embedded nucleotides of EpRE were important for EpRE function could suggest that the initially proposed EpRE consensus sequence be extended to TMnnnRTGAYnCNnGCRnWWW (where M=A or C, R=A or G, Y=C, T or G, W= A or T). On the other hand, while this extended EpRE consensus sequence was shared by some reported functional EpREs (Table 4), the use of a consensus sequence can also be rather misleading. For example, the 5'-flanking nucleotides of EpREs in human GCLM and mice HO-1, and the 3'-flanking nucleotides of EpREs in human NQO-2 and sulfiredoxin-1, do not follow the above consensus sequence. Whether the differences in EpRE sequence requires distinctly different protein binding remains unclear. In addition, the current study also demonstrated that some EpRE core sequences present in genes, although were not involved in response to electrophiles, might be involved in the basal gene expression. Interestingly, these all contained TRE or TRE-like sequences. Finally, the finding that the EpRE 4 could be relocated to positions of non-functional EpRE and maintained both basal and inducible expression of a reporter, suggests that position may not be a regulating feature. This is probably fortunate as the basis for using reporter constructs driven by *cis* elements depends on this lack of positional requirement.

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References

1. Liu S, Pickett CB. The rat liver glutathione S-transferase Ya subunit gene: characterization of the binding properties of a nuclear protein from HepG2 cells that has high affinity for the antioxidant response element. *Biochemistry* 1996;35:11517–11521. [PubMed: 8784208]
2. Li N, Venkatesan MI, Miguel A, Kaplan R, Gujuluva C, Alam J, Nel A. Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element. *J Immunol* 2000;165:3393–3401. [PubMed: 10975858]
3. Joseph P, Xie T, Xu Y, Jaiswal AK. NAD(P)H:quinone oxidoreductase1 (DT-diaphorase): expression, regulation, and role in cancer. *Oncol Res* 1994;6:525–532. [PubMed: 7620221]
4. Mulcahy RT, Wartman MA, Bailey HH, Gipp JJ. Constitutive and beta-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J Biol Chem* 1997;272:7445–7454. [PubMed: 9054446]
5. Moinova HR, Mulcahy RT. An electrophile responsive element (EpRE) regulates beta-naphthoflavone induction of the human gamma-glutamylcysteine synthetase regulatory subunit gene. Constitutive expression is mediated by an adjacent AP-1 site. *J Biol Chem* 1998;273:14683–14689. [PubMed: 9614065]
6. Jaiswal AK. Regulation of genes encoding NAD(P)H:quinone oxidoreductases. *Free Radic Biol Med* 2000;29:254–262. [PubMed: 11035254]
7. Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annual review of pharmacology and toxicology* 2003;43:233–260.

8. Pallardo FV, Markovic J, Garcia JL, Vina J. Role of nuclear glutathione as a key regulator of cell proliferation. *Mol Aspects Med* 2009;30:77–85. [PubMed: 19232542]
9. Shi MM, Iwamoto T, Forman HJ. gamma-Glutamylcysteine synthetase and GSH increase in quinone-induced oxidative stress in BPAEC. *Am J Physiol* 1994;267:L414–421. [PubMed: 7943345]
10. Dickinson DA, Levonen AL, Moellering DR, Arnold EK, Zhang H, Darley-Usmar VM, Forman HJ. Human glutamate cysteine ligase gene regulation through the electrophile response element. *Free Radic Biol Med* 2004;37:1152–1159. [PubMed: 15451055]
11. Forman HJ, Dickinson DA, Iles KE. HNE--signaling pathways leading to its elimination. *Mol Aspects Med* 2003;24:189–194. [PubMed: 12892996]
12. Zhang H, Court N, Forman HJ. Submicromolar concentrations of 4-hydroxynonenal induce glutamate cysteine ligase expression in HBE1 cells. *Redox Rep* 2007;12:101–106. [PubMed: 17263920]
13. Wang W, Jaiswal AK. Nuclear factor Nrf2 and antioxidant response element regulate NRH:quinone oxidoreductase 2 (NQO2) gene expression and antioxidant induction. *Free Radic Biol Med* 2006;40:1119–1130. [PubMed: 16545679]
14. Harper R, Wu K, Chang MM, Yoneda K, Pan R, Reddy SP, Wu R. Activation of nuclear factor-kappa b transcriptional activity in airway epithelial cells by thioredoxin but not by N-acetyl-cysteine and glutathione. *Am J Respir Cell Mol Biol* 2001;25:178–185. [PubMed: 11509327]
15. Zhang H, Liu H, Dickinson DA, Liu RM, Postlethwait EM, Laperche Y, Forman HJ. gamma-Glutamyl transpeptidase is induced by 4-hydroxynonenal via EpRE/Nrf2 signaling in rat epithelial type II cells. *Free Radic Biol Med* 2006;40:1281–1292. [PubMed: 16631518]
16. Wild AC, Moinova HR, Mulcahy RT. Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J Biol Chem* 1999;274:33627–33636. [PubMed: 10559251]
17. Jeyapaul J, Jaiswal AK. Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of gamma-glutamylcysteine synthetase heavy subunit gene. *Biochem Pharmacol* 2000;59:1433–1439. [PubMed: 10751553]
18. Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem* 1991;266:11632–11639. [PubMed: 1646813]
19. Xie T, Belinsky M, Xu Y, Jaiswal AK. ARE- and TRE-mediated regulation of gene expression. Response to xenobiotics and antioxidants. *J Biol Chem* 1995;270:6894–6900. [PubMed: 7896838]
20. Favreau LV, Pickett CB. The rat quinone reductase antioxidant response element. Identification of the nucleotide sequence required for basal and inducible activity and detection of antioxidant response element-binding proteins in hepatoma and non-hepatoma cell lines. *J Biol Chem* 1995;270:24468–24474. [PubMed: 7592662]
21. Wasserman WW, Fahl WE. Functional antioxidant responsive elements. *Proc Natl Acad Sci U S A* 1997;94:5361–5366. [PubMed: 9144242]
22. Nioi P, McMahon M, Itoh K, Yamamoto M, Hayes JD. Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. *Biochem J* 2003;374:337–348. [PubMed: 12816537]
23. Soriano FX, Baxter P, Murray LM, Sporn MB, Gillingwater TH, Hardingham GE. Transcriptional regulation of the AP-1 and Nrf2 target gene sulfiredoxin. *Mol Cells* 2009;27:279–282. [PubMed: 19326073]
24. Dickinson DA, Iles KE, Zhang H, Blank V, Forman HJ. Curcumin alters EpRE and AP-1 binding complexes and elevates glutamate-cysteine ligase gene expression. *Faseb J* 2003;17:473–475. [PubMed: 12514113]
25. Levy S, Jaiswal AK, Forman HJ. The role of c-Jun phosphorylation in EpRE activation of phase II genes. *Free Radic Biol Med* 2009;47:1172–1179. [PubMed: 19666106]

Figure 1.

The constitutive and HNE-inducible activity of putative EpRE sequences in the 5'-flanking region of human GCLC gene. Oligos containing the corresponding EpRE were cloned into pGL-3 promoter vector and the reporters were transfected into HBE1 cells. After being treated with/without 15 μ M HNE for 24h, cells were collected and the luciferase activity was measured. The luciferase activity was normalized with co-transfected β -Gal activity. N=3, * P< 0.05 compared with vehicle vector (v: pGL-3 promoter vector), # P< 0.05 compared with control treatment.

Figure 2.

Effect of EpRE location on EpRE activity. Reporters were constructed as described in the Experimental Procedures. The reporters were transfected into HBE 1 cells and the luciferase activity was measured. The relative luciferase activity was calculated by using the vehicle vector (pGL-3 basic vector) without HNE exposure as the control. N=3, *, P< 0.05 compared with corresponding reporter without HNE exposure; #, P< 0.05 compared with GCLC-Luc without HNE exposure. GCLC-Luc, -3802/+85 GCLC-luc; m4 GCLC-Luc, -3802/+85 GCLC-Luc with EpRE 4 mutated; in EpRE 4/n1-Luc, EpRE 4/n6-Luc, EpRE 4 was relocated to the position of EpRE n1, and n6 of m4 GCLC-Luc, respectively; in EpRE n1/E4-Luc, EpRE n6/E4-Luc and EpRE 3/E4-Luc, EpRE 4 in GCLC-Luc was replaced with EpRE n1, n6, and EpRE 3, respectively.

Table 1

Oligos containing putative EpRE sequences in human GCLC gene

EpRE #	Oligo sequence (sense)	Alias
n1	5'- <u>CGCGT</u> CACAGTCAGTAAGTGATGGAGCCTAGAGTTGAAC <u>C</u> -3'	
EpRE 1	5'- <u>CGCGT</u> CTGACAGGTCATTGCTCTGTCAACACATATTTAT <u>C</u> -3'	ARE 1
n2	5'- <u>CGCGT</u> CAAAGGAGGTTGTTGAAAAGGCTGTGGAAAAATT <u>C</u> -3'	
n3	5'- <u>CGCGT</u> ACCGCTGGGGGAGCTGGTTCAGAATTCGGGGGG <u>C</u> -3'	
EpRE 2	5'- <u>CGCGT</u> CTATGCAGGCAAAGCATTGTCAGAGGGGAGTAAC <u>C</u> -3'	ARE 2
n4	5'- <u>CGCGT</u> ATCGTTACAGCCCTGATGGAGCTGTCTGCTGAAAC <u>C</u> -3'	
n5	5'- <u>CGCGT</u> TCTAGGGAAGAGCTGATTCTGCAAGATATGAGCT <u>C</u> -3'	
n6	5'- <u>CGCGT</u> CCCGGCTCTCCGAGCATGCTCAGCTTGGCCTCAC <u>C</u> -3'	
n7	5'- <u>CGCGT</u> TACGTGGCGGGGGCTGTATCACAACTGCGCG <u>C</u> -3'	
EpRE 3	5'- <u>CGCGT</u> CGGGGGAGGCGGGCCCGCGCAGTCACGTGGCGGGGG <u>C</u> -3'	ARE 3
EpRE 4	5'- <u>CGCGT</u> AGCCCGCACAAAGCGCTGAGTCACGGGGAGGCGCT <u>C</u> -3'	ARE 4
n8	5'- <u>CGCGT</u> CTCGGAACGCGCTGACTCAAGGGAACTGGGAAC <u>C</u> -3'	
n9	5'- <u>CGCGT</u> GTTTTTCCTTATTTGATCTCGCGAACGCGCTGACT <u>C</u> -3'	

Note: only sense oligos were shown. Underlined nucleotides were the cutting sites for Mlu I (5'end) and Xho I (3'end).

Table 2

Putative EpRE sequences in the 5'-flanking region of human GCLC gene

EpRE #	Putative EpRE sequence	Alias
n1	(-407) 5'-TCAGTAAGTGATGGAGCCTAGA -3'(-386)	
EpRE 1	(-848) 5'-ATATGTGTGACAGAGCAATGA -3' (-869)	ARE 1
n2	(-1260) 5'-GAGGTTGTTGAAAAGGCTGTGG-3' (-1239)	
n3	(-2410) 5'-CCGAATTCTGAACCAGCTCCC-3' (-2431)	
EpRE 2	(-2602) 5'-CTCCCCTCTGACAATGCTTTGC-3' (-2623)	ARE 2
n4	(-2728) 5'-TACAGCCCTGATGGAGCTGTCT-3' (-2707)	
n5	(-2781) 5'-GGAAGAGCTGATTCTGCAAGAT-3' (-2760)	
n6	(-2991) 5'-GGCCAAGCTGAGCATGCTCGGA -3' (-3012)	
n7	(-3067) 5'-AGTTGTTGTGATACAGCCCCG-3' (-3088)	
EpRE 3	(-3086) 5'-CCGCCACGTGACTGCGCGGGCC-3' (-3107)	ARE 3
EpRE 4	(-3130) 5'-CCTCCCGTGACTCAGCGCTTT-3' (-3151)	ARE 4
n8	(-3335) 5'-GTTTCCCTTGAGTCAGCGCGTT-3' (-3356)	
n9	(-3374) 5'-TCCTTATTGATCTCGCGAACG-3' (-3353)	

Note: EpREs are numbered from the transcription start site. The orientation of EpRE 1, n3, 2, n6, n7, 3, 4 and n8 were aligned in reverse direction.

Table 3

Effect of point mutation of EpRE sequence on EpRE activity

EpRE	Sequence	Constitutive activity (mean $\pm$ SD)	Induction fold (mean $\pm$ SD)
EpRE4	5'-CCTCCCCG <u>TGACTCAGCG</u> CTTT-3' (wild)	2.16 $\pm$ 0.20*	2.47 $\pm$ 0.36*
	5'-CCTCCCC/ <u>TGACTCAGCG</u> CTTT-3'	1.64 $\pm$ 0.27*	1.16 $\pm$ 0.24
	5'-CCTCCC/ <u>GTGACTCAGCG</u> CTTT-3'	2.05 $\pm$ 0.31*	2.31 $\pm$ 0.48*
	5'-CCTCC/ <u>CGTGACTCAGCG</u> CTTT-3'	2.21 $\pm$ 0.18*	2.15 $\pm$ 0.26*
	5'-CCTC/ <u>CCGTGACTCAGCG</u> CTTT-3'	1.94 $\pm$ 0.28*	2.29 $\pm$ 0.17*
	5'-CCT/ <u>CCCCGTGACTCAGCG</u> CTTT-3'	1.49 $\pm$ 0.12*	1.15 $\pm$ 0.22
	5'-CC <u>c</u> CCCCG <u>TGACTCAGCG</u> CTTT-3'	1.46 $\pm$ 0.13*	1.08 $\pm$ 0.14
	5'-CCTCCCCG <u>TGA</u> <u>at</u> <u>TCAGCG</u> CTTT-3'	1.15 $\pm$ 0.24	0.86 $\pm$ 0.21
	5'-CCTCCCCG <u>TGAC</u> <u>c</u> <u>CAGCG</u> CTTT-3'	1.96 $\pm$ 0.19*	2.13 $\pm$ 0.18*
	5'-CCTCCCCG <u>TGACT</u> <u>i</u> <u>AGCG</u> CTTT-3'	1.06 $\pm$ 0.11	1.17 $\pm$ 0.28
	5'-CCTCCCCG <u>TGACTCA</u> <u>at</u> <u>GCTTT</u> -3'	1.47 $\pm$ 0.27*	1.08 $\pm$ 0.19
	5'-CCTCCCCG <u>TGACTCAGC</u> <u>i</u> <u>CTTT</u> -3'	1.71 $\pm$ 0.25*	1.32 $\pm$ 0.29
	5'-CCTCCCCG <u>TGACTCAGCG</u> <u>i</u> <u>TTT</u> -3'	2.09 $\pm$ 0.21*	2.38 $\pm$ 0.35*
	5'-CCTCCCCG <u>TGACTCAGCG</u> <u>c</u> <u>ccc</u> -3'	1.45 $\pm$ 0.17*	1.22 $\pm$ 0.31
n8	5'-GTTTCCCT <u>TGAGTCAGCG</u> GTT-3'(wild)	1.77 $\pm$ 0.21*	0.82 $\pm$ 0.33
	5'-GTTTCCCT <u>TGAGTCA</u> <u>at</u> <u>GCGTT</u> -3'	1.87 $\pm$ 0.25*	0.97 $\pm$ 0.21
	5'-GTTCCCCG <u>TGAGTCAGCGC</u> <u>i</u> <u>TT</u> -3'	1.96 $\pm$ 0.28*	2.37 $\pm$ 0.34*
EpRE3	5'-CCGCCACG <u>TGACTGCGCGGGCC</u> -3' (wild)	1.51 $\pm$ 0.27*	0.87 $\pm$ 0.24
	5'-CCGCCACG <u>TGACTGC</u> <u>at</u> <u>GGGCC</u> -3'	1.69 $\pm$ 0.27*	1.13 $\pm$ 0.18
	5'-CCTCCACG <u>TGACTGCGCG</u> <u>c</u> <u>cttt</u> -3'	2.03 $\pm$ 0.22*	2.27 $\pm$ 0.36*
n1	5'-TCAGTAAGT <u>GATGGAGC</u> CTAGA-3' (wild)	0.96 $\pm$ 0.13	0.88 $\pm$ 0.31
	5'-TCAGTAAGT <u>GActcagc</u> CTAGA-3'	1.58 $\pm$ 0.27*	1.26 $\pm$ 0.25
	5'-CCTCTAAGT <u>GATGGAGCG</u> <u>T</u> <u>ttt</u> -3'	1.12 $\pm$ 0.17	0.97 $\pm$ 0.17
	5'-CC <u>t</u> cTAAGT <u>GActcaGCg</u> <u>T</u> <u>ttt</u> -3'	1.93 $\pm$ 0.24*	2.35 $\pm$ 0.46*

Note: the constitutive activity is referenced to vehicle vector (pGL3 luciferase promoter vector). Induction fold is the division of luciferase activity of HNE exposure and that of vehicle exposure (ethanol). Putative core EpREs were underlined and mutated nucleotide was in italic lower case. N=3,

* P<0.05 compared with vehicle vector (Constitutive activity section) or without HNE exposure (Induction section).

Table 4

Functional EpRE sequences in Nrf2/EpRE-regulating genes

Genes	Putative EpRE sequence
hGCLC	5'-CCTCCCCG <u>TGACTCAGCG</u> CTTTG-3'
hGCLM	5'-GAAGACAATG <u>ACTAAGC</u> AGAAAT-3'
hNQO1	5'-AGTCACAGT <u>GACTCAGC</u> AGAAT-3'
rNQO1	5'-AGTCACAGT <u>GACTTGGC</u> AAAAATC-3'
mNQO-1	5'-AGTCACAGT <u>GAGTCGGC</u> AAAATT-3'
hNQO2	5'-CAAGGTGGT <u>GATGTTGC</u> ATCAC-3'
mHO-1	5'-CCGGACCT <u>TGACTCAGC</u> AGAAAA-3'
hSulfiredoxin-1	5'-ACTCACCC <u>TGAGTCAGC</u> GACCCGA -3'
rGST-Ya	5'-GCTAATGGT <u>GACAAAGC</u> AACTTT-3'
mGST-Ya	5'-GCTAATGGT <u>GACAAAGC</u> AACTTT-3'
rGST-p	5'-AGTCACTAT <u>GATTCA</u> GCAATAAA-3'
mFerritin	5'-TTCCACCGT <u>GACTCAGC</u> ATTCTG-3'
rGGT-P5	5'-ACCCACAAT <u>GACACAGC</u> AAGAAA-3'

Note: Putative core EpREs were underlined.